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Diversity of Viruses Infecting the Green Microalga *Ostreococcus lucimarinus*

Evelyne Derelle,[a] Adam Monier,[b,c] Richard Cooke,[d] Alexandra Z. Worden,[c,e] Nigel H. Grimsley,[a] Hervé Moreau[a]

CNRS UMR 7232, Sorbonne Universités, Observatoire Océanologique de Banyuls (OOB), Banyuls sur mer, France[a]; University of Exeter, Biosciences, Exeter, United Kingdom[b]; Monterey Bay Aquarium Research Institute (MBARI), Moss Landing, California, USA[c]; Laboratoire Génome et Développement des Plantes, UMR 5096 CNRS, Université de Perpignan Via Domitia, Perpignan, France[d]; Integrated Microbial Biodiversity Program, Canadian Institute for Advanced Research, Toronto, Canada[e]

ABSTRACT

The functional diversity of eukaryotic viruses infecting a single host strain from seawater samples originating from distant marine locations is unknown. To estimate this diversity, we used lysis plaque assays to detect viruses that infect the widespread species *Ostreococcus lucimarinus*, which is found in coastal and mesotrophic systems, and *O. tauri*, which was isolated from coastal and lagoon sites from the northwest Mediterranean Sea. Detection of viral lytic activities against *O. tauri* was not observed using seawater from most sites, except those close to the area where the host strain was isolated. In contrast, the more cosmopolitan *O. lucimarinus* species recovered viruses from locations in the Atlantic and Pacific Oceans and the Mediterranean Sea. Six new *O. lucimarinus* viruses (OlVs) then were characterized and their genomes sequenced. Two subgroups of OlVs were distinguished based on their genetic distances and on the inversion of a central 32-kb-long DNA fragment, but overall their genomes displayed a high level of synteny. The two groups did not correspond to proximity of isolation sites, and the phylogenetic distance between these subgroups was higher than the distances observed among viruses infecting *O. tauri*. Our study demonstrates that viruses originating from very distant sites are able to infect the same algal host strain and can be more diverse than those infecting different species of the same genus. Finally, distinctive features and evolutionary distances between these different viral subgroups does not appear to be linked to biogeography of the viral isolates.

IMPORTANCE

Marine eukaryotic phytoplankton virus diversity has yet to be addressed, and more specifically, it is unclear whether diversity is connected to geographical distance and whether differential infection and lysis patterns exist among such viruses that infect the same host strain. Here, we assessed the genetic distance of geographically segregated viruses that infect the ubiquitous green microalga *Ostreococcus*. This study provides the first glimpse into the diversity of predicted gene functions in *Ostreococcus* viruses originating from distant sites and provides new insights into potential host distributions and restrictions in the world oceans.

Marine viral genomes may encode life's greatest reservoir of unknown biological functions (1, 2). To date, there is little information about how this genetic information is dispersed on a global scale. For example, whether or not this huge diversity of marine viruses is present everywhere (across all oceans) still is debated (3–6). Comparisons and extrapolations from marine metaviromes indicate that at least for phages, viral genetic distance between two distinct geographical locations is never high enough to detect site-specific viral populations, whatever the geographical location (3, 7). However, metagenomics and taxonomic marker-based studies relying on deeper sequencing show that at least a partial biogeography of viral populations can be detected (5, 6, 8–10). Thus, both the existence of a common core set of virus communities and distinct biogeographic patterns have been reported. For example, between contrasting deep-sea environments, such as the Atlantic Ocean and the Mediterranean Sea, a common set of phages constituting the majority of both virus fractions in terms of relative abundance is shared (11). Conversely, comparison of southern New England and Sargasso Sea waters showed that cyanophages that infect one strain of *Synechococcus* have a striking seasonal and spatial biogeographic pattern (6). Across all studies comparing phage diversity at several marine locations, a fraction of viral sequences is systematically found to be shared between the different sampling sites, whereas another fraction appears to be specific to the site where they have been isolated (4, 12). The few studies describing the genetic diversity of viruses infecting marine microbial eukaryotes appear to show similar pictures (13–15).

Several basic questions regarding marine eukaryotic phytoplankton viruses have yet to be addressed, although they are essential for understanding diversity patterns. These include the question of the diversity of genomes of related viruses (e.g., large double-stranded DNA viruses) that infect the same eukaryotic host. More specifically, it is unclear whether diversity is connected to geographical distance and whether differential infection and lysis patterns exist among such viruses that infect the same host

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Address correspondence to N. Grimsley, nigel.grimsley@obs-banyuls.fr, or H. Moreau, h.moreau@obs-banyuls.fr.

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strain. Prasinophyte/prasinovirus systems offer powerful models, because the hosts (prasinophyte algae) are relatively well characterized and because many lytic viruses presenting various host specificities have been isolated on these algae (1, 16–21). The prasinophytes and, more specifically, members of the class *Mamiellophyceae* represent an ecologically important group of unicellular green algae that are found in the euphotic zone of many marine settings (22). Three *Mamiellophyceae* genera, *Bathycoccus*, *Micromonas*, and *Ostreococcus*, are almost always present in environmental clone library studies from coastal environments or quantitative PCR (qPCR)- and terminal restriction fragment length polymorphism (TRLFP)-based studies from coastal and more oligotrophic sites (23–27). These picoplanktonic genera are infected by double-stranded DNA (dsDNA) lytic prasinoviruses (17–20, 28) that can be detected and isolated from down to one infectious particle per liter of seawater using plating approaches for attaining lytic plaques (29).

