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ISOLATION AND CHARACTERIZATION OF A VIRUS THAT INFECTS *EMILIANIA HUXLEYI* (HAPTOPHYTA)[1]

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The isolation and characterization of a virus (designated EhV) that infects the marine coccolithophorid *Emiliania huxleyi* (Lohmann) Hay & Mohler are described. Three independent clones of EhV were isolated from Norwegian coastal waters in years 1999 and 2000. EhV is a double-stranded DNA-containing virus with a genome size of ~415 kilo-base pairs. The viral particle is an icosahedron with a diameter of 160–180 nm. The virus particle contains at least nine proteins ranging from 10 to 140 kDa; the major capsid protein weighs ~54 kDa. EhV has a latent period of 12–14 h and a burst size of 400–1000 (mean, 620) viral particles per cell. A phylogenetic tree based on DNA polymerase amino acid sequences indicates EhV should be assigned to the *Phycodnaviridae* virus family and that the virus is most closely related to viruses that infect *Micromonas pusilla* and certain *Chlorella* species.

Key index words: **algal bloom termination; algal virus; *Emiliania huxleyi*; Haptophyta; large dsDNA viruses; *Phycodnaviridae*; virus ecology**

Abbreviations: **EhV, *Emiliania huxleyi* virus; PBCV-1, *Paramecium bursaria* chlorella virus**

Viruses are the most abundant biological agents in marine aquatic environments (see Fuhrman 1999, Suttle 2000, Wommack and Colwell 2000). However, only recently have investigators begun to examine the influence of viruses on the ecology of marine bacteria and algae. These initial studies indicate that both lytic and latent viruses are important in the life cycles of many, if not all, algae. For example, each of eight species of filamentous brown algae examined (i.e. two species of *Ectocarpus*, three species of *Feldmannia*, and one species each of *Hincksia, Myriotrichia*, and *Pilayella*) has a species-specific lysogenic virus that is intimately involved in its sexual cycle (Müller et al. 1998). Viruses are also involved in the disappearance of algal blooms caused by *Emiliania huxleyi* (Bratbak et al. 1993, 1995, Castberg et al. 2001), *Aureococcus anophagefferens* (Sieburth et al. 1988, Milligan and Cosper 1994), and *Heterosigma akashiwo* (Nagasaki et al. 1994a,b, 1999). The implications from these studies are that in marine environments viruses play important roles in algal bloom dynamics, nutrient cycling, algal community structure, and possibly gene transfer between organisms.

However, despite this accumulating evidence of the ecological importance of algal viruses, only about 12 viruses that infect microalgae have been cultured and even fewer subjected to minimal characterization (Table 1). Most of these viruses are large polyhedral (120–200 nm in diameter) particles containing huge double-stranded (ds)DNA genomes, up to ~560 kb. Recently, however, a couple of viruses have been described that do not fit this general description, for example, a dsRNA virus that infects *Micromonas pusilla* (Brussaard et al. 2001) and a 30 nm in diameter polyhedral virus that infects *Heterosigma akashiwo* (Lawrence et al. 2001). Ultimately, we may discover that algae harbor a variety of virus types.

Emiliania huxleyi (Lohmann) Hay & Mohler and related coccolithophorid species have a worldwide distribution, and every spring and summer they form massive blooms in offshore, coastal, and oceanic waters at mid-latitudes (45–65° N) (Ackleson et al. 1988). Typically, maximum cell concentrations reach about 5×10^6 cells·L^{-1} (Holligan et al. 1983, Ackleson et al. 1988), but concentrations as high as 1.2×10^8 cells·L^{-1} have been reported (Berge 1962). The abundance and wide distribution of *E. huxleyi* and its production of calcium carbonate coccoliths and dimethylsulfoniopropionate (precursor of dimethylsulfide) make it an important species with respect to sediment formation (Berger 1976, Honjo 1976, Westbroek et al. 1989) and to ocean climate and natural acid rain (Charlson et al. 1987).

Electron microscopic studies of *E. huxleyi* blooms provided the first indication that viruses have a significant impact on the native population of the alga (Bratbak et al. 1993, 1995). This report describes the

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TABLE 1. Viruses of eukaryotic microalgae that are in culture.

