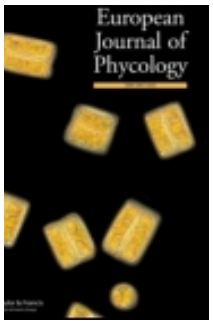

European Journal of Phycology

ISSN: 0967-0262 (Print) 1469-4433 (Online) Journal homepage: http://www.tandfonline.com/loi/tejp20

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To cite this article: Ingo Maier , Susanne Wolf , Nicolas Delaroque , Dieter Müller & Hiroshi Kawai (1998) A DNA virus infecting the marine brown alga Pilayella littoralis (Ectocarpales, Phaeophyceae) in culture, European Journal of Phycology, 33:3, 213-220, DOI: 10.1080/09670269810001736713

To link to this article: https://doi.org/10.1080/09670269810001736713

A DNA virus infecting the marine brown alga *Pilayella littoralis* (Ectocarpales, Phaeophyceae) in culture

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(Received 8 April 1998; accepted 10 June 1998)

A new large DNA virus (PlitV-1) infects the marine filamentous brown alga *Pilayella littoralis*. It was collected in Alaska and infects other *P. littoralis* isolates of different geographic origin. The virus has an icosahedral capsid of *c.* 161 nm in diameter, enclosing an electron-dense core. The genome consists of double-stranded DNA and is approximately 280000 base pairs in size. The virus is latent in somatic cells of the host and is propagated only upon induction of the host's reproductive organs. It causes deformed sporangia, resulting in infertility, and is structurally similar to other brown algal viruses. PCR amplification of a genomic sequence coding for part of a structural glycoprotein of the *Ectocarpus siliculosus* virus EsV-1 produced a fragment of similar size to that obtained with EsV-1.

Key words: algae, Ectocarpales, marine double-stranded DNA virus, Phaeophyceae, phycovirus, *Pilayella littoralis*, PlitV-1

Introduction

Pilayella littoralis is a prominent filamentous brown alga with a wide distribution in intertidal habitats on coasts of cold and cold-temperate zones in both hemispheres. It often forms dense mats on larger algae and on rocks (Cardinal, 1964; Kornmann & Sahling, 1978).

In 1974, Markey reported the occurrence of virus-like particles (VLPs) in electron microscopic preparations of a *Pilayella* specimen collected on the North American Atlantic coast. This study was not pursued due to the lack of living material, and the viral character of the particles could not be confirmed. To our knowledge, no other observations on VLPs in *Pilayella* have been reported.

VLPs and viruses have been reported in a large number of species from diverse eukaryotic algal lines. Apart from viruses infecting *Chlorella*-like green algae exsymbiontic from *Paramecium bursaria* (Ciliata) and *Hydra viridis* (Coelenterata) (Van Etten *et al.*, 1991; Reisser, 1993, 1995), those found in brown algae have been the most intensively studied. Pathogenicity has been confirmed in six virus isolates kept in culture: those of *Ectocarpus siliculosus* (EsV-1), *E. fasciculatus* (EfasV-1), *Feldmannia simplex* (FlexV-1), *F. irregularis* (FirrV-1), *Hincksia hincksiae* (HincV-1) and *Myriotrichia clavaeformis* (MclaV-1) (Kapp *et al.*, 1997; Müller *et al.*, 1998). All these viruses share the following characters: they have icosahedral capsids without appendages, and relatively large genomes (170–340 kbp) of double-stranded DNA (dsDNA). The target cells for infection are the wall-less reproductive cells of the host (gametes or spores). The virus genome is intimately associated with the host genome and goes through an extended latency period with virus propagation restricted to the presumptive reproductive organs of the host (gametangia or sporangia).

We report here an infectious virus disease in *P. littoralis* from the Bering Strait, Alaska, and give a first account of the *Pilayella* virus genome, its interaction with the host and host specificity. A comparison is made with VLPs in *P. littoralis* observed by Markey (1974) and with the other known brown algal viruses. PCR amplification of a gene fragment coding for a structural glycoprotein of EsV-1 (gp-1) has been used as a diagnostic tool to detect virus infections in *Ectocarpus* isolates. It gives positive results with EsV-1 and EfasV-1, but not with other known brown algal viruses (Müller *et al.*, 1996; Sengco *et al.*, 1996). The technique was used to reveal a possible relationship between the *Ectocarpus* viruses and *Pilayella* VLPs.

