Growth Characteristics of *Heterosigma akashiwo* Virus and Its Possible Use as a Microbiological Agent for Red Tide Control

KEIZO NAGASAKI, KENJI TARUTANI, AND MINEO YAMAGUCHI

Harmful Phytoplankton Section, Harmful Algal Bloom Division, National Research Institute of Fisheries and Environment of Inland Sea, 2-17-5 Marushi, Ohno, Saeki, Hiroshima 739-0452, Japan

Received 2 November 1998/Accepted 2 December 1998

The growth characteristics of *Heterosigma akashiwo* virus clone 01 (HaV01) were examined by performing a one-step growth experiment. The virus had a latent period of 30 to 33 h and a burst size of 7.7 × 10² lysis-causing units in an infected cell. Transmission electron microscopy showed that the virus particles formed on the peripheries of viroplasms, as observed in a natural *H. akashiwo* cell. Inoculation of HaV01 into a mixed algal culture containing four phytoplankton species, *H. akashiwo* H93616, *Chattonella antiqua* (a member of the family Raphidophyceae), *Heterocapsa triqueta* (a member of the family Dinophyceae), and *Ditylum brightwellii* (a member of the family Bacillariophyceae), resulted in selective growth inhibition of *H. akashiwo*. Inoculation of HaV01 and *H. akashiwo* H93616 into a natural seawater sample produced similar results. However, a natural *H. akashiwo* red tide sample did not exhibit any conspicuous sensitivity to HaV01, presumably because of the great diversity of the host species with respect to virus infection. The growth characteristics of the lytic virus infecting the noxious harmful algal bloom-causing algae were considered, and the possibility of using this virus as a microbiological agent against *H. akashiwo* red tides is discussed.

**MATERIALS AND METHODS**

Host strains and virus clones. Two strains of *H. akashiwo* were used in this study; *H. akashiwo* H93616 was isolated from the northern part of Hiroshima Bay (Hiroshima Prefecture) in June 1993, and *H. akashiwo* NM96 was isolated from Nomi Bay (Kochi Prefecture) in July 1996 (12). Neither algal culture contained bacteria. The algal strains were grown in modified SWM3 medium (3, 8) enriched with 2 nM Na₂SeO₃ at 20°C by using a cycle consisting of 14 h of cool darkness prior to each experiment.

*H. akashiwo* occurs in coastal waters of subarctic and temperate areas of both the Northern Hemisphere and the Southern Hemisphere. It often kills cultured fish, including salmon, yellowtail, and sea bream, and the damage to aquaculture has been increasing (4, 5). Cage-reared chinook salmon worth seventeen million New Zealand dollars were killed in Big Glory Bay, New Zealand, in 1989 (2). In Japan, cultured yellowtail, amberjack, striped jack, etc. worth 1.0 million and 237 million yen were killed in Kagoshima Bay in 1995 and in Nomi Bay in 1997, respectively (the amounts of damage are estimates made by the Fisheries Agency of Japan). Therefore, practical countermeasures against blooms caused by this noxious microalgae are urgently needed. In order to develop practical countermeasures for eliminating HAB, the algicidal activities of microorganisms have been examined, and algicidal bacteria and viruses have recently been isolated and studied (7).

Any microbiological agent used for elimination of HAB in the natural environment must fulfill the following three requirements: it must be practical in terms of scale, cost, and safety (10). Thus, scale and the specificity of algicidal activity must be considered prior to application of HaV. The objective of this study was to examine the growth characteristics of HaV and the algicidal activity of HaV in mixed algal cultures and in natural waters. Based on the results obtained, the possibility of using HaV as a microbiological agent against *H. akashiwo* is discussed.

Heterosigma akashiwo virus (HaV) is a relatively large DNA virus that infects *Heterosigma akashiwo* (a member of the family Raphidophyceae), which is one of the typical harmful algal bloom (HAB)-causing microalgae (11). The host specificity of HaV, the effects of physicochemical conditions on HaV algicidal activity, and storage techniques for HaV have been examined previously (11–14).

**RESULTS**

Algicidal effects of HaV01 in the mixed algal culture. Exponentially growing *H. akashiwo* H93616, *Chattonella antiqua* OC-B5 (a member of the family Raphidophyceae), *Heterocapsa triqueta* H9104 (a member of the family Dinophyceae), and *Ditylum brightwellii* (a member of the family Bacillariophyceae) cultures were mixed; the initial densities of the cultures used were 2.0 × 10¹, 5.2 × 10¹, 2.2 × 10², and 1.4 × 10³ cells/ml, respectively. HaV01 was inoculated into 50-ml portions of the mixed culture so that the initial HaV01 densities were 6.4 × 10¹ and 6.4 × 10² LCU/ml; viz, the MOI with respect to *H. akashiwo* cells was 3.2 and 0.032, respectively. As a control, an HaV01 suspension was incubated at 100°C for 5 min, cooled, and inoculated into a portion of the mixed culture at an initial density of 6.4 × 10¹ LCU/ml. The incubation conditions were the same as

