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Effect of temperature on the algicidal activity and the stability of HaV (*Heterosigma akashiwo* virus)

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ABSTRACT: The effect of temperature on the algicidal activity and stability of HaV (*Heterosigma akashiwo* virus), which infects the harmful bloom causing alga, *H. akashiwo* (Raphidophyceae), was determined by growing *H. akashiwo* culture inoculated with HaV under various conditions. Temperature and growth stage of the host culture are considered to be important factors determining the algicidal activity of HaV. The optimum temperature for the algicidal activity of HaV ranged from 20 to 25°C. Comparing the viral susceptibility of *H. akashiwo* strains and the algicidal activity of the HaV clones at different temperatures, both were suggested to be phenotypically diverse. Effect of temperature on the stability of HaV was also evaluated. HaV showed a relatively rapid decrease in infectious titer even when preserved at 5°C in the dark. The data is discussed in relation to the behavior of HaV in natural environments and the disintegration mechanism of *H. akashiwo* red tide.

KEY WORDS: HaV · *Heterosigma akashiwo* · Raphidophyceae · Harmful algal bloom · Red tide · Virus · Algicidal activity · Infectivity · Susceptibility · Temperature

INTRODUCTION

Intensive studies on initiation mechanisms of harmful algal blooms (HABs) have proved that the temperature, salinity, irradiation and nutrient conditions are the most important factors in their initiation process (Yamochi 1983, Yamaguchi & Honjo 1990, Yamaguchi et al. 1991, 1997, Yamaguchi 1994). In contrast, the disintegration mechanism of algal blooms has not yet been entirely clarified (Nagasaki et al. 1996). Recently, viral mortality has been highlighted as an important factor in the termination of algal blooms (Bratbak et al. 1993, Nagasaki et al. 1994a, b). Thus, to determine the disintegration mechanism of a red tide, it is necessary to clarify the relationship among the host alga, the virus and the dynamics of their ambient environment.

Especially in studies of plant viruses, temperature is considered to be a significant factor that influences the dynamics of the disease process in the host plant (Aozaki et al. 1989, Mansky et al. 1991, Syller 1991). For example, multiplication of the tobacco mosaic virus infecting tomato is severely reduced at high temperatures due to genetic control by the host for disease suppression (Fraser & Loughlin 1982). The effect of temperature on virus induced lysis in algae is largely unknown although it may be a key factor for understanding their behaviour and activity in natural waters. Temperature may in addition be an important factor for maintaining infectivity in stored virus stocks.

Heterosigma akashiwo virus (HaV) is a relatively large DNA virus infecting *H. akashiwo* (Raphidophyceae), which is one of the typical HAB-causing microalgae in the coastal waters of subarctic and temperate areas of both northern and southern hemispheres, and causes mortality of cultured fish such as salmon, yellowtail and sea breams (Larsen & Moestrup 1989, Hallegraeff 1991, Honjo 1993, Nagasaki & Yamaguchi 1997). HaV was examined for its primary characterization and host specificity, and proved to be highly specific to *H. akashiwo* (Nagasaki & Yamaguchi 1997, 1998). However, physicochemical data on its proliferation has scarcely been accumulated. The present paper details the effect of temperature on the algicidal activity and stability of HaV.

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MATERIALS AND METHODS

Organisms. Two strains of *Heterosigma akashiwo* were used in this study; one was *H. akashiwo* H93616 isolated from northern part of Hiroshima Bay (Hiroshima Prefecture) in June 1993, and the other was *H. akashiwo* NM96 isolated from Nomi Bay (Kochi Prefecture) in July 1996. Both strains were clonal and axenic. They were grown in modified SWM3 medium (Chen et al. 1969, Itoh & Imai 1987) enriched with 2 nM Na_2SeO_3 under a 14 h L:10 h D cycle of ca 45 µmol photons m^{-2} s^{-1} with cool white fluorescent illumination at 20°C prior to each experiment.

