Isolation and Characterization of a Single-Stranded RNA Virus Infecting the Marine Planktonic Diatom Chaetoceros tenuissimus Meunier

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Diatoms are the most abundant group of photosynthetic unicellular organisms; they have a very large biomass and are found in almost all aquatic and semiaquatic habitats (e.g., water, soil, and moist surfaces of rocks). They are of great ecologic importance because they are the principal primary producers in aquatic food webs and sustain the oxygen level in the atmosphere of the planet at about 21% (29). The genus Chaetoceros is highly diverse, with more than 400 species, which are considered to include the key primary photosynthetic producers that sustain the higher forms of aquatic life (17).

Since the 1970s, the significance of viruses as mortality agents for phytoplankton in aquatic systems has been highlighted. A number of algal viruses have been isolated and characterized. There used to be no direct evidence showing the relationship between diatoms and viruses, which led to the idea that the shell covering of diatoms might contribute to a reduced probability of viral infection (30). Later, however, diatoms were found to be infected by viruses similarly to other microalgae, and the first isolated diatom-infecting virus was a single-stranded RNA (ssRNA) virus lytic to a pen-shaped diatom species, Rhizosolenia setigera (R. setigera virus [RsRNAV]) (12). Subsequently, another diatom-infecting virus (Chaetoceros salssugineum nuclear inclusion virus [CsNIV]) lytic to C. salssugineum was isolated (14). Its genome is distinct from those of other viruses, consisting of a covalently closed circular ssDNA (6 kb) that is partly double stranded (997 bases). A third diatom virus, Chaetoceros nuclear inclusion virus (CspNIV), infecting Chaetoceros cf. gracilis, was described by Bettarel et al. (2); however, no data concerning its genome have been reported. The discovery of these diatom-infecting viruses suggested the possibility that viruses have an impact on diatom populations as the key factors regulating the dynamics of diatom blooms in natural waters. Here, we describe the isolation and characterization of a new ssRNA virus infecting Chaetoceros tenuissimus (CtenRNAV).

MATERIALS AND METHODS

Identification of the host diatom strain. The host diatom strain used in the present study (C. tenuissimus 2-10) was isolated from the Maiko Coast, Seto Inland Sea, Japan, on 10 August 2002. It was intensively observed by both optical microscopy and transmission electron microscopy (TEM) (Fig. 1) and was identified as C. tenuissimus Meunier based on its morphology (10, 18). This planktonic diatom species is so small (<10 μm in length) that it is often confused with morphologically similar diatoms, e.g., Chaetoceros neogracilis and C. gracilis.

Algal cultures. The diatoms used as hosts in isolating viral pathogens were as follows: 1 strain each of Asterionella glacilis, C. salssugineum, Chaetoceros socialis, Coscinodiscus sp., Ditylum brightwelli, Eucampia zodiacus, Rhizosolenia setigera, Stephanopyxis sp., Thalassionema sp., and Thalassiosira sp.; 4 strains of Chaetoc-
FIG. 1. *C. tenuissimus* strain 2-10. Shown are optical micrographs of an intact cell (A) and a CtenRNAV01-infected cell (B) and transmission electron micrographs of negatively stained intact cells (C and D).

eros debilis; 2 strains of *C. tenuissimus*; and 13 strains of *Chaetoceros* sp. Each strain was grown in modified SWM3 medium (3, 5) enriched with 2 mM Na₂SeO₃ using a 12-h light/12-h dark cycle with ca. 110 μmol photons m⁻² s⁻¹ provided as cool white fluorescent illumination at 15°C. In the experiments described below, a clonal and axenic culture of *C. tenuissimus* 2-10 was used as the host for virus characterization. To test the interspecies host specificity, clonal strains of *Namnachloropsis* sp. (*Eustigmatophyceae*), *Oltmannsiella viridis* (Chlorophyceae), *Akashio sanguineum*, *Alexandrium钊nominato* (*Euglenophyceae*), and *Heterocapsa triquetra* were used as hosts. *Karenia mikimotoi*, *Prochlorococcus marinus*, *Prochlorococcus marinus*, and *Heterocapsa akashiwo* (Rhaphidophyceae), as well as the 29 diatom strains, were used. They were also incubated under the conditions mentioned above at either 15 or 20°C.

