

# Large double-stranded DNA viruses which cause the lysis of a marine heterotrophic nanoflagellate (*Bodo* sp.) occur in natural marine viral communities

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**ABSTRACT:** A virus (BV-PW1) which causes lysis of 2 strains of a marine heterotrophic nanoflagellate belonging to the genus *Bodo* (strains E1 and E4) was isolated from the coastal waters of Texas, USA. Transmission electron microscopy of ultrathin sections of infected nanoflagellates revealed the presence of intracellular virus-like particles 48 h following infection, concomitant with a decline in flagellate numbers. The virus contains double-stranded DNA, is hexagonal in cross section, ca 230 to 300 nm in diameter and contains an electron dense core. It is morphologically similar to virus-like particles which have been observed in other heterotrophic nanoflagellates, and to viruses which have been isolated which infect eukaryotic phytoplankton. Addition of the virus to cultures of *Pseudobodo parvulus* (ATCC 50091, formerly *Bodo parvulus*) or *Paraphysomonas imperforata* (strain VS1) did not result in lysis. To our knowledge this is the first virus infecting heterotrophic nanoflagellates which has been isolated and maintained in culture. The presence of viruses in seawater which cause lysis of phagotrophic nanoflagellates implies that viruses infect microzooplankton populations in the sea and suggests another important role for viruses in aquatic microbial communities.

**KEY WORDS:** Protozoa · Flagellates · Viruses · Infection

## INTRODUCTION

It is now widely accepted that viruses are an abundant and ubiquitous component of the microbial community in aquatic environments (e.g. Torrella & Morita 1979, Bergh et al. 1989, Proctor & Fuhrman 1990, Hennes & Suttle 1995), ranging in concentration from ca  $10^6$  to  $10^8$  ml<sup>-1</sup> and varying both spatially and temporally (e.g. Børshheim et al. 1990, Bratbak et al. 1990, Cochlan et al. 1993). The high abundance of viral particles in marine environments has stimulated efforts to understand the role of viruses in marine ecosystems, including their impact on host cell populations and the range of organisms which they infect. It is becoming increasingly evident that viruses have a significant effect on natural communities of marine bacteria and

phytoplankton. Viruses which infect heterotrophic bacteria (e.g. Moebus & Nattkemper 1981, Børshheim et al. 1990, Suttle & Chen 1992), cyanobacteria (e.g. Safferman & Morris 1967, Suttle & Chan 1993, 1994, Waterbury & Valois 1993), and eukaryotic phytoplankton (e.g. Mayer & Taylor 1979, Suttle et al. 1990, Cottrell & Suttle 1991, Suttle & Chan 1995) have been isolated from marine environments. Moreover, viruses appear to be responsible for significant mortality in marine microbial communities (Proctor & Fuhrman 1990, Heldal & Bratbak 1991, Steward et al. 1993) and in phytoplankton populations (Suttle & Chan 1994, Cottrell & Suttle 1995), on average causing the lysis of about 20% of bacteria and several % of phytoplankton on a daily basis (Suttle 1994).

Although virus-like particles have been observed in microzooplankton, very little is known about the abundance and occurrence of these pathogens, and few studies have examined the possible occurrence of viral

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pathogens which infect ecologically important grazers. Comps et al. (1991) isolated and purified a virus from batch cultures growing in aerated seawater which infected the rotifer *Brachionus plicatilis*. However, it was not clear whether this viral pathogen was present in the seawater, or if it existed as a persistent infection in rotifers. Nagasaki et al. (1993) observed virus-like particles (VLPs) in an apochlorotic flagellate associated with a bloom of *Prorocentrum triestinum* (Dinophyceae) in Hiroshima Bay, Japan, and calculated that up to 20% of the flagellates contained VLPs. Furthermore, the collapse of enrichment cultures concomitant with the appearance of intracellular VLPs has also been reported in the nanoflagellates *Paraphysomonas* sp. and *Chromophysomonas* sp. (Chrysophyceae) (Preisig & Hibberd 1984), but the putative viral pathogens were not isolated. Observations of VLPs associated with sudden cell lysis in cultures and in microflagellates in nature suggest that viruses may be important in regulating the abundance or composition of microzooplankton communities.

