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A filterable lytic agent obtained from a red tide bloom that caused lysis of *Karenia brevis* (*Gymnodinum breve*) cultures

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ABSTRACT: A filterable lytic agent (FLA) was obtained from seawater in the southeastern Gulf of Mexico during a red tide bloom that caused lysis of *Karenia brevis* (formerly *Gymnodinium breve*) Piney Island. This agent was obtained from <0.2 µm filtrates that were concentrated by ultrafiltration using a 100 kDa filter. The FLA was propagated by passage on *K. brevis* cultures, and the filtered supernatants of such cultures resulted in *K. brevis* lysis when added to such cultures. The lytic activity was lost upon heating to 65°C or by 0.02 µm filtration. Epifluorescence and transmission electron microscopy (TEM) of supernatants of *K. brevis* cultures treated with the lytic agent indicated a high abundance of viral particles (4×10^9 to 7×10^9 virus-like particles [VLPs] ml^{-1}) compared to control cultures (~10^7 ml^{-1}). However, viral particles were seldom found in TEM photomicrograph thin sections of lysing *K. brevis* cells. Although a virus specific for *K. brevis* may have been the FLA, other explanations such as filterable bacteria or bacteriophages specific for bacteria associated with the *K. brevis* cultures cannot be discounted.

KEY WORDS: *Gymnodinium breve* · *Karenia brevis* · Lytic agent · Viruses

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INTRODUCTION

Karenia brevis (Davis) cf. Hansen & Moestrup (= *Gymnodinium breve*) is the causative agent of recurring red tide blooms in the Gulf of Mexico and the southeastern Atlantic USA. Red tide blooms off the coast of Florida caused by *K. brevis* were observed in 21 of 22 yr from 1975 to 1997 (Tester & Steidinger 1997) and have been reported since the Spanish conquests (Gunther et al. 1948). Toxins produced by *K. brevis* can result in massive fish kills, and were implicated in the mortality of 700 bottlenosed dolphins off the east coast of the USA in 1987 (Hersh 1989). More recently, *K. brevis* toxins have been implicated in the mysterious death of 149 Florida manatees in 1995 and 1996 (Landsberg & Steidinger 1998).

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Because of the recurring nature and adverse ecological and economic impacts of red tide blooms, methods to control such blooms are under investigation. The approaches taken have been to use physical/chemical methods or biological control methods to facilitate bloom termination. For example, clay flocculation is being investigated as a mechanism of removal of red tide (*Karenia brevis*) and brown tide (*Aureococcus anophagefferens*) cells (Sengco et al. 2001). Potential biological control agents include protistan grazers, algicidal bacteria and viruses.

Grazers capable of consuming red tide organisms include mixotrophic dinoflagellates such as *Fragilidium mexicanum* (Jeong et al. 1999a) as well as protists such as *Strombodinopsis* (Jeong et al. 1999b). These organisms can usually graze a variety of phytoplankton forms, and not solely one algal type. Thus, competition between more numerous phytoplankton forms

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and *Karenia brevis* can become an issue in bloom control strategies. Recently, intracellular dinoflagellate parasites such as *Ameobophyra* sp. have been shown to infect *Akashiwo sanguinea* (= *Gymnodinium sanguineum*; Gunderson et al. 1999, Yih & Coats 2000).

Much attention has been devoted to algicidal bacteria as agents of bloom termination in nature. Algicidal bacteria that kill *Karenia brevis*, *K. mikimotoi* (Doucette et al. 1999), *Heterosigma akashiwo* (Yoshinaga et al. 1998, 1999), *Chattonella* and *Heterocapsa* sp. (Kondo et al. 1998, Nagasaki et al. 2000) have been described. Some of these are species specific, while others appear to be group specific (i.e. can kill gymnodinoids; Lovejoy et al. 1998).

