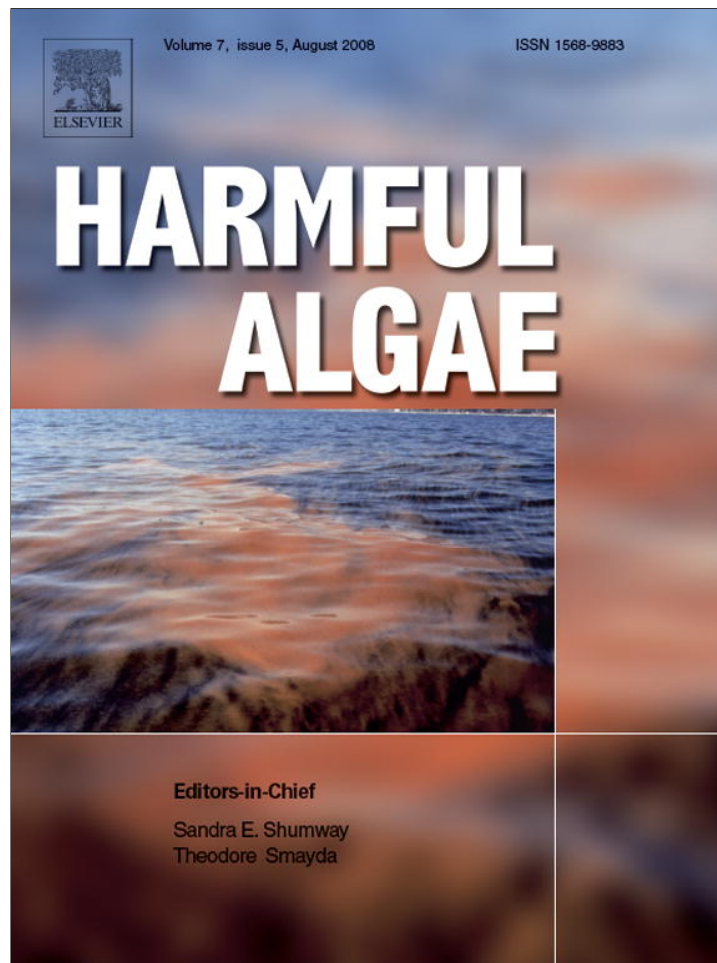


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## Comparative analysis of two algicidal bacteria active against the red tide dinoflagellate *Karenia brevis*

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### Abstract

The red tide dinoflagellate *Karenia brevis* blooms annually along the eastern Gulf of Mexico, USA, and is often linked to significant economic losses through massive fish kills, shellfish harvest closures, and the potential threat to humans of neurotoxic shellfish poisonings as well as exposure to aerosolized toxin. As part of an effort to enhance the strategies employed to manage and mitigate these events and their adverse effects, several approaches are being investigated for controlling blooms. Previous studies have established the presence of algicidal bacteria lethal to *K. brevis* in these waters, and we aim to characterize bacterial–algal interactions, evaluate their role as natural regulators of *K. brevis* blooms, and ultimately assess possible management applications. Herein, the algicidal activity of a newly isolated *Cytophaga/Flavobacterium/Bacteroidetes* (CFB)-bacterium, strain S03, and a previously described CFB-bacterium, strain 41-DBG2, was evaluated against various harmful algal bloom (HAB) and non-HAB species (23 total), including multiple clones of *K. brevis*, to evaluate algal target specificity. Strains S03 and 41-DBG2, which employ direct and indirect modes of algicidal lysis, respectively, killed ~20% and ~40% of the bacteria-containing isolates tested. Interestingly, no bacteria-free algal cultures were resistant to algicidal attack, whereas susceptibility varied occasionally among bacteria-containing isolates of a single algal taxon originating from either the same or different geographic location. The dynamics of *K. brevis* culture death appeared to differ according to whether the algicidal bacterium did or did not require direct contact with algal cells, with the former most rapidly affecting *K. brevis* morphology and causing cell lysis. Both bacterial strains promoted the formation of a small number of cyst-like structures in the *K. brevis* cultures, possibly analogous to temporary cysts formed by other dinoflagellates exposed to certain types of stress. Results were also consistent with earlier work demonstrating that bacterial assemblages from certain cultures can confer resistance to attack by algicidal bacteria, again indicating the complexity and importance of microbial interactions, and the need to consider carefully the potential for using such bacteria in management activities.

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### 1. Introduction

The athecate dinoflagellate *Karenia brevis* (formerly *Gymnodinium breve*; Davis, 1948) G. Hansen and Moestrup is a harmful species often associated with massive fish and marine mammal mortality events in the Gulf of Mexico (Steidinger et al., 1998). As a result, upwards of millions of dollars are lost by the recreation and tourism industries during years with *K. brevis* red tide events (Anderson et al., 2001). In an effort to manage and mitigate the potentially devastating effects of these and other types of harmful algal blooms (HABs), several control strategies

such as chemical algicides, flocculants and other physical manipulations, as well as biological agents, are currently under investigation (Hennes et al., 1995; Doucette et al., 1999; Anderson et al., 2001; Sengco et al., 2001; Kim, 2006). In marine and freshwater ecosystems, biological agents such as bacteria, viruses, protozoans, and fungi have all shown promise as potential algal bloom suppressors (Imai et al., 1998; Doucette et al., 1999; Castberg et al., 2001; Manage et al., 2001; Kang et al., 2005).

Bacteria have significant impacts on aquatic biogeochemical processes such as carbon flux and nutrient regeneration (Azam, 1998; Doucette et al., 1998; Copley, 2002). These microbes are known to be active in the decomposition of freshwater algal blooms (Kang et al., 2005) and may play a similar role in marine systems by influencing the initiation, growth, main-

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tenance, and/or termination of HAB populations (Imai et al., 1998; Kodama et al., 2006). Specifically, a bacterial assemblage can have inhibitory or stimulatory effects on algal growth during a bloom event (Riquelme et al., 1988; Fukami et al., 1997; Simon et al., 2002). Doucette et al. (1999) hypothesized that the relative and absolute abundances of algicidal bacteria, in particular, would increase as a bloom moves through its initiation, development, and maintenance phases. Ultimately, changes in the abundance and composition of the bacterial community may lead to bloom decline as these algicidal bacteria begin to negatively impact algal growth and/or render the alga more susceptible to other factors, such as grazing pressure and nutrient competition. The extent to which bloom termination is a reflection of bacterially mediated algal lysis remains unknown.

