

# Isolation and characterization of two distinct types of HcRNAV, a single-stranded RNA virus infecting the bivalve-killing microalga *Heterocapsa circularisquama*

Yuji Tomaru<sup>1</sup>, Noriaki Katanozaka<sup>2,3</sup>, Kensho Nishida<sup>1</sup>, Yoko Shirai<sup>1</sup>,  
Kenji Tarutani<sup>1</sup>, Mineo Yamaguchi<sup>1</sup>, Keizo Nagasaki<sup>1,\*</sup>

<sup>1</sup>National Research Institute of Fisheries and Environment of Inland Sea, 2-17-5 Maruishi, Ohno, Saeki, Hiroshima 739-0452, Japan

<sup>2</sup>SDS Biotech K.K., 2-1 Midorigahara, Tsukuba, Ibaraki 300-2646, Japan

<sup>3</sup>Present address: Hitec Co. Ltd., 1-8-30 Tenmabashi, Kita, Osaka 530-6025, Japan

**ABSTRACT:** HcRNAV, a novel single-stranded RNA (ssRNA) virus specifically infecting the bivalve-killing dinoflagellate *Heterocapsa circularisquama*, was isolated from the coastal waters of Japan. HcRNAV strains were divided into 2 types based on intra-species host-range tests. The 2 types showed complementary strain-specific infectivity. In the following experiments, typical strains of each type (HcRNAV34 and HcRNAV109), were characterized. Both virus strains were icosahedral, ca. 30 nm in diameter, and harbored a single molecule of ssRNA approximately 4.4 kb in size. Thus, in morphology and nucleic acid type, HcRNAV is distinct from HcV, the previously reported large double-stranded DNA virus infecting *H. circularisquama*. Virus particles appeared in the cytoplasm of the host cells within 24 h post-infection, and crystalline arrays or unordered aggregations of virus particles were observed. The burst size and latent period were estimated at  $3.4 \times 10^3$  to  $2.1 \times 10^4$  infectious particles cell<sup>-1</sup> and 24 to 48 h, respectively. To our knowledge, this is the first report of a ssRNA virus infecting dinoflagellates that has been isolated and maintained in culture. Although HcRNAV is considered to have some similarities with plant viruses belonging to genera *Sobemovirus* and *Luteovirus*, further genomic analysis is required to discuss the detailed classification and nomenclature of HcRNAV. The finding of RNA viruses infecting microalgae such as HaRNAV (*Heterosigma akashiwo* RNA virus) and HcRNAV emphasizes the diversity of algalicidal viral pathogens.

**KEY WORDS:** Dinoflagellate · Phycovirus · ssRNA viruses · *Heterocapsa circularisquama* · Algal virus

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## INTRODUCTION

Viruses, or virus-like particles (VLPs), have been observed in numerous phytoplankton species (Van Etten et al. 1991, Reisser 1993, Proctor 1997, Wommack & Colwell 2000). These observations have led to increased interest in the role of algal viruses in aquatic environments. Some reports suggest that viruses are significant agents sustaining the intra-species diversity of microalgae (Tarutani et al. 2000, Lawrence et al. 2001) and directly controlling the population dynamics of phytoplankton by viral lysis

(Suttle et al. 1990, Bratbak et al. 1993, Nagasaki et al. 1994, Tarutani et al. 2000, Jacquet et al. 2002). In addition, viral lysis of algal cells facilitates material cycle in aquatic environments, and the growth and succession of heterotrophic microbial communities (Gobler et al. 1997, Castberg et al. 2001). Thus, viruses infecting microalgae are regarded as an important member of the microbial community affecting the nutrient cycle in aquatic environments (Fuhrman 1999). To further determine the significance of aquatic viruses, it is necessary to bring more host-virus systems into the laboratory.

\*Corresponding author. Email: nagasaki@affrc.go.jp

So far, at least 14 algal host-virus systems have been reported to be cultured and studied to any extent. Most viruses infecting eukaryotic microalgae that have been isolated so far are large double-stranded DNA (dsDNA) viruses, which are considered as belonging to the family *Phycodnaviridae* (Van Etten 2000, Van Etten et al. 2002). They are icosahedral in shape, large (>100 nm), and harbor a dsDNA measuring 158 to 560 kbp in length (Van Etten & Meints 1999, Sandaa et al. 2001).

Reports of small (<100 nm) viruses and VLPs within algae have been comparatively rare. Brussaard et al. (1996) observed small VLPs (50 to 60 nm in diameter) in *Emiliania huxleyi* (Prymnesiophyceae) that coexisted intracellularly with larger VLPs. Neither of these VLPs were isolated. Recently, Lawrence et al. (2001) isolated a small virus (ca. 30 nm in diameter) infecting the harmful algal bloom-causing microalga *Heterosigma akashiwo* (Raphidophyceae). This virus, called HaNIV, replicates in the nucleus. HaNIV does not appear to be a member of the *Phycodnaviridae*, although its genome has not been characterized yet. Microalgal viruses harboring RNA genomes have recently been isolated, including: a double-stranded RNA (dsRNA) virus, MpRNAV, infecting *Micromonas pusilla* (Prasinophyceae) (C.P.D. Brussaard pers. comm.) and a single-stranded RNA (ssRNA) virus, HaRNAV, infecting *H. akashiwo* (Tai et al. 2003). The former harbors 11 segments of dsRNA and proliferates itself in the cytoplasm, and the latter has ca. 9.1 kb ssRNA and also replicates in the cytoplasm. These findings indicate that the diversity of microalgal viruses is undoubtedly higher than previously envisaged.

*Heterocapsa circularisquama* Horiguchi (Horiguchi 1995) is a harmful bloom-causing dinoflagellate that specifically kills bivalves. It was first observed in Uranouchi Bay, Japan, in 1988. A large dsDNA virus (HcV) infecting *H. circularisquama* was isolated in 1999 that likely belongs to *Phycodnaviridae* on the basis of morphology, genome type, and pathology (Van Etten 2000, Tarutani et al. 2001, Nagasaki et al. 2003). In the present paper we describe the characteristics of a novel ssRNA virus (HcRNAV) infectious to *H. circularisquama*, and discuss its ecological significance. As far as we know, this is the first report of a ssRNA virus that infects and lyses dinoflagellates.

## MATERIALS AND METHODS

**Algal cultures.** Four extracellularly axenic clonal strains of *Heterocapsa circularisquama*, HU9433-P, HA92-1, HCLG-1 and HY9423, were used for isolation of viral pathogens. These strains were isolated from coastal waters of western Japan: Uranouchi Bay in December 1994, Ago Bay in December 1992, Gokasho Bay in August 1999, and Yatsushiro Kai in September 1994, respectively (see Fig. 1). Although *H. circularisquama* generally harbors intracellular bacteria that are considered to have a symbiotic relationship with the host, HU9433-P was also free of intracellular bacteria (Maki & Imai 2001). To test the intra-species host range of the viruses, 52 additional *H. circularisquama* clonal strains were examined as potential hosts. Detailed information about these hosts is provided in Nagasaki et al. (2003). *H. circularisquama* strains were grown in modified SWM3 medium (Chen et al. 1969, Itoh & Imai 1987) enriched with 2 nM Na<sub>2</sub>SeO<sub>3</sub> under a 12:12 h light:dark cycle of 130 to 150  $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup> with cool white fluorescent illumination at 20°C. To examine the inter-species infection specificity of HcRNAV, 32 additional clonal algal strains belonging to the families Bacillariophyceae, Chlorophyceae, Dinophyceae, Euglenophyceae, Eustigmatophyceae, and Raphidophyceae (Table 1) were also tested as potential hosts. These species were cultured under the