The focus of this study was to determine if viral pathogens which infect marine heterotrophic nanoflagellates are present in natural communities of viruses in seawater, and, if so, to isolate and begin characterization of the pathogens.

## MATERIALS AND METHODS

**Nanoflagellate cultures and growth conditions.** The heterotrophic nanoflagellates, strains E1 and E4, were isolated from a sampling site near Yaquina Bay, Oregon, USA (González & Suttle 1993). The unicellular, biflagellate protozoa are 3 to 5  $\mu\text{m}$  in diameter, ellipsoid in shape, and similar in morphology and growth rate, suggesting that they may be related. Nanoflagellate E1 was identified as belonging to the genus *Bodo*. In addition, *Pseudobodo parvulus* (ATCC 50091, formerly *Bodo parvulus*) and *Paraphysomonas imperforata* (strain VS1) were used as host organisms. These isolates were kindly provided by D. Caron of Woods Hole Oceanographic Institution. Nanoflagellate cultures were grown at 20°C in the dark in ultrafiltered (<30 000 molecular weight) seawater which was autoclaved and enriched with 0.01% (w/v) yeast extract. Yeast extract was added to stimulate the growth of a mixed assemblage of bacteria present in the flagellate cultures; bacteria served as the primary food source of the flagellates.

Typically, start-up flagellate cultures used for screening experiments were initiated by adding a small volume of culture to fresh medium. Once exponential growth was reached, flagellates were transferred to fresh medium at an initial concentration of ca  $10^4 \text{ ml}^{-1}$ ,

followed by the addition of viral concentrate or lysate. Abundance of the nanoflagellates was determined by withdrawing an aliquot of culture, staining it with Lugol's fixative and counting the number of flagellates on a hemacytometer; cultures typically reached densities of  $10^6 \text{ flagellates ml}^{-1}$ . The lower detection limit of the hemacytometer counting method was ca  $10^3 \text{ ml}^{-1}$ .

**Virus isolation.** Natural viral assemblages were concentrated by ultrafiltration as described previously (Suttle et al. 1991). Briefly, seawater samples (18 to 120 l) were gently filtered through glass-fiber (MFS GC50, 1.2  $\mu\text{m}$  nominal pore size) then polyvinylidene difluoride (0.2 or 0.45  $\mu\text{m}$  pore size) membrane filters in order to remove the plankton, including most of the bacteria. The remaining particulate material in the filtrate was concentrated to 60–120 ml using a spiral-cartridge ultrafiltration membrane (30 000 molecular weight cutoff), and stored in the dark at 4°C until use. The final concentration factor of the viruses was determined by dividing the final volume of the concentrate by the initial volume of the filtrate. In our study, 19 different seawater samples containing natural viral assemblages were collected at different times of the year during 1989 to 1991 from the western Gulf of Mexico (2 samples), Aransas Pass, Texas (10 samples), and Laguna Madre, Texas (7 samples). The locations of the sampling stations are shown in Suttle et al. (1991).

Concentrated natural viral communities that were collected on different days from the same locations were combined into 3 pooled samples, each representative of a distinct geographic location (i.e. Gulf of Mexico, Aransas Pass, Laguna Madre). The volume from each of the concentrated natural viral communities that was used to make up a pooled viral sample contained the equivalent number of viruses that would be found in 250 ml of seawater assuming a concentration efficiency of 100%. Aliquots of combined samples were added to duplicate 45 ml cultures of each flagellate isolate so that each culture was diluted <10%. Control cultures received no addition. When the addition of viruses resulted in lower growth rates or evidence of cell lysis, an aliquot from the culture was transferred to an uninfected culture in order to propagate any pathogen that was present. The lysate was also tested for infectivity following filtration through 0.2, 0.4, and 1.0  $\mu\text{m}$  pore size polycarbonate filters (Poretics).

