

Isolation and Physiological Characterization of a New Algicidal Virus Infecting the Harmful Dinoflagellate *Heterocapsa pygmaea*

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(Received on July 4, 2012; Revised on July 30, 2012; Accepted on July 30, 2012)

Dinoflagellates are considered one of the most abundant and diverse groups of marine microplankton and viruses are recognized as one of the significant factors affecting the plankton dynamics. Here, we report basic characteristics of a new dinoflagellate-infecting virus, *Heterocapsa pygmaea* DNA virus (HpygDNAV) which infects a toxic dinoflagellate, *H. pygmaea*. HpygDNAV is a polyhedral large virus (ca. 160–170 nm in diameter) propagating in its host's cytoplasm. Because of the virion size, appearance in thin sections, and propagation characteristics, HpygDNAV is assumed to harbor a large double-stranded DNA genome; i.e., HpygDNAV is most likely a nucleocytoplasmic large DNA virus (NCLDV) belonging to the family *Phycodnaviridae*. Its infectivity is strain-specific, rather than species-specific, as is the case for other algal viruses. The burst size and latent period are estimated to be roughly 100–250 infectious units cell⁻¹ and < 96 h, respectively.

Keywords : algal virus, dinoflagellate, *Heterocapsa pygmaea*, NCLDV

Sea water contains diverse organisms and viruses that infect their host. It is assumed that there are 10⁵ to 10⁹ virus particles in one milliliter of sea water. Most of these viruses infect bacteria, cyanobacteria, archaea and other eukaryotic organisms but some portions of these viruses infect macro- or micro algae. These algal viruses are known to play an ecologically significant role in regulating the population dynamics of their phytoplankton hosts (Bratbak et al., 1993; Suttle et al., 1990; Tarutani et al., 2000). Over 50 different viruses or virus like particles (VLPs) infecting marine eukaryotic algae have been isolated and characterized during the last two decades (Nagasaki, 2008; Willson et al.,

2009). To date, many algal host-virus systems were brought into *in vitro* and studied to different extents (Nagasaki, 2008; Van Etten and Meint, 1999; Van Etten et al., 1991).

Among these species, the first and second cultured dinoflagellate-infecting viruses reported were characterized as a large icosahedral double-stranded (ds) DNA virus, *Heterocapsa circularisquama* virus (HcV, Tarutani et al., 2001; Nagasaki et al., 2003), and a small icosahedral single-stranded (ss) RNA virus, *Heterocapsa circularisquama* RNA virus (HcRNAV, Tomaru et al., 2004), infecting *Heterocapsa circularisquama*. Prior to the isolation of these viruses, viral infection in dinoflagellate has been scarcely investigated; there were only three reports concerning the VLPs observed in dinoflagellate by transmission electron microscope in the 1970's (Franca, 1976; Sicko-Goad and Walker, 1979; Soyer, 1978). In this study, we present the new dinoflagellate virus *Heterocapsa pygmaea* DNA virus, HpygDNAV, which infects *Heterocapsa pygmaea* and is newly isolated from Korean coastal waters.

Dinoflagellates are single-celled aquatic organisms with two dissimilar flagella, and are thought to be some of the most abundant and diverse phytoplankton and net primary producers (Graham and Wilcox, 2000). The genus *Heterocapsa* comprises small, marine, gymnodinioid dinoflagellates (Loeblich et al., 1981). *Heterocapsa pygmaea* has been isolated from Hong Kong to Japan (Iwataki, 2008), but this species is newly identified from Korean coastal waters. In the present study, the isolation, growth in culture, life-cycle, stability, and gene content of HpygDNAV, a novel DNA virus, are described. This is the second dinoflagellate-infecting virus isolated from Korean coastal waters.

