Isolation of a novel cyanophage infectious to the filamentous cyanobacterium *Planktothrix agardhii* (Cyanophyceae) from Lake Donghu, China

E-Bin Gao, Xiu-Ping Yuan, Ren-hui Li, Qi-Ya Zhang*

**State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences,
Graduate University of the Chinese Academy of Sciences, Wuhan 430072, PR China**

ABSTRACT: A previously unknown cyanophage, PaV-LD (*Planktothrix agardhii* Virus isolated from Lake Donghu), which causes lysis of the bloom-forming filamentous cyanobacterium *P. agardhii*, was isolated from Lake Donghu, Wuhan, China. PaV-LD only lysed *P. agardhii* strains isolated from Lake Donghu and not those isolated from other lakes. The PaV-LD particle has an icosahedral, non-tailed structure, ca. 70 to 85 nm (mean ± SD = 76 ± 6 nm) in diameter. PaV-LD was stable at freezing temperature, but lost its infectivity at temperatures >50°C. Lysis of host cells was delayed about 3 d after the PaV-LD treatment with chloroform, and the virus was inactivated by exposure to low pH (≤4). The latent period and burst size of the PaV-LD were estimated to be 48 to 72 h and about 340 infectious units per cell, respectively. The regrowth cultures of surviving host filaments were not lysed by the PaV-LD suspension. To our knowledge, this is the first isolation and cultivation of a virus infectious to the filamentous bloom-forming cyanobacterium *Planktothrix* from a freshwater lake.

KEY WORDS: Cyanophage · *Planktothrix agardhii* · Viral infection · Blooms · Cyanobacteria · Freshwater lake

INTRODUCTION

The filamentous cyanobacterium *Planktothrix agardhii* is one of the typical harmful bloom-causing cyanobacteria and can be commonly detected in a large range of aquatic environments (Scheffer et al. 1997, Briand et al. 2002, Davis et al. 2003), including shallow eutrophic freshwater lakes and reservoirs used to provide drinking water. Some strains can produce hepatotoxic microcystins that are toxic to the health of animals including humans (Tonk et al. 2005, Wiegand & Pflugmacher 2005). Consequently, the contamination of freshwater bodies by harmful cyanobacteria is now considered to be an increasing environmental hazard in the world (Chorus & Bartram 1999, Li et al. 2008, www.sciencedaily.com/releases/2008/04/080403140928.htm). Water management usually places a great deal of emphasis on physical and chemical factors that drive the growth and production of harmful cyanobacteria.

Viruses are ubiquitous, dynamic and numerically dominant components of microbial communities in natural aquatic ecosystems (Bergh et al. 1989, Wommack & Colwell 2000, Suttle 2005), and most of them are thought to be infectious to bacteria or cyanobacteria (Proctor & Fuhrman 1990). Since the first isolation of the cyanophage LPP-1, infectious to the freshwater cyanobacterial genera *Lyngbya*, *Plectonema* and *Phormidium* (Safferman & Morris 1963), viruses or virus-like particles have been detected in other genera of cyanobacteria, including *Anacystis*, *Synechococcus*, *Prochlorococcus*, *Anabaena*, *Nostoc* and *Microcystis* (Padan & Shilo 1973, Phlips et al. 1990, Lu et al. 2001, Tucker & Pollard 2005, Yoshida et al. 2006, Wang & Chen 2008). These detections have greatly stimulated the interest in the ecological implications of viral infection for cyanobacterial dynamics in aquatic environments (Suttle & Chan 1994, Suttle 2000). Some reports have suggested that cyanophages are significant bio-

logical agents of cyanobacterial mortality, and play an important role in regulating primary productivity and host population dynamics (Suttle et al. 1990, Hewson et al. 2001, Yoshida et al. 2008).

Despite the significance of viruses in biological control of cyanobacteria blooms, detailed knowledge on the potential effects of viral infection on cyanobacteria is still lacking. This is because it is relatively difficult to select known cyanobacterial species as potential hosts for the isolation of an anonymous virus from natural aquatic environments (Weinbauer 2004). This has largely restricted the isolation and cultivation of cyanobacterial phage–host systems and relevant studies on the potential effects of viral infection on cyanobacterial populations and production. To better understand the important roles of cyanophages on the bloom dynamics and biological control of harmful algae, our laboratory initiated a program in 2004 to investigate the distribution, morphology and abundance of virioplankton in Lake Donghu (Liu et al. 2005,

2006) and isolate the lytic agents of harmful bloom-forming cyanobacteria. In the present study, we focus on the isolation and characterization of a cyanophage that infects and lyses the harmful cyanobacterium *Planktothrix agardhii* from a freshwater lake and look for efficient strategies for the control of cyanobacterial blooms in freshwater environments. This cyanophage was tentatively named PaV-LD (*Planktothrix agardhii* Virus isolated from Lake Donghu) after its host and the location from which it was isolated.