**Electron microscopy.** Duplicate nanoflagellate cultures were infected with a 1% inoculum (v/v) of culture lysate. Subsamples were withdrawn at 18, 48, 66, and 90 h, preserved in 1% glutaraldehyde and harvested by gentle centrifugation ( $6000 \times g$  for 30 min). The pellet was enrobed in 4% agar buffered with 0.1 M cacodylate. Enrobed samples were washed in a sucrose/cacodylate graded series, and fixed with 1%

osmium tetroxide. Fixed samples were dehydrated in a graded ethanol series, washed with propylene oxide and infiltrated with Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and viewed at 80 kV using a Philips EM301 transmission electron microscope.

**Host range.** Host specificity of the isolated virus was tested by adding 1% (v/v) aliquots of culture lysate to exponentially growing cultures of *Bodo* sp. (strain E4), *Paraphysomonas imperforata* (strain VS1), and *Pseudobodo parvulus* (ATCC 50091) and following abundance of flagellates by direct counts using a hemacytometer.

## RESULTS

Of the 4 flagellates initially screened, *Bodo* sp. (strain E1) was the only one which showed a decline in cell numbers in response to the addition of the concentrated natural viral communities. In particular, cell numbers in one of the replicate cultures inoculated with viral communities from Aransas Pass decreased more than 10-fold, relative to controls, 4 d after inoculation (Fig. 1A). When a 1% inoculum from the affected culture was transferred to an exponentially growing culture, the number of flagellates decreased to near detection levels (ca  $10^3$  ml<sup>-1</sup>) within two days

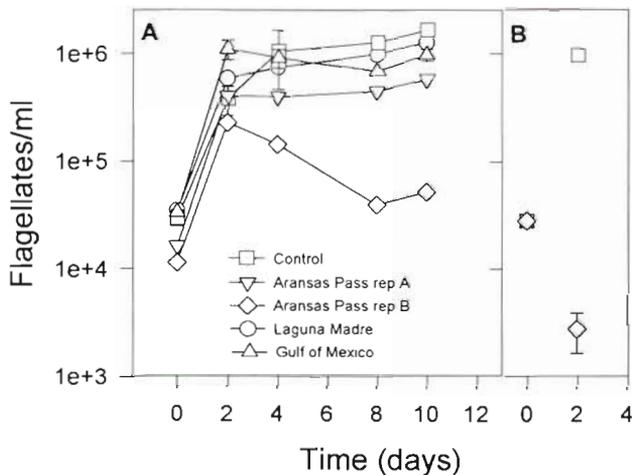


Fig. 1. Effect of adding concentrated natural viral communities to cultures of the nanoflagellate *Bodo* sp. strain E1. (A) Initial screening of pooled concentrated natural viral communities from Aransas Pass (Texas, USA), Laguna Madre (Texas), and the Gulf of Mexico. Symbols and error bars represent the mean and standard deviations of duplicate samples, except from Aransas Pass for which the replicates are plotted separately. (B) Propagation of a 1% (v/v) inoculum of culture lysate from the Aransas Pass replicate B to an exponentially growing uninfected culture

(Fig. 1B). The infective agent was propagated twice more in this manner. The pathogen remained infective after filtration through a 0.2  $\mu$ m pore size polycarbonate filter, suggesting that it was a viral pathogen and not a bacterium (Fig. 2). The infectivity of the pathogen was destroyed by autoclave sterilization and chloroform treatment (data not shown).

Thin sections of infected nanoflagellates (*Bodo* sp. strain E1), 48 and 66 h post infection, revealed the presence of intracellular viruses concomitant with a decline in nanoflagellate numbers (data not shown). The viruses were hexagonal in cross section, suggesting icosahedral symmetry, ca 230 nm in diameter, had double-layered capsids and contained electron dense cores (Figs. 3 & 4). Mature viral particles were associated with a viroplasm located in the cytoplasm and viruses were not observed in the nucleus. The nucleus, as well as the mitochondrion, appeared to remain intact during infection (Fig. 3A). We counted up to 20 viral particles in a single thin section of an infected flagellate, giving a minimum estimate of burst size.

Preparations of viral particles often lost icosahedral symmetry and appeared shriveled, possibly from osmotic shock. Viruses which remained intact occasionally revealed membrane- and tail-like formations (Fig 3C).