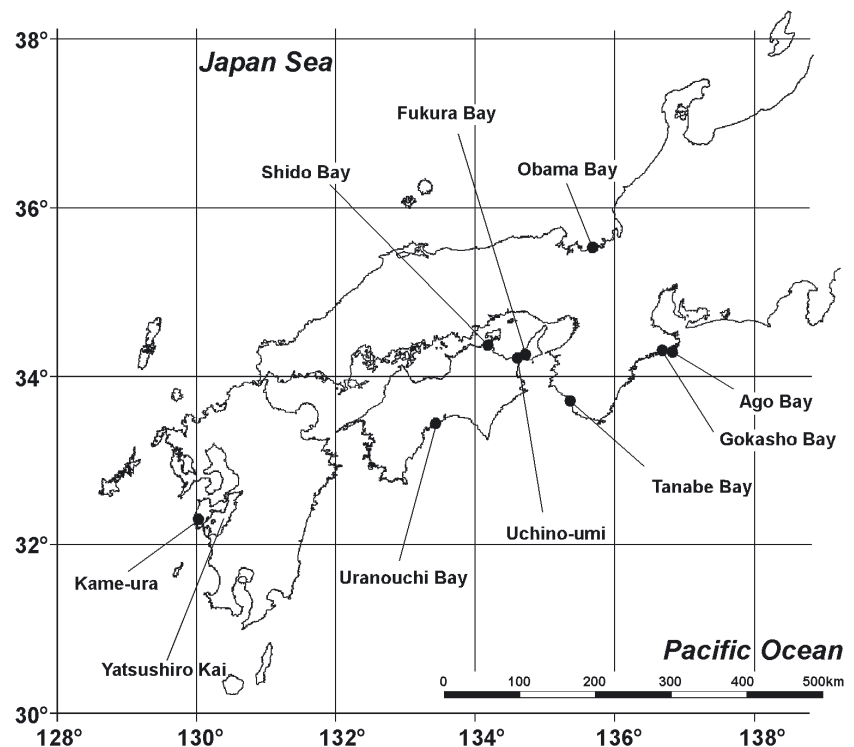


Fig. 1. Locations (●) from which seawater samples were collected for isolation of HcRNAV strains

Table 1. Infection specificities of HcRNAV34 and HcRNAV109 against 36 strains of marine phytoplankton. –: not lysed; +: lysed

Family	Species	Strain code	Temperature (°C)	Strains lysed by HcRNAV34	Strains lysed by HcRNAV109
Bacillariophyceae	<i>Chaetoceros didymum</i>	Ch4	15	–	–
	<i>Ditylum brightwellii</i>	Dity	15	–	–
	<i>Skeletonema costatum</i>	SK-1	15	–	–
	<i>Thalassiosira</i> sp.	Th2	15	–	–
Eustigmatophyceae	<i>Nannochloropsis</i> sp.	SFBB	20	–	–
Chlorophyceae	<i>Oltmannsiellopsis viridis</i>	Olt	20	–	–
Dinophyceae	<i>Alexandrium catenella</i>	ACNG	20	–	–
	<i>Alexandrium tamarense</i>	AKR3	15	–	–
	<i>Gymnodinium catenatum</i>	Gc1ax	20	–	–
	<i>Gymnodinium mikimotoi</i>	G303-ax2	20	–	–
	<i>Gymnodinium sanguinum</i>	GSUR	20	–	–
	<i>Heterocapsa circularisquama</i>	HU9433-P <sup>a</sup>	20	+	–
	<i>Heterocapsa circularisquama</i>	HA92-1 <sup>a</sup>	20	+	–
	<i>Heterocapsa circularisquama</i>	HCLG-1 <sup>a</sup>	20	–	+
	<i>Heterocapsa circularisquama</i>	HY9423 <sup>a</sup>	20	–	+
	<i>Heterocapsa illdefina</i>	CCMP446	25	–	–
	<i>Heterocapsa niei</i>	TG607	25	–	–
	<i>Heterocapsa niei</i>	CCMP447	25	–	–
	<i>Heterocapsa pygmaea</i>	AK23	25	–	–
	<i>Heterocapsa pygmaea</i>	CCMP1322	25	–	–
	<i>Heterocapsa pygmaea</i>	CCMP1490	25	–	–
	<i>Heterocapsa</i> sp.	CCMP451	25	–	–
	<i>Heterocapsa</i> sp.	CCMP450	15	–	–
	<i>Heterocapsa triquetra</i>	Ht	15	–	–
	<i>Heterocapsa triquetra</i>	CCMP448	20	–	–
	<i>Heterocapsa triquetra</i>	CCMP449	15	–	–
	<i>Prorocentrum micans</i>	8304	20	–	–
	<i>Prorocentrum triestinum</i>	Pt-1	20	–	–
	<i>Scrippsiella</i> sp.	SCKR	20	–	–
Euglenophyceae	<i>Eutreptiella</i> sp.	Eut-ax01	20	–	–
Raphidophyceae	<i>Chattonella antiqua</i>	CaTim	20	–	–
	<i>Chattonella marina</i>	CmUR2	20	–	–
	<i>Chattonella ovata</i>	CoV	20	–	–
	<i>Chattonella verruculosa</i>	M	15	–	–
	<i>Fibrocapsa japonica</i>	Fib-1	20	–	–
	<i>Heterosigma akashiwo</i>	H93616	20	–	–

<sup>a</sup>Strain used for virus isolation

conditions given above at the temperatures shown in Table 1.

**Virus isolation.** Seawater was obtained from 9 sampling sites in western Japan (Fig. 1, Table 2): Gokasho Bay on 5 July 2000 (sampling depth and *Heterocapsa circularisquama* cell abundance were 0 m and unknown, respectively), Tanabe Bay on 30 August 2000 (0 m, 270 cells ml<sup>-1</sup>), Ago Bay from 16 July to 28 August 2001 (0.5, 5 and 1 m above the bottom, <0.01 to 2450 cells ml<sup>-1</sup>) (details are shown in Nagasaki et al. 2004, this issue), Fukura Bay on 29 August and 4 September 2001 (0 m, 2500 cells ml<sup>-1</sup> and 0 m, <1 cell ml<sup>-1</sup>, respectively), Shido Bay on 12 September 2001 (0 m, cell abundance unknown), Obama Bay Stn Mamiya-shinju and Stn Ohtsuki-shinju on 12 September 2001 (2 m, 10 cells ml<sup>-1</sup> and 2 m, 10 cells ml<sup>-1</sup>, respectively), Uranouchi Bay on 27 August 2001 (0 m, cell abundance unknown), Uchino-umi Stns 5 and 6 on 15 October

