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Isolation and physiological characterization of a novel virus infecting *Stephanopyxis palmeriana* (Bacillariophyta)

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Recent studies have shown the importance of viruses as potential sources of plankton mortality, which affect primary production and biogeochemical functions of their hosts. Here, we report basic characteristics of a novel virus (*Stephanopyxis palmeriana* virus: SpalV) that causes lysis of a culture of the diatom *S. palmeriana*, which was isolated in Jaran Bay, Korea, in August 2008. SpalV is a round-shaped viral particle ~25-30 nm in diameter that propagates in its host's cytoplasm. In addition, it shows species-specific infectivity among the tested diatom species. The burst size and latent period are estimated to be roughly 92 infectious units cell⁻¹ and <80 h, respectively.

Key Words: algal virus; Bacillariophyceae; *Stephanopyxis palmeriana*

INTRODUCTION

Seawater contains diverse organisms and viruses that infect their host, with ~10⁵-10⁹ virus particles in 1 mL of seawater (Suttle et al. 1990, Suttle 2007). The majority of these viruses infect bacteria, cyanobacteria, archaea, and other eukaryotic organisms, but some of these viruses infect macro- or microalgae. These algal viruses are known to play an ecologically important role in regulating the population dynamics of their phytoplankton hosts (Suttle et al. 1990, Bratbak et al. 1993). Over 50 different viruses or virus like particles (VLPs) infecting marine eukaryotic algae have been isolated and characterized during the last two decades (Van Etten et al. 1991, Nagasaki and Bratbak 2009, Wilson et al. 2009).

Among the primary producers in aquatic ecosystems, diatoms comprise a major group of algae and are the most common phytoplankton. More than 10⁵ species of diatoms are estimated to exist, which account for up to 35-75% of marine primary production in the ocean

(Smetacek 1999). Therefore, lysis of these species by virus infection can reduce primary production. For example, the inoculation of phytoplankton communities of several major algal species with viral concentrates from seawater reduced primary production by as much as 78% (Suttle et al. 1990, Suttle 2007).

Despite the potent roles of diatoms and viruses infecting these hosts in aquatic ecosystems, few studies have examined diatom viruses. Since the first diatom virus was, at least nine diatom viruses have been isolated (Tomaru et al. 2011a, 2011b, 2011c). Although all of these diatom viruses are small icosahedral viruses of 22-38 nm diameter, these diatom viruses are now represented by two recently approved virus genera, namely, *Bacillarnavirus* and *Bacilladnavirus* (International Committee on Taxonomy of Viruses, <http://talk.ictvonline.org/>), as well as several uncategorized members. The genera *Bacillarnavirus* and *Bacilladnavirus* are composed of diatom vi-



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ruses with a linear single-stranded RNA (ssRNA) genome and a circular single-stranded DNA (ssDNA) genome, respectively. Some diatom viruses have a genome type or structure that has not been well characterized. All diatom viruses specifically lyse their respective host diatom species and therefore are thought to represent an important control agent for the dynamics of their respective host population (Avrani et al. 2012).

In the present study, we isolated and characterized a novel virus infecting a diatom, *Stephanopyxis palmeriana*, a cosmopolitan planktonic marine diatom species. The virus, named *Stephanopyxis palmeriana* infecting virus (SpalV), is the first diatom-infecting virus isolated from Korean coastal waters.

MATERIALS AND METHODS

Algal strains

The host organism, *Stephanopyxis palmeriana* (NF-D-SPA-1 strain), was provided by the National Fisheries Research and Development Institute in Korea and verified by sequence analysis of the large-subunit (26S) rDNA D1 / D2 domain and the 18S rDNA gene (Kooistra et al. 2003, Medlin and Kaczmarska 2004, Rynearson et al. 2006). The host was cultured in modified f/2 medium (Guillard 1973) under a 12 L : 12 D cycle of 80-90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with cool-white fluorescent illumination at 20°C.

Isolation of virus particles

Seawater samples were collected at Jaran bay, Korea, between May and October 2008 and prefiltered through 0.8 μm pore-size polycarbonate filters (Nuclepore, Pleasanton, CA, USA). The logarithmic phase cultures of *Stephanopyxis palmeriana* (Fig. 1B) were challenged with the seawater samples filtered through 0.8 μm pore-size polycarbonate filters (Nuclepore) and incubated under the condition mentioned above. Then, to confirm the virus particles, the *S. palmeriana* cultures were checked with the lysate filtered through 0.2- μm -pore-size Dismic-25cs filter (Advantec, Charlotte, NC, USA). Screening and cloning procedure was almost according to the method of Tarutani et al. (2001). In total, 100 mL of *S. palmeriana* logarithmic-phase cultures was inoculated with 20 mL of filtered seawater and incubated under the same conditions described above.