Many genera and classes of algae have been shown to be susceptible to viral infection (Safferman & Morris 1963, Pienaar 1976, Dodds 1979, Milligan & Cosper 1994, Nagasaki et al. 1994a, Zingone 1995). Sieburth et al. (1988) suggested that the termination of *Aureococcus anophagefferens* blooms may have been caused by viruses, and subsequently viruses specific for this organism were isolated (Milligan & Cosper 1994). That red tide organisms such as *Heterosigma akashiwo* could be lysed by viruses (Nagasaki et al. 1994a,b, 1999, Tai & Suttle 2001) has suggested that blooms of such organisms may potentially be controlled by viral infection. Other bloom-forming algae shown to be sensitive to viral infection include *Phaeocystis* (Brussard 2001), *Micromonas pusilla* (Cottrell & Suttle 1991), *Emiliani huxleyi* (Manton & Leadbeater 1974) and *Chysochromulina* (Brussard et al. 1996). Viral infection of bloom-forming algae is often strain specific, resulting in the control of clonal variability in such blooms (Suttle 2000, Taratuni et al. 2000).

The termination of toxic dinoflagellate blooms via viral infection has not been reported, largely because of the lack of reports of viruses infective for dinoflagellates. Tartuni et al. (2001) reported the isolation of a virus infective for the novel shellfish-killing dinoflagellate *Heterocapsa cirularisquama*. In an effort to discover if viruses might cause red tide bloom termination, we have attempted to isolate viruses infective for *Karenia brevis* by concentrating viral fractions from seawater during red tide blooms. Our work suggests that filterable lytic agents (FLAs) found associated with *K. brevis* blooms can lyse this organism.

MATERIALS AND METHODS

Cultures. *Karenia brevis* (Piney Island B4 strain) was grown on L1 medim at 22.5°C and at 50 µmol^{-1} m^{-2} at the Florida Marine Research Institute in St. Petersburg, Florida, USA.

Field sampling for a brevicidal lytic agent. Surface waters were sampled in a *Karenia brevis* bloom during a research cruise aboard the RV ‘Suncoaster’ from December 1 to 5, 1998, and during a bloom off the Mote Marine Laboratory dock in Sarasota on August 24 and September 7, 2001 (Fig. 1). Samples were prefiltered using a 142 mm 0.22 µm filter (Millipore Durapore GV) under positive pressure (10 to 15 pounds per square inch [psi]; 1998 samples) or remained unfiltered (2001 samples). The microbes in the filtered water were concentrated by vortex flow filtration using a Membrex Rotary Biofiltration device (West Borough, MA) fitted with a 100 kDa filter (Paul et al. 1991, Jiang et al. 1992). In this manner, 20 to 40 l samples were prefiltered and concentrated to a retentate volume of 50 to 60 ml.

FLA assay. Twenty-five milliliter aliquots of an actively growing culture of *Karenia brevis* were carefully placed into 25 × 150 mm sterile culture tubes. Culture tubes were cleaned by soaking first in Micro detergent, followed by 5 tap water and 5 deionized (DI) water rinses. The tubes were then soaked in 10% HCl followed by 5 DI water rinses and sterilized by autoclaving. For each assay, 1.0 ml of concentrated retentate from a seawater sample (initial assay) or from a prior assay supernatant was added to each of 3 tubes. Cultures were incubated at 50 µmol^{-1} m^{-2} and 22.5°C on a 12 h light:12 h dark cycle. Control cultures lacked such amendments or were inoculated with 1.0 ml filtered supernatant from a control tube from a prior FLA assay. For most experiments, supernatants from prior FLA experiments were obtained by centrifugation at 15 000 × g for 15 min to remove *K. brevis* cells and

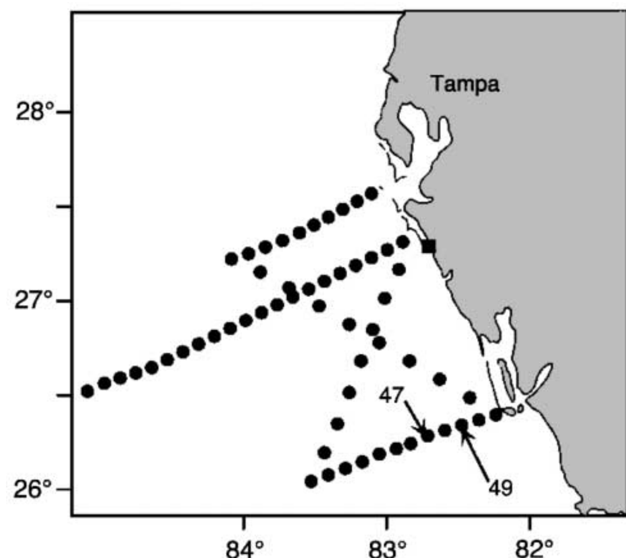

Fig. 1. Cruise track for sampling during red time bloom event in 1998 (●). Stations that yielded the filterable lytic agent (Stns 47 and 49) are indicated. (■) Site of sampling for the red tide bloom in 2001

cell fragments. The supernatants were filtered 3 times through 0.2 µm Acrodisc filtration devices (HT Tuffryn, Pall Corporation, Ann Arbor, MI, USA) immediately prior to use. Cell growth was monitored as fluorescence using a 10 absorption unit (AU) fluorometer (Turner Designs, Sunnyvale, CA, USA).