Algicidal bacteria are classified according to their mode of lysis, which can be either direct or indirect (see reviews by Mayali and Azam, 2004; Salomon and Imai, 2006). The former requires that a bacterium be in direct contact with the target algal species and lysis is thought to result from enzymatic digestion of the algal cell wall/membrane (e.g., Lee et al., 2000). Alternatively, algicidal bacteria exhibiting an indirect killing mechanism release a dissolved lytic agent(s) effective in the absence of physical contact with the target. Based on several recent reviews (Fukuyo et al., 2002; Mayali and Azam, 2004; Hare et al., 2005), at least 56 unique algicidal bacteria have been isolated and classified phylogenetically. Of these, 36 strains were characterized according to their lytic mechanism, with approximately 70% showing an indirect mode of attack and the remaining 30% requiring direct contact with the algal cells. Although the functional significance of cell lysis by algicidal bacteria remains to be determined, this activity clearly enhances the supply of algal-derived organic nutrients that may then provide a competitive advantage to this segment of the microbial community (Doucette, 1995; Mayali and Azam, 2004).

Most algicidal bacteria characterized thus far belong to the *Cytophaga/Flavobacterium/Bacteroidetes* group (Fandino et al., 2001; Yoshinaga et al., 1995) or to the  $\gamma$ -Proteobacteria group (Imai et al., 1995; Yoshinaga et al., 1995). Of the 56 algicidal strains noted above, about 50% belong to the CFB group while about 45% are members of the  $\gamma$ -Proteobacteria, with the remaining strains representing the gram-positive genera *Micrococcus*, *Bacillus*, and *Planomicrobium*. Although there is no conclusive link between phylogeny and the lytic mechanism, the available data suggest that gram-positive bacteria and  $\gamma$ -Proteobacteria employ primarily an indirect mode of attack, while the lytic activity of the CFB group can involve either direct or indirect interactions with target cells.

Considering the overall size of the CFB group, relatively few species are actually algicidal and the mode of attack does not appear to be consistent within or between the various genera. Doucette et al. (1999) isolated an algicidal *Cytophaga* sp., strain 41-DBG2, capable of killing *K. brevis* via the production of a soluble, heat-sensitive, algicidal compound (Twiner et al., 2004), yet a closely related *Cytophaga* sp., strain J18/M01, lyses the raphidophyte *Chattonella antiqua* by direct attack (Imai et al., 1993). Alternatively, *Aquimarina latercula*

(formerly *Cytophaga latercula*; Nedashkovskaya et al., 2006), another marine bacterium within the CFB group, exhibits no algicidal activity and has been used as a negative control in many of our studies.

In this paper, we report the isolation of a new algicidal bacterium belonging to the family *Flavobacteriaceae* (strain S03; GenBank accession no. EU021292) from the Gulf of Mexico and compare its direct mode of algicidal attack with the indirect activity of *Cytophaga* sp. (strain 41-DBG2; GenBank accession no. AF427479) using *K. brevis* isolates determined previously to be susceptible or resistant to these algicidal bacteria. We also assess the effects of exposure to such bacteria on the morphology, growth characteristics, and bacterial succession patterns in the *K. brevis* cultures. The data obtained from this investigation will improve our understanding of the complex interactions between algicidal bacteria and their target algal species, as well as aid in evaluating the possible use of algicidal bacteria as part of a HAB management strategy.

## 2. Materials and methods

### 2.1. Field collections

Water samples were collected from the west Florida shelf in 2001 during the ECOHAB RV Suncoaster cruises (September 20–26; October 20–26). At most stations whole water samples were obtained from three depths, corresponding to surface, middle (mid-water column), and bottom (approximately 1 m from the bottom); occasionally, surface samples were also collected from a continuous flow-through system maintained on the ship's deck. All samples were pre-filtered through 80  $\mu$ m nitex screen and three aliquots of the <80  $\mu$ m filtrate containing both attached and free-living bacteria were amended with glycerol (10% final concentration) and stored in liquid nitrogen. These 'freeze-downs' were transported to the laboratory and used to screen for algicidal activity as outlined below.

### 2.2. Culture conditions and monitoring of growth

A non-axenic (i.e., bacteria-containing) *K. brevis* clonal isolate from Charlotte Harbor, FL, USA (isolate C2; provided by Dr. K. Steidinger, Fish and Wildlife Research Institute, St. Petersburg, FL, USA) was used for the initial screenings of algicidal activity and bacterial isolations. Additional studies employed the non-axenic *K. brevis* clonal isolate NOAA-1 and bacteria-free cultures of *Karenia mikimotoi* isolate G303ax-2 from Suo Nada, Japan (provided, respectively, by Dr. S. Morton, National Ocean Service/CCEHBR, Charleston, SC, USA and Dr. K. Fukami, Kochi University, Japan). The latter isolate was selected based on its phylogenetic similarity to *K. brevis* and its bacteria-free status as demonstrated by both direct microscopic observations and PCR-based methods (Mayali and Doucette, 2002). All algal cultures used in this study were grown in 25 mL of *f/2* medium without silicate (Guillard, 1975) at 20 °C on a 16 h:8 h L:D regime with a photon flux rate of  $\sim 75 \mu\text{mol m}^{-2} \text{s}^{-1}$  (model QSL 100; Biospherical Instruments, San Diego, CA, USA).

*In vivo* fluorescence was monitored every 48 h (unless otherwise specified) using a Turner Designs model 10-AU fluorometer (optical kit 10-040R; Sunnyvale, CA, USA) and used as a proxy for algal growth. A culture was considered to be dead when relative fluorescence units (RFUs) declined below 10% of the controls.

### 2.3. Screening for algicidal activity and isolation of algicidal bacteria

Frozen whole water samples (<80  $\mu\text{m}$  fraction) were thawed, washed twice with sterile 0.22  $\mu\text{m}$ -filtered seawater to remove excess glycerol, and aliquots (100 and 200  $\mu\text{L}$ ) added to exponentially growing *K. brevis* C2 cultures. A negative control of sterile seawater (200  $\mu\text{L}$ ) and a positive control containing  $10^3$  cells  $\text{mL}^{-1}$  of a known algicidal bacterium (strain 41-DBG2) were tested concurrently. When the RFUs declined to below 10% of the negative control, 200  $\mu\text{L}$  from these freshly killed cultures were added to a new exponentially growing *K. brevis* culture. This process, designed to enrich for algicidal bacteria prior to isolation attempts, was repeated until death of *K. brevis* consistently occurred within 4 days of inoculation.