Virus	Host	Particle size[a] (nm)	Genome type and size (kbp)	Major protein (kDa)	Latent period (h)	Burst size (particles·cell^{-1})	References
ChlV	*Chlorella* sp.	190	dsDNA, 330	54	4–6	200–350[b]	Van Etten et al. 1991 Van Etten and Meints 1999
MpV	*Micromonas pusilla*	115	dsDNA, 200	45–54	7–14	—	Cottrell and Suttle 1991 Suttle pers. commun.
MpV-CB9	*Micromonas pusilla*	50	dsRNA, 25	—	—	400	Brussaard et al. 2001
CbV	*Chrysocromulina brevifilum*	145–170	—	—	—	>320	Suttle and Chan 1995
PpV	*Phaeocystis pouchetii*	120	dsDNA, 485	59	12–18	350–600	Jacobsen et al. 1996 Sandaa et al. (unpublished data)
HaV	*Heterosigma akashiwo*	200	—	—	30–33	ca 770	Nagasaki and Yamaguchi 1997
HaNIV	*Heterosigma akashiwo*	30	—	—	42	10^5	Lawrence et al. 2001
AaV[c]	*Aureococcus anophagefferens*	50–55 (tail:70–75)	—	65	—	—	Garry et al. 1998
BtV[c]	*Aureococcus anophagefferens*	140–160	—	—	<24–48	>500	Gastrich et al. 1998
HcV	*Heterocapsa circularisquama*	180–210	—	—	40–56	1300–2440	Tarutani et al. 2001
PoV	*Pyramimonas orientalis*	200	dsDNA, 560	42	14–19	800–1000	Sandaa et al. 2001
CeV	*Chrysochromulina ericina*	150–160	dsDNA, 510	80–82	14–19	1800–4100	Sandaa et al. 2001
EhV	*Emiliania huxleyi*	160–180	dsDNA, 415	54	12–15	400–1000	This study

[a] All particles have polyhedral morphology.
[b] Plaque forming units·cell^{-1}.
[c] AaV and BtV are reported to be the same virus although they differ in size and morphology. AaV is the extracellular form, whereas BtV is the intracellular form (Gastrich et al. 1998).

first isolation and characterization of an *E. huxleyi* virus (EhV), and we suggest that EhV should be assigned to the *Phycodnaviridae* family of viruses. EhV has been maintained in culture for over 2 years.

MATERIALS AND METHODS

Cultures. *Emiliania huxleyi* strain Eh BOF92 was used as the host to isolate virus. Phytoplankton species used to determine the virus host range included *E. huxleyi* strains Eh B and Eh SC91, *Phaeocystis pouchetii*, *Pyramimonas orientalis*, *Chrysochromulina ericina*, and *Micromonas pusilla*. With the exception of *P. pouchetii*, the algae were grown in f/2 medium (Guillard 1975) at 15° C and a 14:10-h light:dark cycle; the light consisted of ~30 μmol photons·m^{-2}·s^{-1} white light from fluorescent tubes. *P. pouchetii* was grown at 8° C. Algal growth was monitored daily by visual inspection.

Isolation of virus. Virus was isolated from water collected at the end of a mesocosm experiment (9 June 1999) after an *E. huxleyi* bloom collapsed from viral lysis (for details see Castberg et al. 2001). Five-milliliter water samples were added to 50 mL

TABLE 2. Explanation for abbreviated virus names.

Abbreviations	Virus	GenBank accession numbers	Virus family
MpV-PB7	*Micromonas pusilla* virus-PB7	U32979	*Phycodnaviridae*
MpV-PB8	*Micromonas pusilla* virus-PB8	U32980	*Phycodnaviridae*
MpV-SG1	*Micromonas pusilla* virus-SG1	U32981	*Phycodnaviridae*
MpV-PL1	*Micromonas pusilla* virus-Pl1	U32982	*Phycodnaviridae*
MpV-PB6	*Micromonas pusilla* virus-PB6	U32978	*Phycodnaviridae*
MpV-GM1	*Micromonas pusilla* virus-GM1	U32977	*Phycodnaviridae*
MpV-SP1	*Micromonas pusilla* virus-SP1	U32975	*Phycodnaviridae*
MpV-SP2	*Micromonas pusilla* virus-SP2	U32976	*Phycodnaviridae*
OTU1	Operational taxonomic units—1 (Chen et al. 1996)	U36931	PCR fragments amplified from environmental samples
OTU2	Operational taxonomic units—2 (Chen et al. 1996)	U36932	PCR fragments amplified from environmental samples
OTU3	Operational taxonomic units—3 (Chen et al. 1996)	U36933	PCR fragments amplified from environmental samples
OTU4	Operational taxonomic units—4 (Chen et al. 1996)	U36934	PCR fragments amplified from environmental samples
OTU5	Operational taxonomic units—5 (Chen et al. 1996)	U36935	PCR fragments amplified from environmental samples
CV-NY2A	*Chlorella* strain NC64A virus NY2A	M86837	*Phycodnaviridae*
PBCV-1	*Chlorella* strain NC64A virus PBCV-1	AF344244	*Phycodnaviridae*
PbiCVA-1	*Chlorella* strain Pbi virus CVA-1	U32985	*Phycodnaviridae*
CbV-PW1	*Chrysochromulina brevifilum* virus PW1	U32983	*Phycodnaviridae*
CbV-PW3	*Chrysochromulina brevifilum* virus PW3	U32984	*Phycodnaviridae*
FsV-1	*Feldmannia* species virus 1	AF013260	
EsV-1	*Ectocarpus siliculosus* virus 1	NC002687	*Phycodnaviridae*
HSV-2	Herpes simplex virus type 2	M16321	*Herpesviridae*
CiV	*Chilo iridescent* virus	AF303741	*Iridoviridae*
EhV-99B1	*Emiliania huxleyi* virus-99B1	AF472534	

