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# Isolation of a virus infectious to the harmful bloom causing microalga *Heterosigma akashiwo* (Raphidophyceae)

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ABSTRACT: A virus infecting the harmful bloom causing microalga *Heterosigma akashiwo* (Raphidophyceae) was isolated from the coastal water of Nomi Bay, Japan, in July 1996. The isolate caused lysis in 2 strains of *H. akashiwo* tested and numerous virus-like particles (VLPs) appeared in the lysed algal culture, whereas virus multiplication was not detected in the healthy culture of *H. akashiwo* without its inoculation. Thus, fulfilling Koch's postulate, it was considered to be a virus and designated HaV (*Heterosigma akashiwo* virus) clone GSNOU-30. The virus particle is icosahedral, lacking a tail, and 202 ± 6 nm (average ± standard deviation) in diameter with an electron-dense roundish core that is distinct from the capsid. The virus stained positive with DAPI, indicating that it possesses a double stranded DNA genome. The virus proliferated in the protoplasm of the host cell as had previously been observed in *H. akashiwo* cells from a natural red tide population. The virus did not cause lysis of *Chattonella antiqua*, *C. verruculosa* or *Fibrocapsa japonica* (Raphidophyceae) as well as 15 strains of phytoplankton belonging to other classes. It is most noteworthy that 3 strains of *H. akashiwo* isolated from Hiroshima Bay, Japan, were resistant to GSNOU-30, suggesting that the viral infectivity is not species-specific but strain-specific. These results suggest that the virus is involved in the population dynamics of *H. akashiwo*, playing a role as a selector to increase genetic diversity of a host species.

KEY WORDS: Red tide · Harmful algal bloom · Lytic virus · *Heterosigma akashiwo* · Raphidophyceae · Host specificity · HaV

## INTRODUCTION

*Heterosigma akashiwo* (Raphidophyceae) is a harmful bloom causing flagellate, which causes mortality of cultured fish such as salmon and yellowtail. Red tides of *H. akashiwo* have been recorded in coastal waters of subarctic and temperate areas of both the Northern and the Southern Hemisphere (Pratt 1959, Larsen & Moestrup 1989, Park et al. 1989, Hallegraeff 1991, Honjo 1993). So far, the physiology and the ecology of *H. akashiwo* have been considerably studied and an elucidation of the mechanism to initiate a *H. akashiwo* red tide has been achieved (Yamochi 1983, 1984, 1989, Honjo 1993). In contrast, the disintegration mechanisms of *H. akashiwo* red tides have only been superficially studied. Several characteristics in the termination process of *H. akashiwo* red tides, e.g. cyst formation, cessation of vertical migration and changes in the growth potential for individual cells, have been reported (Itakura et al. 1996, Nagasaki et al. 1996), but the disintegration mechanisms of red tides have been insufficiently explained up to the present.

VLPs (virus-like particles) have been observed in more than 50 species in at least 12 of the 14 recognized classes of eucaryotic algae (Reisser 1995), including *Heterosigma akashiwo* in a natural red tide population. The proportion of *H. akashiwo* cells harboring VLPs rose specifically in the final stage of red tides. In thin sections, VLPs were 165 to 180 nm in diameter, icosahedral, with an electron-dense roundish core distinct from the capsid, and lacking a tail (Nagasaki et al. 1994a, b). Although, on the basis of these observations, viral mortality has been suggested to be an important factor in the dis-

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integration process of *H. akashiwo* red tides, no evidence for infectivity of the VLP has been obtained.

In recent years, 5 viruses which are infectious to eucaryotic microalgae in the marine environment, *Micromonas pusilla* (Prasinophyceae), *Emiliania huxleyi* (Prymnesiophyceae), *Aureococcus anophagefferens* (Chrysophyceae), *Chrysochromulina* spp. (Prymnesiophyceae) and *Phaeocystis pouchetii* (Prymnesiophyceae) have been isolated, 3 of them originating from natural seawaters where the host species had bloomed (Cottrell & Suttle 1991, Milligan & Cosper 1994, Suttle & Chan 1995, Bratbak et al. 1996, Jacobsen et al. 1996). Lytic activities of the viruses suggest they can be involved in regulating the bloom dynamics of the host species, whereas their tangible role in aquatic ecosystems still remains enigmatic. In the present paper, we report the first data on isolation, lytic activity and host specificity of a virus infecting *H. akashiwo*. To our knowledge, this is the first report on a virus infecting a Raphidophyte.