MATERIALS AND METHODS

Cyanobacterial cultures and growth conditions. Ten cyanobacterial species (24 strains) used for isolation of cyanophages and for host range experiments are shown in Table 1. They were obtained from the Harmful Algae Collection and Algal Resources Collection, Institute of Hydrobiology, Academy of

Table 1. Cyanobacterial strains used in this study, their localities and dates of isolation and their susceptibility to cyanophage PaV-LD infection. FACHB strains used in this study were purchased from the Algal Resources Collection, Institute of Hydrobiology, Chinese Academy of Sciences. HAB strains were obtained from the Harmful Algae Collection, Institute of Hydrobiology, Chinese Academy of Sciences. (+): lysed cells; (–): cells not lysed

Species and strains	Isolation		Susceptibility
	Locality	Date	
Planktothrix agardhii			
HAB0637	Lake Donghu, Wuhan,China	Sep 2006	+
HAB0638	Lake Donghu, Wuhan,China	Sep 2006	+
HAB0642	Lake Donghu, Wuhan,China	Sep 2006	+
HAB0643	Lake Donghu, Wuhan,China	Sep 2006	+
HAB1128	Fusha Down, Guangdong, China	Jun 2006	–
HAB0403	Fuchun River, Zhejiang, China	Sep 2006	–
HAB0169	Hedao, Zhejiang, China	Aug 2006	–
HAB2364	Unknown, Beijing, China	Sep 2006	–
HAB0325	Dianchi, Yunnan, China	Apr 2006	–
Planktothricoides raciborskii			
HAB0306	Fishing Pond, Wuhan,China	May 2006	–
HAB0309	Fishing Pond, Wuhan,China	May 2006	–
Anabaena PCC7120	France	Unknown	–
Anabaena spirioides HAB0502	Fishing Pond, Wuhan,China	Aug 2006	–
Anabaena flos-aquae HAB0337	Fishing Pond, Wuhan,China	May 2006	–
Anabaena variabilis			
FACHB112	Lake Donghu, Wuhan,China	Unknown	–
FACHB113	Lake Donghu, Wuhan,China	Unknown	–
Microcystis aeruginosa			
FACHB569	Lake Donghu, Wuhan,China	Unknown	–
FACHB925	Lake Donghu, Wuhan,China	Unknown	–
FACHB524	Lake Donghu, Wuhan,China	Unknown	–
HAB0125	Lake Taihu, Jiangsu,China	Aug 2006	–
Microcystis smithii HAB0138	Fishing Pond, Wuhan, China	Sep 2006	–
Microcystis botrys HAB0329	Fishing Pond, Wuhan,China	May 2006	–
Aphanizomenon issatschenkoi			
HAB1313	Lake Chidonghu, Wuhan,China	Aug 2006	–
HAB2389	Lake Donghu, Wuhan,China	Sep 2006	–

Sciences, China. Each cyanobacterial strain used in this experiment was clonally grown in medium BG-11 at 26°C under a 14 h light:10 h dark cycle of 30 to 40 µmol photons m^{-2} s^{-1} with cool white fluorescent illumination.

Isolation of cyanophage. A sample of freshwater collected from Lake Donghu located in Wuhan, Hubei Province, China, in June 2007, was sent to the laboratory within 6 h after sampling. A total of 200 µl of the natural freshwater sample were mixed with 5 ml of BG-11 liquid medium, and then were incubated for 2 wk at 26°C under the same lighting conditions as mentioned above. The incubated water sample was filtered through a 0.45 µm pore-size Nucleopore membrane filter to remove zooplankton, phytoplankton, bacteria and cell debris. Aliquots of 0.2 ml of the filtrate were added to 0.8 ml of exponentially growing cultures of *Planktothrix agardhii* strains per well in 24-well culture plates and were incubated under the conditions described above. Control cultures received the 0.45 µm filtrate that had been heat-treated at 100°C for 15 min. The cultures were visually inspected, and checked by optical microscopy every day to examine the changes of filamentous morphology.

