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Identification of causative agents with high mortality in marine bivalve and application of bacteriophage to prevent *Vibrio coralliilyiticus* infection

이매패류의 대량 폐사를 유발하는 원인체들의 규명 및 박테리오파아지를 이용한 ***Vibrio coralliilyticus*** 감염 예방

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A Dissertation for the Degree of Doctor of Philosophy

Identification of causative agents with high mortality in marine bivalve and application of bacteriophage to prevent *Vibrio coralliilyticus* infection

By

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Major in Veterinary Pathobiology and Preventive Medicine

Department of Veterinary Medicine

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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 Pathobiology and Preventive Medicine

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Abstract

Identification of causative agents with high mortality in marine bivalve hatcheries and application of bacteriophage to prevent *Vibrio coralliilyiticus* infection

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This study was conducted to identify the causative agent with high mortalities in marine bivalve hatcheries in Korea and to find out the alternative method against antibiotics abuses.

Since November 2017, high mortalities of larvae of bay scallop (*Argopecten irradians*) were reported in hatcheries located at the southern area of Republic of Korea. Over 90% of larvae aged 5 to 10 days sank to the bottom of the tank and died. The hatcheries could not produce spat and thus artificial seed production industry incurred huge losses. We identified Ostreid Herpesvirus-1 μVar (OsHV-1 μVar) associated with mass mortality by PCR, sequencing and transmission electron

microscopy (TEM). All the samples were positive for OsHV-1 μVar with 99% sequence identity to previously reported OsHV-1 μVar sequences. Partial sequence of ORF-4 of OsHV-1 detected in this study was more closely related to sequences isolated from Europe. This is the first report to confirm the mortality caused by an OsHV-1 infection in the bay scallop.

We first isolated and characterized a high virulent of *Vibrio coralliilyticus* designated as SNUTY-1 that was the cause of Pacific oyster larvae mortality in Korea. SNUTY-1 showed anti-microbial resistance to β-lactams, such as penicillins, cephalosporins, and carbapenems. We sequenced and assembled the complete genome of SNUTY-1 (5,842,676 bp), consisting of two chromosomes (Chr I and Chr II) and two plasmids (pSNUTY1 and pSNUTY2). SNUTY-1 had a unique plasmid (pSNUTY2), which could mean that the Korean isolate is different from other sequenced *V. coralliilyticus* strains from different geographical origins. Toxic proteins such as cytolysin/ hemolysin and extracellular metalloprotease genes were encoded on Chr I and Chr II of SNUTY-1. These data facilitate the control of *V. coralliilyticus* infections in aquaculture by providing valuable insights into the biodiversity of this organism and valuable information for the study of virulence factors.

We isolated and characterized the phage that specifically infects *V. coralliilyticus*. The phage was designated pVco-14 and classified as *Siphoviridae.* We also investigated the potential efficacy of the isolated phage against *V. coralliilyticus* infection. pVco-14 was stable at wide temperature (4-37℃) and pH (5.0-9.0) ranges.

When oyster larvae were pre-treated with the phage before the bacterial challenge, mortality of the phage-treated oyster larvae was lower than that of the untreated control. These results suggest that pVco-14 has potential to be used as a prophylactic agent for preventing *V. coralliilyticus* infection in marine bivalve hatcheries and can reduce the overuse of antibiotics.

Overall, the present study offers important insight into the biodiversity of the OsHV-1 and *Vibrio* spp. and provides valuable information for the aquaculture. Futher studies are required to determine appropriate treatments for preventing OsHV-1 and *V. coralliilyticus* infection-induced mass mortality events at marine bivalve hatcheries. As invertebrates, including bivalves, do not have acquired immune systems, disease prevention methods (e.g. vaccination) are very limited compared to fish. Therefore, the application of bacteriophage may be a solution for the prevention of bacterial disease in invertebrates. The results of our study showed that phage potentially can be used as an effective substitute for antibiotics in the field of bivalve artificial seedling production while reducing the generation of antibiotic-resistant bacteria and environmental pollution.

Key words: Marine bivalve, bay scallop, Pacific oyster, Mass mortality, Ostreid Herpesvirus-1 µVar, *Vibrio coralliilyticus*, Multiple-antibiotic-resistant, Prophylaxis, Bacteriophage (phage)

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Abbreviations

OsHV-1	Ostreid Herpesvirus-1
µVar	Microvariant
Vco	*Vibrio coralliilyticus*
Vco SNUTY	*Vibrio coralliilyticus* strain SNUTY
Vco58	*Vibrio coralliilyticus* strain 58
PCR	Polymerase chain reaction
TEM	Transmission electron microscopy
ORF	Open reading frame
FAO	Food and Agriculture Organization
OIE	World Organisation for Animal Health
AFSW	Autoclaved filtered seawater
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TCBS	Thiosulfate citrate bile sucrose
OVVD	Oyster velar virus disease
PBS	Phosphate buffered saline
UV	Ultra violet
Chr	Chromosome
CFU	Colony forming unit

rRNA	Ribosomal RNA
tRNA	Transfer RNA
ncRNA	Non-coding RNA
KCCM	Korea Culture Center of Microorganisms
ATCC	American Type Culture Collection
COG	Clusters of orthologous gene
FSS	Filtered and sterilized seawater
NCBI	National Center for Biotechnology Information
Phage	Bacteriophage
PFU	Plaque forming unit
Conc.	Concentration
EOP	Efficiency of plating
MOI	Multiplicity of infection
RPM	Revolutions per minute
OD	Optical density
wt / vol	Weight per volume
PEG	Polyethylene glycol
CLSI	Clinical and Laboratory Standard Institute

General introduction

Oysters and scallops are the most consumed shellfish worldwide and account for the largest commodity in the bivalve aquaculture industry. Asia is the major producer of scallops and oysters. In 2016, it produced 2,096,663 tonnes ($ 5.3 millions) out of the 2,126,930 tonnes (98.58%) scallop produced worldwide (1). In addition, 5,435,615 tons, 95.35% of the world production (5,700,911 tons) of oysters, was produced in Asia (1). In case of Korea, scallop production increased dramatically over the years. In 2002, only 5 tonnes scallops were produced in Korea, whereas production has been increased to 2,995 tonnes in 2016 (1). Moreover, Korea is a major producer of Pacific oyster (*Crassostrea gigas*). In 2017, it produced 315,255 tons out of the total 639,030 tons (49.33%) of Pacific oysters produced worldwide (1).

In case of Korea, the artificial seedling production industry for marine bivalve seeds is highly developed in the southern region. However, a mass larval mortality of the marine bivalve occurred since the middle of the 2000s. Causative agents associated with mass mortality were suspected as Ostreid Herpesvirus-1 μVar (OsHV-1 μVar) and *Vibrio* sp.

OsHV-1, which belongs to the family *Malacoherpesviridae* (2, 3), associated with mass mortality of marine bivalve larvae in the spat and juvenile stages. OsHV-1 mass mortality has been reported worldwide including Northeast Asia, Europe, North-America and Oceania (4-15). In addition, it has been reported that OsHV-1 has a

wide host range and is detected by PCR in various kinds of bivalve larvae and spat including Pacific oysters (*Crassostrea gigas*), Eastern oysters (*C. virginica*), European flat oysters (*Ostrea edulis*), Manila clams (*Ruditapes philippinarum*), Carpet shell clams (*Ruditapes decussatus*), French scallops (*Pecten maximus*), Farrer's scallops (*Chlamys farreri*), and *Scapharca broughtonii* (6, 7, 12-15). Since the report of Farley et al. (7), mass mortality associated with OsHV-1 infection have been continuously reported in various kinds of bivalves worldwide, but no treatments are available yet (16). This results in severe economic losses to the bivalve seed production industry every year.

The genus *Vibrio* is composed of ubiquitous aquatic bacteria, including diverse members of planktonic and animal-associated microbial communities (17). Several organisms in this genus (*V. anguillarum*, *V. tubiashii*, *V. alginolyticus*, *V. splendidus* biovar II, and *V. coralliilyticus*) have been associated with mass mortalities in nursery cultures of juvenile bivalves at oyster hatcheries worldwide (18-21). Although all of the abovementioned species have been implicated in bacillary necrosis, recent research indicates that *V. coralliilyticus* is a more significant pathogen of Pacific oyster (*C. gigas*) larvae (22).

V. coralliilyticus is a well-known pathogen of coral, responsible for bleaching, tissue lysis and drastic losses in coral reefs worldwide (23, 24). Moreover, this bacterium has been shown to infect crustaceans, fish (25), and bivalves, including the Pacific oyster (26, 27). Recent sequencing efforts have revealed that *V. coralliilyticus* and *V. tubiashii* are closely related (23, 28) and that some etiological

strains (RE22, RE98, LMG 10953 and ATCC 19105) previously identified as pathogenic *V. tubiashii* are in fact *V. coralliilyticus* (29, 30).

To prevent and control of *Vibrio* spp. infection in marine bivalve hatcheries, various antibiotics are over used and consequently, it causes occurrence of multi drug resistant strains and environmental pollution. Therefore, research on the development of antibiotic substitutes is required.

The application of bacteriophage (phage) has been proposed for the treatment of bacterial diseases, and several studies have reported that phages can be effectively used for the treatment of bacterial infection in humans and livestock (114, 163-165). Application of phages to treat bacterial diseases has also been investigated in fishes (120, 122, 167, 168) and invertebrates (121, 130, 169, 170).

Present study was conducted to identify the causative agents of mass mortality of larvae continuously occurring in the marine bivalve hatcheries in Korea. In addition, to prevent antibiotics overuse causing occurrence of multi-drug-resistant bacteria and environmental pollution, we tried bacteriophage application to prevent *V. coralliilyticus* infection in marine bivalve hatchery.

Literature Review

A. Bivalves

A.1. General description of bivalves

Bivalvia is a class of mollusks that have laterally compressed bodies enclosed by a shell consisting of two hinged parts (30). Those include the oysters, scallops, clams, cockles, mussels and numerous other families that live in seawater, as well as a number of families that live in freshwater (30). Extant bivalves are an important component of marine and freshwater ecosystem, with more than 80% of species inhabiting in ocean environments, and exhibiting varied ecologies (30, 31). Sessile epifaunal bivalves, such as oysters and mussels, attach themselves to hard surfaces using cement or byssal threads, while clams and cockles bury themselves to sand or sediment on the bottom of sea or freshwater environments. In the case of scallops, it lives mainly on the sandy bottom of the sea and use the thrust generated by opening and closing two shells, making it more mobile than other bivalves.

Bivalves are filter feeders and feed primarily on phytoplankton (32). Water passed through the gill from the inhalant opening or the inhalant siphon and enter to body, then is discharged through the exhalant opening or siphon. At this time, phytoplankton is collected by the gills (32).

A. 2. Life history of bivalves

Life cycle of bivalves is shown in **Figure 1** (33). The spawning season of bivalve varies depending on the species and geographic region (31, 33). Spawning may be triggered by various environmental factors such as water temperature, chemical or physical stimulation and water current (30). Bivalves usually spawn only at a particular season of the year. The spawning period is usually short and the entire contents of the gonads are released in a short period of time during spawning. The majority of industrially important bivalves such as oysters, scallop, clams are fertilized by discharging gametes into an open environment. In most bivalves, when spawning ends and the gonads become empty, sex cannot be distinguished even under microscopic observation. At that time, the animal is considered to be in the resting stage (30).

The time taken for embryonic and larval development is species-specific and depends on the water temperature (30, 32). Within 24 hours of fertilization eggs has pass through the multi-celled blastula and gastrula stages and between 24 to 36 hours that develops into a motile trochophore. At this stage, the size of trochophore is about 60-80 μm.

The early stage of developed larvae is called D-shape larvae or veliger. The size of veliger is usually 80-100 μm, with two shells and a complete digestive system, with a specific organ called velum that is peculiar to bivalve larvae (31).

The veliger continues to swim, feed and grow and within a week umbones are developed, which is protuberances of the shell near the hinge. As the larva grows,

the umbone becomes more prominent and this stage is called umbone stage or preveliger. During this stage, the larva grows, the foot development, and the gill rudiments become apparent. Also, a small black eye-spot develops in the center of the larval shell. The preveliger swims in the water and settle and use the foot to crawl on a substrate. When suitable substrate is found, the larva is ready to metamorphose and begins its benthic existence.

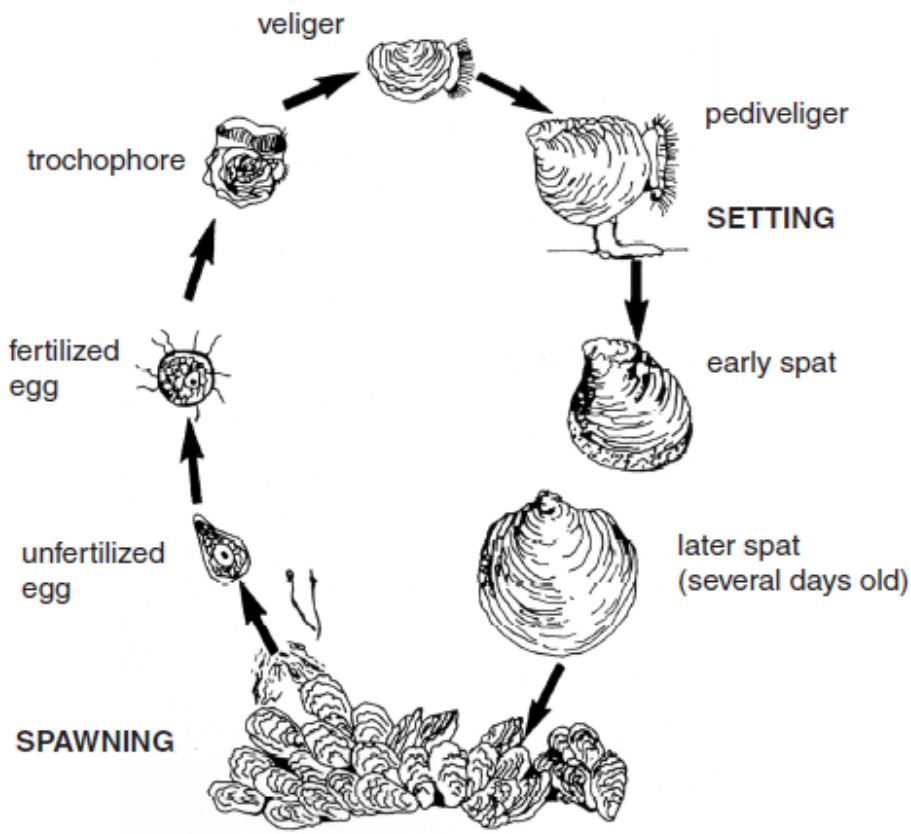

Figure 1. Life cycle of bivalve (33).

A. 3. Aquaculture of bivalves

The first record of bivalves as food source came through the discovery of fossils on the southern coast of Cape South Africa about 16 million years ago (34). Bivalves continue to be an important food source for humanity. Bivalve culture is steadily gaining importance in the aquaculture field. Especially, edible marine bivalve such as oysters, scallops, mussels, clams, cockles and ark shell are culturing in worldwide. In 2018, over 12.3 million tons of edible bivalves were produced worldwide (1). Since 2008, world production amount of bivalve is increasing continuously (1) (**Figure 2**).

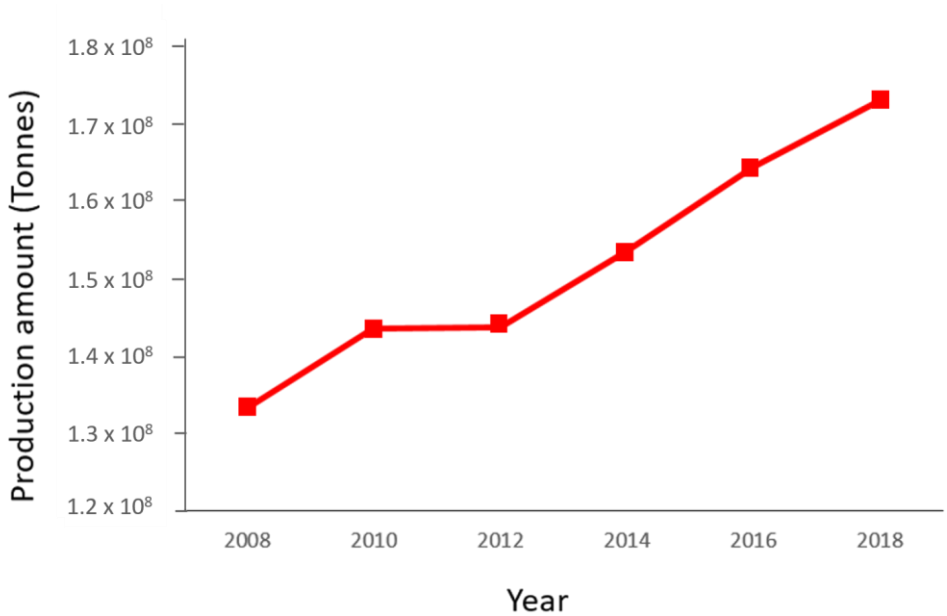

Figure 2. World production amount of bivalves (1).

As mentioned above, the increase in bivalve production worldwide is related to the development of the technology for producing artificial seedling of bivalves (31). In the earlier bivalve culture, the spat was collected through natural seedling; however, the development of artificial seedling production technology has allowed the culture to produce larvae of various bivalve species (30, 31). However, along with the continuous expand in bivalve culture, mass mortality caused by various pathogenic factors. For example, Ostreid Herpesvirus-1, and *Vibrio* spp. are common pathogens, and hence, studies on preventive methods are needed.

B. Ostereid Herpesvirus-1

A.1. *Malacoherpesviridae*

The International Committee on Taxonomy of Viruses named the *Herpesviridae* that infects invertebrates according to the type of host to be infected as *Malacoherpesviridae*. Herpesviruses dataset and phylogenetic tree are shown in **Figure 3** (35). *Malacoherpesviridae* alone contains Ostreid Herpesvirus-1 (OsHV-1) from the genus *Ostreavirus* (2). The core of OsHV-1 virus contains linear double-stranded DNA with an icosahedral capsid (36). Herpesviruses that infect marine mollusks have attracted special research attention due to their economic and ecological impact since occurrence.

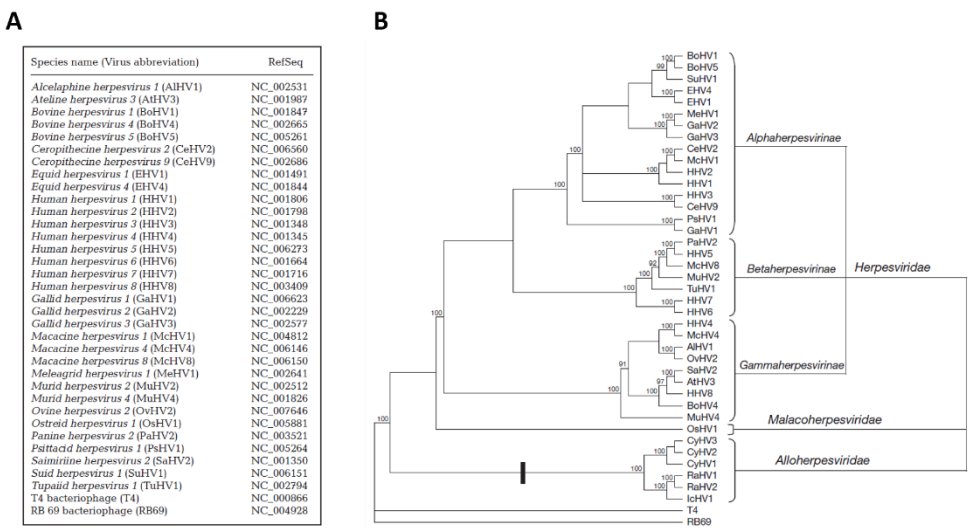

Figure 3. Herpesviruses dataset (A) and phylogenetic tree (B) (35).

