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Variations in paralytic shellfish toxin and homolog production in two strains of *Alexandrium tamarense* after antibiotic treatments

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ABSTRACT: The production and composition of the paralytic shellfish toxins (PSTs) of 2 *Alexandrium tamarense* strains (CI01 and HK9301) in the presence and absence of bacteria were investigated. Attempts were made to produce bacteria-free dinoflagellate cultures using 2 antibiotic treatments. Antibiotic treatment 1 (penicillin-G and streptomycin) partially killed bacteria in 2 dinoflagellate cultures, while antibiotic treatment 2 (penicillin-G, streptomycin, ciprofloxacin, and gentamicin) completely killed bacteria in the 2 dinoflagellate cultures. The toxin production (total toxin yields and cellular toxin content) of the *A. tamarense* CI01 strain increased after antibiotic treatment 1, but decreased after antibiotic treatment 2. Except for the cellular toxin content after mid-stationary phase, the toxin production of the *A. tamarense* HK9301 strain after both antibiotic treatments was higher than that of the control cultures. Over the different growth phases, the toxin composition of the *A. tamarense* HK9301 strain changed when the bacteria were killed by antibiotics, while that of *A. tamarense* CI01 strain remained unchanged. Our findings suggest that the toxins from both strains are produced by the dinoflagellate cells and not by the bacteria associated with them. However, the increase in growth rates and cell density of the 2 dinoflagellates after partial and complete destruction of the bacteria from the cultures implies that bacteria can affect the level of dinoflagellate toxin production by nutrient competition. The reduction of toxin production by the axenic *A. tamarense* CI01 strain and the change in toxin composition of the *A. tamarense* HK9301 strain after antibiotic treatment indicate that bacteria could also affect dinoflagellate toxicity through different mechanisms other than nutrient competition. These potential effects need to be further investigated.

KEY WORDS: *Alexandrium tamarense* · Paralytic shellfish toxin · Bacteria · Antibiotic · Toxin homologs

INTRODUCTION

Dinoflagellates of the genus *Alexandrium* are one of the major groups that cause paralytic shellfish poisoning (PSP) along the coast of China. Several species of *Alexandrium* produce a variety of paralytic shellfish toxins (PSTs), which vary greatly in their toxicity. The cellular concentration of toxins and the combination of PSTs determine the toxicity of these dinoflagellates. The variability in the toxin content of dinoflagellates under different growth conditions has generally been accepted as a major factor contributing to the dramatic change in the toxicity of dinoflagellates among different strains of the same species and/or within the same strain. The toxin composition of dinoflagellates, however, was reported to be relatively stable under varying growth conditions in previous studies (Boyer et al. 1987, Ogata et al. 1987, Kim et al. 1993, Oshima et al. 1993, Sako et al. 1995, Parkhill & Cembella 1999) but were found to vary in the other studies (Boczar et al. 1988, Anderson et al. 1990a, Wang et al. 2005).

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Several research groups investigated the effects of bacteria on dinoflagellate toxicity by killing the dinoflagellate-associated microbiota in the cultures using antibiotic treatments (Gonzalez et al. 1992, Dantzer & Levin 1997, Lu et al. 2000, Hold et al. 2001). The effect of antibiotic treatments on dinoflagellate toxicity varies greatly from no effect to complete loss of algal toxicity. Antibiotic-treated dinoflagellate cultures were reported to produce the same levels of PSTs as those produced by cultures without antibiotic treatments (Dantzer & Levin 1997, Lu et al. 2000). These studies suggested that the dinoflagellate-associated bacteria are not involved in the toxin production in the dinoflagellate cultures. In contrast, Hold et al. (2001) and Gonzalez et al. (1992) reported that the production of PSTs in dinoflagellate cultures increased or decreased after antibiotic treatments, and suggested that the dinoflagellate-associated bacteria could influence toxin production in dinoflagellate cultures. As an extreme case, a marine PST-producing alga (*Alexandrium lusitanicum*) completely lost its ability to produce toxins (Martins et al. 2004). While the effects of bacteria on toxin production are well studied, there are few studies on the effects of bacteria on the toxin composition produced by dinoflagellates.

In this study, the toxin production and composition of 2 local strains (CI01 and HK9301) of the dinoflagellate *Alexandrium tamarense* in the presence and absence of bacteria were investigated. Two antibiotic treatments were applied to develop axenic (bacteria-free) dinoflagellate cultures. Dinoflagellate growth, toxin production, and composition of antibiotic-treated dinoflagellate cultures were compared to those in the control cultures to elucidate the bacterial impacts on toxin production and stability of toxin composition produced by the dinoflagellate cultures.

MATERIALS AND METHODS

Dinoflagellate cultures. The dinoflagellates *Alexandrium tamarense* CI01 (ATCI01) and *A. tamarense* HK9301 (ATHK9301) were isolated from Dapeng Bay, in the northwest coastal region of the South China Sea. Strain ATCI01 produces predominately C2 toxin (C2) with trace amounts of C1 toxin (C1), while strain ATHK9301 produces a variety of PSTs, including C1, C2 and Gonyautoxin (GTX) 1 to 5. The algae were grown in 150 ml of natural seawater supplemented with K medium (Keller et al. 1987) without silica in 250 ml conical flasks. The stationary cultures were maintained at 23.5°C on a 14:10 h light:dark cycle at a light intensity of 100 µmol quanta m^{-2} s^{-1} provided by cool white fluorescent tubes.