Here, we assessed the genetic distance of geographically segregated viruses that infect two *Ostreococcus* species. Four clades corresponding to at least four species and/or ecotypes have been described in the picoeukaryotic genus *Ostreococcus* (30–32), and distributions have been characterized for some (25, 33, 34). We isolated and sequenced viruses that infect *Ostreococcus lucimarinus*, a clade A species that was isolated from coastal California but also is found in many coastal and mesotrophic marine environments (25, 34). We also studied a virus infecting *Ostreococcus tauri*, a host species cultured from a lagoon which appears to be restricted to the northwest Mediterranean Sea (35, 36). Seawater samples from several locations worldwide were tested on these two species by viral plaque plating assays. Viral lysis of *O. tauri* was detected only using samples from the area where the host strain initially was isolated, i.e., northwest Mediterranean Sea, whereas *O. lucimarinus* viruses were found in distant oceanic locations. We present the characterization of the lytic cycles and the comparative analysis of the complete genomes of six *O. lucimarinus* viruses isolated from these distant locations.

MATERIALS AND METHODS

Culture of host algal strains and viruses. *Ostreococcus lucimarinus* (CCMP2972) and *Ostreococcus tauri* (RCC745) were used to screen for the presence of viruses in seawater by host lysis plaques. Preparation of virus suspensions and determination of their infectivity were described previously (17). Seawater was filtered (filter pore size, 0.45 μm) and then concentrated up to 100 times by ultrafiltration with a 50,000-molecular-weight (MW)-cutoff unit (Amicon Ultra; Millipore). Viruses were identified as single lysis plaques after inoculation of 1 to 10 ml of the filtered and concentrated seawater samples onto 0.8% agarose plates with green lawns of each host alga (17). After two rounds of single-plaque purifications, viruses subsequently were produced as described for OtV5 (17) by infecting 2 liters of liquid host culture (12-h light/12-h dark cycle at 100 $\mu E/m^2/s$) at approximately 3×10^7 host cells ml^{-1} (determined by flow cytometry). Briefly, after centrifugation, lysed cultures were passed sequentially through 5-μm and 0.45-μm filters to remove large debris. Virus filtrates were concentrated to a final volume of 1 ml by using the ultrafiltration system mentioned above.

Negative staining and EM pictures. Thirty microliters of viral suspension was deposited onto the shiny side of an electron microscopy (EM) grid for 15 to 45 min to allow the viruses to adsorb to the grid. Using a wedge of filter paper, the sample liquid then was wicked away. Thirty microliters of a 2% uranyl acetate solution then was added and incubated for 45 s at room temperature to stain viruses. The staining solution then was removed using the edge of a filter paper. After drying, the grid was observed on a transmission electron microscope (TEM).

Genome sequencing. Genomic DNA for sequence analysis was prepared by embedding viral particles in agarose strings at approximately 10^{11} particles/ml, lysis of the particles by proteinase K, and migration by pulsed-field gel electrophoresis (PFGE). After migration, a block of agarose containing the viral DNA band was visualized by ethidium bromide staining, cut out, and digested by gelase (Epicentre, Tebu). The DNA was precipitated by adding 2.5 volumes of ethanol (17). Purified viral DNAs were subjected to 454 titanium chemistry sequencing and assembly (GATC, Germany) before gap-filling using custom-made primers and Sanger sequencing.

Sequence annotation. The complete genome sequences were manually annotated using the Artemis software to highlight open reading frames (ORFs). Amino acid sequences of all predicted coding sequences (CDS; defined as open reading frames with appropriate start and stop codons) were screened against the curated Pfam-A profiles for functional motifs using the Perl script Pfam_scan.pl (Wellcome Trust Sanger Institute, United Kingdom) using default settings. The E value cutoff of 10^{-5} was used, as profiles mostly were defined from distantly related sequences, and many functional motifs with relatively high E values are confirmed by significant BLASTP alignments with proteins having the same putative function. Alignments between the seven OlV genomes were performed using Double Act & Act software (http://www.hpa-bioinfotools.org.uk; Wellcome Trust Sanger Institute).

Viral core gene sets. Clusters of orthologs among OlVs and among OlVs plus chloroviruses were detected using orthoMCL v1.4 (37). For OlV orthologs, orthoMCL clustering was run on all results versus those from all BLASTP searches (38) of OlV predicted proteomes using an E value cutoff of 10^{-20}. For OlV and chlorovirus orthologs, which are globally less conserved, an E value cutoff of $1e^{-10}$ was used prior to the orthoMCL clustering.

Phylogenetic reconstructions. The same methodology was applied for both the OlV/chlorovirus core gene set (i.e., 22 concatenated protein sequences) and the phycodnavirus DNA polymerase B phylogenetic reconstructions. The orthologous protein sequences were aligned using M-Coffee, as implemented in T_Coffee v10 (39), and resulting alignments were manually curated. Prior to phylogenetic reconstructions, the substitution model best fitting the data was determined with ProtTest v3 (40) using the Akaike information criterion (AIC). The best maximum-likelihood phylogenetic tree was identified from 100 reconstructions using RAxML v8 (41), and bootstrap supports were computed from 100 maximum-likelihood tree reconstruction replicates.

Genome sequence accession numbers. The six new OlV genomes were submitted to GenBank under the accession numbers HM004431 (OlV1), KP874736 (OlV2), HQ633060 (OlV3), JF974316 (OlV4), HQ632827 (OlV5), HQ633059 (OlV6), KP874737 (OlV7), and KP874735 (OmV1).