A. 2. OsHV-1 µVar

In the past decades, only Ostreid Herpesvirus-1 (reference type) and Ostreid Herpesvirus-1 Var were isolated. Since then, variants with mutations in the C region have been reported and OsHV-1 µVar, emerging variant, was isolated in 2008 (37). OsHV-1 µVar shows deletion of 200 bp at 178204-178404 region which probably compromises the protein coded by ORF5, and there is an insertion of 27 bp which has not been reported in any published sequence (CCCACTGTGATATCATC-GCAAATGAAT). OsHV-1 µVar showed sequence deletions in the microsatellite zone against OsHV-1 reference type when C2/C6 primer pair was used. In the case of OsHV-1, the pattern "CTA" is repeated eight times, but in the case of OsHV-1 µVar, it is repeated four times with deletion (47). Also, the sequence length of OsHV-1 and OsHV-1 µVar were 989 bp and 384 bp, respectively (47). Moreover, in some cases, a deletion with only 10 bp in the same position was also observed. Furthermore, recent analysis of genomic regions revealed the presence of at least nine different genotypes, including two variants close to the OsHV-1 (reference type) (38, 39). In particular, phylogenetic analysis results highlighted the presence of two distinct clusters with a different geographical, European and East Asian, genotypes distribution.

A. 3. Infectivity of OsHV-1 µVar

In vitro infectivity and stability studies of OsHV-1 µVar are very few. Ten picogram per microliter of extracted viral DNA were experimentally detected for 1,

9, and 16 days at 20, 11 and 4°C respectively. And, in a second experiment, 100 pg/µl were detected after 51 days at each of the same temperatures (40). However, the relationship between DNA detection in the PCR and infectivity is still unknown. As with the general rule of viruses that infect most aquatic organisms, OsHV-1 has a longer survival at low temperature (40). Like other herpesviruses, OsHV-1 can exist in a host cell without replication in a potentially infected state and can be reactivated. OsHV-1 was also detected in apparently healthy farmed oysters (10, 41) and, again, in healthy farmed *C. gigas* from the Gulf of California (40); while, regarding the oysters in the wild, the presence of OsHV-1 was recently confirmed in European *C. gigas* (39).

Water is probably the most important natural route of OsHV-1 transmission, since its DNA has been detected in the water around diseased Pacific oysters (42, 43). The experimental infection of Portuguese oyster (*C. angulata*) and Suminoe oyster (*C. rivularis*) was performed by waterborne with extracted OsHV-1 or by cohabitation with infected *C. gigas*, respectively (5). Several researchers have reported the results of larvae and spat showing higher mortality and morbidity than adult oyster, but can infect larvae if an infected adult is stressed by high temperature or other stress (40, 44, 45). Moreover, it is not confirmed if there is a vertical transmission route other than horizontal one since the results were inconclusive (46).

Regarding the infectivity of OsHV-1 on marine mollusks, other than *C. gigas*, only few data are available. The first record of a herpes-like virus in a bivalve mollusk in the USA was described by Farley et al. (6) in *C. virginica*. However,

herpes-like virus have been reported in some molluscs such as *C. ariakensis*, *C. rivularis*, *C. hongkongensis*, *C. sikamea* (10), *C. gigantea* (47), *Ostrea edulis* (48), *Pecten maximus* (5), *Ruditapes decussatus* (5, 12) *R. Philippinarum* (5, 12, 49), *Saccostrea glomerata* (50), *Argopecten irradians* (51) and *Tiostrea chilensis* (52). In many cases, the identification of the virus was based on electronic microscopy and histopathological examinations of infected tissues consistent with the characteristics of herpesviruses, while before 2005 biomolecular examination was not always performed. Recently, Lopez-Sanmartin et al. demonstrated that *O. edulis* could be infected by OsHV-1 µVar by intramuscular injection (53).

B. *Vibrio coralliilyticus*

B.1. Vibriosis in aquaculture

The genus *Vibrio* (Kingdom, Bacteria; Phylum, *Proteobacteria*; Class, *Gammaproteobacteria*; Order, *Vibrionales*; Family, *Vibrionaceae*) are gram-negative, ubiquitous in marine and estuarine ecosystems as well as aquaculture farms, and comprise one of the major microbiota of these ecosystems. Many vibrios such as *V. alginolyticus*, *V. anguillarum*, *V. splendidus* biovar II, *V. tubiashii*, and *V. coralliilyticus*, *V. parahaemolyticus*, *V. harveyi*, *V. anguillarum* are serious pathogens for animals reared in aquaculture (54-58). Vibriosis, caused by infection of *Vibrio* spp, is one of the most prevalent disease in fishes and other aquaculture-reared organisms and is widely responsible for mortality in cultured aquaculture systems worldwide (59, 60).

Some members of the genus *Vibrio* (*V. alginolyticus*, *V. anguillarum*, *V. splendidus* biovar II, *V. tubiashii*, and *V. coralliilyticus*) have been described as the main causative agents of diseases affecting bivalve in all life stages (larval, juvenile, spat and adult) (61-76). The diversity of *Vibrio* species associated with bivalves in different geographical areas has also been the subject of various studies (77-86). Environmental parameters such as water, temperature, and salinity change are well known to affect the diversity of *Vibrio* spp. Those parameters also affect the physiological state of the bivalve and sensitivity to bacterial infection (66, 75, 79-81).

Paillard et al. (66) provided an excellent and complete overview of the bacterial diseases in marine bivalves, including an updated reference of pathogenic *Vibrio* spp. They recognized that the emergence of vibrios as etiological agents in cultured bivalves is likely to increase over the coming years due to ocean warming. Also, Beaz-Hidalgo et al. (85) provides an updated overview of studies performed on the diversity of *Vibrio* species associated with bivalve mollusks. Special point of their study is made on the species that produce disease in the different stages bivalve mollusc's life (larvae, juveniles and a

dults) describing the characteristic signs and diseases caused by *Vibrio* spp. in various hosts.

B.2. Pathogenic *Vibrio* species in bivalve larvae

Evidence of the involvement of a *Vibrio* sp. in the disruption of the velum and internal tissues of the clam larvae *Mercenaria mercenaria*, and resulted in 70% mortality rate (88). Also, Tubiash et al. (89) observed swarms of bacteria on the margins of the larvae, which became progressively denser and after 8 h, and mortality occurred as a result of granular necrosis. This study was the first report to describe the bacillary necrosis affecting numerous bivalves in European oyster, Eastern oyster, Hard clam, Bay scallop.

Major clinical signs of bacillary necrosis included the motility reduction, extension and necrosis of the velum or erratic movements in circles that appeared after 4-5 h of exposure to *Vibrio* spp. (**Figure 4**). *V. coralliilyticus*, *V. tubiashii*, *V.*

splendidus, *V. alginolyticus*, and *V. anguillarum* were reported as the main causative agents of the bacillary necrosis in later studies (90-92).

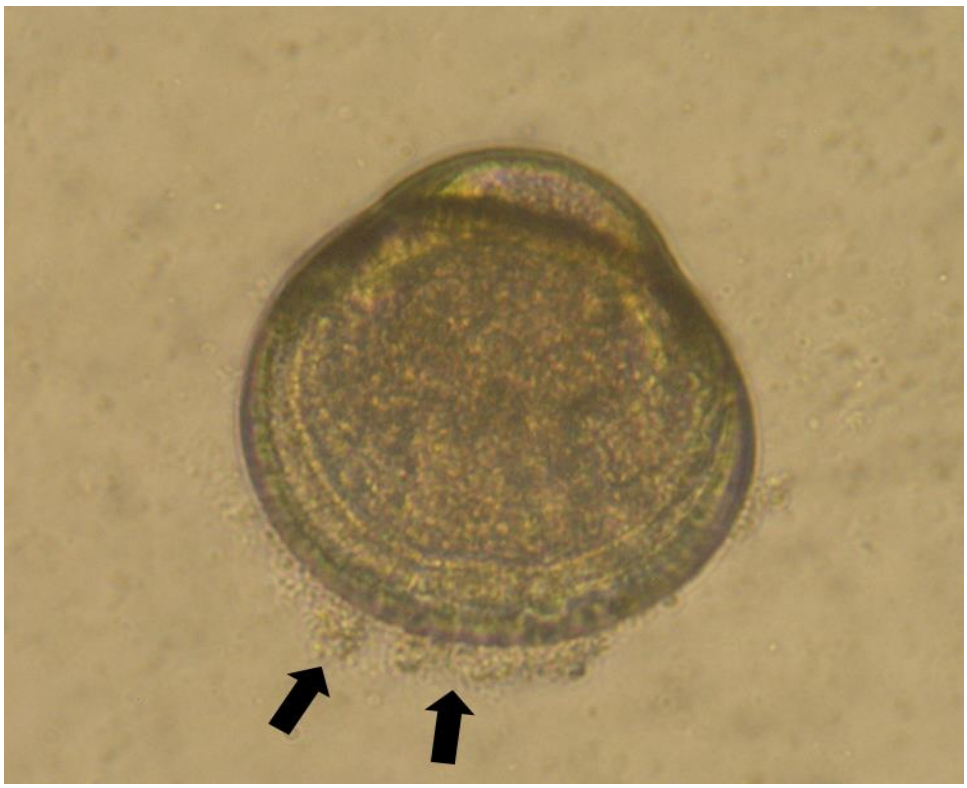

Figure 4. Symptom of bacillary necrosis in Pacific oyster larvae.

Elston and Leibovitz (93) described bivalve larval disease patterns caused by *Vibrio* infection in three pathogenesis stages: I, II and III. Stage of Pathogenesis I affects all larval stages; the larvae become sedentary showing signs of colonization of the mantle and invasion of the visceral cavity. Pathogenesis II affects the early stage of the free-swimming veliger larvae, producing velum disruption and extension and abnormal swimming. The larvae remain active, showing visceral atrophy before

invasion into the organs of the digestive tract occurs. Pathogenesis III affects the pediveliger larva and as in Pathogenesis I the larvae become sedentary. However, in Pathogenesis III has progressive and extensive visceral atrophy and lesions in the organs of the digestive tract. Another specific symptom of larval vibriosis in hatcheries is the appearance of the phenomenon called 'spotting', defined as an accumulation of moribund larvae agglutinated at the bottom of the tanks (94). All the studies mentioned so far have described typical and clinical symptoms of bivalve larvae infected with *Vibrio* spp.

B.3. *Vibrio coralliilyticus*

V. coralliilyticus, well-known pathogen of coral, responsible for tissue lysis, bleaching, and drastic losses in coral reefs, was first identified by Ben-Haim et al. (22). Some strains of *V. coralliilyticus* interrupt the symbiotic relationship between the poly-b of coral and their symbiotic dinoflagellates (*Symbiodinium spp*.), which reside primarily within the gastrodermal cells of the poly-p (5, 22, 23).

The loss of dinoflagellates, and bleaching could induce to the death of coral if the algae are not rebuilt because algae provide most of the energy for many coral species through photosynthesis (96, 97). In addition, if sea surface temperature increases up to 27°C, the toxicity of the *V. coralliilyticus* strain appears to be enhanced, causing coral bleaching and death. Ben-Haim et al. (23) demonstrated that *V. coralliilyticus* BAA-450 (referred to as YB1 in the paper) caused coral bleaching of *Pocillopora damicornis* at 24.5-29°C, but not at 20-22°C and tissue lysis occurred at temperatures

over 27°C (23). Moreover, Vidal-Dupiol et al. also confirmed the ability of BAA-450 to cause coral bleaching and tissue lysis in *P. damicornis* when water temperatures were increased to 25-32.5°C, but not when the temperature maintained at 25°C (95).

V. coralliilyticus type strain BAA-450, known to cause coral bleaching, is also reported to induce mortalities in oyster larvae (21). Intermittent occurrences of larval mortalities have plagued marine bivalve hatcheries, especially in the western coast of the United States, and mortalities are attributed to *V. coralliilyticus* (previously identified as *Vibrio tubiashii*) (21, 68, 111). Outbreaks of severe mass mortality events at major Pacific oyster hatcheries in Japan and Korea were attributed to *V. coralliilyticus* and *V. splendidus* biovar II, respectively (92, 112). Such episodes can be common in many hatcheries with some reporting larval mortalities as high as 80%. These extensive mortalities have subsequently led to shortages of seed oysters necessary for commercial shellfish operations (21, 111). Not only Pacific oysters are susceptible to *V. coralliilyticus* infections, but also other shellfish species do apparently vulnerable, including Eastern oyster (*C. virginica*), Kumamoto oyster (*Crassostrea sikamea*), Geoduck clam (*Panopea generosa*), and Greenshell mussel (*Perna canaliculus*) (21, 68, 111-113).

This relatively wide host-range demonstrates the intense potential of this pathogen. Though, it is unclear whether *V. coralliilyticus* strains possess a single pattern of virulence factors capable of causing disease in various hosts, if there are different sets of virulence factors for different hosts, or if each strain contains

different virulence factors which are specific to certain hosts. Infection of marine bivalve larvae by coral-associated strains and a similar response to temperature would suggest an overlap of virulence factors.

C. Bacteriophage

C.1. General description of bacteriophage

Bacteriophage (phage) is bacterial virus that invades bacterial cells. They exist in the various environments including freshwater, seawater, soil and food and harmless to all organisms including mammal, bird, reptile, amphibian, fish and invertebrate except their targeted bacteria (114-116). Phages are classified into lytic phage and lysogenic phage by the differences in life cycles (**Figure 5**).

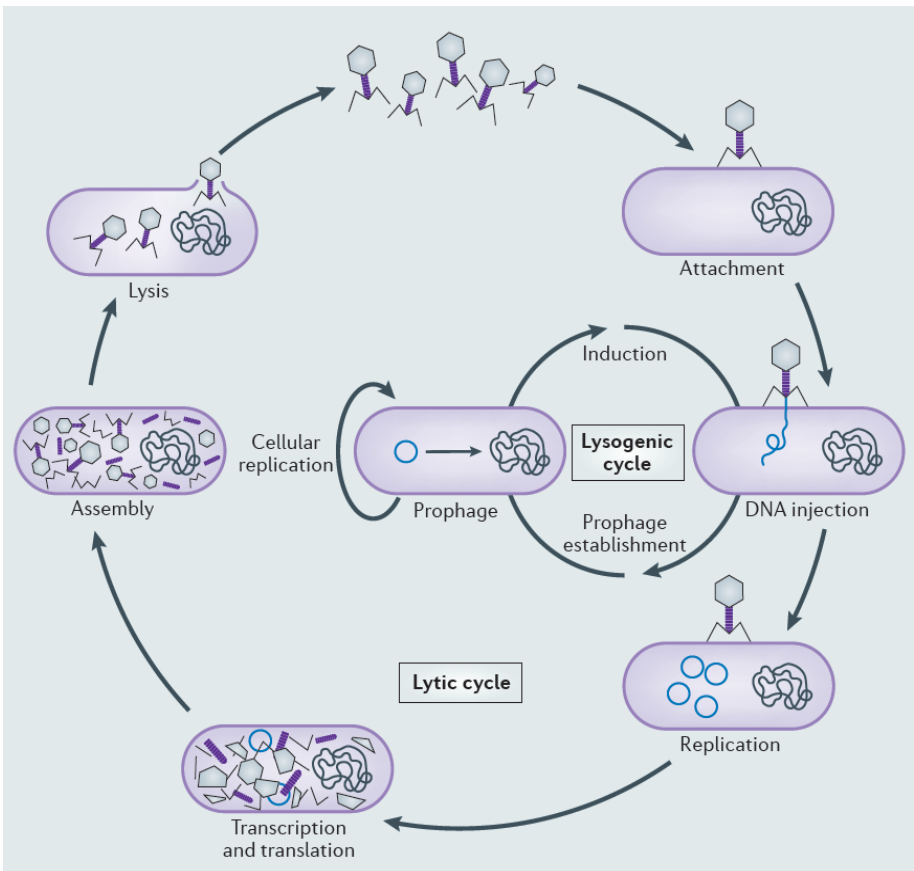

Figure 5. Comparison of life cycle between lytic and lysogenic phages (190).

Lytic phages replicate inside the host cell and progeny viruses that are released, cause cell lysis. This mechanism to destroy host bacteria set the stage for the use of lytic phages as therapeutic or prophylactic agents. In contrast, lysogenic phages infect the host bacteria through the lysogenic cycle. The phage genes remain dormant in the form of a prophage, which is replicated to the host's genome and maintained by a replication cycle. Lysogenic phages can be transformed to a lytic cycle by specific factors (114). Lysogeny and prophages may act as intermediaries to deliver antibiotic resistant genes or other toxin resistance genes to bacteria (117). Therefore, only lytic phages should be used in phage therapy (118).

Phage was discovered by Ernest Hankin (1896) and Frederick Twort (1915). Felix d'Herelle is the first scientist who applied the phage to treat severe dysentery. Since discovery of phage, studies on phage treatment have been actively conducted by various scientists and, several companies have actively developed products to treat bacterial diseases in human. However, since the discovery of Penicillin by Alexander Fleming (1928), phage research has declined with the development of antibiotics research except some Eastern European countries.

Since the discovery of Penicillin, various antibiotics to control bacterial diseases have been studied, and various antibiotics with good antiseptic effects have been actively developed. However, due to the emergence of antibiotic-resistant bacteria, many studies are being actively conducted to find alternatives, and the phage has also been re-examined as a new way to control super bacteria.

C.2. Phage application in aquaculture

According to statistics from the FAO, the aquaculture industry is expanding every year around the world and its output is also rising steadily. It produced 70,203,425 tons of aquaculture products in 2008 and 114,508,042 tons in 2018 (**Figure 6**) (1).

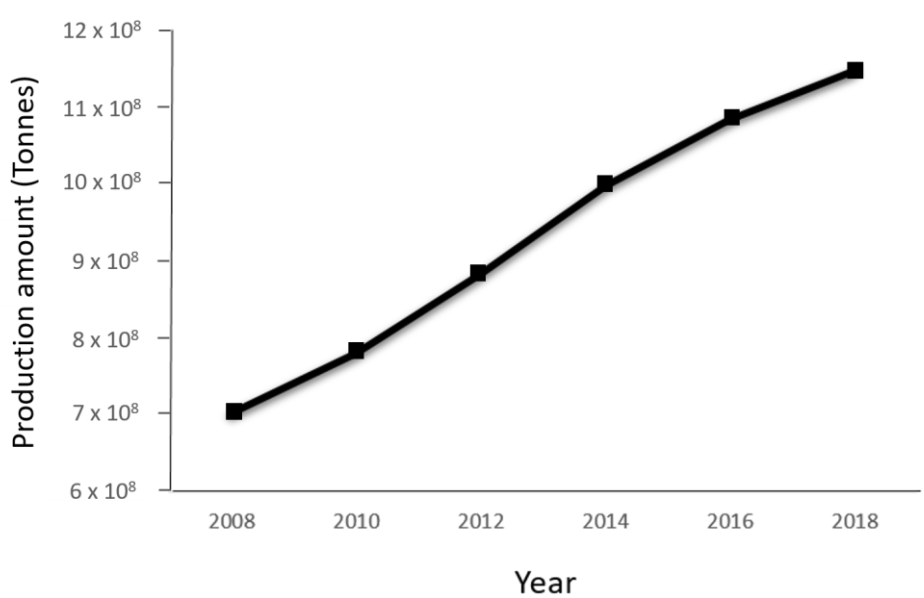

Figure 6. World aquaculture products in the last 10 years (1).

