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Characterization of different viruses infecting the marine harmful algal bloom species *Phaeocystis globosa*

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Abstract

Twelve lytic viruses (PgV) infecting the marine unicellular eukaryotic harmful algal bloom species *Phaeocystis globosa* were isolated from the southern North Sea in 2000–2001 and partially characterized. All PgV isolates shared common phenotypic features with other algal viruses belonging to the family Phycodnaviridae and could be categorized in four different groups. Two main groups (PgV Group I and II) were discriminated based on particle size (150 and 100 nm respectively), genome size (466 and 177 kb) and structural protein composition. The lytic cycle showed a latent period of 10 h for PgV Group I and latent periods of 12 h and 16 h for PgV Group IIA and IIB. Host specificity and temperature sensitivity finally defined a fourth group (PgV Group IIC). Our results imply that viral infection plays an important role not only in *P. globosa* dynamics but also in the diversity of both host and virus community.

Keywords: Algal virus; Characterization; Diversity; *Phaeocystis globosa*; Phycodnaviridae

Introduction

Over nearly two decades, studies have underlined the numerical dominance, ubiquity and worldwide distribution of viruses in marine pelagic environments (Wommack and Colwell, 2000). Algal viruses are diverse and dynamic within the microbial community (Brussaard et al., 2004b; Chen et al., 1996; Cottrell and Suttle, 1991, 1995; Nagasaki et al., 2004; Schroeder et al., 2003; Short and Suttle, 2003; Tomaru et al., 2004). Most of the existing classes of photosynthetic eukaryotic unicellular algae (phytoplankton) have been reported as hosts for virus-like particles (Brussaard, 2004a; Reisser, 1993; Van Etten et al., 1991). Previous studies indicate that algal viruses are relevant mortality agents in marine environments, directly controlling the dynamics of their host population (Bratbak et al., 1993; Brussaard et al., 1996b, 2005a; Evans et al., 2003; Ruardij et al., 2005; Tomaru et al., 2004). Viral lysis of phytoplankton indirectly affects the structure and functioning of the microbial food-webs, especially when it involves bloom-forming algae (Brussaard et al., 2005b; Castberg et al., 2001; Fuhrman, 1999; Wilhelm and Suttle, 1999).

An important bloom-forming phytoplankter is the globally distributed genus *Phaeocystis* (Prymnesiophyceae). *Phaeocystis* has a polymorphic life cycle with flagellated unicellular and non-motile cells that are embedded in colonies. *Phaeocystis* blooms draw down atmospheric CO_2 as well as produce dimethylsulphide (DMS), which is involved in cloud formation (Arrigo et al., 1999; DiTullio et al., 2000; Stefels and Van Boekel, 1993). Thus, *Phaeocystis* is acknowledged as a microalga playing an important role in global climate regulation and is argued to be a key genus influencing the structure and function of marine pelagic environments (Verity and Smetacek, 1996).

Phaeocystis globosa has the potential to generate high biomass blooms in spring occurring in the temperate waters of the southern North Sea (Cadée and Hegeman, 1991; Lancelot et al., 1987). Termination of these blooms can cause excessive production of foam, which becomes a

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nuisance for socio-economical activities like tourism and fisheries (Orton, 1923; Pieters et al., 1980). *P. globosa* is therefore considered a harmful algal bloom species (HAB).

Cell lysis has been found to be an important loss factor for *P. globosa* cells and can account for 75% of the decline of the bloom (Brussaard et al., 1995, 1996a; Van Boekel et al., 1992). Brussaard et al. (2004b) observed that the decline of a natural bloom was accompanied by a considerable increase of putative viruses infecting *P. globosa* (PgVs), suggesting that viruses were a significant source of mortality for this alga. Very recently, a mesocosm study showed that *P. globosa* population dynamics can indeed be virally controlled (Brussaard et al., 2005a). However, to elucidate the ecological implications of viral infection for *P. globosa* dynamics, more detailed knowledge on the interactions between virus and algal host cell and the characteristics of the viruses is needed.

In this study, twelve lytic viruses infecting *P. globosa* (PgVs) are partially characterized. From these twelve PgVs, four groups were distinguished based on their phenotype

