

# Isolation and Characterization of Two Viruses with Large Genome Size Infecting *Chrysochromulina ericina* (Prymnesiophyceae) and *Pyramimonas orientalis* (Prasinophyceae)

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Two lytic viruses specific for *Chrysochromulina ericina* (Prymnesiophyceae) and for *Pyramimonas orientalis* (Prasinophyceae) were isolated from Norwegian coastal waters in June 1998. The lytic cycle was 14–19 h for both viruses; the burst size was estimated at 1800–4100 viruses per host cell for the *Chrysochromulina* virus and 800–1000 for the *Pyramimonas* virus. Thin sections of infected cells show that both viruses replicate in the cytoplasm and that they have a hexagonal cross section, indicating icosahedral symmetry. The *Chrysochromulina* virus had a particle size of 160 nm and a genome size of 510 kbp; the size of the major polypeptide was 73 kDa. The *Pyramimonas* virus had a particle size of 220 × 180 nm and a genome size of 560 kbp; the size of the major polypeptide was 44 kDa. The genome sizes of these viruses are among the largest ever reported for viruses and they are larger than the minimum required for cellular life. The *Chrysochromulina* virus clone CeV-01B and the *Pyramimonas* virus clone PoV-01B described in this study have several properties in common with other viruses infecting microalgae, suggesting that they belong to the *Phycodnaviridae*. © 2001 Academic Press

**Key Words:** algal virus; large dsDNA viruses; *Chrysochromulina ericina*; *Pyramimonas orientalis*; *Phycodnaviridae*.

## INTRODUCTION

Virus and virus-like particles (VLPs) have been observed in at least 44 taxa of eukaryotic algae (Van Etten *et al.*, 1991; Van Etten and Meints, 1999) including many important marine phytoplankton species. However, to date, only 6 different microalgal and 2 different brown algal host–virus systems have been reported to be in culture and studied to any extent. These include viruses infecting the unicellular algae *Chlorella* sp. (Van Etten *et al.*, 1991), *Micromonas pusilla* (Waters and Chan, 1982), *Aurococcus anophagefferens* (Milligan and Cosper, 1994), *Chrysochromulina brevifilum* (Suttle and Chan, 1995), *Phaeocystis pouchetii* (Jacobsen *et al.*, 1996), *Heterosigma akashiwo* (Nagasaki and Yamaguchi, 1997), and the brown algae *Ectocarpus* (Maier *et al.*, 1998) and *Feldmannia* (Henry and Meints, 1992).

The PBCV-1 virus, which infects certain endosymbiotic unicellular *Chlorella*-like green algae, is the prototype of the recently recognized *Phycodnaviridae* family. The characteristic features of this family are large particle size (>100 nm in diameter), polyhedral symmetry, lack of external membrane, cytoplasmic site of assembly, and a large (330–380 kbp) dsDNA genome (Van Etten and Meints, 1999). Many of these properties are shared by other algal viruses but the proper taxonomic affiliation of these viruses is unknown (Van Etten *et al.*, 1991; Suttle, 2000). However, analysis of viral DNA polymerase gene

sequences (DNA *pol*) and total genomic DNA hybridization shows that microalgal viruses infecting *Chlorella* sp., *M. pusilla*, and *C. brevifilum* form a distinct phylogenetic group, suggesting that they all belong to the *Phycodnaviridae* (Chen and Suttle, 1996).

Viruses and VLPs are abundant in aquatic ecosystems and viruses are now recognized to play a significant role in natural microbial communities (Bergh *et al.*, 1989; Proctor and Fuhrman, 1990; Suttle, 2000; Wommack and Colwell, 2000). Most of these particles are presumably bacteriophages, but viruses infecting algae have also been demonstrated to be important agents of phytoplankton mortality in marine ecosystems (Suttle *et al.*, 1990; Bratbak *et al.*, 1993; Suttle, 1994). Viruses may prevent and terminate phytoplankton blooms (Suttle, 2000; Wommack and Colwell, 2000), including blooms of harmful and toxic species (Milligan and Cosper, 1994; Nagasaki *et al.*, 1994b). They affect the marine food web by lysing host cells and thereby turn the carbon and nutrient flow away from the grazing food chain to the benefit of heterotrophic bacteria (Gobler *et al.*, 1997; Bratbak *et al.*, 1998). They may also have a direct impact on world climate by causing increased production of dimethylsulfoniopropionate in algal host cells (Hill *et al.*, 1998; Malin *et al.*, 1998), the precursor of dimethylsulfide, which affects cloud albedo and causes natural acid rain. To learn more about the general features and ecological significance of algal viruses, and to develop molecular methods that may be applied in field studies, it has become necessary to bring more host–virus systems

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into the laboratory where they can be characterized and subjected to experimental studies.