Two HaV clones isolated from seawater samples taken from a *Heterosigma akashiwo* red tide were used in this study; one was HaV01 from Unoshima Fishing Port (Fukuoka Prefecture) in 1996, and the other was HaV08 from Nomi Bay (Kochi Prefecture) in 1996 (Nagasaki & Yamaguchi 1997). Both virus clones contained no bacteria. Immediately prior to each experiment, both virus stocks were inoculated into a fresh culture of *H. akashiwo* NM96, incubated at 20°C for 3 d for multiplication, and the newly obtained viral suspension was used as an inoculum. Virus titer was estimated by means of the extinction dilution method (Nagasaki & Yamaguchi 1997) and MPN (most probable number) was calculated using the computer program developed by Nishihara et al. (1986). As the titration was carried out at the same time as inoculation in each experiment, which required 7 to 10 d before obtaining the titer, we could not equalize the multiplicity of infection (m.o.i.).

Sensitivity of *Heterosigma akashiwo* in relation to the growth phase. In order to design optimal experimental conditions to examine the algicidal activity of HaV against *H. akashiwo*, a preliminary experiment was designed to clarify the difference in sensitivity of *H. akashiwo* to HaV infection in relation to the growth phase of the host culture. Four ml of the culture of *H. akashiwo* H93616 and NM96 either in the late log phase or stationary phase was inoculated with HaV01 or HaV08, setting the m.o.i. at 0.005 and 0.008 to 0.009, respectively. Then the cultures were incubated under the conditions detailed above, and the algal growth was monitored using a fluorometer (Turner Designs). Each assay was run in triplicate.

Effect of temperature and light on viral infectivity. An experiment was designed to evaluate the effect of temperature and light on viral infectivity during storage. The titer of HaV01 suspension was monitored after storage at 5, 10, 15, 20 or 25°C, in the dark or in the light (14 h L:10 h D cycle of ca 45 µmol photons m^{-2} s^{-1} with cool white fluorescent illumination). The virus titer was estimated by means of the extinction dilution method (Nagasaki & Yamaguchi 1997) after 0, 18, 39 and 83 d of storage for each condition.

Effect of temperature on the algicidal activity of HaV. One hundred µl of a vigorously growing culture of each *Heterosigma akashiwo* strain was inoculated into 4 ml of fresh SWM3 and transferred to 4 different temperatures (15, 20, 25 and 30°C). After 3 d of incubation at each temperature for acclimation, aliquots of newly obtained virus suspensions were inoculated, giving final concentrations of HaV01 and HaV08 at 2.9×10^3 and 7.3×10^3 ml^{-1}, respectively. Because the growth rates varied due to different incubation temperatures, the initial m.o.i. ranged from 0.022 to 0.215 (see Fig. 3). Light conditions and algal growth monitoring were as given above. All experiments were triplicated. In each examination, growth of *H. akashiwo* without inoculation of HaV was also monitored in parallel as a control. Lysis of the host algal culture was regarded as caused by viral infection on the basis of visible symptoms (formation of greenish pellet) and the decrease of fluorescence (Nagasaki & Yamaguchi 1997). Here, it should be noted that what we detected in the present experiments was only the outcome of a virus/alga interaction, i.e. effect of virus inoculation on growth of the host alga. In the present paper, we used 'algicidal activity' as a term implying the lethal effect of an algal virus on the host alga involving both virus infectivity and host sensitivity.

RESULTS AND DISCUSSION

At 20°C, *Heterosigma akashiwo* NM96 was sensitive to both HaV01 (m.o.i. = 0.005) and HaV08 (m.o.i. = 0.008) in the late log phase. In contrast, in the stationary phase, it was susceptible to HaV08 (m.o.i. = 0.009) but resistant to HaV01 (m.o.i. = 0.005) (Fig. 1). This result indicates that *H. akashiwo* NM96 became resistant to HaV01 in the stationary phase. Therefore, it is suggested that the susceptibility of *H. akashiwo* NM96 to HaV01 is changeable with its physiological condition. Considering this result, virus inoculation against *H. akashiwo* was carried out in the late log phase in the following experiments. In addition, dissimilarity between HaV01 and HaV08 in terms of algicidal activity against *H. akashiwo* NM96 in the stationary phase is also notable, although further discussion is difficult here because of the different m.o.i. (Fig. 1).

