Growth Characteristics and Intraspecies Host Specificity of a Large Virus Infecting the Dinoflagellate *Heterocapsa circularisquama*

Keizo Nagasaki,^{1*} Yuji Tomaru,² Kenji Tarutani,¹ Noriaki Katanozaka,³ Satoshi Yamanaka,³
Hiroshi Tanabe,³ and Mineo Yamaguchi¹

National Research Institute of Fisheries and Environment of Inland Sea¹ and Japan Society for the Promotion of Science, Ohno, Saeki, Hiroshima 739-0452,² and SDS Biotech K.K., Tsukuba, Ibaraki 300-2646,³ Japan

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The growth characteristics and intraspecies host specificity of *Heterocapsa circularisquama* virus (HcV), a large icosahedral virus specifically infecting the bivalve-killing dinoflagellate *H. circularisquama*, were examined. Exponentially growing host cells were more sensitive to HcV than those in the stationary phase, and host cells were more susceptible to HcV infection in the culture when a higher percent of the culture was replaced with fresh medium each day, suggesting an intimate relationship between virus sensitivity and the physiological condition of the host cells. HcV was infective over a wide range of temperatures, 15 to 30°C, and the latent period and burst size were estimated at 40 to 56 h and 1,800 to 2,440 infective particles, respectively. Transmission electron microscopy revealed that capsid formation began within 16 h postinfection, and mature virus particles appeared within 24 h postinfection at 20°C. Compared to *Heterosigma akashiwo* virus, HcV was more widely infectious to *H. circularisquama* strains that had been independently isolated in the western part of Japan, and only 5.3% of the host-virus combinations (53 host and 10 viral strains) showed resistance to viral infection. The present results are helpful in understanding the ecology of algal host-virus systems in nature.

Heterocapsa circularisquama virus (HcV) is a large double-stranded DNA virus infecting the noxious red tide-causing dinoflagellate *Heterocapsa circularisquama*, which kills bivalves (12, 17–19). HcV infects *H. circularisquama* and replicates in the cytoplasm. Based on the morphological features, genome type, and host range, HcV is considered to belong to the class *Phycodnaviridae* (36, 42). Although dinoflagellates are among the most important groups of phytoplankton, with various interesting properties (37), few data have accumulated on viruses infecting the major algal group. As far as we know, only two viruses infecting dinoflagellates have been isolated and cultured: one is HcV (36), and the other is HcSV (Y. Tomaru, K. Nagasaki, K. Tarutani, and M. Yamaguchi, Abstr. 3rd International Algal Virus Workshop, abstr. O-11, 2002), both of which are infectious to *H. circularisquama*.

The first interesting aspect of the *H. circularisquama*-HcV system is the ecological relationship. The abundance of virus-like particles in the sea was estimated to be 10^5 to 10^9 ml⁻¹, which was much higher than had been estimated before the 1990s (1, 2, 28, 43), and evidence showing the ecological importance of algal viruses has gradually accumulated (21, 33, 35). In the previous study on a large double-stranded DNA algal virus, HaV (22), and its host, *Heterosigma akashiwo*, which causes dense blooms in coastal environments (8, 10), it was shown that HaV has a considerable impact on the dynamics of blooms in the natural environment (20, 21, 35). Thus, the interrelationship between HcV and *H. circularisquama* is of interest from the viewpoint of bloom dynamics, especially the degradation of *H. circularisquama* blooms. Considering that many dinoflagellate species cause red tides, the host-virus sys-

tem is undoubtedly useful material with which to examine viral impact on dinoflagellate blooms.