Antibiotic treatments. Combinations of the 4 antibiotics penicillin-G (Sigma), streptomycin (Sigma), gentamicin (ICN Biomedicals), and ciprofloxacin (ICN Biomedicals) were used to form 2 antibiotic mixtures. These mixtures were added to the dinoflagellate cultures 4 times: at the beginning of the experiments (Day 0) and then on Days 3, 6 and 9, respectively. The amount of antibiotic mixture added each time was the same. In antibiotic treatment 1, algal cultures were supplemented with a mixture of penicillin-G and streptomycin in accordance with Wang et al. (2004). The final concentrations of penicillin-G and streptomycin in the medium were 60 and 134 µg ml^{-1}, respectively. In antibiotic treatment 2, algal cultures were supplemented with a mixture of penicillin-G, streptomycin, gentamicin, and ciprofloxacin in accordance with Hold et al. (2001). The final concentrations of penicillin-G, streptomycin, gentamicin, and ciprofloxacin were 25, 25, 240, and 46 µg ml^{-1}, respectively. Both mixtures were used in the 2 studies to produce axenic algal cultures. The stocks of antibiotics were prepared with Milli-Q water, which was filtered through 0.22 µm membrane filter and stored at –20°C. To prevent fungal growth, amphotericin B (Gibco) at a final concentration of 46 µg ml^{-1} was added to the antibiotic-treated dinoflagellate cultures. The dinoflagellate cultures (15 ml) with and without antibiotic treatment were harvested on Day 12, centrifuged (3600 × *g*, 5 min), washed with autoclaved seawater, and inoculated into 150 ml of sterile K medium. Seven replicates were prepared for each type of the dinoflagellate culture: 2 replicates were used to assess the axenic state of the dinoflagellate cultures, while the remainder were used to determine the dinoflagellate toxicity. All cultures were kept under the same conditions as previously described.

Assessment of the axenic status of antibiotic-treated dinoflagellate cultures. Samples (1 ml each) were taken on Day 6 (mid-exponential phase [MEP]), Day 12 (early stationary phase [ESP]), Day 21 (mid-stationary phase [MSP]), and Day 30 (late stationary phase [LSP]) from dinoflagellate cultures with and without antibiotic treatment and inoculated onto marine agar (MA) 2216 (Difco) plates and into tubes containing 9 ml of the liquid media marine broth (MB) 2216, yeast extract and peptone (YEP) medium (3 g of yeast extract and 5 g of peptone in 1 l of seawater), and YEP medium supplemented with dinoflagellate cell extracts (1.0×10^7 cells in 1 l of dinoflagellates were harvested and lysed). The inoculated MA plates and liquid media were incubated at 23.5°C for 14 d. Continuous shaking (120 rpm) was applied to the inoculated liquid media. In addition, a 1 ml sample was taken and stained with 4,6-diamidino-2-phenylindole (DAPI) (Roche Diagnostics) solution (final concentration = 5 µg ml^{-1}) and sub-

sequently incubated in the dark at room temperature (ca. 23.5°C) for 10 min. The stained dinoflagellate culture was then filtered onto a black polycarbonate 0.22 mm membrane (Osmonics), and photographed under a fluorescent microscope equipped with a camera.

Dinoflagellate growth curves. Samples (1 ml) were taken from dinoflagellate cultures every 3 d and fixed in Lugol's solution and stored at room temperature. They were then diluted in autoclaved seawater and 100 µl of properly diluted samples were transferred into the wells of a 96 well microtitre plate (Corning). In each well, the number of dinoflagellate cells with an intact cell wall was determined by direct count under a light microscope. The growth rates of the dinoflagellates were calculated as described by Wang et al. (2002).

Dinoflagellate toxin production. Dinoflagellate cells in 1 ml samples were harvested by centrifugation at 3600 × *g* for 5 min at room temperature and were resuspended in 0.5 ml of 50 mM acetic acid. Cells were disrupted by freezing and thawing, followed by sonication on ice. The extracts were then centrifuged at 10 000 × *g* for 5 min at 4°C and the supernatants were subjected to toxin analysis.

Toxin analysis was performed using a Hewlett-Packard HP1100 high performance liquid chromatography-fluorescence detection system (HPLC-FLD) as described by Wang et al. (2002). HPLC analytical standards of GTXs were purchased from the National Research Council (NRC) of Canada. Since C1 and C2 standards were not commercially available, we used ATCI01 to produce mg quantities of C1 and C2 toxins (Wang et al. 2002). Toxins were purified using gel filtration columns (Bio-Gel P2, BioRad Laboratories, ~45–90 µm) and a reverse-phase C18 column (125 Å) (Waters Corporation). The purity and concentration were determined using HPLC and Nuclear Magnetic Resonance (NMR) spectroscopy.