RESULTS

Isolation of viruses from distinct geographical origins. Seawater from various worldwide locations was collected and used as source waters to test for lytic viruses infecting *O. lucimarinus* or *O. tauri* (Fig. 1 and Table 1). Lysis plaques were obtained on *O. tauri* plates only using waters from the northwest Mediterranean Sea, i.e., the region where the host strain was isolated (35). This was the case for unconcentrated viral particles and particle concentrates (up to 100-fold) that raised the potential detection limit to 1 lytic viral particle $liter^{-1}$. In contrast, using *O. lucimarinus* as a host, lysis plaques were obtained using waters from geographically distant coastal sites, including the English Channel (eastern-northern Atlantic, two samples), the Canadian Saint Laurent's Bay (western-northern Atlantic), the Chilean (eastern-southern Pacific), and Californian (eastern-northern Pacific, two samples) coasts (Table 1) (29). This greatly expanded the availability of viral

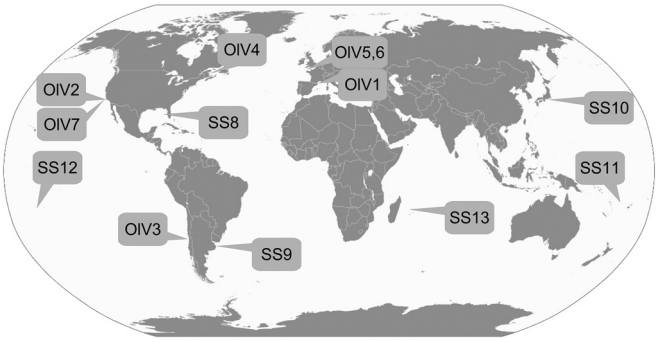


FIG 1 Locations of the sampling sites. The type of *Ostreococcus lucimarinus* virus isolated from each site is indicated. Note that OlV1 was isolated as part of a prior study (18), but samples from that study were used in the present study as well (Table 1).

isolates that infect *O. lucimarinus*, since previously only OlV1 from the French Mediterranean coast was available (18).

Characterization of OlV particles and infectivity. For each of the six positive samples, all lysis plaques were indistinguishable (same size and same aspect). From each plate, one lysis plaque was picked randomly, representing each of the six OlV-positive water samples, and the viruses were amplified for further characterization using the host *O. lucimarinus*. Negative staining pictures of the seven OlVs revealed viral particle structures with icosahedral capsids having a diameter of about 120 to 140 nm without any tail or other protruding structure (Fig. 2). The lytic cycle of each OlV then was characterized using the same multiplicity of infection (MOI) of 10 infectious viral particles (infectivity was determined by the lysis plaque protocol) per host cell (Fig. 3). Similar kinetics of lysis were observed for the seven OlVs on *O. lucimarinus*. Specifically, host cultures were completely lysed within 24 h (Fig. 3A). Release of the first new viral particles occurred approximately 8 h postinfection for the seven OlVs (Fig. 3B) as well as OtV5, as previously reported for the latter (17). Cross infectivity of the seven OlVs was tested against members of other *Ostreococcus* clades/species as well as one *Bathycoccus prasinos* and three *Micromonas* strains (Table 2). None were able to infect the *O. tauri*, *Bathycoccus*, or *Micromonas* strains tested. Moreover, no general pattern of specificity was observed when tested against four *Ostreococcus* clade A strains (to which *O. lucimarinus* belongs) from different host isolation sites (Table 2). All seven OlVs could infect at least one other clade A strain.

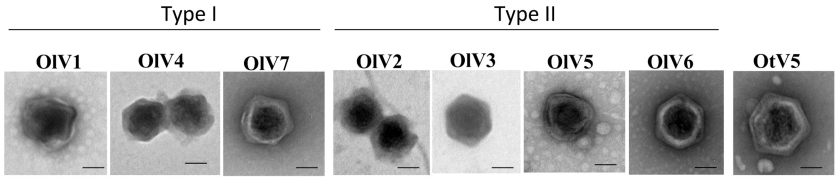
Genome organization of the seven OlVs. Pulsed-field gel electrophoresis (PFGE) of genomic DNA from the six new OlVs indicates that their genomes are similar in size, around 200 kb (Table 3). The total number of putative open reading frames (ORFs) varies from 243 in OlV7 to 269 in OlV2 in their respective genome sequences (Table 3). Two hundred eight genes were orthologous among the seven OlVs, and 34% could be assigned a putative function. Depending on the virus, 3 to 22 ORFs are specific to only one OlV (Fig. 4), resulting in a total of 82 predicted proteins unique to individual OlV genomes. Among these, only five could be assigned a putative function: 1 in OlV3, 1 in OlV4, and 3 in OlV7. Two of these have potential functions directly linked to modification of nucleic acids (1 potential methyltransferase and 1 DNase), and a third [2OG-Fe(II) oxygenase] also may be involved in nucleic acid demethylation (42), while the other two were a putative nucleotide-sugar epimerase and the iron-sulfur cluster assembly protein IscA. The majority of predicted proteins present in at least two but less than seven OlVs are of unknown function. Among those which were assigned a putative function, the 2-oxoglutarate-Fe(II) oxygenase family is highly represented, with 34 genes cumulatively among the seven OlVs. The maximum number of gene copies (9) was seen in OlV3. These genes shared relatively high similarity and are located in a limited region toward the 3′ ends of the genomes. Other genes represented in several copies include methyltransferases (4 to 7 copies) and glycosyltransferases (4 to 6 copies). Genes putatively encoding the phosphate transporter *pho4*, as well as a 6-phosphogluconate dehydrogenase, and a lipase were more sporadically distributed across OlVs but were shared by at least two OlVs. Inteins were not detected in the genome sequences from the OlVs or the *O. tauri* virus.

Gene synteny globally is well preserved, except for an inversion of a conserved 32-kb DNA fragment in half of the viruses isolated (Fig. 5). The inverted fragment codes for 42 (OlV7) to 47 (OlV2) genes, and the inversion itself serves to delineate two groups: OlV1, OlV4, and OlV7 (type I; inverted fragment) and OlV2, OlV3, OlV5, and OlV6 (type II; uninverted fragment) (Fig. 5). These two subgroups also differ in sequence similarities. The global amino acid identities of all orthologous genes inside each

TABLE 1 Worldwide seawater samples tested for viruses infecting *O. lucimarinus* or *O. tauri*