Materials and methods

Algal material

Pilayella littoralis (Linnaeus) Kjellman was collected on intertidal rock platforms at Savoonga and Gambell on Saint Lawrence Island, Bering Strait, Alaska on 5 August 1996. Small fragments of thalli were used for the initiation of unialgal clonal cultures. Specimens of the Gambell collection, designated as PilBS-2 and -3, yielded fertile isolates which formed pluri- and unilocular sporangia simultaneously. Material from Savoonga (designated as PilBS-1) was found to be infected by the virus described in

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the present study. Intraspecific infection experiments were carried out with a sympatric isolate of *P. littoralis* from the Bering Strait (PilBS-3) as well as a strain originating from the German coast (Helgoland, North Sea) and one from the Drake Passage, South Chile (strains PilHel and PilIR; Müller & Stache, 1989). A female partheno-sporophyte of *Ectocarpus siliculosus* from New Zealand (strain NZ4a3; Müller *et al.*, 1990; Sengco *et al.*, 1996) was used in intergeneric infection experiments. Zoospores of *Pilayella* and *Ectocarpus* were obtained by storing mature thalli at 2 °C in darkness overnight. Zoospores were released shortly after the onset of light and transfer of the thalli to fresh culture medium, which was combined with a temperature rise to 18 °C. *E. siliculosus* virus particles (EsV-1) for PCR amplification were isolated from *E. siliculosus* strain NZ-Vic-Z14 (Lanka *et al.*, 1993).

Culture conditions

The culture medium was made up from tap water with a commercial salt mixture (hw Meersalz professional, Wiegandt GmbH, Krefeld, Germany) supplemented with Provasoli ES enrichment (Starr & Zeikus, 1993). In some cultures, ASM-1 (Maier & Calenberg, 1994) was used as a fully synthetic, defined medium with good results. Cultures were illuminated with daylight-type white fluorescent light at an irradiance of 10–15 μmol m^{-2} s^{-1} for 14 h per day at 12 °C. The culture medium was exchanged at 1 to 3 week intervals.

Infection experiments

Dense virus suspensions for infection experiments were obtained following a modification of the technique described by Müller (1991). Filaments of virus-infected *Pilayella* (PilBS-1) were placed in 400 μl droplets of culture medium in the centre of a plastic Petri dish and subjected for a few seconds to a temperature increase from 12 to 37 °C. Discharge of virus masses from *Pilayella* cells was observed with dark field microscopy. One drop of culture medium with swimming spores of the recipient host was added to the virus suspension. After 1 h the spores had settled on the culture dish and the *Pilayella* filaments were removed. Culture medium was added and the host spores allowed to develop. A typical infection experiment started with several hundred spores. After germination and initial development, *c.* 100 juvenile thalli were cultured up to maturity. Infected individuals could easily be recognized and distinguished from those with normal sporangia under low magnification. These experiments were not strictly quantitative, because we were not able to measure the virus titre, and the density of the spore suspensions was not determined.

DAPI staining and light microscopy

For staining with the fluorescent DNA probe DAPI (4′,6-diamidino-2-phenylindole), healthy and virus-infected thalli of the Helgoland strain of *Pilayella* were fixed with 2·5% glutaraldehyde in 70% culture medium, for 1 h at room temperature. Afterwards, specimens were washed three times in culture medium (10 min each) and stained with DAPI (1 μg ml^{-1} in culture medium, containing 0·1% sodium azide) for 2 h in darkness, followed by two washing steps with distilled water. The material was viewed on a Zeiss epifluorescence microscope equipped with a Zeiss Neofluar 100/1·30 with UV excitation at 365 nm. DAPI staining of individual virus particles bound to poly-L-lysine-coated coverslips was performed as described by Maier & Müller (1998). A Zeiss KP 500 filter was used to filter out chlorophyll fluorescence. Low-magnification bright-field micrographs were made with a Zeiss Plan 10/0·22 objective. Photographs were taken on 400 ASA Ilford HP 5 Plus (fluorescence) and on 50 ASA Kodak Technical Pan film (bright field). Cellular dimensions were measured from live cells.

Electron microscopy

Fixation for transmission electron microscopy and flat embedding in Spurr's epoxy resin (Spurr, 1969) between Aclar embedding film (Plano, Marburg, Germany) were carried out as described by Maier *et al.* (1997). Thallus fragments were sectioned longitudinally on a Reichert Om U3 ultramicrotome using a diamond knife. The sections were mounted on Formvar/carbon-coated slot grids and stained with lead citrate (5 min) according to Venable & Coggeshall (1965). The preparations were examined on a Zeiss EM 900 electron microscope and photographed on Agfa Scientia plates. A cross-grating replica (2650 lines mm^{-1}, Agar Scientific) was used as a length standard; all scales given in the figures are $\pm$10%. The mean virus diameter corresponds to the arithmetic mean of calculated caliper diameters, each obtained from the largest diameter and that perpendicular to it of a random sample of median sections. The burst size was calculated from the dimensions of the virus-containing space measured on live cells and the mean virus diameter using the DeHoff & Rhines equation (Williams, 1977).