With the development of the aquaculture industry, mortality caused by various pathogenic factors is also continuously occurring. Various antibiotics for aquaculture are used to control the bacterial diseases, and the aquaculture industry also experiencing problems due to the emergence of antibiotic-resistant bacteria and overuse of antibiotics causing environmental pollution (96). Therefore, studies are actively being conducted on the application of various alternative methods to replace

antibiotics, including the application of vaccines, probiotics, and bacteriophage (26, 120-125).

Like other animals and humans, aquatic animals are also vulnerable to microbial infection. Chemotherapy is a fast and effective way to control or prevent bacterial infection, but abuse of antibiotics led to the occurrence of drug-resistant strains. In particular, indiscriminate use of antibiotics can be serious in the aquaculture industry (119). Vaccination is an ideal way to prevent infectious diseases, but commercially available vaccines are still very limited in the aquaculture field. As fish have lots of infectious diseases, the development of the vaccine is limited. In the case of invertebrates, there is no acquired immune system, so the vaccination cannot be applied. Studies on biological control such as probiotics have been consistently reported in the fish pathology field (124-126). However, as mentioned in the use of probiotics to human, there is considerable difficulty in scientific verification of causal continuity (127).

Problems such as the narrow selection of limited antibiotics licensed for fisheries in aquaculture industry and the emergence of various antibiotic resistant bacteria are being raised. Therefore, there is growing interest in possible use of phage to treat bacterial diseases in aquaculture industry.

Chapter I

Mass mortality in Korean bay scallop (*Argopecten irradians*) associated with Ostreid Herpesvirus-1 µVar

Abstract

Since November 2017, mass mortalities of larvae of bay scallop (*Argopecten irradians*) were reported in hatcheries located at the southern area of Republic of Korea. Over 90% of larvae aged 5 to 10 days sank to the bottom of the tank and died. The hatcheries could not produce spat and thus artificial seed production industry incurred huge losses. We identified Ostreid Herpesvirus-1 µVar (OsHV-1 µVar) associated with mass mortality by PCR, sequencing and transmission electron microscopy (TEM). All the samples were positive for OsHV-1 µVar with 99% sequence identity to previously reported OsHV-1 µVar sequences. Partial sequence of ORF-4 of OsHV-1 detected in this study was more closely related to sequences isolated from Europe. This is the first report to confirm the mortality caused by an OsHV-1 infection in the bay scallop.

Keywords: Ostreid Herpesvirus-1 µVar (OsHV-1 µVar), mass mortality, bay scallop (*Argopecten irradians*) larvae

1.1. Introduction

Asia is the major producer of scallops. In 2016, it produced 2,096,663 tonnes ($ 5.3 millions) out of the 2,126,930 tonnes (98.58%) scallop produced worldwide (1). Since bay scallop was imported from China in 2002, Korea has continuously increased the production of scallops after the development of scallop artificial seed production in the mid-2000s (1). In case of Korea, scallop production increased dramatically over the years. In 2002, only 5 tonnes scallops were produced in Korea, whereas production has been increased to 2,995 tonnes in 2016 (1). The artificial seedling production industry for Pacific oyster seeds is highly developed in the southern region of Korea. Additionally, various other kinds of bivalve hatcheries have also developed including bay scallops in Korea. However, a mass larval mortality of the Pacific oyster occurred in 2011, and one of the associated agents was suspected as Ostreid Herpesvirus-1 μVar (OsHV-1 μVar) (4). In addition, since November 2017, another mass mortality on unknown causes in the bay scallop hatchery is occurring.

OsHV-1, which belongs to the family *Malacoherpesviridae* (2, 3), associated with mass mortality of marine bivalve larvae in the spat and juvenile stages. OsHV-1 mass mortality has been reported worldwide including France, Italy, Ireland, Portugal, Spain, Korea, Japan, China, USA, Australia, and New Zealand (3-14). In addition, it has been reported that OsHV-1 has a wide host range and is detected by PCR in various kinds of bivalve larvae and spat including Pacific oysters (*Crassostrea gigas*), Eastern oysters (*C. virginica*), European flat oysters (*Ostrea edulis*), Manila clams

(*Ruditapes philippinarum*), Carpet shell clams (*Ruditapes decussatus*), French scallops (*Pecten maximus*), Farrer's scallops (*Chlamys farreri*), and *Scapharca broughtonii* (5, 6, 11-14). Since the report of Farley et al. (6), mass mortality associated with OsHV-1 infection have been continuously reported in various kinds of bivalves worldwide, but no treatments are available yet (15). This results in severe economic losses to the bivalve seed production industry every year.

Since November 2017, continuous mass mortalities in the bay scallop commercial hatcheries located in the southern region of Korea has led to huge commercial losses to hatchery farmers resulting in major constraints in the aquaculture of this bivalve. Therefore, the present study was conducted to investigate the causative agent of the mass mortality in bay scallop hatcheries located in the southern region of Korea from November 2017 to May 2018.

1.2. Materials and methods

1.2.1. Clinical examination and bacteria and parasites detection

Dead and moribund larvae that sank to the bottom of culture tanks were collected, preserved at 4℃, and transferred immediately from bay scallop hatcheries located in the southern coast of Korea to the Aquatic Biomedicine Laboratory, College of Veterinary Medicine, Seoul National University. Samples were observed using an optical microscope (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, Japan) to observe the clinical signs. Parasitological, bacteriological, and virological examinations were performed to confirm the causative agent of mass mortalities. For

parasitological examinations, larvae with the seawater used for rearing them were centrifuged at 3,000 *g* for 10 min. After centrifugation, the pellet containing larvae and microorganisms was moved to a plankton counting chamber with 1 ml of autoclaved filtered seawater (AFSW) and observed using an optical microscope. For bacteriological examinations, dead or moribund larvae were washed three times using AFSW and homogenised in AFSW. Cotton swabs dipped in the homogenate were streaked onto Tryptic Soy Agar (TSA; BD Difco, USA) supplemented with NaCl (2.0% final concentration) and thiosulfate citrate bile salts sucrose (TCBS) agar (BD Difco, USA) plates. The plates were incubated at 27℃, which was equal to the water temperature in the hatchery, for 24 h. If dominant colonies were observed on plates, single colony were isolated and re-streaked on TSA and TCBS plate and incubated for 24 h at 27℃ in triplicate to obtain pure colonies. For identifying the bacteria, 16S rRNA gene sequence was carried out at Macrogen, Korea. The bacterial 16S rRNA sequences were subjected to a BLAST search against National Centre for Biotechnology Information (NCBI) databases. Additionally, *Vibrio coralliilyticus* and *V. tubiashii,* which are major causative agents of mass mortality in bivalve larvae, were detected by PCR using *V. coralliilyticus* and *V. tubiashii* specific primer pairs (29, 133).

1.2.2. *Molecular detection of viruses*

Conventional PCR was performed to detect OsHV-1 DNA and diagnose Oyster Velar Virus Disease (OVVD), the major viral disease of bivalve larvae. Fifty

milligrams of dead or moribund larvae was homogenized to break the shells, and total DNA was extracted using the DNeasy® Blood & Tissue Kit (Qiagen, , USA) following the manufacturer's protocol. PCR for OsHV-1 DNA detection was performed using C2/C6 (12), IA1/IA2 (37), and Del36-37F/Del36-37R (134) primer pairs according to the corresponding previously published papers. To measure viral DNA quantities, q-PCR was also performed using C9/C10 primer pairs as previously reported by Pepin et al. (135). To construct a standard curve for the quantification procedure, partial gene of OsHV-1, targeted C9/C10 region was synthesized at gene synthesis company (Macrogen, Korea). Synthesized gene was serially diluted upto 10-fold to determine standard curve and, genomic DNAs from sampled larvae were also used. Real-time PCR was performed following the previously established protocol by Pepin et al. (135). OVVD DNA was also detected using iridovirus specific primer sets previously established by Sudthongkong et al. (136). For gene sequence analysis, PCR products were sent to the genomic division of Macrogen (Korea) where nucleotide sequencing reaction was performed using ABI PRISM 3730XL Analyzer with BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, USA). BLAST was performed to identify the sequences obtained from the specimen, and the hundred most similar sequences were retrieved for phylogenetic analysis. The sequences were aligned using CLUSTALX (137) from GENEIOUS v.9.1.8 (138). Bayesian analysis was performed using MrBayes 3.2.3. (139) based on the GTR+G model chosen as the best substitution model in

jModelTest v.2.0 (140). The Markov Chain Monte Carlo analyses were run for 10 million generations with tree sampling every 1,000 generations.

1.2.3. TEM analysis

Infected larvae were fixed in 2.5% glutaraldehyde (Sigma-Aldrich, USA) and kept overnight at 4℃. Fixed larvae were washed three times with PBS (0.1 M, pH 7.2) and post-fixed in 2% osmium tetroxide (Sigma-Aldrich, USA) for 1 h at room temperature. The samples were then washed three times briefly with PBS and then dehydrated by passing through a graded alcohol solution of 50, 60, 70, 80, 90, 95 and twice in 99.9% ethanol for 10 min each and finally for 1 h in 99.9% and substituted by using propylene oxide (Sigma-Aldrich, USA) for 30 min. Infiltration was conducted by using propylene oxide and EPON diluted epoxy resin (Sigma-Aldrich, USA) mixed with PBS (2:1 for 1 hour and 1:1 for 1 hour). The samples were finally embedded in epoxy resin for 1 h and the mixed samples were loaded into capsules and polymerised overnight at 80℃C. Sections for light microscopy were cut at 1.0 nm and stained with 1% toluidine blue (Sigma-Aldrich, USA) for 45 s. on a hot plate at 80℃. Thin sections were made using an ultramicrotome (MT-XL, RMC products, USA) and collected onto a copper grid. Appropriate areas for thin sectioning were cut at 65 nm and stained with saturated 4% uranyl acetate (Sigma-Aldrich, USA) and 4% lead citrate (Sigma-Aldrich, Saint Louis, USA) before examination with a transmission electron microscope (JEM-1400, JEOL, Japan) operating at 80 kV.

1.3. Results

1.3.1. Clinical examination and bacteria and parasites detection

No specific clinical symptoms such as adhesion of ciliates and velum necrosis on the dead larvae were observed microscopically (**Figure 7a**). Specific clinical signs of *V. coralliilyticus, V. tubiashii* and OVVD infection such as velum necrosis, velar degeneration with loss of cilia and velar epithelium (**Figure 7b**) were not observed. In addition, protozoans including parasitic organisms were also not observed except feeding phytoplankton. Two dominant strains of bacteria were isolated from the larval homogenates. PCR analysis of two dominant strains did not detect *V. coralliilyticus* and *V. tubiashii* DNA. In case of 16S rRNA region PCR, BLAST search of 16S rRNA sequences from the isolates exhibited 99% sequence similarity with corresponding genes of Uncultured *Vibrio* sp. (GenBank accession number: KF758738, JX024158).

1.3.2. Molecular detection of viruses

Examination for OsHV-1 and OVVD was conducted by PCR and sequencing. All samples were negative for OVVD (136). On the other hand, OsHV-1 DNA was detected in all samples by PCR using C2/C6 (12), IA1/IA2 (37), and Del 36-37F/Del 36-37R (134) primer pairs designed for the ORF4, ORF42-43 and ORF35-38 regions of the OsHV-1 sequence. The sequences of the PCR products of Korean OsHV-1 μVar were deposited in GenBank (accession number MK569378, MK569379 and MK591136). Result of real-time PCR showed a linear relationship between the DNA

concentration (X) and the related *Ct* value (Y) using 10-fold diluted synthetic genes (**Figure 8**). Quantities of OsHV-1 µVar DNA in sampled present study were 0.014 ng/ul, 0.05 ng/ul and 0.003 ng/ul. The analysis of the nucleotide sequences of OsHV-1 isolated in this study confirmed that all three sequences were 100% identical. BLAST search confirmed that the sequences isolated in this study exhibited 100% similarity with corresponding genes of three OsHV-1 sequences (KY271630, KY242785, and KF185070). These three OsHV-1s were isolated from Pacific oyster larvae in Europe (France and Ireland). A phylogenetic tree was constructed using the ORF4 gene partial CDs sequence (**Figure. 9**). OsHV-1 variant, isolated in this study was different from previously reported variants from Korea (JQ959597 and JQ959598). Bayesian phylogenetic analysis showed that global OsHV-1 specimen were subdivided into several groups. Although Korea is geographically close to Japan and China, OsHV-1 µVar sampled from Korea formed a monophyletic group with the specimen reported in Europe (e.g., France, Ireland, Italy, Spain, and Portugal). However, the group is poorly supported (bootstrap support=0.78). Further studies are required to reveal epidemiological patterns of OsHV-1.

1.3.3. *TEM analysis*

Viral particles were observed in 7 day-old moribund larvae of bay scallop by TEM (**Figure 10**). Infected cells present numerous vacuoles, and icosahedral nucleocapsids containing a pleomorphic core with an electron-dense structure were observed. Size of viral particles was 96.22±5.8 nm (n=20) (**Figure 10**).

1.4. Discussion

During mass mortality of the bay scallop larvae in the southern regions of Korea, it was observed that 5 to 10-day-old larva sank to the bottom of the tank. Based on this observation, this study focused on OsHV-1, OVVD, *V. coralliilyticus* and *V. tubiashii* infections which were have been reported to demonstrate similar symptoms (6, 89, 92, 141, 142). In this study, specific clinical symptoms such as adhesion of ciliates and velum necrosis, specific clinical signs of *V. coralliilyticus*, *V. tubiashii* and OVVD were not observed and parasitic organisms were also not observed. Although, it could not be confirmed that the isolated *Vibrio* sp. was not the causative agent of the mass mortality, *V. coralliilyticus* and *V. tubiashii* which are reported to cause massive mortality of bivalve larvae were not detected in PCR and the specific clinical signs such as velar degeneration with loss of cilia and velar epithelial cells were not observed (89, 92). Therefore, we ruled out the possibility of the *Vibrio* sp. being the main causative agent of the mass mortality.

According to Segarra et al. (37), OsHV-1 µVar showed sequence deletions in the microsatellite zone against OsHV-1 reference type when using the C2/C6 primer pair. In the case of OsHV-1, the pattern “CTA” is repeated eight times, but in the case of OsHV-1 µVar, it is repeated four times with deletion. As a result of the alignment with reference type (NCBI GenBank accession no. AY509253), isolated OsHV-1 in present study shown only the pattern “CTA” is repeated four times with deletion. Sequence of using IA1/IA2 primer pair showed deletion of one adenine (A) and one substitution of cytosine (C) with thymine (T). Alignment result of present study

showed one ‘A’ deletion but ‘C’ was not replaced by ‘T.’ It means that isolated in Korean OsHV-1 variant was not correct with European OsHV-1 µVar. Using the Del 36-37F/Del36-37R primer pair, Renault et al. (134) reported that the sequence lengths of OsHV-1 and OsHV-1 µVar are 989 bp and 384 bp, respectively and Korean variant showed 384 bp of product size. By comparing the nucleotide sequences of the OsHV-1 detected in the present study with those of the reference type OsHV-1 (accession no. AY509253), we confirmed that the OsHV-1 detected in this study is OsHV-1 µVar (**Figure 11 and Figure 12**).

According to previous studies, OsHV-1 is in the form of a regular icosahedron with a capsid and nucleocapsid with a pleomorphic core; the size of the capsids is also reported to be very similar to what we observed. (5, 143). OVVD capsids are hexagonal and the reported size is 228 nm (141, 144). The viral particles confirmed by TEM observation resembled OsHV-1 (3-5, 53, 143).

We investigated the cause of mass mortality of bay scallop by the exclusion process of parasites, bacteria, and viruses. Additionally, we used PCR to examine OsHV-1, OVVD, *V. coralliilyticus,* and *V. tubiashii* infections. OsHV-1 µVar DNA was detected by PCR and viral capsids were observed by TEM. Other clinical signs or pathogenic factors were not observed. According to the World Organisation for Animal Health (OIE) report, OsHV-1 infected larvae have acute mortality without specific clinical signs (15). Based on this evidence, it was confirmed that the bay scallop larvae died in association with OsHV-1 µVar DNA detection and viral particle observation.

Although OsHV-1 is not designated as an OIE-listed disease, continuous monitoring is required to prevent the prevalence and spread of this disease in order to minimise economic loss to both the domestic and international bivalve industries. OsHV-1 has been reported to be pathogenic to larvae, spat, and juvenile stages, but not to the adult bivalve (41, 145). Arzul et al. (41) suggested that the vertical infection of OsHV-1 detected in the broodstock could cause mass mortality of the seedling culture. Therefore, it is necessary to confirm the presence of OsHV-1 infection in the broodstock before the seedling production of the bivalve. Generally, vaccination is not possible due to the immune system of bivalve, the huge volume of seawater need to use important concentration of virucidal agent and UV treatments of seawater are needed. Degremont (146) suggested that genetic selection appear to be interesting way to control the OsHV-1.

In Korea, OsHV-1 μVar DNA was detected for the first time in adult Pacific oysters in 2011 (147), and it was also detected in the field of Pacific oyster hatcheries, where mass mortality occurred in 2011 (4). So far, there have been reports of OsHV-1 μVar DNA detection only in the southern coast of Korea, and this study also detected OsHV-1 in the artificial seedling production area of bay scallop located in the southern coast of Korea. Korea is surrounded by sea on its three sides, and bivalve hatcheries in each area culture the species of bivalves prominent in that specific area. Because OsHV-1 has a broad host range, it is likely to be present in bivalves in the West and East sea in Korea. Therefore, in order to minimise the damage caused by the occurrence and spread of OsHV-1, continuous monitoring of

bivalve farms and hatcheries located in each sea area is required. In addition, in order to prevent the spread of OsHV-1, local hatcheries should minimise the migration of their broodstock and larvae.

This study investigated the causative agent of mass mortality in bay scallop hatcheries located on the southern coast of Korea. It was confirmed that OsHV-1 μVar was associated with mortality of Bay scallop larvae through PCR, 16S rRNA sequence analyses, and TEM investigation. This is the first report of OsHV-1 μVar DNA detection and viral capsid observation in bay scallops.