The second aspect of interest is related to fisheries and environmental remediation research. Because *H. circularisquama* has caused heavy commercial damage to the aquaculture industry of bivalves such as short-necked clams, blue mussels, Pacific oysters, and pearl oysters in the western part of Japan (17–19), detailed investigations of the biology of *H. circularisquama* have been conducted. In the process, it was shown that *H. circularisquama* has several characteristics distinct from the other representative red tide-causing microalgae, such as members of the genus *Chattonella* and *H. akashiwo*: it can tolerate high temperature and salinity (44), it forms a temporary cyst that is tolerant to the cataclysm of ambient conditions (11, 40) or bacterial attack (26), and it can also attack other phytoflagellates through direct cell contact (39, 40). As HcV is a natural infective agent of *H. circularisquama*, it seemed meaningful to assess the possibility of its use as a microbiological agent for controlling blooms.

On the basis of these backgrounds, the objective of the present study was to examine the growth characteristics and intraspecies host specificity of HcV by elucidating the interaction between HcV and *H. circularisquama* through laboratory experiments.

MATERIALS AND METHODS

Hosts. As a typical host strain, *H. circularisquama* HU9433-P, isolated from Uranouchi Bay (Kochi Prefecture) in March 1994, was used in our experiments to determine the characteristics of HcV. HU9433-P is free from bacterial contamination, and harbors no intracellular bacteria (16). To examine the intraspecies host specificity of HcV, 53 strains of *H. circularisquama* were used, which were isolated from the following locations in western Japan: Ago Bay, Buzen-Kai, Gokasho Bay, Imari Bay, Maizuru Bay, Obama Bay, Uranouchi Bay, and Yatsushiro Kai. Of 13 strains isolated from Ago Bay, 7 were from the water column (HA93-5, HA946, and HcAG1 to HcAG5) and 6 were from the sediment (HAD98-101, HAD102, HAD103, HAD104, HAD106, and HAD108). Seven

* Corresponding author. Mailing address: 2-17-5 Maruishi, Ohno, Saeki, Hiroshima 739-0452, Japan. Phone: 81 829 55 0666. Fax: 81 829 54 1216. E-mail: nagasaki@affrc.go.jp.

strains were isolated from Buzen-Kai (HB1, HB5, HB7, HB9, HB11, HB15, and HB16), 1 was from Gokasho Bay (HG94-4), 2 were from Imari Bay (HI9427 and HI9429), 10 were from Maizuru Bay (MZ1 to MZ10), 10 were from Obama Bay (HO1, HO3, HO4, HO6, HO7, HO11, HO12, HO14, HO15, and HO18), 5 were from Uranouchi Bay (HU9430, HU9433-P, HU9640, HU9641, and HU9643), and 4 were from Yatsushiro Kai (HY9418 to HY9421), all of which were isolated from the water column. All strains were contaminated with extracellular bacteria except for HO4 and HU9433-P. Each *H. circularisquama* strain was grown in modified SWM3 medium (4, 13) enriched with 2 nM Na_2SeO_3 under a 14-h light–10-h dark cycle of ca. $90 \mu\text{mol of photons m}^{-2} \text{s}^{-1}$ with cool-white fluorescent illumination at 20°C.

Viruses. Ten clonal HcV strains used in the present study were free from bacterial contamination: five strains (HcV 01 to 05) were isolated from the surface water of Wakinoura Fishing Port in Fukuoka Prefecture, Japan, on 12 August 1999, and the others (HcV 06 to 10) were from the surface water of Fukura Bay, Hyogo Prefecture, Japan, on 19 August 1999. As a typical lytic virus strain, HcV 03 was principally used in the present experiments. The virus stock was inoculated into a fresh culture of *H. circularisquama* HU9433-P and incubated under the conditions given above, and the newly obtained viral suspension made cell-free by centrifugation (2,000 rpm for 10 min) was used as an inoculum in each experiment. Viral abundance was estimated by the extinction dilution method in our experiments (22, 32), and the most probable number was calculated (27). Thus, although virus abundance was measured immediately after inoculation in each experiment, the multiplicity of infection (MOI) in each inoculation was calculated 10 to 14 days postinoculation.