**Virus isolation.** During June 2004, surface water samples were collected at the mouth of the Shiotsuka River in the Ariake Sound, Japan; sent to the laboratory; and stored at 4°C without fixation within 24 h of sampling. The samples were filtered through a 0.2-μm Dismic-25s filter (Advantage) to remove eukaryotic microorganisms and most bacteria. Algal cultures (0.8 ml) were inoculated with an aliquot (0.2 ml) of the filtrates obtained from the water sample and incubated as described above. The algal cultures inoculated with modified SWM3 served as controls. Consequently, when the host growth was apparently inhibited with the filtrate, we cloned the pathogen through two cycles to extinction using the algae pathogens as controls. An aliquot of the cultures was sampled at 0, 24, 48, and 72 h postinoculation (p.i.) during the first cycle. Cells were gently removed from the surface of the cultures inoculated with an autoclaved pathogen served as the controls. The resultant nucleic acid samples were electrophoresed on a formaldehyde-agarose gel (1.5%; 150 V; 15 min) using an XV Pantera System (DRC Co., Ltd.) or 0.025 M sodium dodecyl sulfate (SDS), 20% glycerol, and 0.005% bromophenol blue and boiled for 5 min. The proteins were separated by SDS-polyacrylamide gel electrophoresis (12.5% polyacrylamide gel; 80 by 40 by 1 mm; 150 V; 50 min) using an Criterion System (Bio-Rad Co., Ltd.). The proteins were visualized using Coomassie brilliant blue stain. Protein molecular mass standards (DRC Co., Ltd.) ranging from 6.5 to 200 kDa were used for calibration.

**Analysis of viral proteins.** The virus suspension was mixed with a fourfold volume of the sample buffer (62.5 mM Tris-HCl, 5% 2-mercaptoethanol, 2% sodium dodecyl sulfate [SDS], 20% glycerol, and 0.005% bromophenol blue) and boiled for 5 min. The proteins were separated by SDS-polyacrylamide gel electrophoresis (12.5% polyacrylamide gel; 80 by 40 by 1 mm; 150 V; 50 min) using an Criterion System (Bio-Rad Co., Ltd.). The proteins were visualized using Coomassie brilliant blue stain. Protein molecular mass standards (DRC Co., Ltd.) ranging from 6.5 to 200 kDa were used for calibration.

**Growth experiment.** A logarithmic-phase culture and a stationary-phase culture of *C. tenuissimus* 2-10 (500 ml) were inoculated with the viral pathogen at an MOI of 11.6 and 12.1, respectively. Host cultures inoculated with an autoclaved viral suspension served as controls. An aliquot of cell suspension was sampled from each culture every 24 h until lysis. The numbers of host cells and viral pathogen were enumerated using optical microscopy and the extinction dilution method (16, 21), respectively.

**Viruses purification.** Five hundred milliliters of viral lysate was sequentially passed through 0.8- and 0.2-μm polycarbonate membrane filters (Nuclepore) to remove cellular debris. The viral suspension was stored at 4°C in the dark overnight. After centrifugation at 75,000 × g for 45°C for 1 h, the viral pellet was resuspended in 20 ml of 10 mM phosphate buffer (pH 7.2) and adjusted to an equal volume of chloroform. After vigorous vortexing, the suspension was centrifuged at 7,500 × g for 20 min at 4°C, the supernatant was centrifuged at 21,000 × g for 4 h at 4°C, and the virus particles were resuspended in 600 μl of ultrapure water.