The purpose of this paper is to describe two new algal viruses infecting the prymnesiophyte *Chrysochromulina ericina* and the prasinophyte *Pyramimonas orientalis*. Both hosts are marine phytoplankton species; they have a worldwide distribution and most commonly occur in low numbers. *C. ericina* have been observed to form blooms together with other *Chrysochromulina* species (Simonsen and Moestrup, 1997), while blooms of *P. orientalis* have not been reported (Daugbjerg and Moestrup, 1993). The viruses we describe have genome sizes of 510 kbp (CeV-01B) and 560 kbp (PoV-01B), which are among the largest genome sizes ever reported for viruses.

## RESULTS AND DISCUSSION

### Viral isolation

Among the 13 host phytoplankton algae used when screening for viruses we isolated viruses infecting *C. ericina* (Prymnesiophyceae) and *P. orientalis* (Prasinophyceae). Both viruses were propagated from the plankton concentrate exposed to UV radiation for 15 s. The same approach for isolation of viruses has been used previously with success (Nagasaki and Yamaguchi, 1997; Bratbak *et al.*, 1996; Jacobsen *et al.*, 1996). By concentrating the plankton fraction from seawater we include infected and virus-producing cells as well as cells that carry a latent virus. Presumably this treatment increases the probability of isolating infectious viruses compared to the alternative approach, which is to remove larger plankton by filtration (usually through a 0.2- $\mu$ m-pore-size filter), concentrate the virus-sized fraction by ultrafiltration (usually on a 10,000–30,000 MW cut off filter), and add this concentrate to selected host cultures (Suttle *et al.*, 1991; Milligan and Cosper, 1994). The UV exposure is intended to induce virus production in cells infected with latent viruses, but the actual mechanism and effect remain unexplored.

VLPs have previously been observed in thin sections of cultured *P. orientalis* isolated from Danish coastal waters (Moestrup and Thomsen, 1974). More recently, Mortimer and Patching (1998, 2000) reported the isolation of a virus infectious to *P. orientalis* from Irish coastal waters. However, in both cases the viral particles were different from those described here (see below). We are not aware of any reports on virus or VLPs in *C. ericina*. Viruses infecting other Prymnesiophytes, i.e., *C. brevifilum* (Suttle and Chan, 1995), *P. pouchetii* (Jacobsen *et al.*, 1996), and *Emiliana huxleyi* (Bratbak *et al.*, 1996) and the Prasinophyte *M. pusilla* (Waters and Chan, 1982) have been isolated and described. The virus clone infecting *C. ericina* was named CeV-01B and the virus clone infecting *P. orientalis* was named PoV-01B.

### Host range

CeV-01B infected three of four *C. ericina* strains and PoV-01B infected one of two *P. orientalis* strains we used in the host range experiment (Table 1). Intraspecies host specificity may thus be common for viruses of phytoplankton, as this also has been described for viruses infecting *H. akashiwo* (Nagasaki and Yamaguchi, 1998) and *M. pusilla* (Sahlsten, 1998). Moreover, the strains of *C. ericina* (IFM, Q17, K-0562) that were sensitive to CeV-01B are all isolated from different locations in Scandinavian coastal waters, while the resistant strain (CCMP 281) originates from the north Pacific Ocean (Table 1). This might reflect geographical differences among *C. ericina*.

### Life cycles

The lytic cycle of the viruses was investigated to determine latent time and burst size (Fig. 1). The initial increase in abundance of free viral particles was for both CeV-01B and PoV-01B observed after 14–19 h, and the maximum abundance was reached after ca. 30 h. The viruses of the related algae *M. pusilla* and *Ph. pouchetti* have comparable latent periods of 7–14 and 12–18 h, respectively (Waters and Chan, 1982; Jacobsen *et al.*, 1996).