SS[b]	Latitude	Longitude	Date (day/mo/yr)	Depth (m)	*O. tauri*[c]	*O. lucimarinus*[c]	Virus[a]
France, Mediterranean Sea	42°49′N	03°01′E	16/01/2008	0	+	+	OlV1 (I)*
France, English Channel	48°43′N	03°58′W	26/09/2008	0	−	+	OlV5 (II)
France, English Channel	48°46′N	03°56′W	20/10/2008	0	−	+	OlV6 (II)
Quebec, north Atlantic	47°21′N	61°45′W	10/11/2008	5	−	+	OlV4 (I)
Florida, north Atlantic (SS8)	25°38′N	80°03′W	03/11/2008	0	−	−	No virus
Argentina, south Atlantic (SS9)	38°28′S	57°41′W	17/12/2008	5	−	−	No virus
California, north Pacific	36°41′N	122°22′W	15/05/2009	0	−	+	OlV2 (II), OlV7 (I)
Japan, north Pacific (SS10)	26°11′N	127°16′E	10/07/2010	0	−	−	No virus
Chili, south Pacific	36°32′S	72°57′W	24/09/2008	15	−	+	OlV3 (II)
Noumea, south Pacific (SS11)	22°21′S	166°13′E	29/09/2008	0	−	−	No virus
Moorea, south Pacific (SS12)	17°30′S	149°56′W	03/11/2008	0	−	−	No virus
Réunion Island, Indian Ocean (SS13)	20°52′S	55°27′E	01/09/2011	0	−	−	No virus

[a] Virus names are provided for OlVs that are described here and that were isolated from the sites shown. The asterisk indicates a genome that has been described previously (18).
[b] SS, sampling site. SS8 to SS13 are sampling sites where no virus could be detected (Fig. 1).
[c] +, at least one lysis plaque was obtained; −, no lysis plaque.



Virus	OlV1	OlV2	OlV3	OlV4	OlV5	OlV6	OlV7	OtV5
Diameter (nm)	118 +/- 9	132 +/- 16	121 +/- 7	118 +/- 12	138 +/- 20	145 +/- 7	138 +/- 8	129 +/- 16
Number of viruses	10	10	6	7	12	11	12	8
Genome size (bp)	194 016	196 300	191790	190920	187 898	185 593	182 309	186 234

FIG 2 Morphology of the seven OlVs compared to that of OtV5. Viruses were observed after negative staining. External morphology might be slightly altered by the preparation but shows an icosahedral structure for all OlVs that is comparable to the morphology of OtV5. No tail or other external structure could be seen. Bar, 50 nm.

group vary from 92% (OlV1, OlV4, and OlV7) to 94 to 97% (OlV2, OlV3, OlV5, and OlV6), whereas between the two groups, the identity for the same set of genes is only 66 to 69% (Table 4). The 5′ and 3′ regions flanking the inversion site show a palindromic symmetry of 42 inverted repeat nucleotides (no mismatch in 14 bp), reminiscent of a meganuclease site (Fig. 6) (43).

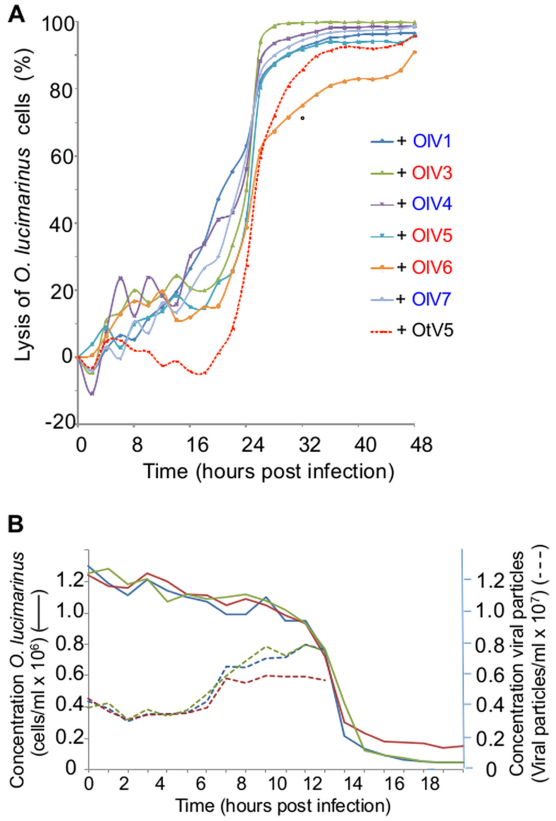


FIG 3 (A) Comparison of the lytic cycles of the OlVs to that of OtV5. Time zero is the time of infection with a multiplicity of infection (MOI) of 10 infectious viruses (measured by a lysis plaque assay) per *O. lucimarinus* cell. *O. lucimarinus* cultures were maintained in a 12-h light/12-h dark cycle, and the culture was infected (time point zero) 1 h after the beginning of the light period. Each experiment was run in duplicate, and curves for each virus represent the averages from these duplicates. Blue, type I viruses; red, type II viruses. (B) Kinetics of lysis of *O. lucimarinus* by OlV5 and of production of viral particles. OlV5 was used as a representative OlV. Continuous lines indicate lysis of *O. lucimarinus* cells (3 independent replicates). Dashed lines indicate the production of free viral particles (three independent replicates). Controls with no viruses added did not show any lysis (not shown). After 9 h, the titer of viral particles did not increase further.

Core genes in viruses of green algae. Genomes of six viruses infecting other *Mamiellophyceae* (*Bathycoccus*, *Micromonas*, and *Ostreococcus*) have been described previously (17–20). In addition, complete genomes of six viruses infecting other green algae, such as *Chlorella* (*Trebouxiophyceae*), are available (44–46). From these and the six new OlV genomes reported here, a core set of viral genes was defined at various taxonomic levels, from the seven OlVs infecting a unique host (*O. lucimarinus*) to a level incorporating all of the complete genome sequences of viruses that are specific to hosts of distinct green lineage classes: the *Mamiellophyceae* (represented by *Bathycoccus*, *Micromonas*, and *Ostreococcus*) and the *Trebouxiophyceae* (represented by *Chlorella*). Core genes were identified by Markov clustering of reciprocal BLASTp hits (orthoMCL; see Materials and Methods). As expected, the number of orthologous genes decreased when the taxonomic distance increased, from 208 (around 80% of the OlV genes) between viruses infecting the same host (*O. lucimarinus*) to 22 orthologous genes shared between prasinoviruses and chloroviruses. The distribution of these orthologous genes on the viral genomes showed preferential (but not exclusive) localization in the central part of the genomes (Fig. 7), as previously observed for a subset of prasinoviruses (18). Interestingly, 11 of these 22 are located in the inverted fragment, although this fragment represents only 17% of the total genome size. Nineteen of the 22 core "green algal" virus genes (viruses infecting hosts in the phylum *Chlorophyta*) (Table 5) have a known potential function, either in DNA replication (9 genes) or in nucleic acid (4 genes), protein (4 genes), sugar (1 gene), and lipid (1 gene) metabolisms. There are 4 hypothetical genes (14%), compared to 92% for the specific genes in one or more (but fewer than 7) of the OlVs.