Effect of filtration on FLA activity. To determine the effect of filtration on FLA activity, a filtered supernatant that had previously shown lytic activity was filtered through a 0.02 µm Anodisc filter (Whatman) or a 0.2 µm Nuclepore filter. One milliliter of such treated culture filtrates was used in the FLA assay described above.

Effect of temperature on FLA activity. To determine the effect of temperature on FLA activity, 0.2 µm filtered FLA was heated to 65 or 95 to 100°C for 10 min by immersion in water baths maintained at those temperatures.

Enumeration of virus-like particles (VLPs). In certain experiments, 1 ml samples of each culture tube were taken daily and fixed with 1% 0.02 µm filtered formalin. Because of the tremendous numbers of viruses produced in certain experiments, it was necessary to dilute these samples 10- to 1000-fold prior to enumeration. Slides were immediately prepared for viral enumeration by epifluorescence microscopy by the method of Noble & Fuhrman (1998), except that SYBR Gold (Molecular Probes, Eugene, OR, USA) was used instead of SYBR Green and that staining time was reduced to 12 min. Bacteria were counted simultaneously in certain samples.

Transmission electron microscopic (TEM) analysis. For analysis of the structure of free viral particles, culture supernatants were diluted 1:10 with DI water, and 1 µl was spotted on Formvar-coated copper TEM grids (Electron Microscopy Supplies, Ft. Washington, PA). The spot was allowed to air dry and then stained with 2% uranyl sulfate (Polysciences, Inc, Warrington, PA, USA) for 30 s.

For thin-section analysis of *Karenia brevis* cells, the methods of Steidinger et al. (1978) were employed.

All TEM samples were examined with a Hitachi 7100 TEM at 75 keV.

RESULTS

Fig. 2A shows the results of incubation of *Karenia brevis* cells with concentrated viral extracts taken from Stns 47 and 49 of the cruise into the southeastern Gulf of Mexico. Growth as indicated by culture fluorescence increased over 9 d, and then fluorescence decreased over the next 2 d (except for the control tubes). To determine if this material (now termed 'filterable lytic agent' or FLA) could be propagated in *K. brevis* cultures, supernatants from each of these treatments were filtered and 1 ml was added to new *K. brevis* cultures (Fig. 2B). The FLA caused a decrease in fluorescence after 8 d while control cultures continued to grow unaffected.

The FLA was serially passaged similarly 16 times with similar results, except that cell lysis occurred usually after 4 d.

Effect of heat treatment and filtration on FLA activity

Fig. 3 shows the effect of heat treatment on FLA activity. Heating at 95 or 65°C apparently inactivated the FLA. Fig. 4A shows the effect of filtration on FLA activity. Filtration through a 0.02 µm filter completely inactivated the FLA activity, whereas filtration through 0.2 µm Acrodisc or Nuclepore filter had no effect on FLA activity.