of exponentially growing *E. huxleyi* cultures; the cultures lysed after 3 to 5 days of incubation. The lysate was filtered through a 0.2-μm syringe filter (FP 30/0,2 CA-S, Schleicher & Schuell GmbH, Dassel, Germany) to remove bacteria and algae, and 5 mL of filtrate was then transferred to 50 mL of a fresh *E. huxleyi* culture. This filtration and inoculation procedure was repeated several times. A clonal isolate of the virus (EhV-99B1) was obtained by adding 10-fold dilutions of the virus lysates to fresh *E. huxleyi* cultures. The procedure was repeated a second time with the lysate from the most diluted sample that produced lysis (Sandaa et al. 2001). Using the same purification procedure, two other isolates of EhV (EhV-2KB1 and EhV-2KB2) were obtained from a similar mesocosm experiment conducted between 6 June and 25 June 2000. However, in the year 2000 experiment, water was collected at the beginning and at the end of the *E. huxleyi* bloom (i.e. 10 June and 20 June, respectively). This algal bloom also collapsed from viral lysis (Jacquet et al. 2002).

Morphology. Fresh virus samples were collected on electron microscope grids by ultracentrifugation and stained with uranyl-acetate as described (Bratbak and Heldal 1993). Cells to be thin sectioned were concentrated by centrifugation, embedded in Na-alginate (1.5%, w/v) in 0.1 M Na-cacodylate buffer (pH 7.3), and gelled in 50 mM $CaCl_2$ (Tamponnet et al. 1988). The embedded cells were fixed with 2.5% glutaraldehyde and postfixed with 2% OsO_4 in Na-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 ultrathin sections were poststained with 2% uranyl acetate and lead citrate (Reynolds 1963) and observed in a Phillips 200 transmission electron microscope (Phillips, Eindhoven, The Netherlands).

Growth. Exponentially growing *E. huxleyi* cultures (1.5 L) were infected by adding 50 mL of fresh lysate; noninfected control cultures received the same volume of f/2 medium. The cultures were incubated as described above and sampled periodically for 45 h. Duplicate samples (1.5 mL) used to count virus particles were fixed in 2.5% glutaraldehyde for 30 min at 4° C and then frozen in liquid nitrogen. The samples were stained with SYBR Green I (Molecular Probes, Eugene, OR, USA) and analyzed using a FACSCaliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) according to the method described (Marie et al. 1999). Algal cells were counted by flow cytometry using fresh, unfixed, and unstained samples (Brussaard et al. 1999).

Host range. A small amount (2.5 μL) of fresh virus lysate was added to 50 mL of an exponentially growing *E. huxleyi* culture. The infectivity of all three EhV isolates was tested against all three *E. huxleyi* strains, and the infectivity of the viruses produced on each *E. huxleyi* strain was then tested against all *E. huxleyi* strains. The ability of EhV-99B1 to infect other algal species was tested by adding 5 mL of fresh virus lysate to 50-mL cultures of exponentially growing algal cultures. The cultures were examined visually and by flow cytometry over a 3-week period for signs of cell lysis and virus production.

Genome size. The size of the virus genome was determined by pulsed-field gel electrophoresis. Cell debris was removed from 80 mL of a fresh lysate by centrifuging twice (30 min at 7500 rpm and 4° C in a Beckman JS-7.5 rotor). Viruses remaining in the supernatant were pelleted by centrifugation for 2 h at 28,000 rpm and 10° C (Beckman SW28 rotor, Beckman Instruments, Inc., Fullerton, CA, USA). Virus particles from natural samples were concentrated by tangential flow filtration using a Vivaflow 200 module with 100,000 MWCO PES membrane (Vivascience, Lincoln, UK) before centrifugation (Larsen et al 2001). The pelleted virus particles were suspended in 400 μL SM buffer (0.1 M NaCl, 8 mM $MgSO_4 \times D7H_2O$, 50 mM Tris-HCl, 0,005% [w/v] glycerol) (Wommack et al. 1999) by incubating at 4° C overnight. Equal volumes of concentrated virus and molten 1.5% InCert agarose (FMC BioProducts, Rockland, ME, USA) were mixed, dispensed into plug molds, and allowed to solidify. The agarose plugs were removed from the molds, placed in 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 proteinase K digestion buffer was decanted, and the plugs were washed three times, 30 min each, in TE buffer (10 mM Tris-Base; 1 mM EDTA, pH 8.0), and stored at 4° C in TE 20:50 buffer (20 mM Tris, 50 mM EDTA, pH 8.0).

