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# Genomic research of bacteriophage and bacteriophage therapy against *Aeromonas hydrophila* and *Vibrio parahaemolyticus*

박테리오파아지 유전체연구 및 *Aeromonas hydrophila*와 *Vibrio parahaemolyticus*에 대한 박테리오파아지 치료법

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**By**

**Jin Woo Jun**

**February, 2014**

**Major in Veterinary Public Health**

**Department of Veterinary Medicine**

**The Graduate school of Seoul National University**

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**A dissertation submitted to the faculty of the Graduate School of Seoul National University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Public Health**

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## Abstract

# Genomic research of bacteriophage and bacteriophage therapy against *Aeromonas hydrophila* and *Vibrio parahaemolyticus*

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*Aeromonas* spp. are primary organisms of normal aquatic microflora, and recently, there has been an increasing appreciation of their role as waterborne pathogens of fish and humans. *Aeromonas hydrophila* is a motile aeromonad that can cause disease in fish, resulting in high rates of mortality wherever outbreaks occur. There has been an increasing incidence of antimicrobial resistance among *Aeromonas* sp. isolated from aquaculture environments. Multiple-antibiotic-resistant *A. hydrophila* exists in aquaculture systems and contributes to the high rate of mortality within the fish industry in Korea. Although the majority of the loach population in Korea is cultured and *A. hydrophila* is one of the main causes of mass mortality in these fish, no effective method has been proposed for the control of *A. hydrophila* infection in aquaculture, except for the application of additional antibiotics. To investigate methods to control the mass mortality of cyprinid loaches (*Misgurnus anguillicaudatus*) caused by multiple-antibiotic-resistant *Aeromonas hydrophila* on a private fish farm in Korea, bacteriophages (phages), designated pAh1-C

and pAh6-C, were isolated from the Han River in Seoul. The two isolated phages were morphologically classified as *Myoviridae* and showed similar infection patterns for *A. hydrophila* isolates. The two phages possessed approximately 55 kb (pAh1-C) and 58 kb (pAh6-C) of double-stranded genomic DNA, and their gDNAs showed different restriction endonuclease digestion patterns. Both phages showed efficient bacteriolytic activity against fish-pathogenic *A. hydrophila* from loaches. The latent periods of the phages were estimated to be approximately 30 min (pAh1-C) and 20 min (pAh6-C), while the burst sizes were 60 PFU/cell (pAh1-C) and 10 PFU/cell (pAh6-C). The phages proved to be efficient in the inhibition of bacterial growth, as demonstrated by their *in vitro* bactericidal effects. Additionally, a single administration of either phage to cyprinid loaches resulted in noticeable protective effects, with increased survival rates against *A. hydrophila* infection. These results suggest that the phages pAh1-C and pAh6-C constitute potential therapeutic agents for the treatment of *A. hydrophila* infection in fish.

*Vibrio parahaemolyticus* is one of the most important causes of gastroenteritis. It is associated exclusively with the consumption of raw or improperly cooked contaminated seafood, especially oysters. *V. parahaemolyticus* is recognized as an important human pathogen globally. East Asians, especially Koreans and Japanese consume a unique diet. Koreans enjoy a wide variety of both raw finfish and shellfish. Although raw oysters have such high densities of *V. parahaemolyticus* that the consumption of raw oysters is known to cause illness in humans, almost all Koreans prefer raw oysters to already cooked oysters because of their fresh taste and high nutritional value. *V. parahaemolyticus* pandemic strains, such as O3:K6, are responsible for the current pandemics in many countries. Emergence of *Vibrio* species that are resistant to multiple antibiotics has been recognized as a serious global clinical problem. Recently isolated *V. parahaemolyticus* pandemic

strains have displayed multiple antibiotic resistance, increasing concerns about possible treatment failure. Alternatives to conventional antibiotics are needed, especially for the multiple-antibiotic-resistant *V. parahaemolyticus* pandemic strain. A bacteriophage, designated pVp-1, that was lytic for *V. parahaemolyticus* was isolated from the coast of the Yellow Sea in Korea. The phage showed effective infectivity for multiple-antibiotic-resistant *V. parahaemolyticus* and *V. vulnificus*, including *V. parahaemolyticus* pandemic strains. The therapeutic potential of the phage was studied in a mouse model of experimental infection using a multiple-antibiotic-resistant *V. parahaemolyticus* pandemic strain. Phage-treated mice displayed protection from a *V. parahaemolyticus* infection and survived lethal oral and intraperitoneal bacterial challenges. This is the first report, to the best of the knowledge, of phage therapy in a mouse model against a multiple-antibiotic-resistant *V. parahaemolyticus* pandemic strain infection.

The complete genome sequence of a novel marine siphovirus pVp-1, which was isolated from the coastal water of the Yellow sea in Korea and infects *V. parahaemolyticus*, was reported. The double-stranded DNA genome of pVp-1 is composed of 111 kb with a G + C content of 39.71%. The genome encodes a total of 157 ORFs. Genome sequence analysis of pVp-1 and comparative analysis with the homologous phage T5 revealed that there is a degree of similarity between pVp-1 and T5, thus indicating a close genetic relatedness between pVp-1 and T5. Genomic comparison of pVp-1 with the phage T5 revealed that these two phages are highly similar in gene inventory.

Based on these results, it can be concluded that *Aeromonas* phages that infect antibiotic-resistant *A. hydrophila* strains could be considered as alternative therapeutic or prophylactic candidtates against *Aeromonas* infections in aquaculture. In addition, phage treatment trials in the mouse model for *V. parahaemolyticus* CRS 09-17 demonstrated that

the application of pVp-1 can protect from a *V. parahaemolyticus* infection, and pVp-1 can be used as a therapeutic agent to reduce the impact of epidemics caused by multiple-antibiotic-resistant pandemic strains.

**Key words:** *Aeromonas hydrophila,* Bacteriophage (phage), *Vibrio parahaemolyticus*, Multiple-antibiotic-resistant pandemic strains, Therapeutic agent.

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# Abbreviations

| | |
|---|---|
| CFU | Colony Forming Unit |
| EOP | Efficiency Of Plating |
| FDA | Food and Drug Administration |
| IP | Intra Peritoneally |
| LC-MS/MS | Liquid Chromatography-tandem Mass Spectrometry |
| MOI | Multiplicity Of Infection |
| OD | Optical Density |
| ORF | Open Reading Frame |
| pAh | Phage against *Aeromonas Hydrophila* |
| PFU | Plaque Forming Unit |
| pVp | Phage against *Vibrio Parahaemolyticus* |
| SDS-PAGE | Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis |
| SPSS | Statistical Package for the Social Sciences |
| TEM | Transmission Electron Microscopy |
| TSA | Tryptic Soy Agar |
| TSB | Tryptic Soy Broth |

# General introduction

The genus *Aeromonas* is a member of the family *Aeromonadaceae* that are primarily aquatic organisms found in water. Some *Aeromonas* sp. are pathogenic for humans as well as fish (27). The organisms in this family produce a clear zone of β-hemolysis on blood agar (11). *A. hydrophila* is a member of motile aeromonads and it can cause disease in fish, resulting in high mortality (1, 2, 5, 6, 18). There has been an increasing incidence of antimicrobial resistance among *Aeromonas* sp. isolated from aquaculture environments (24, 25, 26). Five classes of genetically distinguishable tetracycline resistance determinants, designated A through E, have been described among aerobic enteric gram-negative bacteria (20). Several studies have shown *tet*E to be the predominant determinant among the different classes of tetracycline-resistant genes (4, 19, 25).

The loach (*Misgurnus* spp.) is a member of the Cobitidae family (Lacepede, 1803) and inhabits freshwater systems by nature (13). Two species of loaches (*Misgurnus* spp.), the mud loach (*M. mizolepis*) and the cyprinid loach (*M. anguillicaudatus*), are cultured mostly for food and sometimes for Buddhism ceremonies in Korea (12). The annual demand for loaches in Korea and Japan was over 100,000t in 2004 due to its high nutritional value and use in folk medicine (8). Aquaculture of loach in 2008 was over 432t in Korea (14). Jeollabuk-do province in Korea is famous for the aquaculture of loaches, with over 384t in 2008, which was 89% of the total loach aquaculture in the entire country (7).

There have been few reports about *A. hydrophila* in Korea since the previous publication about isolation of *A. hydrophila* from rainbow trouts in Korea (16). Although the majority of the loach population is cultured and *A. hydrophila* is one of the main causes of its mass mortality in Korea, there was only little knowledge of this bacterium from

cyprinid loach.

*Vibrio parahaemolyticus* is one of the most important causes of gastroenteritis (3). It is associated exclusively with the consumption of raw or improperly cooked contaminated seafood, especially oysters (3). *V. parahaemolyticus* is recognized as an important human pathogen globally (3, 9, 22). According to the official statistics issued by the Korea Food and Drug Administration (10), *V. parahaemolyticus* is one of the most common causes of food-borne disease in Korea, and caused 17 outbreaks with a total of 663 patients in 2005, 25 outbreaks (547 patients) in 2006, 33 outbreaks (634 patients) in 2007, 24 outbreaks (329 patients) in 2008, and 12 outbreaks (106 patients) in 2009.

East Asians, especially Koreans and Japanese consume a unique diet. Koreans enjoy a wide variety of both raw finfish and shellfish. Although raw oysters have such high densities of *V. parahaemolyticus* that the consumption of raw oysters is known to cause illness in humans (3), almost all Koreans prefer raw oysters to already cooked oysters because of their fresh taste and high nutritional value. Also, seafood cross-contaminated with raw oyster can cause high risk for *V. parahaemolyticus* infections in the United States (3). In Korea especially, all seafood in fishery markets, including oysters, is sold in the same seawater and tanks, causing cross-contamination.

Virulence of *V. parahaemolyticus* is commonly associated with the *tdh* and *trh* genes, encoding thermostable direct haemolysin (TDH) and tdh-related haemolysin (TRH), respectively (15). Open reading frame 8 (ORF8) is considered as a potential factor causing epidemics and a genetic marker for *V. parahaemolyticus* O3:K6 strains (23). *V. parahaemolyticus* pandemic strains such as O3:K6 strains exhibit a unique *toxRS* sequence responsible for the current pandemic in many countries (17).

Increasingly there have been more reports of antibiotic resistance in *Vibrio* species.

Emergence of microbial resistance to multiple drugs is a serious clinical problem in the treatment, increasing the fatality rate (21).

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# Literature Review

## A. *Aeromonas hydrophila*

### A.1. Perspective of *Aeromonadaceae*

The genus *Aeromonas* (Kingdom, *Bacteria*; Phylum, *Proteobacteria*; Class, *Gammaproteobacteria*; Order, *Aeromonadales*; Family, *Aeromonadaceae*) has undergone a number of taxonomic and nomenclature revisions in the past. Although originally placed in the family *Vibrionaceae* (104), which also included the genera *Vibrio*, *Photobacterium*, and *Plesiomonas*, subsequent phylogenetic investigations indicated that the genus *Aeromonas* is not closely related to vibrios but rather forms a monophyletic unit in the gamma-3 subgroup of the class *Proteobacteria* (63, 89). These conclusions necessitated the removal of *Aeromonas* from the family *Vibrionaceae* and transfer to a new family, the *Aeromonadaceae* (20). Similarly, only five species of Aeromonas were recognized 25 years ago (47), three of which (*A. hydrophila*, *A. sobria*, and *A. caviae*) existed as phenospecies, that is, a named species containing multiple DNA groups, the members of which could not be distinguished from one another by simple biochemical characteristics. Subsequent systematic investigations have resulted in the number of valid published genomospecies rising to 14 (50), and it is anticipated that additional species will be described because rare strains have been identified that do not reside in any established *Aeromonas* species. Except for one species, *A. salmonicida*, they are motile by means of a single polar flagellum. On the other hand, approximately 30 motile *Aeromonas* spp. (*A. allosaccharophila*, *A. aquariorum*, *A. bestiarum*, *A. bivalvium*, *A. cavernicola*, *A. caviae*, *A.*

*diversa*, *A. encheleia*, *A. enteropelogenes*, *A. eucrenophila*, *A. fluvialis*, *A. hyrophila*, *A. jandaei*, *A. media*, *A. molluscorum*, *A. piscicola*, *A. popoffii*, *A. rivuli*, *A. sanarellii*, *A. sharmana*, *A. schubertii*, *A. simiae*, *A. taiwanensis*, *A. tecta*, *A. trota*, *A. veronii* biovar *sobria*, and *A. veronii* biovar *veronii*) were identified and those species have been associated with various human infections such as gastro-enteritis and wound infections, causing primary and secondary septicemia in immunocompromised persons (31), and have also been implicated as the causative agents of various fish diseases (49).

**A.2. *Aeromonadaceae* species**

From the creation of the genus *Aeromonas* in 1943 through the mid-1970s, aeromonads could be broken down roughly into two major groupings, based upon growth characteristics and other biochemical features (47). The mesophilic group, typified by *A. hydrophila*, consisted of motile isolates that grew well at 35 to 37 °C and were associated with a variety of human infections. The second group, refered to as psychrophilic strains, caused diseases in fish, wers nonmotile, and had optimal growth temperatures of 22 to 25 °C. This group contained isolates that currently reside within the species *A. salmonicida*.

Beginning in the mid-1970s and continuing for almost 10 years thereafter, several groups, including the Institut Pasteur in Paris, the Centers for Disease Control and Prevention (CDC) in Atlanta, GA, and the Walter Reed Institute of Research in Washington, DC, spearheaded an effort to redefine the mesophilic group based upon DNA relatedness studies. Over that span of time, DNA hybridization investigations revealed that multiple hybridization groups (HGs) existed within each of the recognized mesophilic species (*A. hydrophila*, *A. sobria*, and *A. caviae*) (30, 84). These unnamed HGs were represented by reference strains, since in each case they could not be separated unambiguously from each

other by simple biochemical means.

### A.3. *Aeromonas* and ecosystems

Aeromonds are essentially ubiquitous in the microbial biosphere. They can be isolated from virtually every environmental niche where bacterial ecosystems exist. These include aquatic habitats, fish, foods, domesticated pets, invertebrate species, birds, ticks and insects, and natural soils, although extensive investigations on the latter subject are lacking. The vast panorama of environmental sources from which aeromonads can be encountered lends itself readily to constant exposure and interactions between the genus *Aeromonas* and humans.

The relative environmental distributions of *Aeromonas* species in selected settings, as currently known, are presented in previous publications. Earlier studies have indicated that three *Aeromonas* genomospecies (*A. hydrophila*, *A. caviae*, and *A. veronii* bv. *sobria*) are responsible for the vast majority ($\geq$ 85%) of human infections and clinical isolations attributed to this genus (46). The same pattern observed clinically appears to repeat itself in most environmental samples, with *A. salmonicida* included as a predominant species in fish and water samples. In some studies, less frequently encountered species have been found to predominate in environmental samples, such as *A. schubertii* in organic vegetables (67). For newly described species such as *A. aquariorum* and *A. tecta*, no data exist on their relative distributions in the environment outside their initial taxonomic description, and extremely limited data are available on many other taxa described since 2004. Finally, the techniques and methods used to identify *Aeromonas* isolates to the species level vary considerably from one study to the next.

### A.4. Virulence factors of *Aeromonas*

Virulence of *Aeromonas* sp. is multifactorial and not completely understood (29). *Aeromonas* sp. have been reported to elaborate exotoxins (hemolysins, cytotoxins, and enterotoxins), hemagglutinins, adhesins, several hydrolytic enzymes, and invade tissue in culture (10, 36, 45, 57). The hemolysin produced by some *Aeromonas* sp. (also known as aerolysin) has been shown to have both hemolytic and enterotoxic activity (5, 16). Burke et al. (9) found that 97% of the hemolysin-producing strains were able to secrete enterotoxins. Other investigators also reported a correlation between hemolysin and cytotoxin production (82). The hemolytic enterotoxin shares significant homology with the cytotoxic enterotoxin (Act), and two cytotonic toxins (Alt and Ast) (19). Rahim et al., (85) tested 32 act gene probe-positive and 31 randomly selected act gene probe-negative *Aeromonas* isolates for enterotoxicity in a suckling mice assay (SMA), for haemolytic activity on sheep blood agar plates, for the presence of CAMP-like factors, and for cytotoxicity in a Vero cell line. This study indicated the role of Act in the pathogenesis of *Aeromonas* infections and that the enterotoxic potential of *Aeromonas* sp. could be assessed by simply performing a CAMP-haemolysin assay.

Enterotoxigenic isolates of *A. hydrophila* showed hemagglutination (HA) which was not sensitive to mannose (i.e. mannose-resistant [MRHA]) and fucose, but *Aeromonas* strains that were HA-sensitive to mannose or showed no hemagglutination (NHA) were non-toxic strains of *A. caviae* commonly isolated from nondiarrhoeal infection ot the environment (10). In enteric bacteria, hemagglutination of erythrocytes is associated with the ability to adhere to human epitherial cells.

### A.5. *Aeromonas* and aquatic environments

Groundbreaking studies conducted over 30 years ago by Terry Hazen and associates identified viable *Aeromonas* in 135 of 147 (91.8%) natural aquatic habitats sampled in the United States and Puerto Rico (38). *Aeromonas* numbers were higher in lotic than in lentic systems and were higher in thermal gradients ranging from 25 to 35 °C (38, 39). *A. hydrophila* grew over a wide range of temperatures, conductivities, pHs, and turbidities, with only those habitats with extreme ranges of these parameters (extremely saline environments, thermal springs, and highly polluted waters) failing to yield aeromonads.

Today, the genus *Aeromonas* is considered to be almost synonymous with water and aquatic environments, being isolated from rivers, lakes, ponds, seawaters (estuaries), drinking water, groundwater, wastewater, and sewage in various stages of treatment. Concentrations of aeromonads in these sites have been reported to vary from lows of <1 CFU/ml (groundwater, drinking water, and seawater) to highs of $10^8$ CFU/ml or more, in crude sewage or domestic sewage sludge (41). Although primarily a freshwater resident, *Aeromonas* species can be recovered from the epipelagic layer (<200 m) of the ocean (as opposed to benthic regions), most often in estuaries, existing as free-living bacteria or in association with crustaceans. Estuaries are ideally suited for aeromonads, since salinity concentrations are substantially lower there than in the deeper (benthic) regions of the ocean. One study from the Italian coast found aeromonad numbers varying from $10^2$ to $10^6$ CFU per 100 ml throughout the year (32).

### A.6. *Aeromonas* and fish diseases

The role of aeromonads as a causative agent of fish diseases has been known for decades, longer than their comparable role in causing systemic illnesses in humans. Two major groups of fish diseases are recognized. *A. salmonicida* causes fish furunculosis, particularly in salmonids. The disease has several presentations, ranging from an acute form characterized by septicemia with accompanying hemorrhages at the bases of fins, inappetence, and melanosis to a subacute to chronic variety in order fish, consisting of lethargy, slight exophthalmia, and hemorrhaging in muscle and internal organ (6). Mesophilic species (*A. hydrophila* and *A. veronii*) cause a similar assortment of diseases in fish, including motile *Aeromonas* septicemia (hemorrhagic septicemia) in carp, tilapia, perch, catfish, and salmon, red sore disease in bass and carp, and ulcerative infections in catfish, cod, carp, and goby (49). Mesophilic *Aeromonas* species, most notably *A. hydrophila*, have been linked to major die-offs and fish kills around the globe over the past decade, resulting in enormous economic losses. These die-offs included over 25,000 common carp in the St. Lawrence River in 2001 (71), 820 tons of goldfish in Indonesia in 2002, resulting in a 37.5 million dollars loss, and a catfish die-off in Minnesota and North Dakota in 2007. In many of these instances, *Aeromonas* species were sole or copathogens causing invasive secondary infections in immunosuppressed fish due to spawning or environmental triggers, such as high temperatures or low water levels.

### A.7. Antimicrobial resistance

In the last two decade, high rates of resistance to commonly used, cheap oral antibiotics among enteric pathogens has been reported from several developing countries (86, 91, 103). The same story can be said for *Aeromonas* sp., particularly those isolated from

clinical sources and to a lesser extent from foods and water. The ease of which antimicrobial agents can be obtained in these countries has been blamed for this problem (2, 90). High resistance rates to antimicrobial agents appear to be common among aeromonads isolated from fish in developing countries. Antimicrobial agents are used extensively in fish farms to treat and prevent fish diseases and also as feed additives. Such practice has been shown to increase drug resistant bacteria as well as R plasmids (37, 108). However, variation in the resistance rates of aeromonads to different antimicrobial agents in different developing countries can be observed. Such differences in the frequency of resistance may well be related to the source of the *Aeromonas* isolates and the frequency and type of antimicrobial agents prescribed for treating *Aeromonas* infections in different geographical areas (97).

Resistance of most aeromonads to ampicillin is generally considered to be intrinsic or chromosomal mediated (4). Several studies have shown that patients taking ampicillin for reasons other than diarrhoea may predispose them to infection with *Aeromonas* (33, 72). Moyer (72) reported that for the susceptible host, antibiotic therapy, and drinking of untreated water are two significant risk factors for infection with *Aeromonas*. However, gastrointestinal infections with *Aeromonas* are generally self-limiting. Although treatment of patients with symptoms of infectious diarrhea with antibiotics remains controversial, antimicrobial therapy should be initiated for those who are severely ill and for patients with risk factors for extraintestinal spread of infection after obtaining appropriate blood and fecal cultures (40). The current accepted treatment of all acute infectious diarrhoeal diseases is rehydroation, antibiotic treatment, and nutritional therapy (90).