2001 (1 m, 373 cells ml<sup>-1</sup> and 0 to 5 m, 405 cells ml<sup>-1</sup>, respectively), and Kame-ura on 10 December 2001 (2 m, 65 cells ml<sup>-1</sup>). The water samples were processed within 24 h of sampling. Each sample was filtered through a 0.8 µm nominal pore-size polycarbonate membrane filter (Nuclepore) (0.8 µm filtrate). The 0.8 µm filtrate was then diluted in modified SWM3 medium in a series of 10-fold dilution steps. Aliquots (100 µl) of each dilution step were added to 8 wells in cell culture plates with 96 round-bottom wells (Falcon) containing 150 µl of an exponentially growing host culture. *H. circularisquama* strains HU9433-P, HA92-1, HCLG-1 and HY9423 were used as potential hosts. In addition, each sample was filtered through a 0.2 µm nominal pore-size membrane filter (0.2 µm filtrate) and aliquots (1 ml) were inoculated on 1 ml of the host culture (HU9433-P, HA92-1, HCLG-1 and HY9423). To control cultures, a 0.2 µm filtrate treated at 100°C for

Table 2. HcRNAV strains isolated in the western part of Japan from July through August 2000 at Gokasho Bay and Tanabe Bay, and from July through December 2001 at Ago Bay, Fukura Bay, Shido Bay, Obama Bay, Uranouchi Bay, Uchino-umi and Kame-ura. Only the strain numbers are shown. Note that all the strains isolated using HU9433-P or HA92-1 were categorized as UA-type, and those isolated by use of HCLG-1 or HY9423 were CY-type (refer to Fig. 2)

Host strain used for isolation	Isolation site of virus strains								
	Gokasho Bay	Tanabe Bay	Ago Bay	Fukura Bay	Shido Bay	Obama Bay	Uranouchi Bay	Uchino-umi	Kame-ura
HU9433-P		15, 16	19, 20, 27–30, 43–46, 59–62, 69–72, 77–79	85, 86, 89, 90, 93, 94	97, 98		113–116	121, 122	
HA92-1		17, 18	21, 22, 31–34, 47, 49, 50, 63–66, 73–76, 81–84	87, 88, 95, 96	99, 100			123, 124	130
HCLG-1	11–14		23, 24, 38, 51, 67		101, 102	105–108	117–120	125, 126	131
HY9423			39–42, 68	91, 92	103, 104	109–112		127, 128	132

5 min was added. The cell-culture plates were incubated as described above for the *H. circularisquama* cultures. The occurrence of algal lysis was monitored for 10 to 14 d by optical microscopy. We scored algal lysis when at least 90% of host cells in a well were lysed. In most cases when 0.8 µm filtrate of a seawater sample caused algal lysis, its 0.2 µm filtrate also showed an algicidal activity. For virus isolation, we used an algicidal pathogen from algal lysate induced with 0.8 µm filtrates as a rule. The algicidal pathogen to HCLG-1 in Kame-ura water had to be obtained from 0.2 µm filtrate, as we could not obtain it from a 0.8 µm filtrate. As for seawater samples from Gokasho Bay and Tanabe Bay, only 0.2 µm filtrates were examined. Clonal isolates from these algal lysates were obtained by 2 cycles of the extinction-dilution procedure (Nagasaki & Yamaguchi 1997), using the same host strain of *H. circularisquama* that was initially used to obtain the pathogen. To eliminate bacterial contamination the lysate in the most diluted wells of the second assay was filtered through a 0.1 µm nominal pore-size polycarbonate membrane filter, transferred to an exponentially growing culture of the same host, and incubated as described above for the *H. circularisquama* cultures. The resultant lysate was regarded as a clonal pathogen suspension. To check the bacterial contamination in the lysate and stainability of the pathogen with DAPI (4',6-diamidino-2-phenylindole), each clonal pathogen suspension was filtered onto a 0.02 µm pore-size filter (Anodisc 25, Whatman International), stained with DAPI (1 µg ml<sup>-1</sup> final concentration), and observed at 1000× magnification under UV excitation. Algicidal pathogens negatively stained with uranyl acetate were also observed by transmission electron microscopy. Briefly, algicidal pathogen suspension was mounted on a grid (No. 780111630,

JEOL DATUM) for 30 s and excess water was removed by filter paper (No. 1, Toyo Roshi Kaisha); 4% uranyl acetate was then mounted on the grid for 10 s and the excess dye was removed with a filter paper. After drying the grid in a desiccator for 10 min, negatively stained pathogens were observed by transmission electron microscopy (JEOL JEM-1010) at an acceleration voltage of 80 kV.

**Host-range test.** To examine the intra-species host specificity of the pathogens, clonal pathogens were screened against putative hosts to test their infectivity. Clonal pathogen suspensions were centrifuged at 4500 × *g* for 5 min at 4°C; an aliquot of each lysate was then inoculated (1%, v/v) independently into exponentially growing cultures of the 56 *Heterocapsa circularisquama* strains mentioned above. The inter-species host specificity of HcRNAV34 and HcRNAV109 was also tested by adding 5% (v/v) aliquots of each suspension to duplicate cultures of the exponentially growing algal strains listed in Table 1. The culture plates were incubated under the same conditions of light and temperature as described above for the *H. circularisquama* cultures. The occurrence of algal lysis was monitored by optical microscopy. Growth of host cultures without pathogen inoculation was also monitored to serve as a control. Algal lysis was scored when an aggregation of lysed cells was observed on the bottom of culture vessels. Algal cultures that were not lysed at 14 d after inoculation were scored as unsuitable hosts to the inoculum.

Based on the results of the tests, *Heterocapsa circularisquama* strains HU9433-P and HCLG-1 were used in the following experiments as the host of the tested virus strains HcRNAV34 and HcRNAV109, respectively.

**Growth experiments.** To examine the algicidal effect of HcRNAV34 and HcRNAV109, aliquots (2.5%, v/v) of

the pathogen suspension were filtered through a 0.1  $\mu\text{m}$  pore-size polycarbonate membrane filter (Nuclepore) and added to exponentially growing cultures of *Heterocapsa circularisquama* strains HU-9433-P and HCLG-1, respectively. Cultures inoculated with an autoclaved pathogen suspension (121°C, 15 min) served as controls. Each treatment was run in triplicate. The titer of the pathogen was measured by means of the extinction-dilution method (Suttle 1993, Nagasaki & Yamaguchi 1997), and the most probable number of lytic viruses was calculated (Nishihara et al. 1986). Host cell abundance was estimated by counting unfixed cells under an optical microscope using a Sedgewick-Rafter chamber.

In the 1-step growth experiment, 340 ml of exponentially growing cultures of *Heterocapsa circularisquama* strains HU9433-P and HCLG-1 were inoculated with HcRNAV34 and HcRNAV109 at a multiplicity of infection (moi) of 900 and 70, respectively. (Because we could not estimate virus titers before the experiments using the extinction dilution method, which requires 10 to 14 d for results, it was difficult to make the moi even.) Cultures of HU9433-P and HCLG-1 inoculated with an autoclaved pathogen suspension (HcRNAV34 and HcRNAV109, respectively) served as controls. An aliquot of cell suspension was sampled from each culture at 0, 12, 24, 33, 48 and 72 h after inoculation, and used to monitor the host cell abundance and pathogen titer as well as for transmission electron microscopic (TEM) observations. Pathogen titer was determined by the extinction-dilution method (Suttle 1993, Nagasaki & Yamaguchi 1997). For TEM observations, *H. circularisquama* cells were harvested by centrifugation at  $860 \times g$  for 10 min at 4°C and fixed with 1% glutaraldehyde at 4°C. The cell pellets were post-fixed for 3 h in 2% osmic acid in 0.1 M phosphate buffer (pH 7.2 to 7.4), dehydrated in a graded ethanol series, and embedded in Quetol 653 resin (NISSHIN EM). Thin sections were stained with 4% uranyl acetate and 3% lead citrate, and observed at 80 kV using a JEOL JEM-1010 TEM. The proportion of host cells harboring the small intracellular VLPs was estimated by counting at least 50 cells ( $n = 50$  to  $\sim 76$ ). Care was taken to ensure the same cells were not repeatedly counted in different sections. Lysed cells were not included in the counts.