Seawater samples were collected at Jaran Bay, Korea, between April and October 2009 and filtered through 0.2 µm pore-size Dismic-25cs filters (Advantec, Charlotte, NC). The host organism, labeled as *Gymnodinium* sp. (NF-F-GYM-SP-1 strain), was obtained from the National Fisheries Research and Development Institute. However, recent

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taxonomic study on *Gymnodinium* sp. established a new genus, *Heterocapsa* (Iwataki et al., 2008; Pennick and Clarke, 1977; Tamura et al., 2005). Therefore, the host alga was identified by the sequence analysis of the large-subunit RNA gene (D1-D2 region) and the 18S rDNA region and transmission electron microscopic observation. Sequences of the D1-D2 region and the 18S rDNA region showed 100% sequence identity to those of *Gymnodinium* sp. USA29-9 (accession no. AF201747) which had been reclassified as *Heterocapsa* spp. (Iwataki et al. 2008). The host was further identified as *H. pygmaea* based on the morphological observation showing the typical characteristics of the species including the cell size, body scale size, and the number of pyrenoid among them. The host alga was cultured in modified f/2 medium (Gillard, 1973) under a 12L:12D cycle of 80 to 90 $\mu\text{mol photons/m}^2/\text{s}$ with cool white fluorescent illumination at 20°C. Five hundred milliliter of logarithmic-phase cultures of *H. pygmaea* were inoculated with 20 ml of filtered sea water and incubated at the same condition as above. Cultures and cells of *H. pygmaea* 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 controls, which remained healthy (Fig. 1A, B). Further cloning of a virus strain was performed according to the method of Tarutani et al. (2001), and the isolated virus was named as HpygDNAV.

The host range of HpygDNAV was examined by adding 50 μl of the lysate 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 by light microscopy. HpygDNAV was not lytic to any microalgal species tested other than *H. pygmaea*; moreover, it was not lytic to all strains of *Gymnodinium* sp.

tested (Table 1). The infectivity of HpygDNAV is therefore considered not only 'species-specific' but also 'strain-specific', as observed in the case of other algal viruses (Tomaru et al., 2004a, 2004b, 2008).

The replication parameters of HpygDNAV were determined by growth experiments. Cultures containing 500 ml of exponentially growing host cells were inoculated with 20 ml of lysate containing HpygDNAV at a viral titer of 7.0×10^4 estimated by most probable number (MPN); cell density and virus titer were then respectively measured by light microscopy and the extinction dilution method (Tarutani et al., 2001) every 24 h until 140 hours post-inoculation (hpi; Fig. 2). There was a gradual decrease of host cell numbers from 20 to 72 hpi followed by a remarkable decline. In accordance with the changes in host cell number, there was slight increase of virus titer until 72 hpi followed by sharp increase. Therefore, the lytic cycle of HpygDNAV was predicted to be shorter than 36 h. The slow increase of the virus titer from 36 to 72 hpi could be related to low infection efficiency of the virus and relatively small burst size, which was estimated as 100–250 infectious units/cell. The latent period of HpygDNAV is between those of two previously reported dinoflagellate infecting viruses, HcV and HcRNAV, which were 40–56 h and 24–48 h, respectively and longer than those of other microalgal viruses (Nagasaki et al., 2003). However, the estimated burst size is much smaller than those of HcV and HcRNAV, 1,800–2,440 and 3,400–21,000, respectively.

The morphology of HpygDNAV was observed by using a transmission electronmicroscope. *H. pygmaea* cultures were inoculated with HpygDNAV and samples (10 ml) were collected at 0, 24, 48, 72, 80, and 92 hours post-inoculation, 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, then post-fixed for an additional 1 h with 2% osmium