The PaV-LD suspension from these positive samples was obtained by the 3 cycles extinction dilution procedure (Nagasaki & Yamaguchi 1997). In brief, the supernatant of the lysed cultures was diluted with BG-11 medium in a series of 10-fold dilution steps. Aliquots of 0.2 ml of each dilution were transferred into successive 1 ml drops of fresh, exponentially growing *Planktothrix agardhii* cultures with 4 replicates in 24-well culture plates and incubated under the conditions described above. The culture lysate in the highest dilution of the first assay was centrifuged at 8000 × *g* for 15 min at 4°C to remove cell debris, and the supernatant was carried over to the second extinction dilution assay. This process was repeated 3 times. The lysate in the highest dilution of the third assay was inoculated to a fresh, exponentially growing culture of *P. agardhii*. The resultant lysate was used as the PaV-LD suspension in the present study. The number of infectious units of the PaV-LD suspension was estimated by using the most-probable number technique (Saitoh et al. 2003). Furthermore, to rule out potential lysis by bacteria the PaV-LD suspension was also tested for infectivity by filtration through 0.2 µm pore-size syringe filters.

Host range test. The host range specificity of the virus was determined by adding 0.2 ml aliquots of the PaV-LD suspension to triplicate 0.8 ml cultures of the exponentially growing cyanobacterial species listed in Table 1. Growth of host cultures without the PaV-LD suspension was also monitored to serve as a control. The cultures were incubated under the same condi-

tions of light and temperature as described for the growth of *Planktothrix agardhii* cultures. The occurrence of host cells lysis was daily monitored by optical microscopy. Strains that were not lysed after 14 d of incubation were considered to be not susceptible hosts for the virus.

Based on the results of these host tests, *Planktothrix agardhii* strain HAB0637, which was isolated from Lake Donghu in 2006 and identified on the basis of morphological description in existing literature (Suda et al. 2002), was used as the main host in the following experiments.

Growth experiment. Cultures, 100 ml in volume, of exponentially growing *Planktothrix agardhii* HAB0637 were inoculated in duplicate with 10% (v/v) of the PaV-LD suspension. Cultures without the PaV-LD suspension served as the controls. Growth of *P. agardhii* cultures was determined fluorometrically *in vivo* by measuring the chlorophyll content of host cells using a microplate reader (SpectraMax M2, Molecular Devices) on a daily basis. Fluorescence was determined with an excitation of 400 nm wavelength, emission of 800 nm and a cut-off filter of 695 nm. During the experiments, aliquots of the infected and non-infected host cultures were prepared for transmission electron microscopy.

To estimate the latent period and burst size of PaV-LD, a 1-step growth experiment was designed according to the method described by Yoshida et al. (2006). The new phage suspension was appropriately diluted with liquid BG-11 medium and inoculated into 100 ml of exponentially growing *Planktothrix agardhii* HAB0637 culture. The initial density of host cells was about 10^5 cells ml^{-1}, the titer of the PaV-LD was about 10^5 infectious units ml^{-1} and the multiplicity of infection was about 0.45. After inoculation, the density of host cells was periodically determined by direct counting under a microscopic, and the titer of the PaV-LD was estimated by the most-probable number technique (Saitoh et al. 2003). The incubation conditions were as described above.

Viral infectivity of survived filaments. To characterize *Planktothrix agardhii* cells that survived in the lysed culture after viral infection, the surviving filaments were collected and inoculated into fresh BG-11 medium. The sensitivity of regrowth cultures of *P. agardhii* to viral infectivity was tested by adding 20% (v/v) aliquots of fresh viral suspension to exponentially growing cultures. Cultures were monitored for evidence of lysis by optical microscopic observation.

Transmission electron microscopy. The infected or non-infected *Planktothrix agardhii* cultures were both withdrawn 48 h after inoculation and fixed with 2.5% glutaraldehyde. Fixed cells were harvested by centrifugation at 800 × *g* for 5 min at 4°C. The cell pellets

were post-fixed with 2% osmium tetroxide in sodium cacodylate buffer for 1 to 2 h, dehydrated in a graded acetone series, and embedded in LR White acrylic resin. Ultra-thin sections were post-stained with 2% uranyl acetate and 3% lead citrate and observed at 100 kV with a JEOL 1230 transmission electron microscope.