**Analysis of HcRNAV nucleic acid and protein.** A total of 300 ml of exponentially growing *Heterocapsa circularisquama* HU9433-P and HCLG-1 ( $1.0 \times 10^4$  to  $2.0 \times 10^4$  cells  $\text{ml}^{-1}$ ) were inoculated with 3 ml of HcRNAV34 and HcRNAV109 ( $\sim 10^7$  infectious titer  $\text{ml}^{-1}$ ), respectively, and lysed. The resulting lysates were sequentially passed through 8.0, 0.8, and 0.2  $\mu\text{m}$  filters to remove cellular debris. Polyethylene glycol 6000 (Wako) was added to the filtrates to obtain a final con-

centration of 10% (w/v); the resultant suspension was then stored at 4°C in the dark overnight. After centrifugation at  $57\,000 \times g$  for 1.5 h, the viral pellet was washed with phosphate buffer (10 mM  $\text{Na}_2\text{HPO}_4$  and 10 mM  $\text{KH}_2\text{PO}_4$  in distilled water) and again centrifuged at  $217\,000 \times g$  for 4 h to collect the virus particles; they were then resuspended in 500  $\mu\text{l}$  of 10 mM phosphate buffer. The viral suspension was treated with Proteinase K (final conc. = 1 mg  $\text{ml}^{-1}$ , Nippon Gene) in 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.4), 1 mM EDTA and 1% sarcosyl (International Technologies) at 55°C for 1.5 h (Yamada et al. 1991). Nucleic acids were extracted by phenol-chloroform extraction (Yamada et al. 1991) and digested for 1 h with RNaseA (final conc. = 0.1  $\mu\text{g} \mu\text{l}^{-1}$ , Nippon Gene) at 37 or 98°C, or DNaseI (final conc. = 0.2  $\mu\text{g} \mu\text{l}^{-1}$ , Promega) at 37°C. RNase treatment at 37°C digests ssRNA but not dsRNA, while dsRNA denatures into ssRNA at 98°C and thus it is digestible with RNase. Nucleic acid extractions held on ice without enzymatic treatment served as controls. A formaldehyde-agarose gel (1%,  $15 \times 20$  cm, Seakem® Gold Agarose, BMA Inc.) was loaded with 20  $\mu\text{l}$  of nucleic acid and electrophoresed at 50 V for 14.5 h. Nucleic acids were visualized by SYBR-Green II staining (Molecular Probes).

The virus suspension was mixed with a 4-fold volume of sample buffer (62.5 mM Tris-HCl, 5% 2-mercaptoethanol, 2% sodium dodecyl sulphate [SDS], 20% glycerol and 0.005% bromophenol blue) and boiled for 5 min; then the proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) ( $80 \times 40 \times 1.0$  mm, 10 to 20% gradient polyacrylamide gel, 150 V) using an XV PANTERA SYSTEM (DRC). Proteins were visualized by Coomassie brilliant blue staining. Protein molecular weight standers (DRC), ranging from 6.5 to 200 kDa, were used for size calibration.

## RESULTS AND DISCUSSION

### Isolation of viruses and their host-range

In the present study, pathogens to *Heterocapsa circularisquama* were isolated from 9 coastal waters of western Japan (Fig. 1, Table 2). Cell lysis was detected in cultures within 10 d of inoculation. As a result, 107 clonal pathogens were isolated: 4 from Gokasho Bay, 4 from Tanabe Bay, 52 from Ago Bay, 12 from Fukura Bay, 8 from Shido Bay, 8 from Obama Bay, 8 from Uranouchi Bay, 8 from Uchino-umi, and 3 from Kameura (Table 2). No DAPI-stainable free particles were observed in any of the pathogen samples. This indicates that the pathogens are different from a large dsDNA virus HcV (Tarutani et al. 2001).



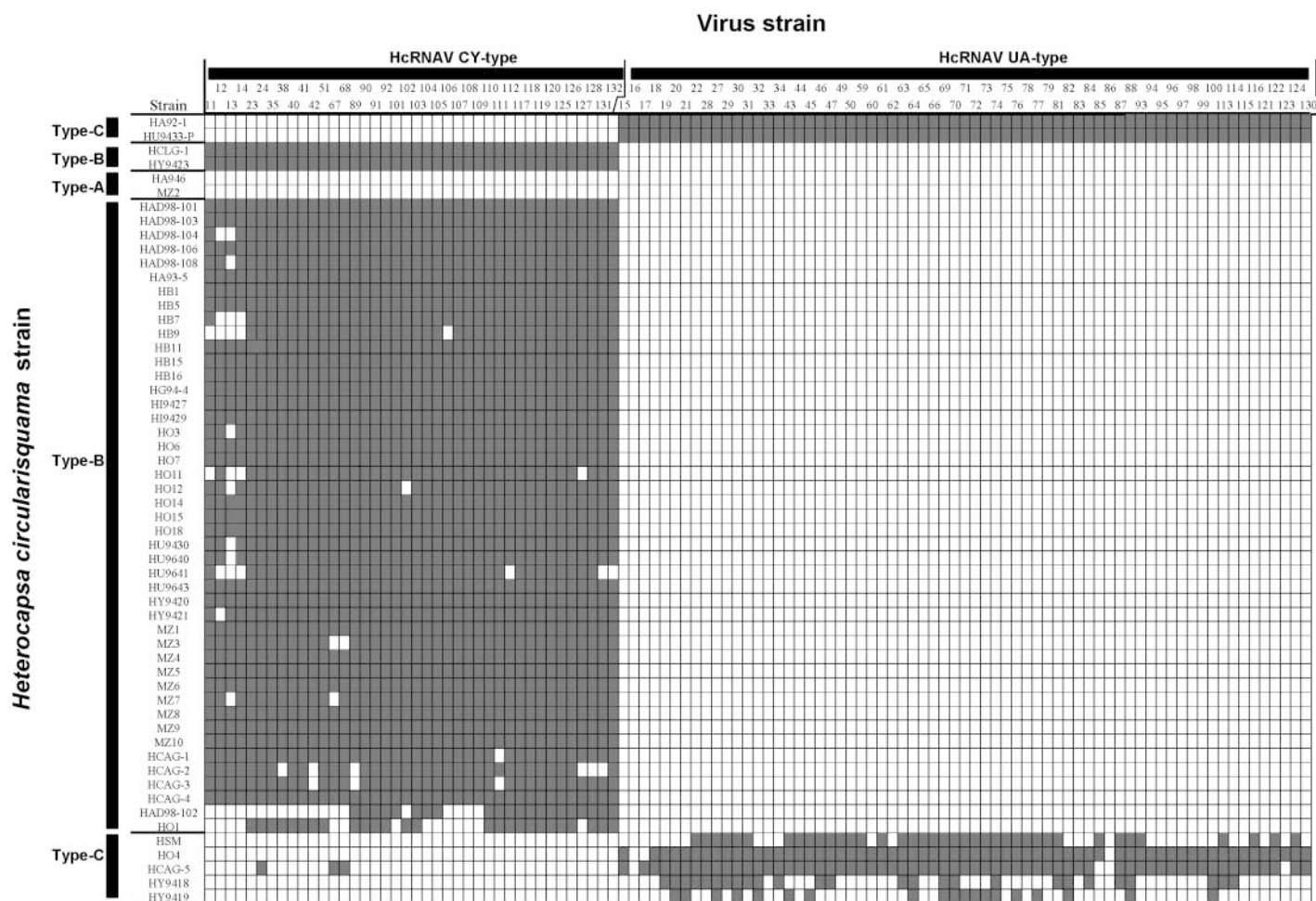


Fig. 2. Viral susceptibility spectra of 56 *Heterocapsa circularisquama* strains to 107 HcRNAV strains. All host and virus strains were isolated from the western part of Japan (Nagasaki et al. 2003, see our Fig. 1 and Table 2). Shaded and open columns indicate susceptibility (with cells lysed) and resistance (with cell growth equal to that of the controls) of host strains to each HcRNAV strain, respectively