Virus sensitivity and growth conditions of host cultures. Preliminary experiments were designed to define the difference in sensitivity of *H. circularisquama* to HcV infection in relation to the growth phase of the host culture. Samples (3 ml) of *H. circularisquama* HU9433-P culture in the late log phase and stationary phase were inoculated with HcV 03 at an MOI of 29 and 4.9 infectious units cell^{-1} , respectively, which were sufficiently high to make most of the cells in the cultures simultaneously exposed to viral attack. These assays were carried out under the conditions given above. In parallel, the growth of *H. circularisquama* HU9433-P without viral inoculation was also monitored as a control. Algal growth was determined with a Turner Designs fluorometer (model 10-005R) equipped with a 436-nm excitation filter and >650-nm emission filter. The fluorescent unit indicates the relative biomass of the host alga. Each assay was run in triplicate.

Furthermore, a semicontinuous culture experiment was designed to verify whether the physiological conditions were related to the virus sensitivity of the host cells. *H. circularisquama* HU9433-P was inoculated into eight series of flasks and incubated for 3 days under the conditions described above, and 0, 33, 50, or 67% of the culture was replaced with fresh SWM3 every 24 h. After 6 days of semicontinuous dilution, a fresh HcV suspension with or without heat-treatment (100°C, 5 min) was added to each host culture to give an MOI of 0.49 infectious units cell^{-1} in all eight flasks. Thereafter, the flasks were incubated without semicontinuous dilution. Throughout the experiments, the abundance of host cells was monitored by direct counting under an optical microscope.

Effect of temperature on algicidal activity of HcV. One hundred microliters of a vigorously growing culture of *H. circularisquama* HU9433-P was inoculated into 3 ml of fresh SWM3 and transferred to four different temperatures (15, 20, 25, and 30°C). After 5 days of acclimation at each temperature, when they were in the exponential growth phase, an aliquot of the new virus suspension was inoculated to give an initial MOI of 73, 29, 24, and 21 infectious units cell^{-1} at 15, 20, 25, and 30°C, respectively. Light conditions were as given above, and host growth was monitored by the use of a Turner Designs fluorometer (model 10-005R). All experiments were run in triplicate. In parallel, the growth of *H. circularisquama* HU9433-P without viral inoculation was also monitored as a control.

One-step growth experiment. In order to estimate the latent period and the burst size of HcV, one-step growth experiments were designed. In the experiments at 20°C and 25°C, algal host cultures were inoculated with HcV 03 at an initial MOI of 64 and 198 infectious units cell^{-1} , respectively. Light conditions were as defined above. The abundances of host cells and total infectious centers (free viruses and infected cells) were monitored periodically by microscopic direct counting and the extinction dilution method, respectively (22, 32). On the basis of the changes in algal cell abundance and the viral abundance, the burst size and latent period were calculated.

During the experiments, aliquots of the algal culture at 20°C were periodically prepared for transmission electron microscopy by a previously reported method (9, 36). Thin sections were stained with uranyl acetate and lead citrate and observed under a JEOL JEM-1010 transmission electron microscope.

Intraspecific host specificity. The 53 *H. circularisquama* strains were independently inoculated with each of the 10 HcV strains. First, 0.6 ml of exponentially

FIG. 1. Algicidal effects of HcV03 on growth of *H. circularisquama* HU9433-P at 20°C. HcV strain 03 was inoculated at the exponential growth phase (on the third day) at an MOI of 29 (●) or stationary phase (on the 20th day) at an MOI of 4.9 (▲). As a control experiment, host growth without viral inoculation is also indicated (○). Bars indicate the standard deviation ($n = 3$).

growing host culture was inoculated with 0.2 ml of a fresh virus suspension diluted 24 times with SWM3 after the surviving cells had been excluded by centrifugation (7,000 rpm for 5 min) and incubated as described above. Lysis of the host algal culture was regarded as being caused by viral infection on the basis of visible characteristics (formation of a pale greenish pellet). Host-virus combinations with indistinct results were reexamined. The resultant data sets were converted to a Euclidean distance matrix and analyzed by unweighted pair-group method analysis of clustering in PHYLIP (Phylogeny Inference Package, version 3.5 [6]). A bootstrap analysis (100 replicates) was used to test the robustness and stability of the branching.