Growth experiment

Cultures containing 500 mL of exponentially growing host cells were inoculated with 20 mL of lysate containing SpalV at a viral titer of 3.0×10^6 estimated by the most probable number; cell density and virus titer were then respectively measured using light microscopy and the extinction dilution method (Tarutani et al. 2001) every 24 h until 120 h postinoculation (hpi) (Fig. 2). And the host range of SpalV was examined by adding 50 μL of the original pathogen suspension to each 1 mL culture of exponentially growing algal strains listed in Table 1. Each culture was incubated under the culture conditions described above and observed under a light microscope.

Transmission electron microscopy (TEM)

The morphology of SpalV was observed using a TEM. *S. palmeriana* cultures were inoculated with SpalV, samples (10 mL) were collected at 0, 24, 48, 72, 80, and 92 hpi, and fixed with 1% glutaraldehyde in f/2 for 2 h at 4°C. Cells were harvested by centrifugation at 3,000 rpm for 20 min, and then post-fixed for an additional 1 h with 2% osmium tetroxide at 4°C. Following two rinses with phosphate buffered saline buffer, the pellet was dehydrated in a graded ethanol series (20-100%) and embedded in Poly/Bed 812 resin (Polyscience, Inc., Warrington, PA, USA). Thin sections were stained with 4% uranyl acetate and 3% lead citrate, and observed using a TEM (JEOL JEM-1010; Jeol, Tokyo, Japan) with an acceleration voltage of 60 kV.

The VLPs were also observed in negative staining with 4% uranyl acetate using a transmission electron microscope. Briefly, a drop of purified virus suspension was mounted on a grid for 30 s, and excess water was removed using filter paper. Then, a drop of 4% uranyl acetate was mounted on the grid for 10 s, and removed using filter paper. After the grid was dried, the grids were observed using TEM with the same conditions as above. Particle diameters were estimated using the negatively stained images.

Storage

The effect of storage temperature on SpalV infectivity was examined according to the method of Tomaru et al. (2005). An exponentially growing culture of *S. palmeriana* was inoculated with the virus and incubated for 7 days. Then the resultant lysate was sequentially passed through 0.8- μm and 0.22- μm filters to remove cell debris. The titer of the fresh lysate estimated using the extinction dilu-