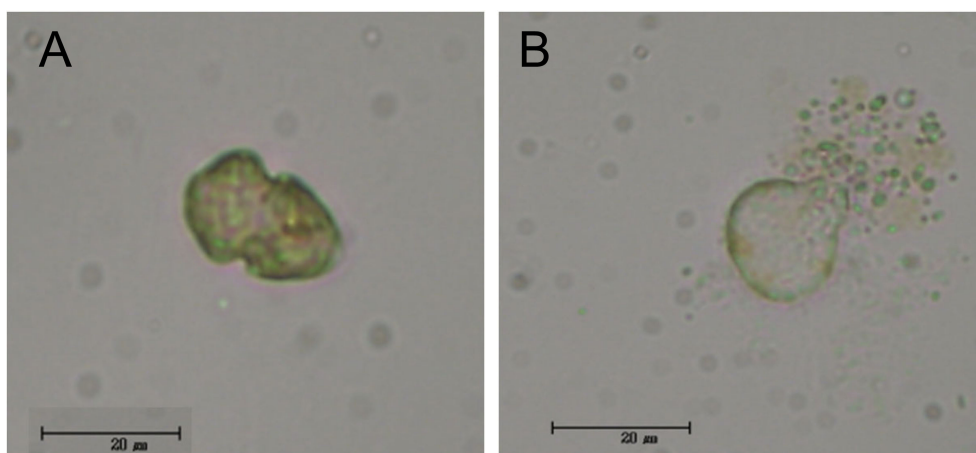


Fig. 1. Images of healthy culture and *Heterocapsa pygmaea* DNA Virus (HpygDNAV) -inoculated culture. Light micrographs of a *H. pygmaea* culture at 0 day (A) and 4 days (B) post-inoculation with HpygDNAV. Scale bars represent 20 μm .

Table 1. Infection specificity of *Heterocapsa pygmaea* DNA Virus (HpygDNAV) against 30 strains of marine phytoplankton

Class	Genus & Species	Strain	Lysis by HpygDNAV
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>Skeletonema palmeriana</i>	NF-D-SPA-1	–
	<i>Skeletonema costatum</i>	ME-SCM-1	–
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-AKA-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>Heterosigma akashiwo</i>	PK-F-HAK-2	–
	<i>Chattonella antiqua</i>	NF-F-CAN-1	–

+: lysed; –: not lysed

tetroxide at 4°C. Following two rinses with PBS buffer, the pellet was dehydrated in a graded ethanol series (20 to 100%) and embedded in Poly/Bed® 812 resin (Polyscience, Inc.). Thin sections were stained with 4% uranyl acetate and 3% lead citrate and observed by transmission electron microscopy (JEOL JEM-1010) with an acceleration voltage of 60 kV. Intracellular virus particles were observed mainly in cytoplasm and the icosahedral virus particles were about 150–200 nm in diameter (Fig. 3B, C). No typical crystalline array formation was observed. Virus particles observed by negative staining was 160 ± 10 nm in diameter and no outer membrane or tail-like structures were observed (Fig. 3D). The size, icosahedral morphology, and cytoplasmic site of virus assembly are properties common among algal virus VLPs (Van Etten et al., 1991), including *Emiliania huxleyi* (170–200 nm; Bratbak et al., 1993) and

Pyramimonas orientalis (180–220 nm; Sandaa et al., 2001).

The effect of storage temperature on HpygDNAV infectivity was examined according to the method of Tomaru et al. (2005). An exponentially growing culture of *H. pygmaea* was inoculated with the virus and incubated for 7 d. 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 dilution method was 7.0×10^4 infectious units/ml. Aliquots of the lysate were stored at 20, 15, 4 and –196°C (liquid nitrogen) in light or dark conditions with or without 10 or 20% dimethyl sulfoxide as cryoprotectant, and re-titrated by extinction dilution method. After 2 weeks of storage at 4, 15, and 20°C in the dark, the estimated titers were 5.1×10^4 , 1.9×10^4 , and 2.3×10^3 infectious units/ml, respectively, but were below the detection limit ($< 3.0 \times 10^1$ infectious units/ml) after 1 month

