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New lipid envelope-containing dsDNA virus isolates infecting *Micromonas pusilla* reveal a separate phylogenetic group

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ABSTRACT: Viral infection of phytoplankton has major implications for biochemical and energy cycles, community dynamics, and microbial evolution in the marine environment. The non-bloom forming picoplankter *Micromonas pusilla*, a significant component of the plankton community worldwide, is known to be susceptible to infection by both dsDNA and dsRNA viruses. Logically, comprehensive knowledge of the ecology of *M. pusilla* requires a better understanding of the diversity and infection mechanisms of their viruses. Here, we investigated 19 new *M. pusilla*-specific viruses (MpVs) isolated from different locations and years. We performed partial characterization of those MpVs including structural characteristics, genome size, phylogenetic analysis based on partial DNA polymerase gene sequences, host range, and stability at different temperatures and upon exposure to chloroform. Combined, these characteristics allowed classification of the MpVs into 2 groups. Exposure to chloroform led to loss of infectivity by all MpVs in one group, which suggests the presence of an outer lipid envelope. In addition, all except one of the members in that group formed a monophylogenetic clade that was distinct from all other MpV isolates. The distinctive characteristics of the 2 MpV groups suggest different infection strategies, which may have important implications for the ecology of both host and virus populations in the environment. Knowledge gained from our study adds value to the MpV isolates as a scientific resource as it will aid in developing and testing in the laboratory new hypotheses about the ecological and biogeochemical implications of *M. pusilla* viral infection in the environment.

KEY WORDS: *Micromonas pusilla* · Virus diversity · Phycodnaviridae · NCLDV· Characterization · Lipids

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INTRODUCTION

Viruses are the most abundant biological entities in marine environments (Bergh et al. 1989, Danovaro et al. 2001) and are major players in the microbial food web. Viruses facilitate the movement of nutrients from organisms to pools of dissolved and nonliving particulate organic matter, a process termed the viral shunt (Wilhelm & Suttle 1999). The viral shunt affects microbial turnover rates and hence energy and material fluxes. Furthermore, viruses have a profound effect on microbial population dynamics and in shap-

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ing microbial evolution mainly through DNA or RNA transduction (Thingstad 2000, Brüssow et al. 2004, Martínez Martínez et al. 2006, Rohwer & Vega-Thurber 2009).

Viral infection constitutes a significant source of phytoplankton mortality in environmental aquatic communities and can even be responsible for the demise of large phytoplankton populations within a time scale of days (Bratbak et al. 1993, Brussaard et al. 1996, Evans et al. 2003, Baudoux et al. 2006, Martínez Martínez et al. 2012). Viruses have been isolated that infect many taxa of marine eukaryotic phytoplankton including *Micromonas pusilla* (Mayer & Taylor 1979, Cottrell & Suttle 1991, Brussaard et al. 2004), a member of the class Prasinophyceae which is considered to be the most primitive in the green lineage from which all other green algae and land plant classes have risen (Sym & Pienaar 1993). *M. pusilla* is a purely planktonic, naked, highly motile, non-blooming unicellular picoflagellate (1 to 3 µm in diameter) and is ubiquitous in coastal and oceanic marine waters throughout temperate and cold oceanic regions where it can occur as a prominent constituent of the picoplankton community (Manton & Parke 1960, Zingone et al. 1999, Not et al. 2004, Šlapeta et al. 2006). Predicted climate change outcomes such as increased water column stratification and reduced nutrient concentrations in ocean surface waters can favor picoplankton growth (Schaum et al. 2013), and thus *M. pusilla* dynamics may be useful indicators of ecosystem change.

The *M. pusilla* virus originally isolated from seawater samples collected in the Strait of Georgia, British Columbia (Mayer & Taylor 1979) was the first algal virus ever isolated. Many other dsDNA *M. pusilla* viruses have subsequently been isolated from numerous distant geographic locations (Waters & Chan 1982, Cottrell & Suttle 1991, Sahlsten 1998, Zingone et al. 2006, Bellec et al. 2009). Additionally, a dsRNA MpV *M. pusilla* virus (Mimovirus MpRV) member of the family Reoviridae was isolated from a Norwegian coastal seawater sample using *M. pusilla* strain LAC38 (Brussaard et al. 2004). All *M. pusilla* viruses isolated so far are icosahedral and large in particle size (90 to 95 nm, MpRV; 100 to 135 nm, dsDNA *M. pusilla* viruses). The dsDNA *M. pusilla* viruses belong to the algal virus family Phycodnaviridae, which is part of a group of viruses known as nucleocytoplasmic large dsDNA viruses (NCLDVs) that replicate in the cytoplasm and in some cases partly in the nucleus of their eukaryotic host cells (Iyer et al. 2001). Research stemming from independent studies of *M. pusilla*-specific viruses has revealed significant genetic diversity within dsDNA *M. pusilla* viruses (Cottrell & Suttle 1991, 1995, Bellec et al. 2014), some knowledge of their infection strategy and effect on host physiology (Mayer & Taylor 1979, Waters & Chan 1982), variation in strain specificity (Sahlsten 1998, Brussaard et al. 2004), and evidence for significant cospeciation between these viruses and their hosts in addition to host switches (Bellec et al. 2014). Furthermore, investigations of their dynamics in the environment have shown seasonal and spatial variation in abundance (Cottrell & Suttle 1991, Sahlsten 1998, Zingone et al. 1999) and have indicated the importance of viruses as mortality agents of *M. pusilla* (Evans et al. 2003). Better understanding of the ecology of *M. pusilla* under current and future oceanic conditions is directly linked to better insight into the diversity and infection mechanisms of specific viruses as drivers of the dynamics and evolution of *M. pusilla*. In the era of 'cultivation-independent omic' methods, having well characterized model virus-host systems in culture is still essential to investigate the environmental parameters that affect viral replication success and specificity as well as to aid in understanding the mechanisms of virus-host interactions and their ecological and biogeochemical consequences through hypothesis driven experimental manipulation.