## B. *Vibrio parahaemolyticus*

### B.1. Perspective of *Vibrio*

The genus *Vibrio* (Kingdom, *Bacteria*; Phylum, *Proteobacteria*; Class, *Gammaproteobacteria*; Order, *Vibrionales*; Family, *Vibrionaceae*) has played a significant role in human history. Outbreaks of cholera, caused by *Vibrio cholerae*, can be traced back in time to early recorded descriptions of enteric infections. Indeed, the path of human history has been influenced significantly by this organism (83, 107, 109). First described by Pacini (80) while he was a medical student in Italy and at a time when the germ theory of disease was in dispute, *V. cholerae* was subsequently identified and described in greater detail by Robert Koch (54, 55), to whom credit for the discovery of the causative agent of cholera traditionally has been given.

The germ theory of disease was developed in the 19th century, based on the British queen's physician John Snow's tracing an 1849 cholera outbreak to a single contaminated well in the Broad Street area of central London; it remains a canonical example of epidemiology. Snow's demonstration was an important mile-stone in public health, correctly identifying the fecal-oral route to human infection and offering powerful arguments for the germ theory (96). Many advances in the prevention and treatment of infectious diseases during the latter half of the 19th century and the first half of the 20th century follow directly from the acceptance of Snow's point of view.

The vibrios have also received the attention of marine microbiologists who observed that the readily cultured bacterial populations in near-shore waters and those associated with fish and shellfish were predominantly *Vibrio* spp. For example, the "gut group" vibrios were described by Liston (58, 59), working at the Marine Laboratory in Aberdeen,

Scotland. Fish diseases caused by vibrios have been reviewed extensively by marine investigators and, among the many fish pathogens, *Vibrio anguillarum* has been recognized historically as a major pathogen of marine animals.

**B.2. Viewpoint of *Vibrio parahaemolyticus***

*Vibrio parahaemolyticus* is a Gram-negative, halophilic asporogenous rod that is straight or has a sinple, rigid curve. It has a single polar flagellum and is motile when grown in liquid medium (7). This bacterium is a human pathogen that occurs naturally in the marine environments and frequently isolated from a variety of seafoods including codfish, sardine, mackerel, flounder, clam, octopus, shrimp, crab, lobster, crawfish, scallop, and oyster (60). Consumption of raw or undercooked seafood, particularly shellfish, contaminated with *V. parahaemolyticus* may lead to development of acute gastroenteritis characterized by diarrhea, headache, vomiting, nausea, abdominal cramps, and low fever. This bacterium is recognized as the leading cause of human gastroenteritis associated with seafood consumption in the United States and an important seafood-borne pathogen throughout the world (53).

Although the gastroenteritis caused by *V. parahaemolyticus* infection is often self-limited, the infection may cause septicemia that is life-threatening to people having underlying medical conditions such as liver disease or immune disorders. Two deaths were among three cases of wound infections caused by *V. parahaemolyticus* in Louisiana and Mississippi after Hurricane Katrina in 2005 (14).

**B.3. Occurrence of *Vibrio parahaemolyticus***

The distribution of *V. parahaemolyticus* in the marine environments is known to relate to the water temperatures. Studies have shown that the organism was rarely detected in seawater until water temperatures rose to 15 °C or higher. Ecological study of *V. parahaemolyticus* in the Chesapeake Bay of Maryland found that *V. parahaemolyticus* survived in sediment during the winter and was released from sediment into water column when water temperatures rose to 14 °C in late spring or early summer (51). Another survey of nine U.S. coastal states conducted between 1984 and 1985 reported an average low density of *V. parahaemolyticus* in seawater when water temperatures dropped below 16 °C (26). However, the densities of *V. parahaemolyticus* in seawater could increase when water temperatures increased to around 25 °C (26, 51). A recent study of occurrence of *V. parahaemolyticus* in Oregon oyster-growing environments between November 2002 and October 2003 also found a positive correlation between *V. parahaemolyticus* in seawater and water temperatures with the highest populations of *V. parahaemolyticus* in water being detected in the summer months (24).

The degree of *V. parahaemolyticus* contamination in raw shellfish is also known to relate to the water temperatures. Therefore, it is more likely to detect *V. parahaemolyticus* in oysters harvested in the spring and the summer than in the winter. *V. parahaemolyticus* can multiply rapidly in oysters upon exposure of elevated temperatures. Studies have shown that populations of *V. parahaemolyticus* in unrefrigerated oysters could increase rapidly to 50-790 folds of its original level within 24 h of harvest if oysters were exposed to 26 °C (35). A survey of 370 lots of oysters sampled from restaurants, oyster bars, retail and wholesale seafood markets throughout the US between June 1998 and July 1999 found a seasonal distribution of *V. parahaemolyticus* in market oysters with high densities being

detected in the summer months (21).

### B.4. Incidence of *V. parahaemolyticus* food poisoning

*V. parahaemolyticus* was first recognized as a cause of food-borne illness on Osaka, Japan in 1951 (23). It caused a major outbreak of 272 illness and 20 deaths associated with consumption of sardines. Since then, *V. parahaemolyticus* has been reported to account for 20-30% of food poisoning cases in Japan (1) and identified as a common cause of seafood-borne illness in many Asian countries (17, 25, 110). *V. parahaemolyticus* was the leading cause of food poisoning (1710 incidents, 24,373 cases) in Japan between 1996 and 1998 (44) and accounted for 69% (1028 cases) of total bacterial foodborne outbreaks (1495 cases) reported in Taiwan between 1981 and 2003 (3) and 31.1% of 5770 foodborne outbreaks occurred in China between 1991 and 2001 (61).

In contrast to Asian countries, *V. parahaemolyticus* infections are rarely reported in European countries. However, sporadic outbreaks have been reported in countries such as Spain and France. Eight cases of *V. parahaemolyticus* gastroenteritis related to fish and shellfish consumption were reported in Spain in 1989 (70). An outbreak of 64 illnesses associated with raw oysters consumption occurred in 1999 in Galicia, Spain (62). A serious outbreak affecting 44 patients associated with consumption of shrimps imported from Asia accurred in France in 1997 (88). A more recent outbreak involving 80 illnesses of *V. parahaemolyticus* infection among guests attending weddings in one restaurant was reported in Spain in July 2004 (64). Epidemiological investigation associated the outbreak with consumption of boiled crab that had been processed under unhealthy conditions.

*V. parahaemolyticus* was first identified as an etiological agent in the US in 1971 after three outbreaks of 425 cases of gastroenteritis associated with consumption of improperly

cooked crabs occurred in Maryland (69). Since then, sporadic outbreaks of *V. parahaemolyticus* infections related to consumption of raw shellfish or cooked seafood were reported throughout the US coastal regions. Between 1973 and 1998, approximately 40 outbreaks of *V. parahaemolyticus* infections were reported to the Centers for Disease Control and Prevention (CDC) (22). Among them, four major outbreaks involving more than 700 cases of illness associated with raw oyster consumption occurred in the Gulf Coast, Pacific Northwest, and Atlantic Northeast regions between 1997 and 1998. In the summer of 1997, 209 cases (including one death) of *V. parahaemolyticus* infections associated with raw oyster consumption occurred in the Pacific Northwest (Oregon, Washington, California and British Columbia of Canada) (12). In 1998, two outbreaks occurred in Washington (43 cases) and Texas (416 cases) were associated with consumption of raw oyster (27). In addition, a small outbreak of eight cases of *V. parahaemolyticus* infections was reported in Connecticut, New Jersey, and New York between July and September in 1998 as a result of eating oysters and clams harvested at Long Island Sound of New York (13). Recently, 14 passengers on board a cruise ship in Alaska developed gastroenteritis after eating raw oysters produced in Alaska in the summer of 2004 (66). More recently, an outbreak of *V. parahaemolyticus* involving 177 cases occurred in the summer of 2006 was linked to contaminated oysters harvested in Washington and British Columbia (15). The occurrence of these outbreaks indicates that contamination of *V. parahaemolyticus* in oysters is a safety concern in the US.

**B.5. Virulence factors**

It is known that most strains of *V. parahaemolyticus* isolated from the environment or seafood are not pathogenic (77). Clinical strains of *V. parahaemolyticus* are differentiated from environmental strains by their ability to produce a thermostable direct hemolysin (TDH), an enzyme that can lyse red blood cells on Wagatsuma blood agar plates. The hemolytic activity of TDH, named the Kanagawa phenomenon, has been reported to be commonly associated with strains isolated from humans with gastroenteritis but were rarely observed in environmental isolates (48). Therefore, the TDH has been recognized the major virulence factor of *V. parahaemolyticus* (68, 100).

Despite epidemiological investigations revealed a strong tie between the Kanagawa phenomenon (KP) and the pathogenicity of *V. parahaemolyticus*, KP-negative strains that did not produce TDH but a TDH-related hemolysin (TRH) had been isolated from outbreak patients (42, 43). Shirai et al. (92) examined 215 clinical strains of *V. parahaemolyticus* isolated from patients with diarrhea for presence of genes encoding TDH (*tdh*) and TRH (*trh*) and found that 52 strains (24.3%) carried only the *trh* gene. These results indicate that TRH is also a virulence factor of *V. parahaemolyticus*. The genes encoding TDH (*tdh*) and TRH (*trh*) have been cloned and sequenced (52, 75, 101). Oligonucleotide probes for both *tdh* and *trh* genes have been developed for detection of virulent strains of *V. parahaemolyticus* (52, 76).

**B.6. Pandemic strains of *V. parahaemolyticus***

Most outbreaks of *V. parahaemolyticus* infections were caused by *V. parahaemolyticus* of diverse serotypes. However, increased incidences of gastroenteritis caused by *V. parahaemolyticus* serotype O3:K6 have been reported in many countries since 1996 (18,

34, 64, 106). This serovar was first identified during a hospital-based active surveillance study of *V. parahaemolyticus* infections in Calcutta, India between 1994 and 1996 (79). The survet identified a sudden increase in incidences associated with this serovar, which accountered for 63% of total *V. parahaemolyticus* strains isolated from patients in Calcutta between September 1996 and April 1997. This highly virulence strain was subsequently recovered at a high rate in other Southeast Asian countries and was isolated from travelers arriving in Japan from various countries in the Southeast Asia (18, 79 106).

*V. parahaemolyticus* O3:K6 was first identified in the US in 1998 and caused the largest outbreak (416 person) associated with oyster consumption in the US history (23). The same serovar wa later involved in another outbreak related to shellfish consumption in Connecticut, New Jersey, and New York (13). Since then, a pandemic spread of this clone to other continents has been reported. In 2004, *V. parahaemolyticus* O3:K6 was isolated from victims of outbreaks occurred in Chile (34) and Spain (64). The isolation of the O3:K6 strain from US outbreaks raised concern about increased risks of *V. parahaemolyticus* infections from US consumption. However, this serovar has not been linked to illness resulted from consuming raw oysters in the US since 1999.

## C. Bacteriophage (phage)

### C.1. General description

Phages are viruses that invade bacterial cells and, in the case of lytic phages, disrupt bacterial metabolism and cause the bacterium to lyse. They were discovered by Ernest Hankin (1896) and Frederick Twort (1915) who described their antibacterial activity. However, Felix d'Herelle (1919) was probably the first scientist who used phages as a therapy to treat severe dysentery. At that time, several companies then actively started up the commercial production of phages against various bacterial pathogens for human use. However, phage production was quickly displaced by the discovery of antibiotics in most of the Western world. Nevertheless, phage therapy is still an on-going practice in Eastern Europe and countries from the former Soviet Union. Several institutions in these countries have been involved in phage therapy research and production, with activities centralized at the Eliava Institute of Bacteriophage, Microbiology and Virology (Tbilisi, Georgia) and the Hirszfeld Institute of Immunology and Experimental Therapy (Wroclaw, Poland). Their work in this field has recently been extensively reviewed (56).

The current threat of antibiotic-resistant bacteria has renewed the interest in exploring phages as biocontrol agents in Western countries (65, 98). In fact, some products based on phages are already commercially available ('PhageBioderm', 'Bacteriophagum Intestinalis Liquidum', 'Pyobacteriophagum Liquidum'). Additionally, some care centers are particularly specialized in phage therapy (for example, Southwest Regional Wound Care Center, Texas).

Besides phage therapy, the use of phages as antimicrobial agents and tools for detecting pathogens in feed and foodstuffs is also expanding with several campanies having been

created recently (**Table I**).

**Table I.** Companies involved in phage application.

| Company | Country | Website |
|---|---|---|
| BIO*phage* PHARMA inc. | Canada | http://www.biophagepharma.net |
| BIOPHARMA LIMITED | Georgia | http://www.biopharmservices.com |
| CJ CheilJedang | Korea | http://www.cj.co.kr |
| CTCBIO INC. | Korea | http://www.ctcbio.com |
| MICREOS Food Safety | Netherlands | http://www.ebifoodsafety.com |
| Exponential Biotherapies Inc. | European Union | http://www.expobio.com |
| GangaGen, Inc. | European Union | http://www.gangagen.com |
| Hexal-gentech | Germany | http://www.hexal-gentech.com |
| Intralytix, Inc. | European Union | http://www.intralytix.com |
| Komipharm International Co. | Korea | http://komilab.com |
| Novolytics | United Kingdom | http://www.novolytics.co.uk |
| Omnilytics, Inc. | European Union | http://www.phage.com |
| Phage Biotech Ltd. | Israel | http://www.phage-biotech.com |

Fields of application comprise of water and food safety, agriculture and animal health. An example is OmniLytics, Inc. that gained US Environmental Protection Agency approval for the use of its product AgriPhage against plant pathogenic bacteria. In food manufacturing industry, EBI Food Safety recently marketed Listex$^{TM}$ P100 for controlling *Listeria* in meat and cheese products (11). In August 2006, the US Food and Drug Administration (FDA) approved the use of a phage preparation targeting *Listeria*, LMP 102 (Intralytix, Inc.), in ready-to-eat meat and poulty products.

**C.2. Phage application in aquaculture**

Cultured fish and shellfish, like other animals and humans, are constantly threatened by microbial attacks. Although chemotherapy is a rapid and effective method to treat or prevent bacterial infections, frequent use of chemotherapeutic agents has allowed drug-resistant strains of bacteria to develop. In particular, this problem in chemotherapy may be serious in fishery industries (74). Needless to say, vaccination is an ideal method for preventing infectious diseases, but commercially available vaccines are still very limited in the aquaculture field. This is partly due to the fact that many different kinds of infectious diseases occur locally in a variety of fish and shellfish species. Studies on biological control such as probiotics have been sporadically reported in the field of fish pathology (28, 78, 105); however, they involve substantial difficulties in scientific demonstration of the causal sequence, as mentioned in human use of probiotics (102). In view of a scientific demonstration of phage treatment, the causal effect of phages in successful phage therapy can be definitively proven by confirming an increase in phage particles in the number or the presence of phages in the survivors, which is the result of the death of host bacterial cells. The feasibility of this demonstration distinguishes phage treatment from other biological controls, which fail to utilize scientific methodology in demonstrating causal relationships. Under these circumstances, phages, as specific pathogen killers, could be attractive agents for controlling fish bacterial infections. Phages of some fish pathogenic bacteria, such as *Aeromonas salmonicida*, *A. hydrophila*, *Edwardsiella tarda*, *Lactococcus garvieae*, *Pseudomonas plecoglossicida*, and *Yersinia ruckeri*, have been reported (73, 74, 81).

**C.3. Phage therapy**

From the continued observations and experiences treating patients, d'Herelle quickly realized that not all phage preparations were effective and that care had to be taken both when preparing and when applying phages. At the same time, the advent of effective chemical antibiotics in the 1930s and 1940s led to the therapeutic use of phages in the West being curtailed. However, clinical use continued in the countries of the former Soviet bloc.

Interest in phage therapy was reignited by increasing concern over antibiotic resistance and also by publication of successful results achieved by of Smith et al. (93, 94, 95), even demonstrating the apparently superior efficacy of phage therapy compared to antibiotics in a mouse model of *E. coli* infection (93). The advent of multi-drug resistant pathogens has forced the re-examination of phage therapy, with work being carried out to modern regulatory standards.

A large amount of indicative data supporting the effectiveness of phage therapy is available from studies involving human patients in Eastern Europe, with few reported adverse events. This evidence for safety, while not up to current regulatory standards, is further supported by the exposure of humans to high levels of phages via everyday activities because of the ubiquitous nature of phages in the environment.

Human safety trials have also been performed with increasing frequency including extensive safety trials undertaken on Staphage Lysate by Delmont Laboratories (USA) (98). This product, which contains high concentrations of antistaphylococcal phages, was administered to humans intranasally, topically, orally, subcutaneously, and intravenously. In over 12 years of use in humans, only minor side effects were observed (98, 99). In a formal safety study, Harold Brussow based at the Nestle Research Center, Switzerland, demonstrated no safety concerns when phages targeting *E. coli* were administered to

human volunteers (8).

An FDA-approved phase I physician-led trial has been completed at a wound care center in Lubbock, Texas (87). Jusing a mixture of phages targeting *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *E. coli* and also showed no safety concerns.

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# Chapter I

## Protective effects of the *Aeromonas* phages pAh1-C and pAh6-C against mass mortality of the cyprinid loach (*Misgurnus anguillicaudatus*) caused by *Aeromonas hydrophila*


### Abstract

To investigate methods to control the mass mortality of cyprinid loaches (*Misgurnus anguillicaudatus*) caused by multiple-antibiotic-resistant *Aeromonas hydrophila* on a private fish farm in Korea, bacteriophages (phages), designated pAh1-C and pAh6-C, were isolated from the Han River in Seoul. The two isolated phages were morphologically classified as *Myoviridae* and showed similar infection patterns for *A. hydrophila* isolates. The two phages possessed approximately 55 kb (pAh1-C) and 58 kb (pAh6-C) of double-stranded genomic DNA, and their gDNAs showed different restriction endonuclease digestion patterns. Both phages showed efficient bacteriolytic activity against fish-pathogenic *A. hydrophila* from loaches. The latent periods of the phages were estimated to be approximately 30 min (pAh1-C) and 20 min (pAh6-C), while the burst sizes were 60 PFU/cell (pAh1-C) and 10 PFU/cell (pAh6-C). The phages proved to be efficient in the inhibition of bacterial growth, as demonstrated by their *in vitro* bactericidal effects. Additionally, a single administration of either phage to cyprinid loaches resulted in noticeable protective effects, with increased survival rates against *A. hydrophila* infection. These results suggest that the phages pAh1-C and pAh6-C constitute potential therapeutic

agents for the treatment of *A. hydrophila* infection in fish.

**Keywords:** Cyprinid loach, *Misgurnus anguillicaudatus*; Multiple-antibiotic-resistant; *Aeromonas hydrophila*; Bacteriophage; Therapeutic agent.

## 1.1. Introduction

The loach (*Misgurnus* spp.) is a member of the Cobitidae family (Lacepede, 1803) and inhabits freshwater systems throughout the world (17). Two species of loaches, the mud loach (*M. mizolepis*) and the cyprinid loach (*Misgurnus anguillicaudatus*), are cultured for a variety of uses, primarily for food, in Korea (16). The annual demand for loaches in Korea is one of the highest for fresh-water fish, due to their high nutritional value and use in both folk medicine and Buddhist ceremonies.

*Aeromonas* spp. are primary organisms of normal aquatic microflora, and recently, there has been an increasing appreciation of their role as waterborne pathogens of fish and humans (31). *Aeromonas hydrophila* is a motile aeromonad that can cause disease in fish, resulting in high rates of mortality (2, 3, 8, 12, 15, 22). There has been an increasing incidence of antimicrobial resistance among *Aeromonas* sp. isolated from aquaculture environments (15, 33, 36, 37). Multiple-antibiotic-resistant *A. hydrophila* exists in aquaculture systems and contributes to the high rate of mortality within the fish industry in Korea (15). Although the majority of the loach population in Korea is cultured and *A. hydrophila* is one of the main causes of mass mortality in these fish, no effective method has been proposed for the control of *A. hydrophila* infection in aquaculture, except for the application of additional antibiotics.

The use of phages has been proposed for the treatment of infectious disease, and several studies have shown that phages can be used successfully for the treatment of bacterial infections in both humans and animals (4, 6, 20, 21, 40). Phage therapy is advantageous in that it is natural and relatively inexpensive, and so far, no serious or irreversible side effects have been described (10, 38). Although phage studies of some fish-pathogenic

bacteria have been conducted (23, 24, 25, 26, 30, 34, 41), there have been few attempts to use phages to control bacterial infections in fish (29). This study aimed to isolate and characterize *A. hydrophila*-specific phages and to evaluate their therapeutic potential during experimental infections of loaches with *A. hydrophila*.

## 1.2. Materials and methods

### *1.2.1. Bacterial strains and culture media*

The bacterial strains ($n$ = 17) used in this study included 5 *A. hydrophila* strains (3 fish-pathogenic clinical isolates and 2 environmental isolates), 4 strains of other *Aeromonas* spp. (3 fish-pathogenic clinical isolates and 1 environmental isolate), and 8 additional strains of different genera; these strains are listed in **Table 1.1**. Laboratory stock strains (**Table 1.1**) were used to determine the host range of the isolated phages. Tryptic soy broth (TSB) and tryptic soy agar (TSA) were used for bacterial culture and phage PFU assays.

### *1.2.2. Phage isolation and host range*

Phages were isolated from natural water of the Han River in May 2010 by the enrichment technique. A water sample (100 ml) was filtered through a 0.45 μm pore-size membrane filter, and the filtrate was mixed with 100 ml of TSB containing fish-pathogenic *A. hydrophila* (JUNAH) as the indicator organism. After 24 h of growth at 25 ℃ with gentle agitation, the culture was centrifuged (13,000 rpm/10 min) and filtered again. The phage activity in the supernatant was then detected with a spot assay (5). The phages were further purified by a CsCl continuous density gradient (35).

The plaques were classified into the following three categories according to the degree

of clarity: clear, turbid, and no reaction. A plaque-forming unit (PFU) assay was performed using the double-agar-layer method (30). The number of PFU was determined after 24 h of incubation at 25 °C, and the efficiency of plating (EOP) was also calculated. The host ranges of the two phages were determined by the double-agar-layer method.