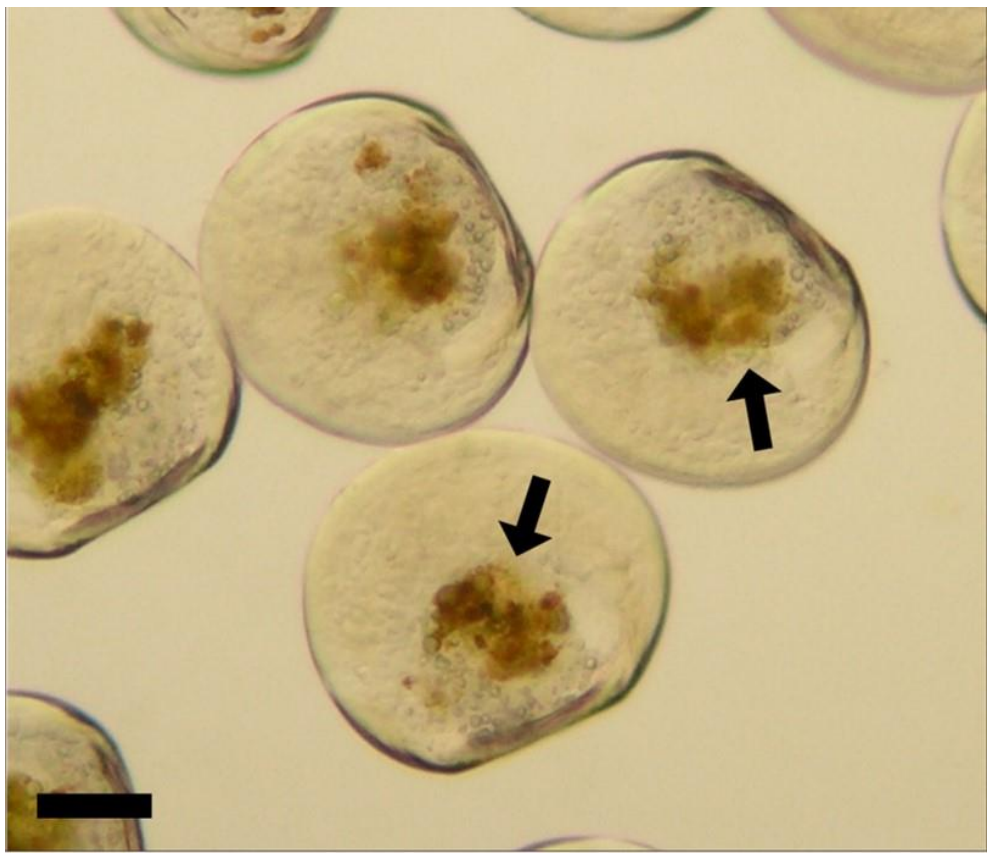

Figure 7. Microscopic observation of OsHV-1 infected bay scallop larvae. The larvae died without any clinical signs and arrows indicate undigested phytoplankton in the digestive gland (Bar: 30 um).

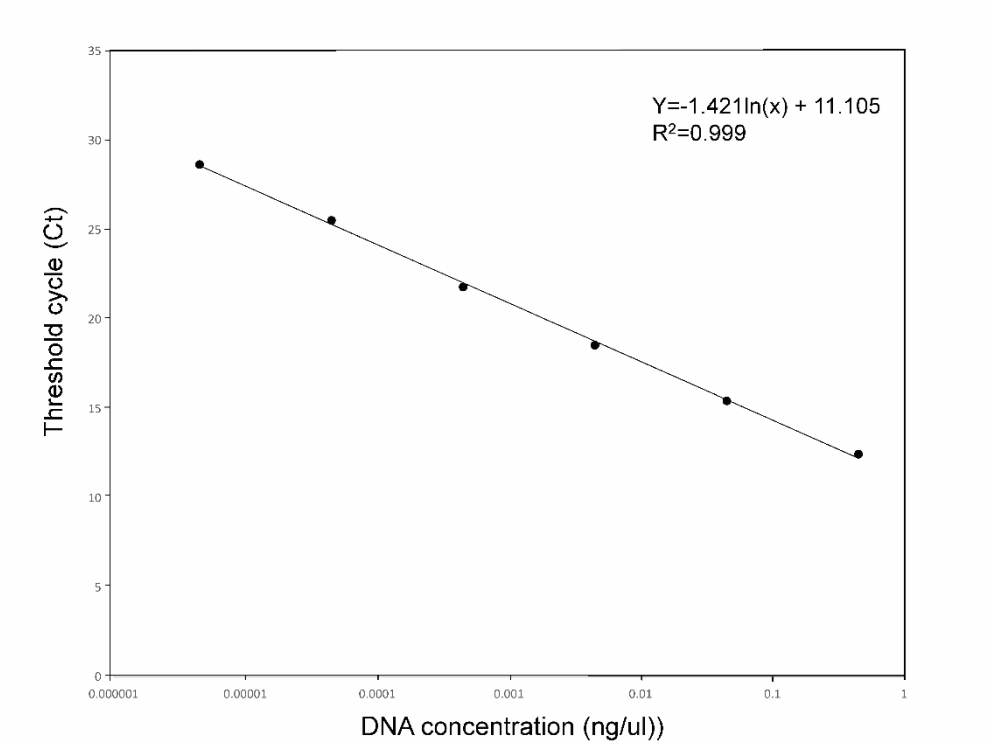

Figure 8. Standard curve of diluted synthesized OsHV-1 partial sequence using C9/C10 primer pair.

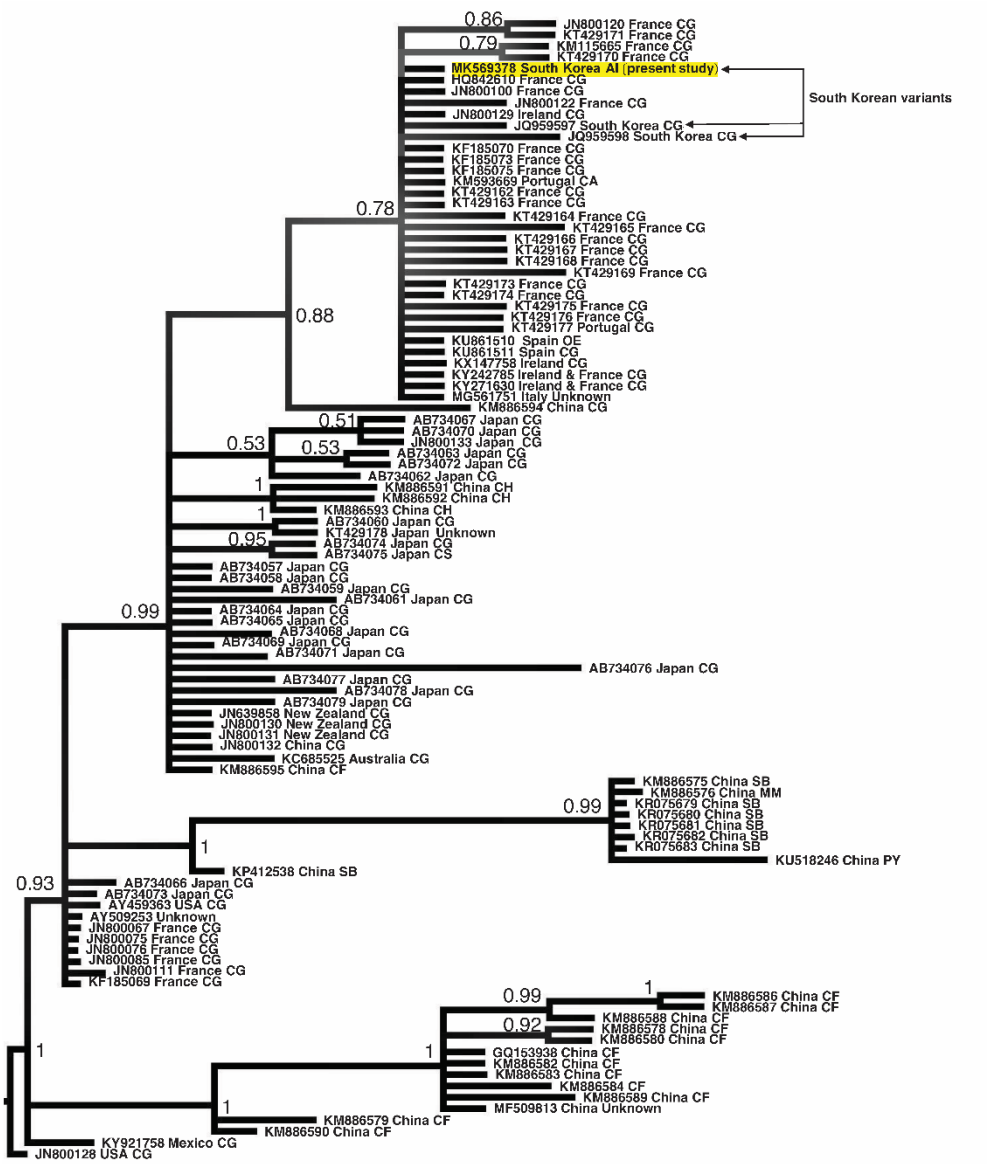

Figure 9. Phylogenetic analysis of Korean OsHV-1 variants. A total of 100 sequences from GenBank were used. Nodal supports are shown next to each node. The labels of the terminal branch are shown in the order of accession number,

locality and host. The abbreviation of hosts are as follows, CG: *Crassostrea gigas*, AI: *Argopecten irradians*, CA: *C. angulata*, OE: *Ostrea edulis*, CH: *C. hongkongensis*, CS: *C. sikamea*, CF: *Chlamys farreri*, SB: *Scapharca broughtonii*, MM: *Meretrix meretrix*, PY: *Patinopecten yessoensis*. Arrows indicate the sequences identified in South Korea.

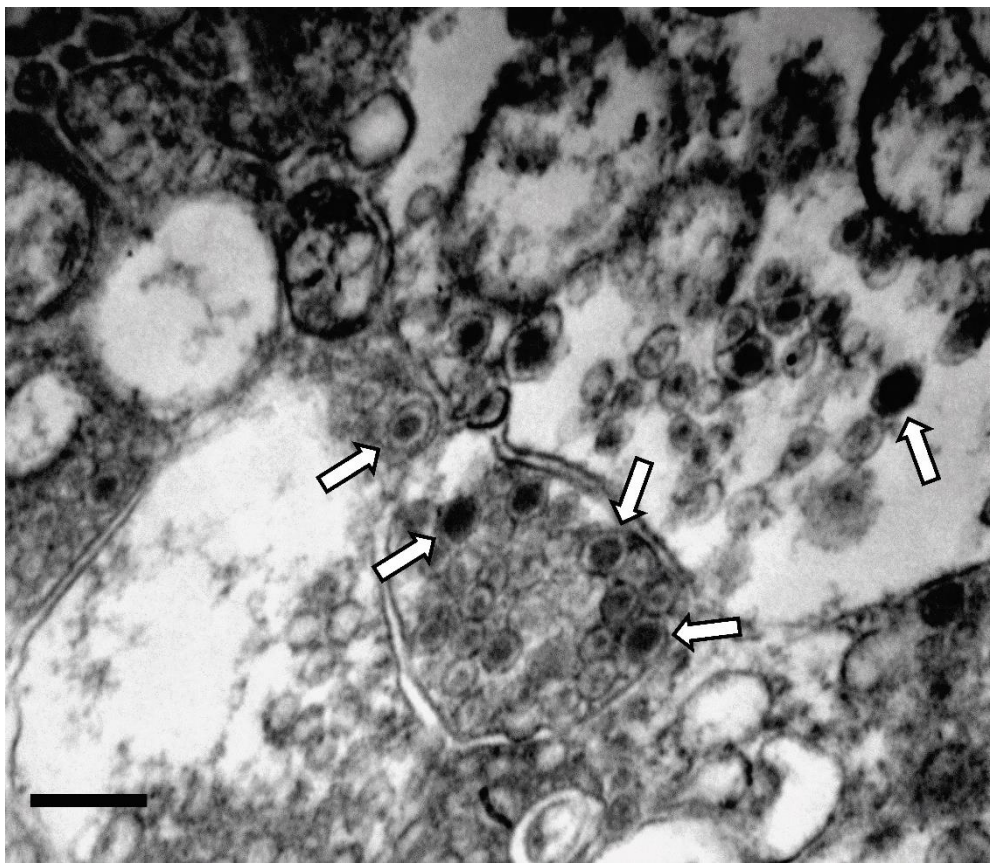

Figure 10. Enveloped virions containing pleomorphic cores (arrows) were observed in the lysed cell (Bar: 500 nm).

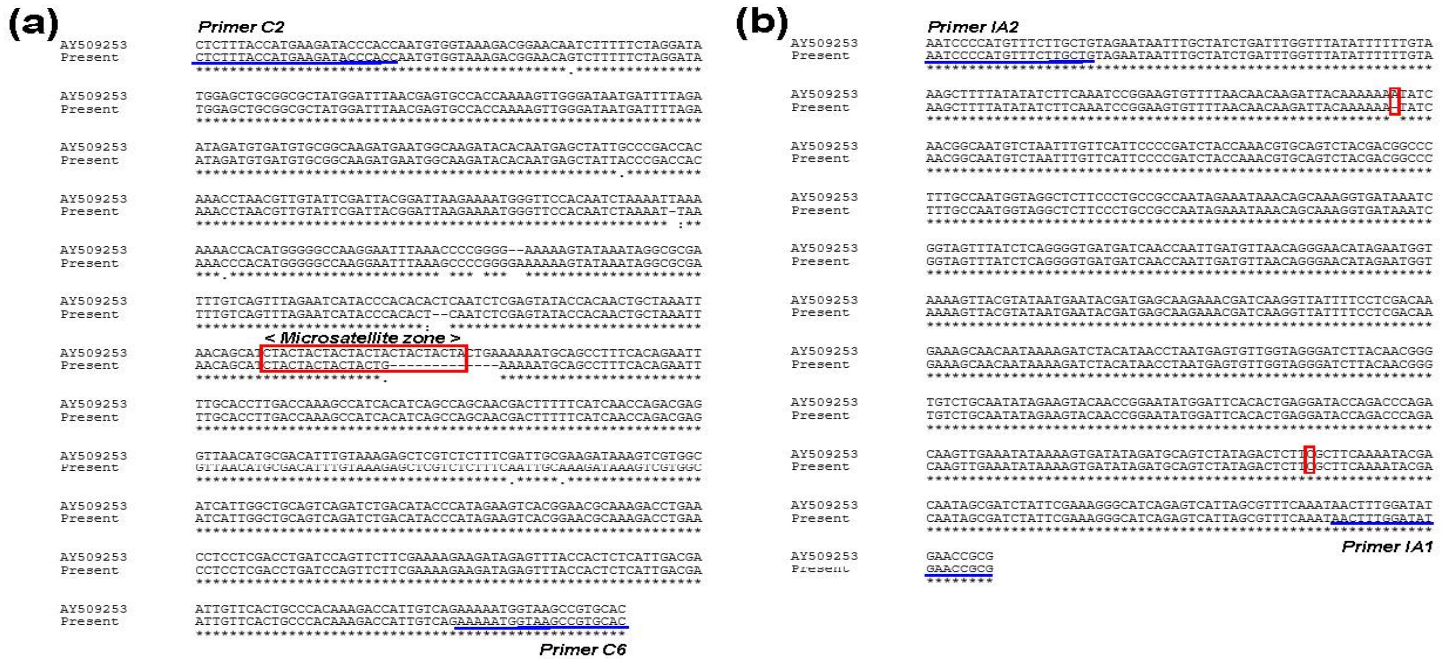

Figure 11. Comparison of sequences of isolated variant and reference type. The locations of each primer are underlined. (a) Result of C2/C6 region alignment. The location of the microsatellite zone was framed. (b) Result of A1/IA2 region alignment. The deletion “A” and not substitute “C” to “T” positions are framed.

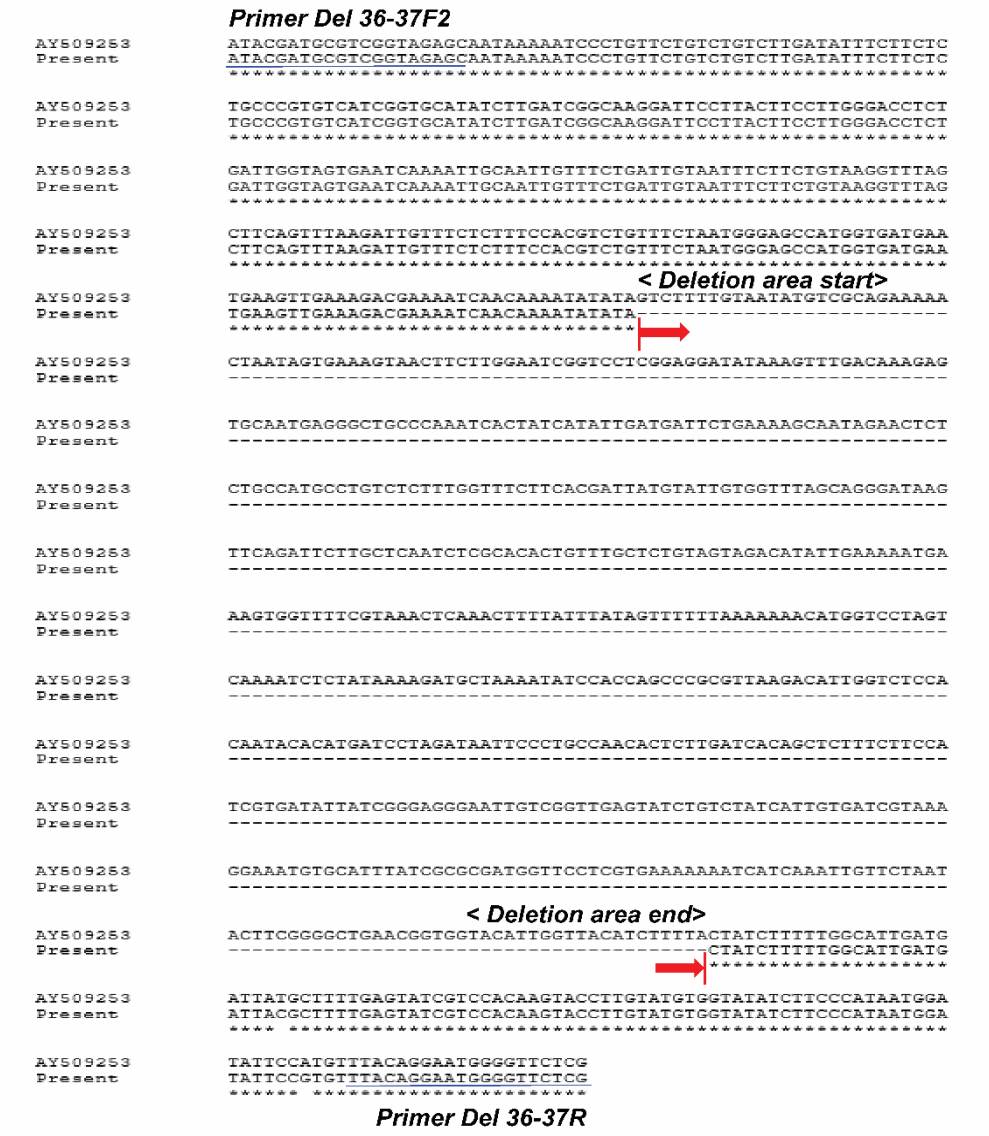

Figure 12. Result of Del36-37 alignment between reference type and OsHV-1, isolated in present study. The location of primer Del36-37F/Del36-37R are underlined with blue color. The location of the deletion area against reference type was marked by arrow.

Chapter II

Identification and genome analysis of *Vibrio coralliilyticus* causing high mortality of Pacific oyster (*Crassostrea gigas*) larvae

Abstract

Vibrio coralliilyticus is known as a coral pathogen that also infects marine bivalve larvae worldwide. It is considered to be one of the major constraints in artificial marine bivalve seed production as it causes mortality. In this study, we first isolated and characterized a high virulent of *V. coralliilyticus* designated as SNUTY-1 that was the cause of Pacific oyster larvae mortality in Korea. In the pathogenicity test, exposure to 2.14×10^5 CFU/mL for 24 h caused mortality to 88.65 ± 2.4% of the tested healthy Pacific oyster larvae. SNUTY-1 showed anti-microbial resistance to β-lactams, such as penicillins, cephalosporins, and carbapenems. We sequenced and assembled the complete genome of SNUTY-1 (5,842,676 bp), consisting of two chromosomes (Chr I and Chr II) and two plasmids (pSNUTY1 and pSNUTY2). The COG functional analysis confirmed that Chr I had more genes associated with basic cellular functions in comparison to Chr II. The results of the phylogenetic trees based on OrthoANI values indicated that the SNUTY-1 was closely related to *V. coralliilyticus* strains. SNUTY-1 had a unique plasmid (pSNUTY2), which could mean that the Korean isolate is different from other sequenced *V. coralliilyticus*

strains from different geographical origins. Toxic proteins such as cytolysin/hemolysin and extracellular metalloprotease genes were encoded on Chr I and Chr II of SNUTY-1. These data facilitate the control of *V. coralliilyticus* infections in aquaculture by providing valuable insights into the biodiversity of this organism and valuable information for the study of virulence factors.