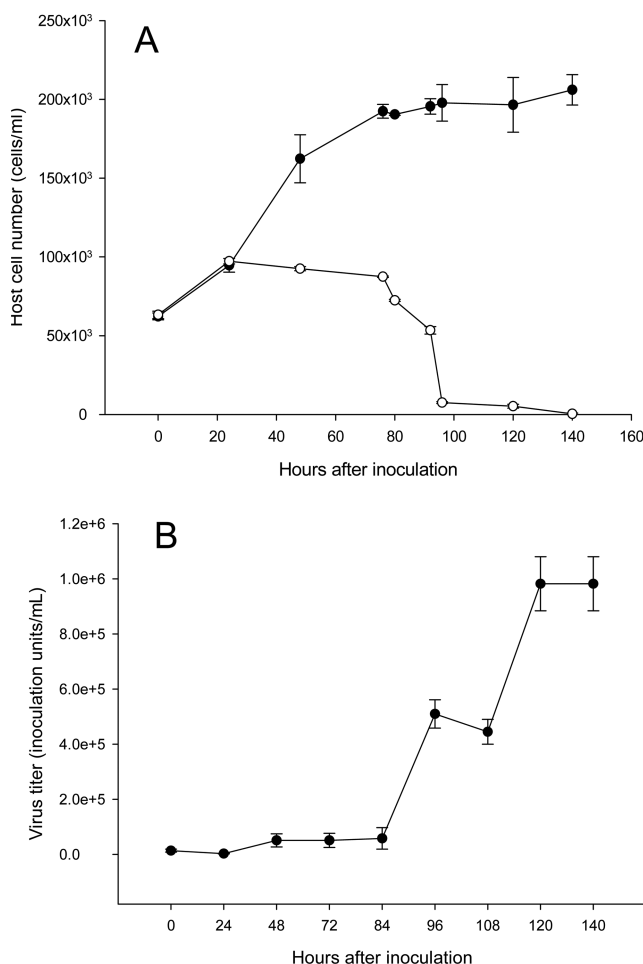


Fig. 2. Multiplication of *Heterocapsa pygmaea* DNA Virus (HpygDNAV) in inoculated host. (A) Changes of host cell numbers with (●) or without (○) inoculation of HpygDNAV. (B) Virus titer in the inoculated culture calculated by extinction dilution method. The data are average from three replications.

of storage at each temperature. The decrease of infectious titers is between those of previously reported viruses, *Micromonas pusilla* virus (MpV) and *Teleaulax amphioxieia* virus (TampV), which showed considerable decay in even cold and dark conditions (Cottrell and Suttle, 1995; Nagasaki et al., 2009). Significant loss of infectivity may therefore occur in natural environments. Cryopreservation conditions for HpygDNAV were optimized similar to the methods of Nagasaki & Yamaguchi (1999) and Tomaru et al. (2005); the highest remaining titers (1.9×10^4 infectious units/mL, or 27%) after 1 month of storage were recorded when the viral suspension was preserved in liquid nitrogen (-196°C) with 10% dimethyl sulfoxide as cryoprotectant (initial concentration = 100%, Merck).

Nucleic acid was extracted by standard phenol-chloroform methods and ethanol precipitated, and the resultant pellet was dissolved in 100 μl of DEPC-water. Aliquots (5