Virus preparation

Suspensions of virus particles were obtained from bulk cultures of PilBS-1 (15–20 g fresh weight) and *E. siliculosus* (infected by EsV-1) with the temperature shock method described by Kapp *et al.* (1997). After low-speed centrifugation (Sorvall RC 28S, HB-6 rotor, 11 200 *g*, 15 min) to remove cellular debris, viruses were precipitated from the supernatant with 8% polyethylene glycol, 1 M NaCl (final concentrations) for 1 h on ice and then sedimented by centrifugation (as above).

Pulsed-field gel electrophoresis

Precipitated virus particles (see above) were resuspended in a small volume of seawater and embedded in low

melting point agarose (Biozym, Hameln, Germany; 1% final concentration). Virus DNA was deproteinized within the agarose plugs by treatment with proteinase K (Boehringer, Mannheim, Germany; 1 mg ml^{-1}) in TE buffer (10 mM Tris, 5 mM EDTA, pH 8·0) containing 1% SDS for 48 h at 55 °C, followed by washing with TE buffer (three times for 1 h each, 55 °C). DNA in the size range of 50–500 kbp was separated in 1% agarose (Seakem, FMC, Rockland, USA) by pulsed-field gel electrophoresis in 40 mM Tris acetate, 1 mM EDTA, pH 8·0, using a BioRad CHEF mapper apparatus. The following run parameters were used: voltage gradient 6 V cm^{-1}, included angle 120°, linear switch time ramp 5·8–38·5 s, run time 19·5 h. Phage λ concatemers (New England Biolabs, Schwalbach, Germany) were used as size markers.

Polymerase chain reaction (PCR) amplification

Precipitated viruses (see above) were resuspended in seawater and deproteinized with 1% SDS, 0·2 mg ml^{-1} proteinase K for 1 h at 55 °C. DNA was extracted with phenol/chloroform, precipitated with isopropanol and after centrifugation resuspended in TE buffer. About 200 ng of DNA (1 μl solution) was employed per PCR mix (50 μl). A genomic product, which corresponds in size to a 692 bp fragment of a gene encoding glycoprotein gp-1 of the *E. siliculosus* virus EsV-1 (Klein *et al.*, 1995), was amplified from PlitV-1 using the PCR protocols and primers described by Bräutigam *et al.* (1995) and Sengco *et al.* (1996). The annealing temperature was 64 °C. EsV-1 DNA prepared by the same method was used for comparison.

