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Microbial community interactions and population dynamics of an algicidal bacterium active against *Karenia brevis* (Dinophyceae)☆

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Abstract

The population dynamics of *Cytophaga* strain 41-DBG2, a bacterium algicidal to the harmful algal bloom (HAB) dinoflagellate *Karenia brevis*, were investigated in laboratory experiments using fluorescent in-situ hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE). Following its introduction into non-axenic *K. brevis* cultures at concentrations of 10^3 or 10^5 bacterial cells per milliliter, 41-DBG2 increased to 10^6 cells per milliliter before initiation of its algicidal activity. Such threshold concentrations were not achieved when starting algal cell numbers were relatively low (10^3 cells per milliliter), suggesting that the growth of this bacterium may require high levels of dissolved organic matter (DOM) excreted by the algae. It remains to be determined whether this threshold concentration is required to trigger an algicidal response by 41-DBG2 or, alternatively, is the point at which the bacterium accumulates to an effective killing concentration. The ambient microbial community associated with these algal cultures, as determined by DGGE profiles, did not change until after *K. brevis* cells were in the process of lysing, indicating a response to the rapid input of algal-derived organic matter. Resistance to algicidal attack exhibited by several *K. brevis* clones was found to result from the inhibition of 41-DBG2 growth in the presence of currently unculturable bacteria associated with those clones. These bacteria apparently prevented 41-DBG2 from reaching the threshold concentration required for initiation of algicidal activity. Remarkably, resistance and susceptibility to the algicidal activity of 41-DBG2 could be transferred between *K. brevis* clones with the exchange of their respective unattached bacterial communities, which included several dominant phylotypes belonging to the α-proteobacteria, γ-proteobacteria, and Cytophaga–Flavobacterium–Bacteroides (CFB) groups. We hypothesize that CFB bacteria may be successfully competing with 41-DBG2 (also a member of the CFB) for nutrients, thereby inhibiting growth of the latter and indirectly providing resistance against

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algicidal attack. We conclude that if algicidal bacteria play a significant role in regulating HAB dynamics, as some authors have inferred, bacterial community interactions are crucial factors that must be taken into consideration in future studies.
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1. Introduction

The toxic dinoflagellate *Karenia brevis* (formerly *Gymnodinium breve*; Daugbjerg et al., 2000), causative organism of neurotoxic shellfish poisoning, blooms on an annual basis in the Gulf of Mexico, causing fish kills and other deleterious events (Anderson, 1994). Much of the current research on this and other harmful algal bloom (HAB) species is aimed at elucidating the mechanisms that regulate the dynamics of bloom stages, defined as initiation, growth, maintenance, and termination (Steidinger et al., 1998). These mechanisms may include physical effects such as winds and currents (Tester and Steidinger, 1997), internal physiological responses (Van Dolah and Leighfield, 1999), and biological interactions with other organisms, including bacteria (Doucette et al., 1999). Several bacteria isolated in association with various HAB species, including dinoflagellates and raphidophytes, have been identified as algicidal (Fukami et al., 1991; Yoshinaga et al., 1995; Kim et al., 1998), which is operationally defined as leading to rapid (1–2 days) algal cell lysis following their introduction into algal cultures. Based on an increase in the concentration of algicidal bacteria during late bloom stages, these and other investigators have proposed that such bacteria may play a role in HAB termination.

Two algicidal bacteria (strains 41-DBG2 and ANSW2-2), native to west Florida shelf waters where *K. brevis* blooms occur, were isolated previously (Doucette et al., 1999). These strains were found to be phylogenetically closely related to algicidal bacteria originating from other locations worldwide (Mayali, 2001), with the majority of these bacteria belonging to the Cytophaga–Flavobacterium–Bacteroides (CFB) group and the gamma (γ) subdivision of the proteobacteria. An increase in algicidal bacteria concentrations during the termination stage of a *K. brevis* bloom has thus far not been documented. Nonetheless, such a scenario is indeed possible, given the phylogenetic similarity between algicidal bacteria active against *K. brevis* and those from other ecosystems where increases in the algicidal component of the microbial community have been reported (Kim et al., 1998; Imai et al., 2001).

A primary objective of this study was to examine the population dynamics of algicidal bacterium, *Cytophaga* sp. (strain 41-DBG2), and the dinoflagellate, *K. brevis*, in non-axenic laboratory cultures, using ribosomal RNA (rRNA) fluorescent in-situ hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE). Earlier data suggested that the time to death of a *K. brevis* culture following the inoculation of 41-DBG2 was dependent upon the initial algicidal bacteria concentration (Doucette et al., 1999). In addition, studies of closely related algicidal bacteria indicated that such bacteria consume algal-derived organic matter (Mitsutani et al., 1992) and may depend on the algae for growth. FISH and DGGE techniques were therefore employed to investigate the effects of different initial concentrations of both algicidal bacteria and target algae on algal cell death and bacterial growth using strain 41-DBG2 and *K. brevis* culture isolate C2 (Charlotte Harbor, FL, USA). We reasoned that a thorough examination of such interactions between strain 41-DBG2 and its algal target would contribute to a more complete description of the mechanisms potentially involved in *K. brevis* bloom termination. Also, since 41-DBG2 is closely related to algicidal bacteria associated with other HAB species, including *Heterocapsa circularisquama* (Nagasaki et al., 2000), *Chattonella antiqua* (Imai et al., 1993), *Heterosigma akashiwo* (Yoshinaga et al., 1998), and *Skeletonema costatum* (Maeda et al., 1998), our findings would also have application to the understanding of factors regulating the population dynamics of other HAB species.

Interestingly, over the past several years, apparent loss of the algicidal activity of bacterium 41-DBG2 against several *K. brevis* isolates has been observed. This phenomenon may reflect a change in the algicidal properties of bacterium 41-DBG2 over time, or

possibly a loss of susceptibility to or acquisition of resistance by these *K. brevis* isolates to algicidal attack. Here we used an approach that involved exchanging bacterial communities between algal isolates to examine the possibility that antagonistic interactions (e.g., allelopathy, nutrient competition, etc.) between 41-DBG2 and the ambient bacterial flora of a *K. brevis* culture mediated the resistance of an isolate to attack by algicidal bacteria. Distinguishing between the contributions of bacterial–algal and intra-bacterial community interactions to the algicidal susceptibility of HAB species may help clarify the respective roles of these interactions in bloom dynamics and explain the scarcity of algicidal bacteria sometimes reported to precede or accompany HAB events (Imai et al., 1998a).

2. Materials and methods

2.1. Culture conditions

Three *K. brevis* clonal isolates were used during this study: isolates C2 (Charlotte Harbor, FL, USA) and C5 (Mexico Beach, FL, USA) donated by Dr. K. Steidinger, and isolate NOAA1 (from Charlotte Harbor, FL, USA) provided by Dr. S. Morton. Algal cultures were grown in 25 ml of Guillard's *f*/2 medium without silicate (Guillard, 1975) in 50 ml glass tubes at 20 °C on a 16:8 h light:dark cycle with a photon flux density of 75 μmol m^{-2} s^{-1} from cool-white fluorescent tubes. Growth was monitored by in vivo fluorescence with a Turner Designs model 10-AU fluorometer (Sunnyvale, CA, USA), and algal cells were enumerated in a Sedgwick–Rafter counting chamber after staining with Lugols iodine. Algicidal bacterium 41-DBG2 was stored frozen in 10% glycerol in LN_2. For use in experiments, this strain was grown in dinoflagellate bacterial growth medium (DBG/5; Doucette et al., 1999) at 20 °C in a shaking water bath. Before addition into algal cultures, exponentially growing algicidal bacteria cells were washed and resuspended in autoclaved and 0.22 μm filtered seawater. Washed bacteria were stained with DAPI (4′,6-diamidino-2-phenylindole; Porter and Feig, 1980) and counted using epifluorescence microscopy to determine the addition required to achieve a desired starting cell concentration.

