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Virus adsorption process determines virus susceptibility in *Heterosigma akashiwo* (Raphidophyceae)

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ABSTRACT: We compared the adsorption kinetics of *Heterosigma akashiwo* virus clones 01 (HaV01) and 53 (HaV53) to several other *H. akashiwo* strains with differing viral susceptibility spectra. When a HaV strain was inoculated into its suitable host strain, only infectious virus particles (3 to 4% of direct count estimates) adsorbed to the host cells, and the adsorption coefficient was estimated to be 4.02 to 5.44×10^{-8} ml min^{-1}; in contrast, virus adsorption to an unsuitable host strain was not detected. This suggests that virus adsorption is an important first step in determining the sensitivity or resistance of *H. akashiwo* strains to viral infection, and that it determines the strain-specific host specificity of HaV.

KEY WORDS: Algal virus · *Heterosigma akashiwo* · Host range · Intraspecies host specificity · Viral adsorption

INTRODUCTION

Heterosigma akashiwo virus (HaV) is a large (~0.2 µm), double-stranded DNA (dsDNA) virus infecting the harmful bloom-forming flagellate *Heterosigma akashiwo* (Hada) Hada (Nagasaki & Yamaguchi 1997, Nagasaki et al. 1999a). Although *H. akashiwo* is a single species belonging to the genus *Heterosigma* (Throndsen 1996), its susceptibility to HaV is diverse among clonal strains (Nagasaki & Yamaguchi 1998a, Nagasaki et al. 1999b, Tarutani et al. 2000, Tomaru et al. 2004b). Based on field surveys and cross-reactivity tests between the host clones and HaV clones, Tarutani et al. (2000) and Tomaru et al. (2004b) suggested that this strain-dependent infection may play an important role in determining the clonal composition and maintaining the intraspecies diversity of natural *H. akashiwo* populations.

Strain-specific host ranges have been found for other viruses infecting eukaryotic microalgae: *Chlorella* virus (CV; Reisser et al. 1988), *Micromonas pusilla* virus (MpV; Cottrell & Suttle 1991, Sahlsten 1998), *Heterosigma akashiwo* nuclear inclusion virus (HaNIV; Lawrence et al. 2001), *Heterocapsa circularisquama* virus (HcV; Nagasaki et al. 2003), *H. circularisquama* RNA virus (HcRNAV; Tomaru et al. 2004a), and *Rhizosolenia setigera* RNA virus (RsRNAV; Nagasaki et al. 2004). However, few studies have determined the mechanisms of intraspecies host specificity. Onimatsu et al. (2004) identified a surface protein from a large dsDNA virus (CV) that interacts with the host cell wall. Nagasaki et al. (2005a) reported that the intraspecies host specificity of a dinoflagellate-infecting single-stranded RNA virus (HcRNAV) may be determined by the nano-structures on the virus surface, which influence the binding to suitable *H. circularisquama* clones.

In the present study we compare the kinetics of viral adsorption to several *Heterosigma akashiwo* strains with different viral susceptibility spectra. Our principal aim is to verify the strain-specific host range of HaV during the viral adsorption process.

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MATERIALS AND METHODS

We used 2 HaV clones and 4 *Heterosigma akashiwo* strains. HaV01 was isolated from Unoshima Fishing Port (Fukuoka Prefecture, Japan), and HaV53 was isolated from Itsukaichi Fishing Port (northern Hiroshima Bay, Japan). Axenic strains of *H. akashiwo* (strains H93616, H94608, H98518-4 and H98527-6) were isolated from Itsukaichi Fishing Port. These were selected because they display differing viral susceptibilities to HaV01 and HaV53 (Table 1; Nagasaki & Yamaguchi 1998a, Tarutani et al. 2000, K. Tarutani et al. unpubl. data). Algal cultures were grown in modified SWM3 medium enriched with 2 nM Na_2SeO_3 (Chen et al. 1969, Itoh & Imai 1987, Imai et al. 1996), and incubated at 20°C using a 12:12 h light:dark cycle (light provided by cool white fluorescent illumination FL40S D EDL D65, Toshiba, at ~50 µmol photons m^{-2} s^{-1}).