The increase in virus abundance occurs earlier than the corresponding decrease in host cell abundance, suggesting that some virus producing cells do not immediately lyse and release free viral particles but remain intact for some time and are therefore included in the algal counts. This makes it difficult to obtain a reliable estimate of the burst size. Assuming that all cells actually did lyse and contribute to the increase in viral abundance, we can estimate a burst size of 1800 for CeV-01B and 800 for PoV-01B. Using only the observed decrease in cell abundance, however, we estimate burst sizes at 4100 and 1000, respectively. These estimates are in any case higher than those found for other phytoplankton, which range from 70 to 770 virus particles per cell (Van Etten *et al.*, 1991; Waters and Chan, 1982; Suttle and Chan, 1995; Bratbak *et al.*, 1996; Jacobsen *et al.*, 1996; Nagasaki *et al.*, 1999).

### Characteristics

Observations by TEM show that both viruses are tailless, that they lack an external membrane, and that they have a hexagonal cross section, suggesting icosahedral symmetry (Figs. 2 and 3). The diameter of CeV-01B particles is about 160 nm while that of PoV-01B particles is about 220  $\times$  180 nm. Thin sections of infected *C. ericina* and *P. orientalis* cells indicate that both viruses replicate in the cytoplasm (Fig. 3). The size, icosahedral morphology, and cytoplasmic site of virus assembly are properties that these viruses have in common with many other

TABLE 1  
List of Phytoplankton Used When Screening for Virus and in the Host Range Experiments

Species	Strain code <sup>a</sup>	Strain origin if not Scandinavian coastal waters	Culture temperature (°C)	Strains used for virus screening	Strains lysed by PoV-01B <sup>b</sup>	Strains lysed by CeV-01B <sup>b</sup>
Basillariophyceae						
<i>Cylindrotheca closterium</i>	IFM		15	x		
Chrysophyceae						
<i>Pseudopedinella pyriformis</i>	IFM		15	x		
Haptophyceae						
(Prymnesiophyceae)						
<i>Chrysochromulina</i> sp.	IFM		15		—	—
<i>Chrysochromulina ericina</i>	IFM		15	x	—	+
<i>C. ericina</i>	Q17	15		—	+	
<i>C. ericina</i>	K-0562		15		—	+
<i>C. ericina</i>	CCMP281	North Pacific Ocean	15		—	—
<i>Chrysochromulina</i> sp.	CCMP284	North Pacific Ocean	8		—	—
<i>Chrysochromulina kappa</i>	EN3		8		—	—
<i>Chrysochromulina polylepis</i>	B11		8		—	—
<i>Chrysochromulina hirta</i>	C.hi-1		8		—	—
<i>Phaeocystis globosa</i>	IFM	Dutch coast	15	x	—	—
<i>Prymnesium parvum</i>	AL		15	x	—	—
<i>Emiliania huxleyi</i>	Br		8		—	—
<i>E. huxleyi</i>	D2		8	x	—	—
Chryptophyceae						
<i>Hemiselmis</i> sp.	IFM		15	x		
<i>Teleaulax amphiol</i>	Dan		15	x		
Prasinophyceae						
<i>Pyramimonas orientalis</i>	IFM		15	x	+	—
<i>P. orientalis</i>	K-0003		15		—	—
<i>Pyramimonas amyliifera</i>	IFM		15		—	—
<i>Pyramimonas cordata</i>	IFM		15		—	—
<i>Pyramimonas</i> sp.	IFM		15	x	—	—
<i>Nephroselmis rotunda</i>	IFM		15		—	—
<i>Tetraselmis</i> sp.	IFM			x		
Dinophyceae						
<i>Katodinium rotundatum</i>	Br		15	x		
Cyanophyceae						
<i>Synechococcus</i> sp.	Dan		15	x		

<sup>a</sup> All cultures were obtained from the culture collection at the University of Bergen (IFM), Norway.

<sup>b</sup> —, not lysed; +, lysed.

algal virus VLPs (Van Etten *et al.*, 1991), including *E. huxleyi* (Bratbak *et al.*, 1993), *C. brevifilum* (Suttle and Chan, 1995), *Ph. pouchetti* (Jacobsen *et al.*, 1996), *H. akashiwo* (Nagasaki and Yamaguchi, 1997), and *A. anophagefferens* (Gastrich *et al.*, 1998). Moestrup and Thomsen (1974) observed VLPs in thin sections of *P. orientalis* that were 60 and 200 nm in diameter and located in the nuclear region. The virus particles observed in *P. orientalis* by Mortimer and Patching (1998, 2000) were 74 nm in diameter.