In the storage experiment, the infectious titer of HaV01 after 18 d of storage under a light/dark cycle at all temperatures tested (5 to 25°C) was too low to be estimated by the extinction dilution method (results not shown). In contrast, the replicates preserved in the dark at the lower temperatures (5 or 10°C) had a detectable titer even after 39 d of storage (Fig. 2). These results indicate that the HaV01 infectivity decreases following exposure to light and temperatures above

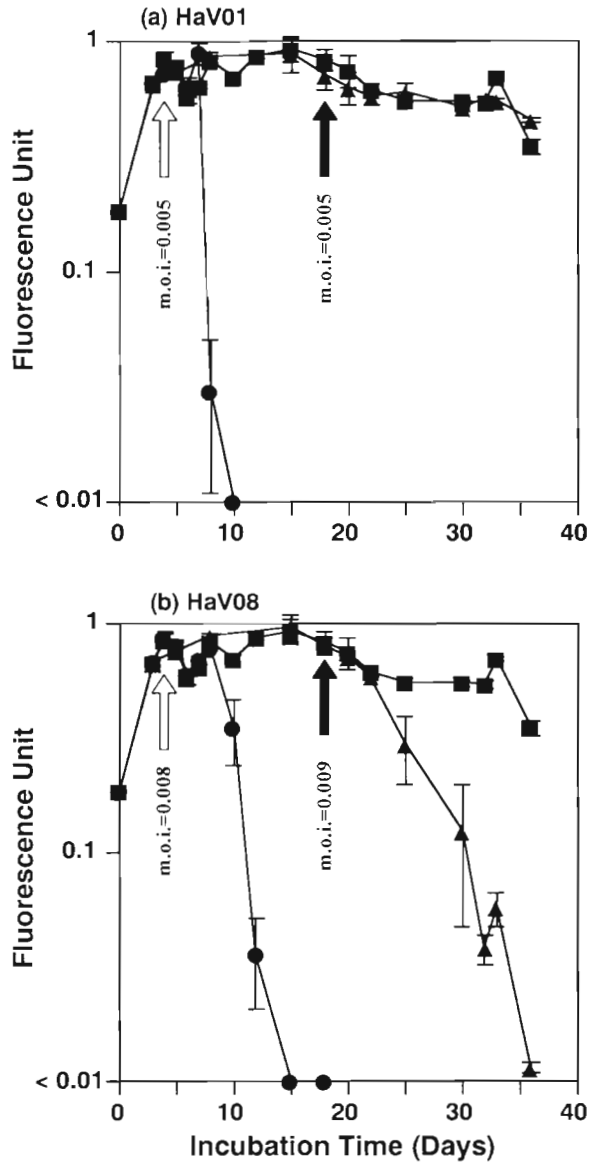

Fig. 1. Growth of *Heterosigma akashiwo* NM96 without viral inoculation (■) and with inoculation of (a) HaV01 or (b) HaV08 in the late log phase (●: white arrows) or in the stationary phase (▲: black arrows). Multiplicity of infection (m.o.i.) at which HaV01 or HaV08 was inoculated is shown under each arrow. Bars indicate standard deviation

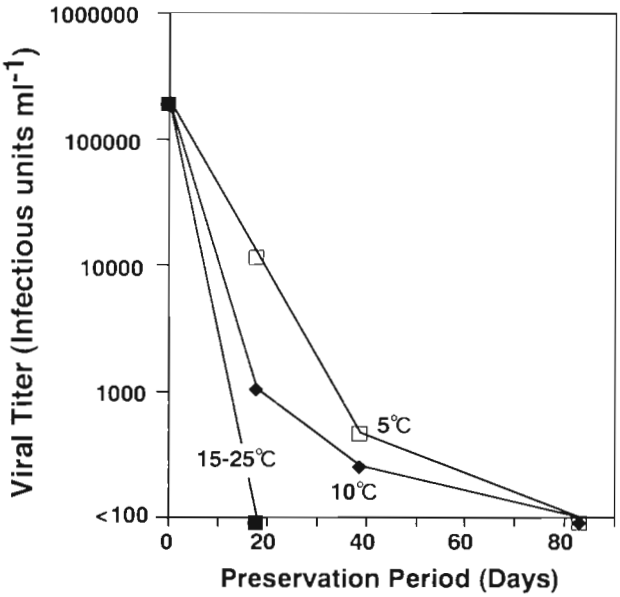

Fig. 2. Decay of HaV01 infectivity incubated in the dark at 5 (□), 10 (◆) and 15 to 25°C (■)

5°C; indeed, Cottrell & Suttle (1995) observed that *Micromonas pusilla* virus (MpV) showed considerable decay even when kept cold in the dark conditions. In contrast, PBCV-1, a virus which infects the *Chlorella*-like green alga, did not show a notable decrease in viral titer even after 1 yr of storage at 4°C (Van Etten et al. 1991). Compared with PBCV-1, HaV appears highly unstable in maintaining an infectious titer.