The virus-agarose plugs, together with phage lambda concatamers (Bio-Rad, Richmond, CA, USA), were placed in wells of a 1% SeaKem GTG agarose (FMC BioProducts, Rockland, ME, USA) gel in 1 × TBE gel buffer (90 mM Tris-borate and 1 mM EDTA, pH 8.0) and overlaid with molten 1% agarose. Samples were electrophoresed in a Bio-Rad DR-II CHEF Cell (Bio-Rad, Richmond, CA, USA) at 200 V with pulse ramps from 20 to 40 s at 14° C for 22 h in 0.5 × TBE tank buffer (45 mM Tris-borate and 1 mM EDTA, pH 8.0). After electrophoresis the gels were stained for 1 h with SYBR Green I according to the manufacturer's instructions and digitally scanned for fluorescence using a FujiFilm FLA2000 laser fluoroimager (Fuji Film, Japan).

Proteins. Virus particles were concentrated from fresh lysates by centrifugation for 2 h at 25,000 rpm and 4° C in a Beckman SW28 rotor, resuspended, and layered on linear 10%–40% sucrose gradients equilibrated with PBS buffer and then centrifuged for 20 min at 2,0000 rpm and 4° C in a Beckman SW28 rotor. A single, compact, light-scattering band was obtained, indicating that the virus remained intact during the purification process. The presence of intact viruses and absence of contaminating cells was confirmed by epifluorescence microscopy after staining the virus concentrate with Sybr Green (Noble and Fuhrman 1998). The viruses were resuspended in PBS buffer and pelleted by centrifugation for 60 mim at 41,000 rpm and 4° C in a Beckman SW41 rotor. The pellets were resuspended in SDS sample buffer (2.5% SDS, 5% glycerol, 0.025% bromophenol blue, 62.5 mM Tris, pH 6.8, and 6.25 mM dithiothreitol) and heated to 100° C for 10 min. Fifteen microliters of the protein suspension were loaded on 4%–20% acrylamide/Tris-glycine gels (10 cm × 10 cm × 1 mm thick, FMC Bioproducts) and electrophoresed for 1 h at 200 V. Proteins were visualized with Coomassie brilliant blue.

DNA polymerase gene. Assuming EhV-99B1 had a DNA polymerase gene similar to other algal viruses (Chen and Suttle 1996), we synthesized a degenerate primer set (AVS1 and POL; Chen and Suttle 1995) designed to amplify a portion of this gene by PCR. DNA from purified virus preparations was amplified following the protocol for PCR as described (Chen and Suttle 1995). The resultant PCR product was cloned into a Bluescript T-vector (Promega, Madison, WI, USA) and sequenced in both directions at the Nebraska Center for Biotechnology DNA sequencing core facility. The sequences were subject to GeneBank BLASTN and BLASTX searches. The EhV DNA polymerase gene sequence is deposited in GenBank under accession number AF472534.

Phylogenetic analyses. Phylogenetic analyses were performed on the predicted DNA polymerase amino acid sequences. A total of 171 amino acid residues of the EhV encoded protein was aligned to related DNA polymerase sequences (Table 2) and used to construct a phylogenetic tree. Multiple amino acid sequence alignments of translated nucleic acid sequences were done with ClustalX (1.5b, NCBI, National Center For Biotechnology Information, http://www.ncbi.nlm.nih.gov/). The alignments were visually inspected and corrected in GeneDoc (www.psc.edu/biomed/genedoc); a neighbor-joining tree was constructed by ClustalX with 1000 bootstraps.