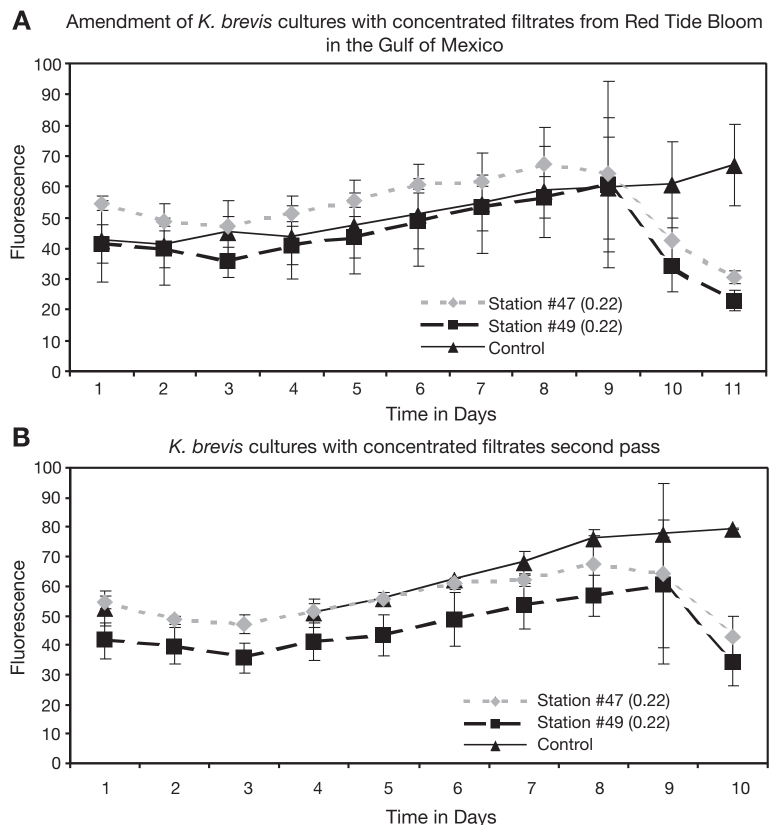

Fig. 2. *In vivo* fluorescence monitoring of *Karenia brevis* cultures amended (A) with viral concentrates from Stns 47 and 49 or (B) with filtered supernatants of cultures previously treated with the viral concentrate. Each point is the mean fluorescence of 3 replicate culture tubes ± SD

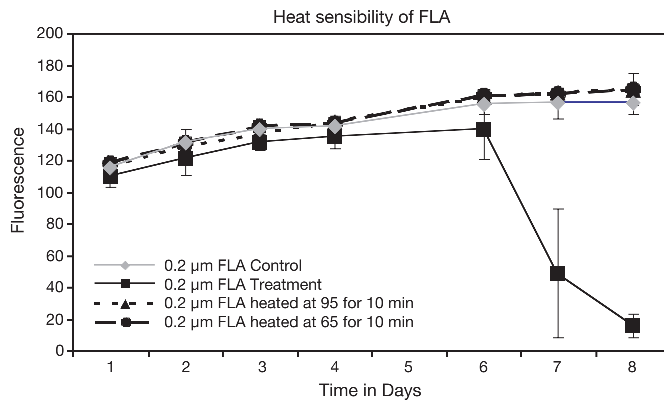

Fig. 3. Heat sensitivity of the filterable lytic agent (FLA) as measured by *in vivo* culture fluorescence of treated and control *Karenia brevis* cultures. *K. brevis* cultures were amended with filtrates of control cultures ('0.2 FLA control'), filtrates from cultures that were lysed by the FLA ('0.2 FLA Acrodisc'), the same filtrates but heated for 10 min at 95°C ('FLA heated at 95 for 10 min') or the same filtrates but heated at 65°C for 10 min ('FLA heated at 65 for 10 min')