The HPLC method used in this study followed that described by Oshima (1995), which was modified by Anderson et al. (1996) for better separation and higher sensitivity of C toxins. By optimizing reaction conditions, the detection limit of PSTs attained by HPLC can be as low as 17 to 110 fmol (Oshima 1995). In our laboratory, similar detection limits, i.e. ca. 10 to 230 fmol of toxin standards, can be achieved. The lowest C2 cellular content of ATCI01 is about 20 fmol cell^{-1} and the lowest cellular PSTs of ATHK9301 are: 0.15 (GTX2/3) to 20 (GTX1/4) fmol cell^{-1}. Therefore, the total amount of toxin in the samples with lowest cell density (~1000 cells ml^{-1}) is sufficiently high to produce sharp and clear peaks and gain good results. Briefly, 2 mobile phases (G- and C-buffers) were used for the separation of GTXs and C toxins, respectively. The C-buffer was made from 2 mM tetrabutylammonium phosphate (Sigma) adjusted to pH 5.8. The G-buffer was made from 2 mM sodium 1-heptanesulfonate (Fluka) and 30 mM ammonium phosphate (pH 7.1). In the post column reaction system (PCRS), the column eluate was mixed with an oxidizing solution made from 7 mM periodic acid (Sigma) in 50 mM potassium phosphate buffer (pH 11.0), and then mixed with 0.5 M acetic acid. The temperatures of the post column reaction for C toxins and for GTXs were 45 and 85°C, respectively. The flow rates for the column separation and PCRS were adjusted to 0.8 and 0.4 ml min^{-1}, respectively. Five µl of each sample was injected into the HPLC by the auto-sampler.

Statistical analyses. For each treatment experiment, cell density, toxin yield, cellular toxin content, and toxin composition were measured. Data from Days 6, 12, 21 and 30 corresponding to MEP, ESP, MSP, and LSP were analyzed using 1-way ANOVA followed by Tukey's multiple comparison tests (Tukey's HSD) to assess the effects of antibiotic treatments on dinoflagellate cell growth and toxicity. For toxin composition of ATHK9301 cultures, 1-way ANOVA and Tukey's HSD were also used to check the changes in PST homologs over different growth phases.

RESULTS

Assessment of residual bacteria

The results of bacterial counts indicate that antibiotic treatment 1 killed 60 to 98% of the cultivable bacteria in both the ATCI01 and ATHK9301 cultures (Table 1). Bacterial growth, however, gradually resumed in the absence of antibiotics. No bacterial growth was detected by either the plating method or liquid media inoculation in the ATCI01 and ATHK9301 cultures with antibiotic treatment 2 (Table 1). Using epifluorescence microscopy, bacteria were still observed in the dinoflagellate cultures after antibiotic treatment 1, but not after antibiotic treatment 2 (Figs. 1 & 2). These results further confirmed that the dinoflagellate cultures were axenic after antibiotic treatment 2.

Dinoflagellate growth

The growth rates in log phase with ATCI01 and ATHK9301 cultures increased after antibiotic treatments (Table 2). All the dinoflagellate cultures grew exponentially and reached the highest growth rate on Day 6. Thereafter, cell division in the control culture ceased and entered a distinct stationary phase, while

Table 1. Assessment of residual bacteria in cultures of *Alexandrium tamarense* CI01 and HK9301 (ATCI01 and ATHK9301) by cultivating algal samples in marine agar 2216 (MA), marine broth 2216 (MB), medium containing yeast extract and peptone (YEP), and YEP medium supplemented with algal extracts. Total bacterial count: $\times 10^6$ colony forming units ml^{-1}; % of bacteria removed by antibiotic treatments = (bacterial count from control culture – bacterial count from antibiotic treated culture)/bacterial count from control culture ×100. +: bacterial growth observed; –: bacterial growth not observed; MEP: mid-exponential phase (Day 6); ESP: early stationary phase (Day 12); MSP: mid-stationary phase (Day 21); LSP: late stationary phase (Day 30)

Culture	Sample	ATCI01				ATHK9301			
		MA	MB	YEP	YEP + ATCI01 extract	MA	MB	YEP	YEP + ATHK9301 extract
Control									
	MEP	0.88	+	+	+	0.56	+	+	+
	ESP	2.76	+	+	+	2.16	+	+	+
	MSP	5.47	+	+	+	4.73	+	+	+
	LSP	5.32	+	+	+	2.98	+	+	+
Antibiotic treatment 1									
	MEP	0.02 (98%)	+	+	+	0.001 (99.8%)	+	–	–
	ESP	0.26 (91%)	+	+	+	0.09 (96%)	+	–	–
	MSP	0.89 (84%)	+	+	+	0.27 (94%)	+	+	+
	LSP	2.11 (60%)	+	+	+	0.96 (68%)	+	+	+
Antibiotic treatment 2									
	MEP	0 (100%)	–	–	–	0 (100%)	–	–	–
	ESP	0 (100%)	–	–	–	0 (100%)	–	–	–
	MSP	0 (100%)	–	–	–	0 (100%)	–	–	–
	LSP	0 (100%)	–	–	–	0 (100%)	–	–	–

cell division in all cultures after antibiotic treatments were extended for 6 to 9 d before entering a stationary phase.

The cell density in ATCI01 and ATHK9301 cultures after antibiotic treatments was clearly higher than that of the control culture throughout the growth curve (Fig. 3). One-way ANOVA showed, in general, that there were significant differences between the control cultures of both algal strains and cultures after 2 different antibiotic treatments, in different growth phases (Table 3). A significant difference in cell density was also observed between antibiotic treatments 1 and 2 in cultures of strain ATHK9301 but not with those of strain ATCI01 (Table 3). Compared to the control culture, cell density of ATCI01 cultures increased by 14 to 107 and 24 to 153% with antibiotic treatments 1 and 2, respectively (Table 2). Similarly, the cell density in the ATHK9301 cultures increased by –4 to 52 and 52 to 79% in antibiotic treatments 1 and 2, respectively (Table 2). These results showed that the destruction of bacteria by antibiotics increased the growth rates and cell density in the dinoflagellate cultures.