2.2. Experimental design (population dynamics)

A full factorial design experiment with two initial concentrations of *K. brevis* clone C2 was carried out (treatments A–F outlined in Table 1) to investigate algal and bacterial population dynamics. Initial algal cell concentrations were approximately 1000 and 6000 cells per milliliter, representing early and late exponential growth phases, respectively. Starting concentrations of algicidal bacterium 41-DBG2 were adjusted to 0, 10^3, and 10^5 cells per milliliter. Triplicate tubes from each of the six treatments were sacrificed daily, beginning with Day 0 (for a total of 142 replicate tubes). However, due to a lack of algicidal activity over the first 4 days in the low initial *K. brevis* concen-

Table 1
Experimental design for population dynamics experiment utilizing *K. brevis* clone C2 and algicidal bacterium 41-DBG2 (bacteria added on Day 0)

Treatment	Initial [algal] (cells per milliliter)	[41-DBG2] added	figure*	Day of kill	[41-DBG2] at kill ± S.D. (cells per milliliter)
A	1000	0	1a	n/a	
B	1000	10^3	1b	8	$3.9 \pm 1.1 \times 10^6$
C	1000	10^5	1c	8	$3.8 \pm 1.3 \times 10^6$
D	6000	0	1d	n/a	
E	6000	10^3	1e	4	$8.4 \pm 1.0 \times 10^6$
F	6000	10^5	1f	3	$5.7 \pm 1.3 \times 10^6$

Data are graphically illustrated in Fig. 1. Day of kill was defined as the first day that algal cell concentrations were significantly lower than the no bacterial addition control algal concentrations, according to a Tukey–Kramer HSD test. Each treatment included 24 replicates, three of which were sacrificed at each sampling time. Treatments A–C were sampled on Days 0–4, 6, 8, and 10 while treatments D–F were sampled on Days 0–5. Standard deviations (S.D.) were calculated from three independent replicates. Data not applicable to a given treatment are given as 'n/a'.

tration treatments and a limited number of replicate tubes (enough for sampling on 8 days), samples from these treatments were taken every 2 days after Day 4. For each independent sample, the following variables were measured: in vivo fluorescence, *K. brevis* cell concentrations, total bacteria concentrations (DAPI), algicidal bacteria concentrations (FISH), and microbial community profiles (DGGE). Cell count data for Day 4 were analyzed with two-way analysis of variance (ANOVA) and data for all other days with a one-way ANOVA with Tukey–Kramer honestly significant differences (HSD) multiple comparisons procedure for differences among means.

2.3. Experimental design (effect of microbial flora)

Previous experiments in our laboratory repeatedly demonstrated that *K. brevis* clone C2 was susceptible to algicidal bacteria attack and that *K. brevis* clones NOAA1 and C5 were resistant (unpublished data). To investigate this phenomenon, the unattached bacterial communities from *K. brevis* clones C2, C5, and NOAA1 were separated from the algae by filtration through a 5 μm filter. Filtrates were shown to be *K. brevis*-free following immediate microscopic examination and repeated observations of subsequent month-long incubations in fresh *f*/2 medium under algal culture conditions. After filtration, the unattached communities were immediately re-introduced into *K. brevis* cultures previously treated with antibiotics and shown to be bacteria-free with DAPI counts (C. Mikulski personal communication). Treatments included *K. brevis* clones with their original unattached bacterial flora added (treatments 3 and 6), *K. brevis* inoculated with bacterial flora from other clonal cultures (treatments 4, 5, and 7), and *K. brevis* C2 inoculated concurrently with bacterial communities from both a susceptible and a resistant culture (treatment 8). In addition, algal cultures with no added unattached bacterial communities were tested for comparison (treatments 1 and 2). All treatments (Table 2) included six replicates inoculated with 10^5 algicidal bacteria (41-DBG2) cells per milliliter as well as six controls with no added algicidal bacteria. Duplicate tubes were sacrificed and sampled for in vivo fluorescence, DAPI staining, FISH, and DGGE immediately prior to algicidal bacteria inoculation (Day 0), before algicidal activity began (Day 3), and as algal cells were beginning to lyse (Day 5). Several dominant DGGE phylotypes from the two 41-DBG2-resistant communities (NOAA1 and C5) were sequenced, as described in the DGGE section below. Additionally, replicate bacterial community treatments in bacterial medium DBG/5 without dinoflagellates were also included (treatments 9 and 10) to compare the growth of 41-DBG2 and the microbial community structure with the treatments containing *K. brevis*. A subsequent experiment

Table 2
Experimental design to determine the effect of unattached bacteria associated with individual *K. brevis* isolates on the algicidal activity of 41-DBG2

Treatment	*K. brevis* isolate	Bacterial community	Algicidal activity	[41-DBG2] on Day 5 (cells per milliliter)	[Total bacteria] on Day 5 (cells per milliliter)
1	C2	None	+	7.4×10^6	7.4×10^6
2	NOAA1	None	+	3.0×10^6	4.1×10^6
3	C2	C2	+	4.9×10^6	2.5×10^7
4	C2	NOAA1	−	1.1×10^5	1.3×10^6
5	C2	C5	−	4.1×10^4	6.2×10^6
6	NOAA1	NOAA1	−	4.9×10^4	4.3×10^5
7	NOAA1	C2	+	3.0×10^6	1.5×10^7
8	C2	C2 and NOAA1	−	2.1×10^5	3.0×10^6
9	None	C2	n/a	$>10^{10}$	$>10^{10}$
10	None	NOAA1	n/a	$>10^{10}$	$>10^{10}$

Algicidal activity reveals the consequence of adding 41-DBG2 to the algal culture (+, culture death; −, no effect), as compared to similar cultures not inoculated with algicidal bacteria. The concentrations of 41-DBG2 on Day 5 (when *K. brevis* cells in susceptible cultures were beginning to lyse) were measured by fluorescent in-situ hybridization with probe DBG65, while total bacteria were quantified after staining with DAPI. Data not applicable to a given treatment are given as 'n/a'.

comprised of treatments 3, 4, 6, and 7 was repeated and monitored with in vivo fluorescence to confirm any changes in susceptibility to algicidal bacteria attack. Due to the clear difference between algal cultures either resistant or susceptible to algicidal attack, no statistical procedures were carried out for this design.

2.4. Fluorescent in-situ hybridization (FISH)

FISH was used to quantify algicidal bacterium 41-DBG2 in all experiments. Probe DBG65 (5′-CAAGCACTCCTGTTACCCC-3′) conjugated to a Cy-3 label (Operon Technologies, Alameda, CA, USA), was designed to specifically detect 41-DBG2 based on its 16S rRNA gene sequence (Mayali, 2001). Samples for bacterial counts were processed according to the methods of DeLong et al. (1999) and Glöckner et al. (1996). Briefly, samples were fixed in 3.7% formaldehyde (final concentration) for at least 30 min at room temperature or overnight at 4 °C, and an aliquot (varying in volume due to bacterial growth over time) was added to 2 ml sterile seawater. This dilution was filtered onto a 25 mm diameter 0.22 μm white polycarbonate membrane (Millipore, Bedford, MA, USA), overlaid with 50% ethanol saline (2% NaCl) for 2 min, and then re-filtered. The filters were then air dried and stored in 12-well plates at −20 °C until analysis. FISH conditions were as follows: 18 μl of hybridization buffer (0.9 M NaCl, 20 mM Tris–HCl (pH 7.5), 0.01% sodium dodecyl sulfate (SDS), 35% formamide, 50 ng probe) were overlaid on filters previously cut into quarters. Samples were incubated for 1.5 h at 46 °C in a chamber equilibrated with hybridization buffer to minimize evaporation, followed by a 20 min wash at 48 °C in buffer (180 mM NaCl, 20 mM Tris–HCl (pH 7.5), 5 mM EDTA, 0.01% SDS). After a rinse in distilled deionized H_2O (ddH_2O), filters were incubated with DAPI (Porter and Feig, 1980) for 5 min to permit visualization of all bacterial cells, mounted on glass slides with Slowfade® (Molecular Probes, Eugene, OR, USA), examined and counted (minimum three 9216 μm^2 grids) using an Axiovert S100 epifluorescent microscope (Zeiss Inc., Thornwood, NY, USA), and photographed with a Spot digital camera (model 1.4.0, Diagnostic Instruments Inc., Sterling Heights, MI, USA).