Key words: *Vibrio* species, Pacific oyster (*Crassostrea gigas*), genome analyses, Chr I, Chr II, SNUTY-1.

2.1. Introduction

Oysters are the most consumed shellfish worldwide and account for the largest commodity in the bivalve aquaculture industry. The Republic of Korea is a major producer of Pacific oyster (*Crassostrea gigas*). In 2017, it produced 315,255 tons out of the total 639,030 tons (49.33%) of Pacific oysters produced worldwide (1). However, since the middle of the 2000s, there have been perpetual occurrences of mass mortality owing to bacillary necrosis in Korean oyster seedling production hatcheries. *Vibrio* sp. has been speculated to be the cause, however, there have been no definite investigation until now.

The genus *Vibrio* is composed of ubiquitous aquatic bacteria, including diverse members of planktonic and animal-associated microbial communities (16). Several organisms in this genus, such as *V. alginolyticus*, *V. anguillarum*, *V. splendidus* biovarII, *V. tubiashii*, and *V. coralliilyticus,* have been associated with mass mortalities in nursery cultures of juvenile bivalves at oyster hatcheries worldwide (17-20, 92). Although all of the abovementioned species have been implicated in bacillary necrosis, *V. tubiashii* has been identified as one of the most critical marine bacteria causing the well characterized adverse effect (93). While *V. tubiashii* and *V. coralliilyitcus* exhibit similar virulence in the Eastern oyster, *V. coralliilyticus*-induced mortality is far greater than *V. tubiashii*-induced mortality in the Pacific oyster (21).

V. coralliilyticus, a Gram-negative, rod-shaped bacterium, is a well-known pathogen of coral, responsible for tissue lysis, bleaching, and drastic losses in coral

reefs worldwide (22, 23). Moreover, this bacterium has been shown to infect fish (24) and bivalves, including the Pacific oyster (25, 26). Therefore, *V. coralliilyticus* could be considered as one of the major pathogens that causes economic damage by infecting various aquatic organisms.

In 2015, continuous mass mortalities in the Pacific oyster hatchery located in the southern area of Korea, led to huge commercial losses to hatchery farmers and aquaculture. For this reason, the present study was conducted to investigate the cause of the mass mortalities that continuously occur in Pacific oyster seedling production in Korea. We characterized the isolated *Vibrio* sp., analyzed its genome sequence to compare with previously reported strains, and investigated its virulence and antibiotic resistance genes.

2.2. Materials and methods

2.2.1. Sampling and clinical examination

Samples of five day-old dead and inactive Pacific oyster larvae (100–120 μm) that had sunk to the bottom of the culture tanks from a Pacific oyster hatchery at Tongyeong (34˚47’13”N, 128˚25’22”E), and healthy larvae (100–140 μm) from Geoje island (34˚47’07”N, 128˚32’40”E) were preserved at 4℃ and immediately transported to the Aquatic biomedicine laboratory, College of Veterinary Medicine, Seoul National University (**Figure 13**). Prior to the start of the experiment, larvae were washed three times using filtered and sterilized seawater (FSS; 33 psu, 0.22 μm, 121℃ for 15 min) in order to eliminate potential infections from other

microorganisms. Washed samples were observed for clinical signs using an optical microscope (Olympus BX41, Olympus Optical Co., Ltd., Japan). In addition, moribund larvae and the seawater were moved to a plankton-counting chamber to verify the existence or nonexistence of parasitological organisms using an optical microscope.

2.2.2. *Molecular detection of pathogen*

Conventional polymerase chain reaction (PCR) was carried out to detect OsHV-1, *V. coralliilyticus*, and *V. tubiashii* that have been reported to be the main causative agents of mass mortalities in marine bivalve hatcheries worldwide. Fifty milligrams of moribund larvae were placed in a 1.5 mL centrifuge tube and the shells were broken by using a disposable tissue homogenizer. Total genomic DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, USA) following the manufacturer's protocols. OsHV-1 was diagnosed using template DNA extracted from inactive larvae. PCR diagnosis of OsHV-1 DNA detection was conducted using C2/C6 primer pair, designed at the ORF-4 region following a previous study (12). PCR for *V. coralliilyticus* and *V. tubiashii* were performed using specific primer pairs vcpAF/vcpAR, designed at the zinc-metalloprotease gene region of *V. coralliilyticus* (148) and protease and hemolysin gene region of *V. tubiashii* (111).

2.2.3. Bacteria isolation and identification

To isolate bacterium, fragmentized inactive larvae using a disposable tissue homogenizer plated on Marine Agar 2216 (BD Difco, USA) and Thiosulfate Citrate Bile Sucrose agar (BD Difco, USA) were incubated at 27℃ for 48 h. Colonies on both agar plates were re-spread onto Tryptic Soy Agar (TSA; BD Difco, USA) supplemented with NaCl (2.0% final concentration) and incubated at 27℃ for 24 h. The dominant single colony was then re-streaked onto TSA (2% NaCl) and incubated for an additional 24 h at 27℃ to obtain pure colonies. Gram staining was then performed using the isolated bacterium. Further, bacterial genomic DNA of the pure cultured bacteria was extracted using DNeasy Blood & Tissue Kit (Qiagen, USA) following the manufacturer's protocols. Analysis of 16S rRNA gene sequence was carried out using an ABI PRISM Big Dye TM Terminator Cycle Sequencing Kit (Applied BioSystem Inc., USA) at Macrogen Inc. (South Korea). Acquired 16S rRNA of bacterium sequence was subjected to BLAST search software supplied by the National Center for Biotechnology Information (NCBI).

2.2.4. Pathogenicity test of isolated bacterium

Healthy Pacific oyster larvae, 5 days old (100-140 μm) and preserved at 4℃, were immediately transported from the oyster hatchery at Geoje to the laboratory. Healthy larvae were washed three times using FSS in order to eliminate other microorganisms. To confirm that the larvae were not infected by a pathogen, such as OsHV-1, *V. coralliilyticus* or *V. tubiashii*, PCR diagnosis was performed before the

challenge test. Washed healthy larvae were placed into 6-well cell culture plates (SPL, Korea) with 10 mL of FSS. The density of the larvae was adjusted to 5±2 larvae/ml. Cultured bacteria in Tryptic Soy Broth, adjusted to 2% NaCl (BD Difco, USA) at 27°CC for 24 h were centrifuged at 3000 x *g* for 10 min and washed three times using FSS. Bacterial suspensions in FSS were adjusted from 2.14×10^3 to 2.14×10^7 colony-forming unit (CFU)/mL and inoculated into each wells for 24 h incubation at 27°C. For the control, healthy larvae were placed into the wells without bacteria inoculation. We then investigated the cumulative mortality of the larvae for 24 h at 6 h intervals using an inverted microscope (Olympus CKX31, Olympus Optical Co., Ltd., Japan). The larvae without cilia movement and intestinal motility were verified to be dead by microscopic observation. The pathogenicity test was conducted in triplicate under the same conditions. For the challenge test, we attempted to re-isolate the inoculated bacterium from dead larvae following the same protocols as described above.

2.2.5. *Anti-microbial susceptibility test and biochemical analysis*

The antimicrobial susceptibility test of the isolated bacterium was performed using 21 varieties of antibiotic disks (Oxoid, UK) that are recommended by the Clinical and Laboratory Standard Institute (CLSI) guideline. The list of antibiotics is summarized in **Table 1**. Standard disk diffusion method was conducted on Muller Hinton Agar (BD Difco, USA) at 27°C for 24 h. Susceptibility and resistance were also determined according to the CLSI guideline (18). *Escherichia coli* (ATCC

25922) was used in the experiment to clarify the strain criteria. For the biochemical analysis, VITEK®2 System (bioMérieux®, France) was performed using a gram-negative colorimetric identification card following manufacturer's protocols. *V. coralliilyticus* 58, which is reported to be a strain with high virulence to Pacific oyster larvae (92, 150), was also analyzed using the anti-microbial susceptibility test and biochemical test to compare with the isolated bacterium.

2.2.6. *Genome sequencing*

The isolated bacterium was cultured overnight on TSA (2% NaCl) and then incubated at 27℃ for 24 h. Bacterial genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, USA) following the manufacturer's manual. Genome sequencing was conducted by Macrogen Inc. (South Korea) by the PacBio RS II system (Pacific Biosciences, USA), following construction of a 20 kb SMRTbell template library. The sequences generated (518,621,132 bp; 69,449 reads) were assembled using the Hierarchical Genome Assembly Process (HGAP) version 3.0 (https://github.com/PacificBiosciences/BioinformaticsTraining/wiki/H-GAP), and the genome annotation was performed with the NCBI Prokaryotic Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/books/NBK174280). Genome annotation was conducted using the National Center of Biotechnology Information Prokaryotic Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/books/NBK174280/), and PHASTER (http://phaster.ca/) analyzed to detect prophages. To assess the genomic relatedness to other *Vibrio* species, the average

nucleotide identity was analyzed using OrthoANI (http://www.ezbiocloud.net/tools/orthoani). Potential virulence genes and antimicrobial resistance genes were preliminarily screened by searching against the Virulence Factor Database (http://www.mgc.ac.cn/VFs/) and the ARG-ANNOT database (http://en.mediterranee-infection.com/article.php?laref=283&titre=arg-annot-), respectively, and were then ultimately identified by manual comparisons with those reported for other *V. coralliilyticus* strains in the GenBank database.

2.3. Results

2.3.1. Identification of causative agent

The healthy larvae from Geoje island showed active mobility using their cilia, whereas the inactive larvae from Tongyeong were observed to be lethargic and had poor motility compared to healthy larvae. Almost all of the moribund and dead larvae showed specific clinical symptom of being infected by *V. coralliilyticus* and *V. tubiashii*, such as velum necrosis and adhesion of ciliates when observed under the microscope (17, 20, 21). Except for feeding phytoplankton, no other parasitic protozoa were observed in the moribund larvae group or in the cultured water. Molecular detection of OsHV-1, *V. tubiashii* showed negative reaction. Whereas, targeted size of product was detected when we used *V. coralliilyticus* specific primer pair. Bacteria isolation from the moribund and dead larvae revealed a dominant gram-negative and curved rod-shaped strain. According to the PCR and sequencing of isolated bacterium results of the 16S rRNA region, over 99% of the sequence were

similar to four corresponding genes of *V. coralliilyticus* (GenBank accession number: CP009617, CP009264, CP016556, and CP031472) from the BLAST search at NCBI. Therefore, the isolated strain in this study was considered to very likely be *V. coralliilyticus* and was designated as *V. coralliilyticus* SNUTY-1. To more clearly confirm the identification of the isolate, whole genome sequence analysis was performed.

2.3.2 *Pathogenicity of isolated V. coralliilyticus SNUTY-1*

The healthy Pacific oyster larvae transported from Geoje island were shown to test negative for OsHV-1, *V. coralliilyticus*, and *V. tubiashii* using PCR. Therefore, we conducted a pathogenicity test with *V. coralliilyticus* SNUTY-1 using the Pacific oyster larvae (pathogen free) sampled from Geoje. All of the larvae in the negative control and the group treated with 2.14×10^3 CFU/mL for 24 h survived. Larvae treated with 2.14×10^4 CFU/mL and 2.14×10^5 CFU/mL for 24 h resulted in 22.2 ± 0.84% and 88.65 ± 2.4% cumulative mortalities, respectively. At concentrations of 2.14×10^6 CFU/mL and 2.14×10^7 CFU/mL, 100% of the larvae died within 18 h (**Figure 14**).

2.3.3. *Anti-microbial susceptibility and biochemical analysis of V. coralliilyticus SNUTY-1*

The results of the antibiotic disk diffusion test are summarized in **Table 1**. *V. coralliilyticus* SNUTY-1 had strong antibiotic resistances to ampicillin, amoxicillin-

clavulanate, ampicillin-sulbactam, piperacillin, cefepime, cefotaxime, cefoxitin, ceftazidime, and meropenem. *V. coralliilyticus* SNUTY-1 was confirmed to be resistant to 37.5% of the antibiotics tested in this study. In the biochemical test, 64 biochemicals were tested. The results from *V. coralliilyticus* SNUTY-1 were comparable with *V. coralliilyticus* 58, except for Ala-Phe-Pro-arylamidase and L-lactate alkalinization. Results of the biochemical test are presented in **Table. 2**.

2.3.4. *Genome of V. coralliilyticus SNUTY-1*

General features of the *V. coralliilyticus* SNUTY-1 strain are summarized in **Table 3**. The fully assembled and closed *V. coralliilyticus* SNUTY-1 genome comprised of 5,842,676 bp, consisting of two chromosomes named Chr I (3,474,874 bp) and Chr II (1,976,676 bp), and two plasmids designated as pSNUTY1 (254,703 bp) and pSNUTY2 (136,423 bp). The two chromosomes exhibited similar G+C content (45.7% and 45.1%, respectively) and had similar percentages of the coding regions (87.9% and 87.6%, respectively). The annotated genome included 5,527 genes, 5,370 coding sequences, 37 rRNAs (5S, 16S, and 23S), 116 tRNAs, and four non-coding RNAs. The majority of the predicted tRNAs (n = 111), rRNA (n = 37), and ncRNA (n = 4) genes were encoded on Chr I, except for the 5 tRNA genes that were mostly on Chr II (**Table 4**). In addition, a total of four prophage regions (two intact and two incomplete) were identified. The clusters of orthologous genes (COG) functional category analysis of *V. coralliilyticus* SNUTY-1 confirmed that Chr I had a higher percentage of genes associated with basic cellular functions compared to

Chr II (**Figure 15a**). Functional genes encoded on Chr I were primary involved in the COG categories of J (translation, ribosomal structure, and biogenesis), L (replication, recombination, and repair), D (cell cycle control, cell division, and chromosome partitioning), M (cell wall/membrane/envelope biogenesis), N (cell motility), U (intracellular trafficking, secretion, and vesicular transport), O (post-translational modification, protein turnover, and chaperones), C (energy production and conversion), F (nucleotide transport and metabolism), I (lipid transport and metabolism), and H (coenzyme transport and metabolism). On the other hand, Chr II possessed a higher percentage of genes involved in K (transcription), V (defense), T (signal transduction mechanisms), W (extracellular structures), X (mobilome, prophages, and transposons), G (carbohydrate transport and metabolism), E (amino acid transport and metabolism), P (inorganic ion transport and metabolism), and Q (secondary metabolites biosynthesis, transport, and catabolism). However, both of the two chromosomes contained genes involved in S (function unknown in COG database), and a match to 10.9% and 13.9% of the predicted genes on Chr I and Chr II, respectively, could not be found in the database. As expected, the search for a match in the database for most of the functional genes encoded on the plasmids pSNUTY1 (31.1%) and pSNUTY2 (47.4%) was unsuccessful (**Figure 15b**).

2.4. Discussion

In general *V. coralliilyticus*, *V. tubiashii* and OsHV-1 are known as main causative agents of the mass mortality of marine bivalve larvae (12, 89, 92). Investigations made in this study on the mass mortality of Pacific oyster larvae specifically revealed that dead and moribund larvae experienced symptoms of bacillary necrosis and adhesion of ciliates. In the cases where larvae died from OsHV-1 infection, the dead larvae were not reported to have experienced the above-mentioned specific symptoms (15). Molecular detection of OsHV-1 was diagnosed by conventional PCR using specific primer pairs. However, moribund and dead larvae showed a negative reaction, hence OsHV-1 could be dismissed as a major causative agent of the mass mortality. In contrast, Sugumar et al. (92) and Tubiash et. al. (89) reported that dead larvae infected with *V. coralliilyticus* and *V. tubiashii* displayed these particular symptoms. Therefore, we focused on *V. coralliilyticus* and *V. tubiashii* infections that could be related to the mass mortality of Pacific oyster larvae in Korea. We conducted PCR using both *Vibrio* spp. specific primer pairs. PCR results using a primer pair specific to *V. tubiashii* were negative, indicating that these bacteria were not present in the dead and moribund larvae (111). Whereas, a positive DNA band was detected in the DNA electrophoresis using *V. coralliilyticus* primer pair vcpAF/vcpAR, designed at zinc-metalloprotease gene region of *V. coralliilyticus* (148). Therefore, it is very likely that the mass mortality can be associated with *V. coralliilyticus* infection.

Pathogenicity test results concurred with what has been previously reported for high pathogenic *V. coralliilyticus* strains (21, 92, 129). In the pathogenicity test, moribund and dead larvae showed similar clinical signs with naturally infected samples. Moreover, an intense motile bacterial swarm was observed around the shell margin and cilia of the larvae. Inoculated *V. coralliilyticus* SNUTY-1 was re-isolated from all of the challenged larvae. The results of the experiment are confirmed as they coincide with Koch's postulates.

The VITEK®2 System (bioMérieux®, France) method is based on the ability of microbial substrates to identify and compare them with existing database (152). In this study, we used VITEK®2 system to identify the isolated strain based on its biochemical characteristics. As pointed out earlier by O'Hara et al (153), it was impossible to identify the species using the data stored in the VITEK data base system. However, the result of the VITEK method (**Table 2**) could be considered as preliminary data for further biochemical characterization of *Vibrio* spp.

Currently, complete genomes of *V. coralliilyticus* OCN014 (101), RE98 (28), RE22 (154), and 58 (150), and *V. tubiashi* ATCC 19109 (155) are available in the GenBank database. Therefore, the OrthoANI algorithm (156) was applied to assess the overall genome similarities between *V. coralliilyticus* SNUTY-1 and the other related *Vibrio* strains. OrthoANI values were acquired and phylogenetic trees were constructed on the basis of the OrthoANI analysis of the four strains of *V. coralliilyticus* (58, OCN014, RE22 and RE98), the one strain of *V. tubiashii* (ATCC 19109), and other associated *Vibrio* spp. utilizing the orthologous average nucleotide

identity tool. The result of phylogenetic trees on the basis of OrthoANI values for strain SNUTY-1 and other associated strains indicated that the Korean isolate, SNUTY-1, was closely related to the *V. coralliilyticus* strains (**Figure. 16**). Moreover, the plasmids pSNUTY1 and pSNUTY2 showed no continuous sequence identity to other plasmid sequences in the GenBank database using BLAST search. Furthermore, plasmid pSNUTY1 was most similar to plasmids p337 (from *V. coralliilyticus* RE22, 99.2% identity) and plasmid p319 (from *V. coralliilyticus* RE98, 98.9% identity), with more than 50% coverage. However, plasmid pSNUTY2 was only similar to plasmid pLMB143 (from *V. campbellii* LMB29, 92.2% identity) with less than 10% coverage, and did not show any similarity to other plasmids found in *V. coralliilyticus* strains. These results strongly support the uniqueness of the Korean isolate from other sequenced *V. coralliilyticus* strains from different geographical origins, including strain 58 that was isolated from Japan (150).