### *1.2.3. Electron microscopy*

Phage particles were negatively stained with 2% uranyl acetate, and electron micrographs were captured with a JEM 1010 transmission electron microscope (JEOL, Akishima, Japan) and a LIBRA 120 energy-filtering transmission electron microscope (Carl Zeiss, Germany). The phage size was considered to be the average of 5-7 independent measurements.

### *1.2.4. One-step growth*

One-step growth curves were obtained for pAh1-C and pAh6-C according to the method of Verma et al. (39). The optical density (OD) of mid-exponential host bacterial cultures (*A. hydrophila* JUNAH) at 600 nm was adjusted to a corresponding cell density of $8.0\times10^6$ CFU/ml. The phage suspension (10 μl) was added to 10 ml of the bacterial culture to obtain a multiplicity of infection (MOI) of 0.001. The phages were allowed to adsorb for 5 min at room temperature. The mixture was then centrifuged (13,000 rpm/3min), and the resulting pellet was resuspended in 20 ml of TSB. Samples (100 μl each) were collected at 5 min intervals and subjected to phage titration.

### *1.2.5. Phage DNA isolation and restriction endonuclease analysis*

Phage DNA was extracted from pure stocks as previously described (35) and subjected to nuclease treatment using DNase I, RNase A, and Mung bean nuclease according to the supplier's instructions (Takara Bio Inc., Japan). In addition, the size estimation and restriction analysis of phage DNA were performed by pulsed-field gel electrophoresis, as described previously by Kim et al. (19).

### *1.2.6. Phage structural protein analysis*

Phage structural proteins were analyzed by SDS-PAGE as previously described (19). After electrophoresis, the protein bands were visualized by staining the gel with Coomassie blue R-250 and identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) at the National Instrumentation Center for Environmental Management (NICEM) at Seoul National University.

### *1.2.7. Phage stability test*

Phage stability tests were conducted as described elsewhere (39), with some modifications. Briefly, to evaluate the stability of the phages in organic solvents, equal volumes of phage solution ($10^5$ PFU/ml) and appropriate organic solvents, such as chloroform, ethanol, and ether, were mixed and incubated at 25 °C for 1 h. To evaluate the stability of the phages at different pH values, the pH of the TSB was adjusted with either 1 M HCl or 1 M NaOH to obtain solutions with pH values of 3, 5, 7, 9, and 11. The pAh1-C and pAh6-C phage suspensions were adjusted to obtain a final concentration of $10^7$ PFU/ml and were subsequently incubated at 25 °C for 1 h. Phage suspensions maintained at pH 7 were used as controls. The stability of the phages at various temperatures (20, 25,

30, 37, 50, and 65 °C) was determined by incubating the phages ($10^5$ PFU/ml) at the respective temperatures for 1 h. After incubation, the phage titer was estimated by the double-agar-layer method.

***1.2.8.*** *Host cell lysis test*

The bactericidal effect of the phages on *A. hydrophila* (JUNAH) was determined by measuring the viable bacterial counts throughout the incubation period. TSB (20 ml) was inoculated with 0.1 ml of an overnight *A. hydrophila* (JUNAH) culture and incubated at 25 °C with shaking until the early exponential phase was reached ($OD_{600\ nm}$ = 0.1) ($8.0\times10^6$ CFU/ml). The phages were added at the indicated MOIs (multiplicity of infection), and the changes in OD were monitored for 24 h.

***1.2.9.*** *Preparation of phage-resistant A. hydrophila variants*

An *A. hydrophila* (JUNAH) culture was treated with an undiluted suspension ($10^{10}$ PFU/ml) of the pAh1-C and pAh6-C phages. Colonies appearing on the plate after 2 days of incubation at 25 °C were purified on TSA, and the selection for phage-resistant variants was repeated. Finally, the cultures that produced no PFU after the addition of $10^{10}$ PFU/ml of each phage were used as phage-resistant variants.

***1.2.10.*** *Phage treatment of infected fish*

A total of 2,400 healthy cyprinid loaches (*Misgurnus anguillicaudatus*) (average weight: 14.98 g) were divided into 60 groups in 7 L fiber plastic tanks at 25 ± 1 °C. In the 1st experiment, all fish were infected intraperitoneally (IP) with *A. hydrophila* (JUNAH), and the treatment groups were immediately injected with pAh1-C and pAh6-C. The

concentrations of bacteria injected were $2.6\times10^6$ CFU/fish for the 1st trial and $2.6\times10^7$ CFU/fish for the 2nd trial. The concentrations of phages used were $3.0\times10^7$ PFU/fish for pAh1-C and $1.7\times10^7$ PFU/fish for pAh6-C. In the 2nd experiment, all conditions were the same as in the 1st experiment, but the treatment groups were fed pellets (0.5% based on body weight) that had been impregnated with the phage suspensions containing pAh1-C and pAh6-C.

Six groups of 40 loaches were used to study the virulence of the phage-resistant *A. hydrophila* (JUNAH) variants. The mortality rates of the fish were recorded daily for 7 days, and the kidneys of both the dead and surviving fish were subjected to a bacterial isolation study, as previously described (15). Every experiment was performed on three separate occasions, and the results shown represent the mean of these three observations ± the standard deviation (SD). All animal experiments were performed according to the guidelines of the Animal Ethical Committee of Seoul National University, Seoul, Republic of Korea.

### ***1.2.11.*** *Statistical analysis*

The experimental results were analyzed for statistically significant differences using Student's *t*-test. A *P* value of less than 0.05 was accepted as statistically significant. The SPSS statistical software package version 13.0 (SPSS, Inc., Chicago, IL) was used for all statistical analyses.

## 1.3. Results

***1.3.1.*** *Morphology, host range, and one-step growth patterns of the Aeromonas phages pAh1-C and pAh6-C*

From May 2009 to December 2010, 22 phages were isolated from water samples from the Han River in Seoul. From the isolated phages, two *Aeromonas* phages, designated pAh1-C and pAh6-C, produced clear plaques and were selected for further studies (**Figure 1.1**). pAh1-C formed small plaques (average diameter: 1.6 mm) in *A. hydrophila* JUNAH, while pAh6-C formed large plaques (average diameter: 3.8 mm). Both phages had isometric heads, necks, and contractile tails, with tail fibers. Based on their morphologies and according to the classification system of Ackermann (1), both phages belong to the family *Myoviridae* (**Figure 1.1**). The tail lengths were 113 ± 6 nm for pAh1-C and 124 ± 6 nm for pAh6-C (mean ± SD) ($n$ = 10), while the widths were 14 ± 3 nm for pAh1-C and 18 ± 1 nm for pAh6-C ($n$ = 10). The head diameters were 41 ± 3 nm for pAh1-C and 63 ± 3 nm for pAh6-C ($n$ = 10).

To evaluate the host ranges of pAh1-C and pAh6-C, they were tested on various *Aeromonas* spp. and *A. hydrophila* strains. Among the 9 *Aeromonas* sp. tested, the two phages inhibited the growth of all *A. hydrophila* strains ($n$ = 5), with pAh1-C producing clear plaques on two strains and pAh6-C producing clear plaques on three strains. The EOP values varied among the *Aeromonas* spp., and no strain showed a value higher than that of the indicator host strain JUNAH (**Table 1.1**). pAh1-C and pAh6-C failed to lyse any of the other 8 bacterial strains used in this study. Additionally, a one-step growth test showed that the latent periods were approximately 30 min (pAh1-C) and 20 min (pAh6-C) and that the burst sizes were 60 PFU/cell (pAh1-C) and 10 PFU/cell (pAh6-C) (**Figure 1.2**).

### *1.3.2. Genomic and proteomic characteristics of the Aeromonas phages pAh1-C and pAh6-C*

The DNA of both phages was completely digested by DNase I but not by RNase A or Mung bean nuclease; thus, both phages were presumed to be double-stranded DNA phages (**data not shown**). In addition, the DNA of pAh1-C was digested by *Msp*I, *Nco*I, and *Hpa*II, and its size was estimated to be approximately 55 kb based on the distinct fragments resulting from *Nco*I digestion. Likewise, the DNA of pAh6-C was digested by *Msp*I, *Sma*I, *Nco*I, and *Hpa*II, and its size was estimated to be approximately 58 kb based on the distinct fragments resulting from *Hpa*II digestion (**data not shown**).

To further characterize pAh1-C and pAh6-C, their structural protein compositions were analyzed by SDS-PAGE and LC-MS/MS. At least 11 (pAh1-C) and 19 (pAh6-C) distinct protein bands, with molecular masses ranging from 11 to 105 kDa, were separated. Six (pAh1-C) and eight (pAh6-C) major protein bands were then subjected to LC-MS/MS for peptide sequencing (**Table 1.3**).

The results revealed differences between the structural protein compositions of the two phages. Although pAh6-C possessed similar structural proteins as other *Aeromonas* phages reported, such as the *Aeromonas* phages Aeh1, 25, and 31, the structural proteins of pAh1-C were similar to those of phages from species other than *Aeromonas* (**Table 1.3**).

### *1.3.3. Phage stability test*

Ethanol treatment resulted in a much greater loss of the activity of both phages compared to treatment with either chloroform or diethyl ether (**Figure 1.3a**). Both phages exhibited similar infection capabilities after incubation at a pH range of 3-11, although

pAh6-C was found to be slightly more pH-sensitive than pAh1-C. The optimal stability of the phages was found at pH 7.0 (**Figure 1.3b**), and they were relatively heat stable over a period of 1 h at 20 ~ 25 °C. However, the following reductions in phage activity were observed for pAh1-C and pAh6-C, respectively: 8.5 ± 0.2% and 19.4 ± 0.6% at 30 °C, 78.7 ± 2.3% and 64.7 ± 0.3% at 37 °C, and 97.9 ± 0.1% and 95.3 ± 0.7% at 50 °C. Incubation at 65 °C for 1 h resulted in the complete inactivation of both phages (**Figure 1.3c**).

### ***1.3.4.*** *Host cell lysis test*

The bactericidal effects of pAh1-C and pAh6-C were tested on early-phase *A. hydrophila* JUNAH cultures (**Figure 1.4**). The $OD_{600}$ values of the uninfected control culture continued to increase during the incubation period. In contrast, infection with pAh1-C or pAh6-C inhibited the bacterial growth at MOIs of 0.01, 1, and 100 until 24 h. However, the bactericidal activity of pAh1-C contrasted with that of pAh6-C. The bacterial growth of cultures infected with pAh1-C was inhibited until 12 h, regardless of MOI, and then increased due to the growth of phage-resistant *A. hydrophila*. In contrast, the bactericidal activity of pAh6-C varied depending on the MOI. Although the $OD_{600}$ values of the cultures infected with pAh6-C increased after 6 h regardless of MOI, the bacterial growth was inhibited most effectively at an MOI of 1. In addition, the $OD_{600}$ values of cultures infected with an MOI of 100 were higher than those of cultures infected with an MOI of 1, due to the growth of phage-resistant bacteria.

### ***1.3.5.*** *Phage-resistant bacterial variants and pathogenicity in fish*

Variant R1, which emerged after exposure to pAh1-C, was also resistant to pAh6-C, while Variant R2, which emerged after exposure to pAh6-C, was sensitive to pAh1-C.

Variant R1 (resistant to both pAh1-C and pAh6-C) and Variant R2 (resistant only to phage pAh6-C) were used in the pathogenicity tests. The pathogenicities of these variants and their parent *A. hydrophila* (JUNAH) strain in loaches are shown in **Table 1.2**. The phage-sensitive parent *A. hydrophila* (JUNAH) caused 100% mortality in the loaches following intraperitoneal injection with $1.8\times10^7$ CFU/fish. All of the fish died within 24 h of injection, and hemorrhages were observed on their surfaces. The inoculated bacteria were isolated in pure cultures from the kidneys of the dead fish. In contrast, the variants that were resistant to pAh1-C and/or pAh6-C were not pathogenic at the same dose ($10^7$ CFU/fish) or at a higher dose ($10^8$ CFU/fish).

### *1.3.6. Protective effect of phage administration*

The protective effects of intraperitoneal injection and oral administration of the phages against experimental *A. hydrophila* (JUNAH) infection are shown in **Figure 1.5**. In the first experiment, the fish were challenged with two different doses of bacterial suspension and were administered two types of phages by IP injection. The fish in the control groups that were not treated with phages began to die at 1 day post-infection, and the cumulative mortalities over seven days were 39.17 ± 3.82% (1st trial, $2.6\times10^6$ CFU/fish) and 100% (2nd trial, $2.6\times10^7$ CFU/fish). In contrast, the fish treated with phages showed lower mortality rates; in the 1st trial, no mortality was observed in the groups treated with pAh1-C or pAh6-C, and in the 2nd trial, the cumulative mortalities were 43.33 ± 2.89% (pAh1-C) and 16.67 ± 3.82% (pAh6-C) (**Figure 1.5a, b**).

In the second experiment, the fish were challenged with the bacterial suspension as in the first experiment but received phage-impregnated feed rather than an intraperitoneal injection. The fish in the control groups showed mortality rates (1st trial, 38.33 ± 2.50%;

2nd trial, 95.83 ± 3.82%) that were similar to those in the first experiment. However, the fish treated with phages showed lower mortality rates than those of the control group. In the 1st trial, the cumulative mortality rates were 17.50 ± 2.50% following treatment with pAh1-C and 11.67 ± 3.82% following treatment with pAh6-C. In the 2nd trial, the cumulative mortality rates were 46.67 ± 3.82% following treatment with pAh1-C and 26.67 ± 2.89% following treatment with pAh6-C (**Figure 1.5c, d**). The administration of a high dose ($10^{10}$ PFU/fish) of either phage to the experimental fish did not affect their physical condition or survival during a one-month period of observation. The bacteria were re-isolated from all of the dead fish except survived fish from the phage-administrated groups, indicating that the mortalities and protective effects were caused by *A. hydrophila* and the phages (pAh1-C and pAh6-C), respectively. In addition, bacteria isolated from the fish that had received the phage and died were still susceptible to both phages.

## 1.4. Discussion

In the current study, the indicator host strain JUNAH was determined to be the causative agent of the mass mortality of cyprinid loaches in a private fish hatchery in Korea (15). The JUNAH strain has demonstrated resistance to multiple antibiotics and was therefore able to cause the death of more than 50% of all fish reared on the farm despite the various antibiotic treatments administered. To discover an effective control agent against *A. hydrophila* infection in aquaculture, water samples were collected over a period of one year for phage isolation.

An increasing incidence of antimicrobial resistance has become a threat to aquaculture environments (9). The emergence of microbial resistance to multiple drugs due to the

liberal and possibly inappropriate use of antibiotics is a serious problem in aquaculture and causes therapeutic failures (9, 27). Alternatives to conventional antibiotics are urgently needed for the control of *A. hydrophila* in aquaculture. Although there have been some previous reports regarding the isolation of *A. hydrophila* phages (7, 23, 24, 25, 26), there is currently a paucity of studies reporting realistic assessments of their therapeutic applications.

Previously, the therapeutic potential of phages in aquaculture environments was described (18, 28, 29). In the current study, two phages were isolated from water of the Han River in Seoul. Although both phages showed a similar host range, they formed differently shaped plaques and burst sizes, and their gDNAs showed different restriction endonuclease digestion patterns. In addition, the two phages exhibited different patterns of bactericidal activity. The method of experimental infection by IP injection was successfully performed in all experimental groups, in the same manner as previously reported (13, 32). The challenge trials using various methods such as bath immersion, cohabitation with infected fish, and oral administration of *A. hydrophila*-incorporated feed have not resulted in reduced mortality rates. The *in vivo* experiments evaluating the protective effects of the two phages indicated that pAh6-C controlled *A. hydrophila* infection more effectively than pAh1-C. Although the phage-resistant bacterial variants were not particularly pathogenic, the $OD_{600}$ values of the pAh1-C-infected bacteria gradually increased after 12 h post-infection.

Wu et al. (41) isolated an *A. hydrophila* phage, AH1, and performed pathogenicity testing using loaches. However, these authors infected *A. hydrophila* with the phage 3 h before injection of the loaches, and this experiment was performed to indicate that the pathogenicity of *A. hydrophila* was eliminated after phage infection and to demonstrate the

potential prophylactic use of the phage. Several *A. hydrophila* phages, such as Aeh1, Aeh2, PM1, PM2, PM3 and 18, have been reported (7, 23, 24, 25, 26). However, there have been no reports demonstrating the successful use of phages for the treatment of *A. hydrophila* infection or providing evidence of their potential as therapeutic agents. In the current study, the successful phage treatment of *A. hydrophila* infection emphasizes the potential of this therapeutic approach. In addition, pAh1-C and pAh6-C were able to inhibit *A. hydrophila* strains from various sources, including three fish-pathogenic isolates (SNUFPC-A6 from the sailfin molly *Poecilia latipinna*, SNUFPC-A8 from the cherry salmon *Oncorhynchus masou masou*, and JUNAH from the cyprinid loach) and two environmental isolates (SNUFPC-A20 from a river and SNUFPC-A21 from sewage); these results suggest the broad application of phages for the treatment of various fish species. Treatment of fish by IP injection resulted in noticeable protective effects, with increased survival rates observed for experimental fish compared to the controls. However, treating fish by IP injection in a fish culture environment is labor-intensive and time-consuming. Therefore, the protective effect of an oral method of administration was also evaluated for the purpose of identifying an effective and realistic route of administration on a larger scale, as required on a fish farm, and this treatment method also improved survival rates considerably.

Despite the improvements in the survival of the phage-treated cyprinid loaches, a considerable degree of mortality remained. For the successful phage control of *A. hydrophila* infection, a combination of both isolated phages may help to increase the possibility of efficient phage therapy.

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**Table 1.1.** Bacterial strains used in this study and infectivity of phage pAh1-C and pAh6-C.

| Bacterial species (*n*) | Strain | Host range[a] (EOPs[b]) | | Source[c] |
|---|---|---|---|---|
| | | pAh1-C | pAh6-C | |
| *A. hydrophila* (*5*) | SNUFPC-A6 | + (0.14 ± 0.02) | ++ (0.64 ± 0.06) | 1 |
| | SNUFPC-A8 | + (0.34 ± 0.02) | + (0.41 ± 0.08) | 1 |
| | SNUFPC-A20 | + (0.11 ± 0.08) | + (0.15 ± 0.10) | 1 |
| | SNUFPC-A21 | ++ (0.87 ± 0.03) | ++ (0.94 ± 0.02) | 1 |
| | JUNAH | ++ (1.00) | ++ (1.00) | 2 |
| *A. media* | SNUFPC-A22 | - | - | 1 |
| *A. salmonicida* | ATCC 33658 | - | - | 5 |
| *A. sobria* | Aro | - | - | 6 |
| *A. veronii* | SNUFPC-A17 | - | - | 1 |
| *Streptococcus iniae* | ATCC 29178 | - | - | 5 |
| *S. agalactiae* | ATCC 27956 | - | - | 5 |
| *Enterococcus faecium* | CCARM 5192 | - | - | 3 |
| *E. faecalis* | CCARM 5168 | - | - | 3 |
| *Vibrio vulnificus* | ATCC 27562 | - | - | 5 |
| *V. parahaemolyticus* | ATCC 17802 | - | - | 5 |
| *V. alginolyticus* | ATCC 17749 | - | - | 5 |
| *Escherichia coli* | DH10B | - | - | 4 |

[a] ++, clear plaque; +, turbid plaque; -, no plaque.

[b] The EOP (efficiency of plating) values were shown as the mean of observations at three different occasions.

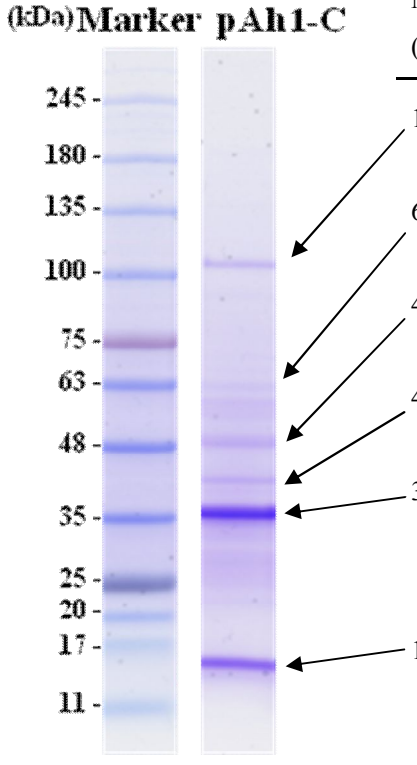
[c] 1, Han et al. (11); 2, Jun et al. (15); 3, obtained from the Culture Collection of Antimicrobial Resistant Microbes in Korea; 4, purchased from Invitrogen; 5, purchased from the American Type Culture Collection; 6, Jun et al. (14).

**Table 1.2.** Pathogenicities of phage-sensitive parent cells and phage-resistant variants of *A. hydrophila* (JUNAH) for loach[a].