2.5. Denaturing gradient gel electrophoresis (DGGE)

DGGE was used to monitor temporal changes in microbial community structure and to compare communities associated with different *K. brevis* cultures. Due to the identical DGGE profiles of 10 and 1 ml samples from the same algal culture (data not shown), volumes of 1.5 ml were deemed to be sufficient to sample the microbial assemblages in 25 ml cultures. Samples were centrifuged at 6500 × *g* for 10 min to concentrate both bacterial and algal cells, the supernatant was removed, and the tubes were frozen at −80 °C for no more than 1 week. A standard CTAB (hexadecyltrimethyl ammonium bromide) protocol (Ausubel et al., 2000) was used to extract DNA using sequential lysozyme (1 mg ml^{-1}) and proteinase K (100 μg ml^{-1} in 0.5% SDS) digestions at 37 °C, followed by incubation in 1% CTAB and 0.7 M NaCl at 65 °C. Samples were sequentially extracted with chloroform:isoamyl alcohol (CI 24:1), phenol:chloroform:isoamyl alcohol (PCI 25:24:1) and again with CI, precipitated with ethanol overnight, and re-suspended in 50 μl TE buffer (1 mM EDTA, 10 mM Tris–HCl, pH 7.5). The V3 region of the 16S rRNA gene was amplified by PCR in 50 μl reactions using primers 341F with a *GC* clamp (5′-*CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG*CCTACGGGAGGCAGCAG-3′) and 517R (5′-ATTACCGCGGCTGCTGG-3′) as in Riemann et al. (1999) under the following conditions: 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2.25 mM $MgCl_2$, 0.2 mM each dNTPs, 0.5 μM each primer, and 1.25 units Taq polymerase (Life Technologies, Rockville, MD, USA). An initial denaturation of 5 min was followed by a touchdown PCR, with annealing temperatures (1 min) decreasing by one degree every two cycles from 65 to 50 °C throughout 32 cycles. Denaturation and extension were constant at 94 and 72 °C, respectively (1 min each), and the final extension time (72 °C) was 7 min.

PCR products were checked for length with 2% agarose gel electrophoresis, and 20 μl samples were loaded on 8% polyacrylamide gels (37:1 acrylamide:bisacrylamide). These gels contained denaturant concentrations of 35–60% in most cases (100% is 7 M urea and 40% v/v deionized formamide). Electrophoresis was performed in a D-code system (BIORAD Inc., Hercules, CA, USA) at 60 °C for 16 h

at 70 V in 0.5× TAE buffer (20 mM Tris, 10 mM acetate, 0.5 mM Na_2-EDTA, pH 7.4). Gels were stained for 15 min with SYBR® Gold (Molecular Probes) in 0.5× TAE buffer and photographed with a standard UV transilluminator using a SYBR Green/Gold photographic filter (S-7569) and Polaroid® 55 black and white film. In some cases, bands were excised with a sterile razor blade, washed in 500 μl ddH_2O for 1 h, and placed in 200 μl TE buffer overnight at 4 °C to elute the DNA. Dilutions of these samples were re-amplified and run on another DGGE gel to confirm that only one phylotype was isolated. PCR products were quantified by 2% agarose gel electrophoresis against a size standard of known DNA concentrations (100 bp ladder, Promega, Madison, WI, USA), and sent to Amplicon Inc. (Pullman, WA, USA) for purification and direct sequencing. Sequences were assigned to their closest relatives with the BLAST search on Genbank (Altschul et al., 1990).

3. Results

3.1. Population dynamics: treatments A–F

The cell concentration data for each of the six treatments (Table 1) are outlined in Fig. 1. As exemplified by the two control treatments (no addition of algicidal bacteria: Fig. 1a and d), *K. brevis* cells at the time of algicidal bacteria inoculation (Day 0) were in early exponential growth phase ($\mu = 0.31\,d^{-1}$ at Day 1) in the low algal concentration treatments (cells growing through Day 10; Fig. 1a) and in late exponential growth phase in the high algal concentration treatments (stationary phase achieved by Day 3; Fig. 1d). Thus, differences in the susceptibility of *K. brevis* to algicidal attack between these two treatments could be attributed either to starting algal cell concentrations or to different physiological properties associated with algal growth phase. To determine if initial algal cell concentrations had significant effects on time to death, we analyzed the *K. brevis* cell numbers from Day 4 (the last day all six treatments were sampled together) with a two-way ANOVA. The two independent variables were initial algal cell concentration and initial algicidal bacteria concentration and the response variable was algal cell concentration, normalized to its initial concentration (Day 0) in order to satisfy the assumptions of ANOVA. All three possible sources of variation were significant in the model ($n = 18$, $r^2 = 0.82$, $P = 0.0003$): initial algal concentration ($P = 0.0082$), initial algicidal bacteria concentration ($P = 0.0007$), and the interaction term ($P = 0.0043$). Further a priori contrast analysis of the interaction term revealed that on Day 4, *K. brevis* numbers from high initial algal concentration treatments with added algicidal bacteria were significantly lower than those from low initial algal concentration treatments. In other words, by Day 4, algicidal bacterium 41-DBG2 had killed *K. brevis* in the high algal concentration treatments but not in the low algal concentration treatments.

To determine if initial algicidal bacteria concentrations had significant effects on time of algal death over the entire course of the experiment, normalized *K. brevis* cell numbers from days other than Day 4 were analyzed with one-way ANOVA followed by *a posteriori* Tukey–Kramer tests for comparison of multiple means. In this case, low and high initial algal concentration treatments were analyzed separately, as were days, due to the fact that high algal treatments were not sampled after Day 5 (treatments with added algicidal bacteria were dead after Day 4). For the high initial algal concentration, *K. brevis* numbers for the 10^5 cells per milliliter algicidal bacteria treatment (treatment F) were significantly lower on Day 3 than the control, but those from the 10^3 cells per milliliter initial algicidal bacteria treatment (treatment E) were not (at the 0.05 level). This indicated that higher initial inoculations of algicidal bacteria killed correspondingly high initial *K. brevis* concentrations significantly faster than lower algicidal bacteria initial inoculations. However, under low initial algal concentrations (treatments B, C), there were no significant differences between the two initial algicidal bacteria concentration treatments on all days sampled. On Day 6, the three treatment means (control, 10^3 and 10^5 cells per milliliter) were still not significantly different from one another, and on Day 8 both algicidal bacteria treatment means were lower than the control.