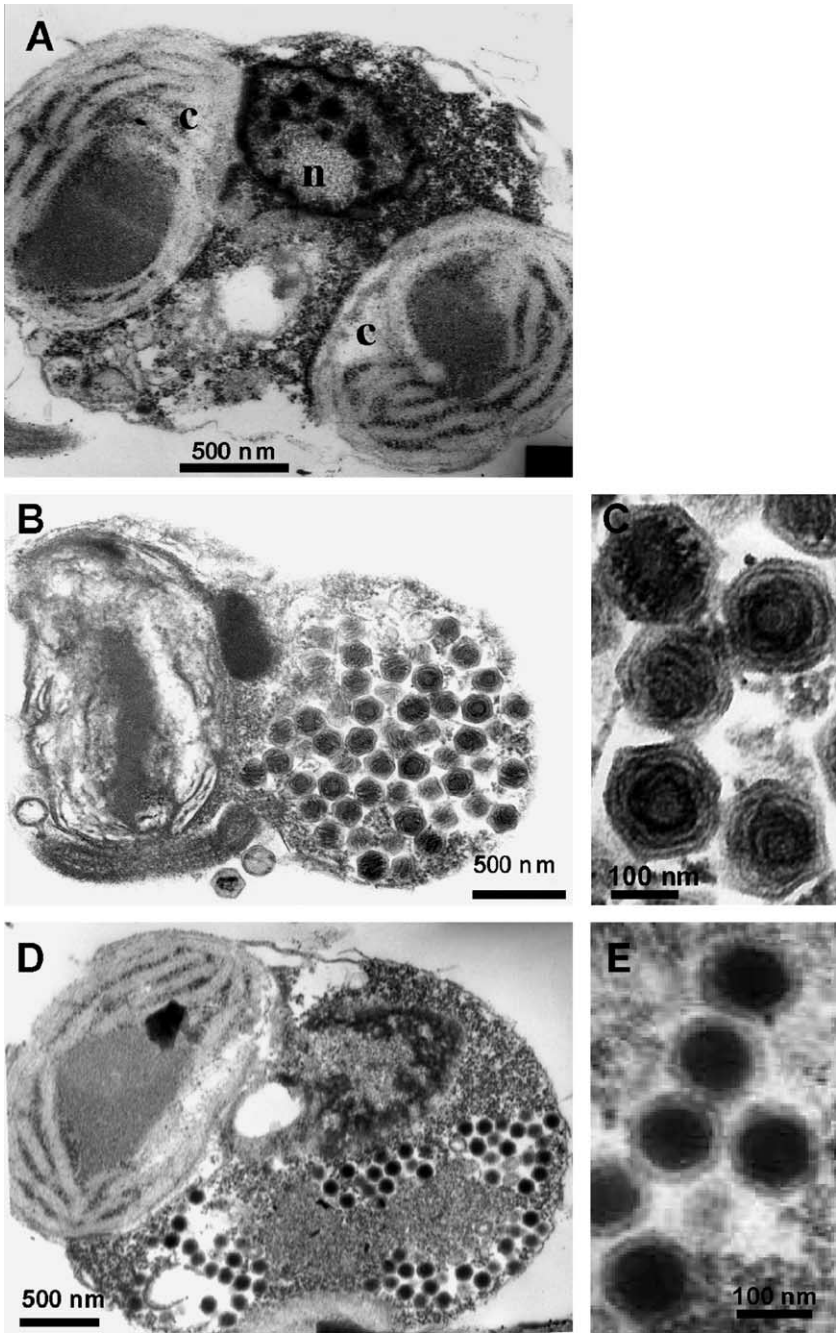

Fig. 1. Transmission electron micrographs of thin sections of infected and noninfected *P. globosa* Pg-I. For all virus isolates, TEM micrographs were obtained, but only representatives are shown here. *P. globosa* noninfected (A), infected with representative virus for PgV Group I (B and C) and *P. globosa* infected with representative virus for PgV Group II (D and E). Nucleus (n) and chloroplast (c) are indicated in the noninfected *P. globosa*.

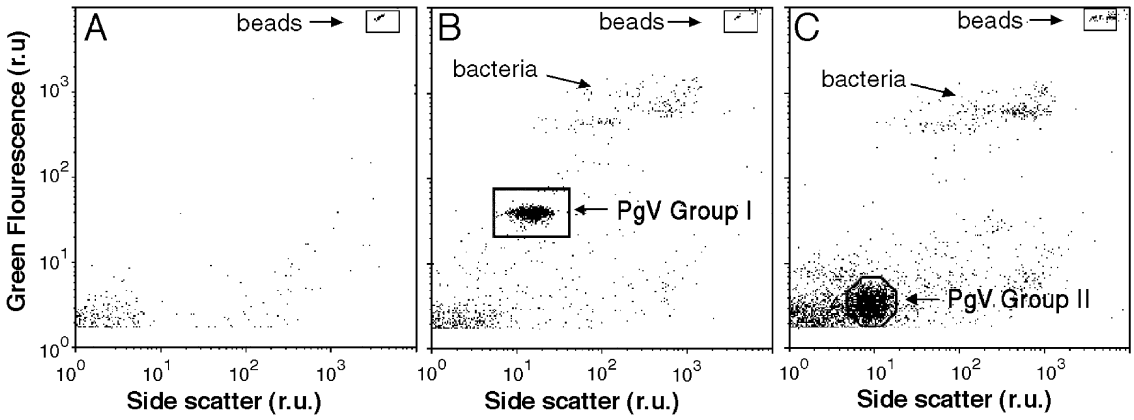

Fig. 2. Flow cytometric signatures of PgV after staining with the nucleic acid-specific dye SYBR Green I of (A) noninfected algal control, (B) representative virus PgV-09T of PgV Group I) and (C) representative virus PgV-11T of PgV Group II. Green fluorescence and side scatter are expressed in relative units (r.u.).

(morphology, genome size, structural proteins, latent period, host range and temperature sensitivity).

Results

Viral morphology and flow cytometric signatures

For all virus isolates, virus-like particles were observed in the cytoplasm of the host cell using TEM. Representatives of the two different virus morphologies are shown in Fig. 1. Both types of viruses were tailless, non-enveloped and with a hexagonal outline suggesting an icosahedral symmetry. The first morphological type (PgV Group I), with a diameter of approximately 150 nm (mean value 153 ± 8 nm) and a thin outer layer surrounding a layered inner core (Figs. 1B and C), was shared by 6 of the virus isolates (PgV-06T, PgV-07T, PgV-09T, PgV-12T, PgV-13T and PgV-14T). The other half of the virus isolates (PgV-01T, PgV-03T, PgV-04T, PgV-05T, PgV-10T and PgV-11T) had the second morphological type (PgV Group II). These viral particles were characterized by a diameter of 100 nm (mean value 106 ± 7 nm) and a thick outer layer surrounding an electron-dense inner core (Figs. 1D and E).

A similar grouping of the virus isolates could be made on the basis of their flow cytometric signature after staining with a green fluorescent nucleic acid-specific dye (representatives are shown in Fig. 2). The larger sized virus particles (PgV Group I, Fig. 2B) had a strongly enhanced green fluorescence compared to the relatively smaller sized virus particles (PgV Group II, Fig. 2C).

Genome size and type

The isolates in PgV Group I harbored a large genome, on average 466 kb ± 4 kb (Fig. 3). Those in Group II harbored a genome of smaller size, on average 177 kb ± 3 kb (Fig. 3). All the viral genomes could be digested with DNase RQ1, indicating their genetic nature to be DNA (data not shown). The large size of the viral genomes, the DNA nature of the genomic material and the staining with DAPI imply that the viral genomes consisted of double stranded DNA.