RESULTS AND DISCUSSION

To improve the chances of obtaining infectious algal viruses from seawater, investigators typically either use centrifugation or filtration to concentrate suspected virus samples (Milligan and Cosper 1994, Suttle and Chan 1995) or they expose the samples to UV radiation to activate lysogenic viruses (Jacobsen et al. 1996, Nagasaki and Yamaguchi 1997, Sandaa et al. 2001) before adding the samples to the test algal cultures. However, neither treatment was required to iso-

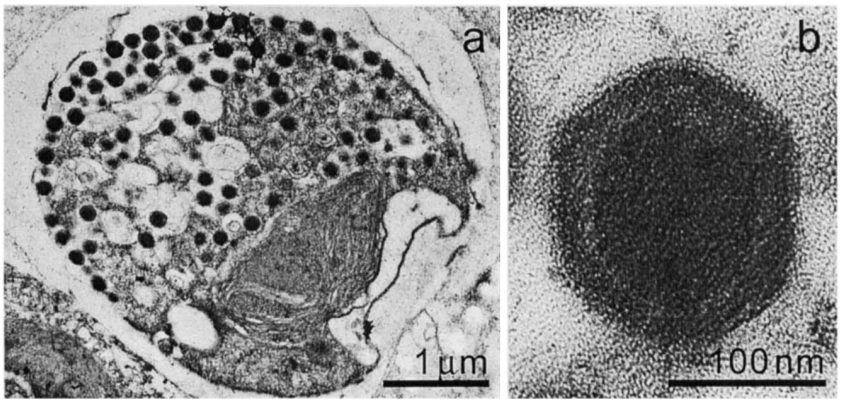

FIG. 1. (a) TEM image of a thin-sectioned *Emiliania huxleyi* cell showing localization of the viral particles in the cytoplasm. (b) Closeup of intracellular virus particle.

late EhVs, and three independent clonal isolates (named EhV-99B1, EhV-2KB1, and EhV-2KB2) were obtained directly from untreated seawater. Isolate EhV-99B1 was obtained from water collected at the end of an *E. huxleyi* bloom in June 1999 and isolates EhV-2KB1 and EhV-2KB2, respectively, were obtained from water collected near the beginning and at the end of an *E. huxleyi* bloom in June 2000. Presumably, the high concentration of large virus-like particles in the native samples ($>10^7$ particles·mL^{-1}; Castberg et al. 2001) rendered such enrichment procedures unnecessary.

Electron micrographs of EhV virions indicate that the particles lack a tail and an external membrane and that they have icosahedral symmetry. Micrographs of thin sections of infected *E. huxleyi* cells indicated that EhV capsid assembly and DNA packaging occurred in the cytoplasm (Fig. 1a). The diameter of the virions is 160–180 nm (Fig 1). The three EhV isolates are morphologically identical and resemble viruses previously reported for *E. huxleyi* (Manton and Leadbeater 1974, Bratbak et al. 1993, 1995, 1996, Brussaard et al. 1996). (Note: Manton and Leadbeater [1974] erroneously reported the virus particles had a diameter of 22 nm instead of 200 nm.) The icosahedral morphology of EhV is similar to other viruses and virus-like particles that infect algae (Table 1, Van Etten et al. 1991).

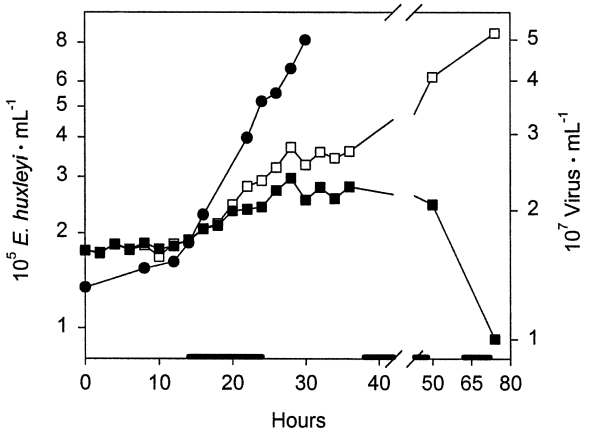

FIG. 2. Growth of noninfected (□) and virus-infected (■) *Emiliania huxleyi* cultures. Free EhV-99B1 virus in the infected culture (●). Bars on the *x* axis indicate light/dark cycles.

Addition of EhV lysates to actively growing *E. huxleyi* at a ratio of ~75 virus particles per cell resulted in an increase in virus particles beginning 12–14 h postinfection (p.i.) without an obvious effect on the number of algal cells (Fig. 2). However, the number of *E. huxleyi* cells in the inoculated culture reached a plateau 20–30 h p.i. and then decreased rapidly at ~50 h p.i. We interpret these results to mean EhV has a replication cycle of 12–14 h and the first burst or release of nascent virus particles accounts for the initial increase. However, because no corresponding decrease in *E. huxleyi* cells occurred at 12–14 h p.i., we conclude that the number of infectious virus particles in the original inoculum was low, that is, only a small fraction of the cells were infected initially. Because of this low infectivity, about three rounds of virus replication (i.e. 40–45 h) are required before there is sufficient virus to infect all the *E. huxleyi* cells. Because of the coccoliths covering the *E. huxleyi* cells, it may be very difficult for a virus to infect the cell, and the cells may be susceptible to infections only at a particular growth phase. In a separate experiment the average burst size for EhV was calculated from the overall net increase in viral particles and the concurrent decrease in *E. huxleyi* cells; a value of 620 (SD = 220, $n = 8$, range, 400–1000) was obtained.