In this study, we report the partial characterization including host range, genome sizing, phylogenetic reconstruction based on the DNA polymerase B gene, heat-inactivation experiments, and effect of exposure to chloroform for 19 new dsDNA *M. pusilla* virus isolates from several locations throughout the North Sea and Dutch coastal waters, the Mediterranean Sea, and the English Channel. Our findings add to the accumulating scientific knowledge on this group of interesting viruses and reveal new aspects of *M. pusilla* virus diversity with potentially significant implications for the ecology of the *M. pusilla*-virus system.

MATERIALS AND METHODS

Algal cultures

Single strain cultures of the prasinophyte *Micromonas pusilla* (Butcher) Manton and Parke were obtained from (1) the Roscoff Culture Collection (RCC strains — see Table 2 for clades), (2) the National Center for Marine Algae and Microbiota at Bigelow Laboratory for Ocean Sciences (strain CCMP 1545 — clade C), and (3) the Culture Collection at the Marine Research Center of Göteborg Uni-

Table 1. *Micromonas pusilla*-virus (MpV) isolates data including genome size as estimated in this study and GenBank accession number for their partial DNA polymerase B sequences. [a]From Chen & Suttle (1996)

MpV strain	Geographical origin	Isolation date	Isolation host	Genome size (Kbp)	GenBank accession #
02T	North Sea 54° 29′ N; 04° 38′ E	July 2003	*M. pusilla* CCMP1545	195	KJ955609
04T	North Sea 59° 34′ N; 00° 30′ E	July 2003		194	KJ955611
05T	North Sea 57° 83′ N; 07° 42′ E	July 2003		189	KJ955612
07T	North Sea 56° 43′ N; 06° 00′ E	July 2003		186	KJ955614
13T	North Sea 59° 00′ N; 00° 00′ E	July 2003		187	KJ955620
40T	English Channel 48° 45′ N; 03° 57′ W	April 2006		194	KJ955624
41T	English Channel 48° 45′ N; 03° 57′ W	April 2006		194	KJ955625
SP1	Pacific Ocean 27° 50′ N; 97° 04′ W	October 1990		186	U32975[a]
03T	North Sea 55° 46′ N; 02° 56′ E	July 2003	*M. pusilla* LAC38	212	KJ955610
06T	North Sea 57° 83′ N; 07° 42′ E	July 2003		200	KJ955613
08T	North Sea 56° 43′ N; 06° 00′ E	July 2003		208	KJ955615
09T	Dutch Coast 52° 10′ N; 04° 16′ E	April 2003		204	KJ955616
10T	Dutch Coast 52° 10′ N; 04° 16′ E	April 2003		210	KJ955617
11T	Dutch Coast 52° 10′ N; 04° 16′ E	April 2003		203	KJ955618
12T	Dutch Coast 52° 10′ N; 04° 16′ E	May 2003		200	KJ955619
14T	North Sea 54° 29′ N; 04° 38′ E	July 2003		209	KJ955621
38T	Mediterranean Sea 41° 40′ N; 02° 48′ W	March 2006		205	KJ955622
39T	Mediterranean Sea 41° 40′ N; 02° 48′ W	March 2006		207	KJ955623
42T	English Channel 48° 45′ N; 03° 57′ W	April 2006		204	KJ955626
43T	English Channel 48° 45′ N; 03° 57′ W	April 2006		205	KJ955627

versity (strain LAC 38 — clade A). The algal cultures were maintained at 15°C and kept at mid-exponential growth phase (approx. 1–2 × 10^6 cells ml^{-1}) by periodically transferring 5 to 10% (v/v) culture in a fresh 1:1 mixture of f/2-Si medium (Guillard 1975) and enriched artificial seawater (ESAW) (Cottrell & Suttle 1991). Light (50 to 100 µmol photons m^{-2} s^{-1}) was supplied by fluorescence tubes under a light-dark cycle of 16:8 h. Cell abundances were calculated by flow cytometry (FCM) on fresh samples as described by Marie et al. (1999) using a FACScalibur flow cytometer (Becton Dickinson), equipped with an air-cooled laser providing 15 mW at 488 nm and with standard filter set-up. The trigger was set on red chlorophyll autofluorescence.

Virus isolation and propagation

Lytic viruses infectious to *M. pusilla* were isolated during spring and summer of different years from seawater samples originating from several geographical locations in the North Sea, the Dutch coastal waters, the English Channel, and the Mediterranean Sea (Table 1). Isolation was conducted by adding filtered (GF/F filters, Whatman, GE Healthcare Europe) seawater to exponentially growing *M. pusilla* strains CCMP1545 and LAC38 cultures (10% v/v) in clear borosilicate tubes. The cultures were incubated for up to 14 d under the light and temperature conditions described above, and were inspected daily for lysis. Lysis was determined by color comparison to non-inoculated control cultures. Lysed cultures were filtered through 0.2 µm pore size polyestersulfone (PES) filters (Minisart® High-flow, Sartorius) and used to reinfect fresh exponentially growing algal cultures. *M. pusilla* virus isolates were made clonal (henceforth referred to as MpVs) by end-point dilution as described by Brussaard et al. (2004). Virus strain MpVSP1 was provided by Dr. C. Suttle, UBC, Canada, and originated from waters off Southern California (Cottrell & Suttle 1991).

Virus production was quantified using flow cytometry. For FCM analysis, samples were fixed with glutaraldehyde (0.5% final concentration, EM grade, Sigma-Aldrich) during 30 min at 4°C followed by freezing in liquid nitrogen and storage at –80°C until analysis. Thawed samples were diluted in TE 10:1 buffer (10 mM Tris-Base, 1 mM EDTA, pH 8.0) and stained with the nucleic acid-specific dye SYBR Green I (Life Technologies) as described by Brussaard (2004). The identity of the MpV population discriminated by FCM after infection and subsequent lysis was verified by comparison to MpV-free cultures at mid-exponential and stationary growth phase, which showed no virus-like particles in the 'MpV-region' (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/a074p017_supp.pdf).

Transmission electron microscopy (TEM)

Aliquots of virus lysates (20 ml, filtered through 0.2 µm pore size filters) were concentrated by ultracentrifugation (142 000 × *g* for 2 h at 8°C, in a Centrikon T-1080 Ultracentrifuge, with a TFF55.38 rotor, Kontron Instruments). The viral pellets were resuspended in 100 µl of TE 10:1 buffer. The concentrated suspensions were fixed with formaldehyde and centrifuged onto electron microscope nickel grids with carbon-coated formvar film in a Beckmann airfuge for 20 min at 30 psi. The grids were negatively stained with 2% uranyl acetate and viewed in a Philips CM12 transmission electron microscope.