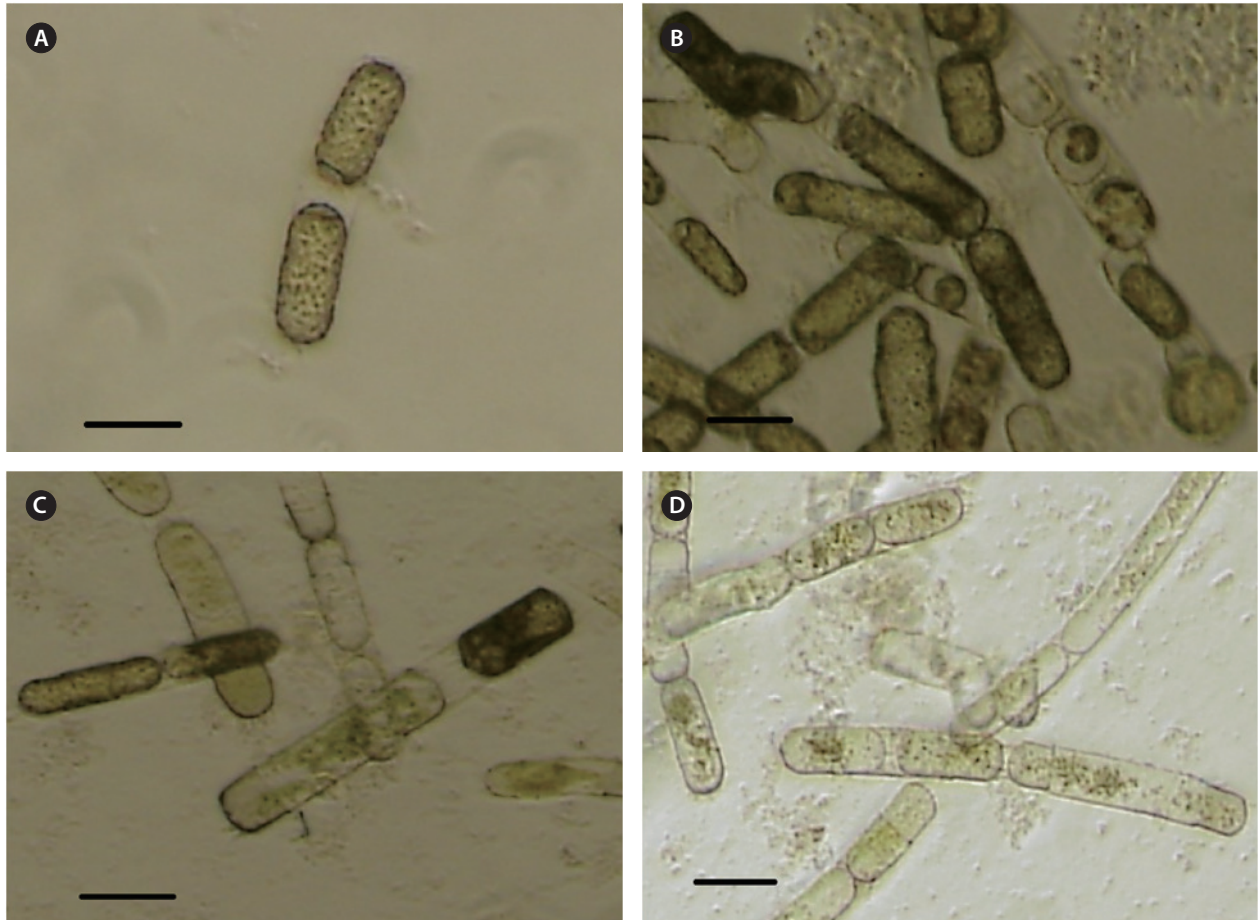


Fig. 1. Images of healthy culture and *Stephanopyxis palmeriana* virus (SpalV)-inoculated culture. Light micrographs of a *S. palmeriana* culture at 0 day (A), 1 day (B), 2 days (C), and 4 days (D) post-inoculation with SpalV. Scale bars represent: A-D, 100 μ m.

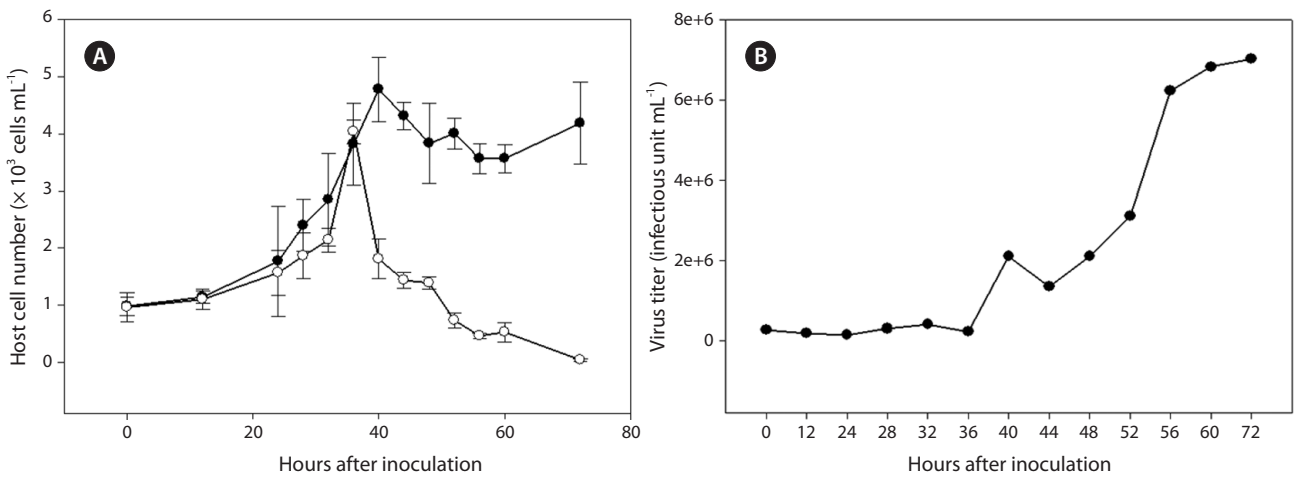


Fig. 2. Multiplication of *Stephanopyxis palmeriana* virus (SpalV) in inoculated hosts. (A) Changes in host cell numbers with (●) or without (○) inoculation of SpalV. (B) Virus titer in the inoculated culture was calculated using the extinction dilution method.

tion method was 2.6×10^7 infectious units mL^{-1} . Aliquots of the lysate were stored at 20, 15, 4, and -196°C (liquid nitrogen) under light or dark conditions with or without 10 or 20% dimethyl sulfoxide (DMSO) as cryoprotectant, and re-titrated using the extinction dilution method.