## MATERIALS AND METHODS

**Algal cultures.** The strains of *Heterosigma akashiwo* and the other microalgae used in this study are listed in Table 1. All of them are clonal, established by the micropipetting method or an extinction dilution method. *H. akashiwo* strains were grown at 20°C and the other algal strains were grown at 15 or 20°C in modified SWM3 medium (Chen et al. 1969, Itoh & Imai 1987) enriched with 2 nM $Na_2SeO_3$ under a 14:10 h light:dark cycle of ca 45 µmol photons $m^{-2}$ $s^{-1}$ with cool white fluorescent illumination.

**Isolation of lytic viruses.** A seawater sample collected in Nomi Bay, Kochi Prefecture, Japan, on 11 July 1996, containing 35400 cells $ml^{-1}$ of *Heterosigma akashiwo*, was initially kept at 4°C and sent to the laboratory within 24 h. Treatment in the laboratory was modified from the method of Bratbak et al. (1996) and Jacobsen et al. (1996); five 50 ml aliquots of the natural seawater were placed in petri dishes, exposed to UV radiation (254 nm wavelength, Toshiba GL15) for 0, 30, 60, 90 and 120 s and incubated for 2 d at 20°C as mentioned above. Then, each sample was filtered through a 0.2 µm Nuclepore membrane filter. 200 µl of each filtrate was inoculated into a 5 ml culture of *H. akashiwo* GS95, which was incubated under the conditions mentioned above. The cultures were checked by light microscopy every day to examine whether cell lysis occurred or not.

Algicidal factors were isolated by the 2-times extinction dilution method (Suttle & Chan 1993). 10-fold dilutions of the supernatant of each lysate were made into

Table 1. Susceptibility of algal strains against HaV GSNOU-30 infection. +: lysed by HaV GSNOU-30; –: not lysed

| | Species | Isolation: Locality | Isolation: Date | Susceptibility |
|---|---|---|---|---|
| Raphidophyceae | *Heterosigma akashiwo* GS95 | Gakasho Bay | May 1995 | + |
| | *Heterosigma akashiwo* UR94 | Uranouchi Bay | May 1995 | + |
| | *Heterosigma akashiwo* H94222 | Hiroshima Bay | Feb 1994 | – |
| | *Heterosigma akashiwo* H94608 | Hiroshima Bay | Jun 1994 | – |
| | *Heterosigma akashiwo* H95623 | Hiroshima Bay | Jun 1995 | – |
| | *Fibrocapsa japonica*-1[a] | Harima Nada[b] | Oct 1985 | – |
| | *Chattonella antiqua* OC-B5 | Osaka Bay | Sep 1985 | – |
| | *Chattonella verruculosa* M | Hiroshima Bay | May 1993 | – |
| Dinophyceae | *Heterocapsa triquetra* H9104 | Hiroshima Bay | Apr 1991 | – |
| | *Heterocapsa circularisquama* HA92-1 | Ago Bay | Dec 1992 | – |
| | *Gymnodinium mikimotoi* G303-ax2 | Suo Nada | Jul 1985 | – |
| | *Prorocentrum triestinum* H9109[a] | Hiroshima Bay | Sep 1991 | – |
| | *Prorocentrum dentatum* H9608[a] | Hiroshima Bay | Aug 1996 | – |
| | *Alexandrium tamarense*-1[a] | Hiroshima Bay[b] | Nov 1995 | – |
| Prymnesiophyceae | *Isochrysis galbana* | Unknown | Unknown | – |
| | *Pavlova lutheri* | Unknown | Unknown | – |
| Cryptophyceae | *Rhodomonas ovalis* | Coast of Fukuyama | Jun 1967 | – |
| Bacillariophyceae | *Skeletonema costatum* SK-1 | Hiroshima Bay | Mar 1989 | – |
| | *Thalassiosira* sp. Th-2 | Hiroshima Bay | Feb 1989 | – |
| | *Chaetoceros didymus* Ch-4 | Hiroshima Bay | Mar 1989 | – |
| | *Ditylum brightwellii* | Hiroshima Bay | Mar 1989 | – |
| Prasinophyceae | *Pyramimonas* sp.[a] | Hiroshima Bay | Jun 1994 | – |
| Chlorophyceae | *Oltomannsielopsis viridis*[a] | Osaka Bay | Oct 1993 | – |