Additionally, we used TEM to screen thin sections from *M. pusilla* cells infected (4 to 12 h after infection) with 2 randomly selected isolates for each host to confirm that the virus particles observed by FCM and TEM in lysate samples were indeed infectious to *M. pusilla*. Infected algal cells were fixed by addition of glutaraldehyde to a final concentration of 0.1% and after 30 min, and cells were pelleted by centrifugation at 3200 × *g* for 5 min. Cells were resuspended in 2% gelatin, pelleted again, and the pellet was cut in small cubes of approximately 0.5 mm. These samples were further fixed for 2 h in 2% glutaraldehyde/3% paraformaldehyde in phosphate/citrate buffer (0.1 M $Na_2HPO_4 \times 2H_2O$ and 9.7 mM citric acid, pH 7.2) containing 2.5 mM $CaCl_2$, washed 6 × 10 min in phosphate/citrate buffer and fixed with 1% osmium tetroxide in phosphate/citrate buffer for 1 h. Samples were washed, dehydrated in a graded series of ethanol (50 to 100%), and infiltrated with LR White (London Resin company). Samples were transferred to gelatin capsules filled with resin and polymerized for 48 h at 55°C. Sections were cut using a Leica Ultracut S ultramicrotome and stained with 2% uranyl acetate and lead citrate (Reynolds) before examination in a Philips CM12 transmission electron microscope. Average capsid sizes were determined by measuring 10 virus particles for each isolate.

Genome size

Freshly produced MpV lysates were partially purified by removing cell debris and bacteria by centrifugation (7500 × *g* for 30 min at 4°C, using a fixed-angle rotor F-34-6-38 in a 5810R centrifuge, Eppendorf). The genome size of the individual MpV isolates was determined by Pulse Field Gel Electrophoresis (PFGE) as described in Baudoux & Brussaard (2005). Briefly, the clarified supernatant was decanted and viral particles were pelleted by ultracentrifugation (142 000 × *g* for 2 h at 8°C, in a Centrikon T-1080 Ultracentrifuge, with a TFF55.38 rotor, Kontron Instruments) and resuspended in 150 µl SM buffer (0.1 M NaCl, 8 mM $MgSO_4 \cdot 7\ H_2O$, 50 mM Tris-HCl, 0.0005% (w/v) glycerine). Plugs (at least 3 per virus isolate) were prepared by mixing equal volumes of virus concentrate and molten 1.5% (w/v) InCert agarose (Cambrex Bioscience) in plastic molds. Once hardened, the plugs were incubated overnight at 30°C in 800 µl of lysis buffer (250 mM EDTA, 1% SDS (v/v), 1 mg ml^{-1} proteinase K, Sigma-Aldrich). After decanting the digestion buffer, the plugs were washed in TE 10:1 buffer (10 mM Tris–Base, 1 mM EDTA, pH 8.0). The plugs were stored at 4°C in TE 20:50 (20 mM Tris, 50 mM EDTA, pH 8.0) until analysis. Plugs were loaded alongside DNA Lambda ladder plugs (Bio-Rad) onto 1% SeaKem GTG agarose gels (Cambrex Bioscience) prepared in 1 × TBE gel buffer (90 mM Tris–Borate and 1 mM EDTA, pH 8.0). Samples were electrophoresed in a Bio-Rad CHEF DR-II Cell unit filled with 0.5 × TBE buffer (45 mM Tris–Borate and 0.5 mM EDTA, pH 8.0), at 6 V cm^{-1} with pulse ramps of 20 to 45 s at 14°C for 22 h. Gels were subsequently stained for at least 1 h with SYBR Green I (1×10^4 of commercial solution, Molecular Probes) and visualized in a FluorS imager (Bio-Rad Instrument). Genome sizes were estimated based on the migration of each individual genomic DNA band compared to a molecular size marker (note that this method offers only approximate size values and complete genome sequencing is required for accurate genome sizing).

Viral DNA purification

Fresh MpV lysates (25 ml) were centrifuged at 10 000 × *g* for 30 min at 4°C to remove most bacteria and cellular debris. The clarified lysate was incubated at 4°C overnight with NaCl (2 M final concentration) and PEG6000 (final concentration 10 wt%) (CalBiochem). The viruses were subsequently pelleted from the supernatant by centrifugation at 9000 × *g* for 25 min at 4°C, the supernatant was decanted and the pellet was air-dried for ~10 min. DNA was extracted by adding 500 µl of prewarmed lysis buffer (0.5% SDS, 20 µg ml^{-1} proteinase K) to the pellet, and incubating at 55°C for 30 min. Then, 80 µl of 5 M NaCl and 100 µl CTAB-buffer (10% hexadecyltrimethyl ammonium bromide [CTAB] in 0.7 M NaCl) were added and the mixture was incubated again at 65°C for 15 min, after which 500 µl of Chloroform: Isoamylalcohol (24:1) was added and mixed well. The sam-

ples were then centrifuged at 20 000 × *g* for 5 min and aqueous phase containing the DNA was transferred to a new tube. Finally, DNA was precipitated using a standard isopranol precipitation method and resuspended in 50 µl of TRIS (50mM, pH8). DNA yield and quality were checked using a NanoDrop® ND-1000 (Thermo Scientific).

PCR amplification and sequencing of viral DNA polymerase B gene fragments

Virus DNA templates were diluted 5000-fold in molecular-grade water and DNA polymerase fragments were amplified from each template using previously described PCR and thermocycling conditions with AVS1-AVS2 primers (Chen & Suttle 1995a,b) modified by adding a M13 tag (5′-CAC GAC GTT GTA AAA CGA C [primer]-3′) to both primers. Two separate PCRs were run, one with the AVS1-M13 and AVS2, and one with the AVS1 and AVS2-M13 primers. Finally, the PCR amplicons were cleaned up using Genscript PCR Clean-up kit (Genscript) according to the manufacturer's recommendations, prior to BigDye cycle sequencing on an ABI prism 310 Genetic analyser at the Royal Netherlands Institute for Sea Research. *M. pusilla* virus DNA polymerase sequences generated in this study were deposited into GenBank (accession numbers in Table 1).