Marine *Vibrio* species are known to produce toxic proteins such as cytolysins, exopolysaccharides, lipases, and proteases (157). According to Hasegawa et al. (158), cytolysin/hemolysin (vthB/A) and extracellular metalloprotease (vtpA) have been described as virulence factors in *V. tubiashii*, and similar sequences were reported in *V. coralliilyticus* RE22 (101). Similarly, the *V. tubiashii*-homologous cytolysin/hemolysin (vthB/A, 98.0% sequence identity) and metalloprotease genes (vtpA, 98.0% sequence identity) were encoded on Chr I and Chr II in *V. coralliilyticus* SNUTY-1, respectively. Moreover, another thirteen and six predicted cytolysin/hemolysin and metalloprotease, respectively, were found in the SNUTY-1

genome. Interestingly, we were able to detect the plasmid pSNUTY1-encoded hemolysinD gene, which was almost identical (>99%) to other plasmids (p337 and p319) found in *V. coralliilyticus* RE22 and RE98, thus suggesting that the virulent plasmid could also be associated with the pathogenicity of *V. coralliilyticus.* Additionally, the SNUTY-1 genome possessed antimicrobial-resistant genes involved in the resistance of β-lactams (MBL fold metallo-hydrolase and PBPs), fluoroquinolone (*qnrVv*), and phenicol (*catB3*). Moreover, the genome has been found to have homologues of tet34 and tet35, which has recently been described in tetracycline resistance of *Vibrio* species (159). In contrast, results of the disc diffusion tests showed that the tested antibiotics were susceptible, except for β-lactams. The disc diffusion test was performed in this study use limited antibiotics from each antibiotic family following CLSI guideline. Although results of disc diffusion test did not match with genome analysis, tested antibiotics were chosen from various antibiotic family. Therefore, further study on resistant antibiotics based on antibiotic resistance genes should be carried out.

In conclusions, a continuous mass mortality event occurred at a Pacific oyster hatchery in Korea. We isolated *V. coralliilyticus*SNUTY-1 and confirmed that it was the causative agent through PCR analysis, whole genome sequence analysis, a challenge test, and clinical observation of signs such as velum necrosis and the adhesion of ciliates. A phylogenetic tree based on Ortho ANI values of SNUTY-1 showed that the Korean isolate was closely related to *V. coralliilyticus* strains. Results of a pathogenicity test revealed that SNUTY-1 was very pathogenic to

Pacific oyster larvae. Whole genome sequence analysis of SNUTY-1 identified cytolysin/hemolysin, and extracellular metalloprotease encoded genes. Those genes might raise mortality in marine bivalve larvae. Thus, SNUTY-1 may potentially cause appreciable problems in marine bivalve hatcheries. In addition, genes that can encode for antibiotics resistance were also identified. The present study offers important insight into the biodiversity of the *Vibrio* sp. and provides valuable information for the study of virulence and antibiotic resistance factors, which will facilitate control of *V. coralliilyticus* in aquaculture. Further studies are required to determine appropriate treatments for preventing *V. coralliilyticus* infection–induced mass mortality events at marine bivalve hatcheries.

Table 1. Antimicrobial susceptibility test of *V. coralliilyticus* strains (SNUTY-1 and 58).

Antibiotics		Strain		Antibiotics		Strain	
		SNUTY-1	58			SNUTY-1	58
B-Lactams	Ampicillin	R*	R	Amphenicols	Chloramphenicol	S	S
	Amoxicillin-clavulanate	R	I*	Amino-glycosides	Amikacin	S*	I
	Ampicillin-sulbactam	R	R		Gentamicin	S	S
	Piperacillin	R	R	Tetracyclines	Tetracycline	S	S
	Piperacillin-tazobactam	S	I	Fluoroqui-nolones	Ciprofloxacin	S	S
	Cefepime	R	R		Levofloxacin	S	S
	Cefotaxime	R	R		Ofloxacin	S	S
	Cefoxitin	I	R	Sulfonamides	Sulfonamides	S	S
	Ceftazidime	R	R		Trimethoprim-sulfamethoxazole	S	S
	Cefuroxime sodium	S	S				
	Imipenem	S	S				
	Meropenem	R	R				

*R: Resistant, I: Intermediate, S: Susceptible.

Table. 2. Biochemical characteristics of *V. coralliilyticus* SNUTY-1 and 58 using VITEK®2 System.

Biochemistry	Strain		Biochemistry	Strain	
	SNUTY-1	58		SNUTY-1	58
Ala-Phe-Pro-ARYLAMIDASE	+	-	D-TREHALOSE	-	-
ADONITOL	-	-	CITRATE (SODIUM)	-	-
L-Pyrrolydonyl-ARYLAMIDASE	-	-	MALONATE	-	-
L-ARABITOL	-	-	5-KETO-D-GLUCONATE	-	-
D-CELLOBIOSE	-	-	L-LACTATE alkalinisation	+	-
BETA-GALACTOSIDASE	-	-	ALPHA-GLUCOSIDASE	-	-
H2S PRODUCTION	-	-	SUCCINATE alkalinisation	-	-
BETA-N-ACETYL-GLUCOSAMINIDASE	-	-	Beta-N-ACETYL-GALACTOSAMINIDASE	-	-
Glutamyl Arylamidase pNA	-	-	ALPHA-GALACTOSIDASE	-	-
D-GLUCOSE	-	-	PHOSPHATASE	-	-
GAMMA-GLUTAMYL-TRANSFERASE	-	-	Glycine ARYLAMIDASE	-	-
FERMENTATIO/GLUCOSE	-	-	ORNITHINE DECARBOXYLASE	-	-
BETA-GLUCOSIDASE	-	-	LYSINE DECARVOXYLASE	-	-
D-MALTOSE	-	-	L-HISTIDINE assimilation	-	-
D-MANNITOL	-	-	COUMARATE	-	-
D-MANNOSE	-	-	BETA-GLUCORONIDASE	-	-
BETA-XYLOSIDASE	-	-	O/129 RESISTANCE (comp. vibrio.)	-	-
BETA-Alanine arylamidase pNA	-	-	Glu-Gly-Arg-ARYLAMIDASE	-	-
L-Proline ARYLAMIDASE	+	+	L-MALATE assimilation	-	-
LIPASE	-	-	ELLMAN	-	-
PALATINOSE	-	-	L-LACTATE assimilation	-	-
Tyrosine ARYLAMIDASE	-	-	D-SORBITOL	-	-
UREASE	+	+	SACCHAROSE/SUCROSE	-	-
D-TAGATOSE	-	-			

Table 3. General features of *V. coralliilyticus* SNUTY-1 and MIGS mandatory information.

Items	Description
Classification	Domain *Bacteria*
	Phylum *Proteobacteria*
	Order *Vibrionales*
	Genus *Vibrio*
	Species *coralliilyticus*
	Strain: SNUTY-1
General features	
Gram stain	Gram negative
Cell shape	Curved rod
Motility	Motile with polar flagella
Temperature	4–37℃
Pigmentation	Non-pigmented
MIGS data	
Investigation_type	Bacteria_archaea
Project_name	Genome sequence of *Vibrio coralliilyticus* SNUTY-1
Lat_lon	34˚47’13”N, 128˚25’22”E
Geo_loc_name	South Korea: Tongyeong
Collection_date	2015-04
Env_biome	landlocked sea [ENVO:00,000,219]
Env_feature	coastal water [ENVO:00,002,150]
Env_material	oyster [ENVO:02,000,079]
Num_replicons	4
Extrachrom_elements	2
Estimated_size	5,842,676
Ref_biomaterial	PMID: 9684317
Source_mat_id	KCCM 43251
biotic_relationship	Infectious (or commensal)
host	*Crassostrea gigas* (Pacific oyster) larvae
host_disease	bacillary necrosis
health_state	inactivated
Pathogenicity	Pathogenic in Pacific oyster (*Crassostrea gigas*)
Trophic_level	Chemoorganotroph
Rel_to_oxygen	Facultative anaerobic
Isol_growth_condt	PMID: 9684317
Seq_meth	PacBio RSII
Annot_source	GenBank
Finishing_strategy	Complete; 160× coverage, 4 contigs
Genome assembly data	
Assembly method	HGAP
Assembly name	HGAP algorithm ver. 3
Genome coverage	237×
Sequencing technology	PacBio RSII

Table 4. Genomic features of *V. coralliilyticus* SNUTY-1.

Attribute	Value			
	Chromosome I	**Chromosome II**	**Plasmid pVs58**	**Plasmid pVs58**
Size (bp)	3,474,874	1,976,676	254,703	136,423
Coding regions (%)	87.9	87.6	81.9	84.2
G+C content	45.7	45.1	49.3	44.7
Total genes	3,358	1,783	254	130
tRNA genes	111	5	-	-
rRNA genes	37	-	-	-
ncRNA genes	4	-	-	-

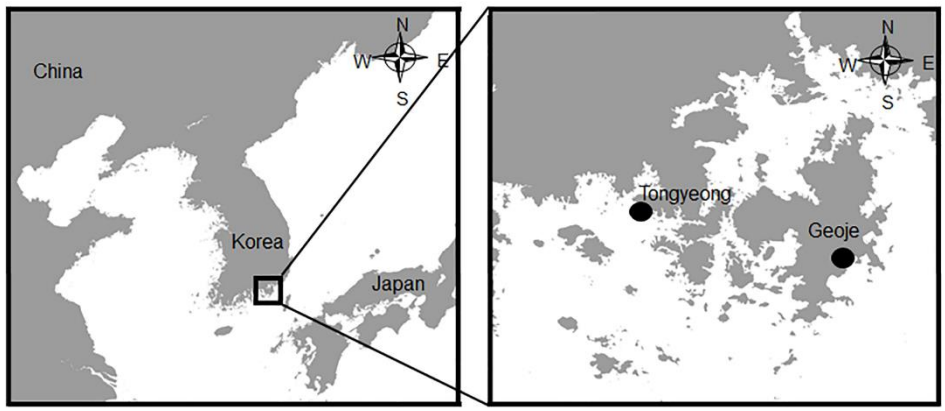

Figure 13. Sampling location of Pacific oyster larvae from Tongyeong (inactive larvae) and Geoje (healthy larvae) located at southern region of Korea.

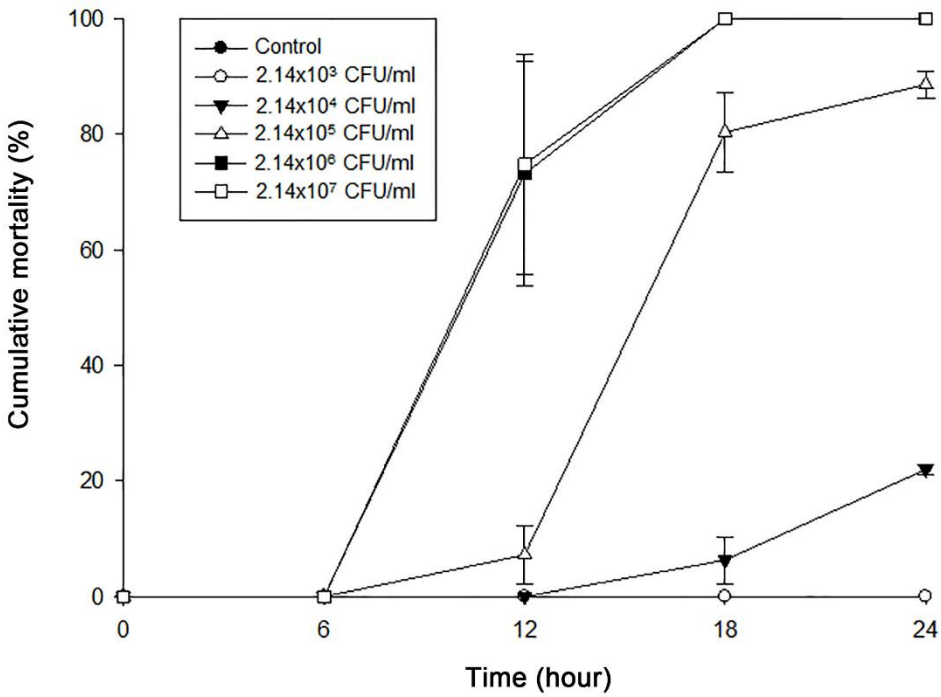

Figure 14. Pathogenicity of *V. coralliilyticus* SNUTY-1. Control indicates only larvae with FSS.

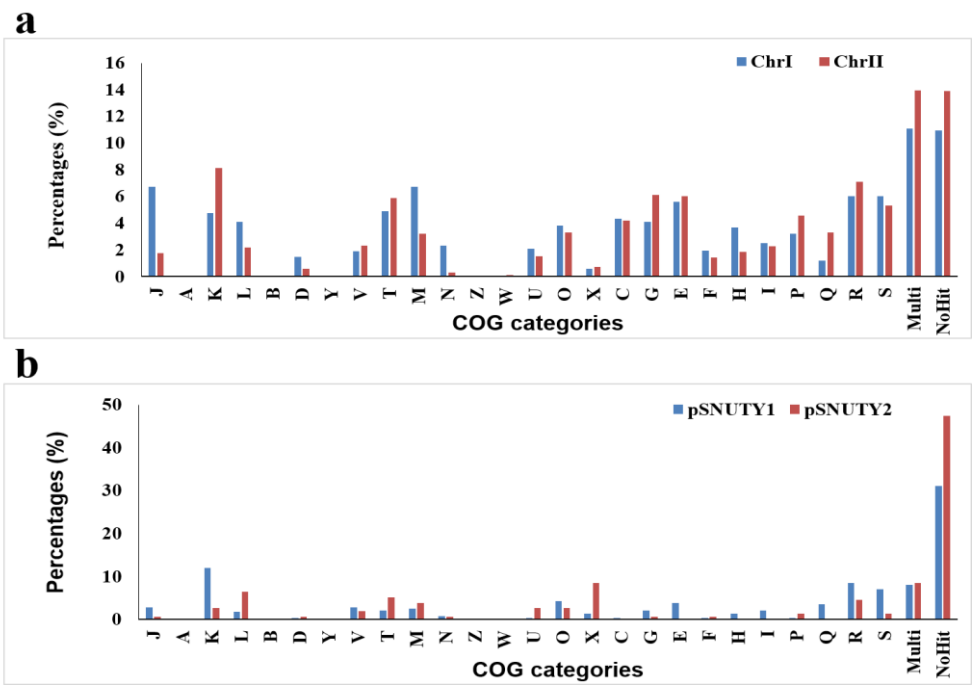

Figure15. Comparison of COG functional categories of Vco SNUTY-1. (a) Two chromosomes of *V. coralliilyticus* SNUTY-1. (b) Two plasmids of *V. coralliilyticus* SNUTY-1. J; translation, ribosomal structure and biogenesis, K; transcription, L; replication, recombination and repair, D; cell cycle control, cell division, chromosome partitioning, V; defense, T; signal transduction mechanisms, M; cell wall/membrane/envelope biogenesis, N; cell motility, W; extracellular structures, U; intracellular trafficking, secretion, and vesicular transport, O; posttranslational modification, protein turnover, chaperones, X; mobilome: prophages, transposons, C; energy production and conversion, G; carbohydrate transport and metabolism, E; amino acid transport and metabolism, F; nucleotide transport and metabolism, H; coenzyme transport and metabolism, I; lipid transport and metabolism, P; inorganic ion transport and metabolism, Q; secondary metabolites biosynthesis, transport and catabolism, R; general function prediction only, S; function unknown.

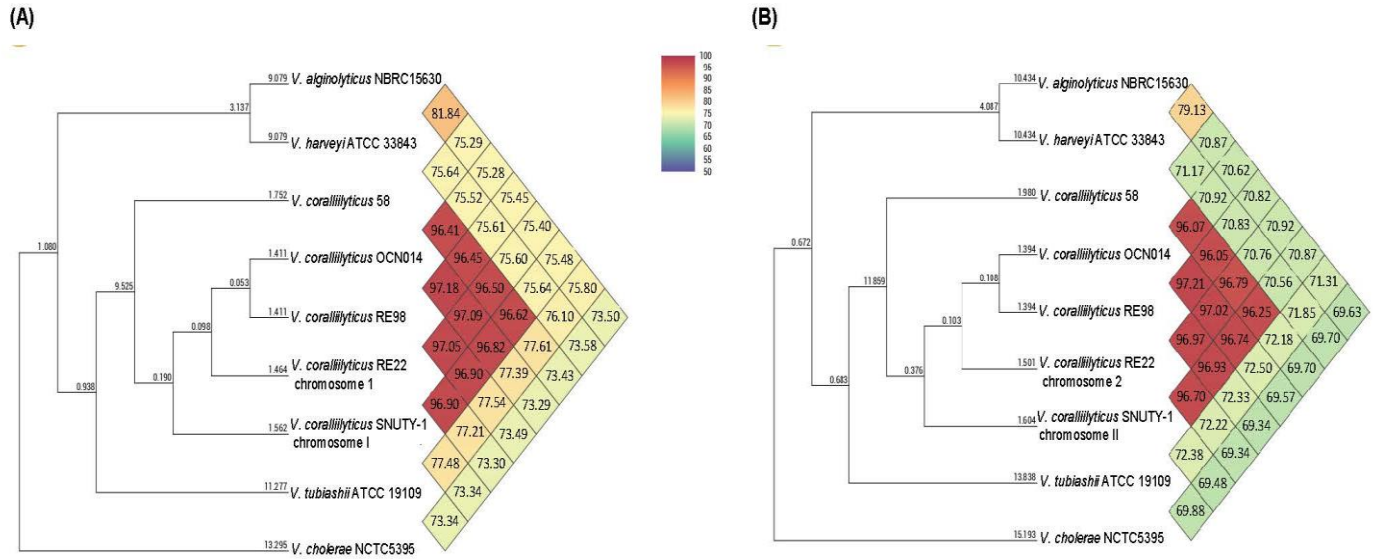

Figure 16. Phylogenetic position of *V. coralliilyticus* SNUTY-1 (A: ChrI, B: Chr II). Ortho ANI values calculated from the OAT software (30).

Chapter III

Application of the bacteriophage pVco-14 to prevent *Vibrio coralliilyticus* infection in Pacific oyster (*Crassostrea gigas*) larvae

Abstract

Vibrio coralliilyticus infects a variety of shellfish larvae, including Pacific oyster (*Crassostrea gigas*) larvae worldwide, and remains a major constraint in marine bivalve aquaculture practice, especially in artificial seed production facilities. In this study, we isolated and characterized the bacteriophage (phage) that specifically infects *V. coralliilyticus*. The phage was designated pVco-14 and classified as *Siphoviridae*. We also investigated the potential efficacy of the isolated phage against *V. coralliilyticus* infection. We conducted a survey to replace the overuse of antibiotics, which generate multi-antibiotic-resistant strains and causes environmental pollution. The latent period of pVco-14 was estimated to be approximately 30 min, whereas the burst size was 13.3 PFU/cell. The phage was found to infect four strains of tested *V. coralliilyticus*. pVco-14 was stable at wide temperature (4–37°C) and pH (5.0–9.0) ranges. Eighty-one percent of oyster larvae died in an immersion challenge at a dose 1.32×10^5 CFU/ml of virulent *V. coralliilyticus* (strain 58) within 24 h. When oyster larvae were pre-treated with the phage before the bacterial challenge (bacterial conc.: 1.32×10^4 and 1.32×10^5

CFU/ml), mortality of the phage-treated oyster larvae was lower than that of the untreated control. These results suggest that pVco-14 has potential to be used as a prophylactic agent for preventing *V. coralliilyticus* infection in marine bivalve hatcheries and can reduce the overuse of antibiotics.

Key words: Pacific oyster larvae, *Vibrio coralliilyticus*, Mass mortality, Bacteriophage, Marine bivalve hatchery.