Lytic cycle

The isolates belonging to PgV Group I, with the large particle diameter and genome size, had a latent period of around 10 h, according to their lytic cycle (Fig. 4A). The decline in algal host abundance in the infected culture was slightly delayed compared to the increase in extracellular free viruses (Fig. 4B). For the viruses of PgV Group II,

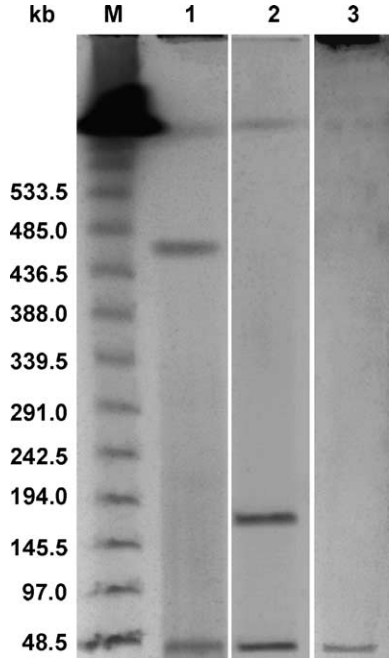

Fig. 3. Genome sizes of all PgV isolates were determined by PFGE. Shown here are representatives for PgV Group I and II. Lane M: Lambda concatamers ladder, Lane 1: representative PgV-09T of PgV Group I, Lane 2: representative PgV-11T of PgV Group II, Lane 3: noninfected culture of *P. globosa*. The small-sized band (approximately 45 kb) as seen in lanes 1–3 corresponds to bacteriophages since the algal cultures were not axenic.

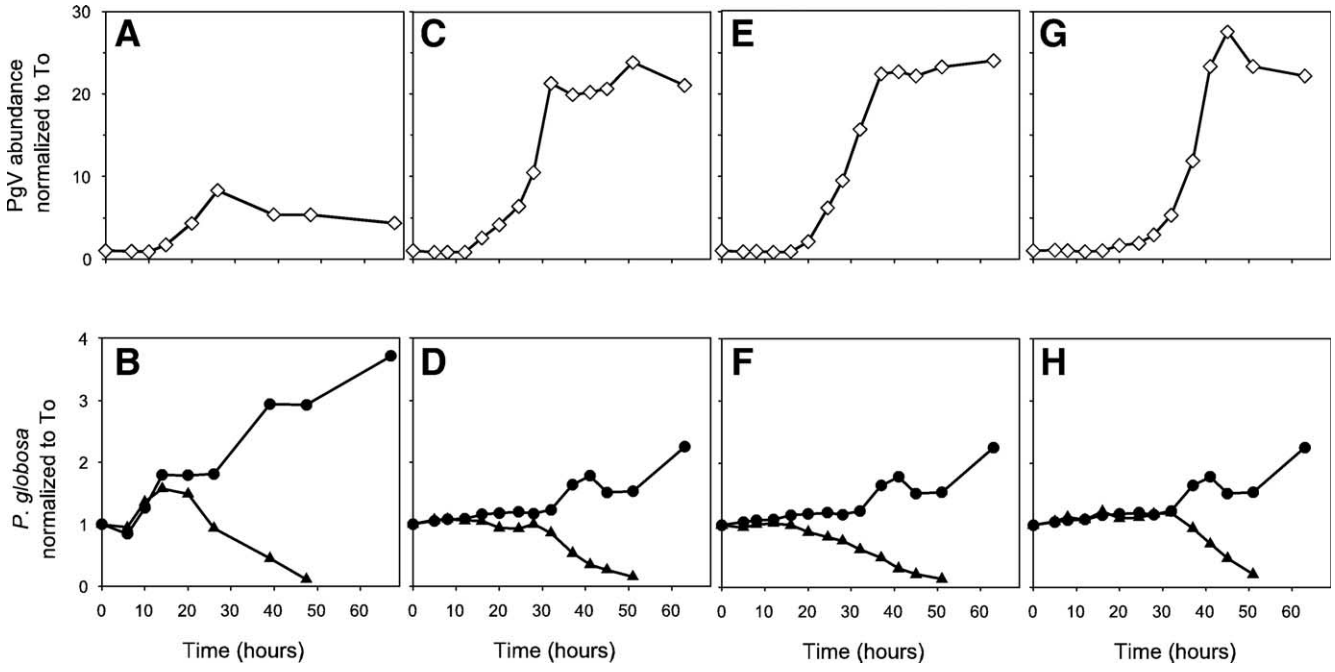

Fig. 4. Abundance of free viral particles (A, C, E and G) and algal host *P. globosa* Pg-I (B, D, F and H). Open diamonds represent PgV abundance, closed circles represent *P. globosa* abundance in the control cultures, and closed triangles represent *P. globosa* abundance in the infected cultures. Viral growth cycles were determined for all viral isolates. Presented here are representatives for PgV Group I (A and B, PgV-09T) and PgV Group II (C and D, PgV-03T; E and F, PgV-11T; G and H, PgV-01T). The length of the latent period for PgV Group I was 10 h and for PgV Group II 12 h (represented by PgV-03T) or 16 h (represented by PgV-11T and PgV-01T).

with the relatively small particle diameter and genome size, two different latent periods were detected: 12 h for PgV-03T and PgV-05T (PgV Group IIA; Fig. 4C) and 16 h for the other viruses (PgV Group IIB; Figs. 4E and G). The production of free viral particles as well as algal lysis was, however, delayed for PgV-01 (Figs. 4G and H) when compared to the other PgV Group IIB isolates (Figs. 4E and F).

From the maximum net decline in algal cell abundance and the concurrent maximum increase in viral abundance, an average burst size for the PgV Group I of 248 viruses *P. globosa* cell^{-1} was estimated. There was, however, considerable variation in burst sizes for the different isolates belonging to PgV Group I despite the fact that the algal host cells were in exponential growth phase the moment of infection (127, 356, 77, 337, 252 and 337 viruses *P. globosa* cell^{-1} for PgV-06T, 07T, 09T, 12T, 13T and 14T). The burst sizes of the virus isolates belonging to PgV Group II were less variable (274, 415, 378, 410, 376 and 360 viruses *P. globosa* cell^{-1} for PgV-03T, 05T, 01T, 04T, 10T and 11T), with an average of 345 viruses *P. globosa* cell^{-1} for PgV Group IIA and 381 viruses *P. globosa* cell^{-1} for PgV Group IIB.