[a]Not axenic; [b]isolated from incubation of a sediment

successive 150 µl drops of *Heterosigma akashiwo* GS95 cell suspension with 8 replicates at each dilution. Supernatant of the algal lysate in the highest dilution of the first assay was carried over to the second extinction dilution assay. Finally, supernatant of the lysate in the highest dilution of the second assay was inoculated to a 50 ml fresh culture of *H. akashiwo* GS95. The resultant lysate was combined with 0.2% of sodium azide, stored at 4°C in the dark, and tentatively designated as 'an original viral suspension' in the present study. All incubation was carried out under the conditions mentioned above. In parallel, on the basis of the resultant numbers of wells with evidence of lysis in each dilution, the concentration of algicidal factors was calculated as MPN (most probable number) in each assay, using the computer program developed by Nishihara et al. (1986).

Virus particles in the lysates were trapped onto a 0.02 µm pore size filter (Anodisc 25, Whatman International Ltd), stained with DAPI (4',6-diamidino-2-phenylindole) and observed under UV excitation. Virus particles negatively stained with uranyl acetate were also observed by transmission electron microscopy.

**Host range.** The host range of the isolated virus was tested by adding 50 µl of the original viral suspension: (1) without any treatment, (2) filtered through a 0.2 µm pore size filter (DISMIC-25, Advantec), (3) filtered through a 0.1 µm pore size filter (Anotop™25, Anotec) or (4) treated at 100°C for 5 min, to 1 ml cultures of the exponentially growing algal strains listed in Table 1. The cultures were observed by light microscopy. Cultures that were not lysed after 10 d were considered to be unsuitable hosts for the virus.

**Viral effect on *Heterosigma akashiwo* cultures.** 50 µl aliquots of the original viral stock suspension were treated as in (1) to (4) above and inoculated into triplicate cultures (4 ml) of *H. akashiwo* GS95 and *H. akashiwo* UR94 (Table 1) in the exponentially growing phase. Growth of *H. akashiwo* was monitored using a fluorometer (Turner Designs). After lysis of algal cells, an aliquot of each culture was prepared for transmission electron microscopy according to the method of Hara & Chihara (1982).

## RESULTS AND DISCUSSION

In the first screening process, cell lysis was detected in the *Heterosigma akashiwo* GS95 cultures inoculated with the natural seawater exposed to UV light for 0, 30 and 60 s, but not 90 or 120 s. Supernatants of the lysed cultures were carried over to the first extinction dilution assay. The resultant algal lysates contained $1.90 \times 10^6$ to $5.10 \times 10^6$ ml$^{-1}$ of algicidal factors. Each lysate in the highest dilution of the first assay was again carried over to the second extinction dilution assay to be isolated; the probability that more than 1 algicidal factor occurred in the final lysates is <0.0106.

The resultant algal lysates contained numerous particles stainable with DAPI. On the other hand, the particles were not observed in the healthy culture of *Heterosigma akashiwo*. Thus, the particles possessing double stranded DNA (dsDNA) appear capable of proliferating in *H. akashiwo* cultures. Then, they were tentatively designated GSNOU-0, GSNOU-30 and GSNOU-60 according to the UV irradiation time, respectively, and GSNOU-30 was further examined in the present study.