The identification of 22 core genes enabled us to evaluate nucleotide sequence identity levels across different taxonomic levels. The average identity was similar for the 22 genes shared by the 7 OlVs (88% ± 5%) and for all *Ostreococcus* viruses (85% ± 5%). Identities were lower for the prasinoviruses as a whole (62% ± 11%) and chloroviruses (59% ± 12%). Across the prasinoviruses and chloroviruses, the core genes shared 30% ± 11% nucleotide identity.

Phylogeny of green algal viruses. Identification of a core set of proteins common to all currently available green algal viral genomes enabled us to reconstruct evolutionary relationships among these viruses (Fig. 8). The phylogenetic relationships between the chloro- and prasinoviruses are similar using a concat-

TABLE 2 Specificity of viruses infecting *O. lucimarinus*[a]

	Ostreococcus									*Bathycoccus*	*Micromonas*		
	Clade A					Clade B	Clade C		Clade D		Clade A		Clade C
Virus	CCMP2972	RCC755	RCC756	RCC802	RCC754	RCC809	RCC745	RCC1108	RCC2590	RCC682	RCC299	RCC372	RCC373
OlV1 (I)	+	−	−	+	+	−	−	−	−	−	−	−	−
OlV2 (II)	+	−	−	+	+	−	−	−	−	−	−	−	−
OlV3 (II)	+	+	+	−	+	−	−	−	−	−	−	−	−
OlV4 (I)	+	−	+	−	−	−	−	−	−	−	−	−	−
OlV5 (II)	+	+	+	−	−	−	−	−	−	−	−	−	−
OlV6 (II)	+	+	+	−	−	−	−	−	−	−	−	−	−
OlV7 (I)	+	+	+	−	−	−	−	−	−	−	−	−	−

[a] Countries are listed if samples were collected in their domain. CCMP2972, Pacific (*O. lucimarinus*, Southern Californian Bight, USA); RCC755 and RCC756, north Atlantic (English Channel); RCC802, Mediterranean Sea (Sicily); RCC754, Atlantic (Morocco); RCC809, tropical Atlantic; RCC745, Mediterranean Sea (*O. tauri*, Thau lagoon, France); RCC1108, Mediterranean Sea (Banyuls Bay, France); RCC2590, Mediterranean Sea (Thau lagoon, France); RCC682, North Sea (Germany); RCC299, equatorial Pacific; RCC372, Mediterranean Sea (Naples, Italy); RCC373, Baltic Sea. Precise coordinates of the origins of the strains can be found at the Roscoff culture collection site (http://www.sb-roscoff.fr/Phyto/RCC/). −, no lysis; +, lysis of the host tested.

enated alignment of the 22 core proteins (Fig. 8) or DNA polymerase B alone (not shown). In both trees, the 7 OlVs do not group according to their geographical origin but rather according to the two inversion-delineated subgroups, OlV1, OlV4, and OlV7 (type I) and OlV2, OlV3, OlV5, and OlV6 (type II), with OtV2 (see Discussion). The other *Ostreococcus* viruses (infecting *O. tauri* [OtV1 and OtV5] or *O. mediterraneus* [OmV1]) are sister type II OlVs, with type I OlVs being in a more basal position (Fig. 8).

DISCUSSION

Oceanic viruses strongly influence the ecology and evolution of their eukaryotic hosts; they manipulate the marine environment (47), but their diversity and distributions still are not well characterized. Viruses infecting the abundant and widespread *Mamiellophyceae* (and other prasinophytes) are known as prasinoviruses. Because of the primary production roles of their hosts, these viruses may exert a specific and important role in microbial ecosystems (14). To date, little is known about the extent to which viruses from distant habitats infect the same host species and/or strain, and if so, how they vary at the genomic level. This type of information is important for understanding viral biogeography, host specificity, and genomic diversity. The distribution of the *Ostreococcus* viruses described here shows a link between the overall distribution of their hosts and the presence of infective viruses. Viruses infecting *Ostreococcus* clade A, represented by cultured isolate *O. lucimarinus* strain CC9901 (34, 35), were not recovered from our low-latitude samples (e.g., between 27°N and 27°S) (Fig. 1 and Table 1). Clade A has been observed at considerable abundances in both coastal and mesotrophic environments (25), but the abundance of this host has not been characterized at the low-latitude sites we investigated. The apparent lack of OlVs in these warmer water environments corresponds with results that show *O. lucimarinus* is present in waters with temperatures of 14 ± 3°C, while a different *Ostreococcus* clade (represented by open-ocean strain RCC809) is present at sites with temperatures of >20°C (25), although seasonal dynamics also could play a role. In contrast to samples from our lower-latitude sites, samples coming from several geographically distant midlatitude sites rendered viruses that infect this host, especially coastal and mesotrophic sites, as opposed to oligotrophic waters, again corresponding well with what is known about host distributions (Fig. 1).