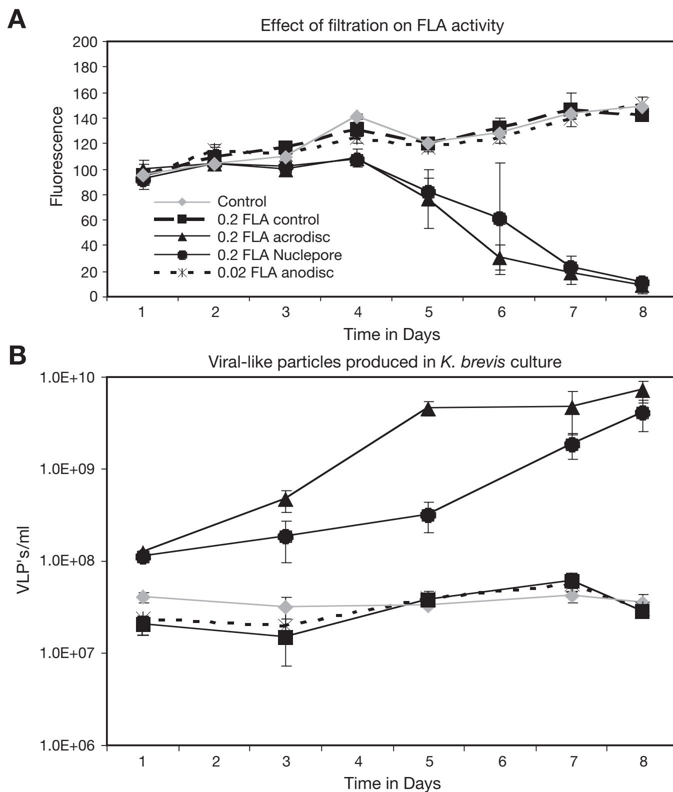

Fig. 4. Effect of filtration treatment of FLA on (A) *in vivo* culture fluorescence or (B) virus-like particle (VLP) production. Treatments were no addition to the *Karenia brevis* culture ('Control'), *K. brevis* cultures amended with filtrates of control cultures ('0.2 FLA control'), cultures amended with 0.2 µm filtered supernatants of cultures that were lysed by the FLA ('0.2 FLA Acrodisc' or '0.2 FLA Nuclepore') and cultures amended with 0.02 µm filtered supernatant ('0.02 FLA Anodisc'). Each point is the mean of 3 fluorescence readings (Panel A) or 9 slides (3 per tube) ± 1 SD

Enumeration of VLPs in cultures

Fig. 4B shows enumeration of VLPs in experiments using the FLA. Cultures inoculated with the FLA had nearly twice the levels of VLPs at the initiation of the experiment and nearly 2 orders of magnitude more than controls at the conclusion of the experiment (4×10^9 to 7×10^9 vs 3×10^7 to 4×10^7).

TEM analysis of FLA

Fig. 5 shows TEM analysis of uranyl sulfate-stained preparations of FLA samples. A dominant siphoviridae was observed in FLA preparations (Fig. 5A,B). This phage had a distinct morphology, with a 'stack of coins'-like tail, and a head 37.3 ± 2.3 nm in diameter and a tail 249 ± 2.0 nm in length. Other VLPs were also present (Fig. 5C,D), but these were encountered less frequently than the stacked-coin siphoviridae.

TEM analysis of *Karenia brevis* cells treated with the FLA

Fig. 6 is a composite TEM photomicrograph of thin-sectioned preparations of *Karenia brevis* cells. Fig. 6A shows a healthy, uninfected *K. brevis* cell while Fig. 6B to D show cells treated with the FLA. It was difficult to locate cells in such preparations that still maintained a recognizable cell wall. Typically such cells were highly vacuolated (Fig. 6B) and had many bacteria-like bodies associated with them. No cells were observed to contain numerous viral particles although an occasional virus particle was observed (Fig. 6D).

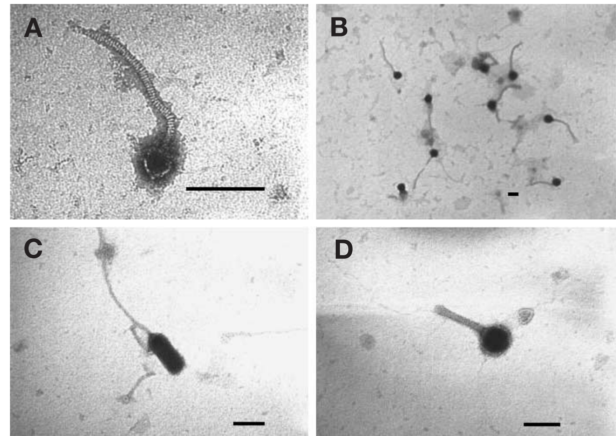

Fig. 5. Transmission electron photomicrographs of viruses found in supernatants of *Karenia brevis* cultures treated with FLA. (A,B) Dominant siphoviridae found in FLA-treated cultures with 'stack of coins' tail morphology. (C,D) Examples of other viruses occasionally encountered in FLA-treated *K. brevis* cultures. Scale bars = 100 nm