DNA sequences from our MpV isolates were aligned with an additional 35 *M. pusilla* virus DNA polymerase B partial sequences available through GenBank and previously published (Chen & Suttle 1996, Bellec et al. 2014). Five *Ostreococcus* virus and 2 *Bathycoccus* virus DNA polymerase B partial sequences (Bellec et al. 2014) were included as an outgroup. The sequences were initially aligned using the ClustalX algorithm (Larkin et al. 2007) within MEGA version 6 (Tamura et al. 2013). The alignment was manually refined and a sequence mask was applied to retain regions of unambiguous alignment; only those positions were included in subsequent phylogenetic analyses. The alignment was subjected to a Bayesian Inference (BI, generally more suitable for phylogenetic reconstruction of highly similar sequences) using MrBayes, version 3.2.1 (Ronquist et al. 2012). We used the general time-reversible (GTR) model of substitution (Lanave et al. 1984, Tavare 1986, Rodriguez et al. 1990), considering invariants and a gamma-shaped distribution of the rates of substitution among sites. We used 4 chains of 1 000 000 generations and trees were sampled every 100 generations using a Markoff Chain Monte Carlo (MCMC) analysis. Chain parameters appeared to be stationary after 100 000 sampled trees; the first 200 000 trees (20 %) were discarded as burn-in for the tree topology and posterior probability. The consensus tree was viewed and edited in MEGA version 6 (Tamura et al. 2013).

Host range

Susceptibility of 17 *M. pusilla* strains, originated from different geographical locations (see Table 2), to the 20 MpV isolates was determined using fresh cultures and virus lysates. Briefly, each MpV isolate was added to exponentially growing cultures of each *M. pusilla* strain (10 % v/v, in triplicate). The inoculated cultures were gently mixed daily to encourage virus adsorption and prevent host cells sedimentation. Host growth for each *M. pusilla*-MpV combination was monitored daily over a 14 d period. Lysis was determined by color comparison to non-inoculated control cultures and virus production was verified by FCM. FCM analysis of non-infected controls proved the absence of a MpV-like group in these cultures. Cultures that had not lysed 14 d after the addition of an MpV innoculum were considered resistant to that virus strain.

In addition, a strain of the prasinophyte *Ostreococcus tauri* RCC475 (obtained from the Roscoff Culture Collection) was included in the study to test if any of the MpV isolates were able to infect a different, but closely related, phytoplankton species.

Thermal stability and sensitivity to chloroform

To determine the ability of the viral isolates to remain infectious after exposure to freezing temperatures, duplicate 0.5 ml (0.2 µm-filtered) aliquots of each virus lysate were stored (without addition of any cryoprotectant) at −196°C, −80°C, and −20°C for 24 h. The aliquots were thawed at room temperature immediately prior to their addition to 5 ml of exponentially growing host cultures (either *M. pusilla* strain CCMP 1545 or LAC38 used for original isolation) in borosilicate tubes. To determine viral stability at higher temperatures (4°C, 15 to 40°C in 5°C increments, 50°C, and 60°C), duplicate 0.5 ml aliquots of each virus lysate were incubated at the specified temperatures for 10 min in a waterbath, followed by 5 min cooling on ice prior to addition to 5 ml of the appropriate host culture. Duplicate virus-free cultures of both host strains served as negative controls. Inoculated

and virus-free cultures were incubated under the normal culturing conditions described above and monitored daily for lysis over a 14 d period.

MpV lysate infectivity was also tested following 10 min incubation with 10% and 50% (v/v) chloroform. The chloroform was separated from solution by centrifugation at 4000 × *g* for 5 min. The aqueous phases containing the viruses were transferred to clean tubes. These tubes were left with lids open at 4°C overnight to allow for evaporation of any remaining chloroform. Exponentially growing cultures (specific *M. pusilla* strain used for isolation) were inoculated with the treated viruses (10% v/v). The degree of infectivity was determined by end-point dilution with 3 replicates and twelve 10-fold dilution levels for each virus strain. Host samples that received the addition of 10% v/v of fresh culture medium or untreated MpVs served as controls. The experiment was performed using 96-well plates (approximately 200 µl volume per well) and borosilicate tubes (in 5 ml volume assays) to rule out possible container or volume effects. Viral production in the last dilution that caused lysis was confirmed by FCM.

RESULTS

Ultrastructure analysis

TEM of thin sections of *Micromonas pusilla* cells (strains CCMP1545 and LAC38, used for original MpV isolations [Table 1]) from cultures inoculated with viruses (4 to 12 h post-infection) revealed degradation of nuclear material and the accumulation of virus particles in the cytoplasm, which was not present in non-inoculated cultures (Fig. 1A–D). TEM analysis of lysed cultures revealed free virus particles uniform in shape, size, and staining intensity that were comparable to those in the thin section photographs. Viruses isolated using either *M. pusilla* strain (MpVs) were tailless, with icosahedral symmetry as suggested by their hexagonal outline, and approximately 125 ± 6 nm in diameter. The virus particles showed a thick outer layer surrounding an electron-dense inner core (Fig. 1E).

Genome size and type

PFGE analysis revealed MpV genomes around 200 Kb (Table 1, Fig. S2 in the Supplement). The MpVs isolated using *M. pusilla* strain CCMP1545 (CCMP1545-MpV isolates hereafter) had smaller genomes, on average 191 ± 4 Kb, compared to isolates obtained using *M. pusilla* strain LAC38 (LAC38-MpV isolates hereafter), which were, on average, 206 ± 6 Kb. Since our cultures were not axenic, bands smaller than 48.5 Kb seen in some of the samples likely correspond to phages with small capsid and genome sizes present in the lysate.

Nucleic acid extraction and direct PCR amplification of the DNA polymerase B gene (considered a good phylogenetic marker for most members of the NCLDV group (Chen & Suttle 1996, Yutin et al. 2009) confirmed that all virus isolates in this study contained dsDNA genomes (see below).