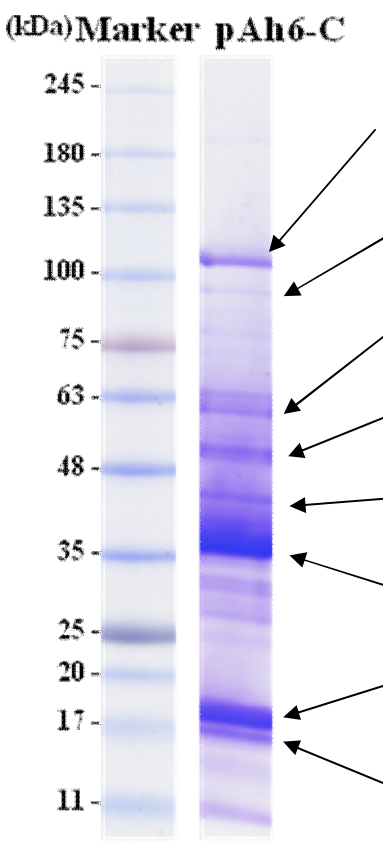
| Strain injected | No. of experiments | Dose (CFU/fish) | No. of dead/total fish | Mortality (%) |
|---|---|---|---|---|
| Parent *A. hydrophila* (JUNAH) | 1st | $1.8\times10^{6}$ | 14/40, 16/40, 15/40 | 37.50 ± 2.50 |
| | 2nd | $1.8\times10^{7}$ | 40/40, 40/40, 40/40 | 100 ± 0 |
| Variant R1 | 1st | $1.4\times10^{7}$ | 0/40, 0/40, 0/40 | 0 |
| | 2nd | $1.4\times10^{8}$ | 0/40, 0/40, 0/40 | 0 |
| | 3rd | $1.4\times10^{9}$ | 6/40, 8/40, 6/40 | 16.67 ± 2.89 |
| Variant R2 | 1st | $1.0\times10^{7}$ | 0/40, 0/40, 0/40 | 0 |
| | 2nd | $1.0\times10^{8}$ | 0/40, 0/40, 0/40 | 0 |
| | 3rd | $1.0\times10^{9}$ | 4/40, 6/40, 5/40 | 12.50 ± 2.50 |

[a] Fish were intraperitoneal (IP)-injected with bacteria and were cultivated at 25 ± 1 °C and observed for 7 days. Variant R1 was resistant to both pAh1-C and pAh6-C; variant R2 was resistant only to pAh6-C.

**Table 1.3.** SDS-PAGE profiles of pAh1-C and pAh6-C virion and their protein profiles by liquid chromatography-mass spectrometry (LC-MS/MS) analysis.




| MW (kDa) | Protein name [Species] | Sequence coverage | Accession number |
|---|---|---|---|
| 105 | e.6 conserved hypothetical protein [*Enterobacteria* phage T4] | 14.7% | NP049742 |
| 62 | hypothetical protein A2p47 [*Lactobacillus* phage A2] | 13.7% | NP680526 |
| 49 | hypothetical protein EFP gp220 [*Enterococcus* phage phiEF24C] | 33.3% | YP001504329 |
| 41 | gp23 [*Mycobacterium* phage 244] | 26.3% | ABD57998 |
| 36 | bacteriophage protein [*Escherichia coli* DEC4D] | 35.1% | EHV05232 |
| 15 | gp7 [*Mycobacterium* phage BarrelRoll] | 28.0% | AEO94149 |




| MW (kDa) | Protein name [Species] | Sequence coverage | Accession number |
|---|---|---|---|
| 105 | NrdA-A aerobic NDP reductase large subunit [*Aeromonas* phage Aeh1] | 56.2% | NP943927 |
| 95 | gp7 base plate wedge initiator [*Aeromonas* phage Aeh1] | 53.9% | NP944089 |
| 61 | hypothetical protein Aeh1p299 [*Aeromonas* phage Aeh1] | 49.9% | NP944177 |
| 51 | hypothetical protein Aeh1p026 [*Aeromonas* phage Aeh1] | 49.2% | NP943904 |
| 44 | gp6 base plate wedge component [*Aeromonas* phage 25] | 44.8% | YP656367 |
| 36 | base plate tail tube cap [*Aeromonas* phage 31] | 41.5% | YP238911 |
| 18.5 | gp68 [*Aeromonas* phage 31] | 58.6% | YP238885 |
| 17 | gp61.1 conserved hypothetical protein [*Aeromonas* phage 25] | 90.3% | YP656265 |

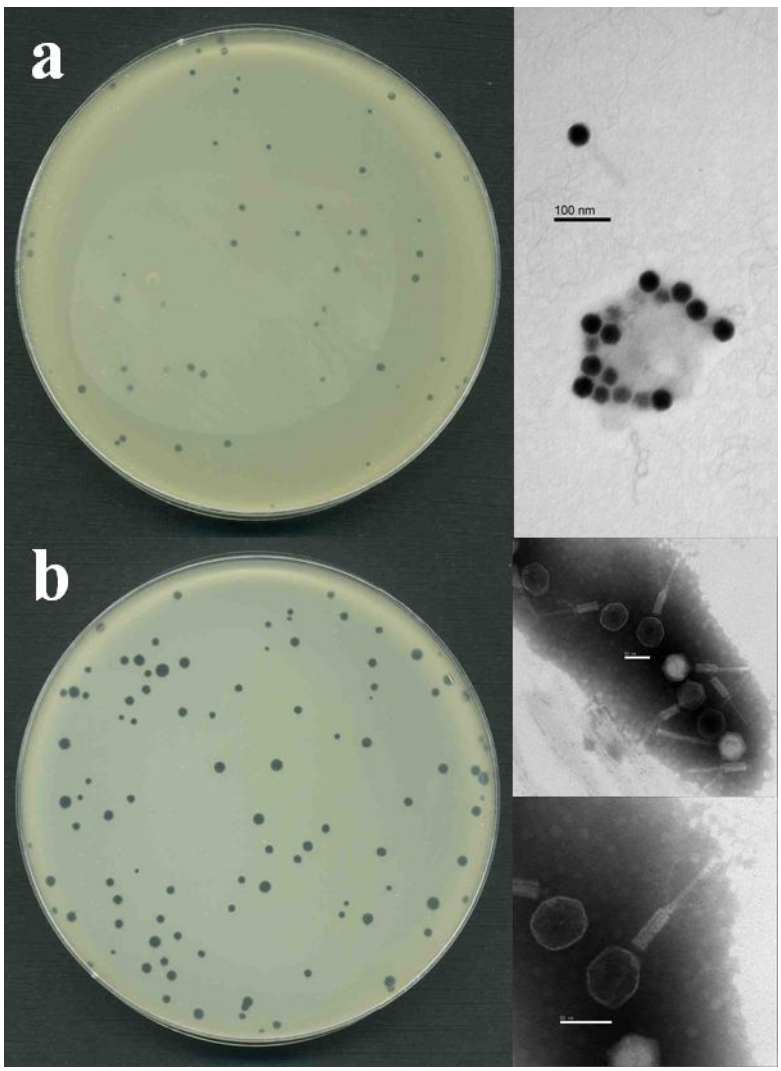



**Figure 1.1.** Phage plaques formed in double layer agar plates with the indicator host strain, *A. hydrophila* JUNAH, and electron micrographs of two phages: (a) pAh1-C (bar = 100 nm) and (b) pAh6-C (bars = 50 nm).

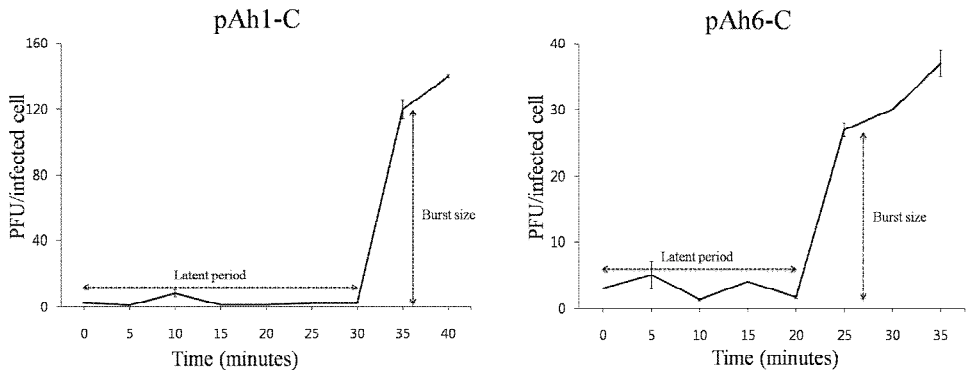



**Figure 1.2.** One-step growth curve of pAh1-C and pAh6-C. The error bars indicate standard deviations.

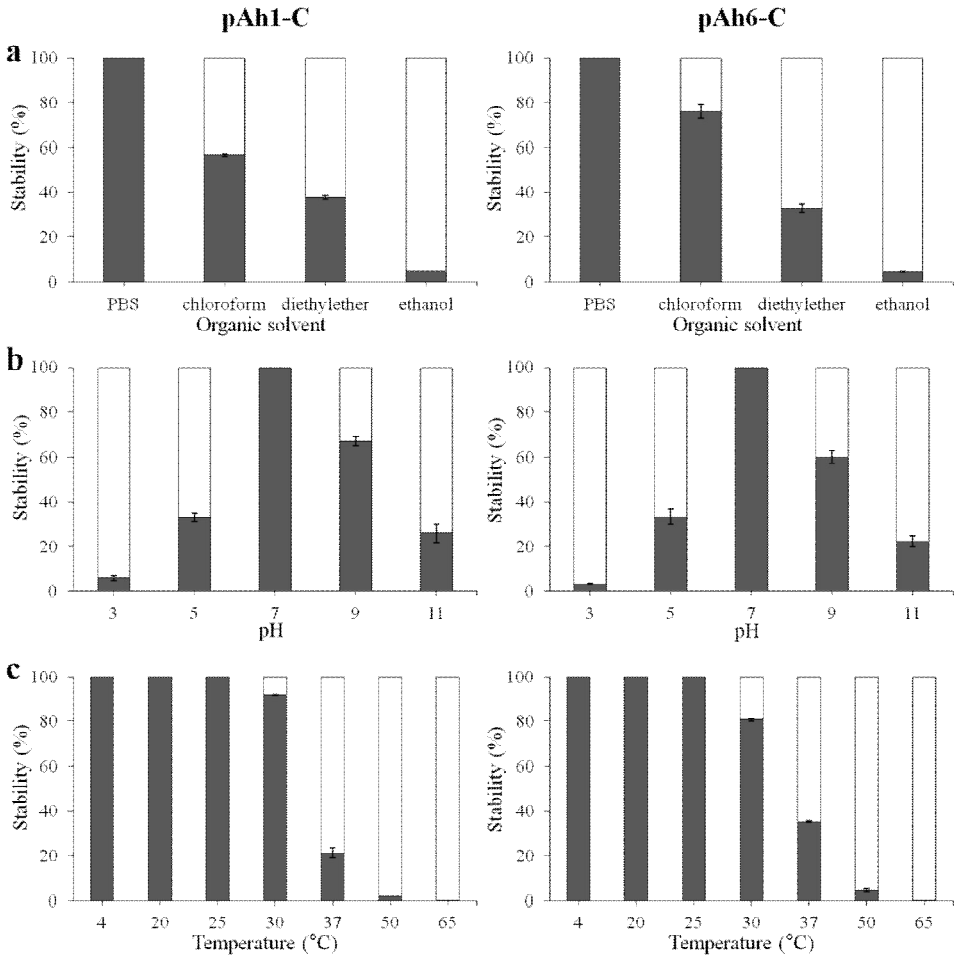



**Figure 1.3.** Stability of pAh1-C (left) and pAh6-C (right) in the presence of various organic solvents (a), pHs (b), and temperatures (c). To test the stability of these phages in response to the different factors, optimal conditions such as sterile PBS (a), pH 7 (b), and 4 °C (c) were used as controls. All values represent the mean of three experiments performed in triplicate on separated occasions, with error bars representing the standard deviations (SD; $n = 3$).

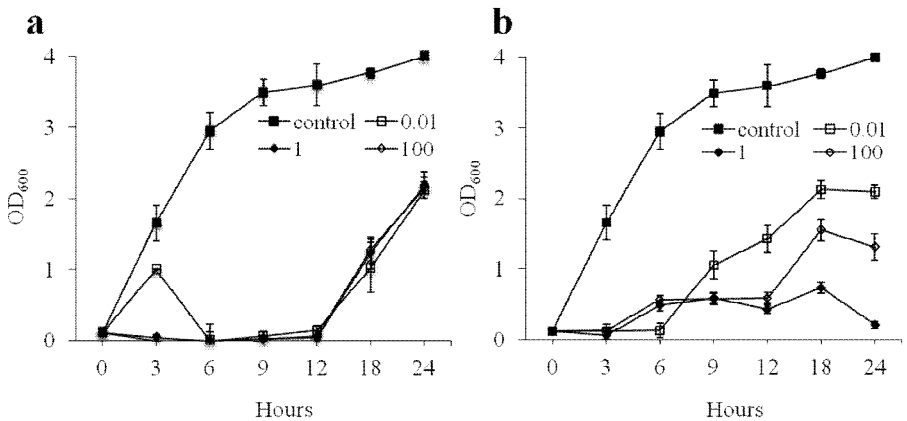



**Figure 1.4.** Bactericidal effects of pAh1-C (a) and pAh6-C (b) against *A. hydrophila* JUNAH. Early exponential-phase cultures of *A. hydrophila* JUNAH were co-cultured with pAh1-C (a) and pAh6-C (b) at MOIs of 0, 0.01, 1, and 100. The results are shown as the mean ± standard deviations from triplicate experiments.

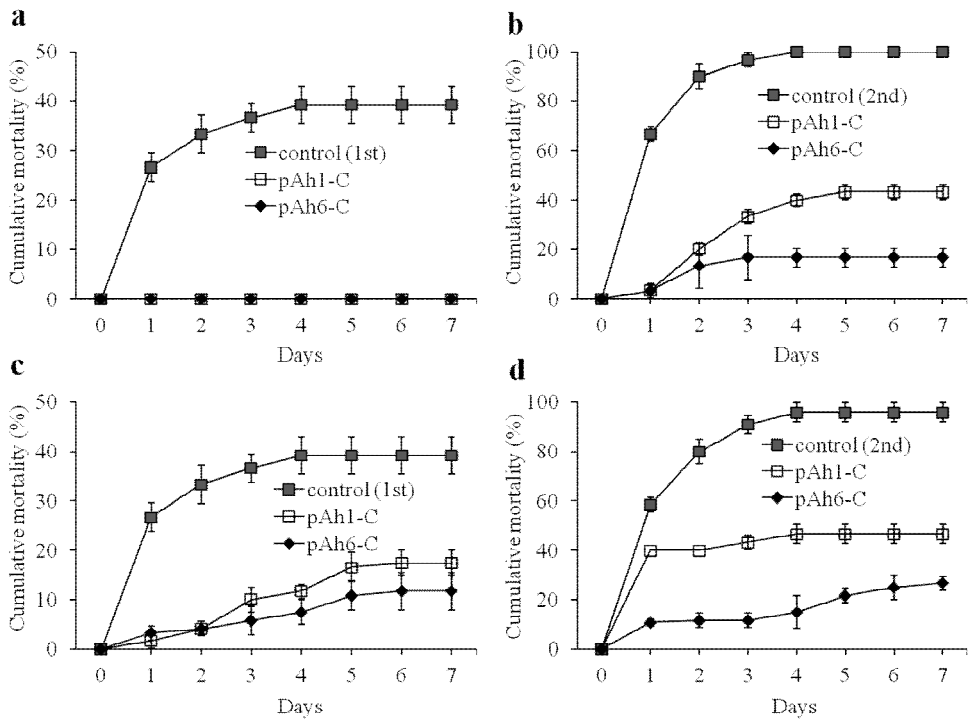



**Figure 1.5.** Cumulative mortality of different treatment groups following challenge with *A. hydrophila* via IP injection. (a) and (b) were treated by IP injection; (c) and (d) were treated by oral administration of phage-coated feed. control: injected with *A. hydrophila* (1st, $2.6\times10^6$ CFU/fish; 2nd, $2.6\times10^7$ CFU/fish) but not treated; pAh1-C: treated with pAh1-C following *A. hydrophila* injection; pAh6-C: treated with pAh6-C following *A. hydrophila* injection. All results are shown as the mean of triplicate experiments, and error bars represent the SD ($n$ = 3).

# Chapter II

## Bacteriophage Therapy of a *Vibrio parahaemolyticus* Infection Caused by a Multiple Antibiotic Resistant O3:K6 Pandemic Clinical Strain


### Abstract

Recently isolated *Vibrio parahaemolyticus* strains have displayed multiple antibiotic resistance. Alternatives to conventional antibiotics are needed, especially for the multiple-antibiotic-resistant *V. parahaemolyticus* pandemic strain. A bacteriophage, designated pVp-1, that was lytic for *V. parahaemolyticus* was isolated from the coast of the Yellow Sea in Korea. The phage showed effective infectivity for multiple-antibiotic-resistant *V. parahaemolyticus* and *V. vulnificus*, including *V. parahaemolyticus* pandemic strains. The therapeutic potential of the phage was studied in a mouse model of experimental infection using a multiple-antibiotic-resistant *V. parahaemolyticus* pandemic strain. Phage-treated mice displayed protection from a *V. parahaemolyticus* infection and survived lethal oral and intraperitoneal bacterial challenges. This is the first report, to the best of knowledge, of phage therapy in a mouse model against a multiple-antibiotic-resistant *V. parahaemolyticus* pandemic strain infection.



**Keywords:** *Vibrio parahaemolyticus*; Bacteriophage; pVp-1; Pandemic strains.

## 2.1. Introduction

*Vibrio parahaemolyticus*, a gram-negative marine bacterium, is one of the most important causes of gastroenteritis associated with consumption of raw oysters (7). *V. parahaemolyticus* pandemic strains, such as O3:K6, are responsible for the current pandemics in many countries (14). Emergence of *Vibrio* species that are resistant to multiple antibiotics has been recognized as a serious global clinical problem (17). Recently isolated *V. parahaemolyticus* pandemic strains have displayed multiple antibiotic resistance, increasing concerns about possible treatment failure (11).

Theoretically, bacteriophages (phages) can be used to treat infectious diseases both in humans and animals (2, 4, 5, 13, 20, 24). Phages display an effective bacteriolytic activity and possess several advantages over other antimicrobial agents, and no serious side effects of phage therapy have been described thus far (8, 20). Because all isolated *V. parahaemolyticus* strains have exhibited resistance to a broad variety of commercial antibiotics, it has been previously noted that alternatives to conventional antibiotics are needed (11).

In this study, one lytic *Siphoviridae* phage, designated as pVp-1 (12) and infects *V. parahaemolyticus* pandemic strains, was isolated and characterized. It was aimed to determine whether this phage could be suitable for therapeutic use in a mouse model of a multiple-antibiotic-resistant *V. parahaemolyticus* pandemic strain.

## 2.2. Materials and methods

### *2.2.1. Bacterial strains*

*V. parahaemolyticus* ATCC 33844 was used as the host bacterial strain for phage isolation and amplification. CRS 09-17 (isolated from a patient with diarrhea; *V. parahaemolyticus* new O3:K6 pandemic strain) (11) was used to evaluate its therapeutic potential.

### *2.2.2. Electron microscope examination*

Phage particles were negatively stained with 2% uranyl acetate, and electron micrographs were taken using a Zeiss TEM EM902.

### *2.2.3. One-step growth*

The one-step growth curve of pVp-1 was determined according to the method of Verma et al. (23). Ten microliters of phage suspension was added to 10 ml of the mid-exponential host bacterial culture (ATCC 33844, $8.0\times10^6$ CFU/ml). The mixture was then centrifuged and the pellet was resuspended in 20 ml of TSB. Samples (100 μl) were taken at 5 min intervals and subjected to phage titration.

### *2.2.4. Phage stability*

Phage stability tests were conducted as described elsewhere (23), with modifications. Briefly, phage stability to various conditions such as organic solvents (chloroform, ethanol, and ether; 25% of total volume), pH (3, 5, 7, 9, and 11), temperature (20, 25, 30, 37, 50, and 65°C), and UV light (30 cm from the UV-C, 253.7 nm; Sankyo Denki, Japan), was

evaluated after 1 h incubation at 25°C (except for the temperature test). After incubation, the phage titer was estimated by the double-agar-layer method.

### *2.2.5. Host cell lysis*

The bacteriolytic effect of the phage on *V. parahaemolyticus* CRS 09-17 was observed by determining viable bacteria counts throughout the incubation period. The phage was added to the early exponential phase ($OD_{600nm}$ = 0.1; $8.0\times10^6$ CFU/ml) of CRS 09-17 at the indicated multiplicity of infection (MOI), and the change in optical density (OD) was monitored for 24 h.

### *2.2.6. Ethics statement*

Specific pathogen-free BALB/c female mice (8-weeks-old) were used with the approval of the Institutional Animal Care and Use Committee, Seoul National University, Seoul, Republic of Korea (Reg. No. SNU-120602-1). All animal care and experimental protocols were performed according to the guidelines of the Animal Ethical Committee, Seoul National University.

### *2.2.7. Induction of V. parahaemolyticus infection in mice*

To determine the 50% lethal dose ($LD_{50}$), *V. parahaemolyticus* CRS 09-17 was diluted with PBS to a range of $2.0\times10^6$ to $2.0\times10^8$ CFU per mouse in 200 μl and was administered by either the intraperitoneal (IP) or orogastric route (orally). Five mice were used for each concentration. The survival rate of mice was recorded until 7 days post-infection. Mice inoculated with CRS 09-17 were observed for their state of infection based on several clinical signs, including ruffled fur, hunchback moribund, and partially closed eyes. The

experiment was replicated three times.

### *2.2.8. Kinetics of phage in mice*

A phage in vivo kinetic assessment was performed as previously described (23), with several modifications. First, between the two groups, with each group composed of 21 mice, one group was given an IP injection, while the other group was orally given the phage preparation ($2.0\times10^8$ PFU/mouse). Second, the two groups (seven mice per group) were given an IP injection or was oral administered a heat-inactivated (65°C, 2 h) phage suspension as the negative control. Finally, at appropriate time intervals, four mice (three test mice and one control mouse) from the IP and oral groups were euthanized, and phage titers were determined from their organs.

### *2.2.9. Treatment of bacteremic mice with phage pVp-1*

The efficacy of phage therapy was evaluated in two separate experiments using the *V. parahaemolyticus* CRS 09-17 infection mouse model. In the first experiment, two groups of mice (control/treatment; five mice in each group) were challenged by an IP injection of a $LD_{50}$ of CRS 09-17. Each mouse was treated with a single IP injection of phage pVp-1 ($2.0\times10^8$ PFU per mouse) or PBS, 1 h after the bacterial challenge ($2.0\times10^7$ CFU per mouse).