Aliquots of HaV were inoculated into 25 ml exponentially growing cultures of *Heterosigma akashiwo* at a multiplicity of infection of ~0.1. This corresponds to a total virus to host ratio of ~3, because 3 to 4% of the HaV particles stainable with DAPI (4′-6-diamidino-2-phenylindole) were considered to be infectious (see 'Results and discussion'). As a control, an algal culture without viral inoculation was passed through a glass fiber filter (Whatman GF/F) and the same volume as the HaV suspension (25 ml) was inoculated. Each experiment was conducted in triplicate. Samples were taken immediately after gentle mixing, and then at 30 min intervals over a period of 120 min; they were then diluted 10-fold with SWM3 medium and centrifuged at 3000 rpm (800 × *g*), at 4°C for 3 min to remove algal cells with adsorbed viruses. An aliquot of the resulting supernatant was fixed with glutaraldehyde at a final concentration of 1%. The unadsorbed virus particles were directly counted using epifluorescence microscopy following the protocol of Weinbauer & Suttle (1997) with partial modifications. Briefly, DAPI solution — 15 µg ml^{-1} DAPI in Tris buffer (10 mM Tris-HCl, 10 mM EDTA-Na, 100 mM NaCl, 10 mM 2-mercaptoethylamine hydrochloride, pH 7.4; Hamada & Fujita 1983) — was added to each fixed sample (final concentration of DAPI = 1 µg ml^{-1}) and the samples were incubated in the dark for 30 min. The stained samples were collected on 0.02 µm Al_2O_3 filters (Anodisk 25, Whatman). The damp filters were mounted on a glass slide with a drop of low-fluorescence immersion oil and covered with a cover slip and another drop of the immersion oil. A minimum of 20 to 100 particles positively stained with DAPI were counted in 20 fields using an Olympus BX50 microscope equipped with a wide UV filter set; thus, the direct count estimates of HaV were calculated. The most probable number (MPN) of unadsorbed viruses was also enumerated using the extinction dilution method (Suttle 1993, Tarutani et al. 2000). Briefly, the remaining supernatant was diluted with SWM3 in a series of 2- to 10-fold dilutions, and aliquots of each dilution were added to 8 wells in 96-well microtiter plates containing exponentially growing *H. akashiwo* H93616 cultures. The cultures in microtiter plates were incubated under the conditions given above and checked daily for 10 to 14 d for cell lysis. The dilution of culture wells in which cell lysis occurred was scored, and the MPN of infectious virus particles was determined using the BASIC program (Hurley & Roscoe 1983). The adsorption coefficient (C_d; ml min^{-1}) was determined as follows (Cottrell & Suttle 1995):

$$C_d = \frac{\alpha - \alpha_c}{N}$$

where α (min^{-1}) is the slope, α_c (min^{-1}) is the slope determined by linear regression for the natural logarithm of the percentage of free viruses in control culture against sampling time, and N (cells ml^{-1}) is the host cell number.

Table 1. *Heterosigma akashiwo*. Viral susceptibility (+: lysed, –: not lysed) of 4 *H. akashiwo* strains to HaV01 and HaV53