3.1. Introduction

Since the development of bivalve artificial seed production technologies, a number of countries have been continuously producing marine bivalves, including Pacific oyster (*Crassostrea gigas*). However, in the development of the oyster hatchery industry, there have been frequent occurrences of mass mortality due to *Vibrio* spp. infection in the production fields of oyster larvae. *Vibrio coralliilyticus* and *V. tubiashii*, particularly, have been reported as major causative agents of mass oyster larva mortality globally (21, 89, 92, 111, 151), resulting in use of various antibiotics to prevent mass mortality in bivalve hatcheries (92, 160-162). Many reports of antibiotic use have shared a concern about the occurrence of multiple-antibiotic-resistant bacteria and environmental pollution and suggested the development of a substitute for antibiotics to prevent these problems. There is a need to explore an eco-friendly disease preventative method to prevent antibiotic overuse.

The application of bacteriophage (phage) has been proposed for the treatment of bacterial diseases, and several studies have reported that phages can be effectively used for the treatment of bacterial infection in humans and livestock (114, 163-165). Application of phages to treat bacterial diseases has also been investigated in fishes (120, 122, 167, 168) and invertebrates (121, 130, 169, 170).

Here, we isolated and characterized the phage specifically infecting *V. coralliilyticus*, which causes massive mortality of Pacific oyster larvae. We also evaluated the potential efficacy of the isolated phage used prophylactically against *V. coralliilyticus* infection in Pacific oyster larvae to prevent antibiotic overuse.

3.2. Materials and methods

3.2.1. Bacterial strains and culture media

Twelve bacterial strains were used in this study including four *V. coralliilyticus* strains previously isolated from inactive oyster larvae (92), seven strains of other *Vibrio* spp., and a strain of another genus, *Escherichia coli* (ATCC 25922) (**Table 5**). These strains were used to examine the host range of the phage. Among them, *V. coralliilyticus* 58 (designated as Vco58), reported as a highly virulent strain infecting Pacific oyster larvae in an earlier study (3), was used as the standard strain. Tryptic soy agar (TSA; BD Difco, USA) and tryptic soy broth (TSB; BD Difco, USA) supplemented with NaCl (2.0% final concentration) were used for bacterial culture and phage plaque forming unit (PFU) assay.

3.2.2. Animals

Five-day-old healthy Pacific oyster larvae (100-120 μm) were transferred immediately from the oyster hatchery located in Tongyeong, Korea, to the Aquatic Biomedicine laboratory, College of Veterinary Medicine, Seoul National University at a temperature maintained at 4℃. Larvae were washed 3 times using filtered and sterilized seawater (FSS) to eliminate other microorganisms before use.

3.2.3. Pathogenicity confirmation of Vco58

Oyster larvae (n = 50 ± 11 per each well) were placed in a 6-well cell culture plate (SPL, Korea) with 10 ml of FSS (32 practical salinity unit; psu). After being

acclimated for 1 h in an incubator adjusted at 27℃ (the same temperature as that of the hatchery tank), Vco58 was inoculated to each well and final concentrations were adjusted to 1.32×10^3, 1.32×10^4, 1.32×10^5, and 1.32×10^6 CFU/ml and incubated at 27℃ for 24 h. We investigated the cumulative mortality of each well at 6 h intervals using inverted light microscope, BX41 (Olympus, Japan). Before inoculation, Vco58 cultured in TSB was washed 3 times using FSS to eliminate TSB components. In the control group, larvae were placed into the well with FSS and were not inoculated with Vco58. We performed the experiment in triplicate under the same conditions.

3.2.4. *Phage isolation, purification, and high titer preparation*

Seawater (450 ml per sample), was collected from oyster hatchery tanks, sewages of oyster hatchery, larval tanks, broodstock tanks, harbors, and beaches located on southern coast of Korea. Seawater was filtered through 0.45 μm pore-size membrane filters and mixed with 50 ml of 10X TSB. After gently mixing, seawater was incubated in a shaking incubator at 150 rpm for 24 h at 27℃. Following that, enrichments were centrifuged at 13,000 *g* for 10 min and supernatant lysates were filtered through 0.22 μm pore-size membrane filter. A colony of Vco58 was cultured on TSA.

The phage activity test was performed via spot assay as previously described by Cerveny et al. (171) using filtered enrichment. Purification and cloning of the isolated phage were performed by the double layered agar method (172) in triplicate

to obtain pure phage. Cloned phages were purified and concentrated by the CsCl density gradient method (173), and the phage titer was calculated using the number of PFU by double layered agar assay.

3.2.5. *Characterization of isolated phage*

3.2.5.1. Host range and efficiency of plating (EOP)

Infectivity of isolated phage was evaluated by spot assay using 12 bacterial strains used in this study (**Table 5**). Ten microliters of purified phage suspension (2.14×10^8 PFU/ml) were dropped onto double layered agar plates inoculated with each strain and incubated at 27°C for *V. coralliilyticus* strains; *Vibrio* spp. and *E. coli* were incubated at their respective optimal culture temperatures (**Table 5**). After incubation for 24 h, the presence or absence of plaque formation was observed. The double layered agar method was conducted on susceptible strains from the host range test to calculate ratio of PFU quantification with the indicator strain to get the EOP value.

3.2.5.2. Electron microscopy

To determine the morphological classification of the isolated phage, purified 10^9 PFU/ml of phage lysate was negatively stained using 2% uranyl acetate onto a copper grid for 1 min, washed 3 times using double-distilled water, and dried for 10 min in a desiccator. Pretreated phage was observed using a transmission electron

microscope, JEM1010 (JEOL, Japan), operating at 80 kV. The size of the phage was measured using an image analysis program (Motic 2.0, China).

3.2.5.3. One step growth

The one step growth curve of isolated phages was performed according to the method of Verma et al. (174). Ten microliters of phage suspension (3.14×10^8 PFU/ml) were added to 10 ml of early exponential phase bacterial culture (2.4×10^8 CFU/ml) to obtain a multiplicity of infection (MOI) of 0.001. The phages were absorbed for 5 min at 25℃, and the mixture was centrifuged at 13,000 *g* for 3 min. After removal of the supernatant, 10 ml of fresh TSB was added and cultured in a shaking incubator at 27℃ with 150 rpm, and 100 µl of phage lysates were collected every 10 min for phage titration by double layered agar method.

3.2.5.4. Stability test

Phage stability test was performed as described previously (174). The stability of the phage at various temperatures, 4, 15, 20, 27, 37, 50, and 60℃, was tested in specific incubators. Phage was incubated at 10^7 PFU/ml in each adjusted incubator for 1 h, and phage titer was calculated by double layered agar method. To evaluate the stability against pH, phage suspensions (10^7 PFU/ml) were adjusted to pH values 3, 5, 7, 9, and 11 using 1 M HCl and 1 M NaOH. Each pH phage suspension was incubated at 27℃ for 1 h and phage titer was calculated.

3.2.5.5. Host cell lysis test

Vco58 was used to evaluate the bactericidal efficacy of the isolated phage. The early exponential phase of Vco58 was diluted in fresh TSB and final OD_{600} value was 0.038 (4.7×10^6 CFU/ml). MOI of each group was adjusted to 0, 0.1, 1, and 10 with purified phage (2.08×10^8 PFU/ml) administration. The absorbance ($OD_{600\ nm}$) was measured at 0, 3, 6, 9, 12, and 24 h after inoculation.

3.2.5.6. Preparation of phage-resistant variants of V. coralliilyticus

One hundred microliters of Vco58 cultured in TSB mixed with phage suspension (10^{10} PFU/ml) was spread on TSA and incubated at 27℃ for 2 days. If colonies were observed on the plate, they were re-cultured and the above procedure was repeated. Finally, bacterial cultures that produced no PFU after administration of phage (10^{10} PFU/ml) were chosen as phage-resistant variants.

3.2.6. *Phage preparation for in vivo test*

To prevent growth of Vco58 due to the nutrient contents of TSB in the phage solution during prophylactic efficacy test, 10% (wt / vol) polyethylene glycol 8000 (PEG, Sigma, USA) and 1 M sodium chloride (NaCl) were added to the phage lysates. After dissolving PEG and NaCl, they were incubated at 4℃ for 12 h and centrifuged at 13,000 *g* for 30 min. After centrifugation, the supernatant was removed and FSS was filled and vortexed to mix the pellet and FSS. The phage

solution substituted with FSS was used for *in vivo* test after confirming the phage concentration by double layered agar method.

3.2.7. Prophylactic efficacy of phage

The experimental groups for prophylactic efficacy test of pVco-14 are listed in Table 3.2. Oyster larvae (n= 50 ± 14) were placed in a6-well plate with 10 ml of FSS and purified phage suspension (eliminated TSB contents) was administered to each well and final concentrations were adjusted to 1.87×10^4, 1.87×10^5, 1.87×10^6, and 1.87×10^7 PFU/ml and adapted at 27℃ for 1 h. Vco58 was inoculated to each well, final concentration was adjusted to 1.32×10^3, 1.32×10^4, 1.32×10^5, and 1.32×10^6 CFU/ml, and the plate incubated at 27℃ for 24 h. We investigated the cumulative mortality in each well at 6 h intervals using an inverted light microscope, BX41 (Olympus, Japan). Two controls, one without a phage group and one without a bacteria group were used in this study. We performed the experiment in triplicate with same conditions.

3.3. Results

3.3.1. Pathogenicity of Vco58

In the pathogenicity test, whole larvae survived both in the negative control group and when the Vco58 concentration was 1.32×10^3 CFU/ml. In contrast, when the concentration of Vco58 was 1.32×10^4 and 1.32×10^5 CFU/ml, the mortality rates were 42.86 ± 7.78% and 81.44 ± 6.99% for 24 h, respectively. When the

concentration was 1.32 × 10^6 CFU/ml, a high mortality rate of 98.66 ± 1.16% was observed 24 h later (**Figure. 17**).

3.3.2. *Phage isolation and morphology*

Phage pVco-14 which specifically infects Vco58 was isolated from the sewage at the oyster hatchery located at Tongyeong, Korea. Spot assay showed that growth of bacteria was inhibited in the area where pVco-14 was dropped on the Vco58 lawn, and a transparent zone was formed. In the double layered agar method, pVco-14 formed plaques with an average diameter of 2.7 mm (**Fig. 18A**). pVco-14 possessed an isometrical head (diameter of 43.92±1.56 nm) and a long non-contractile tail with a length of 121.06 ± 6.72 nm (n= 10). Based on the morphology of pVco-14 according to the classification system of Ackermann (189), it belongs to the family *Siphoviridae* (**Fig. 18B**).

3.3.3. *Characterization of pVco-14*

3.3.3.1. Host range and one step growth

pVco-14 was tested using various *Vibrio* spp. including *V. coralliilyticus* strains, to evaluate the host range. pVco-14 inhibited the multiplication of not only Vco58 but also other *V. coralliilyticus* strains but was not inhibitory to other *Vibrio* spp. and *E. coli* (**Table 5**). As a result of EOP test based on Vco58 used as the standard strain, the remaining three strains of *V. coralliilyticus* also showed very similar EOP values, confirming that *V. coralliilyticus* strains used in this study are very susceptible to

pVco-14 (**Table 5**). In one step growth, pVco-14 showed that the latent phase was approximately 30 min and burst size was 13.3 PFU/cell (**Figure. 19A**).

3.3.3.2. Stability test

pVco-14 was relatively stable in the temperature range of 4–27℃. Phage activity was reduced in accordance with increasing temperature (100% at 4℃, 95.21 ± 4.75% at 15℃, 93.51 ± 5.18% at 20℃, 92.85 ± 5.51% at 27℃, 67.18 ± 8.02 at 37℃, 21.18 ± 1.1% at 50℃, and 0% at 60℃) (**Figure 19C**). In pH, pVco-14 exhibited stability greater than 90% at pH 7.0 (100%) and pH 9.0 (93.41 ± 3.59%). At pH 5, it showed 79.69 ± 7.59% stability, while at pH 3.0 and pH 9.0, it did not exhibit any activity (**Figure 19D**). As a result, it was confirmed that pVco-14 did not exhibit activity at temperatures above 50℃ or in strongly acidic and strongly basic conditions.

3.3.3.3. Host cell lysis and phage-resistant variants

The bactericidal effect of pVco-14 against Vco58 is shown in **Figure 19B**. In case of the control group (MOI: 0), the OD_{600} value continuously increased during the incubation period. Contrastingly, the phage treated groups (MOI: 0.1, 1, and 10) exhibited decreased OD_{600} values (0.038) before phage administration. As a result of induction of phage-resistant variants, Vco58 colonies resistant to pVco-14 were not observed.

3.3.3.4. Prophylactic efficacy of phage

Results of efficacy of pVco-14 as a preventative against Vco58 infection are shown in **Table 6** and **Figure 20**. Phage-treated groups exhibited significantly higher survival compared to untreated groups. When inoculated, *V. coralliilyticus* concentration was 1.32×10^4 CFU/ml, and 24 h later, the average mortality of pVco-14 treated groups was 6.06 ± 2.14% and that of the untreated control was 42.86 ± 7.78%. Vco58 groups inoculated at 1.32×10^5 CFU/ml also showed similar prophylactic efficacy results: the phage treated group exhibited 21.77 ± 5.41% and untreated control exhibited 81.44 ± 7.3% mortality. In contrast, when inoculated *V. coralliilyticus* was 1.32×10^6 CFU/ml, mortality rates were not dramatically lower. The phage treated and untreated groups exhibited mortality rates of 80.53 ± 3.63% and 98.66 ± 13%, respectively (**Table 6 and Figure 20**).

3.4. Discussion

V. coralliilyticus strains used in this study previously were classified as *V. splendidus* biovar II (92). These *Vibrio* spp. strains were isolated from inactive oyster larvae and several *Vibrio* spp. induced mass mortality. Virulent *Vibrio* strains were identified to be *V. splendidus* biovar II by biochemical tests. After the development of various molecular diagnostic techniques, numerous bacteria were re-classified using 16S rRNA gene sequence analysis and PCR using the characteristic gene region of species (29, 148, 158, 175-179). Kim et al. (150) used next generation

sequencing (NGS) to analyze the entire genome sequence of Vco58 and re-classified it to *V. coralliilyticus*. Three strains of *V. coralliilyticus* 58, 59, 60 used in this study were reported to be highly virulent bacteria that induced mass mortality (92). We also confirmed the high pathogenicity of Vco58 through a challenge test; results concurred with the results of a previous study (**Figure 17**) (92). Therefore, Vco58 strain was selected as the indicator and phage host in this study.

Various antibiotics have been used in marine bivalve hatcheries and they have been effective in controlling bacterial infection (92, 161, 180, 181). However, antibiotics also induce bactericidal activity against various essential microbes and cause contamination of water. In addition, although antibiotic use is advantageous for treatment of many bacterial diseases, overuse has generated multiple-antibiotic-resistant bacteria (182, 183). Vco58 has been reported as a multi-antibiotic-resistant strain (92). We performed the disk diffusion test for Vco58 following Clinical and Laboratory Standard Institute guidelines (149) and confirmed that Vco58 was resistant to 9 kinds of antibiotics out of the 24 tested (data not shown), which may have resulted in mortality at the hatchery. Therefore, various studies are being conducted to replace antibiotics with phage, probiotics, and ovoglobulin (26, 120-123, 168).

In contrast with antibiotics, bacteriophage has a narrow host range. pVco-14 infected only the four *V. coralliilyticus* strains tested in this study, confirming its narrow host range. The narrow host range of the phage is advantageous as the phage can be used to target only specific harmful bacteria without affecting beneficial

microbes distributed in the water environment. Hence, various studies are being conducted to apply phages to the aquaculture industry (120-122, 130, 131, 167-169). Cohen et al. (130) and Jacquemot et al. (169) isolated and characterized *V. coralliilyticus* specific phages pathogenic to coral. They also tested the efficacy of isolated phages against *V. coralliilyticus* infection in the coral *Symbiodinium* sp., confirming the mitigation capacity of the phages. We focused on the isolation and characterization of *V. coralliilyticus*-specific phage to prevent bacillary necrosis in marine bivalve larvae caused by *V. coralliilyticus* infection. Consequently, we isolated pVco-14 and conducted studies of its efficacy to reduce mass mortality of Pacific oyster larvae.

In Pacific oyster seedling hatcheries, the larval stage is usually cultured at 24-28℃ and at a pH ranging from 7.0 to 9.0. In the stability tests, pVco-14 was extremely stable at 4-27℃ and pH 7-9 (**Figure 19C, D**), confirming that pVco-14 is stable in the larval culture environment and can be applied to hatcheries as a prophylactic agent to prevent *V. coralliilyticus* infection. In the host cell lysis test, pVco-14 exhibited noticeable bacteriolytic effect. Like the cell lysis test results, pVco-14 resistant variants *V. coralliilyticus* were not induced. In prophylactic efficacy test, pVco-14 showed a strong preventative effect when Vco58 concentrations were 1.32×10^4 or 1.32×10^5 CFU/ml (**Table 6 and Figure 20**). However, when concentration of Vco58 was raised to 1.32×10^6 CFU/ml, the phage treated group did not show the reduced mortality as for the lower bacterial concentrations. Thus, we suggest that pVco-14 is efficacious when the Vco58

concentration is below 1.32 x 10^6 CFU/ml. Usually, a single bacterium does not multiply over 10^6 CFU/ml in the natural environment, so the stability and prophylactic efficacy tests showed that pVco-14 has potential to reduce or prevent *V. coralliilyticus* infection in bivalve hatcheries.

Production facilities for bivalve artificial seedlings are increasing, but damage caused by *V. coralliilyticus* infection continues in the hatcheries. The indiscriminate use of antibiotics has had a significant impact on the occurrence of multiple-antibiotic-resistant strains and environmental pollution such as antibiotic spills from hatchery directly to the sea. As invertebrates, including bivalves, do not have acquired immune systems, disease prevention methods (e.g. vaccination) are very limited compared to fish. Therefore, the application of bacteriophage may be a solution for the prevention of bacterial disease in invertebrates. The results of our study showed that phage potentially can be used as an effective substitute for antibiotics in the field of bivalve artificial seedling production while reducing the generation of antibiotic-resistant bacteria and environmental pollution.

Table 5. Bacterial strains used in this study and the infectivity of phage pVco-14.

Bacterial species	Strain	Host range[a] (EOPs[b])	Culture temp. (℃)	Source[c]
V. coralliilyticus	58	++ (1)	27	(92)
V. coralliilyticus	59	++ (1.2±0.03)	27	(92)
V. coralliilyticus	60	++ (0.89±0.08)	27	(92)
V. coralliilyticus	Q1	++ (0.93±0.07)	27	(92)
V. tubiashii	ATCC 19109	-	26	ATCC
V. parahaemolyticus	CRS09-17	-	37	(184)
V. alginolyticus	rM8402	-	27	(185)
V. anguillarum	HT7602	-	27	(186)
V. cholerae	PS-7701	-	25	(187)
V. harveyi	ATCC 14126	-	26	ATCC
V. vulnificus	ET7618	-	27	(188)
Escherichia coli	ATCC 25922	-	37	ATCC

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

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

[c] Number, references; ATCC, purchased from the American Type Culture Collection.