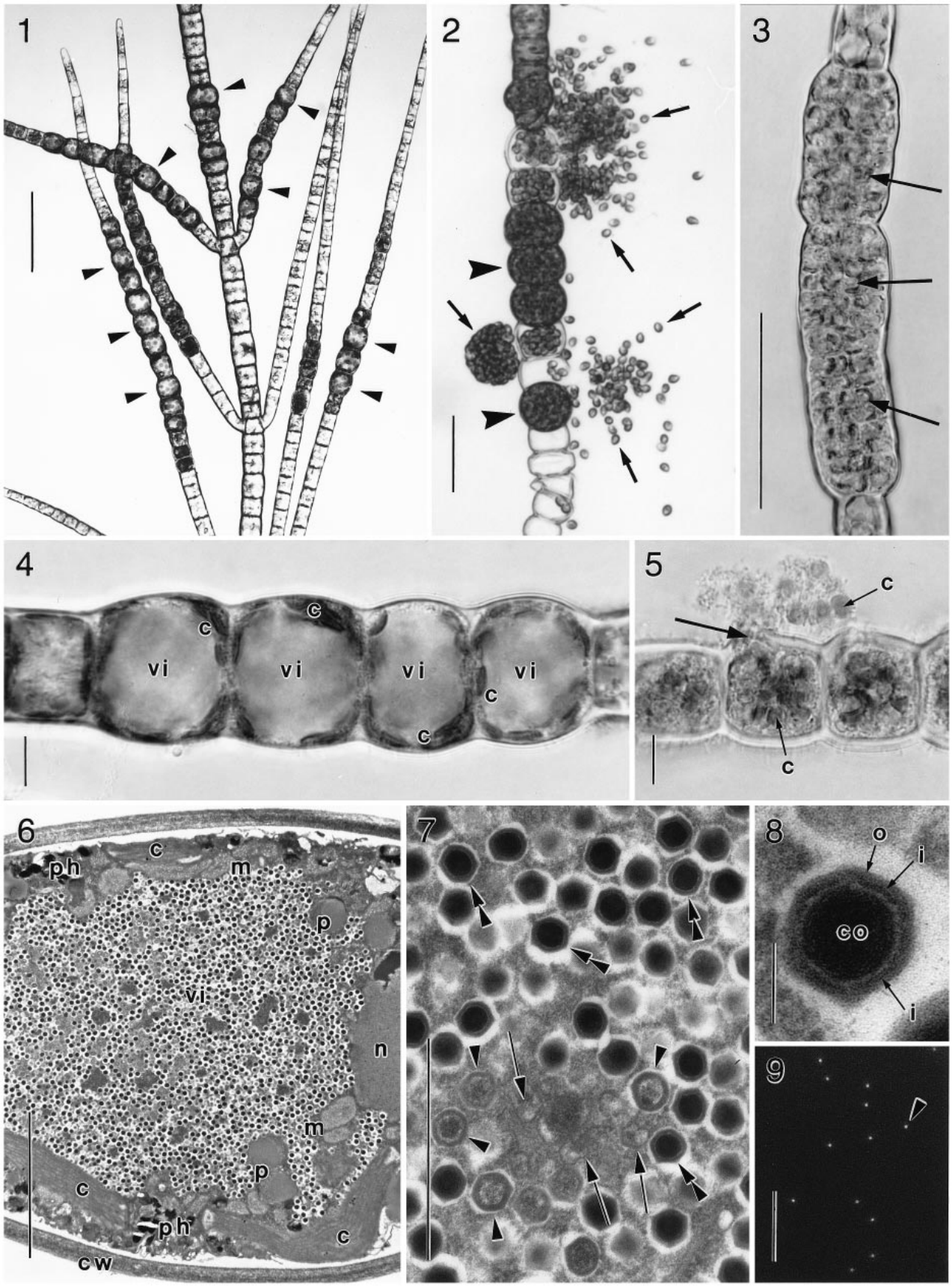
Results

The overall morphology of our isolate of *Pilayella littoralis* from Savoonga (PilBS-1; Fig. 1) was similar to that of specimens originating from Gambell (PilBS-2, -3), with characters agreeing well with the descriptions of the species (Cardinal, 1964). However, while PilBS-2 and -3 formed zoospores in intercalary uni- and plurilocular sporangia (Figs 2, 3), strain PilBS-1 was sterile. Older thalli of the latter formed intercalary, catenate series of swollen, barrel-shaped cells with reduced pigmentation in side branches and subterminal parts (Figs 1, 4). They were 17·6 ± 2·7 μm long and 23·0 ± 2·3 μm wide (mean ± SD, $n = 75$ cells). Their shape and arrangement suggested that they were homologous to unilocular sporangia in normal thalli. Most of the volume of these apparently diseased cells was occupied by a central mass of unstructured, opaque material (Fig. 4). They could frequently be seen to burst and release their contents through a lateral aperture, leaving behind aggregates of discoid chloroplasts and cellular remnants within and outside the cells (Fig. 5). This was induced by the same experimental stimuli that trigger spore release from sporangia, namely onset of the light period and addition of fresh medium combined with an increase in temperature.

Transmission electron microscopy revealed the presence in the central cytoplasm of numerous virus-like particles (VLPs), pentagonal or hexagonal in cross-section (Figs 6–8). The central cytoplasmic region appeared translucent and amorphous in the light microscope (Fig. 4). The VLPs were composed of a strongly electron-dense core and a coat separated from the core by a clear matrix. No outer appendages were observed on the coat (Figs 7, 8). Within the coat a membrane-like 6 nm bilayer could be distinguished from an amorphous outer layer (Fig. 8). The particle diameter on electron micrographs was 161 ± 5 nm, and the core measured 108 ± 3 nm (mean ± SD, $n = 100$). Each VLP was surrounded by a clear cytoplasmic space. The typical burst size of PilBS-1 was estimated to be approximately 250 000 $cell^{-1}$. VLP formation was cytoplasmic or rather occurred in a confluence of cyto- and nucleoplasm after disintegration of the nuclei (Figs 6, 16–21). Empty capsids were assembled on specialized, membranous structures ('virus assembly sites'; Fig. 7) before packaging of the electron-dense core. Cell organelles, i.e. chloroplasts with pyrenoids, mitochondria and remnants of nuclei with associated Golgi bodies as well as the numerous physodes (globular bodies containing phenolics), were confined to the periphery of the cells (Fig. 6). No VLPs were detected in somatic filament cells.