For isolation of algicidal bacterium S03, samples taken directly from killed *K. brevis* C2 cultures were streaked onto agar plates of either dinoflagellate bacterial growth medium (DBG/5; Doucette et al., 1999) or seawater complete medium (SWC: 2.5 g tryptone, 1.5 g yeast extract, 3 mL glycerol, 500 mL 30 ppt natural seawater; Haygood and Neelson, 1985) and placed under the same conditions as described above for algal cultures. Individual bacterial colonies were selected and re-streaked to demonstrate purity. Algicidal activity of the S03 strain was confirmed by adding pure colonies directly to exponentially growing *K. brevis* C2 cultures and monitoring RFUs.

### 2.4. DNA extraction and 16S rDNA sequencing

Bacterial genomic DNA was extracted using a standard CTAB (hexadecyltrimethyl ammonium bromide) protocol with the modifications described in Mayali and Doucette (2002). The 16S rDNA was amplified using the universal prokaryotic primer 27f and the Eubacteria-specific primer 1492r (Long and Azam, 2001) under the following conditions: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM  $\text{MgCl}_2$ , 0.2 mM each dNTP, 0.5  $\mu\text{M}$  each primer, and 2.5 units Taq polymerase (Invitrogen; Grand Island, NY, USA). An initial denaturing step of 5 min at 92 °C was followed by 30 cycles of denaturing (92 °C), annealing (45 °C), and extension (72 °C) each for 45 s. A final extension at 72 °C ran for 7 min. Direct sequencing of purified amplicons was performed by SeqWright DNA Technology Services (Houston, TX, USA) on an ABI Prism<sup>TM</sup> 3730xl sequencer (Applied Biosystems, Foster City, CA, USA). Multiple internal primers (Lane, 1991) were employed to ensure that all regions were sequenced redundantly in both directions. A BLAST sequence similarity search was run on the consensus sequence in GenBank (Altschul et al., 1990; [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).

### 2.5. Strain S03 concentration-dependent algicidal activity

Pure isolates of algicidal strain S03 were grown in SWC broth, washed, enumerated, and added to bacteria-free *K. mikimotoi* cultures at concentrations (final) ranging from  $10^2$  to  $10^6$  cells  $\text{mL}^{-1}$ . Triplicate *K. mikimotoi* cultures were inoculated on Day 1 and monitored every 48 h via *in vivo* fluorescence. A negative control of sterile seawater was added at a volume equivalent to the  $10^6$  S03 cells  $\text{mL}^{-1}$  treatment. A culture was considered killed when the RFUs declined to <10% of the negative control.

### 2.6. Screening algal isolates for resistance/susceptibility to algicidal activity

To assess the degree of taxonomic specificity for algicidal strains S03 and 41-DBG2, six dinoflagellate species (not including *Karenia* spp.), two diatoms, and one raphidophyte were screened against one or both bacteria (Table 1). Ten strains of *K. brevis* and four strains of *K. mikimotoi* were also tested. Exponentially growing cultures were treated with strain S03 at  $10^5$  cells  $\text{mL}^{-1}$  ( $n = 3$ ) or strain 41-DBG2 at  $10^5$  cells  $\text{mL}^{-1}$  ( $n = 2$ ) and monitored for 10 days. Previous studies showed that these concentrations of the respective bacteria elicited culture death within 4 days of inoculation, thereby providing a more closely comparable time course. Negative controls were treated with an equivalent volume of sterile seawater.

### 2.7. Determination of algicidal mechanism

The mode of algicidal attack for strain S03 was examined by inoculating 25 mL cultures of *K. brevis* C2 with this bacterium ( $10^3$  cells  $\text{mL}^{-1}$ ,  $n = 4$ ). Additional *K. brevis* C2 cultures were treated with an equal volume of sterile seawater and served as controls ( $n = 4$ ). Once algal cell lysis occurred in the C2/S03 co-culture replicates, each was passed sequentially through 5  $\mu\text{m}$  polycarbonate and 0.22  $\mu\text{m}$  cellulose acetate filters. The collected filtrate was then amended with *f/2* nutrients and used as the growth medium for bacteria-free *K. mikimotoi* cultures. Half of the *K. mikimotoi* cultures (treatment,  $n = 2$ ) were inoculated with strain S03 ( $10^3$  cells  $\text{mL}^{-1}$ ), while the remaining cultures received sterile seawater (control,  $n = 2$ ). The *in vivo* fluorescence of all cultures ( $n = 2$ ) was monitored every 48 h for 10 days.

### 2.8. Comparative time course of algicidal effects

To compare the effects of strains S03 and 41-DBG2 on two clonal *K. brevis* isolates (C2 and NOAA-1) culture growth, algal morphology, and changes in the microbial community were monitored periodically over a 7-day time course (Days 1, 2, 3, 5, and 7). Treatment with a closely related, non-algicidal bacterium, *A. latercula*, was included as a negative control. All bacteria were added at a concentration of  $10^5$  cells  $\text{mL}^{-1}$  for comparability. An ANOVA was performed in the JMP statistical package (ver. 6; SAS, Cary, NC, USA) and, when necessary, the *a posteriori* Tukey's honestly significant

Table 1  
Susceptibility of various algal species to lysis by the algicidal bacteria strains S03 and 41-DBG2

Species	Isolate	Origin	Bacteria-free	S03 <sup>a</sup>	41-DBG2 <sup>a</sup>
<i>Karenia</i> spp.					
<i>Karenia brevis</i>	B4	Piney Island, FL, USA	No	–	R
<i>Karenia brevis</i>	B5	Corpus Christi, TX, USA	No	R	R
<i>Karenia brevis</i>	C2	Charlotte Harbor, FL, USA	No	S	S
<i>Karenia brevis</i>	C2	Charlotte Harbor, FL, USA	Yes	S	S
<i>Karenia brevis</i>	C5	Mexico Beach, FL, USA	No	R	R
<i>Karenia brevis</i>	NOAA-1	Charlotte Harbor, FL, USA	No	R	R
<i>Karenia brevis</i>	NOAA-1	Charlotte Harbor, FL, USA	Yes	S	S
<i>Karenia brevis</i>	PNS	Pensacola, FL, USA	No	R	R
<i>Karenia brevis</i>	SP3	Corpus Christi, TX, USA	No	R	R
<i>Karenia brevis</i>	Wilson	Tampa, FL, USA	No	R	R
<i>Karenia mikimotoi</i>	CCMP 429	Plymouth, England	No	–	S
<i>Karenia mikimotoi</i>	NOAA-2	Sarasota, FL, USA	No	R	R
<i>Karenia mikimotoi</i>	1651	Unknown	No	–	R
<i>Karenia mikimotoi</i>	G303ax-2	Suo Nada, Japan	Yes	S	S
Other dinoflagellates					
<i>Akashiwo sanguinea</i>	95003	Florida Bay, FL, USA	No	R	R
<i>Alexandrium tamarense</i>	OK905-5	Okkirai Bay, Japan	No	S	S
<i>Alexandrium tamarense</i>	ATFE-10	Fal Estuary, England	No	R	S
<i>Alexandrium monilatum</i>	AM01	Gulf Port, MS, USA	No	R	R
<i>Alexandrium monilatum</i>	AM06	Gulf Port, MS, USA	No	R	S
<i>Alexandrium fundyense</i>	GTCA 29	Gulf of Maine, NH, USA	No	S	S
<i>Gymnodinium catenatum</i>	SE-GC	Senzaki Bay, Japan	No	R	R
<i>Gymnodinium simplex</i>	CCMP 420	Gulf of Tehuantepec, Mexico	No	–	S
Diatoms					
<i>Pseudo-nitzschia multiseriis</i>	CCMP 1660	Prince Edward Is., Canada	No	–	S
<i>Skeletonema costatum</i>	CCMP 1332	Milford, CT, USA	No	S	S
Raphidophyte					
<i>Heterosigma akashiwo</i>	CCMP 1870	Long Beach, CA, USA	No	R	R