Growth of *Heterosigma akashiwo* H93616 and NM96 with or without inoculation of HaV01 and HaV08 at various temperatures is shown in Fig. 3. Although HaV01 was clearly lytic to both *H. akashiwo* H93616 and NM96 at 15°C, notably the algicidal activity against the latter was less acute. In contrast, no algal lysis was caused by HaV08 at 15°C (Fig. 3A, B). At 20 and 25°C, both virus clones caused complete lysis of both algal strains (Fig. 3C–F). At 30°C, *H. akashiwo* H93616 was lysed by both virus clones (Fig. 3G), but no lysis was observed in *H. akashiwo* NM96 (Fig. 3H). These results indicate that the algicidal activity of HaV is highly affected by temperature and the optimum temperature for infection and replication appears to be in the temperature range of 20 to 25°C. The mechanism by which temperature prevents algal lysis is still unknown. Indeed, in studies on terrestrial plant viruses, it is reported that the hosts' resistance against virus replication is changeable with temperature (Fraser & Loughlin 1982). However, further examination at a molecular biological level would be required in order to elucidate effects of temperature on both infectivity of HaV and sensitivity of *H. akashiwo* (Aozaki et al. 1989, Syller 1991).

Although HaV08 was inoculated at higher m.o.i. than HaV01 in the experiment at 15°C, HaV08 caused no algal lysis of both strains (Fig. 3A, B), showing phenotypic diversity between the 2 HaV clones in terms of algicidal activity under relatively low temperature condition (15°C). In addition, comparing the growth curves of *Heterosigma akashiwo* strains infected with HaV01 and HaV08 at 20 and 25°C, there is an apparent tendency that HaV01 requires a shorter period to complete algal lysis (Fig. 3C–F) although HaV01 was