Strain	HaV01	HaV53
H93616	+	+
H98518-4	+	–
H94608	–	+
H98527-6	–	–

RESULTS AND DISCUSSION

There was a pronounced difference in the number of HaV in the stock suspension enumerated by the 2 methods; direct count estimates by epifluorescence microscopy and MPNs by the extinction dilution method (MPN assay). The MPN of HaV01 was 1.2 ± 0.2 × 10^6 ml^{-1} (n = 5) and was ~3% of the direct count estimate at 3.8 ± 0.5 × 10^7 ml^{-1} (n = 5). The HaV53 stock suspension had an MPN estimate of 0.96 ± 0.15 × 10^6 ml^{-1} (n = 5), which was ~4% of the direct count estimate at 2.2 ± 0.4 × 10^7 ml^{-1} (n = 5). Similar differences have been reported for other algal viruses: MpV (Cottrell & Suttle 1995), *Phaeocystis pouchetii* virus (PpV; Bratbak et al. 1998), and HcV (Y. Tomaru et al. unpubl. data). The direct count method detects all of the virus particles with dsDNA (stainable with DAPI) whether they are infectious or not, whereas the MPN assay detects only 'infectious' virus particles. This indicates that only a small fraction of the HaV particles in the lysed cultures were infectious.

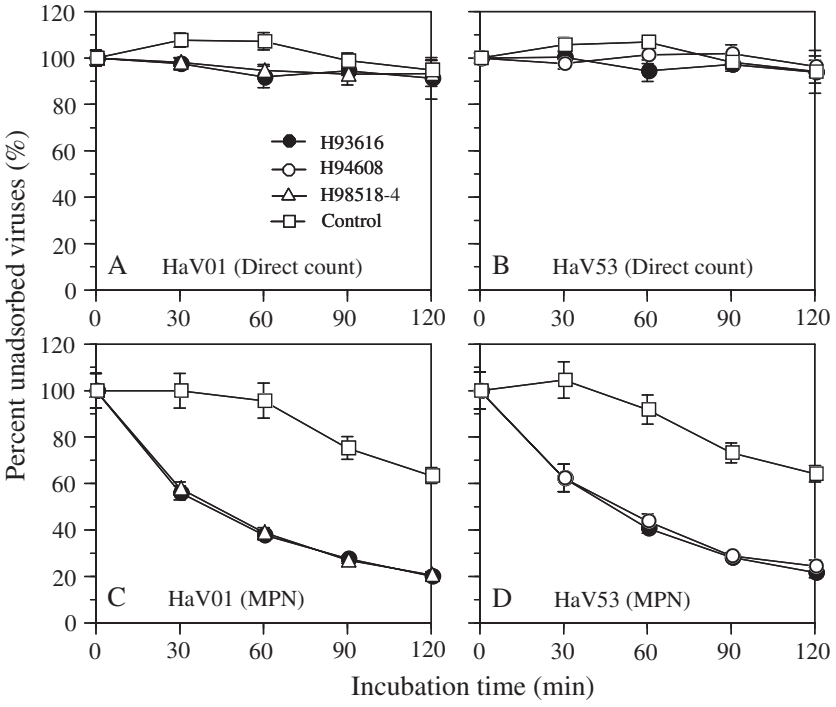

Fig. 1. *Heterosigma akashiwo*. Changes in the relative proportion of (A,C) HaV01 and (B,D) HaV53 particles that were unadsorbed to their suitable host strains as measured by (A,B) the direct count method or (C,D) the extinction dilution method. Vertical bars indicate standard deviation (n = 3). For the control experiments see 'Materials and methods'

When HaV01 was inoculated into a suitable host culture (H93616 or H98518-4; Table 1), the direct count estimates of free HaV01 particles were not significantly different from those of the control cultures (Fig. 1A). In contrast, the MPN of infectious virus particles showed a significant decrease during the experiments, and at 120 min post-inoculation the abundance was ~20% of the initial value (Fig. 1C). Similar results were obtained with HaV53 and its suitable host strains (H93616 and H94608; Table 1), i.e. no significant decline in the direct count estimates (Fig. 1B) but a noticeable decline in the MPN (Fig. 1D). As mentioned above, MPN of infectious HaV particles made up only a small proportion (3 to 4%) of the direct count estimates in these cultures. There may have been no remarkable decrease in the direct count estimates as the proportion of HaV particles adsorbed to *Heterosigma akashiwo* cells was too small to be reflected in the direct count estimates; indeed, the range of decrease in free virus numbers due to adsorption to host cells was less than the counting errors of direct count estimates (Fig. 1A,B). Based on these observations, we concluded that only the 'infectious' HaV particles were able to adsorb to their suitable host cells and cause infection.