In the second experiment, all conditions were similar to those of the first study except that the bacterial challenge ($2.0\times10^7$ CFU per mouse) and phage treatment ($2.0\times10^8$ PFU per mouse) were administered orally. Both experiments were repeated five times, and the health condition of the mice was monitored for 72 h.

In an additional study, two groups (five mice per group) were not challenged with

bacteria and received only phage ($2.0\times10^{11}$ PFU per mouse) by IP and oral routes, as described previously (22). The state of health of these mice was monitored for 28 days.

***2.2.10.*** *Quantitative analysis of V. parahaemolyticus / phage in mouse organs*

During the phage treatment experiment described above, three mice from each group were euthanized at 0, 1, 3, 6, 12, 24, 36, 48, and 72 h post-treatment. As the main target organs of gastroenteritis, the stomach and intestine were removed and homogenized to quantify viable bacteria and phage. Bacterial and phage counts were normalized by organ weight when the organs were halved and processed for histopathological examination. A selective medium (CHROMagar$^{TM}$ Vibrio containing resistant antibiotics) was used for the enumeration of *V. parahaemolyticus*, as described previously (11). This experiment was repeated three times.

***2.2.11.*** *Histopathology of organs*

A portion (one-half) of the stomach and intestine was fixed and cut by a standardized method and placed in tissue cassettes for further processing. Slides of hematoxyline-eosin stained tissues were prepared and observed for histopathology by microscopic examination. Histopathology was examined for severity in a blinded manner.

***2.2.12.*** *Measuring phage immune response*

Mice were immunized using an IP injection of phage ($2.0\times10^{10}$ PFU per mouse) at intervals of 0, 4, 6, and 8 weeks, as described previously (22). At various times, the sera from five mice were prepared and indirect enzyme-linked immunosorbent assays were

performed as previously described (9). Immunoglobulins were detected with goat anti-mouse Ig (immunoglobulin) M- or IgG-specific antibodies.

### *2.2.13. Statistical analyses*

Statistically significant differences in all of the experiments were determined using Student's *t*-test. The SPSS statistical software package version 16.0 (SPSS, Chicago, IL) was used for all statistical analyses.

## 2.3. Results

### *2.3.1. Characterization of Vibrio phage pVp-1*

The *Vibrio* phage pVp-1 formed small plaques (average diameter, 1 mm) in a lawn of *V. parahaemolyticus* ATCC 33844. Based on its morphology, the phage was assigned to the family *Siphoviridae*, according to the classification system of Ackermann (1) (**Figure 2.1**). Additionally, one-step growth of pVp-1 showed that it had a latent period of approximately 15 min with a burst size of 47 PFU/cell (**Figure 2.2**).

The pVp-1 was sensitive to organic solvents. After 1 h of incubation in chloroform, diethylether, and ethanol, phage activity decreased to 37.7%, 33%, and 56.6%, respectively. However, no effect on phage activity was observed within a pH range of 5-11, and the activity remained at a high level (94.9%) at pH 3. In addition, the phage was relatively heat stable over a temperature range of 20-37°C, and no loss in activity was observed, although phage activity decreased to 3.3% at 50°C and 0% at 65°C. Upon exposure to UV light, a complete inactivation of pVp-1 at approximately 45 min was observed (**Figure 2.9**).

### *2.3.2. Phage therapeutic application on pandemic clinical strain*

The bacteriolytic effect of pVp-1 was tested on an early exponential phase culture of *V. parahaemolyticus* CRS 09-17 (**Figure 2.3**). When the culture was not infected by pVp-1 (control), the $OD_{600}$ value continued to increase throughout the incubation. In contrast, bacterial growth induced by pVp-1 was apparently retarded at an MOI of 0.1, 1, and 10 until 24 h. Bacterial growth was properly inhibited at an MOI 1 and 10, whereas the $OD_{600}$ value at an MOI of 0.1 increased gradually and reached 1.0 after 9 h.

In animal experiments, invalidism and the wellness of the animals were measured using four criteria: physical condition, survival rate, CFU per gram of target organs (stomach/ intestine), and histopathology of target organs (stomach/intestine). The $LD_{50}$ of *V. parahaemolyticus* CRS 09-17 was examined using IP and oral routes of administration. The $LD_{50}$ of the IP and oral routes were between $2.0\times10^6$ and $2.0\times10^7$ CFU per mouse (**Figure 2.4**). The IP and oral mouse infection model was an acute death model; all mortality occurred within 36 h. After 36 h post-infection, mice that survived entered the recovery stage.

To examine in vivo kinetics, a pVp-1 kinetic analysis was performed in mice treated by IP and oral administration (**Figure 2.5**). In the IP group, a titer of $7.9\times10^5$ PFU/ml in blood during the first hour increased to a titer of $6.4\times10^6$ PFU/ml over the next 6 h. However, by 12 h of inoculation, the titer decreased to $2.1\times10^5$ PFU/ml. Likewise, pVp-1 titers in the stomach and intestine were also estimated. In the stomach, a titer of $1.3\times10^4$ PFU/g at 1 h increased to a titer of $3.4\times10^5$ PFU/g at 6 h and decreased to $4.5\times10^3$ PFU/g at 12 h. However, in the intestine, a titer of $9.2\times10^4$ PFU/g at 1 h increased to a titer of $6.1\times10^6$ PFU/g at 12 h and decreased to $4.8\times10^4$ PFU/g at 24 h.

In the group receiving the oral administration, a titer of 0 at 1 h in blood increased to a

titer of $3.8\times10^6$ PFU/ml at 12 h and decreased to $6.7\times10^5$ PFU/ml at 24 h. In the stomach, a titer of $1.1\times10^3$ PFU/g at 1 h increased to a titer of $1.2\times10^5$ PFU/g at 3 h and decreased to $1.1\times10^4$ PFU/g at 6 h. In the intestine, a titer of $2.9\times10^5$ PFU/g at 1 h increased to a titer of $5.4\times10^5$ PFU/g at 3 h and decreased to $3.4\times10^5$ PFU/g at 6 h. There was a gradual fall in the titer thereafter and after 48 h of inoculation, pVp-1 became undetectable. During the experiment, all mice were healthy and in normal condition.

To determine whether phage pVp-1 could treat a CRS 09-17 infection, pVp-1 was administered by IP and oral routes 1 h after a CRS 09-17 challenge (**Figure 2.6**). After 6 h of infection, all control (infected but not phage-treated) mice were visibly ill, lethargic, and scruffy. The control group fatality rate was 56% (IP) and 52% (oral) within 36 h. The stomachs and intestines of control mice contained high levels of bacteria (stomach, $1.0\times10^4$ CFU/g; intestine, $5.3\times10^3$ CFU/g) until 12 h post-infection (**Figure 2.6**). In contrast, phage treatment resulted in excellent protection in terms of all four criteria. The phage-treated mice appeared to be only slightly ill and were protected up to 92% (IP) and 84% (oral) from the lethal infection induced by CRS 09-17 ($2.0\times10^7$ CFU) after the administration of a single dose of purified pVp-1 of $2.0\times10^8$ PFU (**Figure 2.6**). The stomach/intestine CFU and histopathologic features were improved by phage treatment. In the IP treatment group, a decrease in stomach/intestine CFU was obtained with the increased phage titer at 12 h post-infection, $1.7\times10^2/3.7\times10^2$ CFU/g and $5.7\times10^3/2.3\times10^4$ PFU/g (**Figure 2.6**). In the oral treatment group, the maximum CFU and PFU values of the stomach/intestine were observed at experimental onset, and the values gradually decreased (**Figure 2.7**). In addition, the histopathologic features demonstrated that the *V. parahaemolyticus* infection significantly damaged the intestinal tract in as observed by the H&E stain (**Figure 2.8C-D**) but not in the gastric region (**data not shown**). Severe destruction of the histologic

structure of the colon was accompanied by a thinning of the wall, enterohemorrhage, and loss of crypts in the mucosal layer (**Figure 2.8C-D**). However, treatment with phage ameliorated the histological damage in the colon. The histopathological examination of the colon of the phage-treated mice revealed a significant recovery in the destruction of the intestinal wall and crypts, hemorrhages and inflammation after both IP and oral treatment (**Figure 2.8E-F**). This suggests that phage-treated animals showed strongly reduced infection severity and could survive a lethal bacterial challenge. In addition, the administration of a high dose ($2.0\times10^{11}$ PFU/mouse) of pVp-1 alone did not affect the physical condition or survival during 28 days of observation.

### *2.3.3. Immune response to phage pVp-1*

After the fourth phage induction in a series of phage injections in mice, titers of IgG and IgM against the phage increased above background levels by 170-fold and 50-fold respectively (**Figure 2.10**). No anaphylactic reactions, changes in physical condition or adverse events were observed during the course of these multiple injections of phage.

## 2.4. Discussion

Based on the morphological analysis, pVp-1 was classified into the family *Siphoviridae*, and it demonstrated a broad host range. This differs from a prior finding that *Siphoviridae* phages are generally considered to have restricted host ranges (26). The phage infected 74% (20 / 27) of all multiple antibiotic resistant *V. parahaemolyticus* strains used in this study, including the two pandemic strains CRS 09-17 and CRS 09-72. Interestingly, pVp-1 infected the *V. parahaemolyticus* clinical isolate CRS 09-17 from a patient with diarrhea,

which represents a multiple-antibiotic-resistant new O3:K6 pandemic strain (*tdh*+, ORF8+, *toxRS/new*+) (11). The results obtained from pVp-1 showed its lytic nature with a latent period and a large burst size. Full genome sequencing of pVp-1 was identified no similarity match with lysogenic or phage integrase-related genes (considered as markers of temperate phages), indicating that it is a novel, newly isolated lytic phage (12). This result emphasizes the potential of pVp-1 as a therapeutic agent, as described previously by Gutiérrez et al. (8), who regarded lytic phages as more suitable phages for therapy. Furthermore, the stability of pVp-1 over a wide range of pH (3-11) and temperature (20-37°C) clearly indicates that pVp-1 would be highly stable in the body.

Current analyses show that the search for new antibiotics conducted by pharmaceutical companies is becoming more and more restricted due to the growing costs of conducting the appropriate trials, low profits, and high risk of the investment, precisely because of the possibility of a rapid acquisition of resistance to the new drug (6, 16, 21, 25). It was hypothesized that phage therapy can be useful, especially in epidemics caused by multiple-antibiotic-resistant pandemic strains. To evaluate the therapeutic potential of pVp-1, a mouse model of *V. parahaemolyticus* CRS 09-17 was used. In the cell lysis test of pVp-1, the growth of CRS 09-17 was apparently inhibited after pVp-1 inoculations at MOIs of 1 and 10, although pVp-1 partially inhibited the bacterial growth at an MOI of 0.1.

Because the route of phage inoculation is important, in vivo phage kinetics were ascertained following an IP and oral administration of pVp-1. To apply pVp-1 at an MOI of 10 (CRS 09-17, $2.0\times10^7$ CFU per mouse), where the maximum effect of cell lysis was examined, kinetic tests were performed with pVp-1 at $2.0\times10^8$ PFU per mouse. Phage titers in the stomach as well as the intestine indicated that pVp-1 was maintained at higher concentrations in these two organs and could prevent *V. parahaemolyticus* infections.

These findings suggest that pVp-1 might be efficacious for prophylactic approach. In blood, pVp-1 reached a high titer within the first hour following IP injection. In contrast, no phage was detected until 1 h, and it took longer to reach a higher titer, when it was administered orally. Moreover, the highest titer in each organ was also much higher in the IP route of administration compared to the oral route of administration. Therefore, it was speculated that the IP route of administration would be the more suitable route of administration.

Phage treatment trials in the mouse model for CRS 09-17 demonstrated that the application of pVp-1 can protect from a *V. parahaemolyticus* infection in all four criteria, and pVp-1 can be used as a therapeutic agent to reduce the impact of epidemics caused by multiple-antibiotic-resistant pandemic strains.

While pVp-1 invoked an immune response in mice, the antibodies raised over the course of repeated injections were not associated with anaphylaxis or other adverse reactions. These experiments were designed as a model for acute human infections, where antibiotics are no longer effective and a single course of phage treatment may rescue the patient. If phages are to be employed repeatedly (e.g., for chronic infections), selection use and phage display may produce phage variants that are less prone to induce an immune response (2).

In 2006, the Food and Drug Administration (FDA) approved the use of a commercial phage cocktail (List-Shield; Intralytix, Inc.) as a biocontrol agent. This is confirmation that the FDA's view of phages is that they are safe for human use and opens the doors for phage commercialization for human applications (10). Despite no reports of significant adverse reactions during the long history of phage administration in humans, phage therapy still needs to gain credibility to overcome the regulatory hurdles facing its adoption in mainstream clinical practice (10). Moreover, it is necessary to establish adequate phage

preparation methodologies such as the purification and removal of endotoxins for safety in phage therapy to prevent anaphylactic responses (3, 15, 18, 19).

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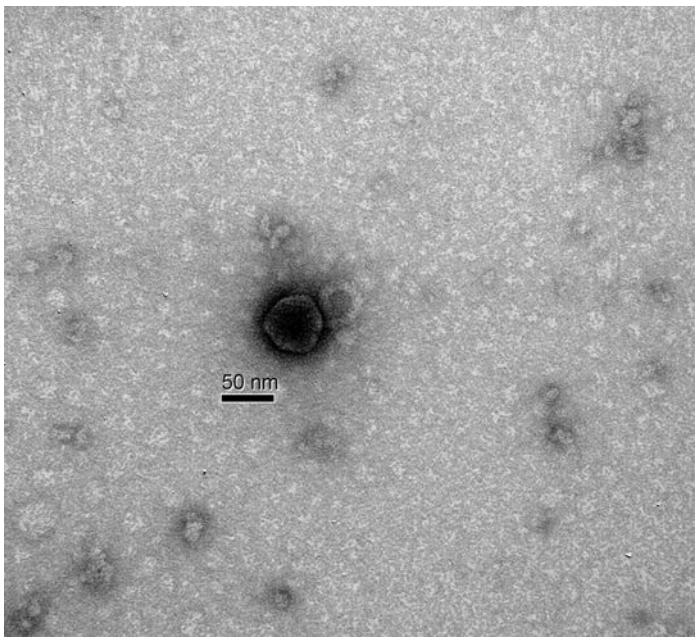


**Figure 2.1.** Electron micrograph of negatively stained phage pVp-1. The bar corresponds to 50 nm.

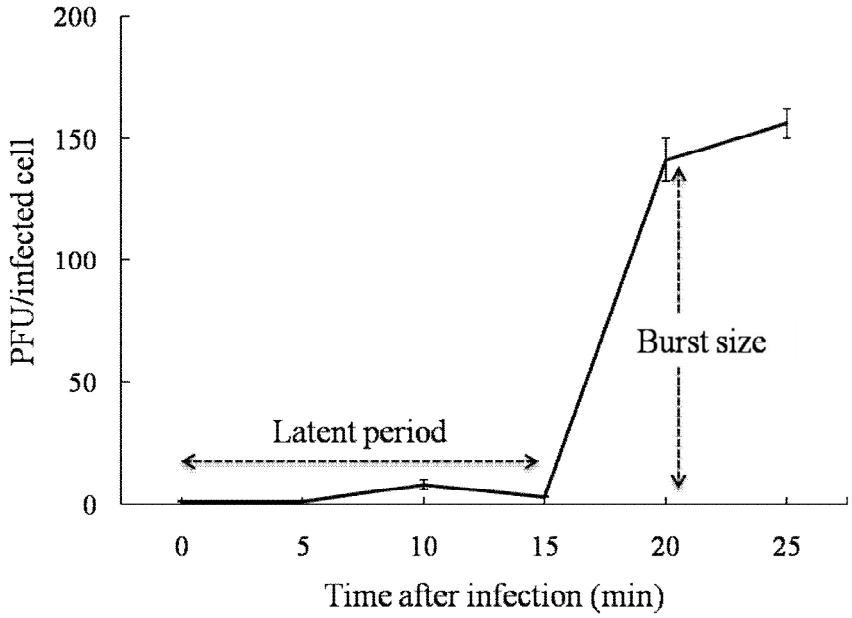


**Figure 2.2.** One-step growth curve of pVp-1. The error bars indicate standard deviations.

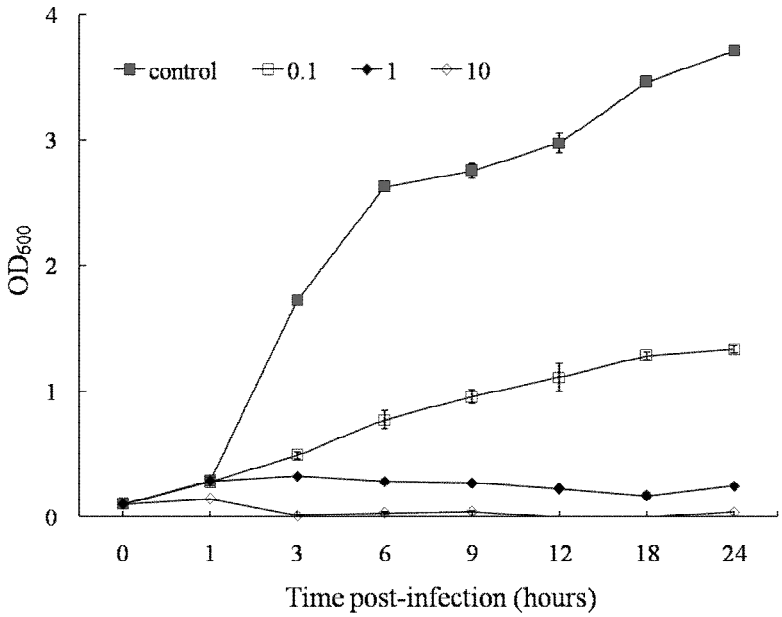


**Figure 2.3.** The bacteriolytic effect of pVp-1 against *V. parahaemolyticus* CRS 09-17. Early exponential phase cultures of *V. parahaemolyticus* CRS 09-17 were co-cultured with pVp-1 at MOIs of 0, 0.1, 1, and 10. The results are shown as the mean ± standard deviations from triplicate experiments.

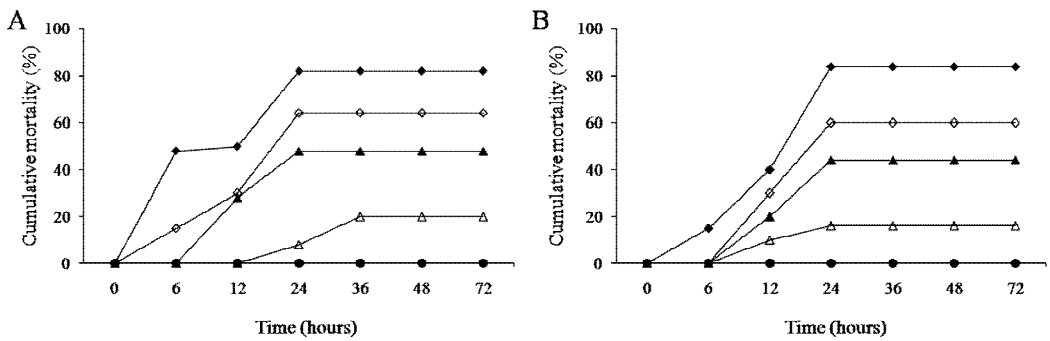


**Figure 2.4.** Experimental infection of mouse model. Experimental infection by IP, *A* or orally, *B*. Four test groups were infected (dose volume 0.2 ml) with $2.0\times10^8$ CFU per mouse (◆), $2.0\times10^7$ CFU per mouse (◇), $2.0\times10^6$ CFU per mouse (▲), and $2.0\times10^5$ CFU per mouse (△) of *V. parahaemolyticus* CRS 09-17. Control (●) group was administered with PBS.

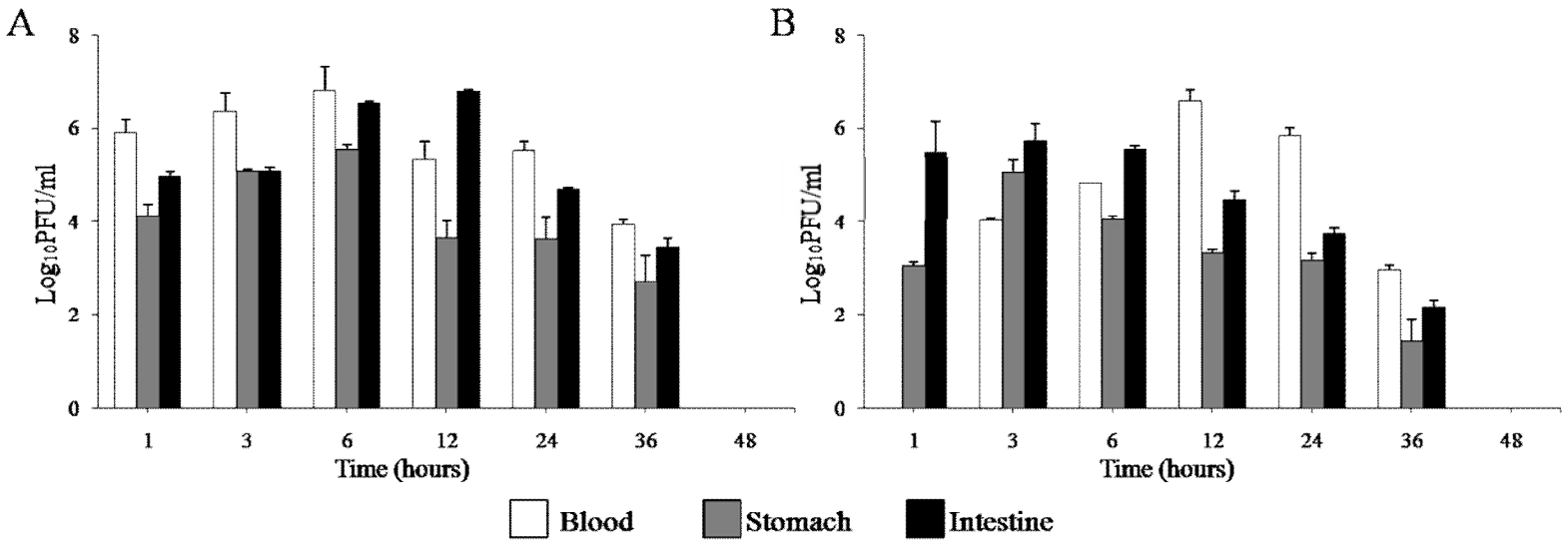


**Figure 2.5.** Kinetics of pVp-1 in the mouse model. Phage was injected via IP, *A* and administered orally, *B* at $10^8$ PFU/mouse. After 1, 3, 6, 12, 24, 36, and 48 h of phage inoculations, blood, stomach, and intestine were removed and their phage titers were estimated. Titers are presented as the means of three experiments performed in triplicate, and the error bars represent the SD ($n = 3$).