Medium from cultures of PilBS-1 that had discharged VLPs was able to cause the same pathological symptoms in offspring of healthy sympatric isolates of *Pilayella* (PilBS-2, -3) when added to motile spores, thus proving the viral nature of the particles. In two experiments, 20% and 65% of, respectively, 87 and 100 grown-on thalli produced diseased cells as described above within 6 weeks in culture. The Bering Strait *Pilayella* virus was also able to infect *P. littoralis* from Helgoland (German Bight, North Sea). Here 9 of 59 thalli showed pathological symptoms. Viruses released from this new host were capable of infecting *P. littoralis* strain PilIR from Chile. The majority of host thalli originating from infection experiments showed complete sterility and total replacement of sporangia by virus-producing cells. In all our experiments, however, we noticed a small proportion of individuals that simultaneously produced virus particles and functional spores in different parts of their thalli. Such spores germinated and developed into thalli again showing infection symptoms; no persistent suppression of virus replication was observed. Our attempts at extra-specific cross-infection failed: in several experiments, zoospores of *E. siliculosus* (strain NZ4a3) were not infected by the *Pilayella* virus. All *Pilayella* strains including the host/virus system could easily be propagated by mechanical fragmentation and grown in mass culture.

Single virus particles could be visualized (Fig. 9) and virus replication followed by fluorescence microscopy after staining with the double-stranded DNA (dsDNA) probe DAPI. Sporogenesis in unilocular sporangia of non-



Figs 1–9. Light and electron micrographs of healthy and virus-infected *Pilayella littoralis* and virus particles. Fig. 1. Habit of *P. littoralis* PilBS-1 infected with PlitV-1. Catenate series of virus-producing cells are indicated by arrowheads. Fig. 2. Unilocular sporangia (arrowheads) in a non-infected thallus (PilBS-2) and release of zoospores (arrows). Fig. 3. A plurilocular sporangium consisting of numerous small cells (arrows), each producing a single zoospore. Fig. 4. A row of virus-producing cells, each showing a clear central region (vi) containing virus particles, and parietal chloroplasts (c). Fig. 5. Dehiscent (arrow) virus-producing cells containing cellular debris.

infected *Pilayella* involves the formation of syncytia containing a large number of nuclei and chloroplasts prior to segregation of zoospores (Figs 10, 11), which is typical of this kind of reproductive cell in brown algae. In contrast, each nuclear division is followed by cytokinesis during the development of plurilocular sporangia, resulting in multiseriate structures (Figs 12, 13). The initial stages in the differentiation of virus-producing cells in *Pilayella* followed the same pattern as that of unilocular sporangia insofar as a series of nuclear divisions without cytokinesis occurred (Figs 14, 15). At the 16–32-nucleate stage, however, the nuclei became hypertrophied and eventually disintegrated (Figs 16, 17). In parallel, strongly increasing DAPI fluorescence indicated massive replication of presumably viral DNA. At maturity, intensive DAPI fluorescence extended over the whole of the cells except for thin peripheral layers of cytoplasm (Figs 18, 19). Virus formation also took place in plurilocular structures (Figs 20, 21), but here the virus-producing compartments were considerably larger than single cells in normal plurilocular sporangia (Fig. 12), probably due to inhibition of cytokinesis after a particular developmental stage.

Intact viral DNA could be obtained with a yield of about 1 μg per gram algal fresh weight. Pulsed-field gel electrophoresis showed that the *P. littoralis* virus has a genome with an approximate size of 280 kbp, which is smaller than the *E. siliculosus* virus (EsV-1) genome included for comparison (Fig. 22). Above a discrete band of full-length linear viral DNA, large-size bacterial DNA was present at the separation limit of the gel system ($\geqslant$ 500 kbp), and some non-migrating DNA failed to enter the gel. Diffuse fluorescence below the bands of viral DNA was caused by fragments originating from DNA breakage during isolation. Extracted virus DNA, but not crude homogenates of virus-infected *Pilayella* thalli, gave a PCR amplification signal with primers designed for a 692 bp fragment of a gene coding for glycoprotein 1 (gp-1) of the EsV-1. The size of the amplification product was similar to that obtained with EsV-1 DNA (Fig. 23).