To determine if algicidal bacteria concentrations were significantly different between treatments at the time of a kill, the above data were used to determine the day algal numbers became significantly lower than their respective no addition control treatments (Table 1, column 5). This turned out to be Days 3, 4, 8, and 8 for treatments F, E, B, and C, respectively. It should be noted that treatments B and C were not

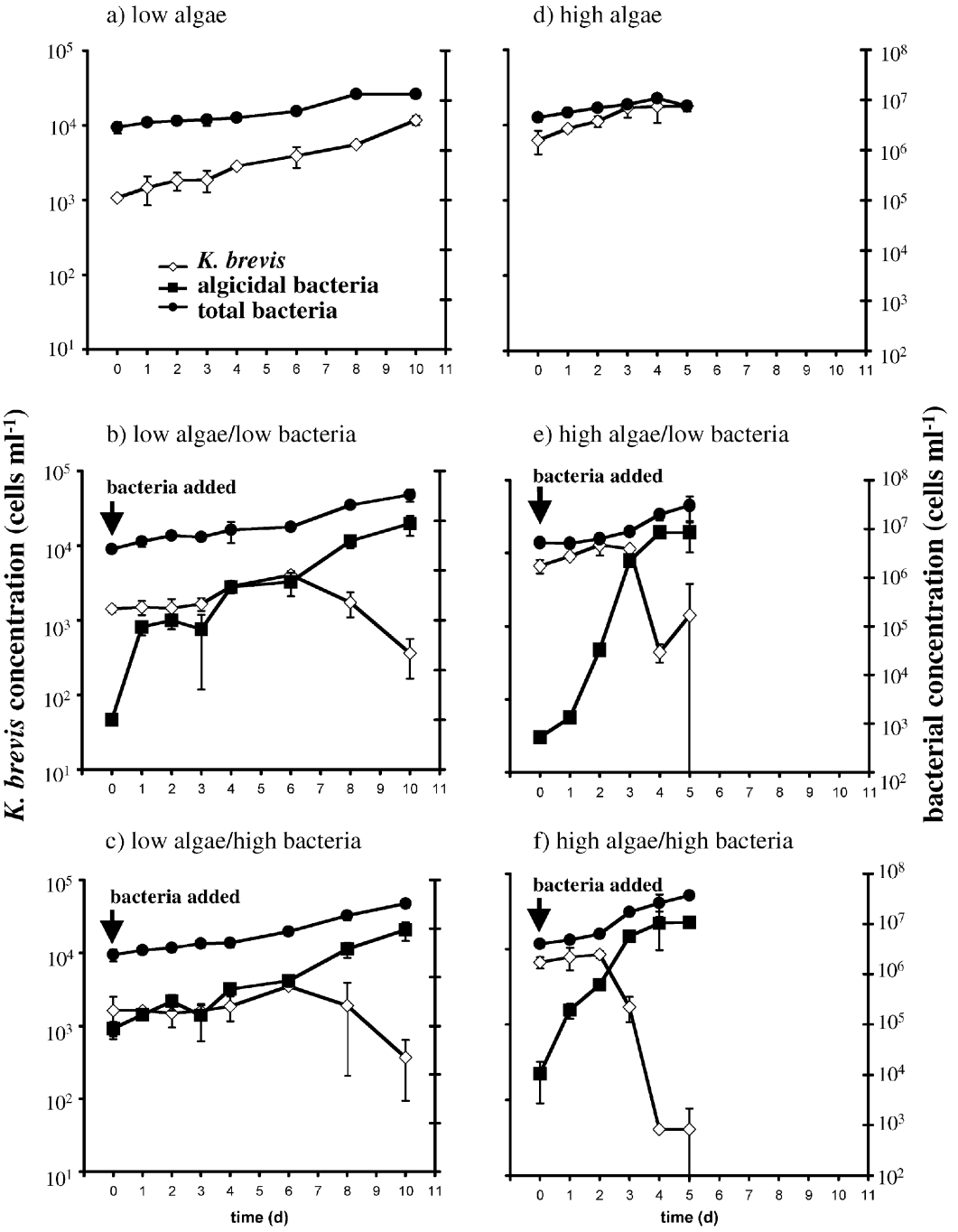

Fig. 1. Data for the six treatments outlined in Table 1 (population dynamics of *K. brevis* clone C2 and algicidal bacterium 41-DBG2) followed over 10 (a–c) or 5 days (d–f). Algal cells were enumerated with direct light microscope counts, while epifluorescence microscopy was used to enumerate total bacterial cells after staining with DAPI and algicidal bacteria following FISH with strain-specific probe DBG65. Error bars are standard deviations of three independent replicates. All treatments with no algicidal bacteria added (a and d) were consistently free of algicidal bacteria.

sampled on Day 7, which may have led to a slight overestimation of killing time. Although algicidal bacteria numbers for treatment E were significantly higher than treatments B and C with a Tukey–Kramer HSD test (treatment F was not significantly different than any of the other three treatments), the range for all four treatments was 2–9 $\times$ 10^6 cells per milliliter (all within one order of magnitude). This indicated a fairly similar algicidal bacteria concentration at the time of a kill throughout the four treatments, regardless of initial algicidal bacteria numbers or algal concentrations.

Although total bacteria increased with time in all six treatments (A–F) independently of algicidal bacteria numbers, they generally increased most rapidly following algal cell lysis. For example, in treatment F, the most rapid increase in total bacteria occurred between Days 2 and 3, while *K. brevis* concentrations were beginning to decrease (Fig. 1f). In this set of experiments, we noted that algal cell numbers and in vivo fluorescence exhibited a close correspondence (data not shown), as revealed by a regression analysis of the low algal concentration treatments ($r^2 = 0.945$, $P < 0.001$, $n = 72$). Based on this result, direct algal cell counts were replaced with in vivo fluorescence measurements for all subsequent experiments.

The DGGE profiles taken at each time point revealed several patterns. Replicate tubes from the same treatment taken on the same day corresponded well, demonstrating the reproducibility of both the culture conditions and the DGGE protocol. There were no discernable microbial community structure differences in the control cultures (no addition of algicidal bacteria) throughout the experiment (Fig. 2), implying that all community changes were attributable to the addition of the algicidal bacteria. For the low algal concentration treatments, the only noticeable microbial community changes occurred between Days 8 and 10 (Fig. 3), after *K. brevis* cell numbers had declined significantly. Results for the high algal concentration treatments were similar as no changes occurred before Day 4, again when *K. brevis* levels were beginning to decline (data not shown).

3.2. Effect of ambient microbial flora: treatments 1–10

Findings from the experiments aimed at elucidating the effect of the unattached bacterial communities

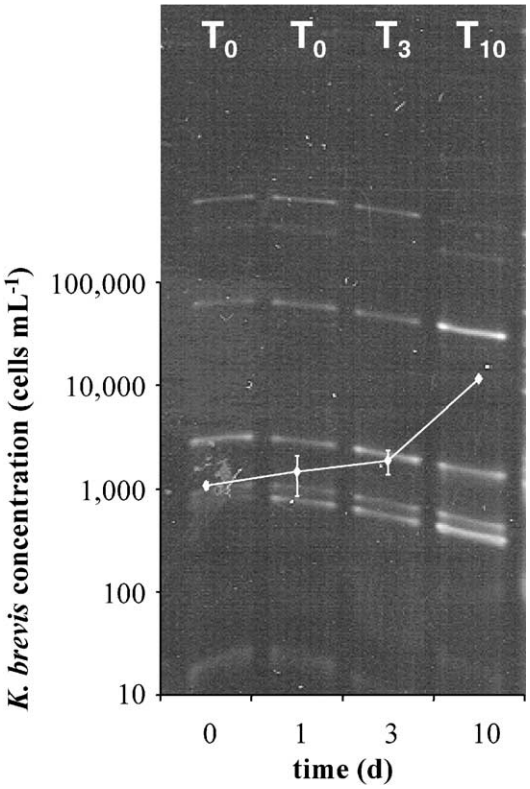

Fig. 2. DGGE profiles from treatment 1 (no addition of algicidal bacteria into low initial algal concentration cultures) demonstrating identical replicate cultures (sampled at T_0) and no microbial community structure changes throughout the length of the experiment. T_n indicates that the sample was taken on day 'n'. Gel has been overlaid with algal cell numbers determined between Days 0 and 10.