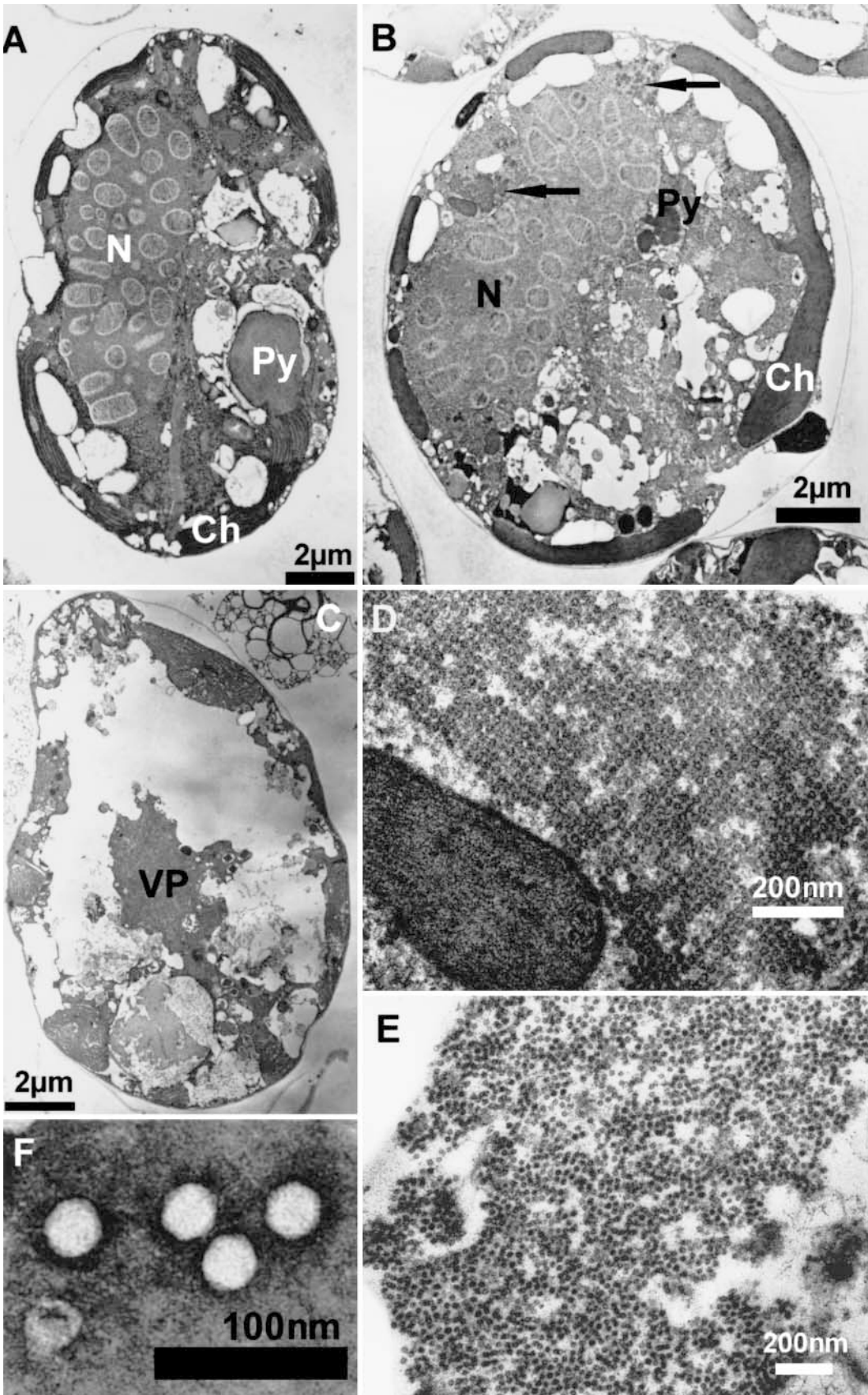
Results of the intra-species host-range assay are shown in Fig. 2. The clonal pathogens were divided into 2 types on the basis of their infection spectra, UA-type and CY-type: the UA-type was composed of HcRNAV strains isolated using HU9433-P or HA92-1 as host strains, and the CY-type was composed of those isolated using HCLG-1 or HY9423 (Fig. 2, Table 2). The infection spectra of UA-type- and CY-type pathogens were complementary to each other (Fig. 2). Cluster analysis, by means of the neighbor-joining method, also supported the division of HcRNAV strains into 2 clusters (data not shown). On the basis of these results, HcRNAV34 and HcRNAV109 were selected as repre-

sentatives of UA-type- and CY-type pathogens, respectively, and thereafter, the 2 typical clonal pathogens were intensively characterized.

Cell lysis occurred in *Heterocapsa circularisquama* cultures inoculated with HcRNAV34 or HcRNAV109 that were filtered through a 0.1  $\mu\text{m}$  pore-size filter. In contrast, algicidal activity was lost by autoclaving the pathogen suspension (data not shown). These data indicate that the pathogens were <0.1  $\mu\text{m}$  in size and were heat-labile.

In 1-step growth experiments, *Heterocapsa circularisquama* cells inoculated with either pathogen contained small VLPs in the cytoplasm (Figs. 3D,E & 4C),

Fig. 3. *Heterocapsa circularisquama* strain HU9433-P infected with HcRNAV34. Transmission electron micrographs. (A) Healthy cell of *H. circularisquama* HU9433-P showing the nucleus (N), chloroplasts (Ch) and pyrenoids (Py). (B) 24 h post-infection showing the development of viroplasm (arrows) in the cytoplasm. (C) 33 h post-infection showing the degradation of organelles and the expanded viroplasm (VP). (D,E) Higher magnification of the (D) crystalline array formation and (E) an unordered aggregation of HcRNAV34. (F) Negatively stained HcRNAV34 particles in the culture lysate