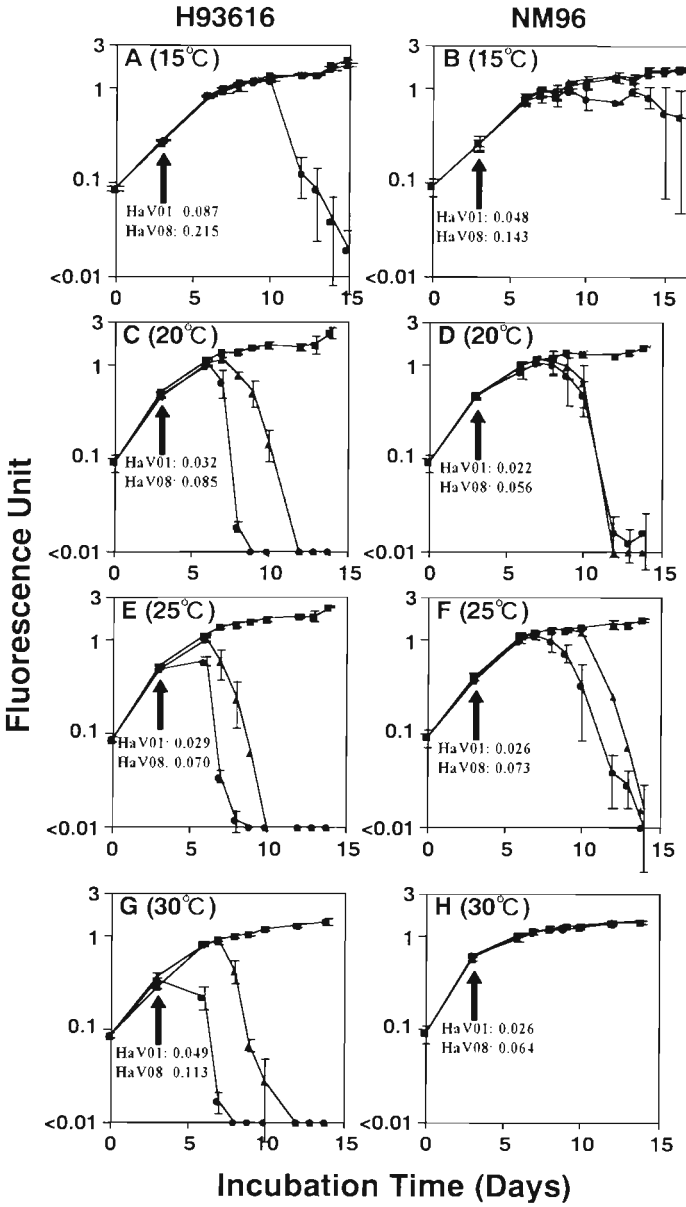

Fig. 3. Growth of *Heterosigma akashiwo* (A, C, E, G) H93616 and (B, D, F, H) NM96 at 15, 20, 25 and 30°C with inoculation of HaV01 (●) and HaV08 (▲) in the late log phase (arrows), and without virus inoculation (■). Multiplicity of infection (m.o.i.) at which HaV01 or HaV08 was inoculated is shown under each arrow. Bars indicate standard deviation

inoculated at a lower m.o.i. than HaV08. This result suggests a difference in the rate of viral replication between the HaV clones. As well, further discussion is indeed difficult due to the different m.o.i., and dissimilarity between *H. akashiwo* H93616 and NM96 in terms of sensitivity against HaV infection under relatively high temperature (30°C) is also noticeable (Fig. 3G, H). These results strengthen our speculation that there is a high diversity among both HaV clones and *H. akashiwo* strains as previously hypothesized on the basis of their intra-species host specificity spectra (Nagasaki & Yamaguchi 1998). Thus, considering the diversity of HaV in terms of infectivity in relation to temperature, it is possible that the effect of selective pressure by viral mortality against microalgal populations is variable depending on temperature conditions *in situ*.

Then, how does HaV behave in natural *Heterosigma akashiwo* blooms? On the basis of field data taken in northern Hiroshima Bay in 1993, the *H. akashiwo* red tide disintegrated when the surface and bottom seawater temperatures were 22 to 23 and 17 to 18°C, respectively. Considering that the optimum proliferation of HaV was observed at 20 and 25°C in the present experiment, there is a reasonable correspondence between the data obtained *in vitro* and *in situ*. This suggests that temperature may be one of the key factors which regulates HaV in nature, leading to mortality in the final stage of *H. akashiwo* red tides. Clarification of the relationships among the host alga, the virus and the other ambient conditions, i.e. salinity, irradiance, nutrient condition, etc., is clearly required. In studies of other algal viruses, some data on the effect of the physiological environment have been detailed. For the effect of irradiance, in light rather than dark conditions, MpV has been shown to lyse its host more rapidly, and the size at which cell rupture occurred in PBCV-1 increased (Waters & Chan 1982, Van Etten et al. 1983). Concerning the effect of nutrient conditions, phosphate concentration is suggested to have a considerable effect on production of viruses in blooms of the marine coccolithophorid *Emiliania huxleyi* (Bratbak et al. 1993, 1996).

Another enigma is how HaV survives, i.e. maintains its infectivity in the natural environment. Considering the instability of HaV01 based on the storage experiment (Fig. 2), it seems unlikely that they are able to remain infectious in the marine environment all the year round. The first possibility of HaV survival tactics is that it has other hosts or can keep latent in them. However, so far, no hosts except for *Heterosigma akashiwo* have been found for HaV. The second possibility is that latency of HaV occurs in *H. akashiwo* cells which can overwinter either as a vegetative cell or as a cyst in the sediments (Yamochi 1989, Imai et al. 1993, Itakura et al. 1996). A latent pathway of algal viruses

infecting marine brown algae is now under elucidation, and is one of the most topical parts of recent literature on marine virus ecology (Müller 1991, Müller et al. 1996). However, it still remains an undetermined link in the red tide disintegration mechanism (Nagasaki et al. 1994a, b). The third possibility is that HaV can remain infectious as a virus particle in the natural environment owing to some unknown preservative effect.

Indeed, since there have been insufficient data accumulated on HaV so far, the present study gives essential information for speculating on the behavior of HaV in the natural environment, for understanding the disintegration mechanism of *Heterosigma akashiwo* red tide, and for designing better countermeasures for controlling *H. akashiwo* red tide by use of HaV.

As well, the present results suggest that incubation temperature should be noted as an important factor both in estimating infectious titer by use of the extinction dilution method and in the procedure of isolating a lytic virus from the natural seawater sample, where viral multiplication is essential. Therefore, when the optimum temperature for viral growth is not known, it would be ideal to prepare several temperatures for incubation.

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