Table 6. Prophylactic efficacy test of pre-phage treatment against Vco58 infection[a]

Expt.	Group	Phage conc. (PFU/ml)	Bacterial conc. (CFU/ml)	Mortality of phage pre-treated larvae after 24 h (%, mean ± SD)
1	Control-1	0	1.32×10^4	42.86 ± 7.78
	Well-1	1.87×10^4	1.32×10^4	3.55 ± 0.57
	Well-2	1.87×10^5	1.32×10^4	5.35 ± 5.04
	Well-3	1.87×10^6	1.32×10^4	6.75 ± 4.25
	Well-4	1.87×10^7	1.32×10^4	8.60 ± 3.20
2	Control-2	0	1.32×10^5	81.44 ± 6.99
	Well-5	1.87×10^4	1.32×10^5	23.33 ± 5.77
	Well-6	1.87×10^5	1.32×10^5	20.10 ± 4.25
	Well-7	1.87×10^6	1.32×10^5	28.24 ± 5.21
	Well-8	1.87×10^7	1.32×10^5	15.40 ± 1.26
3	Control-3	0	1.32×10^6	98.66 ± 1.16
	Well-9	1.87×10^4	1.32×10^6	83.33 ± 28.87
	Well-10	1.87×10^5	1.32×10^6	78.25 ± 6.14
	Well-11	1.87×10^6	1.32×10^6	76.63 ± 16.62
	Well-12	1.87×10^7	1.32×10^6	83.90 ± 6.15

[a] Pacific oyster larvae (n = 50 ± 14) were put into each phage inoculated FSS well and incubated at 27℃ for 1 h, and each concentration of Vco58 was also inoculated to each well. Cumulative mortality of each well was measured at 6 h intervals for 24 h.

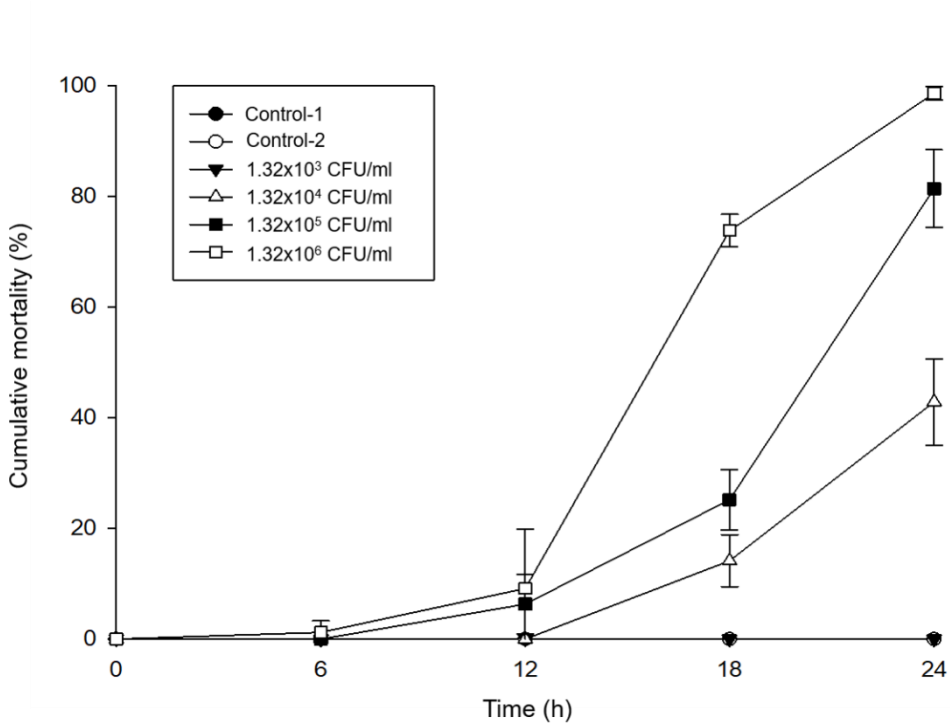

Figure 17. Pathogenicity of Vco58. Control-1 indicates only larvae with FSS, and Control-2 indicates larvae with 1.87×10^7 PFU/ml of phage treated FSS.

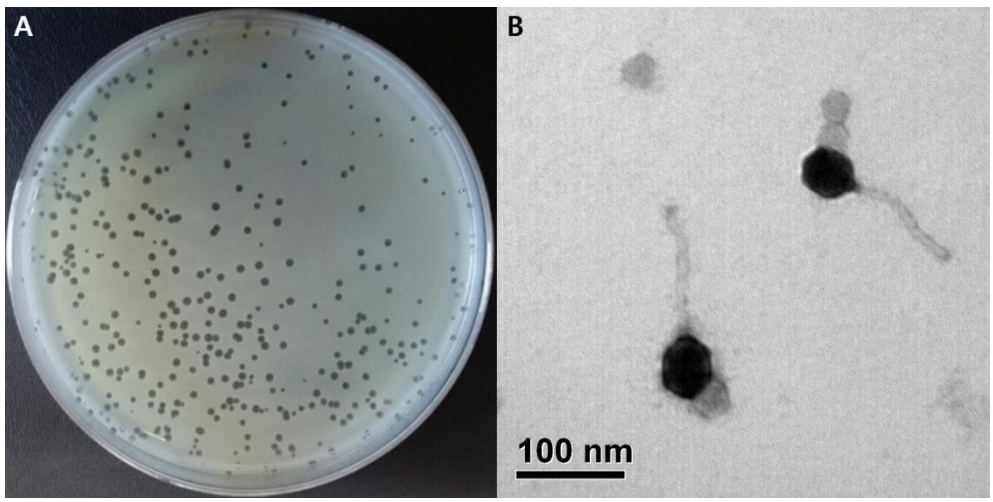

Figure 18. Morphology of phage plaque and isolated phage, pVco-14. A: Phage plaque formed in double layer agar plates, B: Electron micrograph of isolated phage, pVco-14.

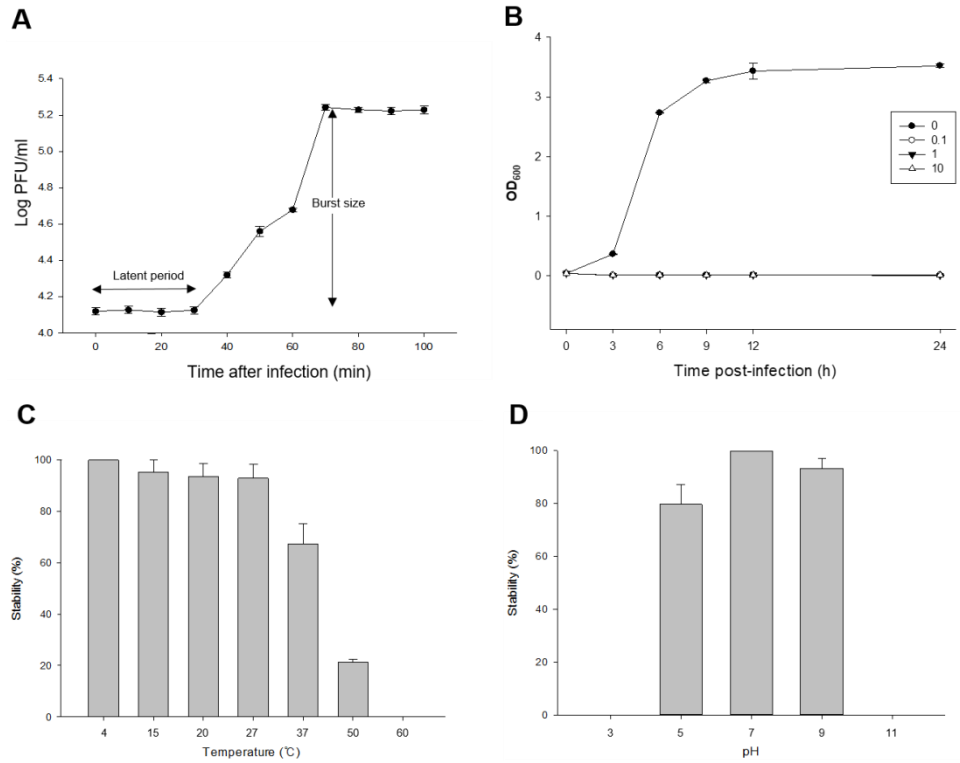

Figure 19. Characterization of pVco-14. A: One-step growth curve of phage pVco-14. The error bars indicate standard deviations. B: Cell lysis effect of pVco-14 against *V. coralliilyticus* 58. The results are shown as mean ± standard deviations from triplicate experiments. C, D: Stability of pVco-14 in the presence of various temperature and pHs. 4℃ and pH 7 were used as controls.

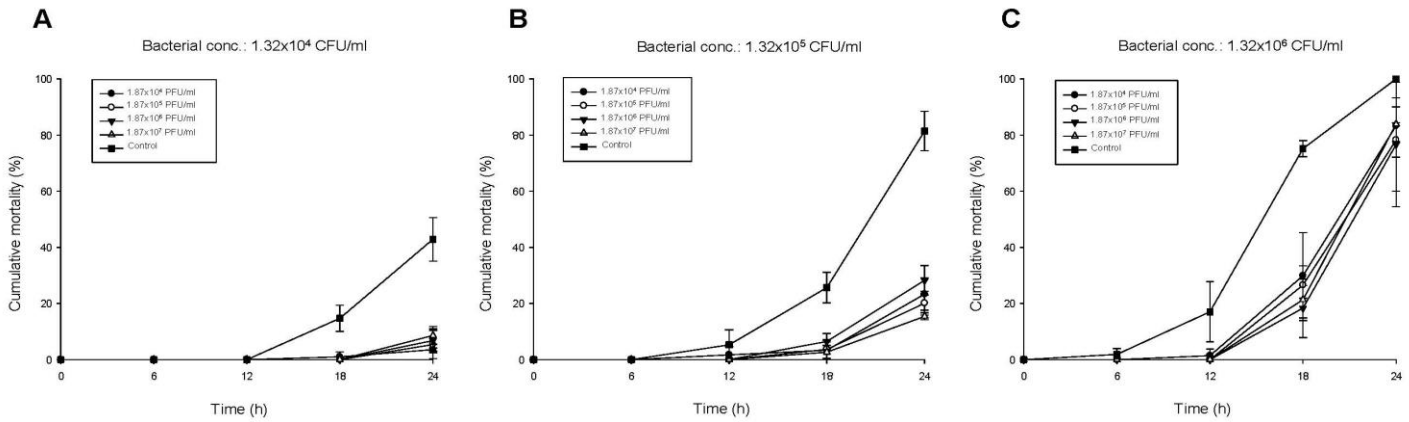

Figure 20. Prophylactic efficacy of various concentration of pVco-14 against various concentration of *V. coralliilyticus* 58 infection in Pacific oyster larvae (A: Concentration of Vco58: 1.32×10^4 CFU/ml, B: 1.32×10^5 CFU/ml and C: 1.32×10^6 CFU/ml).

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GENERAL CONCLUSION

In Korea, OsHV-1 μVar DNA was detected for the first time in adult Pacific oysters in 2011, and it was also detected in the field of Pacific oyster hatcheries, where mass mortality occurred in 2011. So far, there have been reports of OsHV-1 μVar DNA detection only in the southern coast of Korea, and this study also detected OsHV-1 in the artificial seedling production area of bay scallop located in the southern coast of Korea. Korea is surrounded by sea on its three sides, and bivalve hatcheries in each area culture the species of bivalves prominent in that specific area. Because OsHV-1 has a broad host range, it is likely to be present in bivalves in the West and East sea in Korea. Therefore, in order to minimise the damage caused by the occurrence and spread of OsHV-1, continuous monitoring of bivalve farms and hatcheries located in each sea area is required. In addition, in order to prevent the spread of OsHV-1, local hatcheries should minimise the migration of their broodstock and larvae. This study investigated the causative agent of mass mortality in bay scallop hatcheries located on the southern coast of Korea. It was confirmed that OsHV-1 μVar was associated with mortality of Bay scallop larvae through PCR, 16S rRNA sequence analyses, and TEM investigation. This is the first report of OsHV-1 μVar DNA detection and viral capsid observation in bay scallops.

Continuous mass mortality event occurred at a Pacific oyster hatchery in Korea. We isolated *V. coralliilyticus* SNUTY-1 and confirmed that it was the causative agent through PCR analysis, whole genome sequence analysis, a challenge test, and

clinical observation of signs such as velum necrosis and the adhesion of ciliates. A phylogenetic tree based on Ortho ANI values of SNUTY-1 showed that the Korean isolate was closely related to *V. coralliilyticus* strains. Results of a pathogenicity test revealed that SNUTY-1 was very pathogenic to Pacific oyster larvae. Whole genome sequence analysis of SNUTY-1 identified cytolysin/hemolysin, and extracellular metalloprotease encoded genes. Those genes might raise mortality in marine bivalve larvae. Thus, SNUTY-1 may potentially cause appreciable problems in marine bivalve hatcheries. In addition, genes that can encode for antibiotics resistance were also identified. The present study offers important insight into the biodiversity of the *Vibrio* sp. and provides valuable information for the study of virulence and antibiotic resistance factors, which will facilitate control of *V. coralliilyticus* in aquaculture. Further studies are required to determine appropriate treatments for preventing *V. coralliilyticus* infection–induced mass mortality events at marine bivalve hatcheries.

Production facilities for bivalve artificial seedlings are increasing, but damage caused by *V. coralliilyticus* infection continues in the hatcheries. The indiscriminate use of antibiotics has had a significant impact on the occurrence of multiple-antibiotic-resistant strains and environmental pollution such as antibiotic spills from hatchery directly to the sea. As invertebrates, including bivalves, do not have acquired immune systems, disease prevention methods (e.g. vaccination) are very limited compared to fish. Therefore, the application of bacteriophage may be a solution for the prevention of bacterial disease in invertebrates. The results of our

study showed that phage potentially can be used as an effective substitute for antibiotics in the field of bivalve artificial seedling production while reducing the generation of antibiotic-resistant bacteria and environmental pollution.

국문초록

이매패류의 대량 폐사를 유발하는 원인체들의 규명 및 박테리오파아지를 이용한 *Vibrio coralliilyticus* 감염 예방

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Ostreid Herpesvirus-1 (OsHV-1)은 부유유생기부터 부착기 단계 치패의 대량 폐사를 유발하는 병원체 중 하나이다. OsHV-1 의 감염으로 인한 대량 폐사는 전 세계적으로 매년 지속적으로 발생하고 있지만, 현재까지 그 치료법은 개발되지 않고있다. 2017 년 11 월 이후, 한국의 해만가리비 종묘 생산장에서 지속적인 대량 폐사가 발생하였다. 5-10 일령의 유생이 급속하게 수조의 바닥에 가라 앉으며 90% 이상의 폐사율을 기록 하으며, 이러한 대량 폐사의 지속적인 발생으로 인해 한국의 이매패류 종묘 생산장에서는 원활한 유생의 생산이

불가능하게 되어 그 경제적 손실이 매우 크게 발생하였다. 원인 불명의 대량 폐사 원인체를 규명하기 위해 본 연구가 수행되었고, PCR, sequencing 그리고 TEM 을 통한 실험 결과 OsHV-1 이 원인체로 지목되었다. PCR 을 통한 연구에서 폐사한 모든 유생이 OsHV-1 양성반응을 나타냈으며, TEM 을 통한 유생의 조직 검경 결과에서도 viral capsid 가 관찰되었다. 검출된 OsHV-1 의 염기서열을 분석한 결과, 본 연구에서 분리된 OsHV-1 은 OsHV-1 μVar 로 분류되었다. 계통분석을 통해 기존에 보고된 OsHV-1 들과의 근연성을 분석한 결과, 지리적으로 근접한 한국, 중국 그리고 일본에서 보고된 OsHV-1 μVar 보다 유럽의 variants 들과 더욱 근연성을 나타냈었다. 본 연구의 결과는 해만 가리비에서 OsHV-1 μVar 을 분리 및 분석에 대한 최초의 보고이다.

Vibrio spp.는 수생생물의 질병을 유발하는 주요 세균으로 알려져 있으며, 그 중에서도 *Vibrio coralliilyticus* 는 산호와 이매패류 유생의 대량 폐사를 유발하는 주요 원인체로 보고되고 있다. 2000 년대 중반 이후 한국의 참굴 종묘 생산장에서 지속적인 대량 폐사가 발생하였으나 원인체가 *Vibrio* 속 세균으로 추정된다는 연구 결과만 있을 뿐, 그 원인체 분석에 대한 명확한 연구는 현재까지 진행되지 않았다. 따라서 본 연구에서는 국내 최초로 대량 폐사가 발생한

참굴의 종묘 배양장의 유생에서 *V. coralliilyticus* 의 분리 및 동정을 수행하였다. 참굴 유생에 분리된 *V. coralliilyticus* 의 인위감염 실험과 항생제 감수성 검사를 통해 고병원성의 항생제 다재 내성 세균임을 확인하였다. 분리된 *V. coralliilyticus* 의 전체 유전자 염기서열 분석을 통해 2 개의 chromosomes (Chr I, Chr II)과 2 개의 plasmids (pSNUTY1, pSNUTY2)로 구성된 것을 확인하였다. 두 chromosome 의 기능분석 결과, Chr I 에 세포의 기본 기능과 관련된 유전자가 Chr II 에 비해 더 많이 있는 것으로 확인되었으며, 계통수 분석 결과 본 연구에서 분리된 SNUTY-1 은 기존에 보고된 *V. coralliilyticus* strains 와 매우 근연한 것으로 나타났다. SNUTY-1 은 pSNUTY2 라는 독특한 plasmid 를 가지고 있는 것으로 확인되었는데, 이는 한국의 분리주가 다른 지리학적 기원에서 나온 *V. coralliilyticus* 들과는 차이가 있음을 의미한다. 본 연구의 결과는 *V. coralliilyticus* 의 생물 다양성에 대한 중요한 통찰력 및 독성인자 연구를 위한 정보를 제공함으로써 양식산업에서의 *V. coralliilyticus* 감염을 예방하기 위한 데이터로 활용 될 수 있을 것으로 기대된다.

항생제 다재 내성균의 출현과 함께 항생제 대체재 개발에 대한 연구가 활발히 진행되고 있다. 그 중, 박테리오파아지 (파아지)는 자연계에 존재하는 친환경 물질로 항생제의 대안으로 지목되어 활발한

연구가 진행중에 있다. 본 연구에서는 이매패류 종묘 배양장에서 참굴 유생의 대량 폐사를 유발하는 항생제 다재 내성 *V. coralliilyticus* 의 감염을 예방하기 위한 방안으로, *V. coralliilyticus* 에 특이적으로 감염하는 파아지의 분리, 특성 분석 그리고 참굴 유생에 직접 적용하여 그 예방효과를 검증하기 위해 실시되었다. 실험 결과, *V. coralliilyticus*에 감염하는 파아지 pVco-14를 분리하였으며, TEM을 통한 관찰을 통해 *Siphoviridae* 에 속하는 것으로 확인하였다. pVco-14 의 특성 분석 및 안정성 검사에서 실제 양식장의 매우 적합한 것으로 확인되었다. pVco-14 의 예방 효과를 입증하기 위해 *in vivo* test 를 실시한 결과, 파아지를 처리한 실험군에서 참굴 유생이 월등한 생존율을 나타낸 것으로 확인되었다. 이러한 결과는 pVco-14가 해양 이매패류 종묘생산장에서 *V. coralliilyticus* 감염을 예방하기 위한 예방제로 사용될 수 있으며, 항생제의 오남용으로 인한 슈퍼박테리아의 출현 및 환경 오염을 줄일 수 있음을 시사한다.

Key words: 해양 이매패류, 해만가리비, 참굴, 대량폐사, Ostreid Herpesvirus μVar, *Vibrio coralliilyticus*, 항생제 다재 내성, 예방, 박테리오파아지

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