on the resistance of *K. brevis* to algicidal bacteria attack are best illustrated with the in vivo fluorescence data from treatments 3, 4, 6, and 7 (Table 2). The C2 and NOAA1 *K. brevis* clones with their original bacterial communities (treatments 3 and 6) were susceptible and resistant to algicidal bacterium 41-DBG2, respectively (Fig. 4). However, the C2 *K. brevis* clone with the unattached bacteria from the NOAA1 clone became resistant (treatment 4; Fig. 4a), and the NOAA1 *K. brevis* clone with the C2 bacteria became susceptible (treatment 7; Fig. 4b). Results from all treatments (1–10) are summarized in Table 2. Both antibiotic-treated *K. brevis* cultures (C2 and NOAA1) with no added bacterial communities (treatments 1 and 2) were susceptible to

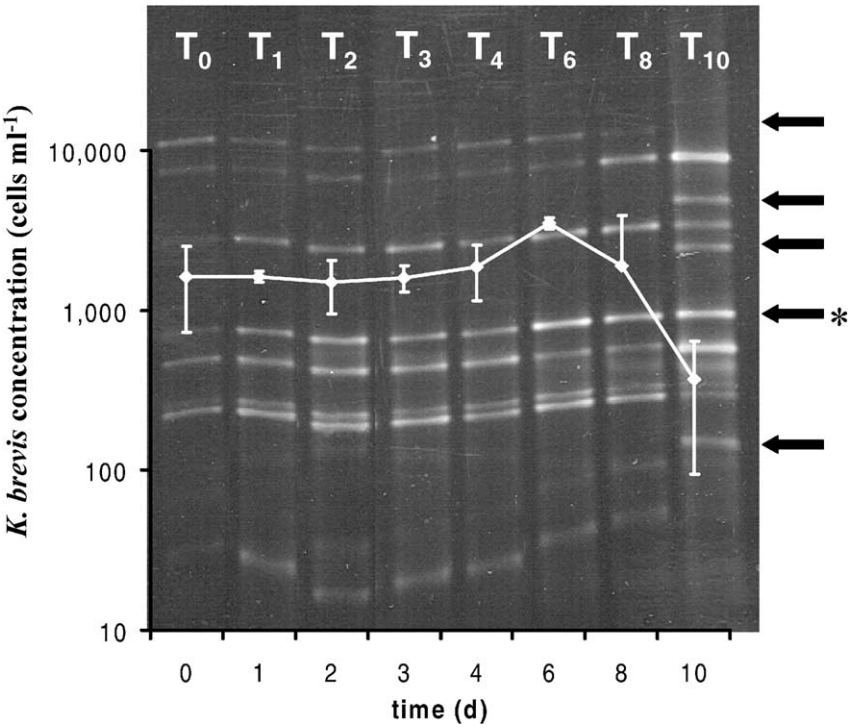

Fig. 3. DGGE profile of treatment 3 (10^5 algicidal bacteria cells per milliliter added on Day 0 at low initial algal concentrations). The gel is overlaid with algal cell numbers taken during the same time period to demonstrate that microbial community changes (between Days 8 and 10) occurred after *K. brevis* cells began to decrease (Day 8). Note the *x*-axis not to scale. Appearances and disappearances of phylotypes are labeled with arrows and the algicidal bacterium 41-DBG2 phylotype is marked with asterisk (∗).

41-DBG2, and the previously susceptible *K. brevis* C2 was rendered resistant with the addition of the bacterial community from *K. brevis* C5 (treatment 5). It should be noted that while the antibiotic-treated C2 culture was bacteria-free before the addition of 41-DBG2, the treated NOAA1 culture either had been contaminated or the antibiotic treatment had not been entirely successful in eliminating all bacteria, as there were bacteria other than 41-DBG2 present on Day 5. Nonetheless, the number of bacteria was greatly reduced (less than 41-DBG2 numbers; see Table 2 for total bacteria and algicidal bacteria concentrations for treatment 2). In addition, the community was sufficiently altered such that these bacteria did not appear to affect resistance or susceptibility to algicidal bacteria attack. To confirm that the novel resistance of the *K. brevis* clone C2 with the added NOAA1 community was not mediated by the absence of its original bacterial community, both unattached bacterial communities (C2 and NOAA1) were inoculated into the bacteria-free *K. brevis* C2 culture (treatment 8). Its subsequent resistance to algicidal attack confirmed that the presence of the unattached bacterial community from NOAA1 was indeed associated with this phenomenon. Finally, the concentration of 41-DBG2 was assessed several days after its inoculation into each treatment (Table 2). Although it was present in all treatments, its concentration was much lower (always $<10^6$ cells per milliliter) in the treatments that were rendered resistant to its algicidal activity (4, 5, 6, and 8), while concentrations were consistently $>10^6$ cells per milliliter in the susceptible cultures. Interestingly, 41-DBG2 grew to very high concentrations ($>10^{10}$ cells per milliliter) in the two treatments without algae (9 and 10), but containing their respective bacterial communities and bacterial medium DBG/5.