Cell lysis was caused in *Heterosigma akashiwo* GS95 and UR94 strains inoculated with the original stock suspension of GSNOU-30 without treatment or with filtration through a 0.2 µm filter. In contrast, this algicidal activity was lost by either filtration through a 0.1 µm filter or heat treatment, indicating that the algicidal factor is sized presumably between 100 and 200 nm and is heat-labile (Fig. 1).

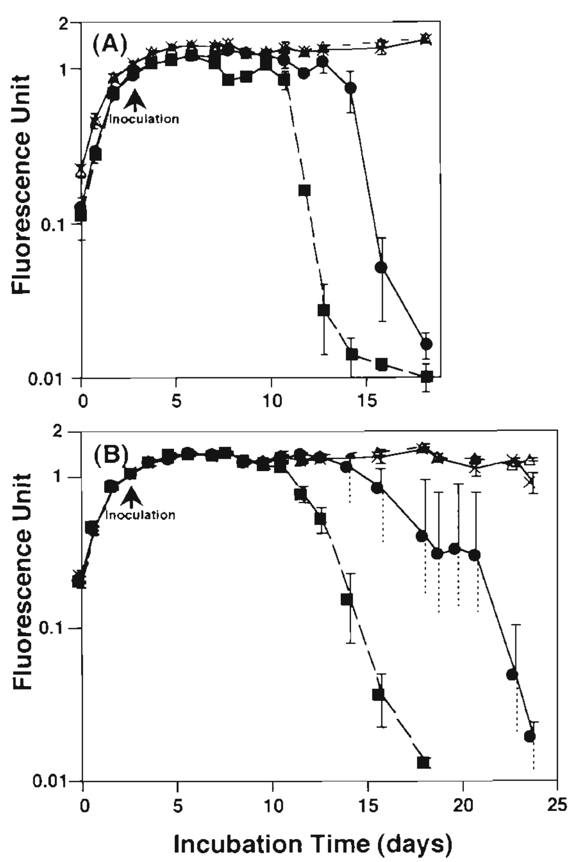


Fig. 1. Growth of (A) *Heterosigma akashiwo* GS95 and (B) *H. akashiwo* UR94 inoculated with HaV GSNOU-30 with no treatment (■), filtered through a 0.2 µm filter (●), a 0.1 µm filter (x) and with heat treatment at 100°C for 5 min (Δ). Error bars indicate SD