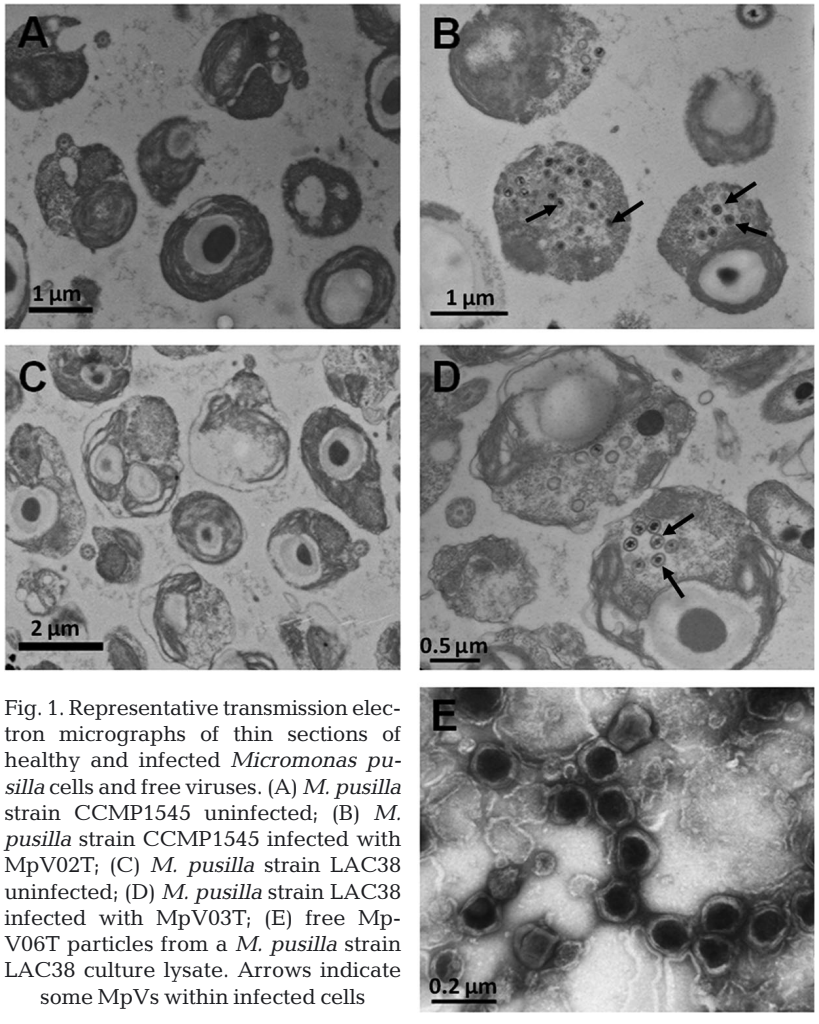


Fig. 1. Representative transmission electron micrographs of thin sections of healthy and infected *Micromonas pusilla* cells and free viruses. (A) *M. pusilla* strain CCMP1545 uninfected; (B) *M. pusilla* strain CCMP1545 infected with MpV02T; (C) *M. pusilla* strain LAC38 uninfected; (D) *M. pusilla* strain LAC38 infected with MpV03T; (E) free MpV06T particles from a *M. pusilla* strain LAC38 culture lysate. Arrows indicate some MpVs within infected cells

Phylogeny

Partial sequences of the DNA polymerase B gene amplified using previously designed AVS1-AVS2 primers (Chen & Suttle 1995a,b) are highly conserved at the nucleotide level among all our MpV isolates. Indeed, several of the newly isolated MpVs have nearly identical partial DNA polymerase B gene sequences. Nonetheless, our Bayesian Inference showed strong support (BI posterior probability = 1) for 2 major clades. One clade contained all the new LAC38-MpV isolates except MpV38T and appeared to be monophyletic, and a second clade contained all remaining MpV, OtV, and OlV sequences from this and other studies. However, the phylogenetic relationships in the second clade were poorly resolved (BI posterior probability = 0.58). Based on the partial DNA polymerase B gene sequence, MpV38T appears phylogenetically closer to a number of MpVs from the English Channel (MicAV27, MicAV28, MicAV31, MicAV32, MicAV34) (Bellec et al. 2014) that were isolated using a *M. pusilla* host that belonged to genetic clade A, like host strain LAC38 used for MpV38T isolation. Two of our CCMP1545-MpVs (MpV41T and MpV05T) were monophyletic to 8 *M. pusila* viruses isolated by Cottrell and Suttle from a variety of geographical locations (1991, 1995) (grey labels, Fig. 2) and to a subset of MpVs isolated in 2009 (Bellec et al. 2014) from English Channel waters, using clade C *M. pusilla* hosts, (MicCV21, MicCV22, MicCV23, MicCV28, MicCV36). Another smaller clade revealed that 2 other 1545-MpVs (MpV40T and MpV02T) were highly similar to MicC497V2. The remainder of our 1545-MpV isolates showed no clear affiliation with any previously-isolated virus. MpV40T and MpV41T were isolated from a single seawater sample collected in 2006 from exactly the same location as that where the MicC-viruses originated.

Host range

The *M. pusilla* strains included in this study showed variable susceptibility to infection, but none was resistant to all MpV isolates (Table 2). In contrast, the closely related prasinophyte *Ostreococcus tauri* strain RCC475 was resistant to infection by all of the 20 MpVs tested (data not shown). Multiple MpV isolates were capable of infecting the same host strains, yet their specificity was highly variable and ranged from MpVs capable of infecting as many as 16 of the 17 *M. pusilla* strains tested (e.g. MpV40T or MpV12T) to as few as 4 *M. pusilla* strains (Mediterranean isolates, MpV38T and MpV39T). Other than a reduced host range for the Mediterranean isolates, no clear pattern in host susceptibility or virus infectivity was observed based

Table 2. *Micromonas pusilla* (host) strain specificity of 19 new *M. pusilla*-virus (MpV) isolates from this study and MpVSP1 (Pac SP1; Cottrell & Suttle 1991). Clear boxes indicate no lysis; grey boxes indicate lysis; • denotes MpV isolated from this host strain. Geographical origins: Pac = Pacific Ocean; Med = Mediterranean Sea; Sar = Sargasso Sea; Sou = Southern Ocean; Nor = North Sea; Eng = English Channel; Bal = Baltic Sea; Dut = Dutch coastal waters