The PaV-LD suspension was absorbed onto carbon-coated copper grids by centrifuging at $100\,000 \times g$ for 1.5 h in a Beckman Optima L-90 K ultracentrifuge in a SW 41 swing-out rotor (Beckman). Grids were stained with a drop of 2% uranyl acetate for 10 min. The excess dye was removed with filter paper. After the grid was dried at room temperature, stained PaV-LD particles were observed at 80 kV with the transmission electron microscope. The diameter of cyanophages was estimated from the stained images.

Detection of physico-chemical characteristics. Heat stability of the PaV-LD was tested with treatments in water baths from 30 to 100°C in steps of 5°C for 15 min each. The treated samples were cooled on ice for 5 min and inoculated in duplicate into exponentially growing cultures of *Planktothrix agardhii*. A culture of *P. agardhii* infected with a non-treated virus lysate was used as a positive control and a non-infected culture of *P. agardhii* served as a negative control. To test the stability of the virus at low temperatures, aliquots of the PaV-LD suspension were stored at –20, –80 and –196°C (liquid nitrogen) in the dark with cryoprotectants. After 1 wk, the frozen samples were thawed at room temperature and added to exponentially growing cultures of *P. agardhii*. A non-frozen sample was used as positive control. The stability of the PaV-LD suspension to acidic or alkalic conditions was tested by exposure to pH levels ranging from pH 3 to pH 10 in 1 pH unit increments. After 1 h incubation at room temperature, the various pH treatments were adjusted to pH 8.0 with 0.1 N HCl or 0.1 N NaOH and were inoculated into fresh cultures of *P. agardhii*. Aliquots of media that were treated in the same way were used as controls. For determination of the stability of chloroform, 1 ml of the PaV-LD suspension was vigorously shaken with an equal volume of chloroform for 5 min. Chloroform was separated by centrifugation at $5300 \times g$ for 5 min, and the aqueous phase containing the PaV-LD was recovered and left for 6 h at room temperature to allow any remaining chloroform to evaporate. The same procedure was carried out with BG-11 media as a control. The chloroform-treated PaV-LD suspension, chloroform-treated media, non-treated PaV-LD suspension and non-treated BG-11 media were added to exponentially growing *P. agardhii* cultures. All cultures were incubated under the conditions described above.

RESULTS AND DISCUSSION

Planktothrix agardhii are planktonic filamentous cyanobacteria with abundant gas vacuoles, and grow by means of binary fission. The color of healthy *P. agardhii* cultures is blue-green. Trichomes are straight or slightly curved. The average length of each normal trichome is 215 µm (SD = 58 µm, n = 35) (Fig. 1A). Cell length is about 3.5 µm (SD = 0.5 µm, n = 35), cell width is about 3.3 µm (SD = 0.3 µm, n = 35) and the ratio of cell length to width is about 1.06:1.

In the first isolating process, within 8 d, lysis of host cells was detected in *Planktothrix agardhii* HAB0637 cultures inoculated with the filtered freshwater. The cultures' color visibly changed from normal healthy green to pale yellow due to the degradation or release of cell contents of the host organisms. The average length of the lysed filaments of hosts became approximately 35 µm (SD = 10 µm, n = 35) (Fig. 1B), which was much shorter than that of the normal filaments. Presumably, viruses simultaneously attacked some parts of a single filament in the early stages of viral infection and then the infected parts were lysed and gradually became discolored (Fig. 1C). Serial inoculation of the viral lysates into fresh exponentially growing *P. agardhii* HAB0637 cultures led to the same algicidal symptoms. The PaV-LD suspension filtered through a 0.2 µm pore-size filter still retained algicidal activity; however, cell lysis of the cultures occurred later compared to the non-treated pathogen suspension (data not shown). It is probable that the lag of algicidal activity is due to adsorption of the partial infectious agents onto filters.

The host range of the PaV-LD was tested on 10 cyanobacterial species (24 strains). The PaV-LD infected and lysed 4 strains of *Planktothrix agardhii* (HAB0637, HAB0638, HAB0642, HAB0643) isolated from Lake Donghu, but failed to cause lysis of any other cyanobacterial species or strains tested, including 5 strains of *P. agardhii* isolated from 4 different lakes throughout China (shown in Table 1). These results demonstrated that the host range specificity of the PaV-LD was correlated with the geographical locations of host isolation. To explain what determines the infection specificity of the PaV-LD against *P. agardhii*, both ecological and physiological studies should be performed by collecting more strains of the host species from different regions.