The host range of the virus (BV-PW1) was tested on 3 other nanoflagellates, *Bodo* sp. (strain E4), *Pseudobodo parvulus* (ATCC 50091) and *Paraphysomonas imperforata* (strain VS1). The virus caused lysis of the nanoflagellate *Bodo* sp. strain E4 but did not infect *Pseudobodo parvulus* or *Paraphysomonas imperforata*.

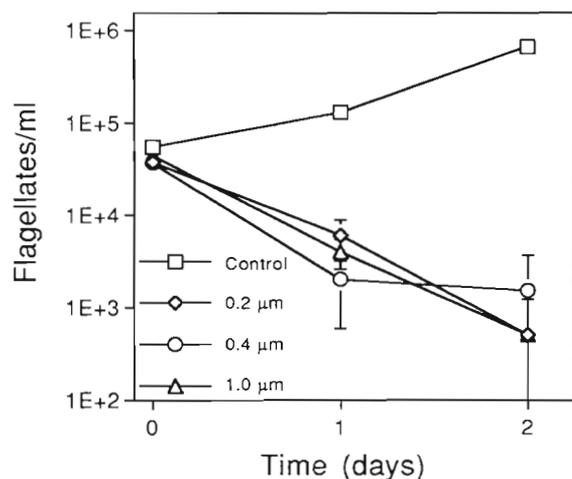


Fig. 2. Effect of passing culture lysate through a 0.2, 0.45 and 1.0  $\mu$ m pore size filters and adding to cultures of *Bodo* sp.; Filtered lysate was added at  $t = 0$ . Detection limit of the hemacytometer counting method is ca  $10^3$  flagellates ml<sup>-1</sup>. Error bars represent the standard deviation of duplicate treatments