Starting bacterial concentrations were  $10^5$  cells  $\text{mL}^{-1}$ ; algal growth (*in vivo* fluorescence) was monitored for 10 days after inoculation of the bacteria.

<sup>a</sup> Algal isolates are denoted as susceptible (S), resistant (R), or not tested (–).

difference (HSD) test was used to identify which treatments were significantly different.

At designated time points, samples from each treatment were fixed with 10% formalin, examined on an Axiovert S100 epifluorescence microscope (Zeiss, Inc., Thornwood, NY, USA), and photographed with a Spot digital camera (model 1.4.0, Diagnostic Instruments, Inc., Sterling Heights, MI, USA).

Genomic DNA was extracted from each treatment, amplified, and analyzed for bacterial community structure using the following DGGE protocol. The 16S rRNA gene was amplified using primers 341f, with a 32-base GC clamp at the 5' end, and 517r (Muyzer et al., 1993). The PCR reaction components and conditions were as described previously by Mayali and Doucette (2002). Eight percent polyacrylamide gels (37:1 acrylamide:bisacrylamide) were prepared with a denaturing gradient of 35–60% (100%: 7 M urea and 40% v/v deionized formamide) and DNA concentrations in all wells were normalized. Gels ran for 16 h at 70 V/60 °C in a BIORAD D-code system (Hercules, CA, USA) containing  $0.5 \times$  TAE buffer and were stained with SYBR<sup>®</sup> Gold (Invitrogen-Molecular Probes, Eugene, OR, USA) upon completion. Gels were imaged using a FluorChem<sup>™</sup> 8900 Imaging System (Alpha Innotech, San Leandro, CA, USA) using a SYBR Green/Gold photographic filter and analyzed with dedicated AlphaEase FC software (ver. 4.0).

### 3. Results

#### 3.1. Isolation of algicidal bacterium strain S03

The initial screening of surface water sample #131 (28° 27.3' N/83° 13.1' W) exhibited putative algicidal activity against *K. brevis* C2; over the course of three consecutive enrichments, culture death was achieved in 4 days versus the initial 25 days. Samples from the last enrichment were plated on SWC agar and a purified colony, herein referred to as strain S03, tested positive for algicidal activity. The same algicidal bacterium, based on 16S rDNA sequence data (see below), was also isolated independently from a second spatio-temporally distinct, whole water sample.

#### 3.2. Identification of strain S03

BLAST results for the 16S rDNA sequence (1435 bp) of strain S03 revealed the most closely related bacterium (98% maximum identity; 99% query coverage) to be an unknown marine bacterium, SCRIPPS\_413 (GenBank accession no. [AF359548](#)); similarly, *Formosa* sp. 51X/A01/134 (GenBank accession no. [AY576730](#)) resulted in a 98% maximum identity against 98% of the target sequence. The top 10 matches were all identified as members of the family *Flavobacteriaceae* and, consequently, members of the CFB group. Interestingly, strain

S03 exhibited a 95% sequence similarity to a previously characterized algicidal bacterium, *Flavobacterium* sp. strain 5N-3 (GenBank accession no. [AB017597](#)),

Mayali (2001) classified strain 41-DBG2 (GenBank accession no. [AF427479](#)) as a member of the CFB complex, sharing a close taxonomic relationship with six other algicidal bacteria and one non-algicidal bacterium, *A. latercula* (formerly *C. latercula*); however, based on recent submissions to GenBank, strain 41-DBG2 is now most similar to the bacterium LPK13 (*Flavobacteriaceae*; GenBank accession no. [EF527872](#)) (95% maximum identity; 100% query coverage). The next closest matches (all 94% similarity) included three unidentified members of the phylum *Bacteroidetes*, as well as the recently named *Dokdonia donghaensis* (Yoon et al., 2005), *Krokinobacter genikus*, *K. diafertikos*, and *K. eikastus* (Khan et al., 2006), all belonging to newly described genera.

### 3.3. Strain S03 concentration-dependent effect on *Karenia mikimotoi*

A concentration-dependent relationship was observed when increasing levels of strain S03 were added to bacteria-free cultures of *K. mikimotoi* (G303ax-2) on Day 0 (Fig. 1). By Day 2, a decrease in *in vivo* fluorescence was observed for all bacterial additions, with the relative rates of decline generally proportional to the strain S03 concentration. On Day 4 all treatments exhibited a fluorescence signal less than 10% of the negative control indicating culture death.

### 3.4. Resistance/susceptibility of algal isolates to algicidal activity

As many as 23 algal isolates were exposed to bacterial strains S03 and 41-DBG2 to determine their susceptibility to algicidal attack; several *K. brevis* isolates as well as other HAB and non-HAB species, including species of *Akashiwo*, *Alexandrium*, *Gymnodinium*, *Heterosigma*, *Pseudo-nitzschia*, and *Skeletonema*, were screened (Table 1). No bacteria-free isolates (*Karenia* spp. only) were resistant to algicidal attack, whereas *K. brevis* C2 was the only bacteria-containing *Karenia*

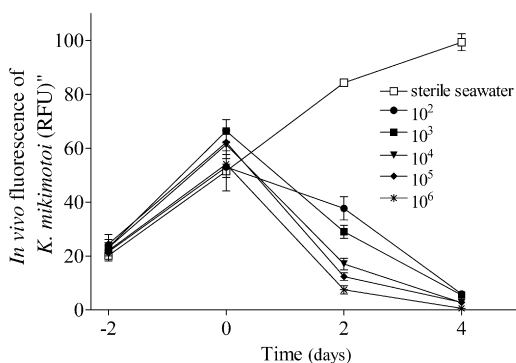


Fig. 1. Effects of algicidal strain S03 inoculation density on growth of bacteria-free *K. mikimotoi* (G303ax-2). On Day 0 strain S03 was introduced into algal cultures at concentrations ranging from  $10^2$  to  $10^6$  cells  $\text{mL}^{-1}$  and *in vivo* fluorescence (mean  $\pm$  1 SE;  $n = 3$ ) monitored over 4 days. A negative control received an equivalent volume of sterile seawater on Day 0.