RESULTS AND DISCUSSION

Virus sensitivity and growth conditions of host cultures.

Although *H. circularisquama* strain HU9433-P was highly sensitive to HcV 03 in the late log phase, it became less susceptible in the stationary phase (Fig. 1). This result suggested that the susceptibility of *H. circularisquama* HU9433-P to HcV 03 varies with its physiological condition. Considering that DNA viruses utilize the biosynthetic function of hosts such as DNA synthesis and protein synthesis, it is probable that host cells in the vigorously growing phase are the most suitable for viral growth because of their high biosynthesis activity.

As reported previously, algal lysis was not complete in both experiments (36), and surviving cells were immobile and roundish, resembling temporary cysts (40). As previous experiments showed that the surviving cells were able to regrow in fresh medium and the recovered cells were sensitive to HcV (36), it was considered that survival was allowed by their physiological status, not by any acquired resistance. Because the MOI was sufficiently high (>4.9) that all of the host cells were exposed to viral attack in the experiment, there must be a mechanism for the surviving cells to resist viral infection. One possible explanation is that the viruses attached to the host cell surface, but since the host cells were not physically active, virus propagation was not effectively completed, as was observed in the case of the typical algal virus *Paramecium bursaria* *Chorella*

FIG. 2. Changes in abundance of *H. circularisquama* HU9433-P in semicontinuous culture experiments. Either 0% (A), 33% (B), 50% (C), or 67% (D) of the cultures was replaced with fresh SWM3 for 6 days before viral inoculation. Arrows indicate the time of viral inoculation (●). As a control, the abundance of the host without viral inoculation is also indicated (○). Bars indicate the standard deviation ($n = 3$).

virus type 1 (PBCV-1) (41), and the other is that the viruses did not attach to the host cells because of the structural change of the cell surface observed in those exposed to bacterial attack (26). In either case, it is presumed that the proportion of cells that were ready to change into temporary cysts increased in the stationary-phase culture.

In the semicontinuous culture experiment, the intermittent replacement of the host algal culture with fresh culture medium should have the following effects: (i) reducing host cell concentration, (ii) reducing the concentration of waste products excreted from host cells, and (iii) supplying the essential growth components in SWM3. On the basis of the data shown in Fig. 2, the relative growth of *H. circularisquama* was estimated by calculating (cell density 5 days after virus inoculation)/(cell density at virus inoculation). When the replacement percentage of the culture was high, the growth activity was high in the control cultures but the host cells were highly sensitive to viral infection, and vice versa (Fig. 3).

These results supported the speculation that sensitivity to HcV is affected by the physiological condition of the host cells. In contrast, in the case of the interaction between PpV and its host *Phaeocystis pouchetii*, the host was susceptible to viral infection in all stages of growth, although the host cells' growth conditions had a significant impact on burst size (3), showing a variety of characteristics among microalgal viruses.

Our speculation is that the physiological condition of *H. cir-*

cularisquama cells was presumably diverse rather than identical (flat) even in a clonal batch culture, and it was closely related with their ability to change into temporary cysts that were more resistant to viral attack. From the exponential growth phase through the stationary phase, it is likely that the

FIG. 3. Relative growth of *H. circularisquama* HU9433-P in semicontinuous culture experiments (see Fig. 2) estimated by calculating (cell density 5 days after virus inoculation)/(cell density at virus inoculation). Solid and open bars indicate experiments with and without viral inoculation, respectively.