In CeV-01B we found one major polypeptide of approximately 73 kDa, at least three minor polypeptides with molecular masses between 27 and 50 kDa, and one polypeptide of 110 kDa (Fig. 4). In PoV-01B we found a single major polypeptide of approximately 44 kDa. Two additional minor polypeptides of approximately 103 and 122 kDa and some very weak bands corresponding to

polypeptides of 20 and 27 kDa were also detected (Fig. 4). The size of the major polypeptide, which we presume is the main capsid protein, is comparable to the size of the major polypeptides found in other algal viruses such as the *Chlorella* virus PBCV-1, 54 kDa (Skrdla *et al.*, 1984); the *Ph. pouchetii* virus PpV-01, 59 kDa (Jacobsen *et al.*, 1996); and the *A. anophagefferens* virus AaV-01, 68 kDa (Garry *et al.*, 1998).

### Genome size

Pulsed-field gel electrophoresis (PFGE) showed that CeV-01B has a genome size of 510 kb while PoV-01B has a genome size of 560 kb (Fig. 5). This is considerably larger than the *Chlorella* viruses, which have genome sizes ranging from 330 to 380 kb (Van Etten and Meints, 1999). Both CeV-01B and PoV-01B DNA were cleaved by

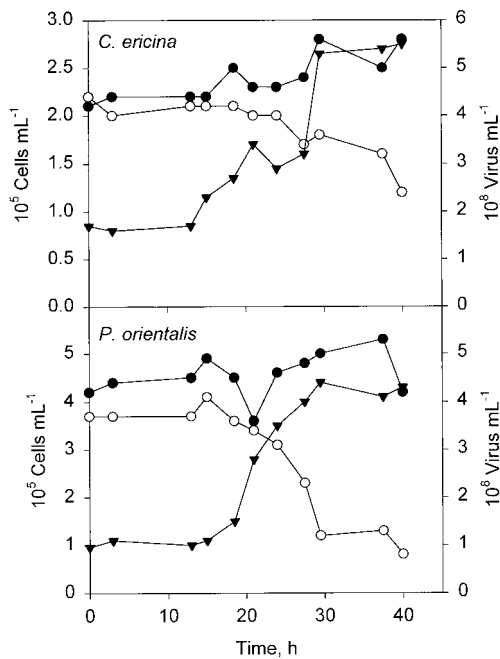


FIG. 1. Growth of *Chrysocromulina ericina* and *Pyramimonas orientalis* in noninfected control cultures and in cultures infected with their respective viruses, CeV-01B and PoV-01B. Algal cells in control cultures (●). Algal cells in infected cultures (○). Virus in infected cultures (▼).

the restriction enzymes *MboI* and *Sau3AI*, showing that they are dsDNA viruses.

The large sizes of the CeV-01B and PoV-01B genomes are surprising. The minimum number of genes sufficient for cellular life has been estimated to be 300 (Hutchison *et al.*, 1999; Mushegian, 1999), which is equivalent to 375 kbp assuming 1.25 kb of DNA per gene (Fraser *et al.*, 1995). The genome of the smallest known microorganism (*Mycoplasma genitalium*) is 580 kbp (Fraser *et al.*, 1995). It is both interesting and thought-provoking that viruses can have a genome size comparable to that of simple living organisms and significantly larger than the theoretical minimum for cellular life.

The largest dsDNA viruses listed by The International Committee on Taxonomy of Viruses (ICTV) are the *Phycodnaviridae* (160–380 kbp), the *Iridoviridae* (140–383 kbp), and the *Poxviridae* (130–375 kbp). The largest virus ever reported is the *Bacillus megaterium* phage G with a dsDNA genome of about 670 kbp (Hutson *et al.*, 1995). Sequencing the *Phycodnaviridae*-type virus PBCV-1 (*Chlorella* virus) has revealed many unexpected and interesting putative genes that encode, e.g., different DNA methyltransferases, DNA site-specific endonucleases, and enzymes involved in manipulating sugars, synthesizing polysaccharides, and metabolizing lipids (Van Etten and Meints, 1999). The size of the CeV-01B and PoV-01B genomes raises the following questions: do these viruses have the same genes as PBCV-1, do they have other or additional genes, do they have many insertions of large repetitive elements as shown for the *Feldmannia*

virus (Lee *et al.*, 1995), or do they have many or large introns? The last possibility seems less likely since many large DNA viruses either lack introns or have only a few that are short, such as virus PBCV-1 (Van Etten and Meints, 1999).