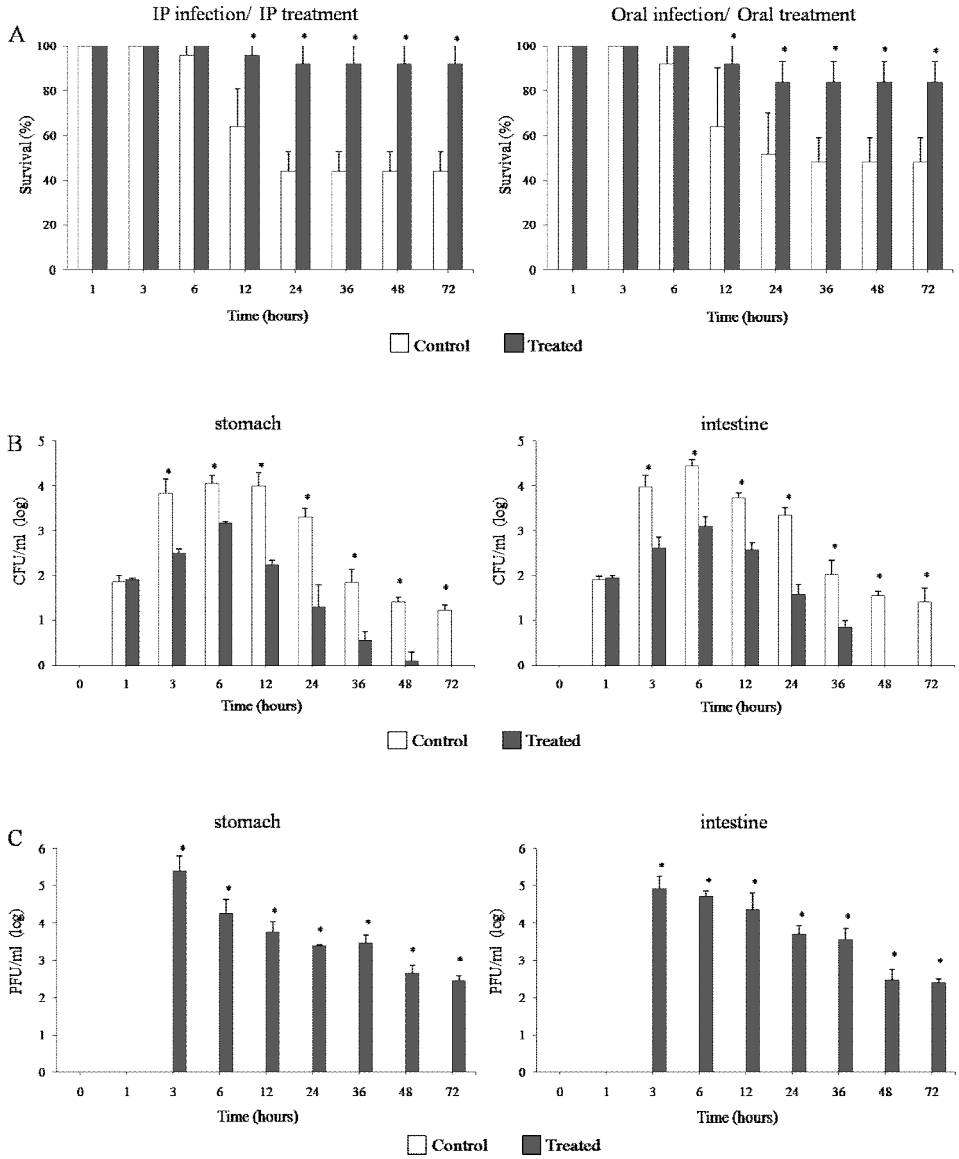


**Figure 2.6.** Effect of phage treatment on *V. parahaemolyticus* infection in mice. *A*, The survival rate of mice in the control (treated with phosphate buffered saline, PBS) and treated (phage treated) groups. The $LD_{50}$ of *V. parahaemolyticus* CRS 09-17 ($2.0\times10^7$ CFU/mouse) that was required to induce an acute death model by way of the IP and oral routes. The phage, pVp-1 ($2.0\times10^8$

PFU/mouse), was applied by IP injection and oral administration after a 1 h CRS 09-17 challenge because the maximum effect of cell lysis was examined at an MOI of 10. *B*, CFU per gram of target organs (stomach/intestine) in the experimental mice through IP infection / IP treatment. *C*, The PFU per gram of target organs (stomach/intestine) in the experimental mice through IP infection/ IP treatment. The bars show the mean, and the error bars show the standard error. Significant differences ($p<0.05$) were observed at various time points (shown with asterisks).

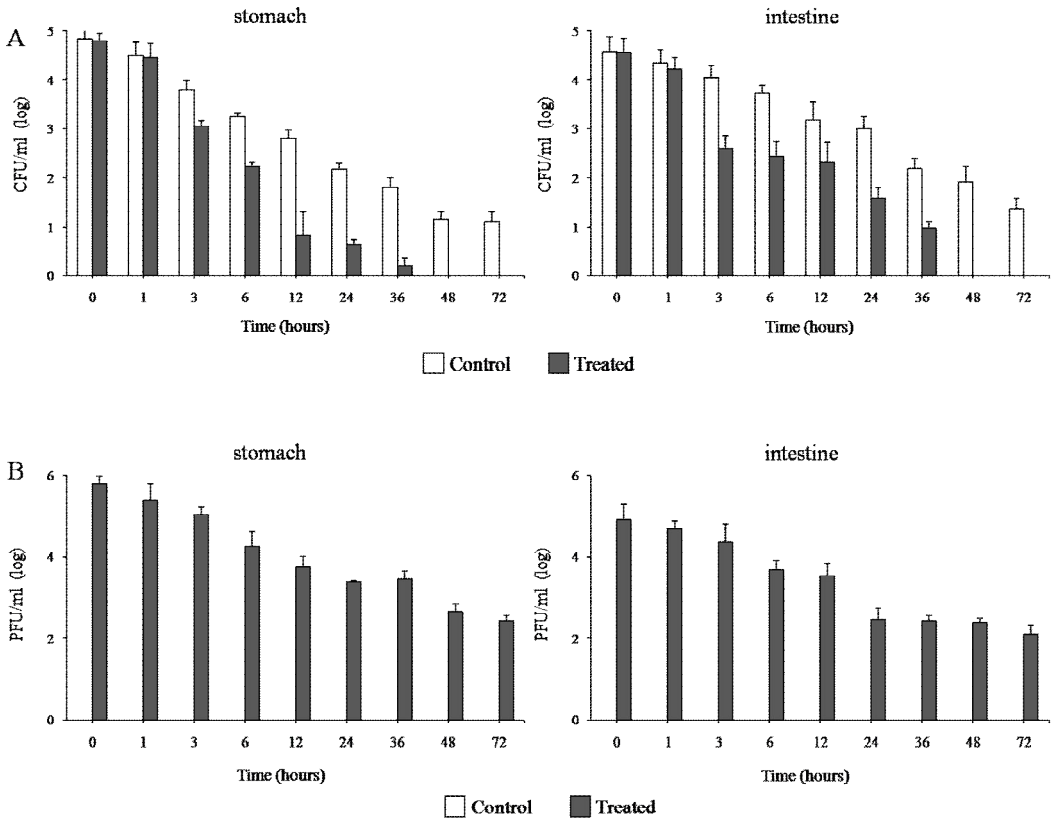


**Figure 2.7.** CFU and PFU values of the stomach/intestine. Effect of phage treatment on *V. parahaemolyticus* infection in mice. *A*, CFU per gram of target organs (stomach/intestine) in the experimental mice through oral infection / oral treatment. *B*, The PFU per gram of target organs (stomach/intestine) in the experimental mice through oral infection/ oral treatment. In the oral infection / oral treatment group, the maximum CFU and PFU values of stomach / intestine were determined at the experimental onset and the values were gradually decreased. The bars show the mean, and the error bars show the standard error.

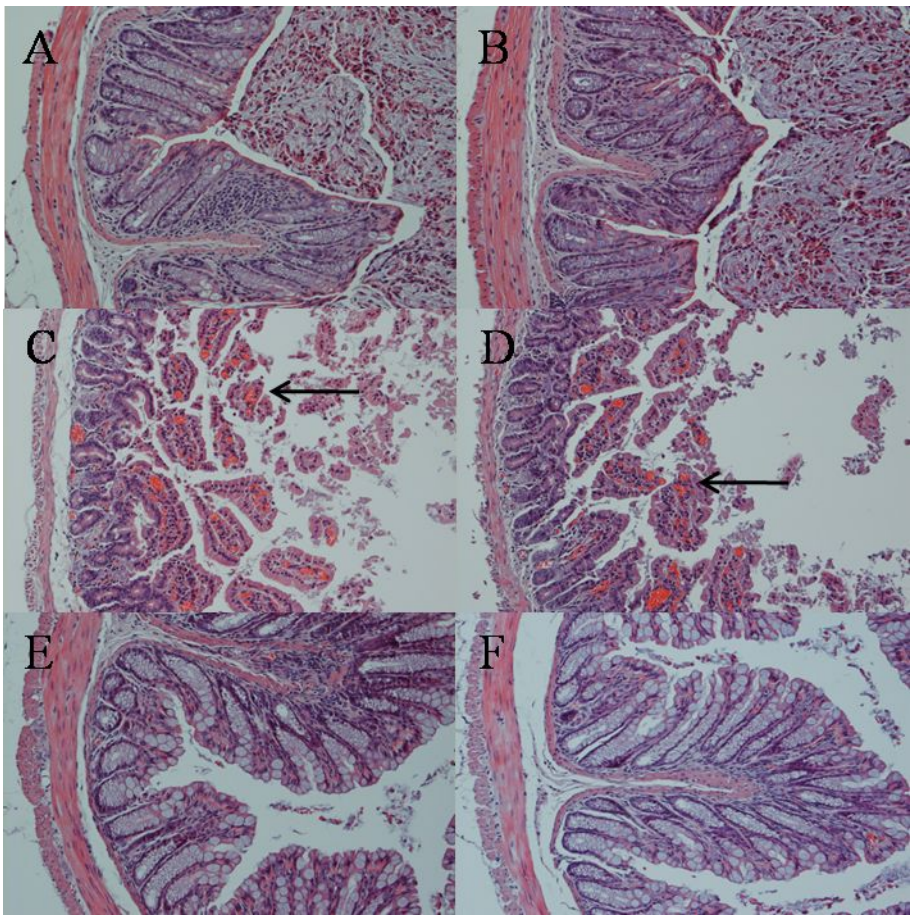


**Figure 2.8.** Histopathologic features of the intestines of mice infected with *V. parahaemolyticus* CRS 09-17 and treated with the phage pVp-1. The micrographs depict the histologic features of the mice from the experiment. *A*, A healthy mouse that only received a PBS IP injection. *B*, A healthy mouse that only received PBS oral administration. *C*, A control mouse (IP infection / no phage treatment). *D*, A control mouse (oral infection / no phage treatment). Deteriorated crypts are indicated in *C* and *D*. *E*, A phage-treated mouse (IP infection / IP treatment). *F*, A phage-treated mouse (oral infection / oral treatment). The phage-treated mice demonstrated the protected morphology of the crypt in both the IP and oral treatment groups. Sections were stained with hematoxylin and eosin and observed at a magnification of ×200.

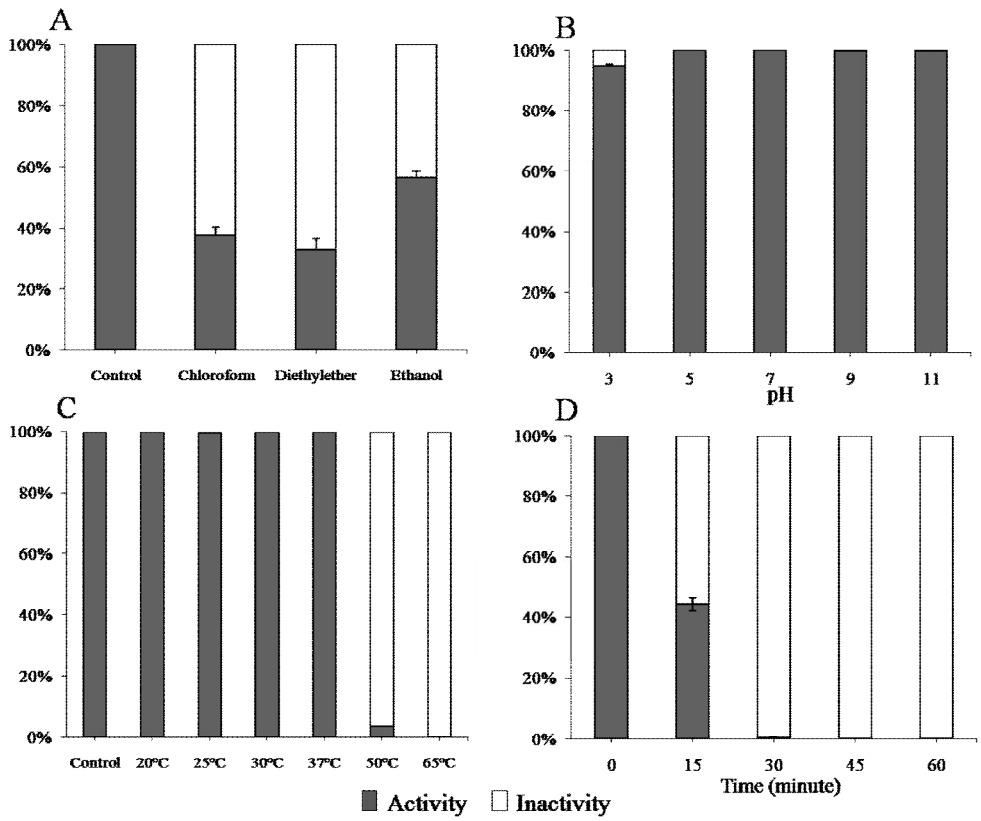


**Figure 2.9.** The sensitivity of pVp-1 to various organic solvents: *A*, pH: *B*, temperatures: *C*, and exposure to UV light: *D*. For sensitivity to various factors, optimal conditions included sterile PBS: *A*, pH 7: *B*, 4°C: *C*, and 0 min: *D* and acted as a control. All values represent the mean of three experiments performed in triplicate on different occasions, with error bars representing the standard deviations (SD; $n = 3$).

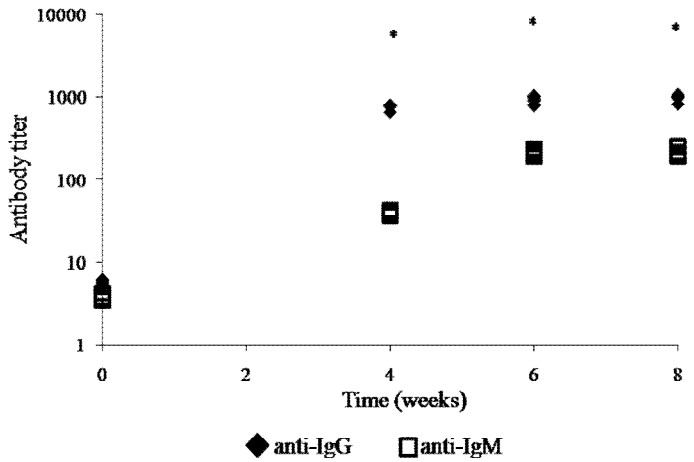


**Figure 2.10.** Antibody titers in mice ($n$ = 5) to repeated injections of phage pVp-1. Phage was IP injected at the indicated time points. The resulting titers of anti-IgG and anti-IgM antibodies are indicated. Significant differences ($p$<0.01) were observed at various time points (shown with asterisks).

# Chapter III

## Complete genome sequence of a novel marine siphovirus pVp-1, infecting *Vibrio parahaemolyticus*


### Abstract

Among the abundant bacteriophages that belong to the order *Caudovirales* in the ocean, the genome sequence of marine siphoviruses are poorly investigated in comparison to myo- or podoviruses. Herein, the complete genome sequence of *Vibrio* phage pVP-1 was reported, which belongs to the family *Siphoviridae* and infects *V. parahaemolyticus* ATCC 33844.



**Keywords:** *Vibrio parahaemolyticus*; Bacteriophage; pVp-1; *Siphoviridae*.

## 3.1. Introduction

Marine viruses are the most abundant biological entities in the ocean (10), thus making its genome analysis essential for a better understanding of its enormous genetic diversity (1). Most reported marine viruses up to date are bacteriophages (phages) that belong to the order *Caudovirales*, which is divided into three families: *Myoviridae*, *Podoviridae* and *Siphoviridae* (10). Among the genome-sequenced marine phages, siphoviruses are relatively poorly investigated (9) and only two of those including phiHSIC (7) and SIO-2 (1) were studied and reported to infect *Vibrio* spp.. Herein, the complete genome sequence of a novel marine siphovirus pVp-1 was reported, which was isolated from the coastal water of the Yellow sea in Korea and infects *V. parahaemolyticus* ATCC 33844, which was isolated from patient with food poisoning.

## 3.2. Materials and methods

Genomic DNA was extracted as previously described (8), and sequenced using standard shotgun sequencing reagents and a 454 GS-FLX Titanium Sequencing System (Roche) by Macrogen in Korea (Approximately 50 × coverage). The full-length genome sequence was obtained by sequence assembly using the SeqMan II sequence analysis software (DNASTAR). The putative open reading frames (ORFs) were predicted using Glimmer 3.02 (2) and GeneMark.hmm (6), and putative ORF functions were analyzed by BLASTP and InterProScan (12). Putative tRNA genes were searched for using tRNAscan-SE (v. 1.21) software (5).

## 3.3. Results

The double-stranded and non-redundant DNA genome of pVp-1 was 111,506 bp in length with a G+C composition of 39.71%. A total of 157 ORFs containing more than 40 amino acid residues and 19 tRNAs (including 1 pseudogene) were identified, suggesting this as the first marine phage genome in the family *Siphoviridae* with a large number of tRNAs capable of infecting *V. parahaemolyticus*. A number of 48 ORFs showed no homology to proteins from the GenBank database, while other 69 and 40 ORFs code for proteins with some homology to known phage and bacteria-related proteins, respectively. Among the 40 bacteria-related genes in phage pVp-1, 5 ORFs (*orf34, orf38, orf79, orf85 and orf97*) were highly homologous to *Vibrio*-related proteins and 35 ORFs shared some similarities with unrelated bacteria spanning a wide range of phyla. The positions, directions, sizes, molecular weights, and putative functions of each pVp-1 ORFs are shown in **Table 3.1**. The results demonstrate that pVp-1 has a unique genomic composition. Lysogeny genes responsible for the lysogenic properties of this phage, such as integrase, were not found in the genome of pVp-1. None of the ORFs showed any similarities with pathogenecity factors that can cause problems in clinical usage.

Bioinformatic analyses were performed for the assignment of putative functions to 69 phage-related ORFs, and those ORFs were clustered together by at least 3 functional roles such as DNA metabolism (*orf2, orf3, orf4, orf6, orf7, orf12, orf14, orf15, orf16, orf17, orf18, orf21, orf28, orf32, orf42* and *orf52*), viral morphogenesis (*orf139, orf141, orf143, orf144, orf148, orf149, orf153, orf155, orf156* and *orf157*) and lytic properties (*orf73, orf82* and *orf83*). Interestingly, most ORFs encoding DNA metabolism and viral morphogenesis genes were clustered together at each ends of the sequenced genome by

functional roles, and were similar (≤ 79%) to those of T5 (11) or T5-like phages (3, 4), thus indicating a close genetic relatedness between pVp-1 and those phages. Genomic comparison of pVp-1 with the phage T5 revealed that these two phages are highly similar in gene inventory. The putative ORFs in the pVp-1 genome were predicted using CoreGenes and had high similarity to the ORFs of the phage T5 (**Figure 3.1**). Additionally, according to the ACT comparison results, two parts of the pVp-1 genome was reversed relative to the order in the T5 genome (**Figure 3.2**).

In contrast, there were no sequence similarities to marine *Vibrio* phages belonging to *Siphoviridae* (phiHSIC and SIO-2), and high proportions of genes in pVp-1 were not similar to other sequenced phages or bacteria.

The genome sequence of *Vibrio* phage pVp-1 was deposited in the GenBank under accession number JQ340389.

## 3.4. Discussion

Based on these results, the newly sequenced *Vibrio* phage pVp-1 could be considered as a novel T5-like virus, and will help to advance the understanding of the biodiversity of marine phages belongs to the family *Siphoviridae*. In addition, a novel virulent phage, pSf-1, capable of inhibiting *V. parahaemolyticus* growth indicated the safety needed for phage therapy against vibriosis.