Dinoflagellate toxin production

The highest toxin production in all cultures of ATCI01 was observed in the MSP or LSP (Fig. 4). With antibiotic treatment 1, the total toxin yields of ATCI01 in stationary phases (ESP, MSP and LSP) were 53 to 70% higher than those of the control cultures (Table 2). The cellular toxin content in the ESP and MSP was also higher (26 to 37%) than in the control cultures. However, the cellular toxin content decreased by 19% compared to the control cultures in LSP (Table 2). With antibiotic treatment 2, except for the total toxin yields on Day 30, the total toxin yields and cellular toxin content in the whole stationary phase were 35 to 42 and 46 to 60% lower than the control cultures (Table 2).

Compared to ATCI01, ATHK9301 produced a greater variety of PSTs, which were mostly produced in the ESP or LSP (Figs. 5 & 6). The total toxin yields and cellular toxin content in ATHK9301 cultures after antibiotic treatment 1 were higher than those of the control cultures (39 to 140 and 37 to 57%) throughout the growth phases except for LSP (Table 2). In antibiotic treatment 2,

the total toxin yields were 43 to 120 % higher than those of the control cultures in the growth stages between MEP and the MSP (Table 2). Thereafter, they dropped to a level lower than those of the control cultures in LSP. The cellular toxin content was higher (16 to 23 %) than that of the control group in the MEP and ESP, but lower (15 to 49 %) than the control cultures thereafter (Table 2). Except in LSP, a significant difference could be observed between control and antibiotic-treated or between antibiotic treatments 1 and 2 of ATCI01 and ATHK9301 cultures throughout the growth phases (Table 3).