No signs of cell lysis or virus production were detected 3 weeks after *P. pouchetii*, *P. orientalis*, *C. ericina*, and *M. pusilla* were inoculated with each of the three EhV isolates. However, all three EhV isolates replicated in the three *E. huxleyi* strains and the viruses produced in each of the host strains infected all three *E. huxleyi* strains. The *E. huxleyi* strain Eh B was isolated in 1991 from Raunefjorden, western Norway; Eh SC91 was isolated in 1990 from Skagerak; and Eh BOF92 was isolated in 1990 from the North Sea at 48° N 12° W. Thus, EhV isolated in two consecutive years infected *E. huxleyi* strains isolated 10 years earlier from three different locations. These results indicate that EhV infects a broad range of *E. huxleyi* strains.

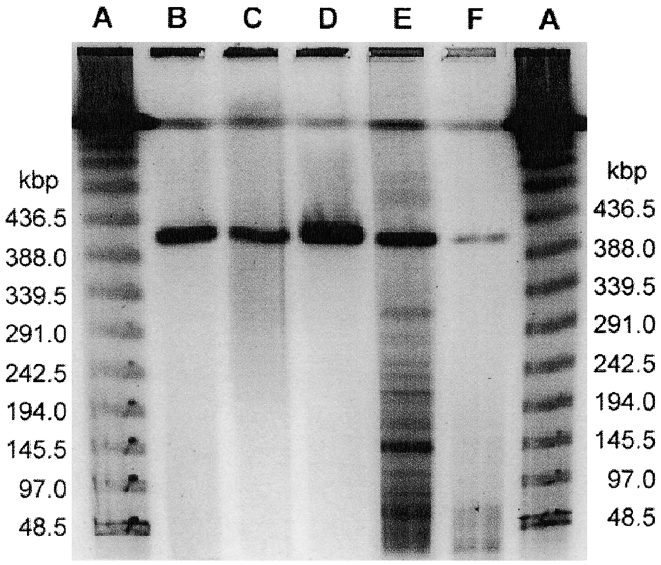

FIG. 3. Pulsed-field gel electrophoreses of DNA isolated from EhVs. Lanes A: Molecular size standards are λ concatamers. Lane B: EhV-99B1. Lane C: EhV-2KB1. Lane D: EhV-2KB2. Lane E: Seawater samples from which EhV-2KB1 was isolated. Lane F: Seawater samples from which EhV-2KB2 was isolated.

The genome sizes of the three EhV isolates were ~415 kb as estimated by pulsed-field gel electrophoresis (Fig. 3). Not surprisingly, DNAs with similar sizes were present in water samples collected from both mesocosm experiments from which the EhVs were isolated (Fig. 3, lanes E and F). In addition, many other faster migrating DNAs (~30–300 kb) were present in these native water samples. These additional DNAs are considered to be of viral origin, and the results indicate the diversity of the viral community from which EhV was isolated (see Steward et al. 2000, Larsen et al. 2001).

The EhV 415-kb genome is among the largest virus genomes described to date (Table 1), considerably larger than the 333-kb genome of the *Phycodnaviridae* type virus, *Paramecium bursaria* chlorella virus 1 (PBCV-1) (Van Etten and Meints 1999). PBCV-1 contains 11 tRNA genes and ~375 protein encoding genes. If the gene density of EhV is similar to PBCV-1, the virus is predicted to have ~470 protein-encoding genes. To put this number in perspective, it exceeds the 250–350 protein encoding genes estimated to be the minimal number required to support life (Hutchison et al. 1999, Itaya 1995, Mushegian and Koonin 1996) and is equivalent to that of the smallest mycoplasma; *Mycoplasma genitalium* has 470 genes (Fraser et al. 1995).

The EhV-99B1 virion contains one major protein of ~54 kDa and four well-defined proteins that weigh between 19 and 45 kDa (Fig. 4). Including faint bands, nine proteins, 10–140 kDa, are detected in the virion. EhV-2KB1 and EhV-2KB2 virions have similar protein profiles as EhV-99B1 (results not shown). We assume the 54-kDa protein is the EhV-99B1 major capsid protein because it is the most abundant and because it has a similar size as the major capsid protein of PBCV-1 and many other algal viruses (Table 1). The PBCV-1 major capsid protein is extensively post-translationally modified, the N-terminal methionine is removed, and the protein is myristoylated and glycosylated (Graves and Meints 1992, Wang et al. 1993, Que et al. 1994). It will be interesting to determine if the EhV-99B1 major capsid protein is also post-translationally modified.