RESULTS AND DISCUSSION

Cultures and cells of *S. palmeriana* were lysed by the filtrate and became pale in color, presumably due to the loss or degradation of photosynthetic pigments. Incubation with the viral lysate caused complete lysis of host cultures within 1 week, in contrast to the controls, which

remained healthy (Fig. 1A-D). Further cloning of the virus strain was performed according to the method of Tarutani et al. (2001), and the isolated virus was named *Stephanopyxis palmeriana* infecting virus (SpalV).

SpalV was not lytic to any microalgal species tested other than *S. palmeriana*; moreover, it was not lytic to other diatom strains including *Chaetoceros* or *Skeletonema* (Table 1). Many algal viruses are reportedly not only “species-specific,” but also “strain-specific” (Tomaru et al. 2008). However, the strain-specific infectivity of SpalV could not be accessed because of the limited availability of *Stephanopyxis* strains.

The replication parameters of SpalV were determined based on growth experiments. An increase in viral ti-

Table 1. Infection specificity of *Stephanopyxis palmeriana* virus (SpalV) against 35 strains of marine phytoplankton

Class	Genus and Species	Strain	Lysis by SpalV
Bacillariophyceae	<i>Asterionella glacialis</i>	NF-F-Asa-1	-
	<i>Chaetoceros curvisetus</i>	ME-CCS-1	-
	<i>Chaetoceros curvisetus</i>	NF-F-CCS-2	-
	<i>Chaetoceros</i> sp.	PK-CH-1	-
	<i>Chaetoceros</i> sp.	PK-CH-HuR-1	-
	<i>Skeletonema costatum</i>	ME-SCM-1	-
	<i>Skeletonema costatum</i>	KMMCC-292	-
	<i>Skeletonema</i> sp.	ME-SCM-2	-
	<i>Skeletonema</i> sp.	KMMCC-226	-
	<i>Stephanopyxis palmeriana</i>	NF-D-SPA-1	+
	<i>Thalassiosira</i> sp.	PK-TH-1	-
<i>Thalassiosira</i> sp.	PK-TH-6	-	
Chlorophyceae	<i>Dunaliella</i> sp.	PK-F-DUN-SP-1	-
Cryptomonadaceae	<i>Rhodomonas</i> sp.	PK-F-RHO-SP-1	-
	<i>Rhodomonas</i> sp.	PK-F-RHO-SP-2	-
	<i>Rhodomonas</i> sp.	PK-F-RHO-SP-3	-
Dinophyceae	<i>Akashiwo sanguinea</i>	NF-F-ACA-1	-
	<i>Alexandrium</i> sp.	NF-F-ALE-SP-1	-
	<i>Alexandrium</i> sp.	NF-F-ALE-SP-2	-
	<i>Alexandrium catenella</i>	NF-F-ACA-1	-
	<i>Alexandrium catenella</i>	NF-F-ATA-3	-
	<i>Amphidinium carterae</i>	PK-F-ACA-1	-
	<i>Cochlodinium polykrikoides</i>	NF-F-CPO-3	-
	<i>Gymnodinium impudicum</i>	NF-F-GIM-1	-
	<i>Gymnodinium</i> sp. (<i>Heterocapsa pygmaea</i>)	NF-F-GYM-SP-1	-
	<i>Gymnodinium</i> sp.	NF-F-GYM-SP-2	-
	<i>Prorocentrum minimum</i>	NF-F-PMI-1d	-
	<i>Prorocentrum minimum</i>	PK-F-PMI-2	-
	<i>Prorocentrum micans</i>	NF-F-PMC-1	-
	<i>Prorocentrum micans</i>	NF-F-PMC-2	-
Euglenophyceae	<i>Eutreptia</i> sp.	NF-F-EUT-SP-1	-
Prymnesiophyceae	<i>Prymnessium parvum</i>	PK-F-Pry-SP-1	-
Raphidophyceae	<i>Heterosigma akashiwo</i>	NF-F-HAK-1	-
	<i>Heterosigma akashiwo</i>	PK-F-HAK-1	-
	<i>Chattonella antiqua</i>	NF-F-CAN-1	-

+, lysed; -, not lysed.