Clade C *O. tauri* was lysed only using seawater collected from the northwest Mediterranean coast (Thau lagoon in this study) (Table 1), a finding that corresponds well with the fact that thus far, this host has been reported only in the northwest Mediterranean. However, this host is lysed by OtV1, which was isolated from the English Channel (19). One potential explanation for this contradictory observation is that OtVs are present outside the Mediterranean Sea but are at concentrations much below our detection limits, since Weynberg isolated OtV1 using much larger water volumes (i.e., 1,000-fold concentrates from 20-liter samples; K. Weynberg, personal communication). Alternatively, these results indicate that *O. tauri* itself is present in other near-shore environments, or that OtV1 is active against other *Ostreococcus*

TABLE 3 Global characteristics of the seven *Ostreococcus lucimarinus* viruses

	Value for:						
Parameter	OlV1 (I)	OlV2 (II)	OlV3 (II)	OlV4 (I)	OlV5 (II)	OlV6 (II)	OlV7 (I)
Genome size (bp)	194,016	196,300	191,790	190,920	187,898	185,593	182,309
ORFs (no.)	254	269	264	256	254	251	243
G+C (%)	40.9	41.2	41.4	40.6	41.5	41.6	41.0
Terminal inverted repeats (bp)	2,150	1,535	1,669	1,611	1,718	1,733	1,731
Gene density (gene per kb)	1.31	1.37	1.38	1.34	1.35	1.35	1.33
Coding proportion (%)	95.2	95.5	95.0	94.7	95.1	94.9	95.1
Average ORF size (bp)	727	697	690	706	703	701	713
tRNA[a]	5	5	5	5	5	5	5

[a] The five tRNAs are the same for the seven OlVs.

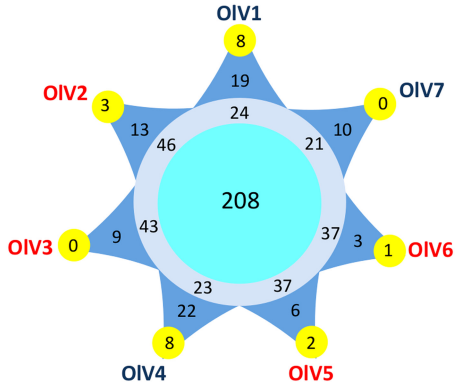


FIG 4 Number of core and specific genes among the seven *Ostreococcus lucimarinus* viruses. Pale blue circle, OlV core genes; gray circle, genes present in more than one OlV but not in all seven of the genomes; dark blue triangles, genes specific to only one OlV; yellow external circles, number of these specific genes shared between this OlV and at least one other prasinovirus. See Materials and Methods for the determination of the orthologous genes.

types present in the English Channel (not investigated in either study) and successfully cross-infects *O. tauri* (30).

The morphology and size of the six new OlVs sequenced here is typical of other characterized prasinoviruses that infect *Micromonas* or *Ostreococcus* (17, 18). The particles also are morphologically similar to the much larger (165 to 190 nm) *Chlorella* viruses except for the spike structure at the vertex, which is not observed in prasinoviruses (45, 48). Globally, they show icosahedral symmetry, like the great majority of the other dsDNA aquatic eukaryote viruses currently described (49), without any tail, in contrast to many archaeal and bacterial bacteriophages (50, 51). The size of their genomes (around 200 kb) and the number of potential ORFs also are similar to those of the other sequenced prasinoviruses (17–20). Several lines of evidence support the delineation of two distinct OlV groups that we term type I and type II. In phylogenetic analysis of 22 proteins shared across the analyzed viruses, the OlVs formed two bootstrap-supported groups (Fig. 8). Concordance of the two reconstructions performed here (Fig. 8) indicates

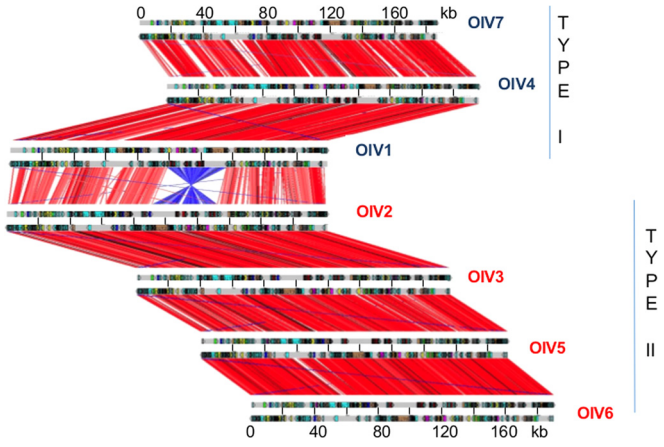


FIG 5 Synteny among the seven *Ostreococcus lucimarinus* virus genomes. Synteny analysis is based on the alignment between annotated open reading frames translated into amino acids for each of the seven OlVs. Each red line represents one orthologous gene. Window, 20 amino acids. Each blue line represents an inverted orthologous gene sequence.

TABLE 4 Percent identity of the core genes between the seven *Ostreococcus lucimarinus* viruses[a]

Virus	% identity to:						
	OlV1	OlV2	OlV3	OlV4	OlV5	OlV6	OlV7
OlV1	100	66	67	**92**	67	67	**92**
OlV2		100	**95**	68	**94**	**94**	69
OlV3			100	68	**95**	**95**	68
OlV4				100	69	68	**92**
OlV5					100	**97**	69
OlV6						100	69
OlV7							100

[a] Boldface numbers indicate compaisons between viruses of the same type.

that PolB is a reliable phylogenetic marker for investigating natural diversity of chlorella- and prasinoviruses (9, 13). One *O. tauri* virus (OtV2) was grouped with OlV type II viruses. However, this virus was isolated using *Ostreococcus* sp. strain RCC393, a clade B strain that is present primarily in oligotrophic waters (25), rather than a strain from clade C (represented by *O. tauri*). Hence, while it is unknown whether the so-called OtV2 also infects clade A or C strains, its name is misleading, since it was not isolated against *O. tauri*. In addition to the observed phylogenetic relationships, nucleotide identities were higher for gene orthologs from OlVs in the same phylogenetic group than between groups, and gene presence/absence patterns were more similar within each group than between groups.