A conserved region in the DNA polymerase gene (*DNA pol*) found in some viruses infecting microalgae has been used to examine the genetic diversity and relationship between these viruses (Chen and Suttle, 1995, 1996; Chen *et al.*, 1996). When we used the same degenerate primer set designed to amplify this region by nested PCR (AVS1, AVS2, and POL; Chen and Suttle, 1995) on CeV-01B and PoV-01B only a diffuse band of approximately 200 bp was obtained (data not shown). With the *M. pusilla* virus MpV-SP1 the expected DNA fragment of ca. 500 bp was obtained (Chen and Suttle, 1995). Amplification with these primers has also been tried on the *Feldmannia* virus with negative results (Chen and Suttle, 1995). However, phylogenetic analysis of the *DNA pol* gene from the *Feldmannia* virus clearly placed the virus in the *Phycodnaviridae* family (Lee *et al.*, 1998). The reason for the negative results obtained with CeV-01B and PoV-01B may be that these viruses lack DNA

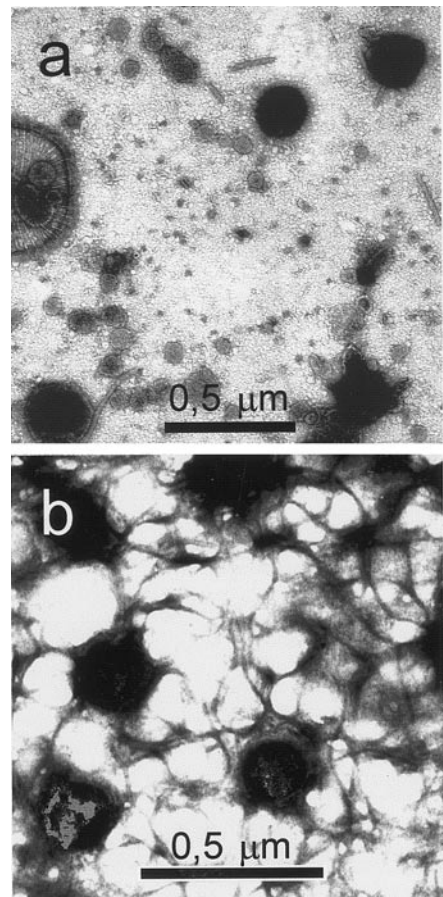


FIG. 2. Transmission electron micrographs of free viral particles. (a) *Chrysocromulina ericina* virus CeV-01B and (b) *Pyramimonas orientalis* virus PoV-01B.

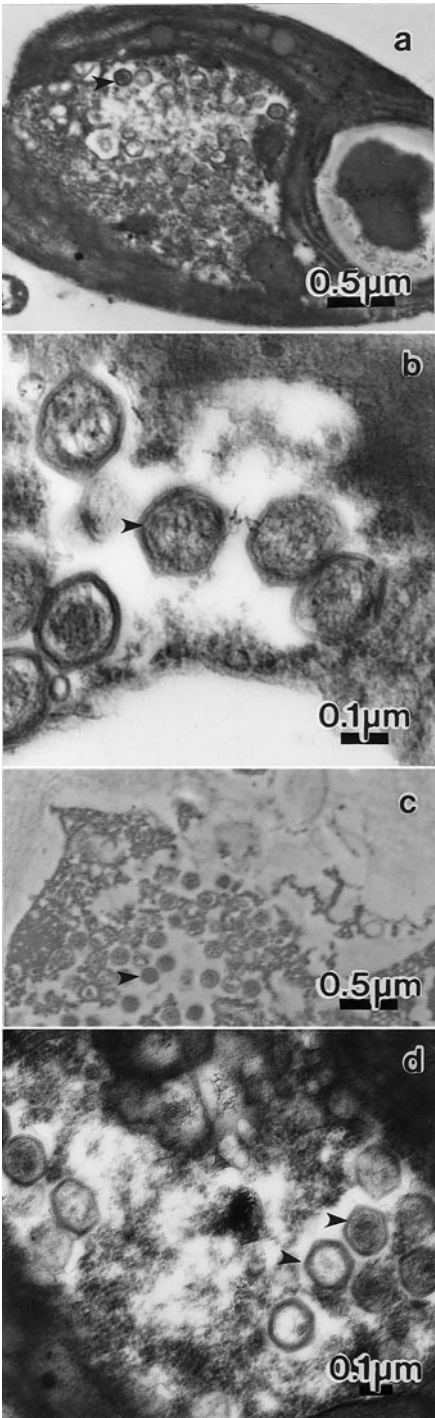


FIG. 3. Thin sections of virus infected cells. (a, b) *Pyramimonas orientalis* infected with PoV-01B virus. (c, d) *Chrysocromulina ericina* infected with CeV-01B.