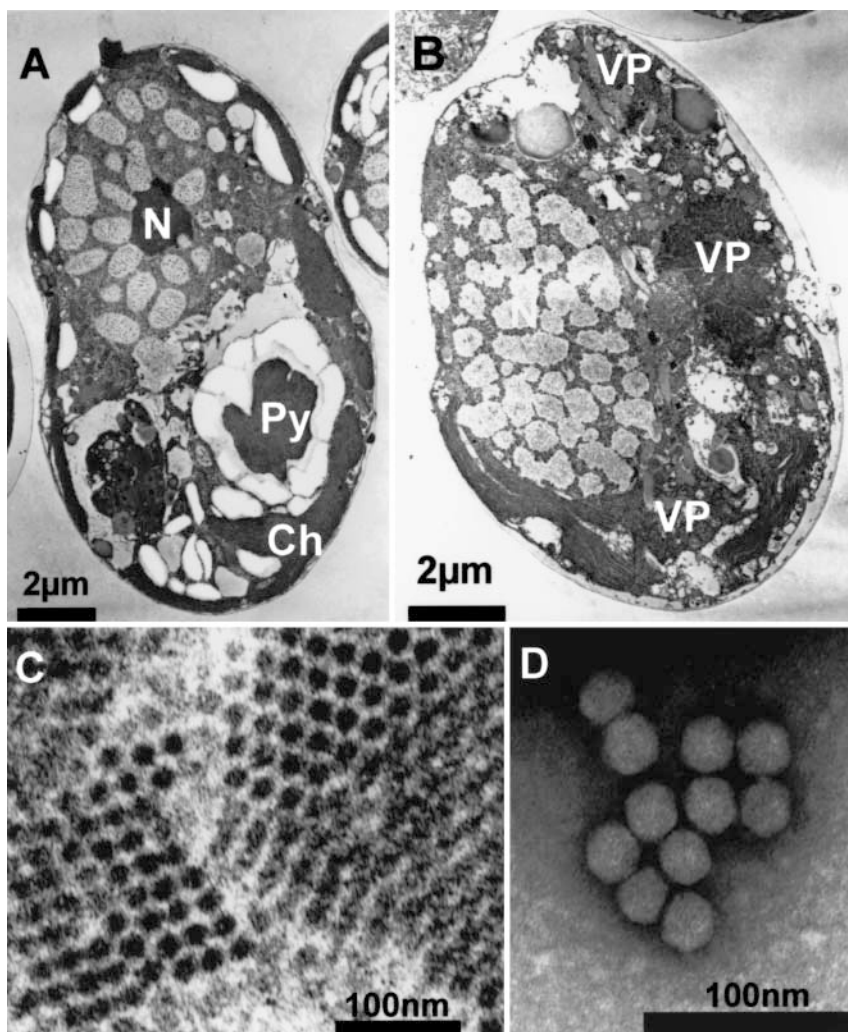


Fig. 4. *Heterocapsa circularisquama* strain HCLG-1 infected with HcRNAV109. Transmission electron micrographs. (A) Healthy cell of *H. circularisquama* HCLG-1 showing the nucleus (N), chloroplasts (Ch) and pyrenoids (Py). (B) 48 h post-infection showing the development of viroplasms (VP) and degradation of organelles in the cytoplasm. (C) Higher magnification of the crystalline array formation of HcRNAV109. (D) Negatively stained HcRNAV109 particles in the culture lysate

and VLPs similar in size were also observed in negatively stained culture lysates (Figs. 3F & 4D). Healthy *H. circularisquama* cells in the control cultures had cytoplasmic structures typical of dinoflagellates with no symptoms of viral infection (Figs. 3A & 4A). Although VLPs formed either crystalline arrays or unordered aggregations in the cytoplasm (Figs. 3D,E & 4C), regular array structures were only occasionally observed in thin sections.

On the basis of these results, it was demonstrated that (1) the pathogenic effect was transferable to a fresh algal culture, (2) VLPs appeared in lysed cultures, and (3) VLPs were not detected in healthy cultures. Koch's postulates were fulfilled, and thus it was concluded that the VLPs observed within the pathogen-inoculated cultures were lytic viruses infecting *Heterocapsa circularisquama*. We designated the pathogen HcRNAV (*Heterocapsa circularisquama* RNA virus), and the isolated virus strains were numbered as shown in Table 2.

Results of the inter-species host-range test are shown in Table 1. HcRNAV34 and HcRNAV109 were not lytic to the 32 strains of marine phytoplankton, including 11 strains of the genus *Heterocapsa*, except for *H. circularisquama*. Considering that each HcRNAV clone was not lytic to all of the *H. circularisquama* strains tested (Fig. 2), HcRNAV is rather strain-specific than species-specific.

Strain-specificities of algal viruses have been reported in some host-virus systems, *Heterosigma akashiwo*-HaV (Nagasaki & Yamaguchi 1998), *H. akashiwo*-HaNIV (Lawrence et al. 2001), and *Micromonas pusilla*-MpV (Sahlsten 1998). Tarutani et al. (2000) reported that the majority of *H. akashiwo* cells surviving after bloom disintegration caused by viral infection were resistant to most simultaneously isolated HaV strains, and suggested that viral control of the clonal composition of *H. akashiwo* populations may occur in natural environments. Thus, viruses are considered to affect not only the quantity (biomass) of the bloom but also the diversity of strains (quality) within the population.



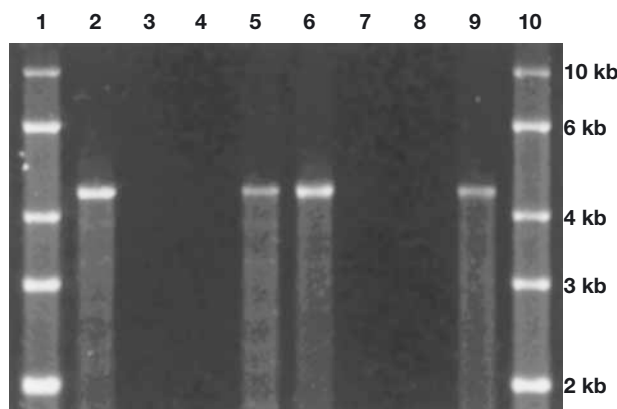


Fig. 5. Nuclease treatments of the nucleic acids isolated from HcRNAV34 (Lanes 2 to 5) and HcRNAV109 (Lanes 6 to 9). HcRNAV nucleic acids are shown untreated in Lanes 2 and 6, after treatment with RNaseA at 37°C in Lanes 3 and 7, after treatment with RNaseA at 98°C in Lanes 4 and 8, and DNaseI at 37°C in lanes 5 and 9, respectively. RNA molecular weight markers are shown in Lanes 1 and 10

### Morphology, genome, and protein of HcRNAV

The particles of HcRNAV34 and HcRNAV109 were revealed to be tail-less and lack an external membrane. They were icosahedral in shape and measured approximately  $30 \pm 2$  nm (mean  $\pm$  SD,  $n = 24$ ) in diameter (Figs. 3D-F, 4C,D). With regard to the similarities in size, icosahedral structure, virus assembly in cytoplasm, and infectivity to microalgae, HcRNAV is considered to be most closely related to HaRNAV, a

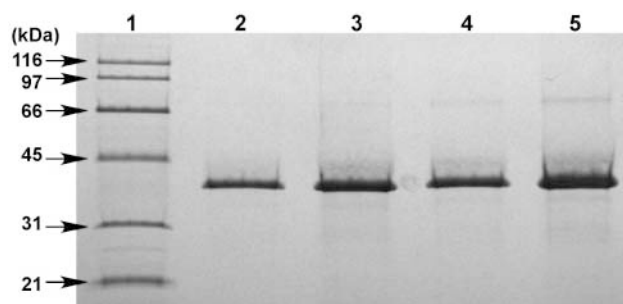


Fig. 6. Major proteins of *Heterocapsa circularisquama* RNA viruses. Proteins extracted from HcRNAV34 were loaded in Lanes 2 (2 µl) and 3 (4 µl), and those from HcRNAV109 were loaded in Lanes 4 (2 µl) and 5 (4 µl). Molecular weight markers are shown in Lane 1

single-stranded RNA virus that infects the harmful algal bloom-causing phytoplankton *Heterosigma akashiwo* (Tai et al. 2003).