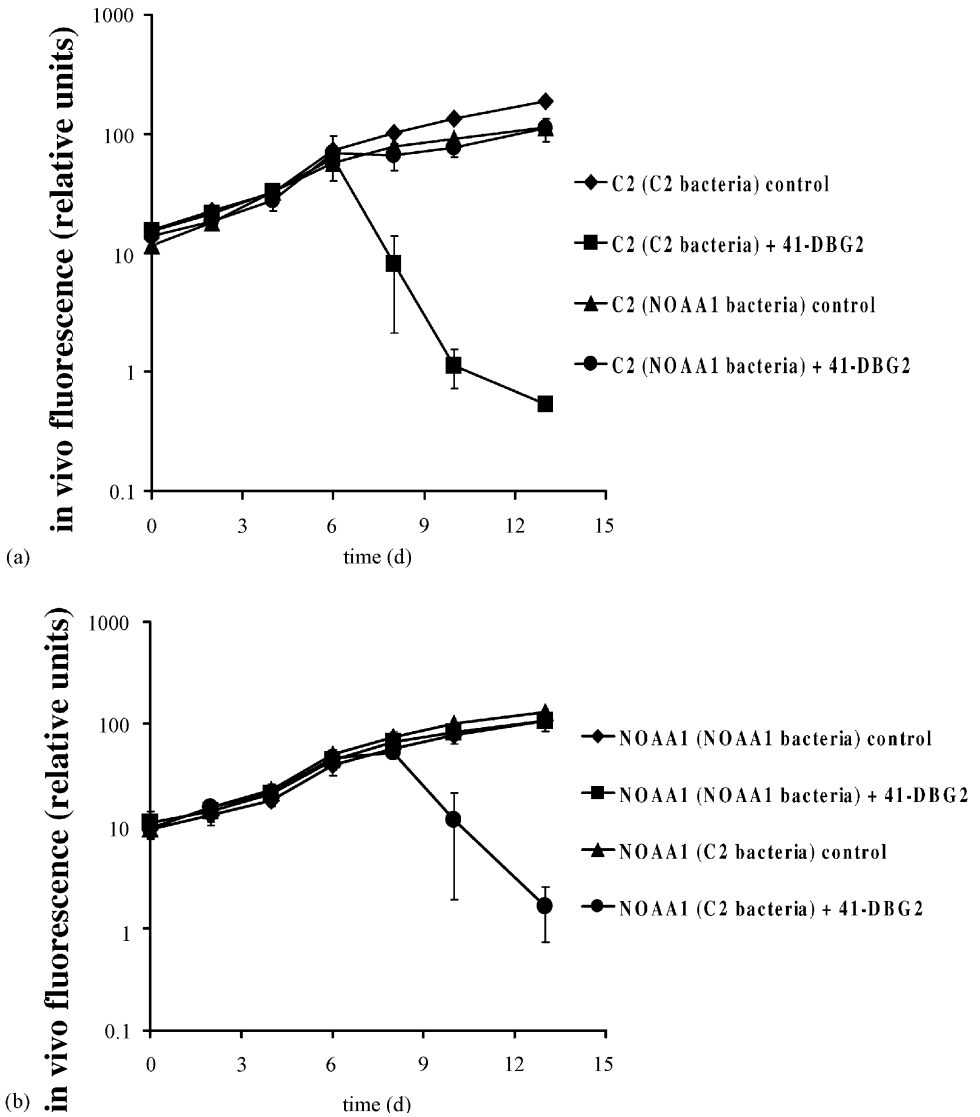

Fig. 4. Response of *K. brevis* clone C2 (a) and clone NOAA1 (b) containing one of two different bacterial communities (C2 or NOAA1 bacteria) to inoculation with algicidal bacterium 41-DBG2 at 10^5 cells per milliliter. No addition controls were also monitored for comparison.

DGGE was used to compare the microbial communities (on Day 3, before the onset of algicidal activity) from the different treatments discussed above (Fig. 5) to determine if the bacterial community switching had been achieved. DGGE profiles from treatments 3 (*K. brevis* C2 with C2 bacteria) and 7 (*K. brevis* NOAA1 with C2 bacteria) were very similar, as expected, as were those from treatments 4 (*K. brevis* C2 with NOAA1 bacteria) and 6 (*K. brevis* NOAA1 with NOAA1 bacteria). The profile from treatment 8 (*K. brevis* C2 with both C2 and NOAA1 bacteria) appeared very similar to the combined profiles of the C2 and NOAA1 bacteria on their own, with the exception of several phylotypes that were not detected. Samples from Days 0 and 5 were also analyzed (Fig. 6), this time with a narrower denaturing gradient (35–60%

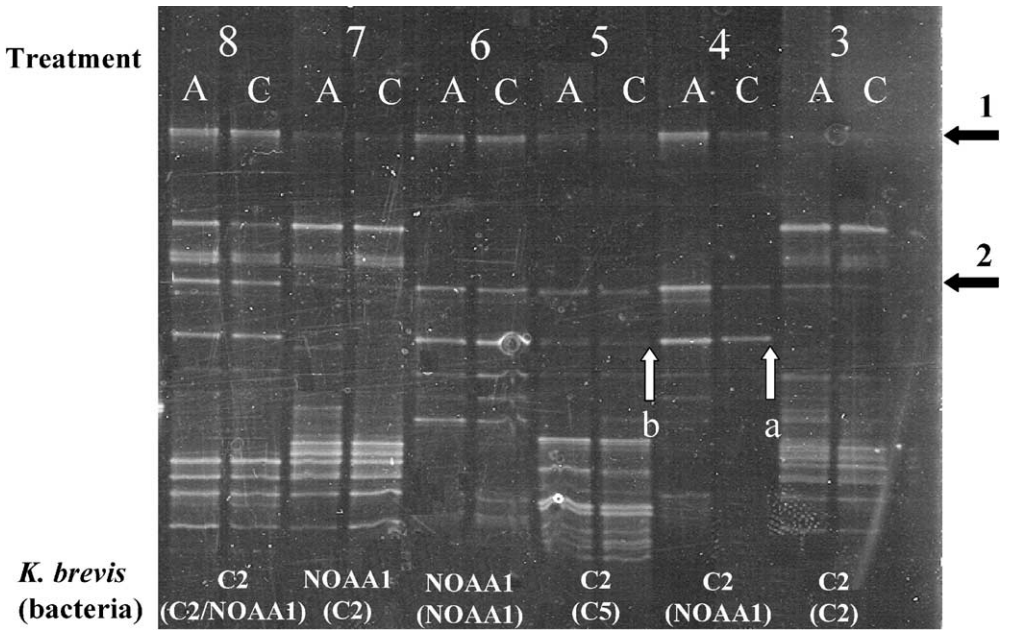

Fig. 5. DGGE profiles from the bacterial community exchange experiment (all samples taken on Day 3, prior to the onset of algicidal activity). Treatments (numbers at top of gel) are outlined in Table 2. C, no addition control; A, algicidal bacteria added on Day 0. *K. brevis* clone and origin of bacterial community (in parentheses) are indicated at the bottom of the gel. Phylotypes 1 and 2 are of dinoflagellate origin (see text). Phylotypes a (KbN1-B1) and b (KbC5-B1) were also sequenced (Table 3).

instead of 25–70%) to try to separate any phylotypes that appeared identical on the initial gel. This analysis was also necessary to compare DGGE profiles within treatments between the beginning (Day 0) and the end of the experiment (Day 5), as well as between control cultures and those into which 41-DBG2 was inoculated. Within each treatment, DGGE profiles were nearly identical between control cultures (no addition of algicidal bacteria, labeled C on Figs. 5 and 6) and those inoculated with 41-DBG2 (labeled A on Figs. 5 and 6), with the notable exception of the 41-DBG2 phylotype present in the latter treatments taken on Day 5 (labeled with asterisk (∗) in Fig. 6). The two communities grown on bacterial medium without *K. brevis* but with 41-DBG2 (treatments 9 and 10) differed considerably from their respective communities grown with *K. brevis*, although several phylotypes were shared between them (Fig. 6b).

We sequenced two dominant phylotypes (labeled 1 and 2 in Fig. 5) present in all three bacterial communities (NOAA1, C2, C5) that showed high sequence similarities to *K. brevis* chloroplast 16S rDNA (98%) and 18S nuclear rDNA (100%). Under a narrower gradient, phylotype 2 further separated into two bands (Fig. 6), demonstrating probable 18S rRNA gene heterogeneity not detected by direct sequencing (one or two bases in this sequence were scored as background noise but may have represented actual amplicons). The presence of bands of dinoflagellate origin demonstrated that the reportedly bacteria-specific primers 341F and 517R (Muyzer et al., 1993) can amplify both chloroplast and mesokaryotic (Rizzo, 1991) rDNA as well as bacterial rDNA with the PCR conditions used in this and other studies (Riemann et al., 1999).

To search for bacteria common to the NOAA1 and C5 communities but not present in the C2 community, three additional dominant phylotypes from the NOAA1 community and three from the C5 community (KbN1-B1, 2, and 3 and KbC5-B1, 2, and 3; Table 3) were sequenced. Although KbC5-B1 and KbN1-B1 initially appeared to migrate to the same spot on the gel (Fig. 5), a narrower gradient