Structural proteins

For the structural protein analysis, at least two representative clonal virus isolates of each PgV group described above were selected. After isopycnic CsCl centrifugation of PgV Group I, 3 bands with a buoyant density of 1.22, 1.23 and 1.275 g ml^{-1} (respectively band 1, 2 and 3 in Fig. 5) were detected for each virus isolate tested. All bands consisted of PgV Group I viruses with their typical high

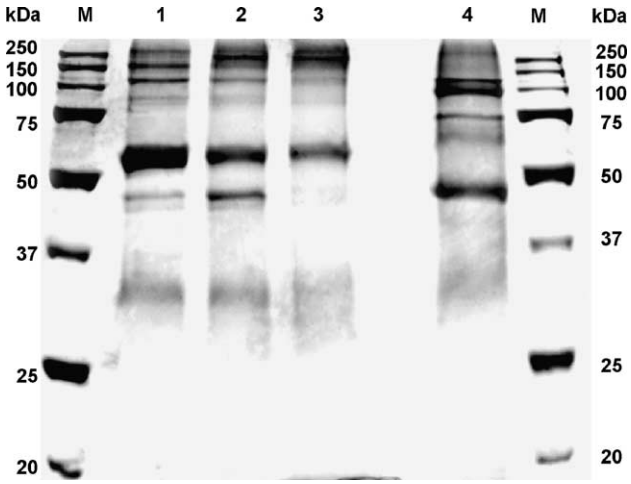

Fig. 5. SDS-PAGE of structural proteins from viral particles purified by isopycnic CsCl centrifugation. Three distinct bands (bands 1–3, with increasing densities) of comparable protein concentration (50–55 μg ml^{-1}) were recorded for two representatives of PgV Group I (PgV-07T and PgV-09T), whereas only one band was recorded for representative viruses of PgV Group II (PgV-01T, PgV-03T and PgV-11T). Lanes M: molecular weight marker, Lane 1: band 1 of representative PgV-09T of PgV Group I, Lane 2: band 2 of representative PgV-09T of PgV Group I, Lane 3: band 3 of representative PgV-09T of PgV Group I, Lane 4: representative PgV-11T of PgV Group II. Not all bands may be visible on the gel shown.

green fluorescence signature after staining with a nucleic acid-specific dye in combination with flow cytometry. The heaviest band 3 was relatively thicker than the other two and contained 70% of the total purified PgV Group I viruses. Besides, most of the protein bands obtained after SDS-PAGE (Fig. 5) were common in all three CsCl fractions which indicates that the bands represent the same virus strain. Either the virus particles are unstable in CsCl and thus the different bands represent different forms of dissociated virus or some of the bands consist of immature virus particles. For all three bands, the regained viruses (after repeated wash steps with sterile seawater or PBS) had lost infectivity.

The lightest density fraction (band 1) of the PgV Group I consisted of four major polypeptides of approximately 257, 161, 111 and 52 kDa and five minor polypeptides with molecular masses of 205, 94, 84, 42 and 41 kDa (Fig. 5). SDS-PAGE did, however, reveal differences in the relative amount of the detected polypeptides for each density fraction of PgV Group I. As compared to band 1, the intermediate (band 2) and the heaviest fractions (band 3) had a substantially higher amount of the 205 kDa protein, in combination with a reduced relative amount of the 161, 111 and 52 kDa proteins. Furthermore, the intermediate fraction showed an enhanced relative amount of the 42 kDa polypeptide.

After CsCl equilibrium centrifugation of PgV Group II representatives, only one band with a buoyant density of 1.37 g ml^{-1} was observed. Also here, the viruses had lost their infectivity. SDS-PAGE of PgV Group II revealed 4 main polypeptides of 119, 99, 75 and 44 kDa and 3 minor polypeptides of 60, 62 and 38 kDa.

Table 1
List of phytoplankton species used to screen for virus-induced lysis by PgV strains in the host range tests

Genus/species	Strains
Prymnesiophyceae	
Phaeocystis globosa	Ph91mf[a]
Phaeocystis globosa	Pg-G (A)[b]
Phaeocystis globosa	Pg-I[b]
Phaeocystis globosa	Pg01MD-02[c]
Phaeocystis globosa	Pg01MD-06[c]
Phaeocystis globosa	SK 35[d]
Phaeocystis globosa	Unknown[d]
Phaeocystis globosa	Ph91hc[a]
Phaeocystis globosa	Pg-G (B)[b]
Phaeocystis globosa	Ph Miller[a]
Phaeocystis globosa	Pg01MD-04[c]
Phaeocystis globosa	Pg Kac 31[e]
Phaeocystis pouchetii	Pp Kac 75[e]
Phaeocystis pouchetii	AJ01[f]
Phaeocystis pouchetii	Pp-13[f]
Phaeocystis antarctica	CCMP1871[g]
Phaeocystis cordata	Phaeonap1[h]
Phaeocystis jahnii	B5[h]
Emiliania huxleyi	Unknown[i]
Isochrysis galbana	CCMP 1323[j]
Pavlova lutheri	Unknown[f]
Chrysochromulina polylepis	Unknown[k]
Bacillariophyceae	
Leptocylindrus danicus	CCMP 469[j]
Asterionellopsis glacialis	Unknown[k]
Chaetoceros socialis	Cs-T01[c]
Thalassiosira weissflogii	CCMP 1049[j]
Ditylum brightwellii	CCMP 358[j]
Chlorophyceae	
Dunaliella sp.	Unknown[c]
Nannochloris sp.	CCAP 251/2[l]
Prasinophyceae	
Tetraselmis sp.	Unknown[c]
Prasinococcus capsulatus	CCMP 1192[j]
Cryptophyceae	
Rhodomonas salina	CCMP 1319[j]
Eustigmatophyceae	
Nannochloropsis salina	CCAP 849/4[l]
Dinophyceae	
Prorocentrum micans	CCMP 1589[j]
Scrippsiella sp.	Unknown[c]
Amphidinium sp.	Unknown[m]
Gymnodinium simplex	Unknown[c]
Cyanophyceae	
Synechococcus sp.	CCMP 839[j]
Synechococcus sp.	CCMP 1334[j]