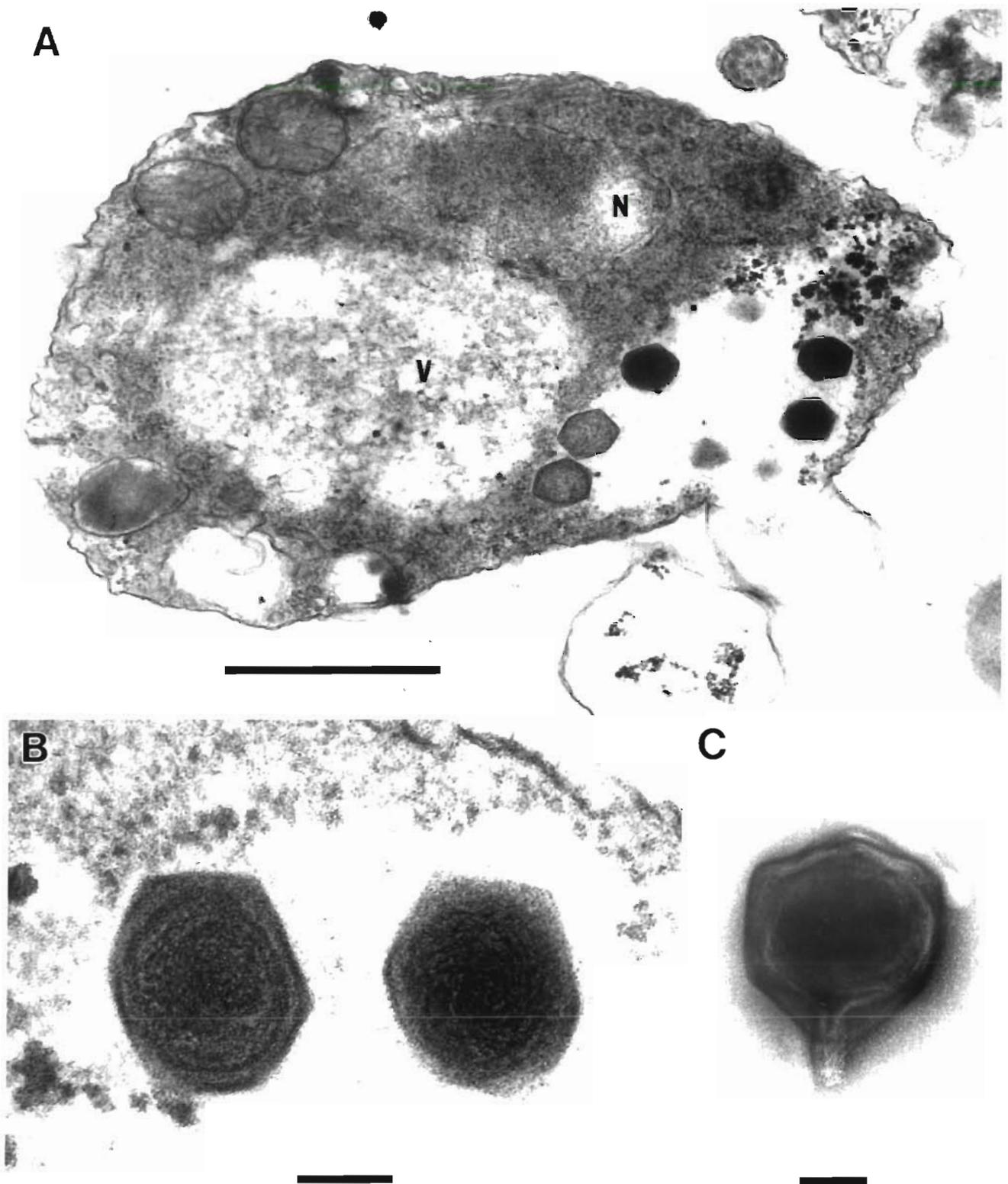


Fig. 3. (A) Electron micrograph of an ultrathin section of an infected nanoflagellate (*Bodo* sp. strain E1) showing viruses associated with a viroplasm (V) outside of the intact nucleus (N); scale bar = 1  $\mu$ m. (B) Higher magnification of ultrathin section showing electron dense cores of viruses; scale bar = 100 nm. (C) Positive stained preparation of an intact virus showing evidence of a tail and viral membrane; scale bar = 100 nm