Host clade	Origin	Strain	*Micromonas pusilla*-viruses (MpVs)																			
			Nor 02T	Nor 04T	Nor 05T	Nor 07T	Nor 13T	Pac SP1	Eng 40T	Eng 41T	Nor 03T	Nor 06T	Nor 08T	Dut 09T	Dut 10T	Dut 11T	Dut 12T	Nor 14T	Med 38T	Med 39T	Eng 42T	Eng 43T
A	Pac	RCC299																				
A	Med	RCC449																				
A	Sar	RCC450																				
A	Sou	RCC658																				
A	Med	RCC372																				
A	Nor	RCC692																				
?	Sou	RCC803																				
B	Med	RCC434																				
B	Eng	RCC418																				
B	Eng	RCC461																				
C	Med	RCC498																				
C	Eng	RCC465																				
C	Eng	RCC834																				
C	Nor	RCC629																				
C	Bal	RCC373																				
C	Eng	CCMP1545	•	•	•	•	•	•	•	•												
A	Bal	LAC38									•	•	•	•	•	•	•	•	•	•	•	•

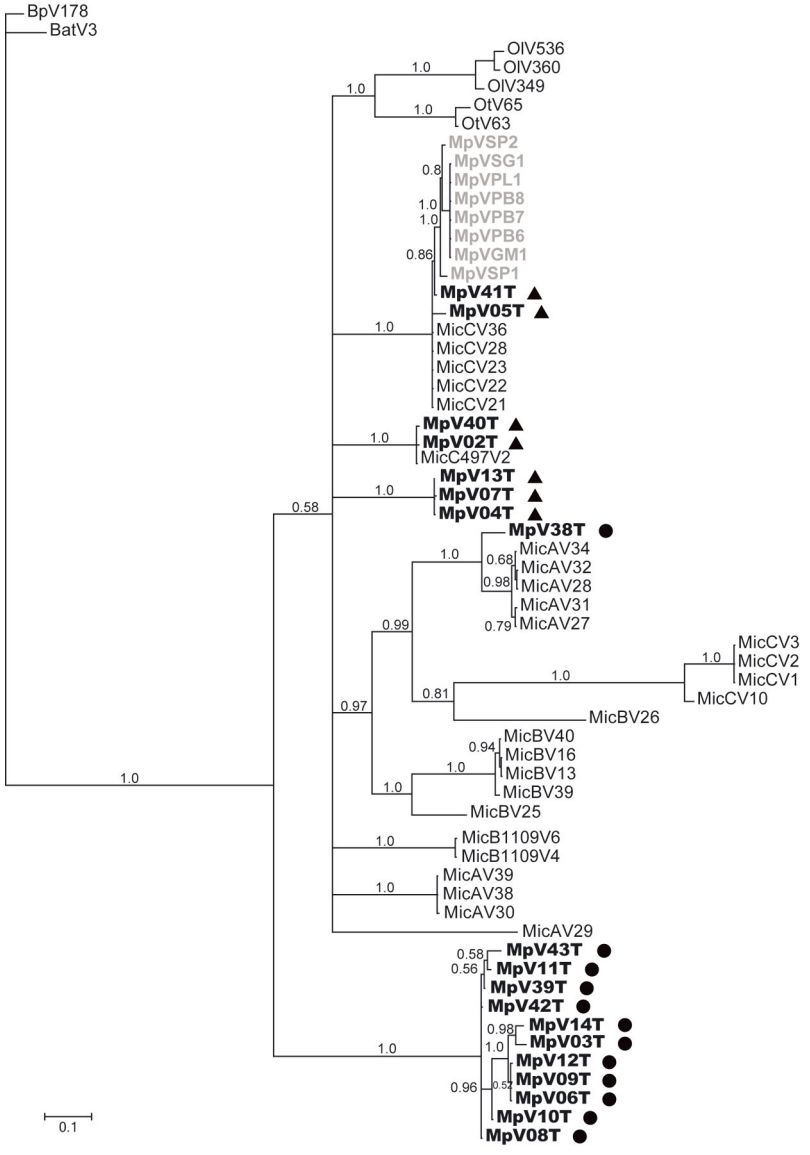


Fig. 2. Phylogenetic tree based on DNA polymerase family B gene fragments. Phylogenetic reconstruction was based on nucleotide sequences using Bayesian Inference. Numbers indicate branch support as posterior probabilities. In bold are novel MpV isolates from this study, isolated with *Micromonas pusilla* strain CCMP1545 (▲) or with *M. pusilla* strain LAC38 (●). In grey are MpV isolates from Cottrell & Suttle (1991), which were isolated with *M. pusilla* Plymouth 27 (from which CCMP1545 originated). Not highlighted are MpVs and other picoeukaryote viruses (OlV and OtV, *Ostreococcus* spp.; BpV and BatV, *Bathycoccus* spp.) from Bellec et al. (2014). Letters A, B, and C within the names of these isolates indicate *M. pusilla* host clade. GenBank accession number for DNA polymerase sequences previously reported can be found within the respective references. The scale bar represents the number of inferred amino acid substitutions per site

on the geographical origin and time of isolation of either the host strains or the viruses. However, it is noteworthy that none of the CCMP1545-MpV isolates were able to infect *M. pusilla* strain LAC38 and vice versa (Table 2).