Notes to Table 1:
[a] OSD-RIKZ, The Netherlands.
[b] Culture collection University of Groningen, The Netherlands.
[c] Culture collection of The Netherlands Institute for Sea Research, The Netherlands.
[d] Alfred Wegener Institute, Bremerhaven, Germany.
[e] University of Kalmar, Sweden.
[f] University of Bergen, Norway.
[g] University Libre of Bruxelles, Belgium.
[h] Stazione Zoologica Anton Dohrn, Naples, Italy.
[i] University of Leiden, The Netherlands.
[j] Provasoli-Guillard National Center for Culture of Marine Phytoplankton, Maine, USA.
[k] University of Oldenburg, Germany.
[l] Culture Collection of Algae and Protozoa, Scotland, UK.
[m] University of Copenhagen, Helsingør, Denmark.

Host range specificity

The virus isolates were specific for *P. globosa* as no other algal species tested, including other *Phaeocystis* species, were infected (Tables 1 and 2). The viruses of PgV Group I had a slightly higher degree of strain specificity than the viruses of PgV Group IIA and IIB (Table 2). PgV-01T was, however, an outlier as it was the only virus isolate causing lysis of all *P. globosa* strains tested (including one from the west coast of the USA, Table 2). This difference, in combination with the delayed algal host lysis and production of viral particles, was striking enough to separate it into a new group (PgV Group IIC).

Table 2
Phaeocystis globosa strains used to screen for virus-induced lysis by different PgV isolates

P. globosa strains	Strain origin if not Southern Bight, North Sea	PgV Groups											
		I						IIA		IIB			IIC
		PgV-06T	PgV-07T	PgV-09T	PgV-12T	PgV-13T	PgV-14T	PgV-03T	PgV-05T	PgV-04T	PgV-10T	PgV-11T	PgV-01T
Ph91mf		+	+	+	+	+	+	+	+	+	+	+	+
Pg-G (A)		+	+	+	+	+	+	+	+	+	+	+	+
Pg-I		+	+	+	+	+	+	+	+	+	+	+	+
Pg01MD-02		+	+	+	+	+	+	+	+	+	+	+	+
Pg01MD-06		–	–	–	–	–	–	+	+	+	+	+	+
SK 35		–	–	–	–	–	–	–	–	–	–	–	+
Pg 1	Unknown	–	–	–	–	–	–	–	–	–	–	–	+
Ph91hc		–	–	–	–	–	–	–	–	–	–	–	+
Pg-G (B)		–	–	–	–	–	–	–	–	–	–	–	+
Ph-Miller	CA, USA	–	–	–	–	–	–	–	–	–	–	–	+
Pg01MD-04		–	–	–	–	–	–	–	–	–	–	–	+
Pg Kac31	Skagerrak	–	–	–	–	–	–	–	–	–	–	–	+

Plus (+) indicates lysis, and minus (−) indicates no lysis of the algal host culture upon infection with PgV (20% v/v).

Thermostability

The representatives of PgV Group I had different sensitivities to heat and freezing treatments when compared to the PgV Group IIA, IIB and IIC (Table 3). PgV Group I became sensitive at temperature ≥35 °C, with a complete inactivation of the virus at 45 °C. The viruses belonging to PgV Group IIA and Group IIB were negatively affected by temperature ≥25 °C, with a complete loss of infectivity at 35 °C for PgV Group IIA and IIB and 30 °C for PgV Group IIC.

All virus isolates remained infective after storage for more than a year at 4 °C in the dark. The PgV Group I representative was stable when frozen for 24 h at all temperatures tested (−20 °C, −50 °C, −80 °C and −196 °C). The viruses of Group IIA and IIB were only stable when frozen at −80 °C and −196 °C. PgV Group IIC was the most sensitive as it could not withstand freezing at any of the temperatures tested.

Discussion

All twelve virus isolates infecting specifically *P. globosa* that were characterized in the present study seem to belong to the virus family Phycodnaviridae: they infect an eukaryotic algal species, are polyhedral in shape, do not have an envelope, lack a tail, are large in diameter (>100 nm) and contain large dsDNA genomes (>175 kb, http://www.ncbi.nlm.nih.gov/ICTVdB/51000000.htm; Brussaard, 2004a; Van Etten and Meints, 1999). Moreover, our suggested classification of the PgVs into the Phycodnaviridae is confirmed by a recent study examining the genetic relatedness among seven PgV isolates of which six are described in the present study (Brussaard et al., 2004b). Based on conservative DNA polymerase (*pol*) gene sequences, which have been shown to be a good phylogenetic marker for inferring genetic relationships among algal viruses, these authors showed that the PgVs formed a closely related monophyletic group within the family Phycodnaviridae. Their results demonstrated that the DNA *pol* fragments of the viruses examined were at least 96.9% identical to each other. Brussaard et al. (2004b), however, did detect variation in the lysis patterns of *P. globosa* based on the in vivo fluorescence algae infected by different PgVs. Our results confirm that, despite the similarity in inferred amino acid sequence phylogeny, PgV isolates differ largely in their phenotypic characteristics. Thus, the present characterization provides relevant additional information for a proper classification of these viruses.