* Corresponding author. Mailing address: Harmful Phytoplankton Section, Harmful Algal Bloom Division, National Research Institute of Fisheries and Environment of Inland Sea, 2-17-5 Marushi, Ohno, Saeki, Hiroshima 739-0452, Japan. Phone: 81-829-55-0666. Fax: 81-829-54-1216. E-mail: nagasaki@nrfaffrc.go.jp.
the densities of 3.3 and HaV01 were inoculated into 100 ml of this natural seawater at initial densities of 6.3 \times 10^3 cells/ml and 1.7 \times 10^5 LCU/ml, respectively; viz., the MOI with respect to H. akashiwo cells was 260. For the control experiments, we used a filtrate obtained by passing an H. akashiwo H93616 culture through a type GF/F filter and a HaV01 suspension that had been incubated at 100°C for 5 min. The effects of the algal culture filtrate and the heat-treated virus suspension on the growth of H. akashiwo H93616 were also examined. The incubation conditions and methods used to monitor algal growth were the same as those described above.

For the second experiment, surface water was collected in Kusatsu Fishing Port in northern Hiroshima Bay on 28 April 1998; diatoms were the dominant organisms in this water, and a HaV01 suspension that had been incubated at 100°C for 5 min. The effects of the virus-synthesizing globular zone, ca. 10^3 virus particles could have been present in a hypothetical globe. This value is comparable to the burst size (7.7 \times 10^5 LCU/ml) calculated by the extinction dilution method in the one-step growth experiment. HaV01 particles were presumably formed on the periphery of the viroplasm (Fig. 3B), as previously observed in H. akashiwo samples obtained from natural waters (15), suggesting that the viruslike particles observed in the previous field studies and the HaV01 particles are identical. HaV01 particles were also observed in the subsurface area, which presumably had been released from the viroplasm and had virus DNA inserted (Fig. 3A, arrowhead).

Algicidal activity of HaV01 in the mixed algal culture. Specific algicidal effects of HaV01 were observed even when C. antiqua, H. triquetra, and D. brightwellii were also present (Fig. 4). Although the rate of disappearance of H. akashiwo was affected by the MOI, H. akashiwo was specifically eliminated even with the lower MOI used in this experiment (0.03). In contrast, HaV01 had no conspicuous effect on the growth of the other three species of phytoplankton.

Algicidal activity of HaV01 in the natural seawater culture. The algicidal effects of HaV01 in natural seawater were examined twice. First, surface water was collected in Kure Port in northern Hiroshima Bay on 8 April 1998; the density of H. akashiwo in this water was 5 cells/ml. H. akashiwo H93616 and HaV01 were inoculated into 100 ml of this natural seawater at initial densities of 3.3 \times 10^3 cells/ml and 2.3 \times 10^5 LCU/ml, respectively; viz., the MOI with respect to H. akashiwo cells (including both natural cells and strain H93616 cells) were 0.70, 0.07, and 0.007, respectively. For the control experiment, we used a filtrate H93616 culture filterate and a heat-treated suspension of HaV01. The incubation conditions and monitoring methods used were the same as those described above.

RESULTS

One-step growth experiment. In the one-step growth experiment, H. akashiwo cells became roundish within 8 h after inoculation of HaV01 (Fig. 1). Almost all of the cells lost motility within 24 h. The H. akashiwo cell density decreased drastically and the culture became transparent 30 to 33 h after inoculation (Fig. 2). After lysis of most of the cells in the host culture at 33 to 47 h after inoculation, a small number of surviving cells were observed; these cells were roundish and had lost motility. At 47 h after inoculation, the host cell density had decreased to less than 10^3 cells/ml, while the density of HaV01 had increased to 9.8 \times 10^5 LCU/ml (Fig. 2). Considering the high initial MOI (2.04) and the synchronous lysis of the host cells, it is probable that most of the H. akashiwo cells did not escape HaV01 infection; that is, this experiment established that one-step growth of HaV01 occurred. The data show that 9.8 \times 10^5 HaV01 particles originating from 1.3 \times 10^5 virus-infected H. akashiwo cells were released into the culture, indicating that ca. 7.7 \times 10^7 infectious particles were produced by each H. akashiwo cell infected with HaV01. The latent period of HaV01 is considered to be 30 to 33 h.

At 24 h after virus inoculation, both mature and immature daughter virus particles were observed in most of the thin sections of H. akashiwo cells. A typical thin section of an infected cell contained ca. 170 sections of virus particles in a circular area with a diameter of 4 \mu m (Fig. 3A). Assuming that virus particles were distributed at the same concentration in a virus-synthesizing globular zone, ca. 10^3 virus particles could have been present in a hypothetical globe. This value is comparable to the burst size (7.7 \times 10^5 LCU/ml) calculated by the extinction dilution method in the one-step growth experiment. HaV01 particles were presumably formed on the periphery of the viroplasm (Fig. 3B), as previously observed in H. akashiwo samples obtained from natural waters (15), suggesting that the viruslike particles observed in the previous field studies and the HaV01 particles are identical. HaV01 particles were also observed in the subsurface area, which presumably had been released from the viroplasm and had virus DNA inserted (Fig. 3A, arrowhead).