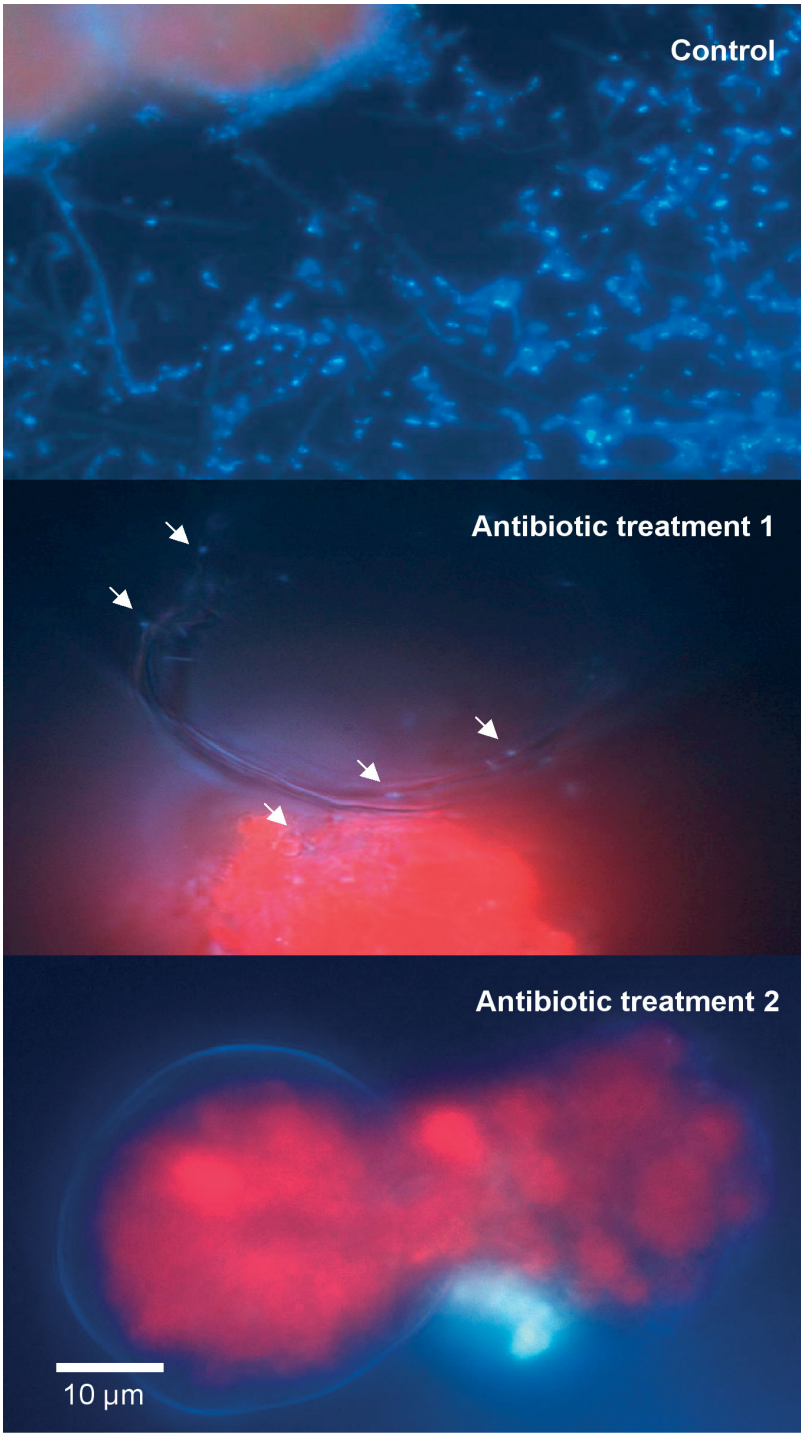

Fig. 1. *Alexandrium tamarense*. Assessment of residual bacteria in the ATCI01 cultures with and without antibiotic treatments by fluorescent microscopy. The axenic nature of the dinoflagellate cultures was checked by direct microscopic observation of the dinoflagellate samples stained with DAPI. Arrows indicate the presence of bacterial cells

Dinoflagellate toxin composition

Two PSTs (C2 at a high level and its epimer derivative C1 at a trace level) were detected in all ATCI01 cultures throughout the growth phases. C1 and C2 made up 100 % of the total toxin produced by ATCI01 with and without antibiotic treatments. This indicates that the identity of C1 and C2 in the ATCI01 cultures did not change with the presence or absence of bacteria.

Seven PSTs including C1/C2, GTX1/4, GTX5 and a trace amount of GTX2/3 (~1 mol % of total toxin) were detected in all ATHK9301 cultures. The mole percentage of toxins in the ATHK9301 cultures significantly changed with different growth phases (Fig. 7, Table 4). A significant difference was observed between the control and 2 antibiotic-treated cultures but not between antibiotic treatment 1 and antibiotic treatment 2 (Fig. 7, Table 4). In all cultures, the mole percentage of GTX1/4 increased gradually while that of C1/C2, GTX5 and GTX2/3 decreased as the cultures aged. The mole percentage of C1/C2 increased from 36 to 50 and 48 % in ESP, and from 18 to 31 and 32 % in LSP after antibiotic treatments 1 and 2. In contrast, the mole percentage of GTX1/4 decreased from 64 to 44 and 45 % in ESP and from 71 to 63 and 62 % in LSP. A slight difference was observed in the mole percentages of GTX5 but not in GTX2/3 (Table 4).

DISCUSSION

The antibiotic mixture in antibiotic treatment 1 containing penicillin-G

and streptomycin killed over 90% of the cultivable bacteria in the dinoflagellate cultures. Bacterial growth was temporarily suppressed, but resumed in the dinoflagellate cultures without the presence of antibiotics. Antibiotic treatment 2 was more efficient in killing bacteria from the dinoflagellate cultures. In fact, this antibiotic mixture (penicillin-G, streptomycin ciprofloxacin and gentamicin) was efficient in killing Gram-negative alpha- and gamma-proteobacteria in dinoflagellate cultures (Hold et al. 2001). No bacterial growth was detected by the plating method in the ATCI01 and ATHK9301 cultures, indicating that the bacterial cell density in the dinoflagellate culture was below the detection limit (10 colony forming units ml^{-1}). Since >95% of marine bacteria may be unculturable (Gallacher & Smith 1999), in this study, the residual bacteria in the antibiotic-treated dinoflagellate cultures were further assessed using a combination of culture-dependent methods (growth in 3 liquid media) and fluorescent microscopy. Therefore, the possibility of detecting residual bacteria in the antibiotic-treated dinoflagellate cultures was increased. No residual bacteria were detected in the ATCI01 and ATHK9301 cultures using these methods. It is possible that there may have been some unculturable bacteria which lived inside the dinoflagellate cells and which survived antibiotic treatment 2, but which could not be detected using fluorescent microscopy. The cell density of the residual unculturable bacteria, if any, should be very low. Therefore the ATCI01 and ATHK9301 cultures in antibiotic treatment 2 could be considered as axenic cultures. To remove the antibiotics, the inocula of experimental dinoflagellate cultures were centrifuged and washed with autoclaved seawater. Although this may cause some stress to the algal cells, both control and antibiotic-treated inocula were subjected to the same stress. The observed variations in growth and toxin production should simply be caused by the axenic or non-axenic quality.