Thermal stability and sensitivity to chloroform

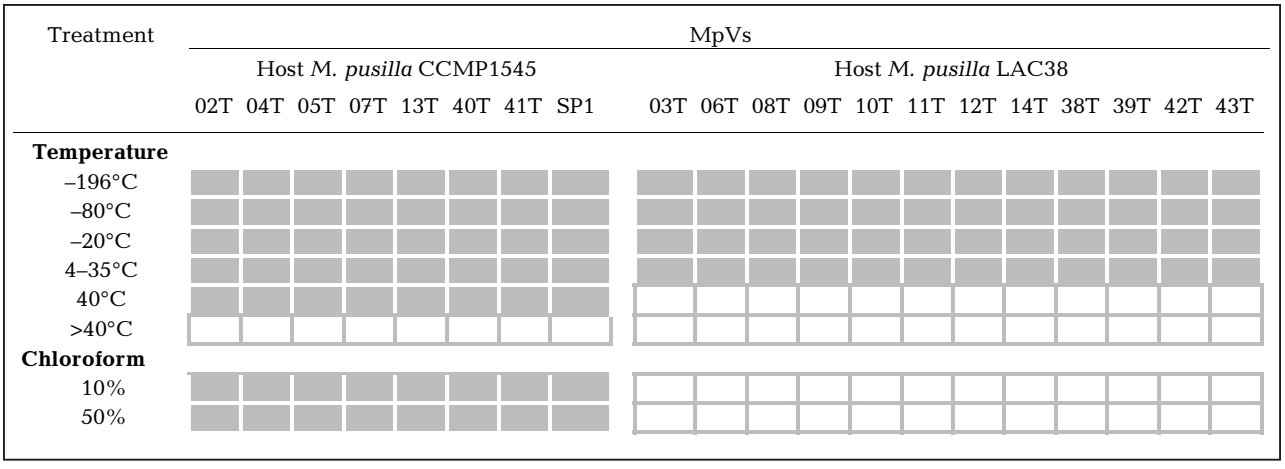
All of our MpV isolates remained infectious after freezing at −20°C, −80°C, and −196°C for 24 h, and slow thawing at room temperature. However, LAC38-MpV isolates were more sensitive to high temperatures and lost infectivity following exposure to temperatures above 35°C, while heat inactivation of CCMP1545-MpVs only occurred above 40°C (Table 3).

Treatment of the MpV isolates with either 10% or 50% chloroform resulted in complete loss of infectivity of all LAC38-MpV isolates, while none of the CCMP1545-MpV isolates lost infectivity (Table 3).

DISCUSSION

All MpV isolates described here appeared to be assembled in the cytoplasm of the host and showed similar hexagonal morphology and particle diameter (125 ± 6 nm). These characteristics are comparable to those of earlier published *Micromonas pusilla* viruses (Mayer & Taylor 1979, Cottrell & Suttle 1991, Zingone et al. 2006). However, we were able to separate the new MpVs into 2 distinct groups based on patterns in (1) average genome size (larger genome size for LAC38-MpVs isolates), (2) susceptibility to viral infection of *M. pusilla* strains CCMP1545 and LAC38 (viruses that lysed one of this host strains could not lyse the other host strain and vice versa), (3) sensitivity to different temperatures treatments (LAC38-MpV isolates more sensitive to high temperature exposure), and (4) loss of infectivity following exposure to chloroform only by LAC38-MpV isolates. The same 2 virus groups could be resolved based on the phylogeny of their partial DNA polymerase B gene sequences, with the exception of MpV38T that was phylogenetically apart from the rest of LAC38-MpVs isolates. Overall, the phylogenetic grouping depending on the host strain and

Table 3. Sensitivity of *Micromonas pusilla*-virus (MpV) isolates to exposure to a wide range of temperatures and to chloroform. Grey boxes indicate lysis by the MpV isolate after the specified treatment. Clear boxes indicate loss of viral infectivity



clade is consistent with that reported by Bellec et al. (2014). Some of our MpV isolates for *M. pusilla* strain CCMP1545 (clade C) are phylogenetically close to 6 dsDNA *M. pusilla* viruses isolated from the English Channel (Bellec et al. 2014) and 8 dsDNA *M. pusilla* viruses isolated from other distant geographical locations (Cottrell & Suttle 1991, Chen & Suttle 1996), one of which, MpVSP1, was included for comparison in our characterization study. The 6 English Channel *M. pusilla* viruses were isolated using *M. pusilla* strain RCC834 and the other 8 *M. pusilla* viruses were isolated and propagated using *M. pusilla* strain Plymouth27 (obtained from the algae culture collection at the University of Texas at Austin). Interestingly, *M. pusilla* strain Plymouth27 was the initial isolate from which strains RCC834 (Roscoff Culture Collection, Station Biologique Roscoff) and CCMP-1545 (National Center for Marine Algae and Microbiota, Bigelow Laboratory) originated (information obtained from culture collection sites). Furthermore, *M. pusilla* strain RCC834 was also included in our host range analysis but it showed different susceptibility to our MpV strains than CCMP1545. Indeed, RCC834 was susceptible to infection by 8 of the 12 LAC38-MpVs. Changes in susceptibility to viral infection by 2 distinct host cultures originating from a common isolate have also been reported for the phytoplankton species *Emiliania huxleyi* (Allen et al. 2007). It is likely that susceptibility differences observed for the 2 *M. pusilla* strains with a common origin, as for *E. huxleyi*, are due to changes in the host, adaptive or not, induced by slight changes in culture conditions under which the alga is maintained for long periods of time (Lakeman et al. 2009). The idea of changes in the host that can affect host range is also supported by the results from a recent study of genomic data that suggests slower evolutionary divergence of prasinoviruses than that of their hosts (Moreau et al. 2010).

MpVs belonging to the 2 groups described here coexist in the environment as several strains were isolated from the same water sample (e.g. MpV02T and -14T; MpV40T, -41T,-42T, and -43T). However, we do not have data about the relative abundance and dynamics of members of both MpV groups that co-occur in the environment. Previous studies have investigated seasonal abundance and dynamics of *M. pusilla* and their viruses in geographically distant locations throughout coastal waters of the Pacific and Atlantic Oceans (Cottrell & Suttle 1991), Scandinavian waters (Sahlsten 1998), and the Mediterranean Sea (Zingone et al. 1999). All 3 studies reported the presence of dsDNA *M. pusilla* viruses at all times of the year, yet they varied among locations and temporally at the same location. In particular, Sahlsten (1998) investigated the abundance of *M. pusilla* viruses infectious to different *M. pusilla* host strains including strains LAC38, CCMP1545, and CCMP491 (a reclone of CCMP1545), and found that viruses infectious to *M. pusilla* strain LAC38 were numerically dominant at all time points. Host range analysis in that study is in accordance with our findings since MpV isolates infectious to *M. pusilla* strain LAC38 appear innocuous to strains CCMP1545 and CCMP-491, and vice versa, under the specific culture and environmental conditions set during these studies. What determines such specificity remains unanswered, especially given the fact that isolates obtained with *M. pusilla* strain LAC38 (clade A) can infect other clade C hosts and CCMP1545-MpVs can lyse

clade A hosts other than LAC38. Further genomic and phenotypic characterization of both *M. pusilla* strains might aid in figuring out this conundrum. Unfortunately, no other parameters in Sahlsten's paper are comparable to those in our study to allow us to place their *M. pusilla* virus isolates into either group as defined here.