Algicidal effects of HaV01 in the mixed algal culture. Specific algicidal effects of HaV01 were observed even when C. antiqua, H. triquetra, and D. brightwellii were also present (Fig. 4). Although the rate of disappearance of H. akashiwo was affected by the MOI, H. akashiwo was specifically eliminated even with the lower MOI used in this experiment (0.03). In contrast, HaV01 had no conspicuous effect on the growth of the other three species of phytoplankton.

Algicidal activity of HaV01 in the natural seawater culture. The algicidal effects of HaV01 in natural seawater are shown in Fig. 5 and 6. HaV01 specifically affected H. akashiwo H93616 in unsterilized natural seawater cultures containing numerous natural microorganisms (data not shown). In addition, HaV01...
had no obvious effect on the growth of diatoms even at an MOI of 260, which in a natural environment would correspond to an extraordinarily high HaV density (Fig. 5). *H. akashiwo* H93616 was specifically eliminated even when the MOI was as low as 0.007 (Fig. 6).

**DISCUSSION**

HaV01 has a latent period of 30 to 33 h and a burst size of $7.7 \times 10^2$ LCU per infected cell. Compared with the other microalgal viruses whose growth characteristics have been studied, the burst size and the latent period of HaV01 are greater and longer, respectively (Table 1). If these data are used, the potential infectivity can be provisionally calculated based on the growth characteristics of HaV01 estimated in the one-step growth experiment. If an HaV01 particle whose latent period and burst size are 33 h and $7.7 \times 10^2$ LCU/ml, respectively, is placed in 1 ml of seawater and constantly supplied with sensitive fresh host cells, ca. $6.0 \times 10^5$ infectious particles should be released into the 1 ml of seawater after two cycles of infection, which should take ca. 66 h. Considering that the highest density of *H. akashiwo* cells in natural red tide water is ca. $4 \times 10^5$ cells/ml (6), this virus density is high enough to potentially infect all of the cells. The highest yield of HaV01 that we have obtained in our laboratory is ca. $10^8$ infectious LCU/ml, which implies that 1 ha of shallow (mean depth, 10 m) coastal water should have an HaV density of ca. 1 particle/ml if 1 liter of the HaV suspension is used. In addition, low-cost small-scale production of HaV (in 2-liter flasks) is possible in a laboratory. Although the calculations described above are merely theoretical, use of HaV as a microbiological agent appears to be promising from the viewpoint of scale and cost. Of course, additional technical improvements would be required for large-scale production of HaV prior to practical...
use of HaV as a microbiological agent for elimination of red tide. The present experiments also showed that HaV01 specifically eliminates H. akashiwo H93616 when other phytoplankton species are present and in natural seawater containing numerous natural microorganisms, even at a low MOI. Thus, HaV01 specifically infects H. akashiwo even when other microorganisms are present in the ambient water. From the viewpoint of the safety of using HaV as a microbiological agent for elimination of red tide, HaV specifically affects the target alga, H. akashiwo, and appears to have little influence on other phytoplankton in the natural environment.

On the basis of the characteristics determined so far, HaV01 is a promising tool for controlling H. akashiwo red tides because (i) it has a high growth rate and thus can be applied to natural environments (i.e., it meets the scale requirement), (ii) it can be produced at a low cost, and (iii) it specifically attacks the target HAB-causing alga and has little or no effect on other organisms, guaranteeing its safety. In addition, HaV01 originates from natural coastal water and has not been genetically manipulated or altered in any way from its natural form (11).

A preliminary examination on the algicidal effects of HaV01 on a natural population of H. akashiwo red tide was performed by using water collected in northern Hiroshima Bay on 18 May 1998, in which H. akashiwo (8.0 × 10^3 cells/ml) and Eutreptiella spp. (8.2 × 10^3 cells/ml) dominated and diatoms were scarce. HaV01 was inoculated into the natural seawater sample at an MOI with respect to the natural H. akashiwo cells of 0.12. However, little specific growth inhibition of H. akashiwo was detected (data not shown). Although it has not been determined why the natural H. akashiwo population was not eliminated, it should be noted that natural populations of both sensitive and resistant cells occur in natural H. akashiwo red tide seawater samples (12). Therefore, the intraspecies specificity of HaV appears to be the most difficult obstacle to the use of this virus as a microbiological agent against H. akashiwo red tides. To solve this problem, what determines the infection specificity of HaV against H. akashiwo must be clarified. Another practical solution would be to prepare a cocktail of HaV clones, each with a different specificity of infection.

In conclusion, although HaV is a possible microbiological agent when scale, cost, and safety are considered, the effects of various HaV clones on natural populations of H. akashiwo must be assessed in more detail before this virus can be used for elimination of H. akashiwo red tides.

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