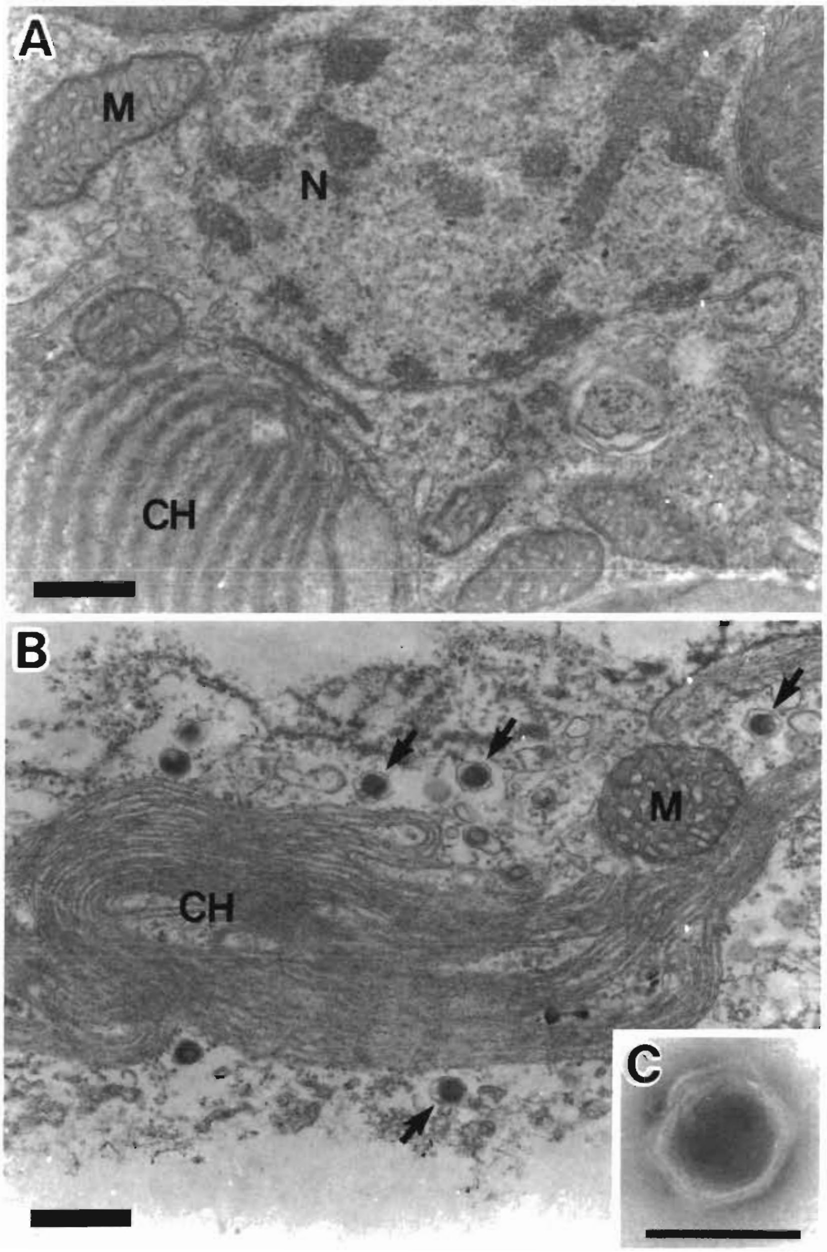


Fig. 2. Transmission electron micrograph of *Heterosigma akashiwo* UR94, 13 d after inoculation with HaV GSNOU-30 (A) with or (B) without heat treatment, and of (C) the negatively stained virus particle. Each organella is intact in (A). In contrast, a chloroplast is highly degraded and virus-like particles (arrows) are observed in (B). Scale bars: (A, B) 500 nm, (C) 200 nm. N: nucleus; CH: chloroplast; M: mitochondrion

VLPs were detected by transmission electron microscopy in the lysed cultures inoculated with the original stock suspension with no treatment (Fig. 2B) and filtration through a 0.2 µm filter. In contrast, no viral replication was detected in the cultures inoculated with the original stock suspension with filtration through a 0.1 µm filter or heat-treated (Fig. 2A). The VLP was 202 ± 6 nm (average ± standard deviation) in diameter (Fig. 2C) and pentagonal or hexagonal in cross-section, indicating icosahedral symmetry (Fig. 2B). It harbored an electron-dense roundish core that was distinct from the capsid and no tail-like structure was observed (Fig. 2B).

These results, (1) VLPs were observed in the lysed culture, (2) the algicidal effect was transferrable to a fresh algal culture and (3) VLPs were not found in healthy culture, fulfill Koch's postulates. Therefore, we concluded that the algicidal factor is both morphologically and physiologically a lytic dsDNA virus. The virus infecting *Heterosigma akashiwo* has been designated 'HaV (*Heterosigma akashiwo* virus) GSNOU-30'.

In the first step of the viral lysis, the infected cell became roundish, lost mobility, settled to the bottom of the incubation chamber, then was lysed and discolored gradually (Fig. 3). On the basis of these observations, it is suggested that infected cells sink to lower layers in the water column as observed in a natural red tide population (Nagasaki et al. 1996). The relationship between the cessation of upward migration of *Heterosigma akashiwo* cells in the final stage of a red tide and the loss of mobility caused by viral infection is noteworthy.