One possible explanation for why only a small portion of HaV particles were infective is that a large proportion of virus particles had adsorbed to algal cell debris generated through the process of host cell degradation (Cottrell & Suttle 1995). This would inactivate newly generated viruses and inhibit their adsorption to new host cells. Another possibility is that a significant proportion of the virus particles released into the environment were immature, i.e. most HaV particles lacked a mature surface structure, and thus were not able to adsorb to the virus receptor of *Heterosigma akashiwo*.

The adsorption coefficients of HaV01 and HaV53 to the sensitive *Heterosigma akashiwo* strains calculated from the changes in the MPN estimate had a range of 4.0 to 5.4×10^{-8} ml min^{-1} (Table 2). These values are higher than those previously reported for other algal viruses, e.g. MpV (1.4×10^{-9} ml min^{-1}; Cottrell & Suttle 1995) and cyanophage S-PM2 (6.2 to 6.7×10^{-9} ml min^{-1}; Wilson et al. 1996). This is presumably due to the larger cell volume of *H. akashiwo* in comparison to the other host algal species.

The adsorption coefficient can be theoretically estimated using diffusive transport of the viruses to the host cells (Murray & Jackson 1992). Assuming the diameters of HaV and *Heterosigma akashiwo* are 0.2 and 15 µm, respectively, and the swimming velocity of *H. akashiwo* is 20 to 160 µm s^{-1} (Smayda 1998), the theoretical adsorption coefficient for *H. akashiwo* and HaV was estimated to be 3.3 to 6.7×10^{-8} ml min^{-1}. This agrees well with the value measured in the present study (4.0 to 5.4×10^{-8} ml min^{-1}). These results suggest that the adsorption kinetics of infectious HaV particles is subject to the diffusive transport theory.

When HaV01 was inoculated into its resistant *Heterosigma akashiwo* strain cultures (H94608 and H98527-6; Table 1), the free virus particles (direct count estimates) did not show any significant decrease in abundance (Fig. 2A). The MPN of infectious virus

Table 2. *Heterosigma akashiwo*. Adsorption coefficient ($\times 10^{-8}$ ml min^{-1}) of HaV01 and HaV53 to 4 *H. akashiwo* strains estimated by the most probable number (MPN) assay. Values show average ± SD (n = 3). –: no significant differences compared to the control culture

Strains	HaV01	HaV53
H93616	5.4 ± 0.3	5.4 ± 0.7
H98518-4	4.8 ± 0.2	–
H94608	–	4.0 ± 0.2
H98527-6	–	–