polymerase or that their DNA polymerases differ from those of the viruses investigated by Chen and Suttle (1995). The third possibility is that CeV-01B and PoV-01B have the same DNA polymerase as other algal viruses but the specificities of the degenerate primers AVS1 and AVS2 are not broad enough to detect the gene in these new viruses. Considering the similarities in particle size,

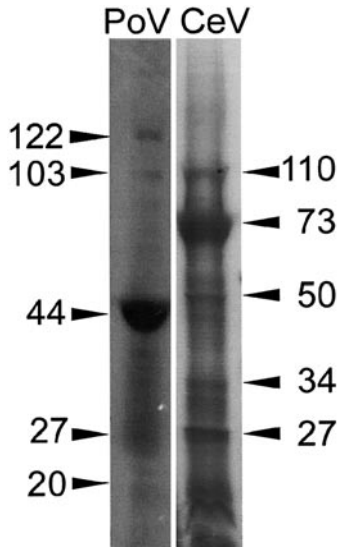


FIG. 4. SDS-PAGE showing major polypeptides of PoV-01B (Lane PoV) and CeV-01B (Lane CeV). Numbers indicate the molecular size in kDa.

morphology, site of assembly, type of nucleic acid, and genome size, we suggest that both CeV-01B and PoV-01B belong to the *Phycodnaviridae*.

Some phytoplankton species form almost monospecific blooms while others occur rather in low numbers as members of more diverse plankton communities. Viral activity may provide an explanation for this difference. Several case studies on bloom-forming phytoplankton,

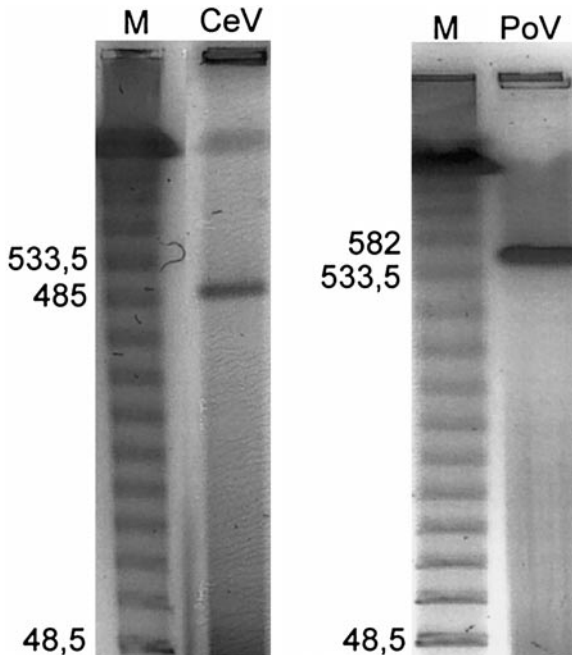


FIG. 5. Virus genome size determined by pulsed-field gel electrophoresis. Lane CeV, CeV-01B. Lane PoV, PoV-01B. Lane M, molecular size standard,  $\lambda$  concatemers. Numbers indicate the molecular size in kb.

including *E. huxleyi* (Bratbak *et al.*, 1993, 1995), *A. anophagefferens* (Sieburth *et al.*, 1988; Milligan and Cosper, 1994), and *H. akashiwo* (Nagasaki *et al.*, 1994a,b), suggest that viruses are important in the termination of blooms. Although the evidence remains largely circumstantial, the most important role for algal viruses has nevertheless also been suggested to be to maintain community diversity by preventing bloom formation (Suttle, 2000; Wommack and Colwell, 2000). The basic difference between the two ideas is that in the first case viruses attack when the host population density is so high that we consider it a bloom, while in the second case viruses attack when the population density is still low. If viruses are the key factor in blooming, the difference between bloom-forming and non-bloom-forming phytoplankton is not due to the properties of the algae themselves, but to their respective viruses. The viruses of bloom-formers may thus be expected to replicate slowly and require a high host population density to propagate efficiently, while the viruses of non-bloom-formers may be expected to have a very efficient propagation strategy that only requires a low host density. *C. ericina* and *P. orientalis* usually occur in low abundance in seawater (Daugbjerg and Moestrup, 1993) and if the viruses we have isolated play a role in the ecology and population dynamics of these species, we may expect them to be of the latter type. Future studies comparing the viruses of bloom-forming and non-bloom-forming phytoplankton that now are available in culture may confirm or reject this hypothesis.