No algal lysis was observed for *Fibrocapsa japonica*, *Chattonella antiqua*, *C. verruculosa* (Raphidophyceae), *Heterocapsa triquetra*, *H. circularisquama*, *Prorocentrum triestinum*, *P. dentatum*, *Alexandrium tamarense* (Dinophyceae), *Isochrysis galbana*, *Pavlova lutheri* (Prymnesiophyceae), *Rhodomonas ovalis* (Cryptophyceae), *Skeletonema costatum*, *Thalassiosira* sp., *Chaetoceros didymus*, *Ditylum brightwellii* (Bacillariophyceae), *Pyramimonas* sp. (Prasinophyceae), *Oltomannsiellopsis viridis* (Chlorophyceae) inoculated with GSNOU-30. On the other hand, 3 strains of *Heterosigma akashiwo* (strains H94222, H94608 and H95623) isolated from Hiroshima Bay were resistant to HaV GSNOU-30 (Table 1). Thus, infectivity of HaV GSNOU-30 is not species-specific but strain-specific, showing the first example of phenotypic diversity among *H. akashiwo* strains in terms of viral infection. If the lytic virus plays a role as a selector to increase genetic diversity of a host species (Suttle & Chan 1993, Waterbury & Valois 1993), both ecological and physiological studies for an algal virus should be designed to use several strains of the host species.

Furthermore, the viruses isolated in the present study and the VLPs observed in the *Heterosigma akashiwo* cells in the red tide seawater sample (Nagasaki et al. 1994a, b) highly resembled each other in morphology (Fig. 2), suggesting the viral mortality in *H. akashiwo* blooms involving viral lytic activity.

What determines the sensitivity and resistance of a host cell remains unclear. Although there is no evidence which demonstrates the lysogeny in algal viruses infectious to eucaryotic hosts, one possible explanation is that immunity against a virus infection could be acquired by lysogeny of the same (or closely related) virus. Another possibility is a biochemical difference at the cell surface of host strains which can result in resistance against viral infection.

It is also indistinguishable whether algicidal activity originated from a lytic virus or a temperate virus in the sample seawater. Indeed, UV treatment was made in order to induce the latter into the former, its practical effect for induction has not been clearly elucidated yet. In the present study, the algicidal activity had already existed in the natural seawater sample. However, it does not necessarily exclude the possibility of induction by UV treatment in the case of GSNOU-30 and GSNOU-60.

It is fascinating to speculate on the mechanism of viral resistance and lysogeny. As well as the 5 microalgal viruses isolated so far, HaV is also expected to be a suitable material for these studies.

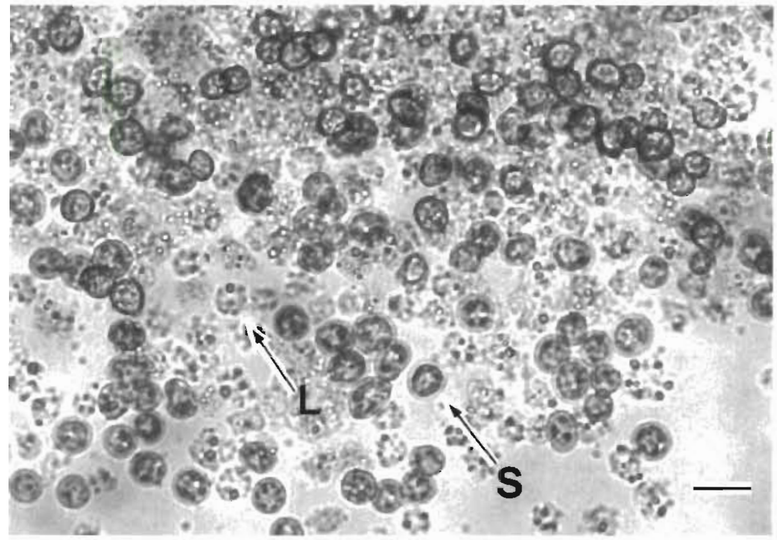


Fig. 3. *Heterosigma akashiwo* GS95 infected with HaV GSNOU-30. Note that a considerable proportion of the cells have been lysed (L). The surviving cells have also lost mobility and become roundish in shape (S). Scale bar = 20 µm

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