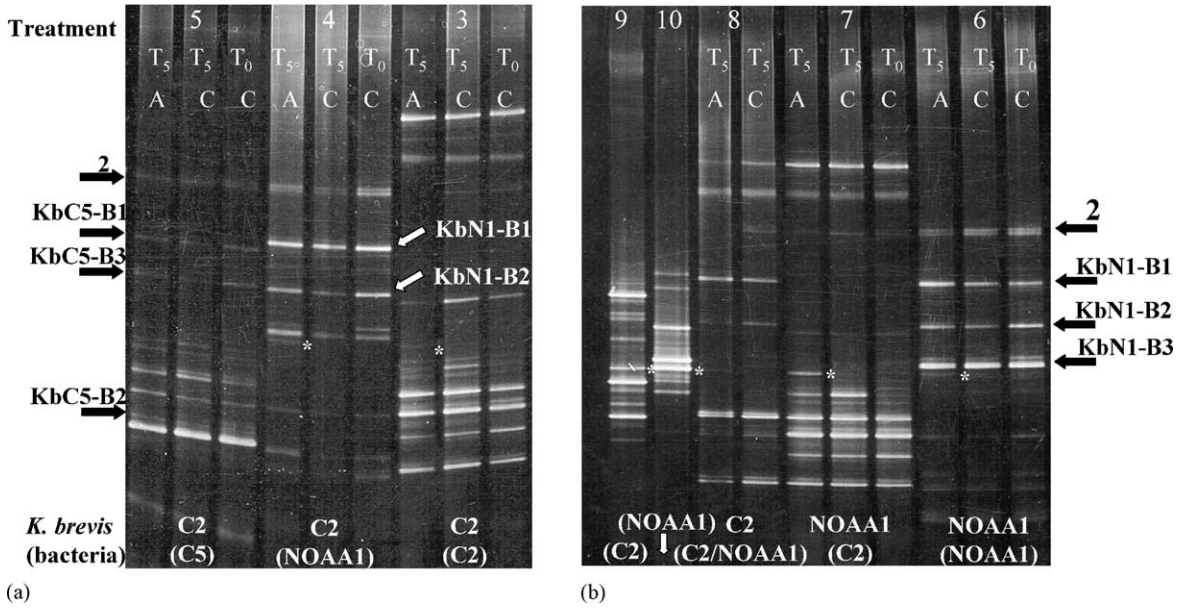

Fig. 6. DGGE profiles from the bacterial community exchange experiment (taken on Days 0 and 5), using a narrower denaturing gradient than employed in Fig. 5. Treatments (numbers at top of gel) are outlined in Table 2 and shown on either gel 'a' (treatments 3–5) or 'b' (treatments 6–10). *K. brevis* clone and origin of bacterial community (in parentheses) are indicated at the bottom of the gel. Note that treatments 9 and 10 contained no *K. brevis*. C, no addition control; A, algicidal bacteria added on Day 0. Phylotype 2 (*K. brevis* DNA) is indicated as in Fig. 5, and sequenced phylotypes (Table 3) are also marked, as is 41-DBG2 asterisk (∗).

Table 3
List of sequenced DGGE phylotypes from algicidal bacteria-resistant microbial communities associated with *K. brevis* laboratory cultures NOAA1 and C5

Phylotype	Accession no.	Closest matching organism (Accession no.)	Similarity (%)	Taxon
KbN1-B1	AF427481	Bacterium BD (Ay030101)	96	CFB
KbN1-B2	AF427484	*Methylophaga* sp. (AB049743)	96	γ-Proteo
KbN1-B3	AF427485	Uncultured marine bacterium HstpL7 (AF159649)	100	α-Proteo
KbC5-B1	AF427483	Uncultured *Microbium* BHC6 (AF090543)	94	γ-Proteo
KbC5-B2	AF427482	*Taxeobacter gelupurpurascens* (Y18836)	90	CFB
KbC5-B3	AF427480	*Marinospirillum minutulum* (AB006769)	94	γ-Proteo

separated them slightly (Fig. 6) and sequence analysis confirmed this difference. Similarly, although phylotypes KbN1-B2 and KbC5-B3 were not separated by a narrower gradient (Fig. 6), sequence analysis confirmed their origins from different bacteria. Based on this analysis, the NOAA1 and C5 bacterial communities did not share any DGGE phylotypes of bacterial origin that were not present in the C2 community.

4. Discussion

To our knowledge, this is the first study to employ molecular biology techniques to investigate algicidal bacteria population dynamics in controlled laboratory experiments utilizing non-axenic algal cultures. By taking this approach, we have uncovered critical aspects of algicidal bacteria ecology that otherwise would not have been detected, such as intra-bacterial

community interactions and how strain 41-DBG2 behaves in a mixed microbial assemblage. Moreover, based on the close phylogenetic affinity of 41-DBG2 to marine algicidal bacteria active against various HAB species (Mayali, 2001), our findings for *K. brevis* from the Gulf of Mexico examined herein may reveal insights into factors regulating the population dynamics and killing activity common to many algicidal bacteria.

4.1. Population dynamics

Our data indicate that algicidal bacterium 41-DBG2 must attain a threshold concentration of at least 10^6 cells per milliliter before any algicidal activity is detectable. Similar results have been found with algicidal bacteria strains J18/M01 (Imai et al., 1993), A5Y (Mitsutani et al., 1992), and 5N-3 (Fukami et al., 1992), members of the same phylum as 41-DBG2 (the Cytophaga–Flavobacterium complex). This pattern suggests that concentration-dependent signaling, such as quorum sensing (Fuqua et al., 1994), may be involved in initiating algicidal activity in this group of bacteria. If quorum sensing is not involved, the threshold concentration may simply be the point at which algicidal bacteria reach an effective killing density or become growth-limited by the algal-derived dissolved organic matter (DOM) in the medium, initiating the active lysis of algal cells as a means to increase their nutrient supply. It is also possible that algal cells may be negatively affected without actually lysing, prior to 41-DBG2 reaching its threshold concentration. Data from the bacterial community exchange experiments (Table 2) revealing that all cultures resistant to algicidal bacteria attack contained concentrations $<10^6$ 41-DBG2 cells per milliliter, while all susceptible cultures exhibited concentrations $>10^6$ 41-DBG2 cells per milliliter, are consistent with the some type of concentration-based mechanism.

Interestingly, the threshold killing concentration of 41-DBG2 is not attained when *K. brevis* cell concentrations are low, in which case the onset of algicidal activity is delayed until the algal culture has grown to higher numbers (Fig. 1b–c). This delay may reflect a requirement for more algal cells and their associated DOM, or perhaps for the succession of cultures from early to late exponential growth phase, accompanied by changes in algal physiology and possibly enhanced susceptibility to algicidal attack. In either case, it strongly suggests that without organic nutrients in the culture medium, 41-DBG2 needs DOM released by *K. brevis* to grow to ecologically relevant numbers. On the other hand, although it does not grow to very high concentrations on algal growth medium containing primarily inorganic nutrients (data not shown), 41-DBG2 can attain concentrations greater than 10^6 cells per milliliter in both bacterial growth medium and in axenic cultures of species not affected by its algicidal properties, such as *Akashiwo sanguinea* (formerly *G. sanguineum*; data not shown). The latter outcome raises thus far unanswered questions about the mechanisms of resistance of unaffected algal species, which will be addressed in future work. These results imply that 41-DBG2 is not an obligate algal predator but can grow on many different kinds of organic substrates, similar to closely related algicidal bacteria strains A5Y and J18/M01 (Mitsutani et al., 1992; Imai et al., 1993). Furthermore, this information is consistent with 41-DBG2's origin from waters with no detectable *K. brevis* cells (Doucette et al., 1999), indicating that it may be ubiquitous in west Florida shelf waters and its algicidal activity triggered only when susceptible target algal cells are present in high numbers.