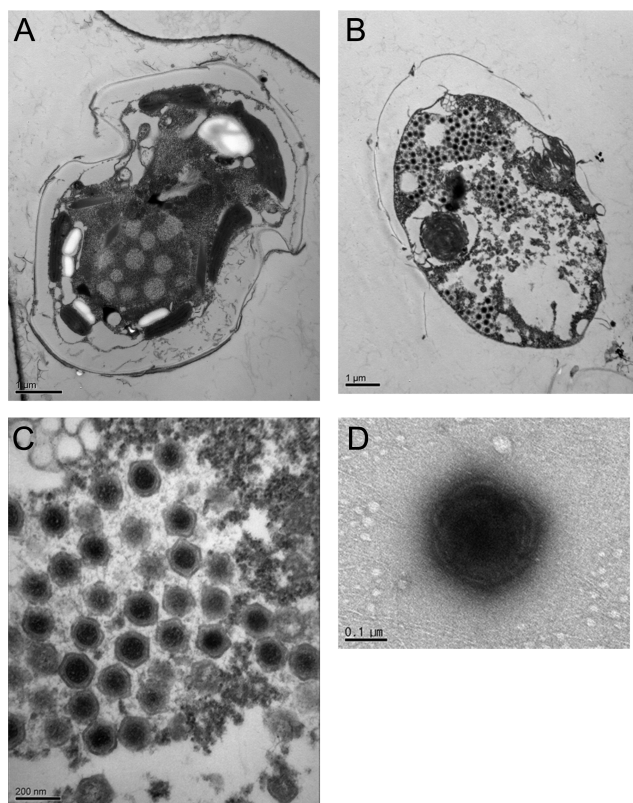


Fig. 3. Electron micrographs of *Heterocapsa pygmaea* DNA Virus (HpygDNAV). (A) Healthy *Heterocapsa pygmaea* NF-F-GYM-SP-1 cells. (B) Thin section of a HpygDNAV-infected cell harboring virus particles. (C) Higher magnification image of virus assembly in the host cytoplasm. (D) Negatively stained virus particles from lysate.

μl) of the nucleic acid solution were incubated with 5 U of DNase I (Takara Bio, Inc.), 60 U of RNase A (Takara Bio, Inc.) at 37°C for 1 h. Nucleic acid samples incubated at 37°C without enzymatic treatment for 1 h served as a control. The viral genome was sensitive to DNase I, but not RNase A (Fig. 4). These data imply that the HpygDNAV genome is double-stranded DNA.

As far as the authors know, this is one of only a few reports describing features of a virus infecting dinoflagellates (Alveolata) to be made after the isolations of HcV and HcRNAV, which infect *Heterocapsa circularisquama* (Nagasaki et al., 2003; Tarutani et al., 2001; Tomaru et al., 2004a). The virion size, shape, the cytoplasm as accumulating site and large dsDNA genome suggest that HpygDNAV is new member of the NCLDV group (Iyer et al., 2006).

Viruses are thought to influence community composition by increased infection of competitively superior host species that are overrepresented in a mixed population and have rapid doubling times (Sandaa, 2008; Wommack and Colwell, 2000). This is a rational explanation of the ecological relationship between the bloom-forming dinoflagellate *H.*

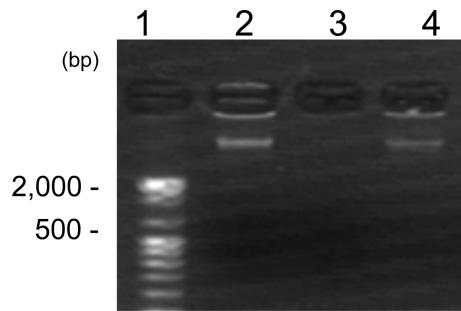


Fig. 4. Characterization of HpygDNAV by digestion with selective nucleases. Purified HpygDNAV nucleic acid without enzyme treatment (lane 2) or treated with DNase I (lane 3), RNase A (lane 4). Samples were electrophoresed in a 1% agarose gel with 100 bp DNA ladder (lane 1).

circularisquama and its infectious virus, HcRNAV, and of the dynamics of host algae observed by a field survey in Ago Bay, Japan (Nagasaki et al., 2003; Tomaru and Nagasaki, 2004).

Although we cannot provide sufficient data to determine the roles of viruses in marine environments or in host population dynamics, *H. pygmaea* itself is newly identified in Korea. Thus, detailed ecological observations and analysis of both host and viral genetic information will be required to fully elucidate their ecological niches.

Acknowledgments

This research was supported by the Pioneer Research Center Program through the National Research Program of Korea, funded by the Ministry of Education, Science and Technology (Grant No. M1071118001-08M1118-00110) and by the project “Environmental-friendly Aquaculture Technology using biofloc technology (RP-2012-AQ-053)”, National Fisheries Research & Development Institute (NFRDI).

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