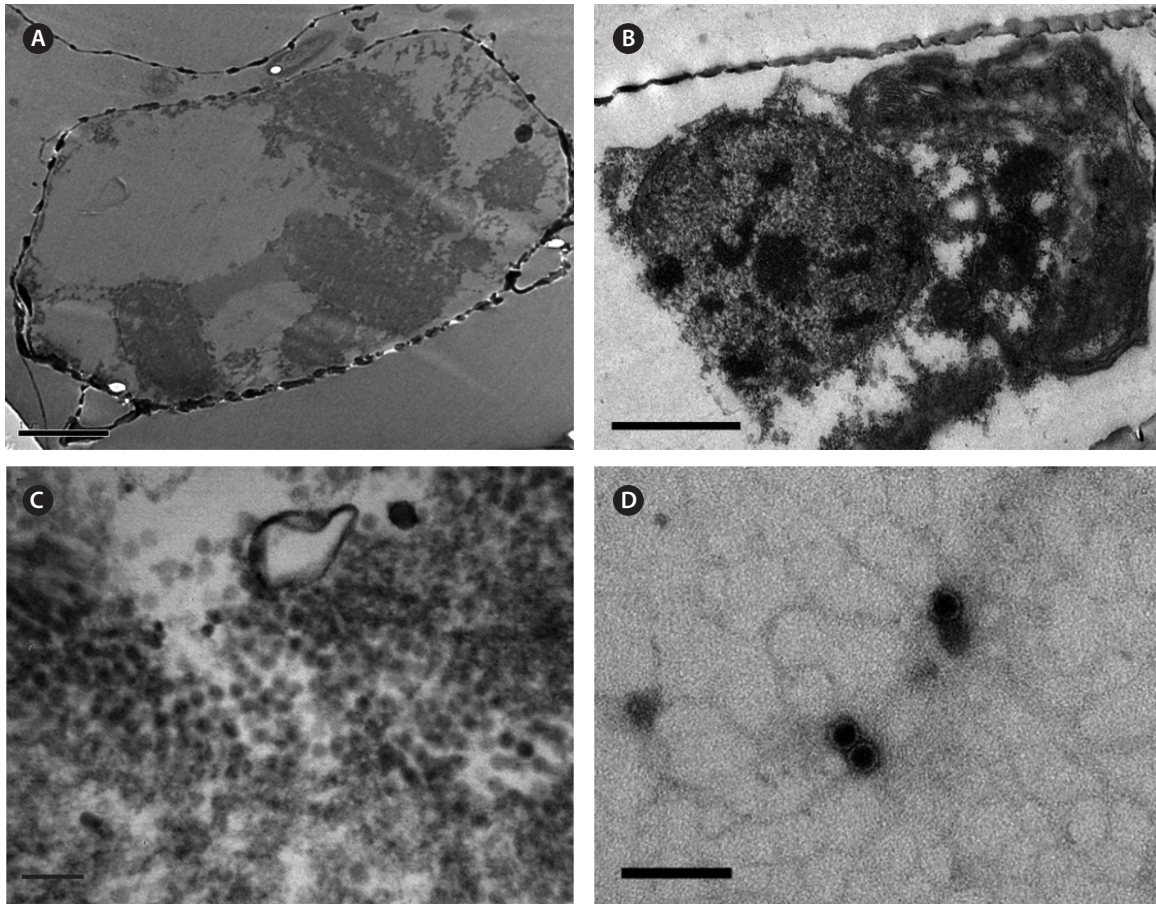


Fig. 3. Electron micrographs of *Stephanopyxis palmeriana* virus (SpalV). (A) Healthy cell, *S. palmeriana* NF-D-SPA-1. (B & C) Cells infected by the pathogen SpalV. (B) Post inoculation (48 h); numerous small dense masses that appears to be fragmenting. (C) Higher magnification image of the virus-like particles in the host cytoplasm. (D) Negatively stained virus-like particles from lysate. Scale bars represent: A & B, 1 μ m; C & D, 100 nm.

ter was detected from 36 to 56 hpi, and the lytic cycle of SpalV was predicted to be shorter than 40 h. Furthermore, by comparing the decrease in host cell number to the increase in viral titer between 48 and 80 hpi (Fig. 2A), the burst size was estimated to be 92 infectious units cell⁻¹ (Fig. 2B). An increase in viral titer during exponential host growth has been commonly observed in *Chaetoceros* host-virus systems, including CtenDNAV, CtenRNAV, Clo-rDNAV, and Csp03RNAV (Shirai et al. 2008, Tomaru et al. 2011b, 2011c, 2013).