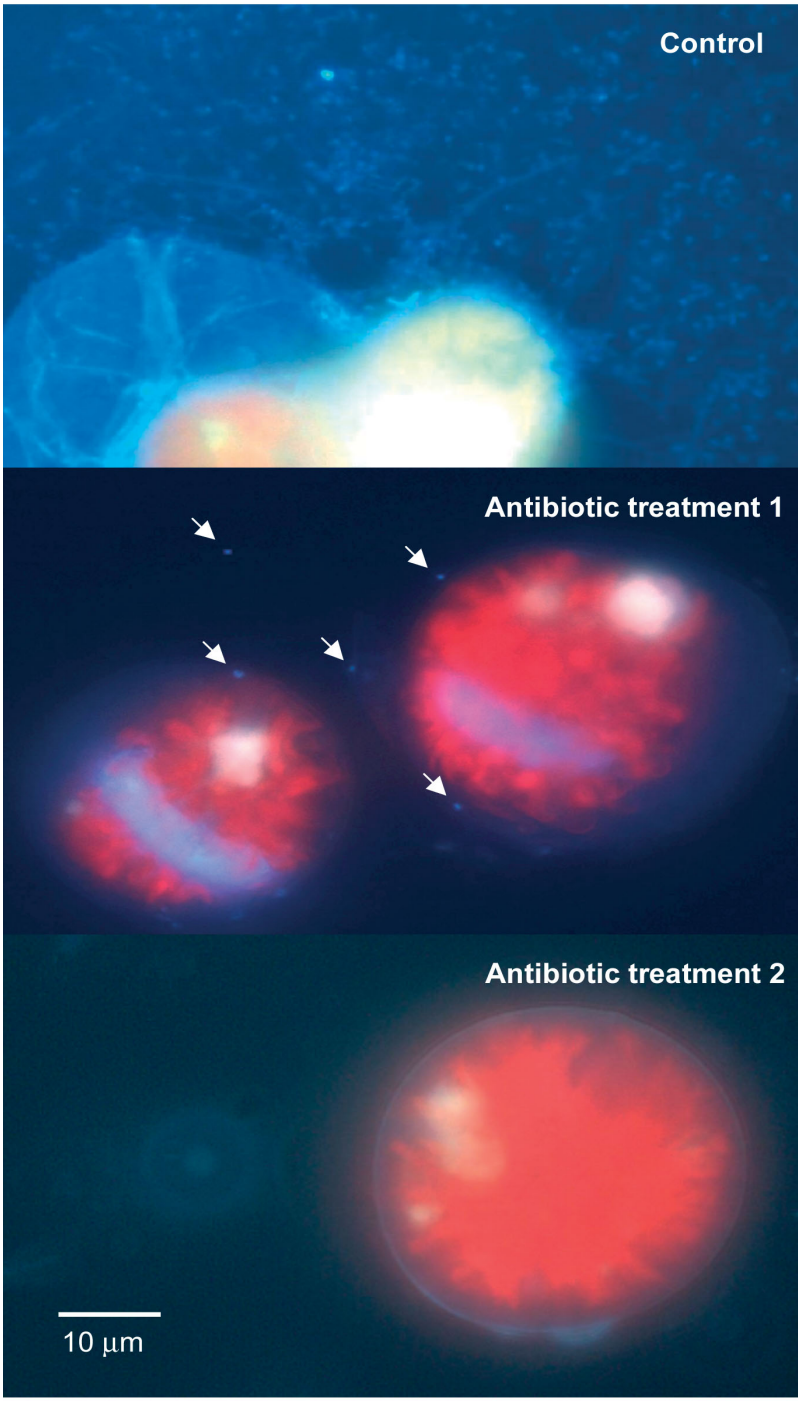

Fig. 2. *Alexandrium tamarense*. Assessment of residual bacteria in ATHK9301 cultures with and without antibiotic treatments by fluorescent microscopy. Axenic nature of dinoflagellate cultures was checked by direct microscopic observation of dinoflagellate samples stained with DAPI. Arrows: presence of bacterial cells

The highest toxin production in all cultures of ATCI01 and ATHK9301 was observed in the stationary phase when phosphorus became a limiting factor. This is in good agreement with the observations of Wang et al. (2002) and Wang & Hsieh (2005), suggesting that

Table 2. Comparisons between growth and toxin production in dinoflagellate cultures with and without antibiotic treatments. MEP: mid-exponential phase (Day 6); ESP: early stationary phase (Day 12); MSP: mid-stationary phase (Day 21); LSP: late stationary phase (Day 30). In parentheses = (antibiotic treated – control)/control × 100. na: not applicable

		Algal growth rate	Algal cell density ($\times 10^6$ cells l^{-1})	Total toxin yield (nmol l^{-1})	Total cellular toxin content (fmol $cell^{-1}$)
ATCI01					
Control	MEP	0.94	14.0	435	31
	ESP	na	15.8	586	37
	MSP	na	16.9	746	44
	LSP	na	8.3	401	48
Antibiotic treatment 1	MEP	1.11 (+18)	15.9 (+14)	542 (+25)	34 (+9)
	ESP	na	19.5 (+24)	994 (+70)	51 (+37)
	MSP	na	20.5 (+21)	1142 (+53)	56 (+26)
	LSP	na	17.1 (+107)	669 (+67)	39 (–19)
Antibiotic treatment 2	MEP	1.22 (+30)	17.4 (+24)	284 (–35)	16 (–48)
	ESP	na	25.1 (+59)	374 (–36)	15 (–60)
	MSP	na	24 (+42)	431 (–42)	18 (–59)
	LSP	na	20.9 (+153)	542 (+35)	26 (–46)
ATHK9301					
Control	MEP	0.61	9.2	636	69
	ESP	na	10.6	638	60
	MSP	na	13.3	883	67
	LSP	na	12.4	1259	102
Antibiotic treatment 1	MEP	1.03 (+69)	8.8 (–4)	885 (+39)	101 (+45)
	ESP	na	16.1 (+52)	1529 (+140)	95 (+57)
	MSP	na	20 (+51)	1828 (+107)	91 (+37)
	LSP	na	13.3 (+8)	1118 (–11)	84 (–17)
Antibiotic treatment 2	MEP	1.31 (+115)	13.9 (+52)	1121 (+76)	81 (+16)
	ESP	na	19 (+79)	1404 (+120)	74 (+23)
	MSP	na	22.3 (+68)	1260 (+43)	57 (–15)
	LSP	na	19.5 (+57)	1002 (–20)	52 (–49)