Optical microscopic observation and chlorophyll fluorescence results show growth of *Planktothrix agardhii* HAB0637 cultures in the absence and presence of the PaV-LD suspension. In the infected cultures, most of the host filaments settled to the bottom of the incubation flask and lost mobility, and then lysis of host cells occurred within 2 to 3 d after the inoculation.
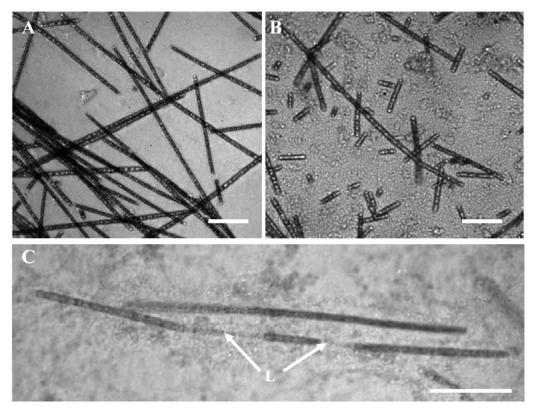
Fig. 1. *Planktothrix agardhii.* Optical microphotographs of (A) healthy cultures, (B) HAB0637 cultures 48 h after inoculation with cyanophage PaV-LD and (C) 2 parts of a single HAB0637 filament lysed (L). Scale bars = 50 µm

In contrast, the healthy cultures kept growing exponentially (Fig. 2). In the 1-step growth experiments, the growth parameters of PaV-LD were estimated. The cell density of *P. agardhii* HAB0637 began to decrease, and the titer of the PaV-LD to increase, 2 d after infection (Fig. 3), indicating that the PaV-LD phage has a latent period of 48 to 72 h. At 6 d after inoculation, the density of host cells decreased to 2.70×10^3 from 8.20×10^5 cells ml^{-1}, while the virus titer increased to $2.78 \times$

10^8 from 3.70×10^5 infectious units ml^{-1}. From the decline in host cell abundance and the concurrent increase in phage abundance, about 340 infectious particles were estimated to be released by each *P. agardhii* cell infected with the PaV-LD phage. Compared with the other cyanophages whose growth characteristics have been studied (Tucker & Pollard 2005, Yoshida et al. 2006), the latent period and average
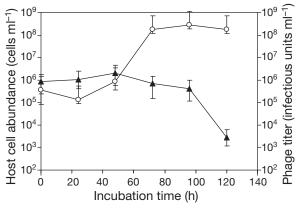

Fig. 2. *Planktothrix agardhii.* Algicidal curves of HAB0637 cultures as measured by chlorophyll fluorescence, showing the changes following inoculation with (■) and without (▲) the PaV-LD suspension. Values are means ± SD (n = 3)

Fig. 3. Growth curves of cyanophage PaV-LD. Changes in abundance of cyanophage PaV-LD (○) and the density of *Planktothrix agardhii* host cells (▲) in a 1-step growth experiment. The initial multiplicity of infection was about 0.45. The error bars indicate 95% confidence limits (○) or SD values (▲)

burst size of the PaV-LD are much longer and greater, respectively. This could be the result of the large size of the host cells. Here, our estimates were only approximations, and the latent period and burst size of this cyanophage may be associated with the growth and physiological status of host cells. Considering the initial mode of infection and host cell lysis, most *P. agardhii* cells did not escape the PaV-LD infection. However, a small number of filaments and short fragments were observed in the culture lysates, indicating that the lysis of the PaV-LD was not complete. These surviving host filaments recovered to an exponentially growing phase within several weeks when they were cultured in fresh BG-11 medium. The re-growth cultures were not lysed by an addition of the PaV-LD suspension. This could be explained by the resistance acquired by the host to the cyanophage (Waterbury & Valois 1993).

Transmission electron microscopy of ultra-thin sections of *Planktothrix agardhii* HAB0637 filaments infected with the PaV-LD suspension revealed that there had been propagation of viral particles inside the thylakoid space and the nucleiod (Fig. 4B). None of the virus-like particles were observed in the healthy filaments (Fig. 4A). Furthermore, numerous virus particles with similar size and morphology were observed in the culture lysates (Fig. 5). The viral particles were uniformly hexagonal or circular in cross section, indicating that the virus had an icosahedral symmetry and was tail-less, unlike most isolated cyanophages that have a contractile tail. The average diameter of viral particles ranged from 70 to 85 nm (mean ± SD = 76 ± 6 nm). Due to the atypical morphology of the non-tailed structure, these cyanophages may have some uncommon features (Bergh et al. 1989, McDaniel et al. 2006), such as nucleic acid gene content. For example, the non-tailed virus particle does not contain a readily identifiable *g20* gene encoding the viral capsid structure, which is a conservative phylogenetic marker for the cyanomyoviruses (Fuller et al. 1999, Zhong et al. 2002, McDaniel et al. 2006). Therefore, further studies are needed to characterize the PaV-LD phage at the gene or genomic level.