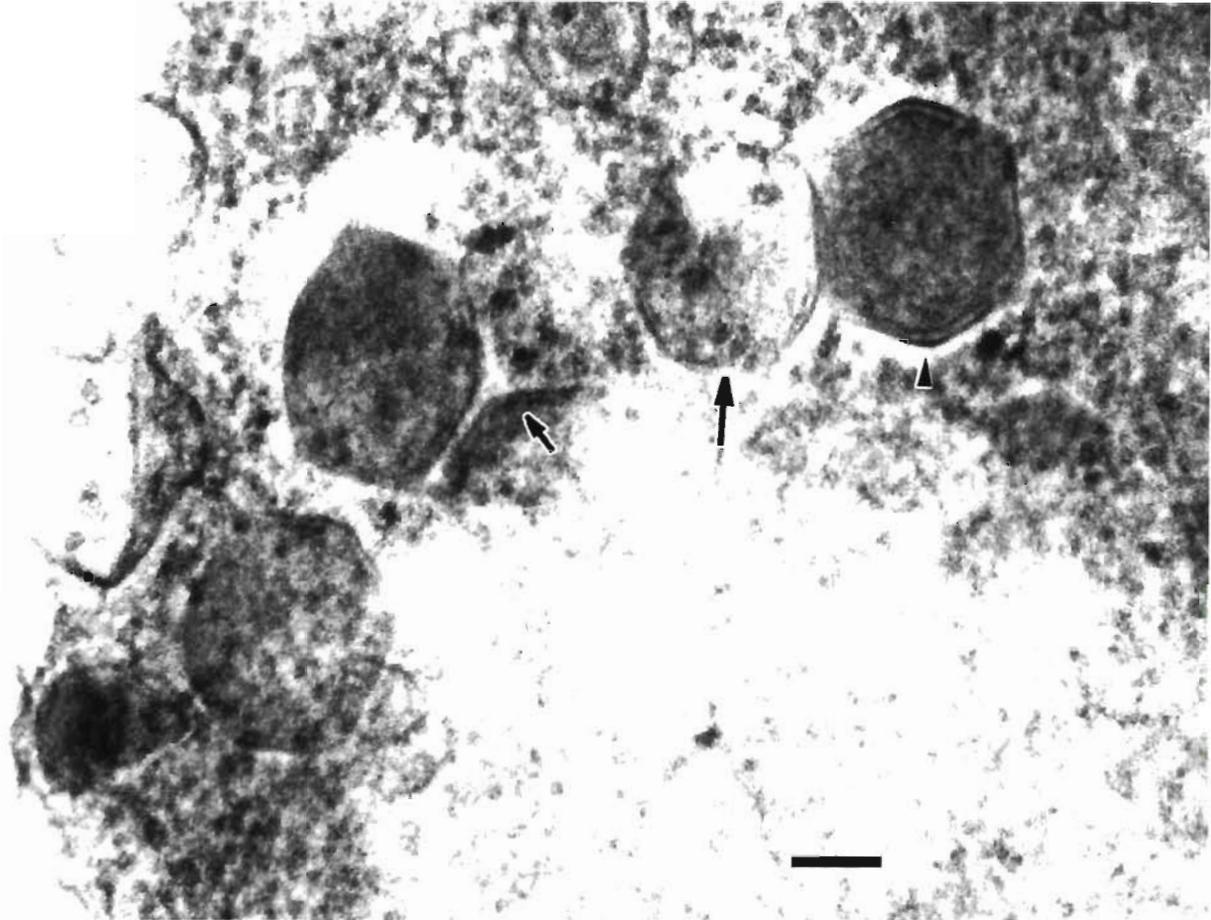


Fig. 4. Ultrathin section showing different stages of virus assembly including initial capsid formation (small arrow), intact capsid (large arrow) and intact capsid with electron dense core (arrowhead) 48 h post infection; scale bar = 100 nm

## DISCUSSION

The results of our investigation are significant because they demonstrate that viruses which infect and cause lysis of *Bodo* sp. occur in natural marine viral communities. Moreover, we were able to isolate and maintain the virus in culture and propagate it indefinitely on a nanoflagellate host, thereby fulfilling Koch's postulate.

### Virus characterization

Addition of filtered lysate to an exponentially growing culture of a *Bodo* sp. (strain E1) resulted in cell lysis within 2 d (Fig. 1B). Regrowth of flagellates was never observed in lysed cultures. Thin sections of infected nanoflagellates showed that the viruses were hexagonal and pentagonal in cross section, suggesting icosahedral symmetry (Fig. 3), and were similar to

VLPs observed in other nanoflagellates (Presig & Hibberd 1984). Up to 20 viruses were observed in a single section. Based on the morphology observed in the ultrathin sections, these viruses contain an electron dense core and are similar to viruses which infect eukaryotic microalgae, including *Micromonas pusilla* (Mayer & Taylor 1979, Cottrell & Suttle 1991), *Chlorella* spp. (Van Etten et al. 1981) and *Chrysochromulina* spp. (Suttle & Chan 1995). The viruses are similar to those which infect *M. pusilla* and *Chrysochromulina* spp. in that they are quite host specific, infecting *Bodo* sp. but not *Pseudobodo parvulus* and *Paraphysomonas imperforata*. Moreover, purified infectious viruses (unpreserved) can be stained with the fluorochrome DAPI (4',6-diamidino-2-phenylindole) (Suttle 1993), indicating the presence of double-stranded DNA.

The free viral particles are chloroform sensitive, suggesting that the capsids contain a lipid component (Rovozzo & Burke 1973) or that a viral membrane is

required for infectivity. In some micrographs the particles appear to be surrounded by a membrane-like structure (Fig. 3C). Since virus assembly is localized in the cytoplasm, it is likely that the viral membrane would be derived from the cytoplasmic membrane, suggesting the potential for viral release by budding. In contrast, there is no evidence for a membrane surrounding viruses that infect microalgae. Moreover, the tail-like structures observed in some viral particles appear to clearly distinguish these viruses from others that have been described which infect photosynthetic protists.

This virus cannot be classified with certainty at this time; yet, the presence of double-stranded DNA and icosahedral symmetry are consistent with characteristics of the Phycodnaviridae, which infect unicellular algae (Van Etten et al. 1991). The Phycodnaviridae, however, are not known to possess a viral membrane or a tail structure. Herpes viruses are also large double-stranded DNA viruses with icosahedral morphology and an external membrane. Yet, in Herpesviridae, replication and capsid assembly typically occur in the nucleus, whereas virus capsid assembly in *Bodo* sp. (strain E1) was observed to take place in a viroplasm located in the cytoplasm. An analysis of inferred amino acid sequences for B-family DNA polymerases revealed that the Herpesviridae is the closest family to the Phycodnaviridae (Chen & Suttle 1996). Although this novel virus shares morphological features with both phycoviruses and herpesviruses, further characterization at biochemical and molecular levels will be required before the genetic relationship among these viruses can be inferred.