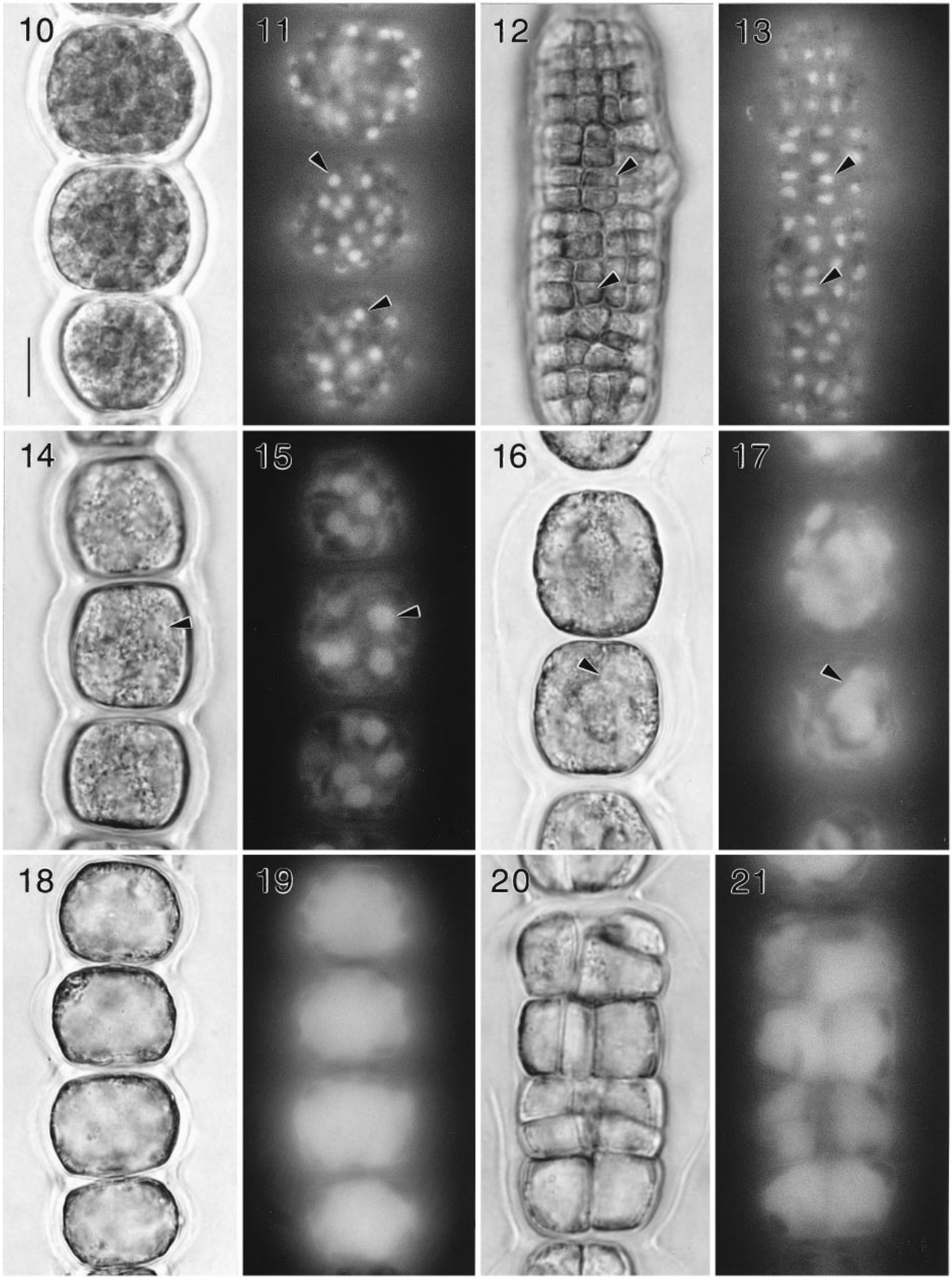
Discussion

Our infection experiments demonstrated the infectivity and thus the viral nature of the particles found in *P. littoralis*. Following the terminology of Müller (1996) we propose the designation '*Pilayella littoralis* virus type 1, PlitV-1' for this pathogen. Its host range includes *P. littoralis* isolates originating from widely spaced geographical locations, including strains from the Arctic Sea, North Atlantic and South Pacific. In our experiments, *E. siliculosus* could not be infected by PlitV-1, which is in agreement with the relatively narrow host specificity encountered in other brown algal viruses (Müller, 1996; Maier *et al.*, 1997; Müller *et al.*, 1998).

PlitV-1 is morphologically very similar to, and might correspond to, the particles observed by Markey (1974) in fixed material of *P. littoralis* from Massachusetts. The pentagonal and hexagonal profiles of sectioned particles are indicative of an icosahedral shape in both cases. Good agreement is also seen in the appearance of the electron-dense core, which is probably composed of DNA and associated proteins, and the coat. Other common features include mass production of virus particles in the cytoplasm of cells that are apparently homologous to unilocular zoosporangia and concomitant nuclear breakdown. The particles are also of similar size. This applies in both cases to dehydrated, embedded particles and the diameter probably differs significantly from that of hydrated virions. The clear cytoplasmic spaces surrounding the virus particles in our electron microscopic preparation are probably a fixation artefact and due to differential shrinkage during fixation and dehydration.