To determine the relationship of EhV to other viruses, a conserved portion of the EhV-99B1 DNA polymerase gene was amplified by PCR, using a degenerate set of primers (AVS1 and POL; Chen and Suttle 1995) designed to distinguish algal viruses (Chen and Suttle 1996). The AVS1 and POL primers generated a PCR product from EhV-99B1 DNA of the expected size (i.e. ~500 base pair) (Chen and Suttle 1995). DNA sequencing revealed that the PCR product was 517 base pair. The EhV-99B1 nucleotide sequence was 97% identical to two EhV isolates from Plymouth, UK (Willie Wilson, personal communication). Of the predicted 171 amino acids, only one amino acid differed between the Norwegian and English EhV isolates. The EhV DNA polymerase has 49% amino acid sequence identity to the DNA polymerase from *M. pusilla* viruses, 45% identity to the chlorella viruses, and 36% identity to the iridovirus *Chilo iridescent* virus (Fig. 5).

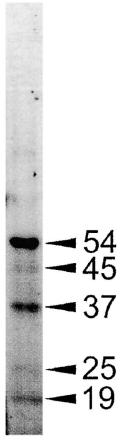

FIG. 4. SDS-PAGE of proteins from EhV-99B1. Numbers are molecular size in kDa.

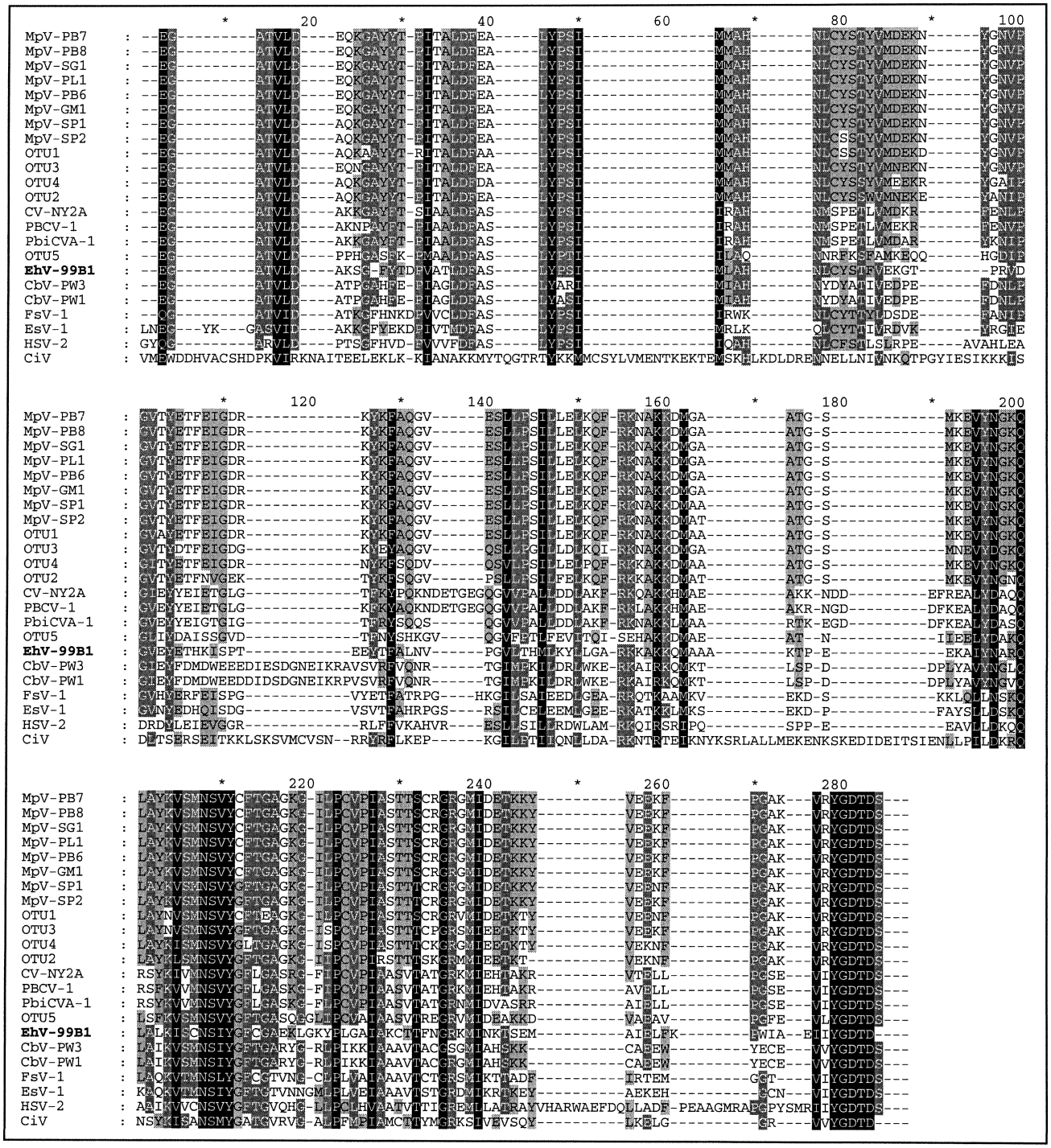

FIG. 5. Alignment of DNA polymerases from dsDNA viruses and related operational taxonomic units (OTUs). The alignment was produced using ClustalX (Jeanmougin et al. 1998) and manually edited using GeneDoc (http://www.psc.edu/biomed/genedoc/). The abbreviation of the virus names and accession numbers used in the figure are listed in Table 2.