### Ecological implications

The discovery of viruses which infect and cause lysis of *Bodo* sp. is significant as it demonstrates that viruses which infect flagellate grazers are present in nature. Furthermore, as these viruses cause lysis of flagellate grazers in culture, even when initially present at very low concentrations, it is reasonable to assume that these or similar viruses also affect flagellate populations in nature when conditions are favorable (i.e. bloom events). These findings augment previous observations by showing that flagellate grazers, in addition to primary producers, are subject to infection and lysis by viruses. Potentially, viruses could be important in regulating and maintaining the diversity of flagellate grazer communities, as viruses should propagate rapidly when host abundance is high (Wiggins & Alexander 1985). Reports of VLPs in natural populations of heterotrophic and autotrophic protists, including during bloom events (Sieburth et al. 1988,

Bratbak et al. 1993, Nagasaki et al. 1993, 1994a, b), support the suggestion that viruses may regulate host cell populations and may even be involved in bloom termination. However, infective agents were not isolated during these other studies, and the nature and transmissibility of these VLPs is unknown.

In our study the abundance of viruses which caused the lysis of the heterotrophic flagellate *Bodo* sp. (strain E1) was generally low, and viruses were isolated only from the combined concentrated virus communities from Aransas Pass. Subsequent screening of the individual concentrates that made up the pooled sample did not result in the isolation of a lytic agent; hence, we cannot provide any information on the temporal and spatial distribution of the pathogen. Moreover, screening of several additional viral concentrates collected from the same areas (Aransas Pass, Gulf of Mexico and Laguna Madre) did not yield any other lytic agents, suggesting that the presence of the viruses is temporally and spatially variable. It is also possible that the concentration procedure is inadequate to sample these viruses, or that the viruses are relatively unstable in the concentrates. Although the recovery efficiency of viruses from seawater using our ultrafiltration procedure can be quite high (Suttle et al. 1991), the flagellate virus BV-PW1 appears to be less stable (authors' unpubl. data), suggesting that we may have underestimated its abundance in nature. Furthermore, the large size of this virus may have resulted in losses during the prefiltration procedure.

If these viruses are rare in nature, it seems unlikely that contact rates with host cells would be adequate to maintain the viral populations given the relatively high turnover rates of most infectious marine viruses in seawater (Suttle & Chen 1992, Suttle & Chan 1994, Cottrell & Suttle 1995). One explanation may be the existence of lysogeny, whereby the viral genome is archived in a quiescent state in the host genome until conditions favor replication (e.g. during a bloom event). Alternatively, the virus may have a refuge such as associations with particulate or colloidal matter which could increase its persistence. It should be stressed, however, that viruses need not be abundant in order to be ecologically significant, as they can propagate rapidly through a population as host abundance and, thereby, contact rates increase.

An interesting caveat of our observations is that one of the flagellate strains (*Bodo* sp. strain E4) that was infected by the viruses also ingests and digests free viral particles in seawater (González & Suttle 1993). As the mechanism of infection by these viruses is unknown, it is not clear whether grazing on viral particles affects the probability that the flagellates will be infected.

This report documents that viruses which infect and cause lysis of heterotrophic flagellates are a component of natural marine viral communities, and that these viruses can be isolated and maintained in culture. These observations imply that viruses may not only be important regulators of bacterial and phytoplankton communities, but may also affect the grazers that consume them. Moreover, although the impact of flagellate grazers on bacteria and phytoplankton has been comparatively well studied, less work has been done on the processes that regulate the abundance and composition of heterotrophic flagellates in nature. These results indicate that viruses can infect bacterivorous flagellates. This identifies another potentially important role of natural viral communities that has implications for our understanding of nutrient and energy cycling in the sea.

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