Types I and II do not appear to correlate with geographical origins of the viruses. This indicates that the inversion and sequence divergence arose before the dispersion of the two groups. Moreover, the fact that the bona fide *O. tauri* viruses grouped with type I OlVs suggests these viruses cospeciated with their hosts from a common ancestor with OlV2, OlV3, OlV5, and OlV6. Among type II OlVs, the percent nucleotide identity between orthologous genes of viruses isolated from the same location (OlV5 and OlV6) (Table 1) is higher (97%) than that with the other type II viruses (94 to 95%), suggesting that inside each subgroup the sequence distance reflects the geographical distance, or that the viruses infecting Mediterranean Sea host strains have become more specialized for these hosts. However, additional sampling of viruses will be required to test this hypothesis. The presence of the sequence inversion in the two virus subgroups suggests that this inversion is an ancient rare event that occurred before the separation of the two groups. This hypothesis also is supported by the sequence divergence observed inside the inverted fragment that is similar to the divergence found in the rest of the OlV genomes. Furthermore, the phylogeny suggests that the most parsimonious explanation is that *O. tauri* viruses have arisen from a type I *O. lucimarinus* viral ancestor (with the inversion) by host switching.

Among the genes which are shared by at least two but fewer than the seven OlVs, 2-oxoglutarate-Fe(II) oxygenase genes (52) are present in multiple copies in several viruses. These highly similar copies are located toward the 3′ ends of the genomes, suggesting gene duplications from a single or a limited number of initial acquisitions. This gene family also has been described in multiple copies in viruses infecting cyanobacteria (53). The authors proposed that these genes were involved in the regulation of the cellular nitrogen metabolism or in DNA repair for the benefit of the virus. However, they also could be involved in controlling host translation during the infection (54, 55). Interestingly, the *pho4*

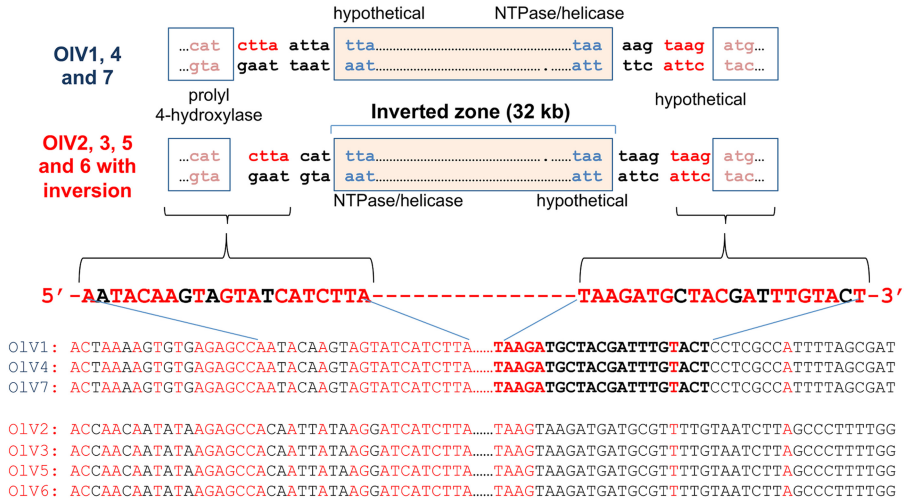


FIG 6 DNA sequences flanking the inverted region. Sequences conserved between the two groups of viruses (OlV1, OlV4, and OlV7 versus OlV2, OlV3, OlV5, and OlV6) and flanking the inverted fragment are represented in red, whereas divergences are in black.

gene, found in six OlVs, also has been found in many other marine eukaryotes and viruses (56). Similarly, a gene that encodes PstS, a protein that is involved in phosphate metabolism, is found in many prokaryotes and cyanophages (57). Multiple independent *pho4* gene transfer events (with retention) have been proposed to occur between marine viruses and their hosts. Thus, manipulation of host phosphate uptake may be an important adaptation for viral proliferation in marine systems, although it is not necessarily an indicator of low ambient phosphate concentrations (10, 54, 58). Methyltransferase genes also are found frequently in viral genomes, where they protect the viral genome against degradation and/or might modify the host genome (59).

Depending on the virus, 3 to 22 ORFs are specific to just one of the OlV genomes and are not found in other prasinoviruses sequenced to date. Since there is no detectable difference between viral replication rates under our laboratory conditions, we speculate that the function of these genes relates to enhancing host growth under certain environmental conditions. Such a metabolic stimulation of viral fitness has been described in cyanobacteria, wherein the host is supplemented with proteins involved in photosynthesis (60). In the closely related prasinophyte *Micromonas*, the virus appears to spare the chloroplast, allowing it to maintain energy production and the photochemical turnover of residual photosystem II complexes during lysis (61). However, in OlVs most of these specific genes have unknown functions, making it more difficult to speculate on possible adaptive functions. Since the OlVs have similar replication cycle kinetics, these virus-specific genes may represent dispensable genome fractions that can be