**Analysis of nucleic acids.** The viral suspension was treated with 1 mg ml⁻¹ proteinase K (Wako Pure Chemical Industries, Ltd.) and 1% sarcosyl (International Technologies, Inc.) at 55°C for 1.5 h. Then, the nucleic acid was extracted by the standard phenol-chloroform method and ethanol precipitated, and the resultant pellet was dissolved in 100 μl of ultrapure water. Aliquots (7 μl) of the nucleic acid solution were incubated with 0.25 U μl⁻¹ DNase I (Promega Corp., Ltd.) or 0.025 μg μl⁻¹ RNase A (Nippon Gene Co., Ltd.) in 0.01 M NaCl and 0.015 mM Na ₃ citrate, pH 7.0, at 37°C for 19 h (19, 22). Nucleic acid samples stored on ice without enzymatic treatment for 1 h served as controls. The resultant nucleic acid samples were electrophoresed on a formaldehyde-agarose gel (1.5%; 150 V; 15 min) using an XV Pantera System (DRC Co., Ltd.) or 0.025 M sodium dodecyl sulfate (SDS) and 0.005% bromophenol blue and boiled for 5 min. The proteins were separated by SDS-polyacrylamide gel electrophoresis (12.5% polyacrylamide gel; 80 by 40 by 1 mm; 150 V; 50 min) using an Criterion System (Bio-Rad Co., Ltd.). The proteins were visualized using Coomassie brilliant blue stain. Protein molecular mass standards (DRC Co., Ltd.) were used for calibration.

**Sequence analysis and phylogenetic analysis.** The sequence analysis was performed as follows. The RNA purified from the virus pellet using the RNAeasy Mini Kit (Qiagen) was reverse transcribed to construct cDNAs with the cDNA Synthesis Kit M-MLV version (Takara Co., Ltd.) using random primers according to the manufacturer’s recommendation. The 5' ends of the resultant double-stranded DNA fragments were phosphorylated using T4 polynucleotide kinase (Promega Corp., Ltd.) and ligated into the plasmid vector pSTV28 (Applied Biosystems). The sequence analysis was performed as follows. The sequence analysis was performed as follows. The sequence analysis was performed as follows. The sequence analysis was performed as follows. The sequence analysis was performed as follows.

Four consensus phylogenetic trees were constructed using the following methods: neighbor-joining (NJ) and maximum likelihood (ML) methods with the Jones-Taylor-Thornton (1) model.
Thornton matrix (JTT model) packaged in the Phylip 3.65 program (6). Amino acid sequences used for comparison in the analyses were as follows, with the database accession numbers (referring to the NCBI database unless otherwise stated): Aichi virus, AB010145; bovine enteric calicivirus, AJ011099; bean pod mottle virus, NC_003496; black queen cell virus, NC_003784; cricket paralysis virus, NC_001834; deformed wing virus, NC_004830; H. akashiwo RNA virus (HaRNAV), NC_005281; Norwalk virus, M87661; human poliovirus 1 Mahoney, V01149; parsnip yellow fleck virus, M83830; rice turgo spherical virus, AAA66056; Sacbrood virus, NC_002066; Schizochytrium single-stranded RNA virus, BAE47143; Triatoma virus, NC_003783; and Taura syndrome virus, NC_003005. Amino acid sequences used for comparison in the analyses were as follows, with the database accession numbers (referring to the NCBI database unless otherwise stated): Aichi virus, AB010145; bovine enteric calicivirus, AJ011099; bean pod mottle virus, NC_003496; black queen cell virus, NC_003784; cricket paralysis virus, NC_001834; deformed wing virus, NC_004830; H. akashiwo RNA virus (HaRNAV), NC_005281; Norwalk virus, M87661; human poliovirus 1 Mahoney, V01149; parsnip yellow fleck virus, M83830; rice turgo spherical virus, AAA66056; Sacbrood virus, NC_002066; Schizochytrium single-stranded RNA virus, BAE47143; Triatoma virus, NC_003783; and Taura syndrome virus, NC_003005.

RESULTS AND DISCUSSION

Isolation and morphological features of the virus. A clonal pathogen causing lysis of C. tenuissimus strain 2-10 was isolated from Ariake Sound. The virus was filterable through a 0.1-μm polycarbonate membrane filter and was serially transferable to 2-10 cells from control cultures showing the normal cytoplasmic organization of cryoprotectant. This is similar to other ssRNA viruses infecting microalgae, e.g., RsRNAV and Heterocapsa circularisquama RNA virus (HcRNAV) (12, 27).

Stability. CtenRNAV01 suspension samples containing 7.02 × 10^10 virus particles were stored at 20, 10, 4, −20, −80, and −196°C in the dark; the titers after 28 days were 3.01 × 10^9, 3.01 × 10^9, 3.85 × 10^9, 2.08 × 10^10, 2.26 × 10^10, and 9.82 × 10^10 infectious units ml^{-1} (relative titer, 4 to 140%), respectively. Hence, CtenRNAV01 was shown to be very stable under any storage conditions without the addition of cryoprotectant. This is similar to other ssRNA viruses infecting microalgae, e.g., RsRNAV and Heterocapsa circularisquama RNA virus (HcRNAV) (12, 27).