isolate susceptible to both strains S03 and 41-DBG2. Considering only the bacteria-containing algal isolates, strain S03 killed 4 of 18 isolates tested (22%), whereas strain 41-DBG2 was effective against 9 of 23 isolates (39%). All five of the isolates susceptible to S03 were also killed by 41-DBG2. Nevertheless, *Alexandrium tamarense* ATFE-10 and *Alexandrium monilatum* AM06 were susceptible only to attack by strain 41-DBG2, yet *A. monilatum* AM01 was resistant to both algicidal strains. In addition, *A. tamarense* OK905-5 (origin—Japan) was killed by both strains S03 and 41-DBG2, unlike *A. tamarense* ATFE-10 (origin—England).

### 3.5. Algicidal mechanism of strain S03

To determine whether algicidal bacterium S03 employs a direct or indirect mechanism of attack, *K. brevis* C2 cultures were grown in the presence ( $10^3$  cells  $\text{mL}^{-1}$ ; treatment) and absence (control) of strain S03 ( $n = 4$ ). Immediately following lysis of the C2/S03 co-cultures on Day 4 (Fig. 2), filtrates from both treatment and control cultures were re-amended with *f/2* nutrients and inoculated with bacteria-free *K. mikimotoi* ( $n = 2$ ) on Day 5. The growth of both control and treatment cultures was not significantly different ( $p = 0.144$ ; Fig. 2).

### 3.6. Comparative effects of strains S03 and 41-DBG2 on *Karenia brevis*

The effects of algicidal bacteria strains S03 and 41-DBG2, and the non-algicidal bacterium *A. latercula*, were examined on *K. brevis* isolates C2 (susceptible; Table 1) and NOAA-1 (resistant; Table 1). Cultures of *K. brevis* C2 inoculated with strain S03 or 41-DBG2 (both  $10^5$  cells  $\text{mL}^{-1}$ ) were lysed by Day 3 and Day 7, respectively (Fig. 3A). According to an ANOVA and an *a posteriori* Tukey's HSD test, the response of cultures treated with either of the algicidal bacteria differed significantly from those receiving *A. latercula* ( $p = 0.0009$ ). *K.*

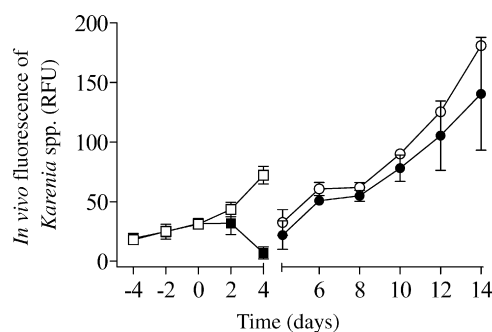


Fig. 2. Growth of *Karenia* spp. in the presence of algicidal strain S03 and in *Karenia*/S03 co-culture filtrates. On Day 0, strain S03 (■) was inoculated into cultures of *K. brevis* isolate C2 at  $10^3$  cells  $\text{mL}^{-1}$ . An equivalent volume of sterile seawater was added to negative controls (□). *In vivo* fluorescence (mean  $\pm$  1 SE;  $n = 4$ ) was monitored over 4 days. Immediately after algal cell lysis in S03 treatments (Day 4; note x-axis scale break), all *Karenia*/S03 co-cultures were filtered ( $0.2 \mu\text{m}$ ), filtrates were re-amended with *f/2* nutrients, and bacteria-free *K. mikimotoi* (G303ax-2) cells were added (●). Negative control culture filtrates from Day 4 were treated identically (○). *In vivo* fluorescence (mean  $\pm$  1 SE;  $n = 2$ ) was monitored over 10 days. Positive control filtrates receiving strain S03 were included (data not shown).

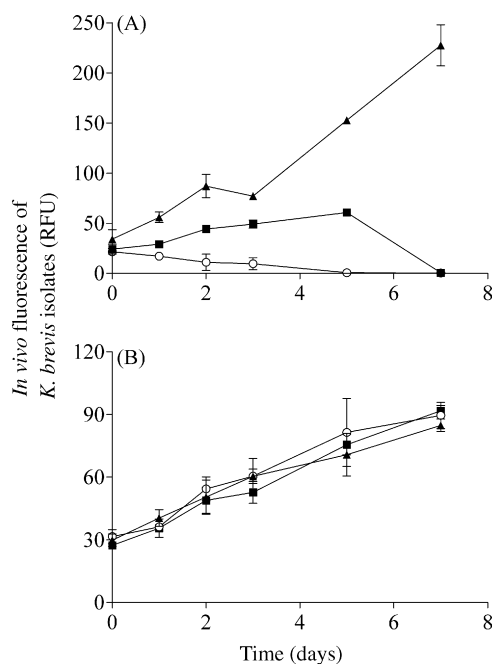


Fig. 3. Growth of *K. brevis* isolates C2 (A) and NOAA-1 (B) exposed to algalicidal or non-algalicidal bacteria. On Day 0, S03 (○), 41-DBG2 (■), and a negative control *Aquimarinia latercula* (▲) were inoculated at  $10^5$  cells  $\text{mL}^{-1}$  and *in vivo* fluorescence (mean  $\pm$  1 SE;  $n = 3$ ) was monitored over 7 days. Subsamples were taken for light microscopic and DGGE analysis on Days 0, 1, 2, 3, 5, and 7 (see text for details).

*brevis* isolate NOAA-1 was resistant to attack by strains S03 and 41-DBG2 (Fig. 3B), showing no significant differences in growth between the control cultures and the two algalicidal treatments ( $p = 0.322$ ). All cultures continued to grow over the 7-day time series.

Throughout this experiment, cells of *K. brevis* isolates C2 and NOAA-1 exposed to *A. latercula* showed normal morphological characteristics (Fig. 4); however, cells of *K. brevis* C2 cultures treated with strain S03 exhibited rounding and reduced motility by Day 1 or 2, with most cells lysed by Day 5. Strain 41-DBG2 also caused noticeable rounding, cytoplasmic blebbing, and/or loss of motility but not until Day 5, with breakdown of intracellular structure, loss of cell wall integrity, and lysis occurring on Day 7 (Fig. 4). Notably, a small number of round, thick-walled cyst-like structures were observed on and after Day 7 in cultures receiving either strain S03 or 41-DBG2, whereas no similar structures were observed in control cultures (Fig. 4). The morphology of *K. brevis* NOAA-1 cells treated with strain S03 or 41-DBG2 were indistinguishable from the controls exposed to *A. latercula* (Fig. 4).