Intracellular virus particles were observed mainly in the cytoplasm, and the roundish virus-like particles were ~25-28 nm in diameter which were not observed in non-infected healthy host cell (Fig. 3A-C). No typical crystalline array formation reported in other diatom viruses was observed (Nagasaki et al. 2005, Tomaru et al. 2008, 2013). Virus particles observed by negative staining were 20 ± 5 nm in diameter, and no outer membrane or tail-like

structures were observed (Fig. 3D). Similar structures in the virus-infected host cells have been reported for other diatom viruses, including viruses infecting *Chaetoceros* cf. *gracilis* (CspNIV 25 nm) (Bettarel et al. 2005), *Chaetoceros debilis* (CdebDNAV 30 nm) (Tomaru et al. 2008), and *Chaetoceros socialis* f. *radians* (CsfrRNAV 22 nm) (Tomaru et al. 2009).

After 1 month of storage at 4, 15, and 20°C in the dark, the estimated titers were 7.02×10^4 , 1.9×10^4 , and 5.1×10^3 infectious units mL⁻¹, respectively, but were below the detection limit ($<3.0 \times 10$ infectious units mL⁻¹) after 3 months of storage at each temperature. The decrease in infectious titers has been also reported for other microalgal viruses such as *Chaetoceros tenuissimus* virus (CtenRNAV) and *Chaetoceros socialis* virus (CsfrRNAV), which showed considerable decay even under cold and dark conditions (Shirai et al. 2008, Tomaru et al. 2009). Based on these results, significant loss of infectivity may occur

in natural environments. Cryopreservation conditions for SpalV were optimized similar to the methods of Nagasaki and Yamaguchi (1999) and Tomaru et al. (2005); the highest remaining titers (7.02×10^5 infectious units mL⁻¹ or 72%) after 1 month of storage were recorded when the viral suspension was preserved in liquid nitrogen (-196°C) with 10% DMSO as cryoprotectant (initial concentration = 100%; Merck, Darmstadt, Germany).

Based on previous reports, we know that phytoplankton viruses smaller than 40 nm in diameter typically contain ssRNA genomes and are related to viruses from the highly diverse picorna-like superfamily (Culley et al. 2003). These are the cases for the ssRNA virus previously found in a diatom (*Chaetoceros socialis*) (Tomaru et al. 2009), the ssRNA virus infecting the raphiophyte *Heterosigma akashiwo* (Tai et al. 2003), and the ssRNA virus of the dinoflagellate *Heterocapsa circularisquama* (Tomaru et al. 2004). A dsRNA virus ranging from 65 to 80 nm in diameter was recently shown to infect the prasinophyte *Micromonas pusilla* (Brussaard et al. 2004). On the basis of virion morphology, it is possible that SpalV is genetically related to ssRNA viruses; however, further work is needed to characterize its genome type and size.

Viruses are thought to influence community composition through increased infection of competitively superior host species that are abundant and are fast growing (Sandaa 2008). Although we do not have sufficient data to determine the roles of viruses in marine environments or in host population dynamics, the interaction between *Phaeocystis pouchetii* and the lytic virus PpV01 in laboratory microcosms and the ecological relationship between the bloom-forming dinoflagellate *Heterocapsa circularisquama* and its infectious virus, HcRNAV, provide a rational explanation of the role of viruses in dynamics of host algae (Nagasaki et al. 2003).

CONCLUSION

We have reported basic characteristics of a novel virus (*Stephanopyxis palmeriana* virus, SpalV) that causes lysis of a culture of the diatom *Stephanopyxis palmeriana*, which was isolated in Jaran Bay, Korea, in August 2008. SpalV is a round-shaped viral particle ~25-30 nm in diameter that propagates in its host's cytoplasm. In addition, it shows species-specific infectivity among the tested diatom species and the burst size and latent period are estimated to be roughly 92 infectious units cell⁻¹ and <80 h, respectively.

To the best of our knowledge, this is the first report of

a diatom virus infecting and lysing *S. palmeriana* which seasonally bloom occurring in Korean coastal water. The roles of viruses in marine environments or in host population dynamics remain unclear, and detailed ecological observations and analysis of both host and viral genetic information are required to fully elucidate their ecological niches.

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