Nucleic acids. Denaturing gel electrophoresis showed that the major nucleic acids isolated from CtenRNAV01 virions were 8.9 and 4.3 kb. The electrophoretic bands were sensitive to RNase A both under high- and low-salt concentrations, but not to DNase I (Fig. 3). These data indicated that both bands were ssRNA. There are other viruses whose genomes consist of two species of linear positive-sense ssRNA (e.g., Sadwavirus and Cheravirus) (8, 9), in which each RNA molecule codes for a different product and both are essential for infection. Sequencing of the total viral genome produced only one contig sequence with a poly(A) tail; it was 9,431 bases long, excluding the poly(A) tail. Because only the 3' end was shown to be polyadenylated, the larger band is unlikely to be circular.
Based on these observations, there may be a considerable overlap between the two RNA molecules; further analysis is essential to clarify the detailed genome structure of this virus.

**Proteins.** The sizes and numbers of structural proteins of the CtenRNAV01 virions were estimated using SDS-polyacrylamide gel electrophoresis. CtenRNAV01 had three major polypeptides of 33.5, 31.5, and 30.0 kDa (Fig. 4). The number of major proteins of CtenRNAV01 was the same as that of the ssRNA diatom virus RsRNAV, whose band masses were 41.5, 41.0, and 29.5 kDa (12).

**Viral replication.** When a logarithmic culture of *C. tenuissimus* was inoculated with CtenRNAV01 at a high MOI, the host culture exhibited a growth curve similar to the control culture (without virus inoculation) until 7 days p.i.; however, the virus titer of the infected culture showed a significant increase (~3 orders of magnitude) within the initial 3 days p.i. Then, at 8 to 9 days p.i., the host culture showed a drastic decrease in cell numbers, and the virus titer reached $10^{10}$ infectious units ml$^{-1}$ at 9 days p.i. (Fig. 5A). Considering the initial rapid increase in the virus titer following virus inoculation, the lytic cycle of CtenRNAV01 was estimated to be less than 24 h. The host cell numbers, however, did not show significant decreases until after 8 days p.i., in spite of the remarkably high MOI; this was repeatedly observed in several preliminary tests (data not shown). When a stationary-phase host culture was inoculated with CtenRNAV01 at a high MOI, the host culture also showed a growth curve similar to that of the control culture until 3 days p.i., and the virus titer showed a significant increase (~3 orders of magnitude) within the initial 3 days p.i.; then, at 3 to 6 days p.i., the host cells showed a rapid decrease in number, and the virus yield reached $10^{10}$ infectious units ml$^{-1}$ at 6 days p.i. (Fig. 5B). The rapid increase in virus titer following inoculation of the stationary-phase host culture also suggests that the lytic cycle is shorter than 24 h. We also observed significant accumulation of CtenRNAV01 genome-size RNA (the 8.9-kb band) in the host culture within 24 h p.i., as observed by extracting the total RNA from the host cell pellet (data not shown). We found the time from virus inoculation to the sudden crash of the host culture was shorter in stationary-phase culture than in logarithmic-phase culture (Fig. 5). This suggests higher sensitivity of stationary-phase cells than logistically growing cells to CtenRNAV01 infection. This is contrary to the other microalgal host-virus systems, in which logistically growing cells are more sensitive to virus infection (12, 15). Considering that viruses utilize the biosynthetic functions of hosts, it seems rational that host cells in the vigorously growing (logarithmic) phase are more suitable for viral growth because of their high biosynthesis activity; hence, future interpretation of these unexpected results will be of great interest.