### 3.7. Denaturing gradient gel electrophoresis (DGGE) analysis of *Karenia brevis* microbial community

The interaction of algalicidal bacteria strains S03 and 41-DBG2 with bacterial communities co-occurring in the non-axenic *K. brevis* isolates C2 and NOAA-1 were evaluated using denaturing gradient gel electrophoresis (DGGE). Fewer bands, each representing at least one bacterial phylotype, were

observed in *K. brevis* NOAA-1 cultures compared to those of the C2 isolate (Fig. 5). The band representing strain S03 was present throughout the time course in *K. brevis* C2 cultures amended with this bacterium. Maximum intensity of this band occurred on Day 3 (Fig. 5, upper left), coincident with a declining *in vivo* fluorescence (cf. Fig. 3A). In contrast, the most intense band representing strain S03 in *K. brevis* NOAA-1 cultures was on Day 0, immediately after being introduced into the dinoflagellate culture. Between Days 0 and 3, this band decreased in intensity until it became undetectable on Day 5.

DGGE analysis of *K. brevis* isolates C2 and NOAA-1 exposed to the strain 41-DBG2 displayed trends similar to those observed for the S03 experiments. In cultures of *K. brevis* C2 the band representing strain 41-DBG2 increased gradually in intensity, achieving a maximum coincident with culture lysis on Day 7 (cf. Fig. 3B). Unfortunately, insufficient amounts of DNA were obtained on Day 0 for analysis. In the resistant *K. brevis* NOAA-1 cultures (cf. Fig. 3B), the band representing strain 41-DBG2 was faintly apparent for the first 3 days (Fig. 5, lower right). On Days 5 and 7 insufficient amounts of DNA resulted in blank lanes for both time points.

## 4. Discussion

Here we report the isolation of a new algalicidal bacterium from the eastern Gulf of Mexico, USA and a comparison of its activity with that of another algalicidal strain originating from this region. Although both bacteria attack the red tide dinoflagellate, *K. brevis*, the dynamics of these interactions, and thus the efficacy of the killing process, clearly differ according to whether physical contact with target algal cells is required. Moreover, such differences in mode of attack may also influence the susceptibility of these bacteria to co-occurring antagonistic bacteria, which have been documented to adversely affect the growth and killing activity of algalicidal strains (Roth et al., in press). These results add to our understanding of how algalicidal bacteria interact not only with their target algal species, but also with the ambient microbial community, and further demonstrate the complex nature of these relationships.

16S rDNA sequence data placed algalicidal strain S03 in the family *Flavobacteriaceae*, with its closest relatives being an unidentified marine bacterium and a member of the genus *Formosa* (both 98% maximum identity). The former was isolated from cultures of the non-toxic, bloom-forming dinoflagellate, *Scrippsiella trochoidea* (Hold et al., 2001), while the latter was isolated from surface and subsurface water samples in the Mediterranean Sea (Agogu e et al., 2005). Nonetheless, given the sequence information available and phylogenetic analysis performed (data not shown), it was not possible to conclusively assign strain S03 to a genus at this time. The sequence similarity was also quite high (95%) between strain S03 and a previously described algalicidal bacterium, *Flavobacterium* sp. (strain 5N-3; Fukami et al., 1992). However, unlike strain S03, which requires direct contact with its target algal cells, strain 5N-3 releases a small (<500 Da) algalicidal compound into the surrounding medium

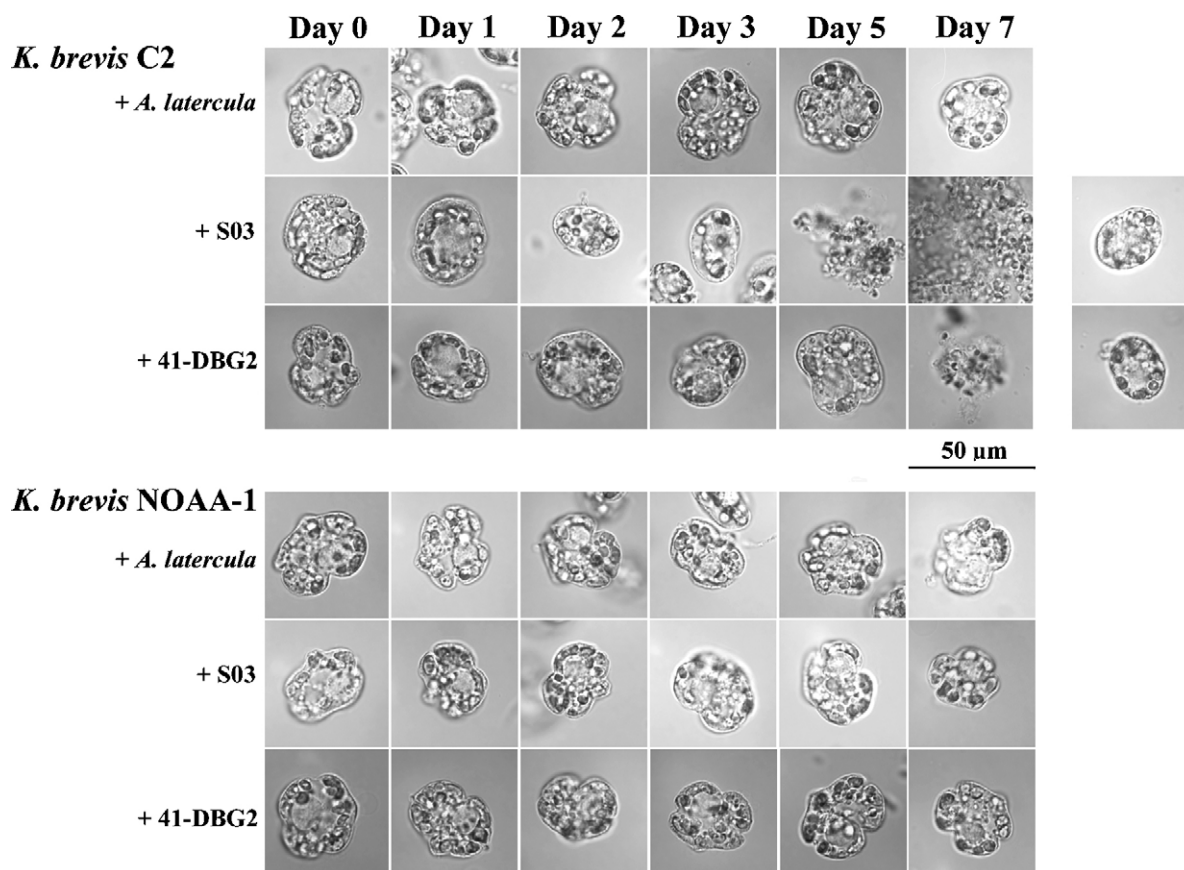


Fig. 4. Photomicrographs of representative *K. brevis* (C2 and NOAA-1) cells exposed to algicidal bacteria strains S03 or 41-DBG2, or the negative control, *Aquimarina latercula*, at  $10^5$  cells  $\text{mL}^{-1}$  starting on Day 0. Cyst-like structures were observed on Day 7 in the *K. brevis* C2 cultures inoculated with either strain S03 or 41-DBG2 (photomicrographs to the right of time series).