The DGGE profiles from this set of experiments demonstrated that the microbial community structure associated with *K. brevis* clone C2 did not change immediately after algicidal bacterium 41-DBG2 was added, but rather after algal cells began to lyse and introduce an additional source of organic nutrients into the culture medium. Alternatively, it may be that changes in the microbial community are a direct result of strain 41-DBG2 reaching 10^6 cells per milliliter and itself exerting effects on co-occurring bacteria. Data from the bacterial exchange experiment revealing both high 41-DBG2 concentrations prior to cell lysis in susceptible cultures (Table 3) and no microbial community change up to that point (Fig. 6) are consistent with the former suggestion. These results lead us to formulate the hypothesis that following its inoculation into a *K. brevis* clone C2 culture, algicidal bacterium 41-DBG2 does not directly compete with nor displace dominant members of the bacterial community, but rather fills a vacant niche. Following its establishment and growth to the threshold concentration necessary for initiation of algicidal

activity, the bacterial community structure does change, likely due to the different nutrient regime resulting from algal cell lysis and the subsequent release of algal-derived DOM. Bacteria adapted for a dilute nutrient system (Bell, 1983) may be outcompeted by those adapted to a more concentrated nutrient system, and any bacteria that maintain a symbiotic relationship with algae, and are thus nutritionally dependent upon living algal cells (Mouget et al., 1995), would also decline to background levels. Results from our short-term laboratory experiments parallel bacterial community changes over the course of natural phytoplankton blooms established by past studies (e.g., Romalde et al., 1990). Interestingly, a more recent study has suggested that *Cytophaga*-like bacteria play a significant role in the decomposition of DOM during a dinoflagellate bloom (Fandino et al., 2001). It remains to be determined whether some of these *Cytophaga*-like bacteria directly contribute to algal mortality in natural bloom systems, as did *Cytophaga* strain 41-DBG2 in our laboratory experiments.

4.2. *Effect of ambient microbial flora*

DGGE analyses from the bacterial community exchange experiments demonstrated that different *K. brevis* clones inoculated with the same unattached bacterial community exhibited nearly identical profiles, confirming that these communities were successfully exchanged. Since we sampled cultures prior to complete algal death, DGGE profiles within treatments were identical at different time points, corroborating the results from the previous population dynamics experiment that no microbial community changes occur before the killing of the algae. The most intriguing result from this set of experiments, however, involved the inhibition of algicidal activity mediated by bacteria from the NOAA1 and C5 communities. To our knowledge, only one previous study has mentioned such an inhibition of algicidal activity (Imai et al., 1998a), its cause suggested to be either viral or bacterial in nature. In our case, we found evidence challenging the possibility that viruses were responsible for the algal resistance to algicidal attack based on the lack of algicidal activity inhibition by $<0.22\ \mu m$ filtrate from the NOAA1 community (data not shown). We have formulated two hypotheses that could explain algicidal activity inhibition due to the apparent inability of 41-DBG2 to reach its threshold concentration of 10^6 cells per milliliter. One (or more) bacterial strain from the resistant (C5 and NOAA1) cultures is either directly inhibiting the growth of 41-DBG2 or indirectly competing with it for nutrients (disruptive vs. interference competition). At this point sufficient evidence to reject either hypothesis is lacking, but a recent study documenting antagonistic interactions among marine bacteria (Long and Azam, 2001) suggests that antibiotic-mediated inhibition may be a widespread strategy in these ecosystems. We believe that bacteria responsible for the inhibition of algicidal activity are currently non-culturable on bacterial growth medium, based on the similar growth exhibited by 41-DBG2 in treatments 9 and 10 (41-DBG2 + C2 bacteria and 41-DBG2 + NOAA1 bacteria, respectively, both in bacterial broth medium). Due to some differences in the DGGE profiles exhibited by samples from this treatment in comparison to those from cultures growing with *K. brevis* (Fig. 6), we concluded that some bacteria from the NOAA1 community cannot be cultured on our bacterial growth medium. DOM is not limiting to bacterial growth in standard bacterial medium whereas it may be in *K. brevis* cultures, meaning that bacterial competition for DOM is probably not occurring in the former. In addition, high nutrient concentrations in the bacterial medium may be suppressing the growth of bacteria adapted to a low nutrient environment. Inhibition of algicidal activity was not observed in a preliminary experiment employing six bacterial strains from the NOAA1 culture isolated on agar plates of the same medium (DBG/5) and subsequently co-cultured with *K. brevis* clone C2 and 41-DBG2 in *f*/2 (data not shown). Based on these results, we determined that the examination of the unculturable bacteria from the unattached bacterial communities able to transmit resistance to algicidal bacteria attack (from *K. brevis* clones NOAA1 and C5) was the next logical step.

The DGGE profiles from the NOAA1, C5, and C2 communities exhibited two phylotypes in common (labeled 1 and 2 on Fig. 5), but subsequent sequence analysis of the two NOAA1 phylotypes revealed them to be of dinoflagellate origin (both chloroplast and nuclear rDNA). Future researchers utilizing these DGGE primers must take this cross-reactivity into account and further optimization of PCR conditions or the re-design of these primers may be necessary. Based on

information from the six other sequenced phylotypes, the C5 and NOAA1 communities associated with resistance to 41-DBG2 algicidal activity were both comprised of members of the γ-proteobacteria (phylotypes KbN1-B2, KbC5-B1, and KbC5-B3) and members of the CFB (phylotypes KbN1-B1 and KbC5-B2). The latter could be competing with 41-DBG2 for nutrients, since they belong to the same major phylogenetic group and may have similar metabolic properties. An exhaustive search for CFB bacteria in the C2 community (susceptible to algicidal bacteria attack) revealing the absence of such bacteria could lend support to this hypothesis. Interestingly, phylotype KbN1-B3 from the NOAA1 community matched 100% with the sequence from an α-proteobacterium found along with diatoms on the leaves of the seagrass *Halophila stipulacea* (Weidner et al., 2000). Our finding of this bacterium in a *K. brevis* culture suggests that it may sustain a symbiotic or commensal relationship with several different marine phytoplankton groups, if not also with marine macrophytes.

5. Conclusions

Based on our findings, it is now clear that intra-bacterial community interactions should be incorporated into any study of bacterial–algal dynamics, whether in the laboratory or under natural bloom conditions. *K. brevis* bloom dynamics have been divided into four stages (Steidinger et al., 1998) and a conceptual model that included potential algicidal bacteria effects on these dynamics was previously described (Doucette et al., 1999). We can now further illustrate the possible progression of a *K. brevis* bloom, incorporating both algicidal bacteria effects on algal dynamics, as well as intra-bacterial community interactions consistent with our laboratory findings, which will serve as a testable hypothesis for future field studies. During bloom initiation, algal cells occur at low concentrations and if algicidal bacteria are present in low numbers, they cannot grow to high levels due to the minimal amount of algal-derived DOM in the water. Over the ensuing growth and maintenance phases of the bloom, algal cells increase in concentration, but the microbial community may comprise bacteria that can successfully compete with and thus impede the growth of algicidal bacteria. At some point during the maintenance phase, these inhibitors of algicidal bacteria growth may disappear or be outcompeted by algicidal bacteria, possibly due to changes in algal-derived DOM quantity and/or quality, enabling the latter to reach their threshold concentration necessary to trigger killing activity and algal cell lysis. The further release of DOM from the lysed algal cells would, in turn, continue to promote the growth and activity of the algicidal bacteria, contributing to the decline of the bloom.

Future research aimed at testing this hypothesis in the field will require the sampling of bacterial communities from at least two distinct bloom stages, preferably maintenance and termination, as well as the isolation of algicidal bacteria from the same bloom. Laboratory experiments utilizing axenic *K. brevis* cultures and algicidal bacteria can then be used to quantify the concentrations of both algicidal bacteria and their inhibitors from the sampled bacterial communities. If this hypothesis is valid, we can speculate on the bloom phase origin for the *K. brevis* cultures used in this study. The culture susceptible to algicidal bacterium 41-DBG2 (isolate C2) may have been isolated from the termination stage of a bloom (algicidal bacteria inhibitors are not present), whereas the cultures resistant to 41-DBG2 (isolates NOAA1 and C5) may originate from a growth or maintenance bloom phase (algicidal bacteria inhibitors are present). Although our conceptual model is inherently simplistic, we feel that it may add significant information to the overall knowledge of HAB ecology if future field studies provide it empirical support. It may also help to explain why algicidal bacteria are at times found in low numbers in certain blooms (Imai et al., 1998b) or in early bloom stages (Fukami et al., 1991; Yoshinaga et al., 1995; Kim et al., 1998).

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