FIG. 4. Algicidal effects of HcV 03 on growth of *H. circularisquama* HU9433-P at 15°C, 20°C, 25°C, and 30°C. Host growth with (●) and without (○) viral inoculation is shown in each graph. Bars indicate the standard deviation ($n = 3$).

proportion of host cells more changeable into temporary cysts increased to cause the low sensitivity to viral infection.

Effect of temperature on algicidal activity of HcV. The algicidal effect of HcV 03 occurred over a wide range of temperatures, 15 to 30°C, and algal lysis was remarkable at 25 to 30°C (Fig. 4). A negative effect of temperature on the algicidal activities of viruses was not as clear as the interaction between HaV and *H. akashiwo* (23).

H. circularisquama bloom outbreaks occur not only in summer but also in late autumn (13). Considering that HcV showed infectivity over a wide temperature range (Fig. 4), viral infection could be one of the notable factors regulating host dynamics throughout the year.

One-step growth experiment. The one-step growth experiments revealed the growth parameters of HcV. To calculate the burst size, the abundance of hosts and viruses of 48 h to 64 h and 32 h to 64 h was determined in the experiments at 20°C and 25°C, respectively. At 20°C, the latent period and burst size were estimated at 56 h and 1,800 infectious particles cell⁻¹, respectively (Fig. 5A). At 25°C, virus propagation was faster, and the burst size was higher; the latent period was 40 h, and the burst size was 2,440 infectious particles cell⁻¹ (Fig. 5B). These data agree with the idea that the more vigorously growing host cells at 25°C are preferable for viral growth because of their higher biosynthesis activity (44). The growth parameters calculated through the one-step growth experiments were similar to those previously estimated by transmission electron microscopy, 48 to 72 h and 1,300 infectious particles cell⁻¹ (36). The burst size of HcV was comparable to that of *Chrysochromulina ercina* virus (CeV) (30), and the latent period was somewhat longer than those of the other microalgal

viruses reported to date (5, 7, 14, 25, 31). Of course, it should be noted that these parameters are affected by the physiological condition of the host cells (3). Even though the burst size of HcV was relatively high among those of the large double-stranded DNA algal viruses, it was smaller than those of the small algal viruses such as *Heterosigma akashiwo* nuclear inclusion virus (HaNIV) (15) and HcSV (Y. Tomaru, K. Nagasaki,

FIG. 5. Changes in abundance of *H. circularisquama* HU9433-P (●) and HcV 03 (■) in one-step growth experiments at 20°C (A) and 25°C (B); the initial MOI was 64 and 198 infectious units cell⁻¹, respectively.

FIG. 6. Transmission electron micrographs of thin sections of *H. circularisquama* HU9433-P at 0 h (A), 8 h (B), 16 h (C), 24 h (D), and 32 h (E) postinfection with HcV 03. (F) Thin section of a surviving cell at 48 h postinfection. Bars: 500 nm (A to D); 2 μ m (E and F).

K. Tarutani, and M. Yamaguchi, Abstr. 3rd International Algal Virus Workshop, abstr. O-11, 2002).

Transmission electron microscopy revealed the process of how HcV replicated in the host cells (Fig. 6). At 8 h postinfection, aggregation of ribosomes to form a characteristic pre-viroplasm structure was noticeable, which was undetectable prior to viral inoculation (Fig. 6A and B). At 16 h postinfection, viral capsids and their components appeared, but mature virus particles were scarcely observed (Fig. 6C). Mature virus

particles appeared at 24 h postinfection, and the electron-lucent virus-producing areas (viroplasms) were clearly distinguished from the ambient cytoplasmic area (Fig. 6D). At 32 h postinfection, viroplasms enlarged to cover a large part of the host cell (Fig. 6E), and finally the infected cells burst.