Denaturing gel electrophoresis revealed that each virus strain had a single molecule of a nucleic acid approximately 4.4 kb in length that was sensitive to RNaseA both at 37 and 98°C, but not to DNaseI (Fig. 5). These data indicate that the HcRNAV genome is ssRNA. Considering that the genome size of the icosahedral RNA viruses smaller than 30 nm ranges from 3.4 to 15.5 kb (Tidona & Darai 2002), the genome size of HcRNAV is relatively small. As the RNAs isolated from HcRNAV34 and HcRNAV109 were not retained by a poly (A) tail purification column but recovered from the wash (data not shown), the HcRNAV genome prob-

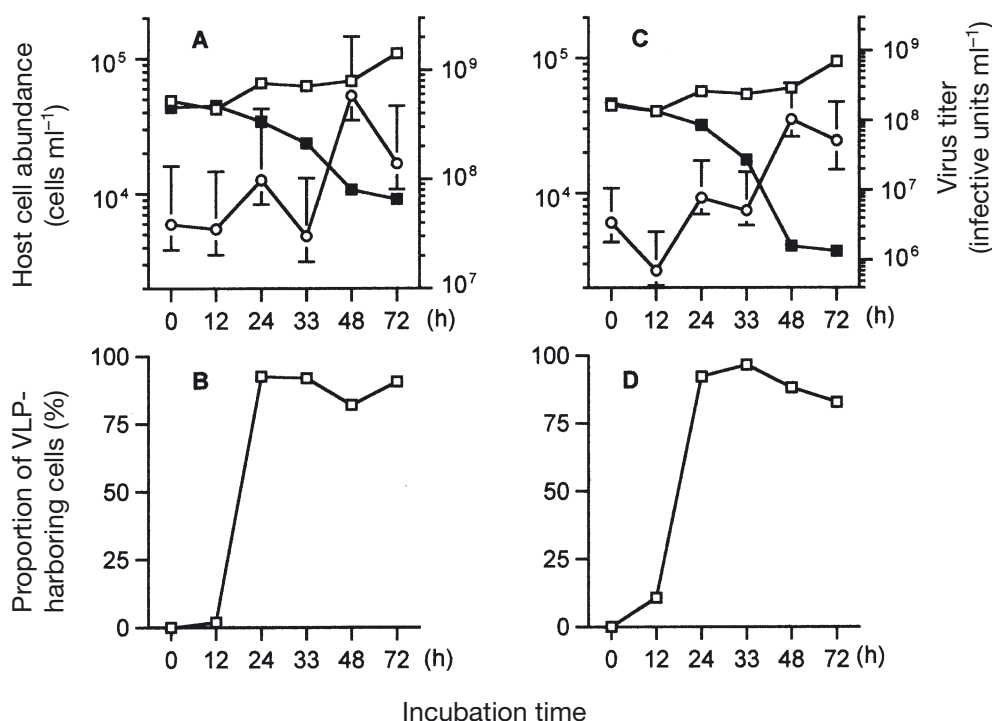


Fig. 7. *Heterocapsa circularisquama*. Changes in density in 1-step growth experiments. (A) HU9433-P vs. HcRNAV34, the initial multiplicity of infection (moi) was 900. (B) % of host cells harboring virus particles in the total surviving cells ( $n > 50$ ) in (A). (C) HCLG-1 vs. HcRNAV109, the initial moi was 70. (D) % of cells harboring virus-like particles in the total surviving cells ( $n > 50$ ) in (C). (A,C) ■: with addition of HcRNAV suspensions filtered through 0.2 µm pore-size filter; □: with addition of an autoclaved filtrate (negative control); ○: viral titer (infectious units ml<sup>-1</sup>; error bars indicate the 95% confidence intervals)

ably does not contain a poly (A) tail. Partial sequences of HcRNAV34 and HcRNAV109 genomes are now under way (data not shown), and indicate that the nucleotide sequences of HcRNAV34 and HcRNAV109 are similar to each other. Although HcRNAV is similar to *Sobemovirus* and *Luteovirus* with respect to morphology (25 to 30 nm, icosahedral), nucleic acid (single molecule of ssRNA, 4.1 to 5.7 kb) and replication site (cytoplasm) (Tidona & Darai 2002), preliminary genome sequence analysis reveals that they are not closely related (data not shown). Detailed reports on the genomic organization and classification of HcRNAV will be published elsewhere.

The protein analysis shows that both HaRNAV strains have 1 major polypeptide with a molecular weight of ca. 38 kDa (Fig. 6). In comparison, the morphologically similar algal virus HaRNAV has several (at least 5) major polypeptides with molecular weights of 24.0 to 33.9 kDa (Tai et al. 2003), and the plant viruses *Sobemovirus* and *Luteovirus* have 3 major proteins (12 to 106 kDa) and 6 major proteins (4 to 60 kDa), respectively (Tidona & Darai 2002).

#### Pathology and growth characteristics of HcRNAV

When cultures of *Heterocapsa circularisquama* were inoculated with an appropriate strain of HcRNAV, infected host cells lost motility and settled onto the bottom of the incubation vessel. The proportion of virus-harboring cells rapidly increased from 12 to 24 h post-inoculation (Fig. 7B,D). Discrimination between virus-harboring cells and healthy cells requires careful scanning by transmission electron microscopy, because intracellular structures of infected cells remain mostly intact in the early stages of infection (Fig. 3A,B). In the present observation, only cells harboring a crystalline array or unordered aggregation of small VLPs in a cytoplasm were regarded as those infected by HcRNAV. The pathological appearances, such as degradation of the cytoplasm structure, organelles, and nucleus, were clear at 33 to 48 h post-infection, and

during this period the newly formed virus particles appeared in the cytoplasm (Figs. 3C & 4B). A decline in cell number was observed from 24 to 48 h post-infection, while virus abundances showed a considerable increase in the medium from 33 to 48 h post-infection (Fig. 7A,C). Hence, the latent period of HcRNAV was estimated to be 24 to 48 h, which is shorter than that of HcV (Tarutani et al. 2001). The increase of viral abundance was not parallel with a decreasing cell number. This makes it difficult to obtain a reliable estimate of the burst size. Viruses just released from lysed cells may form a clustered aggregation, e.g. Fig. 3D,E, and an apparent increase in viral titer may be caused by aggregation degradation. Assuming that all the cells disrupting at 24 to 48 h post-infection contributed to the increase in viral abundance, the burst size of HcRNAV34 and HcRNAV109 was estimated at  $2.1 \times 10^4$  and  $3.4 \times 10^3$  infectious units cell<sup>-1</sup>, respectively, which are 1.6 to 10 times higher than that of HcV. In the present experiments, as there was no dilution step after adsorption period, re-adsorption of the newly produced viruses might have caused an underestimation of the calculated burst size. In addition, these data should be translated with care because the burst size can be affected by moi (Van Etten et al. 1983, Bratbak et al. 1998). Still, it is considered that HcRNAV has a higher replication rate and higher yield than that of HcV (1800 to 2440 infectious units cell<sup>-1</sup>, Nagasaki et al. 2003). The burst size of HcRNAV was 1 to 3 orders of magnitude higher than those ever reported for the large algal viruses (>100 nm in diameter, DNA algal viruses), but 1 to 2 orders of magnitude less than that of the small algal virus HaNIV (reviewed in Castberg et al. 2002).