## 3.5. References


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**Table 3.1.** Predicted genes and gene products of pVp-1.

| Gene | | | Gene product | | | Amino acid Identity (%) | Putative function [organism] (E-value) | Predicted TMH and signal peptide | |
|---|---|---|---|---|---|---|---|---|---|
| ORF No. | Range | Strand | aa size | MW (kD) | pI | | | TMHHM | SignalP |
| 1 | 109-663 | + | 185 | 21.1 | 10.11 | 31 | gp30 DNA ligase [Acinetobacter phage Acj61] (9.7) | 0 | N |
| 2 | 690-1142 | - | 151 | 17.0 | 5.14 | 47 | putative deoxyUTP pyrophosphatase [Enterobacteria phage T5] (4e-26) | 0 | N |
| 3 | 1142-2014 | - | 291 | 33.8 | 4.77 | 52 | flap endonuclease [Enterobacteria phage T5] (2e-71) | 0 | N |
| 4 | 2011-2499 | - | 163 | 18.7 | 8.56 | 53 | D14 protein [Enterobacteria phage T5] (1e-44) | 0 | N |
| 5 | 2492-2785 | - | 98 | 11.0 | 5.72 | 37 | response regulator receiver domain protein [Bacteroides ovatus SD CC 2a] (3.5) | 0 | N |
| 6 | 2778-4652 | - | 625 | 71.4 | 5.72 | 43 | probable exonuclease subunit 2 [Enterobacteria phage T5] (5e-117) | 0 | N |
| 7 | 4642-5625 | - | 328 | 37.9 | 5.65 | 56 | putative recombination endonuclease, subunit D12 [Enterobacteria phage T5] (2e-93) | 0 | N |
| 8 | 5625-6002 | - | 126 | 14.9 | 4.52 | 34 | ATP-dependent DNA helicase RecG [Fusobacterium periodonticum ATCC 33693] (3.0) | 0 | N |
| 9 | 6049-6852 | - | 268 | 29.9 | 4.74 | 31 | D11 protein [Enterobacteria phage T5] (8e-28) | 0 | N |
| 10 | 6849-7052 | - | 68 | 8.2 | 6.55 | 55 | hypothetical protein T5.125 [Enterobacteria phage T5] (8e-12) | 0 | N |
| 11 | 7269 - 7667 | - | 133 | 15.3 | 4.58 | 27 | periplasmic sensor signal transduction histidine kinase [Ruegeria sp. R11] (1.0) | 0 | N |
| 12 | 7700 - 9055 | - | 452 | 51.2 | 8.34 | 55 | putative ATP-dependent helicase [Enterobacteria phage T5] (2e-136) | 0 | N |
| 13 | 9052 - 9567 | - | 172 | 20.5 | 9.62 | 38 | hypothetical protein [Enterobacteria phage T5] (3e-25) | 0 | N |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 14 | 9557 - 12130 | - | 858 | 97.8 | 5.75 | 58 | DNA polymerase [Enterobacteria phage T5] (0.0) | 0 | N |
| 15 | 12228 - 12983 | - | 252 | 29.1 | 6.39 | 43 | DNA primase [Enterobacteria phage T5] (3e-51) | 0 | N |
| 16 | 13087 - 14487 | - | 467 | 52.5 | 5.04 | 39 | putative replicative DNA helicase [Enterobacteria phage T5] (7e-97) | 0 | N |
| 17 | 14548 - 15312 | - | 255 | 27.7 | 6.05 | 50 | D5 protein [Enterobacteria phage T5] (4e-52) | 0 | N |
| 18 | 15317 - 16093 | - | 259 | 28.0 | 8.72 | 52 | NAD-dependent DNA ligase, subunit B [Enterobacteria phage T5] (8e-57) | 0 | N |
| 19 | 16250 - 16777 | - | 176 | 19.8 | 5.26 | 32 | hypothetical protein [Pseudomonas phage KPP10] (8e-15) | 0 | N |
| 20 | 17008 - 17229 | - | 74 | 8.3 | 5.65 | 35 | hypothetical protein [Chlamydomonas reinhardtii] (1.6) | 0 | N |
| 21 | 17229 - 18197 | - | 323 | 36.2 | 5.14 | 52 | DNA ligase [Enterobacteria phage T5] (8e-85) | 0 | N |
| 22 | 18231 - 18497 | - | 89 | 10.2 | 6.82 | 28 | unnamed protein product [Tetraodon nigroviridis] (4.3) | 0 | N |
| 23 | 18497 - 18862 | - | 122 | 13.6 | 4.56 | 40 | putative bacteriophage protein; putative prohead protease [Acinetobacter baumannii AB058] (5.5) | 0 | N |
| 24 | 18862 - 19095 | - | 78 | 8.8 | 4.42 | | | 0 | N |
| 25 | 19125 - 19430 | - | 102 | 11.9 | 4.74 | 58 | hypothetical protein [Enterobacteria phage T5] (7e-23) | 0 | N |
| 26 | 19414 - 19734 | - | 107 | 12.2 | 9.82 | 38 | hypothetical protein [Enterobacteria phage T5] (1e-10) | 0 | N |
| 27 | 19773 - 20183 | - | 137 | 14.7 | 4.66 | 36 | D3 protein [Enterobacteria phage T5] (2e-11) | 0 | N |
| 28 | 20262 - 21092 | - | 277 | 31.8 | 8.73 | 35 | D2 protein [Enterobacteria phage T5] (2e-37) | 0 | N |
| 29 | 21302 - 21514 | - | 71 | 7.8 | 9.59 | 42 | similar to 60S ribosomal export protein NMD3 [Taeniopygia guttata] (2.3) | 0 | N |
| 30 | 21694 – 22626 | - | 311 | 35.7 | 9.32 | 43 | hypothetical protein | 0 | N |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | [Salmonella phage E1] (2e-33) | | |
| 31 | 22587 – 22778 | - | 64 | 7.7 | 10.0 | 33 | phage integrase [Frankia sp. CcI3] (9.7) | 0 | N |
| 32 | 22791 – 25580 | - | 930 | 106.8 | 6.23 | 46 | putative replication origin binding protein [Enterobacteria phage T5] (0.0) | 0 | N |
| 33 | 26246 - 26560 | - | 105 | 12.0 | 6.64 | 35 | hypothetical protein OsI_37948 [Oryza sativa Indica Group] (2.2) | 0 | N |
| 34 | 26834 - 28987 | + | 718 | 81.0 | 5.31 | 58 | anaerobic ribonucleoside triphosphate reductase [Vibrio parahaemolyticus 10329] (0.0) | 0 | N |
| 35 | 29026 - 29238 | + | 71 | 8.3 | 4.94 | 32 | glutaredoxin 2 [Vibrio sp. MED222] (0.071) | 0 | N |
| 36 | 29238 - 30230 | + | 331 | 38.5 | 5.81 | 64 | hypothetical protein ORF027 [Pseudomonas phage PA11] (3e-121) | 0 | N |
| 37 | 30230 - 31924 | + | 565 | 63.4 | 5.34 | 68 | hypothetical protein ORF029 [Pseudomonas phage PA11] (0.0) | 0 | N |
| 38 | 31926 - 32399 | + | 158 | 18.0 | 7.55 | 63 | anaerobic ribonucleoside-triphosphate reductase activating protein [Vibrio parahaemolyticus AQ3810] (7e-50) | 0 | N |
| 39 | 32486 - 32884 | + | 133 | 15.4 | 4.19 | 50 | hypothetical protein [Trichomonas vaginalis G3] (4.4) | 0 | N |
| 40 | 32871 – 33314 | + | 148 | 17.1 | 9.05 | 38 | hypothetical protein [Deftia phage phiW-14] (9e-20) | 0 | N |
| 41 | 33295 - 33522 | + | 76 | 8.4 | 7.81 | 39 | Aldo/keto reductase (ISS) [Ostreococcus tauri] (1.4) | 0 | N |
| 42 | 33503 - 33961 | + | 153 | 16.9 | 5.92 | 46 | ribonuclease H [Enterobacteria phage T5] (4e-31) | 0 | N |
| 43 | 33958 - 34158 | + | 67 | 8.0 | 9.90 | | | 0 | N |
| 44 | 34206 - 34439 | + | 78 | 9.0 | 5.69 | | | 0 | N |
| 45 | 34443 - 34634 | + | 64 | 7.7 | 6.57 | 36 | BcepGomrgp36 [Burkholderia phage BcepGomr] (1.2) | 0 | N |
| 46 | 34703 - 34969 | + | 89 | 10.1 | 4.45 | 53 | hypothetical protein [Enterobacteria phage T5] (0.22) | 0 | N |
| 47 | 35038 - 35313 | + | 92 | 10.3 | 10.05 | 33 | hypothetical protein | 0 | N |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | [Coraliomargarita akajimensis DSM 45221] (9.4) | | |
| 48 | 35313 - 35480 | + | 56 | 6.5 | 4.56 | 36 | hypothetical protein [Acinetobacter phage 133] (0.41) | 0 | N |
| 49 | 35473 - 35736 | + | 88 | 9.9 | 11.51 | | | 0 | N |
| 50 | 35736 - 35933 | + | 66 | 8.0 | 5.26 | 36 | hypothetical protein [Shigella phage SP18] (0.014) | 0 | N |
| 51 | 36071 - 36316 | + | 82 | 9.7 | 6.28 | 68 | hypothetical protein [Vibrio phage KVP40] (5.9) | 0 | N |
| 52 | 36303 - 37061 | + | 253 | 28.1 | 4.82 | 32 | phage exodeoxyribonuclease [Escherichia coli TA280] (5e-16) | 0 | N |
| 53 | 37137 - 37331 | + | 65 | 7.2 | 10.76 | | | 0 | N |
| 54 | 37383 - 37619 | + | 79 | 8.6 | 11.0 | | | 2 | N |
| 55 | 37813 - 37968 | + | 52 | 5.8 | 11.74 | | | 0 | Y |
| 56 | 39330 - 39698 | + | 123 | 14.0 | 9.22 | 35 | hypothetical protein [Klebsiella phage KP32] (9.0) | 0 | N |
| 57 | 40243 – 40425 | + | 61 | 6.8 | 6.54 | | | 0 | N |
| 58 | 40933 – 41337 | + | 135 | 15.2 | 9.12 | 26 | hypothetical protein [Vibrio phage KVP40] (0.10) | 0 | N |
| 59 | 41334 - 41564 | + | 77 | 8.8 | 7.70 | 32 | hypothetical protein [Trichoplax adhaerens] (3.2) | 0 | N |
| 60 | 42004 – 42186 | + | 61 | 7.2 | 10.01 | | | 0 | N |
| 61 | 42571 - 42774 | + | 68 | 8.8 | 10.47 | 48 | hypothetical protein [Aeromonas phage Aeh1] (0.16) | 0 | N |
| 62 | 42800 - 43348 | + | 183 | 20.6 | 4.96 | 41 | hypothetical protein [Staphylococcus carnosus subsp. carnosus TM300] (8e-26) | 0 | N |
| 63 | 43348 - 43980 | + | 211 | 23.0 | 9.33 | 29 | nicotinamide mononucleotide transporter [Enterobacteria phage EPS7] (1e-10) | 6 | Y |
| 64 | 44063 - 44323 | + | 87 | 9.8 | 4.42 | 35 | gp31 protein [Vibrio phage VP58.5] (0.018) | 0 | N |
| 65 | 44316 - 44462 | + | 49 | 5.5 | 4.04 | | | 0 | N |
| 66 | 44524 - 44829 | + | 102 | 11.4 | 4.62 | 38 | hypothetical protein SINV_00549 [Solenopsis invicta] (4.3) | 0 | N |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 67 | 45031 - 45225 | + | 65 | 7.6 | 9.73 | | | 0 | N |
| 68 | 45328 – 45492 | + | 55 | 6.4 | 7.71 | | | 1 | N |
| 69 | 45662 – 46285 | + | 208 | 23.1 | 6.22 | 34 | hypothetical protein [Enterobacteria phage T5] (7e-13) | 0 | N |
| 70 | 46653 - 46919 | + | 89 | 10.1 | 9.16 | 46 | hypothetical protein [Aeromonas phage phiAS4] (3e-12) | 0 | N |
| 71 | 46912 – 47283 | + | 124 | 14.3 | 9.00 | 38 | intracellular protein transport protein (UsoA) [Aspergillus oryzae RIB40] (7.7) | 1 | Y |
| 72 | 47280 - 47573 | + | 98 | 11.4 | 8.42 | 26 | PseT.2 conserved hypothetical protein [Enterobacteria phage T4] (1.2) | 0 | Y |
| 73 | 47563 – 48210 | + | 216 | 24.3 | 4.66 | 30 | deoxynucleoside monophosphate kinase [Enterobacteria phage T5] (2e-10) | 0 | N |
| 74 | 48368 - 48703 | + | 112 | 12.0 | 4.27 | 37 | gp71 [Phage phiJL001] (2e-04) | 0 | N |
| 75 | 48714 - 51221 | + | 836 | 89.8 | 4.90 | 31 | hypothetical protein [Vibrio phage ICP2] (2e-15) | 0 | N |
| 76 | 51235 - 53820 | + | 862 | 93.3 | 4.58 | 30 | hypothetical protein TU18-25_00220 [Vibrio phage ICP3_2009_A] (0.011) | 0 | N |
| 77 | 53859 - 54329 | + | 157 | 16.2 | 4.47 | 35 | hypothetical protein [Synechococcus elongatus PCC 6301] (9e-05) | 0 | Y |
| 78 | 54314 - 54721 | + | 136 | 15.7 | 6.57 | 37 | dipicolinic acid synthetase, A subunit [Geobacillus sp. Y4.1MC1] (4.9) | 1 | Y |
| 79 | 54679 - 55014 | + | 112 | 12.3 | 4.65 | 31 | hypothetical protein VSAK1_25885 [Vibrio shilonii AK1] (5e-07) | 0 | N |
| 80 | 55007 - 55165 | + | 53 | 5.7 | 3.92 | | | 0 | N |
| 81 | 55162 - 55371 | + | 70 | 7.8 | 7.83 | 38 | CheA signal transduction histidine kinase [Geobacter lovleyi SZ] (3.5) | 0 | N |
| 82 | 55595 - 56266 | + | 224 | 25.6 | 9.00 | 39 | putative holing [Enterobacteria phage T5] (2e-39) | 1 | N |
| 83 | 56268 - 56657 | + | 130 | 14.7 | 9.71 | 58 | gp43 protein [Enterobacteria phage K1E] (1e-29) | 0 | N |
| 84 | 56751 – 57212 | + | 154 | 17.9 | 7.10 | 30 | Metal dependent phosphohydrolase [Streptococcus phage 8140] (7e-07) | 0 | N |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 85 | 57269 – 58309 | + | 347 | 39.8 | 7.77 | 29 | possible DNA binding protein [Xanthomonas phage Xp15] (1e-13) | 0 | N |
| 86 | 58382 - 58684 | + | 101 | 12.0 | 9.65 | 30 | tRNA-splicing endonuclease subunit sen34 [Paracoccidioides brasiliensis Pb01] (3.3) | 0 | N |
| 87 | 58704 – 58856 | + | 51 | 6.2 | 7.09 | | | 0 | N |
| 88 | 58841 - 59047 | + | 69 | 7.7 | 6.02 | 37 | Metalloreductase [Cryptococcus gattii WM276] (7.6) | 1 | Y |
| 89 | 59038 – 59409 | + | 124 | 14.7 | 5.11 | 26 | acetyl-CoA hydrolase/transferase [Eubacterium cellulosolvens 6] (0.89) | 0 | N |
| 90 | 59402 – 59626 | + | 75 | 8.4 | 9.41 | 35 | conjugation system ATPase, TraG family [Bacteroides salanitronis DSM 18170] (0.90) | 0 | N |
| 91 | 59628 - 59951 | + | 108 | 12.1 | 5.77 | 33 | (dimethylallyl)adenosine tRNA methylthiotransferase [Azoarcus sp. EbN1] (0.57) | 0 | N |
| 92 | 59935 - 60153 | + | 73 | 8.8 | 10.61 | | | 0 | N |
| 93 | 60150 – 60314 | + | 55 | 5.9 | 4.20 | | | 1 | Y |
| 94 | 60311 - 60622 | + | 104 | 12.0 | 5.34 | 30 | hypothetical protein PTSG_05611 [Salpingoeca sp. ATCC 50818] (1.6) | 1 | Y |
| 95 | 60687 – 61925 | + | 413 | 46.2 | 9.14 | 37 | ABC transporter ATP-binding protein [Corynebacterium kroppenstedtii DSM 44385] (4.2) | 0 | N |
| 96 | 61985 – 62143 | + | 53 | 6.0 | 9.30 | 33 | unnamed protein product [Oikopleura dioica] (7.3) | 1 | N |
| 97 | 62145 – 63392 | + | 416 | 46.3 | 9.07 | 24 | conserved hypothetical protein [Vibrio parahaemolyticus 16] (3e-06) | 1 | Y |
| 98 | 63456 – 63854 | + | 133 | 15.4 | 9.19 | 32 | periplasmic binding protein [Bacteroides salanitronis DSM 18170] (2.0) | 2 | N |
| 99 | 63842 – 64243 | + | 134 | 15.4 | 6.65 | 33 | ABC transporter related protein [Clostridium thermocellum DSM 1313] (2.5) | 0 | N |
| 100 | 64347 – 65087 | + | 247 | 28.4 | 5.33 | 32 | serine/threonine protein phosphatase [Enterobacteria phage T5] (8e-23) | 0 | N |
| 101 | 65149 – 65349 | + | 67 | 7.7 | 5.18 | 35 | thioesterase superfamily protein | 0 | N |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 102 | 65349 - 65645 | + | 99 | 11.3 | 4.57 | 38 | [Desulfococcus oleovorans Hxd3] (9.1)<br>transcriptional regulator<br>[Bradyrhizobium japonicum USDA 110] (8.0) | 0 | N |
| 103 | 65632 - 66015 | + | 128 | 14.9 | 4.65 | 31 | sodium/potassium-transporting ATPase subunit alpha-4<br>[Bos taurus] (5.5) | 0 | N |
| 104 | 66056 - 66574 | + | 173 | 20.5 | 4.66 | 38 | RNA helicase, putative<br>[Trypanosoma brucei gambiense DAL972] (7.1) | 0 | N |
| 105 | 66571 – 66945 | + | 125 | 14.6 | 9.17 | 36 | glutamate synthase, NADH/nadph, small subunit<br>[Paenibacillus polymyxa SC2] (6.2) | 0 | N |
| 106 | 66942 – 67148 | + | 69 | 8.0 | 4.79 | | | 0 | N |
| 107 | 67301 - 68263 | + | 321 | 37.0 | 6.36 | 50 | conserved hypothetical protein<br>[Aeromonas phage PX29] (1e-83) | 1 | N |
| 108 | 68267 – 68827 | + | 187 | 21.1 | 5.52 | 61 | hypothetical protein<br>[Aeromonas phage Aeh1] (3e-54) | 1 | Y |
| 109 | 68902 - 69183 | + | 94 | 11.3 | 4.44 | | | 0 | N |
| 110 | 69183 - 69509 | + | 109 | 13.0 | 9.70 | 36 | conserved hypothetical protein<br>[Candida albicans WO-1] (6.6) | 0 | N |
| 111 | 69547 – 69708 | + | 54 | 6.2 | 6.52 | | | 1 | N |
| 112 | 69755 – 70312 | + | 186 | 21.0 | 8.50 | 24 | ORF090<br>[Staphylococcus phage Twort] (8.7) | 0 | N |
| 113 | 70306 - 70455 | + | 50 | 5.5 | 9.10 | | | 1 | N |
| 114 | 70457 – 71092 | + | 212 | 25.2 | 9.06 | 28 | beta-lactamase domain protein<br>[Dethiobacter alkaliphilus AHT 1] (0.011) | 0 | N |
| 115 | 71153 - 71485 | + | 111 | 13.4 | 4.19 | 33 | hypothetical protein<br>[Enterobacteria phage 933W] (3.9) | 0 | N |
| 116 | 71463 – 71714 | + | 84 | 9.9 | 5.63 | 37 | argininosuccinate lyase<br>[Brevibacillus brevis NBRC 100599] (0.90) | 0 | N |
| 117 | 71692 - 72018 | + | 109 | 12.3 | 4.29 | 31 | retrotransposon protein, putative, Ty3-gypsy subclass<br>[Oryza sativa Japonica Group] (0.83) | 0 | N |
| 118 | 72090 – 72470 | + | 127 | 14.6 | 4.29 | 32 | aminopeptidase P<br>[Reinekea sp. MED297] (4.0) | 0 | N |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 119 | 72472 - 72714 | + | 81 | 9.1 | 8.96 | 25 | unnamed protein product [Oikopleura dioica] (4.4) | 0 | N |
| 120 | 72686 - 72910 | + | 75 | 8.9 | 4.47 | 39 | serine/threonine protein kinase [Methanosarcina barkeri str. Fusaro] (7.3) | 0 | N |
| 121 | 72907 – 73146 | + | 80 | 9.2 | 6.54 | | | 0 | Y |
| 122 | 73113 - 73247 | + | 45 | 5.4 | 8.40 | 48 | similar to Cytosolic carboxypeptidase 1 (ATP/GTP-binding protein 1) (Nervous system nuclear protein induced by axotomy) [Ciona intestinalis] (9.4) | 0 | N |
| 123 | 73514 - 74662 | + | 383 | 44.5 | 8.88 | 28 | hydrolase, haloacid dehalogenase-like family [Bacillus cereus G9241] (3.8) | 0 | N |
| 124 | 74652 – 75011 | + | 120 | 14.4 | 9.40 | 27 | 6-phosphofructokinase [Clostridium difficile QCD-23m63] (2.5) | 0 | N |
| 125 | 75238 - 75378 | - | 47 | 5.6 | 10.22 | | | 0 | N |
| 126 | 76295 - 76516 | - | 74 | 9.0 | 4.51 | 32 | UDP-galactopyranose mutase [Leishmania major strain Friedlin] (5.2) | 0 | N |
| 127 | 76516 - 76710 | - | 65 | 7.8 | 4.51 | 30 | TRAP family transporter, periplasmic substrate binding subunit [Reinekea sp. MED297] (8.7) | 0 | N |
| 128 | 76971 - 77288 | - | 106 | 12.0 | 7.86 | | | 0 | N |
| 129 | 77316 - 77708 | - | 131 | 15.2 | 5.01 | 28 | chromogranin A (parathyroid secretory protein 1) [Monodelphis domestica] (1.0) | 0 | N |
| 130 | 77805 - 78125 | - | 107 | 12.3 | 3.86 | 25 | Transketolase [Salinispora arenicola CNS-205] (3.4) | 0 | N |
| 131 | 78192 - 78314 | - | 41 | 4.7 | 5.96 | | | 0 | N |
| 132 | 79248 - 79718 | + | 157 | 16.8 | 5.07 | 38 | A2 protein [Enterobacteria phage T5] (5e-09) | 0 | N |
| 133 | 79815 - 80186 | + | 124 | 13.8 | 9.37 | 25 | DEAD/DEAH box helicase, putative [Arabidopsis thaliana] (2.9) | 0 | N |
| 134 | 80288 - 81943 | + | 552 | 61.8 | 6.14 | 31 | A1 [Enterobacteria phage T5] (2e-50) | 0 | N |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 135 | 82024 - 82662 | + | 213 | 24.5 | 4.57 | 41 | 5'-deoxyribonucleotidase [Enterobacteria phage T5] (2e-27) | 0 | N |
| 136 | 82810 - 83478 | - | 223 | 25.5 | 8.61 | 28 | hypothetical protein [Enterobacteria phage T5] (8e-08) | 0 | N |
| 137 | 83490 - 85427 | - | 646 | 72.6 | 9.67 | 30 | hypothetical protein EUBDOL_02205 [Eubacterium dolichum DSM 3991] (2.2) | 0 | N |
| 138 | 85549 - 86019 | + | 157 | 17.6 | 5.12 | 49 | hypothetical protein [Enterobacteria phage T5] (8e-27) | 0 | N |
| 139 | 86019 - 87338 | + | 440 | 50.8 | 5.04 | 59 | terminase large subunit [Enterobacteria phage T5] (9e-144) | 0 | N |
| 140 | 87420 - 87890 | + | 157 | 18.1 | 7.75 | 51 | hypothetical protein [Enterobacteria phage T5] (5e-31) | 0 | N |
| 141 | 87893- 89119 | + | 409 | 46.1 | 5.30 | 51 | portal protein [Enterobacteria phage T5] (1e-123) | 0 | N |
| 142 | 89109 - 89354 | + | 82 | 9.6 | 9.24 | 30 | hypothetical protein [Pyrobaculum calidifontis JCM 11548] (9.0) | 0 | N |
| 143 | 89341 - 89946 | + | 202 | 22.3 | 4.78 | 54 | putative prohead protease [Enterobacteria phage T5] (1e-53) | 0 | N |
| 144 | 89954 - 91351 | + | 466 | 51.6 | 5.46 | 60 | major head protein precursor [Enterobacteria phage T5] (2e-142) | 0 | N |
| 145 | 91412 - 91954 | + | 181 | 20.3 | 5.24 | 22 | hypothetical protein [Enterobacteria phage EPS7] (7e-04) | 0 | N |
| 146 | 91954 - 92700 | + | 249 | 28.3 | 9.95 | 45 | hypothetical protein [Enterobacteria phage T5] (1e-52) | 0 | N |
| 147 | 92697 - 93182 | + | 162 | 18.9 | 4.86 | 38 | hypothetical protein [Enterobacteria phage T5] (1e-26) | 0 | N |
| 148 | 93197 - 94612 | + | 472 | 51.7 | 4.68 | 45 | major tail protein [Enterobacteria phage T5] (2e-104) | 0 | N |
| 149 | 94618 - 95502 | + | 295 | 32.7 | 6.14 | 29 | minor tail protein [Enterobacteria phage T5] (8e-17) | 0 | N |
| 150 | 95499 - 95909 | + | 137 | 15.6 | 4.72 | 33 | hypothetical protein [Enterobacteria phage T5] (5e-16) | 0 | N |
| 151 | 95920 - 96312 | + | 131 | 15.2 | 9.06 | 35 | hypothetical protein | 0 | N |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 152 | 96394 - 97152 | + | 253 | 27.6 | 5.62 | 27 | [Enterobacteria phage T5] (9e-14)<br>6-phosphogluconate dehydrogenase [Vibrio salmonicida LFI1238] (3.1) | 0 | N |
| 153 | 97168 - 100239 | + | 1024 | 111.2 | 6.14 | 39 | pore-forming tail tip protein pb2 [Enterobacteria phage T5] (3e-71) | 0 | N |
| 154 | 100404 - 101021 | + | 206 | 23.1 | 4.85 | 54 | hypothetical protein [Enterobacteria phage T5] (1e-55) | 0 | N |
| 155 | 101018 - 103861 | + | 948 | 106.9 | 5.12 | 46 | structural tail protein [Enterobacteria phage T5] (0.0) | 0 | N |
| 156 | 103858 - 106944 | + | 1029 | 111.1 | 6.01 | 46 | tail protein Pb4 [Enterobacteria phage T5] (5e-131) | 0 | N |
| 157 | 106947 - 107729 | + | 261 | 28.0 | 9.44 | 22 | putative phage tail protein [Enterobacteria phage EPS7] (5e-05) | 0 | N |