## MATERIALS AND METHODS

### Algal cultures

The phytoplankton species used for isolation of viruses and for host range experiments are listed in Table 1. The seawater medium we used was f/2 (Gulliard, 1975) and the cultures were incubated at 8 or 15°C (see Table 1) in continuous white light from fluorescent tubes (40–50  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  measured at the surface of the cultures). Cell numbers were determined in a Fuchs-Rosenthal hemacytometer after glutaraldehyde fixation (2.5% final concentration).

### Isolation, cloning, and purification of viruses

A water sample of 40 L was collected on June 23, 1998, in the bay by the Marine Biological Field Station of the University of Bergen, adjacent to Raunefjorden, 20 km south of Bergen, western Norway. The plankton community was concentrated by centrifugation in a Beckman J2-HS centrifuge equipped with a JCF-Z continuous flow rotor (Beckman Instruments, Fullerton, CA) using a speed of 5000 rpm and a flow rate of 600 mL  $\cdot$  min<sup>-1</sup>. This procedure retained particles larger than 100,000S in a final volume of ca. 800 mL. To provoke virus production in

cells that might carry inducible viruses, we exposed 30-mL aliquots of the concentrate to UV radiation (254 nm, Philips fluorescent tube, type 57415 P/40 A6 T UV 15 W, 40-cm distance) for 15, 30, and 60 s, and stored them overnight at 4°C in the dark (Bratbak *et al.*, 1996). The three UV-treated samples plus one untreated sample were then filtered through 0.45- $\mu\text{m}$ -pore-size Supor filters (Pall Gelman Laboratory, Ann Arbor, MI) to remove algae, bacteria, and cellular debris before 1 mL of each were added to 50-mL cultures of exponentially growing algae. Cultures were incubated for 4 days as described above and then visually inspected and compared to noninfected control cultures. One milliliter of any culture showing signs of cell lysis was transferred to fresh algal cultures as above. The presence of viruses in cultures that lysed after several subsequent transfers was confirmed by fluorescence microscopy after staining with SYBR Green I (Noble and Fuhrman, 1998).

Stock cultures of virus were centrifuged twice for 30 min at 7500 rpm and 4°C in a Beckman JS-7.5 rotor to remove larger particles and made axenic by filtration through 0.2- $\mu\text{m}$ -pore-size Meditron syringe filters (Schleicher & Schuell GmbH, Dassel, Germany). The purity of the stock cultures was confirmed by fluorescence microscopy after staining with SYBR Green I (Noble and Fuhrman, 1998).

Clonal virus isolates were obtained by 10-fold dilution from 10<sup>-1</sup> to 10<sup>-12</sup> in microtiter plates containing 100  $\mu\text{L}$  exponential growing host culture per well. The plates were incubated for 14 days as described and the contents of the most diluted wells showing lysis were then transferred to new microtiter plates. This procedure was repeated three times.

### Host range

The host range of each virus was tested by adding 100  $\mu\text{L}$  of fresh lysate to duplicate cultures (20 mL) of 22 different exponentially growing strains of phytoplankton (Table 1). Cultures that did not lyse within 14 days after inoculation were considered to be not susceptible to the virus. Lysed cultures were inspected by light microscopy and by fluorescence microscopy after staining with SYBR Green I (Noble and Fuhrman, 1998) to confirm virus proliferation.

### Infection cycle

Host cultures (200 mL) in late log growth phase were infected by adding 200 mL of fresh lysate to give an initial virus particle to host ratio on the order of 1000. Noninfected control cultures were diluted with f/2 medium. The cultures were incubated as described above and sampled for 45 h. For counting algae and virus, parallel samples (1.5 mL) were fixed in 2.5% glutaraldehyde for 30 min at 4°C and then frozen in liquid nitrogen. Samples were stained with SYBR Green I and analyzed using a

FACSCalibur flow cytometer (Becton Dickinson, Rutherford, NJ) according to Marie *et al.* (1999).