The burst sizes of CtenRNAV01 were estimated to be $1.18 \times 10^6$ and $1.10 \times 10^5$ infectious units cell$^{-1}$, respectively, when virus inoculation was performed in exponential- and stationary-phase cultures; the calculations were based on the changes in host cell numbers and virus titers at 8 to 9 and 3 to 4 days p.i., respectively. These burst sizes are higher than those of the other diatom viruses, $10^3$ infectious units cell$^{-1}$ for RsRNAV and $10^2$ infectious units cell$^{-1}$ for CsNIV (12, 14). A typical thin section of the CtenRNAV-infected cell contained ~3,000 particles. Assuming the virus particles’ aggregation is three dimensional, each cell would contain ~1.5 $\times 10^5$ virus particles. Considering their possible aggregation and partial deficiency, the above estimated burst size (~$1 \times 10^5$ infectious units cell$^{-1}$) appears reasonable.

**BLAST searching and phylogenetic analysis.** By using ORF finder (http://www.ncbi.nlm.nih.gov/projects/orffinder), two open reading frames (ORFs) were identified in the 9.4-kb contig mentioned above. By using BLAST, the upstream and downstream ORFs (ORF-1 and ORF-2) were predicted to be a replicase polyprotein gene and a structural polyprotein gene, respectively. The significant BLAST hits (E value $<1E-60$) of ORF-1 were to the replicase polyprotein of RsRNAV (E value = 0.0) and hypothetical proteins of marine RNA virus JP-A (2E$-109$) and JP-B (6E$-68$); those of ORF-2 were to the capsid polyprotein of RsRNAV (5E$-134$), a hypothetical
protein of marine RNA virus JP-B (4E–87), the capsid polyprotein of HaRNAV (3E–80), and HP of marine RNA virus JP-B (3E–67) (4, 7, 20). Here, we note that JP-A and JP-B are previously unknown RNA viruses whose genomes were assembled from reverse-transcribed whole-genome shotgun libraries originated from British Columbian waters (Canada) (4). The significant BLAST hits may support the hypothesis of Culley et al. (4) that both JP viruses likely have protist hosts.

The RdRp domain was identified in ORF-1 and analyzed as follows. Both the NJ and ML methods were used to assess the phylogenetic relationships among positive-sense ssRNA viruses, including CtenRNAV. Similar topologies were obtained using the two methods; hence, only the ML phylogenetic tree is shown in Fig. 6. The monophyly of CtenRNAV01 and RsRNAV was supported with a bootstrap value of 100% (Fig. 6). This suggests the existence of a diatom-infecting ssRNA virus clade within the positive-sense ssRNA viruses. The tree also suggests lower relatedness of the diatom-infecting ssRNA viruses with those infective to the other marine stramenopile organisms: Schizochytrium single-stranded RNA virus, infecting a fungoid protist, Siccodichytrium minutum (previously reported as Schizochytrium sp.) (23), and HaRNAV, infecting a bloom-forming raphidophyte, H. akashiwo (7).

**Implications.** CtenRNAV is the second ssRNA virus described that infects diatoms. The most remarkable feature of this virus is its distinctively high yield of \(10^{10}\) infectious units ml\(^{-1}\). Generally, the highest yield found in other viruses infecting microalgae is between \(10^7\) and \(10^8\) ml\(^{-1}\); thus, the yield of CtenRNAV01 is 2 to 3 orders of magnitude higher. The high concentration of the host cell in a culture (\(10^6\) cells ml\(^{-1}\)) and the burst size of CtenRNAV01 (\(10^4\) infectious units cell\(^{-1}\)) may reasonably contribute to the high yield. This feature may make this virus suitable for experiments in which large numbers of virions are necessary.

There is insufficient data to determine the roles of ssRNA viruses in marine environments. However, studies of the bloom-forming dinoflagellate H. circularisquama and its infectious ssRNA virus, HcRNAV, provide a rational explanation for the ecological relationship between them. HcRNAV affects the dynamics (especially bloom termination) of host algae and may be preserved in sediments in the absence of its host population (13, 25). The ecology of C. tenuissimus and its viruses is now under examination; some preliminary results of our recent field survey show the existence of virus-like agents that are lytic to C. tenuissimus in both waters and sediments of western Japan, and they increase during blooms of Chaetoceros spp. (Y. Tomaru, unpublished data). This may indicate an intimate
ecological relationship between the host diatom and its viruses, as was observed with H. circularisquama and HcRNAV. Further study will be required to determine the ecological importance of CtenRNAV as an agent affecting the dynamics of C. tenuissimus in natural environments.

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