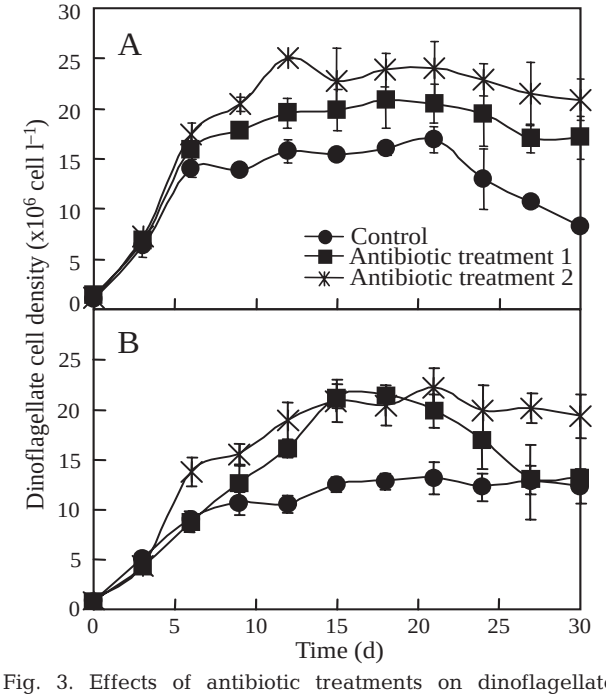

Fig. 3. Effects of antibiotic treatments on dinoflagellate growth in (A) ATCI01 cultures and (B) ATHK9301 cultures. Data are means ± SD of 4 to 5 replicates

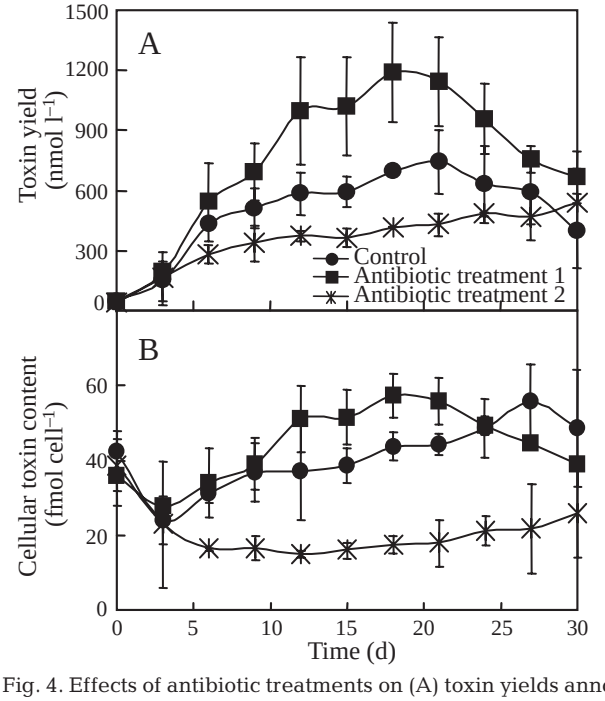

Fig. 4. Effects of antibiotic treatments on (A) toxin yields annd (B) cellular toxin content in ATCI01 cultures. Data are means ± SD of 4 to 5 replicates

Table 3. ANOVA and Tukey's HSD of pooled data of 2 algal strains showing effect of antibiotic treatments on algal cell density, toxin yield and cellular toxin contents at different growth phases. Tukey's HSD: values arranged from left to right in ascending order; those not significantly different (p = 0.05) are connected by a line. MEP: mid-exponential phase (Day 6); ESP: early stationary phase (Day 12); MSP: mid-stationary phase (Day 21); LSP: late stationary phase (Day 30); C: control culture; T1: antibiotic treatment 1; T2: antibiotic treatment 2

Algae	ANOVA						Tukey's HSD Culture		
	Parameter	Growth phase	MS effect	MS error	$F_{2,\ 9\text{-ATCI01}}$ $F_{2,\ 12\text{-ATHK9301}}$	p			
ATCI01	Cell density	MEP	11.633	1.871	6.218	0.020	C	T1	T2
		ESP	85.633	3.635	23.444	0.020	C	T1	T2
		MSP	50.619	3.427	14.773	0.001	C	T1	T2
		LSP	1167.871	4.712	35.630	<0.001	C	T1	T2
	Toxin yield	MEP	68010	2109	32.252	<0.001	T2	C	T1
		ESP	401643	6227	64.497	<0.001	T2	C	T1
		MSP	511819	5774	88.640	<0.001	T2	C	T1
		LSP	74094	5283	14.025	0.002	C	T2	T1
	Cellular toxin	MEP	367	13.085	28.070	<0.001	T2	C	T1
		ESP	1348	31.405	42.913	<0.001	T2	C	T1
		MSP	1522	26.026	58.482	<0.001	T2	C	T1
		LSP	500	30.267	16.513	0.001	T2	T1	C
ATHK9301	Cell density	MEP	40.570	0.845	48.025	<0.001	T1	C	T2
		ESP	90.868	0.470	193.362	<0.001	C	T1	T2
		MSP	110.447	3.018	36.599	<0.001	C	T1	T2
		LSP	74.273	2.063	36.008	<0.001	C	T1	T2
	Toxin yield	MEP	293692	3948	74.391	<0.001	C	T1	T2
		ESP	1163796	5812	200.233	<0.001	C	T2	T1
		MSP	1130375	6565	172.173	<0.001	C	T2	T1
		LSP	82585	3756	21.989	<0.001	T2	T1	C
	Cellular toxin	MEP	1215	78.100	15.559	<0.001	C	T2	T1
		ESP	1524	41.473	36.744	<0.001	C	T2	T1
		MSP	1621	87.085	18.618	<0.001	T2	C	T1
		LSP	3347	91.425	36.612	<0.001	T2	T1	C