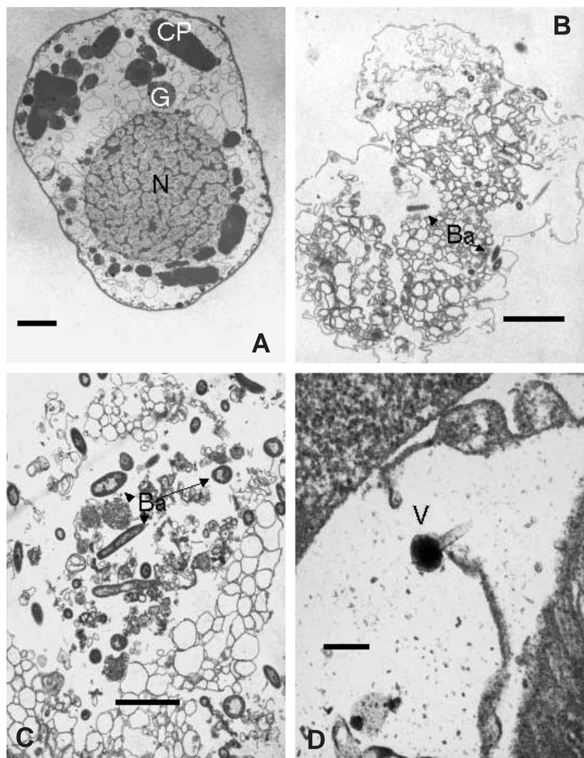

Fig. 6. Transmission electron photomicrographs of thin sections of *Karenia brevis* cells from (A) control and (C,D) FLA-treated cultures. Ba: bacteria; CP: chloroplast; G: golgi body; N: nucleus; V: virus-like particle. Scale bars = 2 µm, except for (D), where it is 200 nm

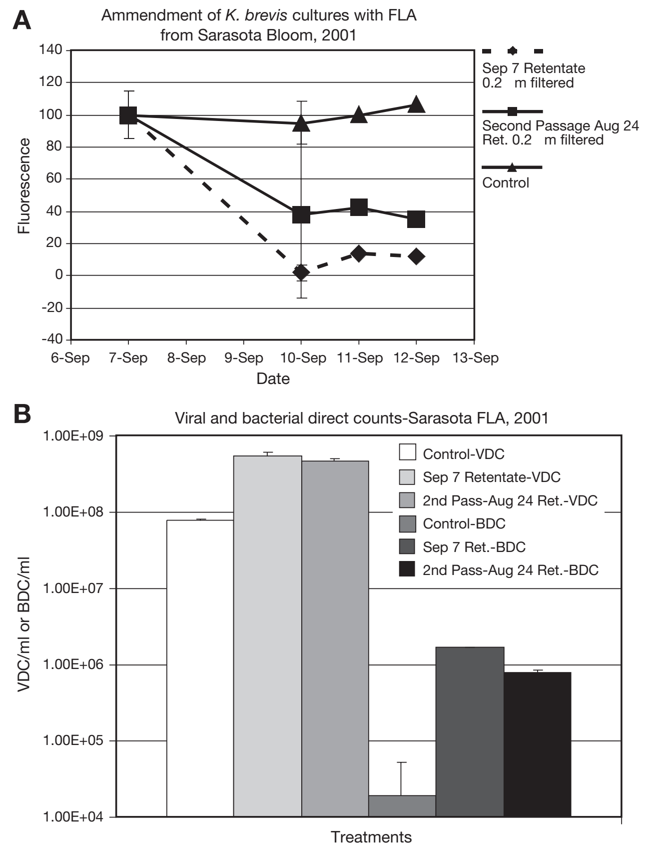

Fig. 7. Filterable lytic agent from Sarasota, Florida, USA in 2001. (A) Effect of FLA on *in vivo* fluorescence of *Karenia brevis* cultures, from the September 7, 2001, sampling event (dashed line with diamonds) or the second passage of the FLA from the August 24, 2001, sampling (squares). (B) Bacterial (BDC) and viral direct counts (VDC) taken from the last sampling of the experiment plotted in (A)

Loss of lytic activity

In December 1999, 1 yr after the original 'isolation' of the FLA, the agent (or culture extracts) lost activity toward *Karenia brevis* after many serial passages in *K. brevis* cultures. Further attempts to 'revive' the lytic agent by passage on fresh *K. brevis* cultures were unsuccessful.

Filterable lytic activity associated with a bloom off Sarasota

Fig. 7A shows the effect of a FLA collected from a bloom off Sarasota, Florida, on *Karenia brevis in vivo* fluorescence. The results shown are for the direct use of a filtered retentate collected on September 7, 2001, and the second passage of a retentate collected on August 24, 2001. This lytic agent lost activity after 3 passages. Fig. 7B shows the viral and bacterial direct counts measured at the termination of the experiment in the control cultures and those treated with the FLA. Both viral and bacterial counts were elevated in cultures treated with the FLA compared to controls.