The significant results of our study indicated that (1) the algicidal factors could be transferred to a fresh host culture, (2) virus-like particles were observed in the lysed culture and (3) virus-like particles were not detected in healthy culture, which are common characteristics of other isolated cyanobacterial viruses. Therefore, the virus-like particles observed within infected cells and in the culture lysates were both morphologically and physiologically considered to be a cyanophage. This cyanophage was tentatively named PaV-LD (*Planktothrix agardhii* Virus isolated from Lake Donghu) after its host and the location from which it was isolated.
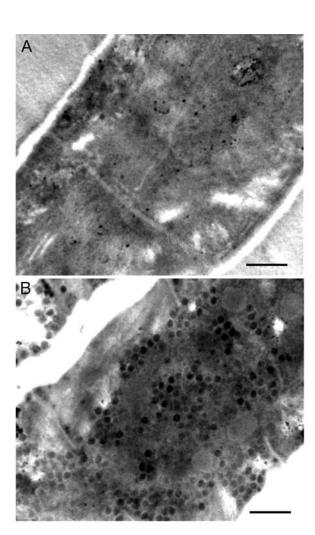

Fig. 4. *Planktothrix agardhii.* Transmission electron micrographs of ultra-thin sections of (A) a healthy filament of HAB0637 and (B) a HAB0637 filament 48 h after infection with PaV-LD. Scale bars = 500 nm
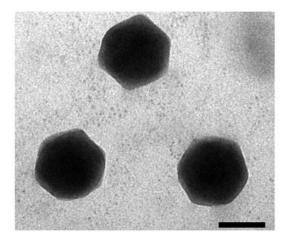

Fig. 5. Transmission electron micrograph of cyanophage PaV-LD particles. Scale bar = 50 nm

The PaV-LD was stable to freezing for 1 wk at −20, −80 and −196°C (liquid nitrogen) in the dark without cryoprotectants, suggesting that the virus can be stored for even longer times for future use. When subjected to temperatures over 50°C, the PaV-LD lost its ability to cause lysis of host cells, indicating that the PaV-LD is heat-labile. The virus was inactivated after exposure to acidic pH (≤4), and resistant to treatment with chloroform, which resulted in about a 3 d delay in lysis of host cells in the present experiment, suggesting lack of a lipid component in the structure of the virus (Fenner et al. 1974).

The filamentous cyanobacterial genus *Planktothrix* is one of the most important planktonic cyanbacteria that form intense blooms that occur periodically or persist all year round (Scheffer et al. 1997, Briand et al. 2002). They are global in distribution and contain a large number of species, including toxin-producing and nontoxin-producing species (Sivonen & Jones 1999). The successful isolation of the PaV-LD shows that viruses that infect and lyse the filamentous cyanobacterium *P. agardhii* are an active component of natural aquatic environments. Considering that the PaV-LD strain was isolated from natural freshwater where its host cyanobacterium *P. agardhii* HAB0637 was isolated, it is probable that there is a close interaction between PaV-LD and *P. agardhii* in the natural environment. Based on results from the present study, cyanophage PaV-LD may be used as a good microbiological agent for causing host mortality because it can be proliferated in the lab at a relatively low cost, specifically attack the target cyanobacterium *P. agardhii* and has no harmful effects on other organisms. If the growth characteristics of PaV-LD estimated in the 1-step growth experiment were used, the potential infectivity could contribute in controlling *P. agardhii* blooms. However, resistance of the host to the phage may be one of the limiting factors of using this infectious agent for cyanobacterial blooms in natural environments. Further studies will be performed to determine the mechanism of viral resistance and lysogeny. Although the host–virus system was recently isolated from natural water, the detailed information about the ecological effects of the PaV-LD is still unknown. More studies need be carried out to address the biological features of the PaV-LD, and its algicidal activity under different environmental conditions, such as light and nutrient levels.

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