### Genome size

The genome size of the viruses was determined by PFGE. Cell debris and bacteria were removed from 80 mL of a fresh lysate by centrifuging twice (2 times 30 min at 7500 rpm and 4°C in a Beckman JS-7.5 rotor). The viruses in the supernatant were then centrifuged for 2 h at 28,000 rpm and 10°C (Beckman SW28 rotor). Pellets were resuspended in 400  $\mu$ L SM buffer (0.1 M NaCl, 8 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 50 mM Tris-HCl, 0.005% (w/v) glycerol) (Wommack *et al.*, 1999) and incubated at 4°C overnight. Equal volumes of virus concentrate and molten 1.5% InCert agarose (BMA, Rockland, ME) were mixed, dispensed into plug molds, and left to solidify. The plugs were then punched out from the molds into a small volume of buffer (250 mM EDTA, 1% SDS) containing 1 mg/mL proteinase K and incubated in the dark at 30°C overnight. The digestion buffer was decanted and the plugs were washed three times for 30 min each in TE buffer (10 mM Tris-base; 1 mM EDTA, pH 8.0). Virus-agarose plugs were stored at 4°C in TE 20:50 buffer (20 mM Tris, 50 mM EDTA, pH 8.0).

Agarose plugs with virus, and with  $\lambda$ -phage concatamers (Bio-Rad, Richmond, CA) that served as molecular weight markers, were placed into wells of a 1% SeaKem GTG agarose (BMA) gel in 1 $\times$  TBE gel buffer (90 mM Tris-borate and 1 mM EDTA, pH 8.0) with an overlay of molten 1% agarose. Samples were electrophoresed using a Bio-Rad DR-II CHEF Cell unit operating at 200 V with pulse ramps from 20 to 45 s at 14°C for 23 h in 0.5 $\times$  TBE tank buffer (45 mM Tris-borate and 1 mM EDTA, pH 8.0). After electrophoresis gels were stained for 30 min with SYBR Green I (Molecular Probes, Eugene, OR) according to the manufacturer's instructions and digitally scanned for fluorescence using a FujiFilm FLA2000 laser fluoroimager.

### Enzyme digestion

Virus DNA bands on the PFGE gels were sliced out of the gels and treated with  $\beta$ -agarase (FMC BioProducts) as recommended by the manufacturer. The liquid agarose-DNA mixture was then concentrated using Microcon Y50 columns (Millipore Corp., Bedford, MA). The DNA concentration was measured using PicoGreen (Molecular Probes) according to the manufacturer's recommended procedure. To characterize the nature of the viral genomes, approximately 0.5  $\mu$ g of viral DNA was digested with RQ1 RNase-free DNase (Promega, Madison, WI) at 37°C for 1 h in a total volume of 20  $\mu$ L following the manufacturer's instructions. The 1-kb DNA ladder (New England Biolabs, Beverly, MA) was used as control. Restriction enzyme digestion of the viruses was performed by digesting 1.0  $\mu$ g of viral DNA with the

restriction enzymes *Mbol* and *Sau3AI* (Promega) in a total volume of 20  $\mu$ L overnight at 37°C. The digest was visualized by PFGE as described above, but with pulse ramps from 8 to 30 s at 14°C for 9 h.

### SDS-PAGE

Viruses were concentrated from fresh lysates by ultracentrifugation as described above, purified on 10–40% sucrose gradients, resuspended in PBS buffer, and pelleted by ultracentrifugation. The pellets were suspended in SDS sample buffer (2.5% SDS, 5% glycerol, 0.025% bromophenol blue, 62.5 mM Tris, pH 6.8, and 6.25 mM DTT) and heated to 100°C for 10 min. Acrylamide/Tris-glycine gels (4–20%, 10  $\times$  10 cm and 1 mm thick, FMC BioProducts) were loaded with 15  $\mu$ L protein suspension and run for 1 h at 200 V. Proteins were visualized by Coomassie brilliant blue staining.

### Transmission electron microscopy

Fresh virus samples were harvested onto electron microscope grids by ultracentrifugation and stained with uranyl acetate as described in Bratbak and Heldal (1993). Samples for thin sectioning of cells were prefixed with 2.5% glutaraldehyde. Cells were concentrated by centrifugation, gently washed, embedded in sodium alginate (1.5%, w/v) in 0.1 M sodium cacodylate buffer (pH 7.3), and gelled in 50 mM  $\text{CaCl}_2$  (Tamponnet *et al.*, 1988). Small amounts of cells embedded in alginate were postfixed with 2% osmium tetroxide in sodium cacodylate buffer (pH 7.3) for 1–2 h, dehydrated in a graded ethanol series, and embedded in LR White acrylic resin (Agar Scientific, Essex, UK). The sections were poststained with 2% uranyl acetate and lead citrate (Reynolds). Ultrathin sections were observed in a Jeol 100S transmission electron microscope.

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