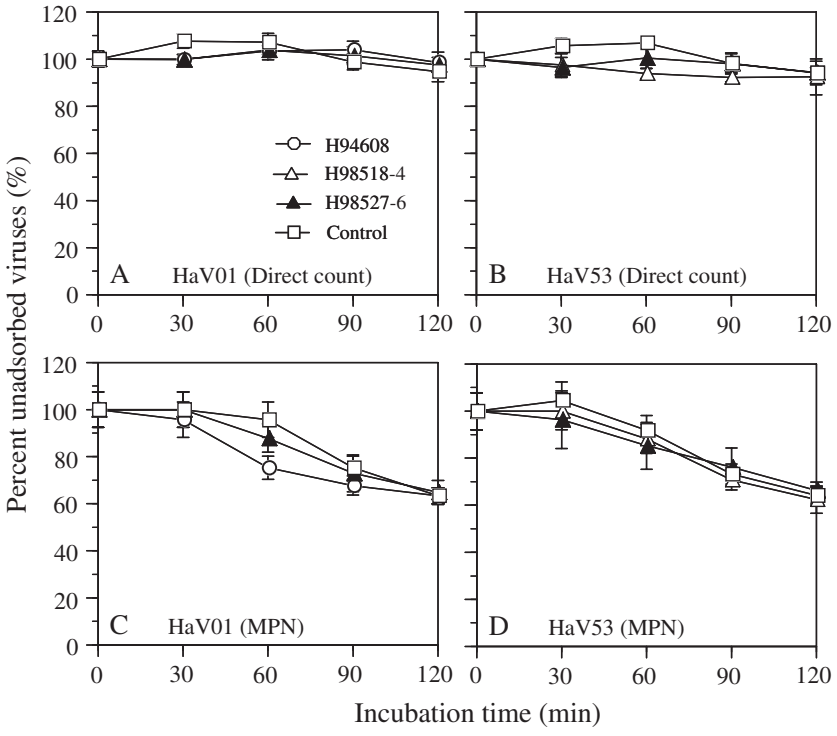

Fig. 2. *Heterosigma akashiwo*. Changes in the relative proportion of (A,C) HaV01 and (B,D) HaV53 particles that were unadsorbed to their unsuitable host strains measured by (A,B) the direct count method or (C,D) the extinction dilution method. Vertical bars indicate ±SD (n = 3). For the control experiments see 'Materials and methods'

particles decreased slightly during the experiments but the change was not significantly different from the control culture (Fig. 2C); this may reflect the gradual loss of HaV infectivity through the incubation under the condition mentioned above (see 'Materials and methods') (Nagasaki & Yamaguchi 1998). In addition, with HaV53 and its resistant host strains (H98518-4 and H98527-6; Table 1), the changes in the number of free viruses were not significantly different from those in the control cultures using either the direct count method or MPN assay (Fig. 2B,D). These results indicate that HaV adsorbed hardly at all to the resistant strains. Furthermore, viral adsorption may be the primary process in determining the sensitivity or resistance of *H. akashiwo* strains to viral infection, which would account for the strain-specific host range of HaV.

CONCLUSION

The data presented here suggest that the strain-specific host specificity of HaV is determined during the viral adsorption process. Development of resistance to viral infection is often related to lysogeny, especially in the case of bacteriophages (e.g. Bisen et al. 1986), which is one of the predominant tactics for bacteriophages to be multiplied in accordance with their hosts' propagation (Wilson & Mann 1997). However, lysogeny is unknown for viruses infecting eukaryotic marine algae except for *Ectocarpus siliculosus* virus-1 (EsV-1) infecting the brown alga *E. siliculosus* (Delaroque et al. 1999). The HaV-DNA polymerase gene fragment is highly conserved among HaV clones and was not detected in the genome of *Heterosigma akashiwo* strain H93616 using PCR experiments, even though it shows apparent resistance to a number of HaV strains (Nagasaki et al. 2005b, Tomaru et al. 2004b); this rules out the possibility of lysogeny as the cause of the virus resistance observed here. A more plausible explanation is that resistance develops at the level of the algal cell wall, as observed in bacteriophages infecting *Streptomyces erythreus* (Donadio et al. 1986). Some biochemical variation may exist on the cell surface among *H. akashiwo* ecotypes with distinct virus sensitivity spectra.

To understand the adsorption mechanism of HaV, it is essential to identify and characterize the communication between the hosts' virus receptor and the viral surface protein. In the case of CV, a viral-surface glycoprotein Vp260 with 13 internal tandem repeats of 61 to 65 amino acids is suggested to cause variations in nature of the viral surface (Que et al. 1994, Chuchird et al. 2002). A putative HaV gene encoding a protein with more than 20 internal tandem repeats of ~74 amino acids has been identified and is now under examination (Y. Shirai unpubl. data). This is of particular interest for determining whether these structures are involved in the host strain specificity of HaV.

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