Also in the one-step growth experiments, about 10% of the host cells survived after viral inoculation (Fig. 5). These data agree with the observations that 17 of the 171 cells in thin sections of the host cells at 40 h postinfection did not harbor

FIG. 7. Dendrogram showing levels of relatedness among the 10 HcV clones based on the algicidal spectra against 53 *H. circularisquama* strains by means of unweighted pair-group method analysis. Neighbor-joining analysis gave a similar tree (data not shown). The bootstrap values from 100 resamplings ($>50\%$) are shown above each branch in italics. The scale bar beneath the tree represents a Euclidean distance. Note that the levels of relatedness among the 10 virus strains are extremely high, although they appear divided into two clusters in the tree.

virus particles. Some of the cells lacking virus particles were indistinguishable from uninfected host cells, and some harbored numerous granules, presumably containing polysaccharide materials (Fig. 6F). Although one possible explanation for the mechanism of how the algal cells avoid infection is a change(s) in the envelope structure to form a thick matrix layer, the surviving cells lacked thick-layered envelopes, as was observed in the cells exposed to bacterial attack (26), suggesting that another mechanism enabled immunity to the viral attack.

Intraspecies host specificity. Viral lysis occurred in 502 of the 530 (53 host strains and 10 virus strains) combinations between virus strains and host strains tested (502 of 530 = 94.7%). Among the 53 *H. circularisquama* strains examined for viral sensitivity, 46 were lysed by all 10 HcV strains. In contrast, HB9, HO4, HcAG-1, HcAG-2, HcAG-3, HcAG-4, and HcAG-5 were resistant to some of them, but there was no host strain that showed complete resistance to all 10 HcV strains tested. It was most notable that the five strains from Ago Bay (HcAG-1 to HcAG-5) showed relatively high resistance to viral infection

and that the sensitivities to HcV strains 01, 04, 07, and 09 were complementary to those of HcV strains 02, 03, 05, 08, and 10, i.e., the HcAG strains that were sensitive to the former group were resistant to the latter, and vice versa.

Although no obvious relationship between host specificity and the locality of virus strains was found with respect to these results, it was notable that the virus sensitivity spectrum of *H. circularisquama* HB9 reflected the locality of viruses; it was sensitive to HcV strains 01 to 05 but resistant to HcV strains 06 to 10. The occurrence of resistant combinations between HcV and *H. circularisquama* was as low as 5.3% (28 of 530), which was about one-fifth of that observed between HaV and *H. akashiwo* (~28%) reported previously (24). This was also certified by analyzing the algicidal activity spectra of the 10 HcV strains by means of unweighted pair-group method analysis, which also proved the low diversity among them (Fig. 7).

Intraspecies host specificity of algal viruses is important from the aspect of their roles and functions in the aquatic environment. The host ranges of *Micromonas pusilla* virus (29), HaV (24), HaNIV (15), *Heterosigma akashiwo* RNA virus (34) and *Emiliana huxleyi* virus (31) are complex, and lysis by individual viral isolates was restricted to specific host strains. Because of the strain specificity, the importance of viruses in maintaining intraspecies diversity in algal populations is highlighted. Although an apparent intraspecies diversity in *H. circularisquama* was found with regard to the infection specificity of HcSV (Tomaru et al., submitted for publication), results of the present cross-assay showed that the HcV was more widely infectious to *H. circularisquama* strains than HcSV.

Future view. Although viruses have recently been considered an important component in aquatic ecosystems (33, 38, 43), it has not yet been sufficiently clarified how HcV regulates *H. circularisquama* populations and how it affects the disintegration of its blooms. Considering that the HcV strains were isolated from natural seawater where *H. circularisquama* dominated, it is probable that there is a close interaction between HcV and *H. circularisquama* in the natural environment. Based on the present study, HcV was shown to be highly effective in infecting vigorously growing host cells at a wide range of temperatures and to have a comparatively high growth activity and a relatively wide host strain range for *H. circularisquama*. This fundamental information on HcV will be helpful in understanding the ecology of the host-virus system in future studies and also in measuring the possibility of its use as a tool for controlling *H. circularisquama* blooms.

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