We categorized two main groups of viruses infecting *P. globosa* (PgV Group I and PgV Group II) which differed largely in genome size, particle diameter and protein composition. The genome size of the PgV Group I viruses

Table 3
Sensitivity of PgV isolates to temperature

Temperature	PgV Groups			
	I	IIA	IIB	IIC
20 °C	–	–	–	–
25 °C	–	+	+	+
30 °C	–	+	+	++
35 °C	+	++	++	++
40 °C	+	++	++	++
45–75 °C	++	++	++	++
−20 °C	–	++	++	++
−50 °C	–	++	++	++
−80 °C	–	–	–	++
−196 °C	–	–	–	++

Sensitivity was classified as not sensitive (−, no loss of infectivity), sensitive but still lysis (+, delayed lysis of the host in comparison of non-treated isolate) and very sensitive (++, complete loss of infectivity). The viral lysate was freshly prepared and added to exponentially growing algal host. Control exposure temperature was set at 15 °C, and viral activity was assayed in duplicate. All PgV isolates stayed infective at 4 °C for at least a year. Treatments were performed on representatives of each PgV groups: PgV-09T for PgV Group I, PgV-03T for PgV Group IIA, PgV-11T for PgV Group IIB and PgV-01T for PgV Group IIC.

was more than 2.5 times larger than the viruses belonging to PgV Group II (466 vs. 177 kb). Complementing this, the particle size was 1.5 times larger (150 vs. 100 nm in diameter), and the maximum size of the main structural proteins was about twice as large (257 vs. 119 kDa). These results make it plausible that virus–host interactions, host range and viral replication might differ significantly. Host range specificity was, however, remarkably comparable for PgV Group I and II, with many of *P. globosa* strains being infected by both groups of viruses. In the case that no other characteristics affect successful infection, viruses with the shortest latent period (PgV Group I) would have a competitive advantage. One of the *P. globosa* strains (Pg01MD-06) was infected by the PgV Group II viruses, but not by the PgV Group I viruses, which in turn might provide a niche for these viruses with a longer latent period. We found relatively high algal host diversity in the field: indeed, three *P. globosa* strains differing in their sensitivity to PgV infection have been isolated in April 2001 (clonal Pg01MD-02, -04, and -06; Table 2). Pg01MD-02 was sensitive to the infection by all PgV groups, in contrast to Pg01MD-06 and Pg01MD-04 which were resistant to PgV Group I, and PgV Group I, IIA and IIB, respectively. Interestingly, Pg01MD-04 had the tendency to flocculate (produce mucus) upon infection, as did all other algal host strains that were not sensitive to infection by PgV Group I, IIA and IIB (with the exception of Pg01MD-06).

Subgroup PgV Group IIC had a much broader host range than all the other PgV isolates, being able to infect all *P. globosa* strains tested regardless of their geographical origin, tendency to flocculate and the presence of flagella (Pg 1 cells did not have flagella for example). This indicates that this type of PgV would have had a higher probability of encountering a suitable host in the field as compared to the other PgV groups, potentially resulting in dominance despite its longer latent period. The PgV group IIC was, nevertheless, isolated only once, whereas the other PgV groups could be isolated more often and regardless of the absolute abundance of *P. globosa* algal host in the waters (unpublished data, CB). Although we cannot rule out that some PgV groups were more easily isolated, it could be that either production of (immature) viral particles or loss of infectivity differed for the various PgV groups. Temperature has, for example, been suggested as a relevant factor reducing the infectivity of phages (Weinbauer, 2004), but little is known about the temperature sensitivity of algal virus model systems. Although scarcely studied, temperature sensitivity of algal virus isolates seems very diverse (Brussaard et al., 2004a; Cottrell and Suttle, 1995; Nagasaki and Yamaguchi, 1998; Van Etten et al., 1991). Of the PgV isolates characterized here, Group IIC was most sensitive to a rise in temperature. Even after 10 min at temperatures above 20 °C, a loss of infectivity was detected. Such temperature was observed in situ during the summer of 2000 (Van Aken, 2001). As the incubation time in situ will be much longer than 10 min, it can be speculated that the dynamics and potential dominance (due to the broad host range) of PgV Group IIC viruses is strongly controlled by temperature.

Although we only investigated the ability of the PgV isolates to withstand freezing for a short period of time (24 h), the possibility for cryopreservation of PgV Group I, IIA and IIB without any additives is remarkable. We have indications that PgV Group I can withstand cryopreservation for even longer periods (preliminary results), which offers the opportunity to store the original virus, isolate the appropriate host and study the model system in detail with time. To our knowledge, there has only been one other account of cryopreservation of an algal virus without the addition of cryoprotectants (a dsRNA virus infecting *Micromonas pusilla*; Brussaard et al., 2004a). Long-term cryopreservation has been reported for two algal viruses (infecting *Heterosigma akashiwo* and *Phaeocystis pouchetii*) after addition of DMSO or sucrose but was found to be difficult and virus isolate-dependent (Nagasaki, 2001). More detailed research is needed to find out which viral characteristics accommodate successful cryopreservation (for example of PgV Group I, IIA and IIB in contrast to PgV Group IIC).

The 4 distinct types of *P. globosa* viruses described during this study were collected within a year from the same geographical location. We found different PgV groups co-occurring in the same water sample, for example, during the decline of the summer bloom in 2000 (PgV Group I and Group IIA). As a direct consequence of coexisting viruses infecting the same host population, viral infection is argued to be one of the most important factors regulating the abundance and clonal composition of phytoplankton population occurring in the same water (Tomaru et al., 2004; Sahlsten, 1998; Tarutani et al., 2000; Brussaard, 2004a). Several *P. globosa* strains, differing in their sensitivity to PgV, were co-occurring with the characterized viruses, which confirms that virus infection may regulate clonal diversity during an algal bloom.

However, our results also indicated that there can be a significant overlap in the host range of the different PgVs. This suggests that distinct PgV groups co-occurring in the same area do compete for the same specific host strain. Little is known about viral competition for the same specific host strain to date, most likely because so far only a few virus systems infecting the same algal host strain have been brought into culture. Thus, to what extent viral competition affects the diversity of the viral community, as well as of the algal host populations in the field remains to be seen.