The *Pilayella* virus fits well into the group of known brown algal viruses (Kapp *et al.*, 1997; Müller *et al.*, 1998). Fluorescence staining by DAPI, which binds specifically to dsDNA (Jeppesen & Nielsen, 1989), indicates the presence of a dsDNA genome in PlitV-1, and the relatively large genome size of about 280 kbp is well within the range of 170–340 kbp found in other brown algal dsDNA viruses. Target cells for infection are the cell-wall-free zoospores of the host and the virus is latent in somatic filament cells. Any fragment of the thallus is able to regenerate into a larger thallus bearing deformed reproductive cells producing virus particles, as in other brown algal virus–host combinations. The mechanism of virus induction is unknown, but apparently related to the initiation of sporangia which in turn is correlated with thallus age and size. Virus formation involves the development of multinucleate cells, nuclear hypertrophy and breakdown and virus assembly in a cytoplasm/nucleoplasm continuum. The formation of empty capsids on membranous virus assembly sites, and subsequent acquisition of the viral core, has been observed by Markey (1974) and bears strong resemblance to virus assembly in the brown alga *Hincksia hincksiae* (HincV-1, Wolf *et al.*, 1998) as well as in an exsymbiontic *Chlorella*-like green alga (PBCV-1, Van Etten *et al.*, 1991). Immature particles of the *E. siliculosus* virus (EsV-1) and *Feldmannia* sp. (FsV) have also been observed in connection with, and are probably formed on, tubular, membrane-bound structures (Müller *et al.*, 1990; Henry & Meints, 1992). Morphologically, and especially with regard to the presence of a membrane in the coat, PlitV-1

Fig. 6. Electron micrograph of a virus-producing cell in longitudinal section. Fig. 7. A virus assembly site in which empty capsids (arrowheads) are formed on membranous structures (arrows), and mature virus particles (double arrowheads). Fig. 8. A virus particle in median section at high magnification, showing the electron-dense nucleoprotein core (co) and the capsid consisting of an inner membrane-like bilayer (i) and an outer coat layer (o). Fig. 9. Fluorescence micrograph of single virus particles (arrowhead) stained with DAPI. Scale bars represent: Fig. 1, 0·1 mm; Figs 2, 3, 50 μm; Figs 4, 5, 9, 10 μm; Fig. 6, 5 μm; Fig. 7, 1 μm; Fig. 8, 100 nm. Abbreviations: c, chloroplast; cw, cell wall; m, mitochondria; n, remnant of nucleus; p, pyrenoids; ph, physodes; vi, central virus-containing region.



Figs 10–21. Fluorescence and corresponding bright-field micrographs of DAPI-stained cells during sporogenesis and virus formation. Figs 10, 11. Unilocular zoosporangia close to maturity, each containing numerous nuclei (arrowheads). Figs 12, 13. A mature plurilocular zoosporangium. In each cell a single spore nucleus is stained (arrowheads). Figs 14, 15. Virus-producing cells at the 16-nucleate syncytium stage. One nucleus is indicated by an arrowhead. Figs 16, 17. Hypertrophied and disintegrated nuclei (arrowheads) in virus-producing cells. Figs 18, 19. Mature virus-producing cells showing strong DAPI fluorescence. Figs 20, 21. Virus formation in a plurilocular structure, indicated by strong DAPI staining over the whole cells. Scale bar represents 10 μm.

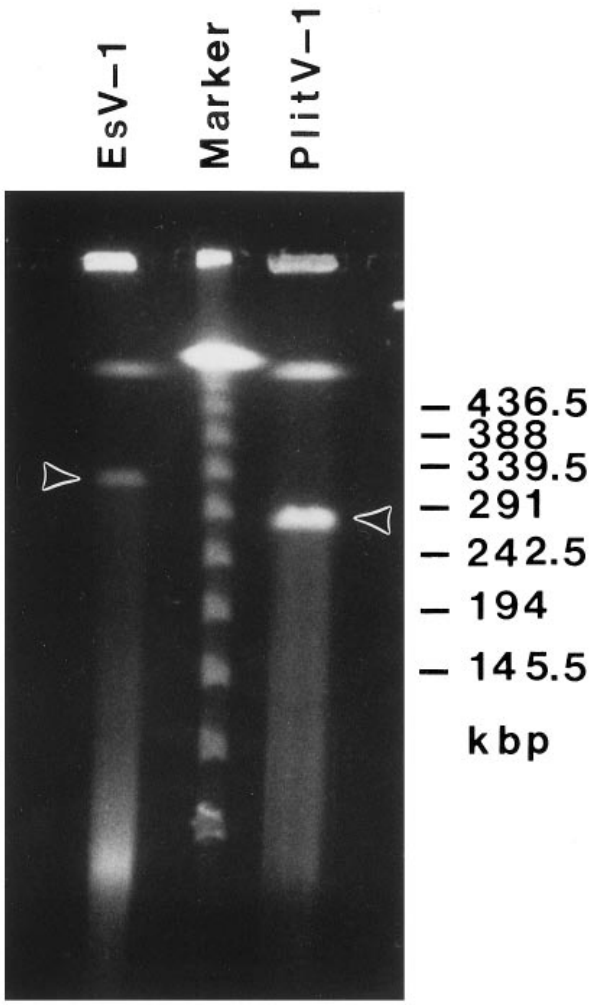


Fig. 22. Pulsed-field gel electrophoresis of viral DNA. Lane 1, *E. siliculosus* virus (EsV-1). Lane 2, phage λ concatemers as length markers with sizes indicated in kilobase pairs (kbp). Line 3, *P. littoralis* virus (PlitV-1). Full-size linear viral DNA is indicated by arrowheads.