DISCUSSION

We have described a FLA obtained from the Gulf of Mexico that resulted in lysis of cell populations of the Piney Island strain B4 of *Karenia brevis*. The FLA was heat sensitive and could be removed by 0.02 µm filtration. This material was passaged in *K. brevis* cultures for nearly a year before lytic activity was lost. The observations of high viral titers in the filtrates of samples after cell lysis suggested the involvement of a virus in this process, and a numerically dominant siphoviridae was observed by TEM in treated culture supernatants. However, no (or very few) viruses were observed in TEM photomicrographs of lysing cells. Instead, bacteria were observed in thin sections of lysing cells. Additionally, the onset of lysis (originally 7 to 9 d after addition of FLA but then 5 d after serial passages) was not consistent with other algal viral infections, which typically take 24 to 48 h to effect lysis

(Cottrel & Suttle 1994, Nagasaki et al. 1994a). A similar observation of a FLA that was active against *Gymnodinium mikimotoi*, *Tetraselmis* sp. and *Alexandrium catenella* was found in seawater from Funka Bay, Hokkaido, Japan (Onji et al. 1999, 2000). This 'virus-like suppressive agent' was also sensitive to heat treatment (50°C for 30 min) and 0.05 µm filtration, as well as RNase treatment, exposure to pH 5.0, UV treatment and protease digestion. As in our study, TEM observation showed severely damaged cells after FLA treatment but no indication of viruses (or bacteria) in bursting cells.

We hypothesize that the lysis observed was not the direct effect of a virus on *Karenia brevis* but rather the result of a viral-bacterial interaction. It is possible that a bacterium performing a positive effector role on *K. brevis* (providing an organic nutrient, cofactor, etc.) was present in the culture and that the virus removed or lowered the concentration of this bacterium. Also, treatment of cultures with such a virus may have selected for a phage-resistant bacterium, which might explain the loss of lytic activity. Alternatively, it may be that the virus caused lysis of a 'neutral' bacterium in the culture that, when lysed, released a lytic agent, enzyme or other algicidal material. It may be that storage of such virus at 4°C resulted in inactivation, as was shown for *Heterosigma akashiwo* virus (HaV); storage of HaV at 5°C in the dark resulted in its rapid inactivation (Nagasaki & Yamaguchi 1998). We feel that the bacterial component of the *K. brevis* cultures used may have undergone a change, such that it was no longer sensitive to the virus in the FLA. Using a bacterial host obtained from the *K. brevis* culture, we isolated a virus that was morphologically similar to the distinctive siphoviridae observed after *K. brevis* lysis. However, preliminary studies suggested that this virus did not result in *K. brevis* lysis. However, *K. brevis* cultures at that time were no longer sensitive to the FLA.

Karenia brevis has not yet been cultivated axenically for any period of time. Our studies show that such cultures are mixtures of *K. brevis* cells, a diverse bacterial community, along with their phages. Preliminary evidence suggests that some of the bacteria in these cultures are lysogens (Paul et al. 2000). Prophage induction may be occurring during the lytic events observed, further complicating the interpretation of our results.

Suttle (2000) noted that a previous report of a particular virus causing the diatom *Navicula* sp. to lyse (Suttle et al. 1990, 1991) was really a bacterium that copurified with the virus (Chan et al. 1997). Suttle (2000) concludes that 'to conclusively demonstrate that a lytic agent is viral, it must be propagated on an axenic host.' If that is not possible, morphologically similar viral particles should be shown to occur in infected cells (Suttle 2000). Neither of these criteria were met in this study. We triple filtered our FLA and tested sterility by observing growth (or the lack of) in nutrient media. This does not preclude the possibility that a small bacterium (or viroid) may have been the lytic agent.

A second FLA was obtained from a *Karenia brevis* bloom in Sarasota nearly 3 yr after we obtained the first FLA. This agent behaved similarly to the original agent, yet seemed to lack the virulence, in that it could be passaged only 3 times before it lost lytic activity. Viral direct counts in cultures treated with the lytic agent were lower (4.6×10^8 to 5.5×10^8 ml^{-1}) than those of the 1998 lytic agent (4×10^9 to 7×10^9 ml^{-1}).

Our results show that FLAs can occur in water supporting red tide blooms and that these agents may cause rapid bloom collapse. We are currently examining other blooms for the presence of FLAs.

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