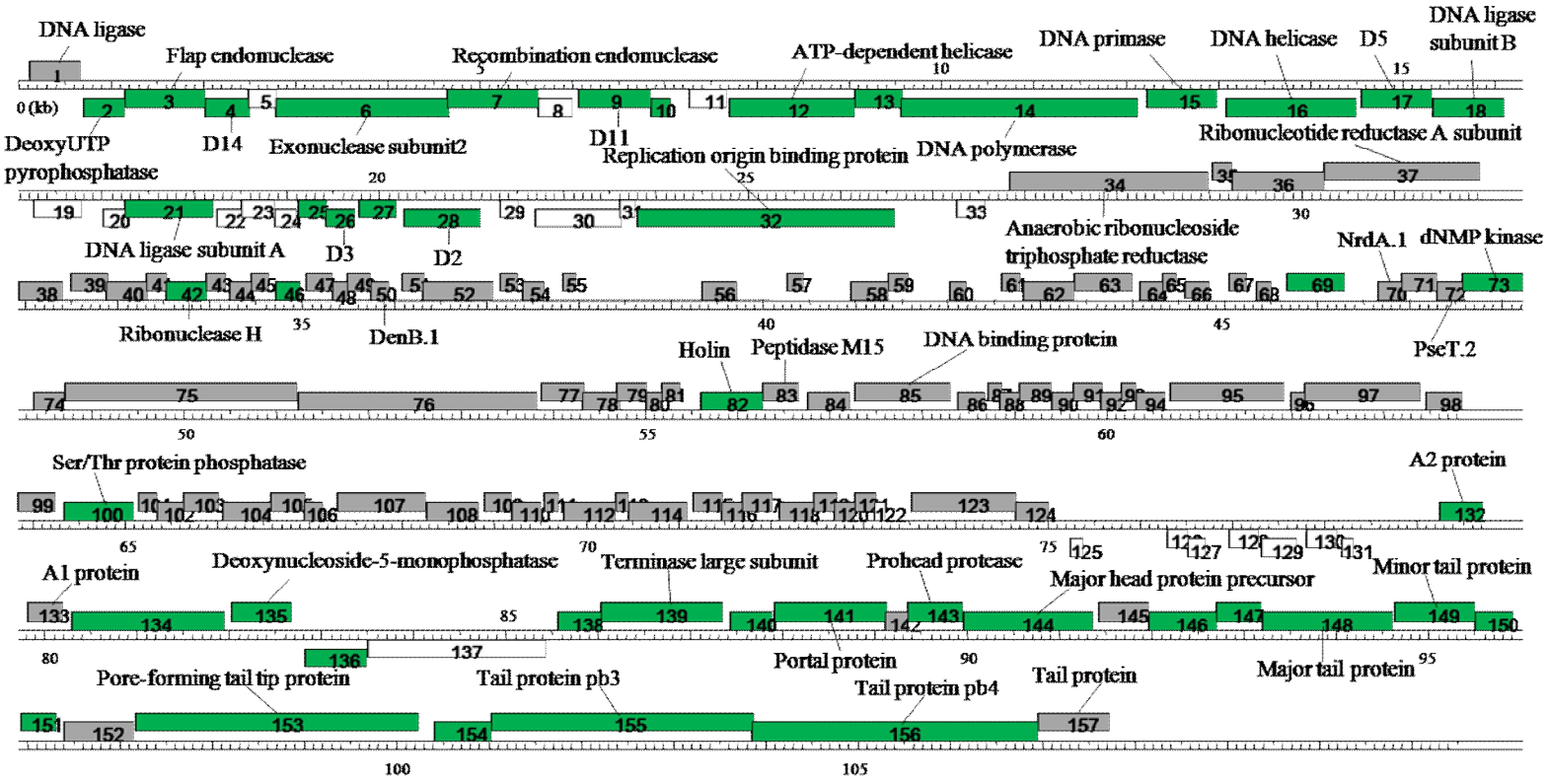


**Figure 3.1.** Genome map of phage pVp-1. Hypothetical functions of encoded proteins were determined through comparison of amino acid sequences to the non-redundant databank using BLASTP. The + and – stranded ORFs were colored as grey and white, respectively. The CoreGenes between phage pVp-1 and phage T5 were colored as green.

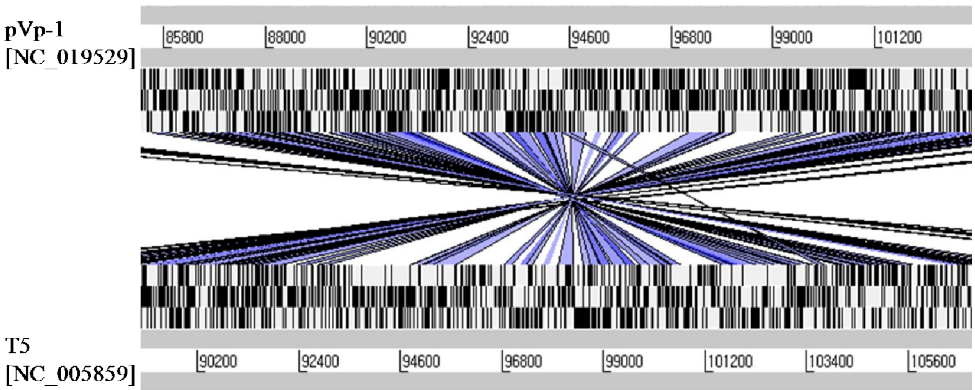


**Figure 3.2.** Genome comparison of phage pVp-1 to its relative phage (T5) using Artemis Comparison Tool (ACT). Translated BLAST (tblastx, score cutoff: 40) was used to align translated genome sequences of phages. The blue lines represent the reverse and forward matches, and color intensity is proportional to the sequence homology. Nucleotide base-pairs were indicated between grey lines for each phage genomes.

# GENERAL CONCLUSION

Recently, there has been an increasing appreciation of their role as waterborne pathogens of fish and humans. There has been an increasing incidence of antimicrobial resistance among *Aeromonas* sp. isolated from aquaculture environments. Multiple-antibiotic-resistant *A. hydrophila* exists in aquaculture systems and contributes to the high rate of mortality within the fish industry in Korea. Although the majority of the loach population in Korea is cultured and *A. hydrophila* is one of the main causes of mass mortality in these fish, no effective method has been proposed for the control of *A. hydrophila* infection in aquaculture, except for the application of additional antibiotics. In the first step, to investigate methods to control the mass mortality of cyprinid loaches (*Misgurnus anguillicaudatus*) caused by multiple-antibiotic-resistant *Aeromonas hydrophila* on a private fish farm in Korea, bacteriophages (phages), designated pAh1-C and pAh6-C, were isolated from the Han River in Seoul. The two isolated phages were morphologically classified as *Myoviridae* and showed similar infection patterns for *A. hydrophila* isolates. The phages proved to be efficient in the inhibition of bacterial growth, as demonstrated by their *in vitro* bactericidal effects. Additionally, a single administration of either phage to cyprinid loaches resulted in noticeable protective effects, with increased survival rates against *A. hydrophila* infection.

*Vibrio parahaemolyticus* is one of the most important causes of gastroenteritis. Although raw oysters have such high densities of *V. parahaemolyticus* that the consumption of raw oysters is known to cause illness in humans, almost all Koreans prefer raw oysters to already cooked oysters because of their fresh taste and high nutritional value. *V. parahaemolyticus* pandemic strains, such as O3:K6, are responsible for the current

pandemics in many countries. Emergence of *Vibrio* species that are resistant to multiple antibiotics has been recognized as a serious global clinical problem. Recently isolated *V. parahaemolyticus* pandemic strains have displayed multiple antibiotic resistance, increasing concerns about possible treatment failure. Alternatives to conventional antibiotics are needed, especially for the multiple-antibiotic-resistant *V. parahaemolyticus* pandemic strain. In the second step, a bacteriophage, designated pVp-1, that was lytic for *V. parahaemolyticus* was isolated from the coast of the Yellow Sea in Korea. The phage showed effective infectivity for multiple-antibiotic-resistant *V. parahaemolyticus* and *V. vulnificus*, including *V. parahaemolyticus* pandemic strains. The therapeutic potential of the phage was studied in a mouse model of experimental infection using a multiple-antibiotic-resistant *V. parahaemolyticus* pandemic strain. Phage-treated mice displayed protection from a *V. parahaemolyticus* infection and survived lethal oral and intraperitoneal bacterial challenges.

In the third step, the complete genome sequence of a novel marine siphovirus pVp-1 was reported, which was isolated from the coastal water of the Yellow sea in Korea and infects *V. parahaemolyticus*. Genomic comparison of pVp-1 with the phage T5 revealed that these two phages are highly similar in gene inventory.

Based on these results, it is clear that phages can be considered as altenative therapeutic or prophylactic candidates against bacterial infections in humans as well as fish. The use of biocontrol method using phages seems to be a promising alternative to conventional antibiotics.

## 국문초록

# 박테리오파아지 유전체연구 및 *Aeromonas hydrophila*와 *Vibrio parahaemolyticus*에 대한 박테리오파아지 치료법

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*Aeromonas* spp.는 정상적인 수중환경에 존재하는 주요 세균으로, 최근 어류뿐만 아니라 인간에게도 피해를 유발하는 수인성 병원균으로서 주목받고 있다. *Aeromonas hydrophila*는 어류에서 질병을 유발하는 운동성 aeromonad의 일종이며 질병 발생시 높은 폐사를 야기한다. 최근 양식 환경에서 분리된 *Aeromonas* sp.에서 항생제 내성 발생 빈도의 증가가 보고되고 있다. 여러 종류의 항생제에 내성을 획득한 *A. hydrophila*는 실제 양식 환경에 존재하고 있으며 한국 어업에 많은 폐사를 일으키고 있다. 국내산 미꾸라지의 대부분은 양식산이며 *A. hydrophila*는 미꾸라지 폐사의 주요 원인으로 알려져 있지만, 양식산업에서의 *A. hydrophila* 감염증에 대한 대책으로 항생제 투여외에는 그 어떤 효과적인 대책도 없는 실정이다. 국내의 어류 양식장에서 발생한 다수의 항생제에 내성을 보이는 *A. hydrophila* 감염에 의한 미꾸라지의 폐사를 막기 위한 방안을 연구하기 위하여, 한강에서 pAh1-C와 pAh6-C라고 명명된 박테리오파아지 (이후 파아지)를 분리하였다. 두개의 파아지는 형태학적으로 *Myoviridae*로 분류되었고 *A.*

*hydrophila* 균주들에 유사한 감염 양상을 나타내었다. 두개의 파아지는 55 kb (pAh1-C)와 58 kb (pAh6-C) 크기의 double-strand DNA를 보유하고 있었으며, 그 두 파아지의 DNA는 제한 효소 이용 분석에서 서로 다른 패턴을 보였다. 두 파아지는 모두 어류에서 병원성을 유발하는 *A. hydrophila*에 대해 효과적인 세균 억제 능력을 갖고 있는 것으로 드러났다. 두 파아지의 latent period는 대략 30 분 (pAh1-C)과 20 분 (pAh6-C)으로 관찰되었고 burst size는 60 (pAh1-C)과 10 (pAh6-C)으로 측정되었다. 생체외 실험실 상에서 관찰된 세균 억제 능력 측정에서 드러난 바와 같이, 본 파아지들은 세균의 성장을 효과적으로 억제하는 것으로 판명되었다. 또한 미꾸라지를 대상으로 한 일회성의 파아지 투여는 *A. hydrophila* 감염증에 대해 괄목할 만한 방어 효과를 나타내며 생존율이 크게 향상되었다. 이러한 연구 결과는 pAh1-C와 pAh6-C, 이 두 파아지가 어류에서 *A. hydrophila* 감염증에 대한 치료제로서의 충분한 가능성을 갖고 있음을 증명하고 있다.

*Vibrio parahaemolyticus*는 위장관질환을 유발하는 주요 원인체이다. 본 감염증은 불완전하게 조리된 해산물, 특히 굴의 생식과 밀접하게 관련되어 있다. 전 세계적으로 *V. parahaemolyticus*는 주요 감염증의 원인으로 알려져 있다. 동아시아인들, 특히 한국인과 일본인은 독특한 식습관을 가지고 있다. 한국인들은 다양한 종류의 어류와 어패류를 생식한다. 생굴은 다량의 *V. parahaemolyticus*를 함유하고 있으며 생굴 섭취는 질병 발생의 위험성이 있음에도 불구하고, 대부분의 한국인들은 그 신선한 풍미와 높은 영양소를 즐기기 위하여 조리된 굴보다 생굴을 더 선호한다. O3:K6 와 같은 *V. parahaemolyticus* pandemic strain은 세계 여

러 국가에서 대규모 식중독의 원인균으로 알려져 있다. 다수의 항생제에 내성을 보유한 *Vibrio* 세균의 출현은 심각한 범세계적 문제로 인식되어 왔다. 최근 분리된 *V. parahaemolyticus* pandemic strain들은 다수의 항생제에 내성을 나타내었으며 이는 감염되었을 경우 치료 실패의 가능성에 대한 우려를 증폭시키고 있다. 이에, 다수의 항생제 내성 *V. parahaemolyticus* pandemic strain에 대한 항생제 대체 방법이 절실히 요구되고 있다. *V. parahaemolyticus*에 감염되며 pVp-1으로 명명된 파아지가 황해 연안에서 분리되었다. 파아지는 *V. parahaemolyticus* pandemic strain을 포함하여, 다수의 항생제 내성 *V. parahaemolyticus*와 *V. vulnificus*에 효과적인 감염성을 나타내었다. 다수 항생제 내성 *V. parahaemolyticus* pandemic strain을 이용한 마우스 감염 실험 모델을 활용하여 본 파아지의 치료제로서의 가능성에 대하여 검증하였다. 파아지를 투여한 마우스는 *V. parahaemolyticus* 감염증에 대하여 충분한 방어력을 보였으며, 식이와 복강 주사를 통한 치사량에 달하는 세균 감염에서도 생존력을 나타내었다. 본 연구는 다수의 항생제 내성 *V. parahaemolyticus* pandemic strain에 대한 파아지 치료의 최초 보고이다.

*V. parahaemolyticus*에 감염되는 신규 해양 siphovirus pVp-1가 황해의 연안에서 분리되었고, 이의 유전체가 보고되었다. pVp-1의 double-strand DNA 유전체는 111,506 bp, 39.71%의 G + C 함량으로 분석되었다. pVp-1의 유전체 분석에서는 pVp-1이 T5 파아지와 높은 유전체적 유사도를 갖고 있다는 것이 증명되었고, 이는 pVp-1과 T5 사이의 밀접한 유전체적 관련성을 시사하는 것이었다. pVp-1과 T5를 대상으로 하는 유전체적 유사성 분석 결과에서는 이 두 파아지가 유

전체 구성에 있어서 높은 유사성을 보유하고 있음이 제시되었다.

이러한 결과를 바탕으로, 항생제 내성 *A. hydrophila*에 감염하는 *Aeromonas* 파아지가 양식 환경에서의 *Aeromonas* 감염증에 대한 치료제 혹은 예방제제으로서의 충분한 가능성을 보유하고 있음이 확인되었다. 또한 *V. parahaemolyticus* CRS 09-17 세균을 이용한 마우스 대상의 파아지 치료 실험은 pVp-1 투여가 *V. parahaemolyticus* 감염증으로부터의 방어를 가능하게 하며, 다수의 항생제 내성 pandemic strain에 의해 야기되는 대규모 전염병의 피해를 최소화하는데 치료제로서 사용될 수 있다는 것이 증명되었다.

**Key words:** *Aeromonas hydrophila*, 박테리오파아지 (파아지), *Vibrio parahaemolyticus*, 다수의 항생제 내성 pandemic strain, 치료제.

**Student number:** 2011-31100

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3. Sang Phil Shin, Ji Hyung Kim, Casiano H. Choresca Jr., Jee Eun Han, **Jin Woo Jun**, Se Chang Park: Comparision and phylogenetic analysis of *Thelohanellus kitauei* with other *Thelohanellus* spp. Japanese Society for Fish Pathology conference, Japan (Shimonoseki) Sep., 2012.
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