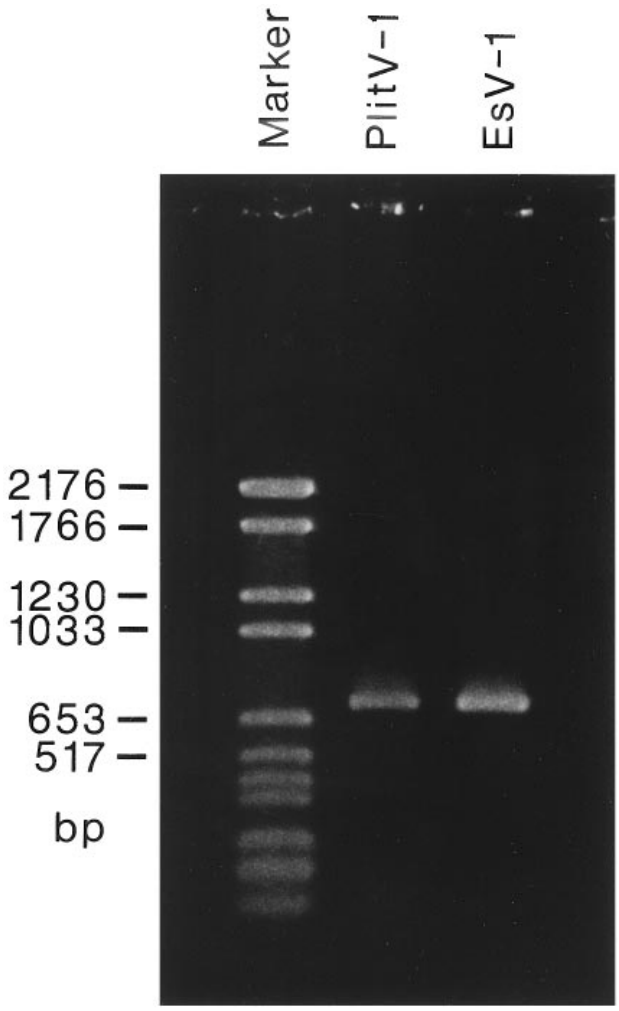


Fig. 23. Gel electrophoresis of PCR-amplified gp-1 gene fragments. Lane 1, markers with sizes in base pairs (bp) indicated; lane 2, *P. littoralis* virus (PlitV-1); lane 3, *E. siliculosus* virus (EsV-1).

is very similar to HincV-1 but differs from EsV-1, which contains an additional membrane-like structure between coat and core (Müller *et al.*, 1990).

PCR analyses aiming at the detection of a DNA sequence homologous to a gene fragment of EsV-1 yielded an amplification product of the same size as that produced with EsV-1 DNA. The genomes of PlitV-1 and EsV-1 thus probably share a gene coding for a viral structural protein (gp-1). The failure of PCR amplification from crude extracts of virus-infected *Pilayella* was probably caused by the presence of interfering substances in the algal homogenates.

In *Ectocarpus* sporophytes infected by EsV-1, meiotic segregation in unilocular sporangia may create virus-free gametophytes (Bräutigam *et al.*, 1995; Sengco *et al.*, 1996). This mechanism of escape from viral infection is probably not possible in *P. littoralis* because no evidence of sexual reproduction has been found in this species (Müller & Stache, 1989). Still, the virus–host interaction between PlitV-1 and *P. littoralis* appears to be well balanced: only a very small proportion of individuals in field populations appears to be affected and, in culture, infected thalli were seen partly to suppress virus formation and to resume formation of normal spores.

With the *P. littoralis* virus described here there are now seven brown algal viruses, all of which have been confirmed to be infectious pathogens and are kept in clonal culture. It can be expected that future investigations including improved isolation and molecular techniques will reveal many more brown algal taxa to be affected by similar viruses. Apart from their interesting genetic and cell biological features, these viruses probably also have significant effects on natural host populations.

Acknowledgements

We thank Eva Kessler for excellent technical assistance and Elke Rometsch for collaboration in initial PCR experiments. This work was supported by the Deutsche Forschungsgemeinschaft (Mu 196/18-3).

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