(Fukami et al., 1992), more similar functionally to strain 41-DBG2, a *Cytophaga* sp. that also produces a dissolved, low molecular weight algicide (Twiner et al., 2004).

The prior assignment of strain 41-DBG2 and several other algicidal bacteria to the genus *Cytophaga* may require re-evaluation, based in part on the recent reclassification of *C. latercula* to *A. latercula* (Nedashkovskaya et al., 2006). In addition, a number of new genera have been included in the CFB group (e.g., Yoon et al., 2005). The 16S rDNA sequence of strain 41-DBG2 is presently most similar (95%) to that of another algicidal marine bacterium within the family *Flavobacteriaceae* (strain LPK13) reported to be capable of inducing temporary cyst formation in the bloom-forming dinoflagellate *Lingulodinium polyedrum* (Mayali et al., 2007).

Most known algicidal bacteria are classified within either the CFB group or the *Alteromonas*–*Pseudoalteromonas* group (Yoshinaga et al., 1995; Kondo et al., 1999; Kitaguchi et al., 2001), with the former often more prevalent in both marine (median 18%; range 2–72%) and freshwater (median 7%; range 0–18%; Glöckner et al., 1999) bacterial communities. Given the frequent dominance of the CFB group and its inclusion of many known algicidal bacteria, it is likely that such bacteria will prove to be more abundant than previously thought. In fact, during this study we identified two spatio-temporally independent Gulf of Mexico samples that contained the algicidal

strain S03. Interestingly, both were surface samples with non-detectable levels of *K. brevis*. This finding is consistent with an earlier conceptual model (Doucette et al., 1999) suggesting that algicidal bacteria are present in the ambient bacterial community at low concentrations during non-bloom conditions, which may allow them to respond rapidly to the appearance of *K. brevis* target cells during bloom initiation.

Algicidal strains S03 and 41-DBG2 exhibited identical specificities for the bacteria-containing *Karenia* isolates screened (when both strains were tested). Neither bacterium had an effect on *K. brevis* isolates C5 and NOAA-1 nor *K. mikimotoi* isolate NOAA-2, while only *K. brevis* C2 was susceptible to both strains. By swapping bacterial communities between susceptible and resistant *K. brevis* isolates, Mayali and Doucette (2002) demonstrated that resistance to algicidal attack was a function of the associated bacterial assemblage and not an intrinsic property of the dinoflagellate cells. Consistent with this argument, Long and Azam (2001) found that over half of the 86 bacterial isolates they examined exhibited some level of inhibitory activity against other bacteria and nine of these were antagonistic, each completely preventing growth of at least 10 other bacterial strains. Indeed, we recently isolated a member of the family *Flavobacteriaceae* from resistant *K. brevis* NOAA-1 cultures capable of reducing both the growth and algicidal activity of strain S03 in a concentration-dependent manner