Combined, the distinct effect of chloroform exposure and maintenance of infectivity at high temperature exposures differed between the MpV groups in this study, suggesting different infection efficiencies under various environmental conditions and even possibly different propagation strategies. To our knowledge, this is the first study that reports the distinction between dsDNA *M. pusilla* viruses either susceptible or resistant to chloroform exposure. Maintenance of infectivity after exposure to chloroform by all of the members in the CCMP1545-MpVs group suggests that they lack a lipid membrane surrounding their capsids, i.e. they are non-enveloped viruses. The opposite argument is the case for LAC38-MpVs. To date, our understanding of the mechanisms of infection in prasinoviruses (dsDNA *M. pusilla* viruses) is limited. In the initial report of *M. pusilla* viruses isolation, Mayer & Taylor (1979) showed an infection mechanism similar to what is known for non-enveloped NCLDV members of the genera *Chlorovirus* and *Phaeovirus* (Van Etten et al. 2002, Wilson & Allen 2009). Briefly, virus adhesion to the host cell surface is followed by fusion of a viral inner-membrane to the host membrane, allowing entry of the virus genome into the host, and leaving an empty extracellular capsid. Progeny particles form in the cytoplasm of the host and are released through localized rupture of the host's cell membrane. This infection mechanism is consistent with more recent observations on 2 *M. pusilla* virus isolates from Mediterranean waters (Zingone et al. 2006). Our TEM analysis did not provide information regarding the entry mechanisms of MpVs in this study, but shows MpV accumulation in the cytoplasm and rupture of the host's cell membrane. Yet, variations of infection strategies exist among members of the NCLDV group in particular regarding viral entry to and exit from the host cell (Iyer et al. 2001, Law et al. 2006, Mackinder et al. 2009, Mutsafi et al. 2013). Such differences occur even within NCLDV families. For example, the double membrane enveloped vaccinia viruses (family *Poxviridae*, animal viruses) can occur in 3 infectious forms with different structures, abundance, and roles (Smith et al. 2002). The vaccinia viruses display a non-fusogenic outer membrane dissolution allowing the inner envelope to fuse with the host's plasma membrane and releasing an intact virion core into the host's cytoplasm (Law et al. 2006, Doceul et al. 2010). *E. huxleyi*-virus EhV-86, another member of the NCLDV group, has a different infection mechanism than that described for other algal viruses (chloroviruses, phaeoviruses, and prasinoviruses). EhV-86's entry and exit strategies are more similar to those of animal NCLDVs. EhV-86 has a lipid membrane-enveloped capsid and, although it is still not clear whether it enters its host via an endocytotic or an envelope fusion mechanism, an intact viral nucleoprotein core with its capsid reaches the host's cytoplasm. EhV-86 progeny is released via a budding mechanism during which the virions become again enveloped with host's plasma membrane (Mackinder et al. 2009). Based on our results, it is tempting to hypothesize that MpVs infectious to *M. pusilla* strain CCMP1545, which retained infectivity after treatment with chloroform, might be non-enveloped and follow an infection mechanism as previously described for other dsDNA *M. pusilla* virus isolates. On the contrary, the chloroform-sensitive viruses that infect *M. pusilla* strain LAC38 might carry lipid membranes around their capsids and have entry and exit strategies similar to the ones described for EhV-86.

Furthermore, the loss of infectivity at temperatures above 35°C by viruses that infect *M. pusilla* strain LAC38, compared to infectivity maintenance up to 40°C by MpVs specific to *M. pusilla* strain CCMP-1545, is in agreement with literature since the 1950s that indicates that non-enveloped viruses are usually more heat-resistant than viruses with a lipid bilayer envelope (Nims & Plavsic 2013). The different sensitivity of MpVs with and without lipid envelopes to heat suggests variable ecological niche adaptation between both groups. Although it is unlikely that viruses get exposed to such high temperatures in the environment, the result may indicate differential ability among both MpV groups to handle longer term exposure to high temperatures, hence allowing niche differentiation and variable propagation success throughout changing environmental conditions, e.g. one MpV group could be more successful during warmer seasons and the other in colder seasons.

Finally, it is worth noting that the genomes of all the MpVs infectious to *M. pusilla* CCMP1545 are less than 200 Kb in size (average 191 ± 4 Kb), while the MpVs that can infect *M. pusilla* strain LAC38 carry genomes of 206 ± 6 Kb on average, as estimated by PFGE. Full genome sequencing of several representatives from each group would conclusively determine whether the observed differences in genome

sizes correspond to a deletion or insertion of one or several genes of ecological significance. It could also shed light on alternative propagation mechanisms as suggested by the differential characteristics we observed among the 2 MpV groups in this study.

Combined, the results in our study add to the known complexity of the *M. pusilla*-virus system (dsDNA and dsRNA *M. pusilla* viruses) by revealing a greater than previously thought diversity of dsDNA *M. pusilla* viruses, based overall on phylogenetic distinction, average genome size, and the indications of the existence of enveloped and non-enveloped dsDNA *M. pusilla* viruses. In addition, the new chloroform-sensitive MpV isolates infectious to *M. pusilla* strain LAC38 suggest that different infection mechanisms may exist among dsDNA *M. pusilla* viruses. Our study also highlights the importance of basic characterization of cultured viruses to reveal traits and key features that might not have been revealed by genome sequencing of a few isolates. Isolation and maintenance of host-virus systems in the laboratory provides extremely valuable resources for the investigation of major plankton-virus ecology questions, biogeochemical consequences of viral infection, microbial evolution, and unveiling their potential for translational and applied science. Often, thorough testing of hypotheses cannot be achieved by cultivation-independent approaches, in particular in the case of viruses, due to the lack of universal phylogenetic markers and the limitations in appropriate methods for *in situ* field measurements. Full benefit from these model systems can only be achieved through comprehensive knowledge of the virus characteristics.

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