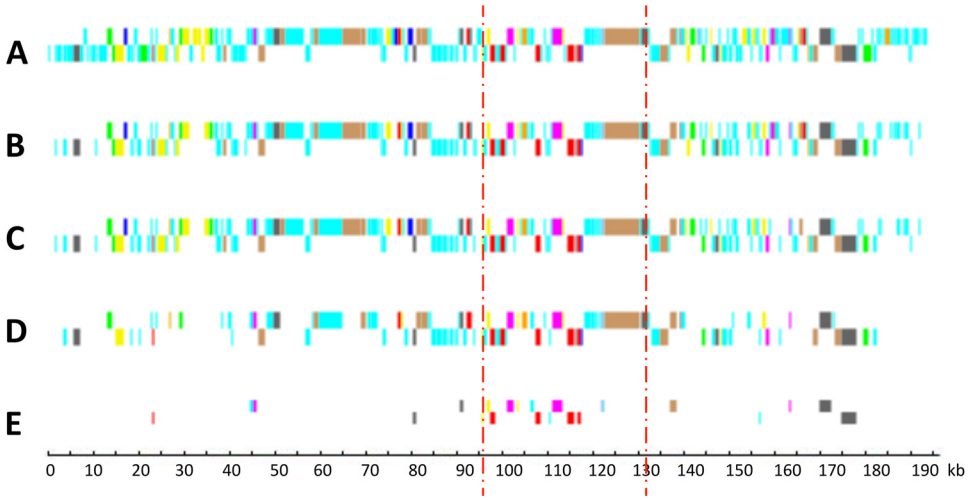


FIG 7 Distribution of core and specific genes in relation to taxonomic levels. (A) OlV1, all genes (254 genes); (B) OlV core genes mapped on OlV1 (208 genes); (C) *Ostreococcus* genus virus core genes mapped on OlV1 (177 genes); (D) *Mamiellophyceae* virus core genes mapped on OlV1 (100 genes); (E) core genes common to *Mamiellophyceae* and *Trebouxiophyceae* (*Chlorella*) viruses (22 genes). Dark blue, DNA methylation and site-specific endonucleases; pale blue, hypothetical proteins; red, transcription; green, sugar metabolism; pink, nucleotide metabolism; yellow, protein synthesis and degradation; gray, DNA replication recombination and repair; orange, signaling; brown, miscellaneous; mauve, lipid and fatty acid metabolism. The zone between the two vertical bars corresponds to the fragment inverted in type II viruses.

TABLE 5 List of the 22 green algal virus core genes

Gene function	Virus gene[a]
DNA replication and transcription	
Transcription factor S-II (TFIIS)	OlV1_030c
ATPase (DNA packaging)	OlV1_95c
PCNA1, proliferating cell nuclear antigen homolog 1	OlV1_111
Putative mRNA-capping enzyme	OlV1_121c
TATA box binding protein (TBP)	OlV1_136c
Transcription activator/SWI/SNF helicase	OlV1_147c
RNase III	OlV1_149c
DNA polymerase B (type II)	OlV1_221
DNA topoisomerase	OlV1_225c
Nucleic acid metabolism	
Thymidylate S	OlV1_061
Ribonucleoside-diphosphate reductase small subunit	OlV1_126
Ribonucleoside-diphosphate reductase large subunit	OlV1_143
dUTPase, deoxyuridine 5′-nucleotide hydrolase	OlV1_209
Protein metabolism	
Prolyl 4-hydroxylase	OlV1_118c
Ubiquitin hydrolase-like cysteine peptidase	OlV1_120
33-kDa *in vitro* translation protein	OlV1_128
Major capsid protein	OlV1_167
Lipid metabolism	
Patatin-like phospholipase	OlV1_158
Hypothetical	
Hypothetical protein NY2A_B350L	OlV1_060
Hypothetical protein FR483_N168R	OlV1_133
Hypothetical protein	OlV1_141c
Hypothetical protein NY2A_B168R	OlV1_199c

[a] The numbering of the genes is based on their position in the OlV1 genome. C, coded on the complementary strand. Genes highlighted in gray are located inside the inverted region.

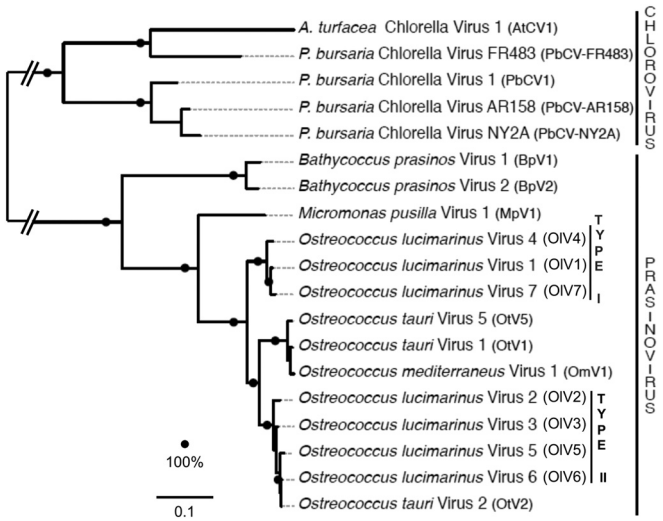


FIG 8 Maximum-likelihood phylogeny of green algal viruses based on prasino- and chlorovirus core genes. The phylogeny was reconstructed from a concatenated alignment of 22 conserved protein sequences (alignment length, 6,688 positions) and using the LG+G+F substitution model. The scale bar reflects substitutions per site; the branch connecting the chlorovirus and the prasinovirus clades was shortened for representation purposes. Bootstrap supports of 100% are indicated as black dots.

employed under nonoptimal conditions. No inteins were identified in these viral genomes, although inteins with homing endonucleases have been observed in prasinoviruses (62), including some *Ostreococcus* viruses (63), and in some green algae, including *Bathycoccus*, with viruses being a proposed mechanism for propagation (64).

Given the wider distribution of *O. lucimarinus* (clade A) viruses, the simplest of several potential scenarios for the evolution of *O. tauri* (clade C) viruses might be that they arose after an initial host switch from *O. lucimarinus* to *O. tauri* of a virus carrying a type I inversion. The OlV ancestor then would have become more specialized in the Mediterranean-adapted clade C host lineage. Further phylogenetic investigations of coastal populations of *Ostreococcus* spp. from different continents should shed light on the origins and evolution of these viruses. Collectively, our studies provide the first glimpse into the diversity of predicted gene functions in *Ostreococcus* viruses originating from distant sites. The results demonstrate that these OlVs are able to infect the same algal host strain, which belongs to a clade that is broadly distributed in coastal and mesotrophic environments. Finally, our results provide new insights into potential host distributions and restrictions in world oceans.

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