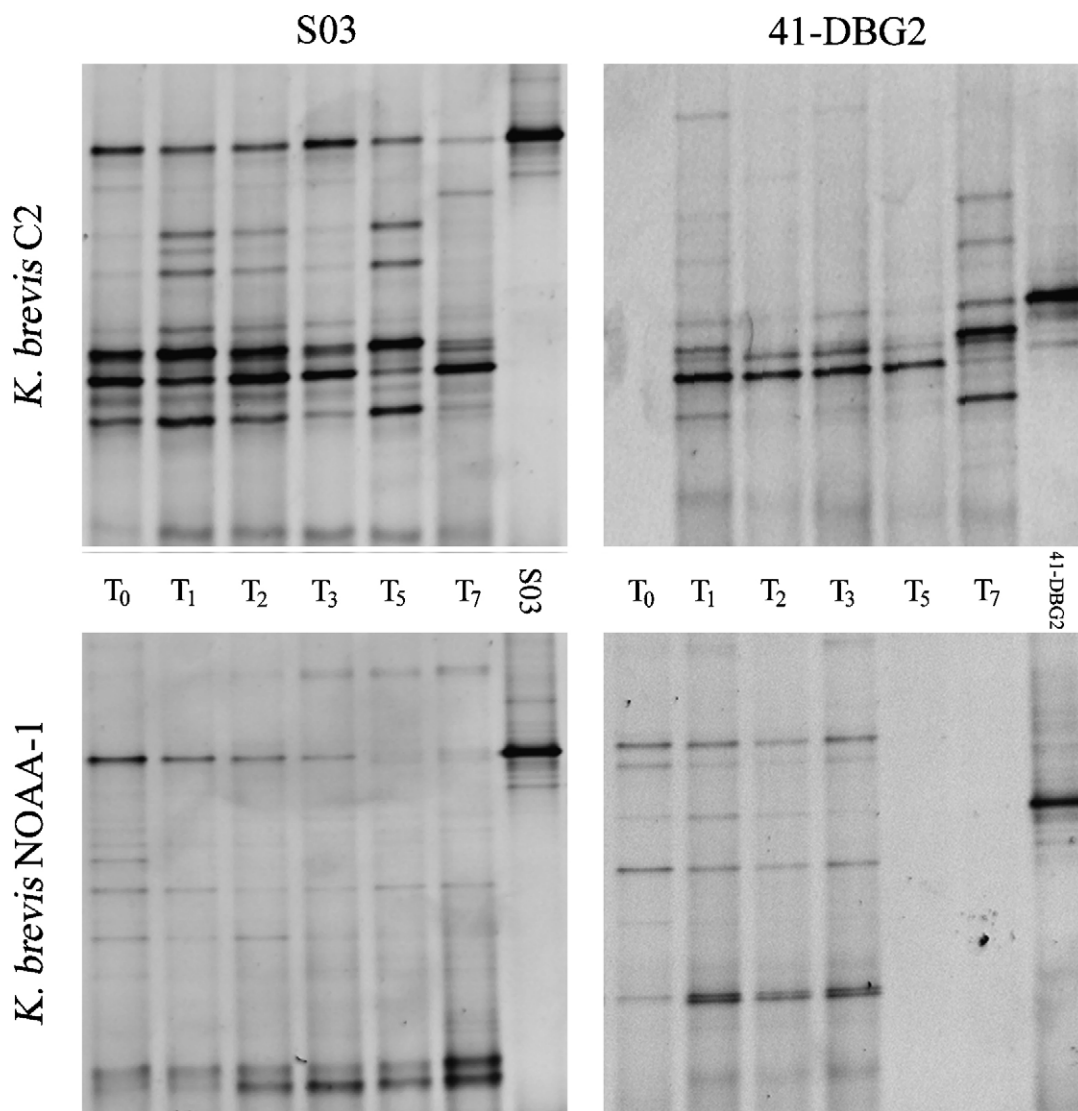


Fig. 5. Denaturing gradient gel electrophoresis (DGGE) images of the bacterial phylotypes in *K. brevis* isolates C2 and NOAA-1 exposed to algicidal strain S03 or 41-DBG2. *K. brevis* cultures were inoculated with either strain S03 or 41-DBG2 on Day 0 at  $10^5$  cells  $\text{mL}^{-1}$  and bacterial DNA from Days 0, 1, 2, 3, 5, and 7 was analyzed. DNA from purified bacterial cultures was run (lane 7) as a reference. The amount of material loaded per well was normalized and ranged from 100 to 150 ng DNA per well. Gel imaging software was used to produce negative images for resolution of fainter bands. Blank lanes represent samples yielding insufficient quantities for DGGE analysis.

(Roth et al., in press). Note that our DGGE data showed the rapid disappearance of algicidal bacteria phylotypes following inoculation into this same *K. brevis* isolate (NOAA-1) and no growth-inhibiting/morphological effects on the dinoflagellate were observed. Additionally, all bacteria-free *Karenia* cultures tested thus far (*K. brevis* isolates C2, NOAA-1; *K. mikimotoi* isolate G303ax-2) have been susceptible to lysis by known algicidal strains, further supporting the idea that a component(s) of the bacterial assemblage is involved in conferring resistance to algicidal attack.

A limited number of non-dinoflagellate taxa were screened for susceptibility to algicidal strains S03 and 41-DBG2. The one raphidophyte tested was resistant to both bacteria, whereas neither of the two diatoms examined showed resistance to algicidal attack; however, the small sample size precludes generalizing about the potential impact of these bacteria on a

given algal group. None of the taxa resistant to algicidal attack, regardless of algal group, were bacteria-free. For dinoflagellates other than *Karenia* spp., two clonal cultures of *A. monilatum* (AM01, AM06) isolated from the same water sample (S. Morton, personal communication) and two *A. tamarense* isolates of different geographic origin were differentially susceptible to one of the algicidal bacteria. Such differences may reflect the association of bacteria “antagonistic” against an algicidal strain (e.g., Roth et al., in press) with resistant cultures at the time of their isolation.

The overall effectiveness of algicidal attack did not appear to be greatly influenced by whether or not contact with algal cells was required. Nonetheless, the mechanism of algicidal attack (direct versus indirect) did appear to modulate the temporal dynamics of interactions with target cells. *K. brevis* C2 cultures treated with strain S03 (direct attack) began to decline (*in vivo*

fluorescence) immediately following exposure, with photomicrographs revealing rounded cells within a day or two of exposure and complete cell lysis shortly thereafter (by Day 5). Culture death also coincided with the presence of a predominant S03 phylotype (band) in the DGGE analysis. Introduction of strain 41-DBG2 (indirect attack) to cultures of this same *K. brevis* isolate caused growth suppression throughout the time course, corresponding to less severe cell rounding, with lysis not occurring until Day 7, which also matched well with the timing of maximum band intensity for the 41-DBG2 phylotype. Members of the CFB complex are known degraders of biomacromolecules such as cellulose (Reichenbach and Dworkin, 1991), a key structural component of dinoflagellate cell walls, which may explain the rapid loss of cell integrity observed with strain S03. Alternatively, the activity of a dissolved algicide released into the growth medium would be diffusion-limited, which could lead to a more protracted killing of an algal culture as was noted for strain 41-DBG2. The exact nature of these dissolved algicidal compounds, and thus their mechanism of action, has yet to be elucidated (reviewed by Skerratt et al., 2002; Twiner et al., 2004), although previous workers have proposed the involvement of ectoproteases (Mitsutani et al., 2001; Lee et al., 2000).

A particularly interesting observation made during this study was the consistent presence of a small number of cyst-like cells later in the time course, but only in those cultures treated with algicidal bacteria. A true resting cyst, generally considered to be the product of gametic fusion in dinoflagellates, has yet to be described for *K. brevis*. Nonetheless, given the circumstances under which these cyst-like structures occurred (i.e., exposure to algicidal bacteria), it is likely that they are analogous to asexual temporary or ecdysal cysts documented previously for other dinoflagellates and considered to be induced by some type of physico-chemical, or possibly biologically mediated, stress (see Steidinger and Garcés, 2006). As noted above, strain LPK13 (*Flavobacteriaceae*), an algicidal bacterium showing the highest sequence similarity (95%) to strain 41-DBG2, is also capable of inducing temporary cyst formation in a dinoflagellate (*L. polyedrum*), which is proposed as a mechanism to eliminate attached, potentially harmful bacteria (Mayali et al., 2007). Scanning electron microscopy as well as germination studies will be needed to better characterize the morphology and assess the viability of the putative *K. brevis* temporary cysts.

To conclude, numerous algicidal bacteria have been identified both in freshwater and marine ecosystems. Nonetheless, continued exploration of how they interact with both their algal target species, as well as the ambient microbial community, is necessary to determine what role, if any, these bacteria play in regulating the growth and termination of HABs. Composition of the microbial community appears to be a key factor influencing the killing activity of certain algicidal strains in cultures of *K. brevis* and this is likely to be the case in natural bloom populations. Moreover, a recent laboratory study of brevetoxin dynamics following attack of *K. brevis* cells by algicidal bacteria has revealed marked changes in the both the size-fractionated distribution of the toxin, accompanied by a decline in overall toxicity (Roth et al., 2007). Although a

difficult task, it will be essential to evaluate the potential outcomes of these complex interactions in field-based studies (e.g., mesocosms) when assessing the potential role of algicidal bacteria not only as natural regulators of bloom dynamics, but as part of a HAB management and control strategy.

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