HcRNAV did not cause complete lysis of host cultures; ca. 10 to 20 % of host cells survived even at 72 h post-infection (Fig. 7A,C). Most of the surviving host cells were immotile and had lost their theca (Fig. 8) and they often re-grew in the culture lysate (data not shown). TEM observations revealed that 9 to 17 % of cells contained no virus particles and showed no symptoms of viral infection, such as degradation of cyto-

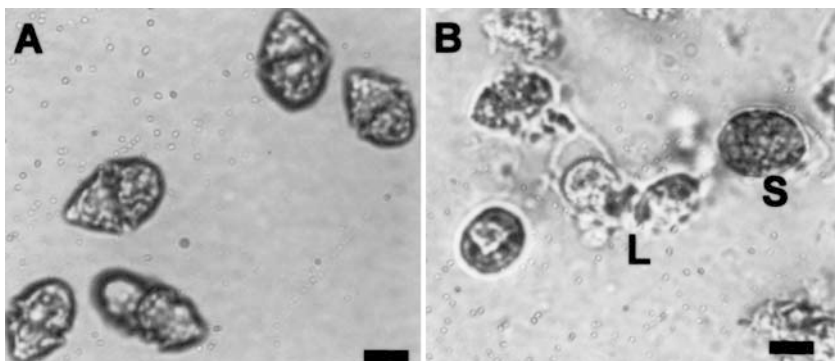


Fig. 8. *Heterocapsa circularisquama*. Optical microphotographs of HU9433-P (A) before and (B) after inoculation of HcRNAV34 (72 h post-infection). S: survived cell; L: lysed cell. Scale bars = 10  $\mu$ m

plasma or organelle at 72 h post-infection of HcRNAV (Fig. 7B,D). On the other hand, these data should be translated with care because it is possible that virus-harboring cells are so delicate that they lyse during TEM preparation (thin sectioning, concentration, dehydration and embedding) (Bratbak et al. 1993). In addition, since TEM observation of a cell gives only the image of a cell section, cells with a few virus-like particles are difficult to detect. Thus, the proportion of virus harboring cells might have been under-estimated.

TEM observation suggested that the surviving cells may act as seeds for the re-growth of host populations. Survival of host cells was also observed during HcV infection (Tarutani et al. 2001, Nagasaki et al. 2003), but the survival mechanism is unknown. To understand the population dynamics of *Heterocapsa circularisquama* in nature, elucidation of this survival mechanism is essential.

### Implications

In the present study, we examined the characteristics of the novel ssRNA virus HcRNAV, which infects the bivalve-killing dinoflagellate *Heterocapsa circularisquama*. As far as we know, this is the second report of ssRNA viruses infecting a microalga, the first being HaRNAV, which infects the fish-killing raphidophyte *H. akashiwo* (Tai et al. 2003). Furthermore, MpRNAV, a dsRNA algal virus which infects the cosmopolitan phytoplankton *Micromonas pusilla*, has been also isolated. It is icosahedral, 50 to 60 nm in diameter, and harbors 11 segments of dsRNA, the total size of which is 24.6 kbp (C. P. D. Brussaard pers. comm.).

Most of the microalgal viruses isolated so far are large dsDNA viruses; MpV and CbV have been classified in the family *Phycodnaviridae* as well as the typical virus PBCV-1 infecting an exsymbiotic *Chlorella*-like alga (Van Etten 2000), and PpV (Jacobsen et al. 1996), BtV (Gastrich et al. 1998), HaV (Nagasaki & Yanaguchi 1997), HcV (Tarutani et al. 2001), EhV (Wilson et al. 2002, Schroeder et al. 2002), PoV, and CeV (Sandaa et al. 2001) are also similar in size, shape, and genome type. Therefore, they may belong to the family *Phycodnaviridae*. Since novel RNA algal viruses such as HcRNAV, HaRNAV (Tai et al. 2003) and MpRNAV (C. P. D. Brussaard pers. comm.) have also been isolated so far, however, a new classification will have to be developed.

Host range studies revealed the diversity in the *Heterocapsa circularisquama* strains as well as that in the HcRNAV strains. Based on the virus sensitivity spectra, *H. circularisquama* strains were divided into 3 types: no lysis for both type of HcRNAV, lysed by HcRNAV CY-type, and lysed by HcRNAV UA-type (Fig. 2). Cluster analysis by means of the neighbor-joining method also

supported this categorization (data not shown). More than a single type of *H. circularisquama* strains were isolated together from a seawater sample; e.g. HO4 and the other HO strains from Obama Bay in August 1997, HY9418-9419 and other HY strains from Yatsushiro Kai in September 1994. The results indicate that studies on algal viruses could lead to an understanding of diversity within the host algal species in natural environments. In addition, multiple types of host and virus strains were isolated together from Ago Bay on 16 August 2001: the host strains HCAG-5 (Type C) and HCAG-2 to 4 (Type B), and the virus strains HcRNAV19 to 22 (UA-type) and HcRNAV23 to 24 (CY-type) (Fig. 2, Table 2). Because multiple types of HcRNAV and *H. circularisquama* coexisted in a given geographical area (Fig. 2, Table 2), it is likely that HcRNAV potentially plays a significant role in determining the composition of host strains, as well as affecting the dynamics of the *H. circularisquama* population in nature (Nagasaki et al. 2004). However, the ecological implications of the diversity of *H. circularisquama* and its virus HcRNAV have not yet been fully explained. To address this issue, it is first necessary to clarify the mechanisms that support and determine diversity among host and virus clones.

**Acknowledgements.** This study was supported by the Industrial Technology Research Grant Program in 2001 from the New Energy and Industrial Technology Development Organization of Japan (NEDO), the Society for Techno-innovation of Agriculture, Forestry and Fisheries (STAFF), and the Ministry of Agriculture, Forestry and Fisheries, Japan. Authors are grateful to Dr. T. Uchida of the Hokkaido National Fisheries Research Institute, Japan, Drs. S. Itakura and Y. Matsuyama of the National Research Institute of Fisheries and Environments of Inland Sea, Japan, and Dr. I. Imai, Kyoto University, Japan, who kindly provided *Heterocapsa* strains. Thanks are also due to Mr. K. Nakanishi and Mr. N. Hata of the Mie Prefectural Science and Technology Promotion Center, Dr. T. Takeuchi of the Wakayama Research Center of Agriculture, Forestry and Fisheries, Mr. T. Ishikawa of the Kochi Prefectural Fisheries Experimental Station, Mr. A. Mizuta of the Hyogo Prefectural Awaji Civil Affairs Bureau, Mr. N. Yoshimura of the Kumamoto Prefectural Fisheries Research Center, and Mr. K. Hibino of the Fukui Prefectural Reinan Promotion Branch Office, who kindly provided seawater samples. Authors are also thankful to Drs. T. Okuno and K. Mise of Kyoto University for their technical advice on viral RNA analysis. Map drawing software was provided by Mapmap 4 (The Geographical Survey Institute, Japan [H13 No. 367]).

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