Phylogenetic analysis (Fig. 6) indicates that EhV-99B1 clusters with the *Phycodnaviridae* family, but it forms a separate branch between the *M. pusilla* viruses and the chlorella viruses (Fig. 6). EhV is more distantly related to representatives of the *Iridoviridae* and *Herpesviridae*. The finding that EhVs belong in the *Phycodnaviridae* family also suggests that EhVs have a long evolutionary history because accumulating evidence suggests that the phycodnaviruses are ancient. For example, an extensive phylogenetic analysis of DNA

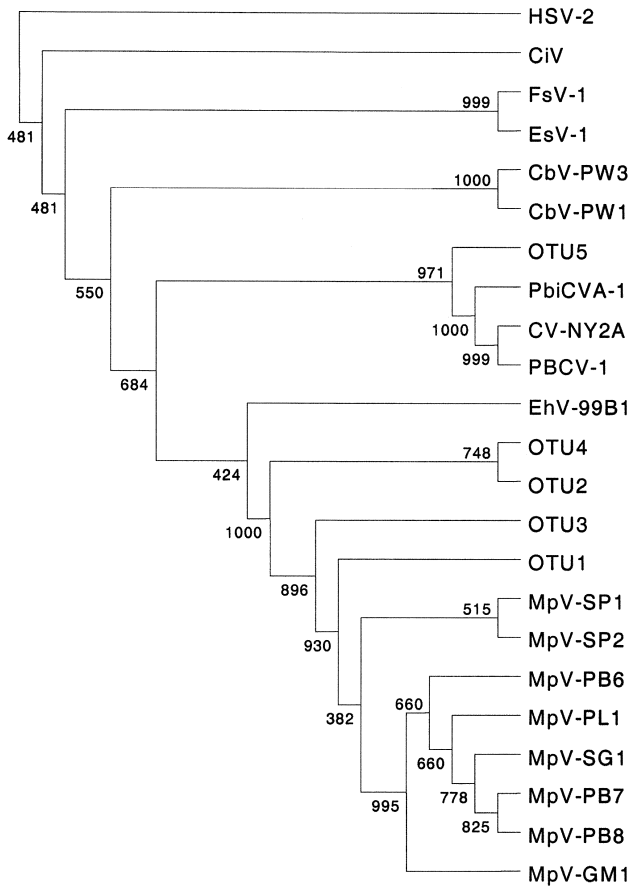

FIG. 6. Neighbor-joining tree showing the relationship between the dsDNA viruses and operational taxonomic units (OTUs) aligned in Fig. 5. The numbers at the nodes indicate bootstrap values (1000 analysis). The abbreviation of the virus names and accession numbers used in the figure are listed in Table 2.

polymerases indicates that the phycodnavirus enzymes reside near the root of all eukaryotic δ DNA polymerases (Villarreal 1999, Villarreal and DeFilippis 2000). Likewise, unicellular algae are considered to be among the oldest eukaryotic cells (e.g. Schopf 1999). Therefore, studies on phycodnaviruses may reveal interesting aspects about the evolution of genes and genomes.

In conclusion, we find that EhV, like its host *E. huxleyi*, appears to be widely distributed, at least in Norwegian and English coastal waters; EhV infects *E. huxleyi* strains isolated at three geographic locations 10 years before isolation of the virus; the ~415-kb EhV genome is among the largest virus genomes described to date; and the biological properties and phylogenetic analyses indicate that EhVs should be assigned to the *Phycodnaviridae* virus family.

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