Material and methods

Algal cultures and growth conditions

The different unialgal *P. globosa* strains and species representatives of different taxonomic groups (not axenic), used for virus isolation and host specificity testing, are listed in Table 1. All algal species, except *P. pouchetii* AJ01 and

all Dinophyceae, were cultured in ESF2 medium, a 1:1 mixture of f/2 medium (Guillard, 1975) and enriched artificial seawater (ESAW, Cottrell and Suttle, 1991; Harrison et al., 1980) with the addition of Tris–HCl and Na_2SeO_3 (Cottrell and Suttle, 1991). The Bacillariophyceae were grown in ESF2 medium completed with silicate (150 μM), *P. pouchetii* AJ01 was cultured in IMR1/2 medium (Eppley et al., 1967) and the Dinophyceae were cultured in a medium specifically for dinoflagellates (Hansen, 1989). All cultures, except *P. pouchetii* AJ01, were incubated under a light:dark cycle of 16:8 h at 100 μmol photons m^{-2} s^{-1}. *P. pouchetii* AJ01 was grown under a light:dark cycle of 14:10 h at 40–50 μmol photons m^{-2} s^{-1}. All cultures were grown at 15 °C, except for *P. pouchetii* AJ01, *P. pouchetii* Pp-13 and *P. antarctica* CCMP1871 which were grown at 8 °C and 2 °C, respectively.

Virus isolation

Lytic viruses infecting *P. globosa* (PgV) were isolated from natural seawater originating from the southern North Sea in 2000 (June–October) and 2001 (April) according to the procedure described in Brussaard et al. (2004b). Briefly, filtered (Whatman GF/F) natural seawater was added to *P. globosa* cultures (10–20%, v/v) and incubated for 10 days at standard culture conditions of the host. Different host strains of *P. globosa* were used for virus isolation in order to maximize the chance of successful virus isolation. Algal growth was monitored via in vivo chlorophyll fluorescence (F_0) using a Turner Designs fluorometer. The cultures that showed signs of lysis as compared to noninfected controls were filtered through 0.2 μm pore size cellulose acetate filters (Schleicher and Schuell GmbH, Dassel, Germany), after which the lysate was used to reinfect an exponentially growing algal host culture. After recurrent lysis and reinfection, viral isolates were made clonal by end-point dilution as described by Brussaard et al. (2004b).

Transmission electron microscopy

The presence of virus-like particles was confirmed for all virus isolates using transmission electron microscopy (TEM). Infected algal cells (10–15 h after infection) were fixed with glutaraldehyde (0.1% final concentration, EM grade, Darmstadt, Germany) for 2 h on ice. Fixed cells were harvested by low speed centrifugation (3200 × *g*, 5 min, with a A-4-62 swing-out rotor and using a 5810R centrifuge, Eppendorf), after which pellets were resuspended in 6% glutaraldehyde (final concentration) prepared in 0.1 M cacodylate buffer and completed with 5 mM $MgCl_2$ and 5 mM $CaCl_2$ (pH 7.2, all products purchased at Sigma-Aldrich). Samples were kept on ice for 2 h and centrifuged as described above. Pelleted cells were resuspended in 0.1% glutaraldehyde (final concentration) and stored at 4 °C until postfixation. Prior to postfixation, cells were harvested, transferred to 1.5 ml microtubes and washed twice in 0.1 M cacodylate buffer (pH 7.2) using a microcentrifuge (6000 × *g*, 5 min, microcentrifuge model 5415C, Eppendorf). Samples were carefully postfixed with 1% osmium tetroxide (Sigma-Aldrich) prepared in 0.1 M cacodylate buffer (1–2 h), after which they were washed 3 times with 0.1 M cacodylate buffer (pH 7.2). The postfixed cells were dehydrated in an ascending ethanol series (from 70 to 100% absolute ethanol v/v, Fluka) and washed twice with propylene oxide (Agar Scientific, Essex, UK). The supernatant was removed, and the pellets were infiltrated into a 1:1 mixture propylene oxide:agar resins (mixture of 12.6 g MNA, 12.6 g DDSA, 24.8 g agar resin and 0.5 g DMP, Agar Scientific, Essex, UK). The samples were left with lid open overnight and placed at 60 °C for 48 h for polymerization of the resin. Once the resins solidified, plugs were thin-sectioned using a Reichert ultramicrotome. The thin sections were post-stained with 2% uranyl acetate and lead–citrate (Reynolds) before examination under a 100 CX transmission electron microscope (final magnification from ×33,500 to ×52,000, JEOL, Tokyo). At least 10 viral-like particles from each isolate were measured to estimate average particle diameter.

Virus growth cycle

A unialgal culture of *P. globosa* Pg-I was used to determine the one-step virus growth cycle for each PgV isolate. This strain was chosen because of its sensitivity to all the viral isolates studied. Exponentially growing *P. globosa* cells (250 ml) were infected with a freshly produced PgV lysate at an initial virus to host ratio of 20. In case of doubt (for example, when finding deviating burst size), the lytic growth experiment was repeated in order to confirm the results. Most probable number examination of the viral lysates showed that the multiplicity of infection (MOI) of the different viral isolates ranged between 13 and 20. Noninfected control cultures of *P. globosa* received equal volume of medium. The samples were incubated at the host culture standard conditions and sampled every 4 h for a total period of 50 h. Algal and viral abundances were monitored by flow cytometry (FCM, Beckton Dickinson FACScalibur equipped with a 15-mW, 488-nm air cooled argon-ion laser). Algal samples were analyzed directly upon sampling, whereas virus samples were fixed with 25% glutaraldehyde (0.5% final concentration, EM grade, Merck) during 30 min at 4 °C followed by freezing in liquid nitrogen and storage at −80 °C. Analysis of the virus samples was performed using flow cytometry after dilution in TE and staining with the nucleic acid-specific dye SYBR Green I (Molecular Probes, Eugene, OR) according to Brussaard (2004b).

Host range

The host specificity of all PgV isolates was tested using a broad range of phytoplankton species, including 12

different strains of *P. globosa* (see Table 1). Freshly produced PgV lysate was added to exponentially growing algal cultures (20% v/v). The natural in vivo fluorescence of the cultures was monitored every 2 days for 10 days at standard culture conditions. Cultures that did not show signs of lysis as compare to noninfected control cultures were considered resistant to the virus tested. Cultures that underwent lysis were inspected for viral proliferation using flow cytometry.

Genome size and nature

For all virus isolates, freshly produced viral lysate was clarified of bacteria and cell debris by low speed centrifugation step (7500 × *g*, 30 min at 4 °C with fixed angle rotor F-34-6-38 and using a 5810R centrifuge, Eppendorf). Supernatant was concentrated by ultracentrifugation (141,000 × *g*, 2 h at 8 °C, with a rotor TFF55.38 and using a Centrikon T-1080 ultracentrifuge, Kontron Instruments). The viral pellets were resuspended in 150 µl of SM buffer (0.1 M NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris–HCl, 0.0005% (w/v) glycerin; Wommack et al., 1999) and stored at 4 °C overnight. Equal volumes of virus concentrate and molten 1.5% (w/v) InCert agarose (Cambrex Bioscience, Rockland, ME USA) were dispensed into plug moulds and left to solidify for 3 min at −20 °C. The plugs were then punched out of the mould into microtubes containing 800 µl of lysis buffer (250 mM EDTA, 1% SDS (v/v), 1 mg ml^{-1} proteinase K, all products were purchased at Sigma-Aldrich) and incubated overnight at 30 °C. Next day, the digestion buffer was decanted, and the plugs were washed 4 times for 30 min each in TE 10:1 buffer (10 mM Tris–Base, 1 mM EDTA, pH 8.0). Virus-agarose plugs were stored at 4 °C in TE 20:50 (20 mM Tris, 50 mM EDTA, pH 8.0) until analysis. To determine the nature of the viral isolates, virus-agarose plugs previously prepared were treated with DNase RQ1 RNase-Free DNase (Promega) during 1 h at 37 °C. Plugged samples and Lambda concatamers plugs (Bio-Rad, Richmond, CA) were loaded onto a 1% SeaKem GTG agarose gel (Cambrex Bioscience, Rockland, ME USA) prepared in 1× TBE gel buffer (90 mM Tris–Borate and 1 mM EDTA, pH 8.0). Wells of the gel were overlaid with 1% molten agarose, and the gel was placed in the electrophoretic cell containing 0.5× TBE tank buffer (45 mM Tris–Borate and 0.5 mM EDTA, pH 8.0). Samples were electrophoresed using a Bio-Rad DR-II CHEF Cell unit operating at 6 V/cm with pulse ramps of 20 to 45 s at 14 °C during 22 h. After electrophoresis, gels were stained for 1 h with SYBR Green I (1×10^4 of commercial solution, Molecular Probes, Eugene, OR) and destained 10 min in MilliQ (Gradient A10, Millipore) before a digital analysis for fluorescence using a FluorS imager (Bio-Rad Instrument).

Protein characterization analysis

A 5 l freshly produced lysate was concentrated using a 30 kDa MWCO ultrafiltration (Vivaflow 200, Vivascience). The virus concentrate was clarified of bacteria and cell debris by low speed centrifugation (7500 × *g*, 30 min at 4 °C, with a fixed angle rotor type F-34-6-38, using a 5810R centrifuge, Eppendorf) and further harvested by ultracentrifugation (141,000 × *g*, 2 h at 8 °C, with a TFT 55.38 rotor using a Centrikon T-1080 ultracentrifuge, Kontron Instruments). The pellets were resuspended in 150 µl SM buffer (0.1 M NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris–HCl, 0.0005% (w/v) glycerin, Wommack et al., 1999). Viruses were purified on a 1.40 or 1.45 g ml^{-1} Cesium Chloride gradient (Molecular Biology grade, Sigma-Aldrich). Samples were ultracentrifuged (111,000 × *g*, 72 h at 8 °C with a SW41Ti swing-out rotor, Beckman and using a Centrikon T-1080 ultracentrifuge, Kontron Instrument). The visible viral bands were extracted, washed twice with PBS (pH 8) using a 30 kDa MWCO centrifugation filter Amicon Ultra (Millipore). The total amount of protein in each cesium chloride bands was estimated using a BCA Protein Assay Kit (Pierce, Rockford, USA) according to the manufacturer's instructions. The purified viral particles were heated 4 min at 95 °C in SDS sample buffer. A subsample of 10 µl was loaded on a SDS-PAGE gel (Ready gel for polyacrylamide electrophoresis, 10% Tris–HCl, Bio-Rad, Hercules, CA, USA) using a Mini Protean 3 Cell (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Protein molecular weight standards (Precision plus protein standard, Bio-Rad, Hercules, CA, USA) were used for size calibration. The gel was stained for 30 min with a solution of Sypro Orange (5×10^{-4} of the commercial stock, Molecular Probes, Eugene, OR) diluted in 7.5% (final concentration) acetic acid. The gel was destained for 5 min in 7.5% acetic acid prior to the analysis using an Imago imager (B&L Systems, Maarssen, The Netherlands).

Stability against physiochemical treatment

To determine the viral stability at low temperatures, duplicates of 0.5 ml viral lysate in 2 ml cryovials were placed at −196 °C (liquid nitrogen), −80 °C, −50 °C and −20 °C for 24 h. Samples were thawed at 30 °C, after which they were quickly added to exponentially growing *P. globosa* Pg-I cultures (10% v/v). Heat stability was tested for temperatures ranging from 15 to 75 °C in steps of 5 °C. A subsample of viral lysate of 1 ml was heated in a waterbath at the specific temperature of interest for 10 min, after which samples were cooled on ice for 5 min. The subsamples were added in duplicate to exponentially growing algal culture of host (10% v/v). All cultures were incubated for 10 days at the standard culture condition of the host. The natural in vivo fluorescence of the algal cultures was monitored during 10 days to detect algal cell lysis. An algal culture infected with a non-treated virus lysate was taken along as a positive control, and a noninfected culture of *P. globosa* Pg-I served as a negative control.

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