the toxins produced by the 2 toxic dinoflagellates were secondary metabolites. The enhancement of PST production by phosphorus-limiting dinoflagellate cultures has also been well documented in other studies (Anderson et al. 1990b, Flynn et al. 1994, Bechemin et al. 1999, John & Flynn 2000, Guisande et al. 2002).

Although toxic dinoflagellates have generally been considered as the sole source of PST, bacteria have recently been reported to be PST producers (Kodama et al. 1988, Gallacher et al. 1997) or capable of significantly affecting algal toxicity (Gonzalez et al. 1992, Doucette & Powell 1998, Hold et al. 2001). In natural environments, bacteria co-exist with harmful algal bloom species. In order to prevent or reduce the events of PST poisoning, it is necessary to study the roles of bacteria in algal toxin production. The easiest way to study the bacterial–algal interaction in algal cultures is to start with laboratory experiments under controlled conditions before applying the knowledge to natural environments.

The influences of algal toxin production by bacteria have been reviewed by Doucette et al. (1998) and Gallacher & Smith (1999). Direct influences include autonomous production or metabolism of PSTs. Indirect influences include production of unknown cofactors which inhibit or stimulate dinoflagellate toxin synthesis, secretion of signaling molecules that control cellular and communicating processes in dinoflagellates and influence nutrient availability which thus affect dinoflagellate metabolism and toxicity. Total toxin production and cellular toxin content of ATCI01 and ATHK9301 cultures after antibiotic treatment 1 were higher than that of the control cultures. This can be explained by killing or inhibiting the growth of bacteria that can degrade algal toxins or compete for nutrients with dinoflagellates in the dinoflagellate cultures (Table 1), which may make more nutrients (e.g. phosphate, Gurung et al. 1999) available and increase the numbers of dinoflagellate cells (Fig. 3), and thus toxin production. It is also possible that certain toxin-producing or stimulative bacteria that were not killed by the antibiotics might have increased their activity due to the elimination of competition by the cultivable bacteria that were killed by the antibiotics. Bacterial

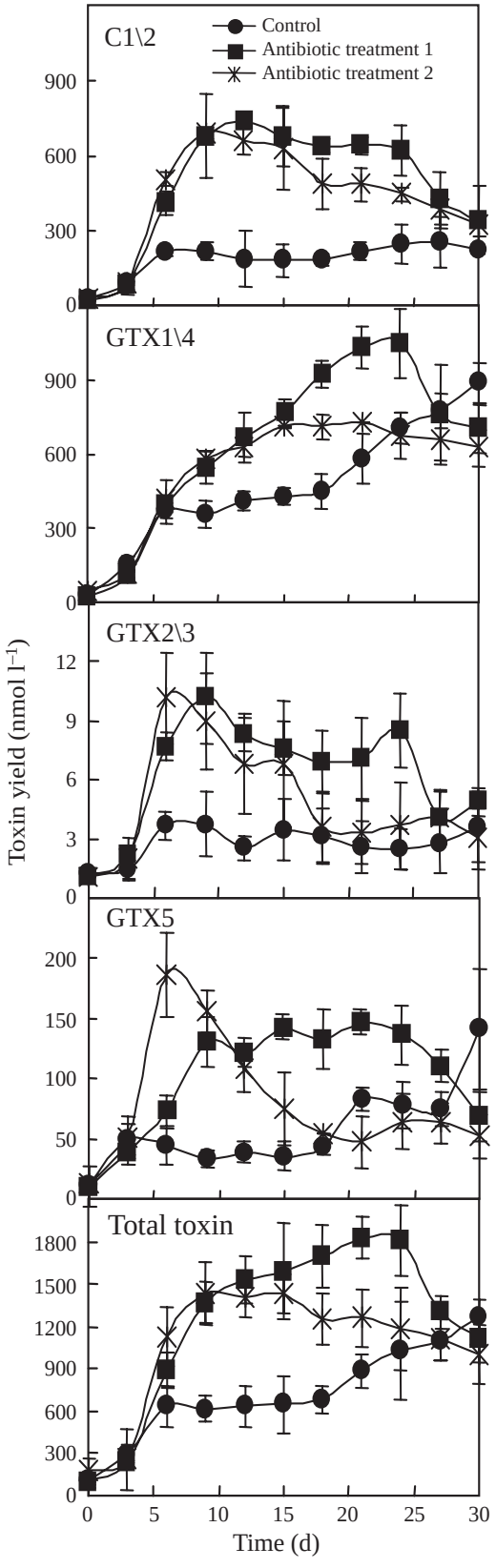

Fig. 5. Effects of antibiotic treatments on toxin yields in ATHK9301 cultures. Data are means ± SD of 4 to 5 replicates

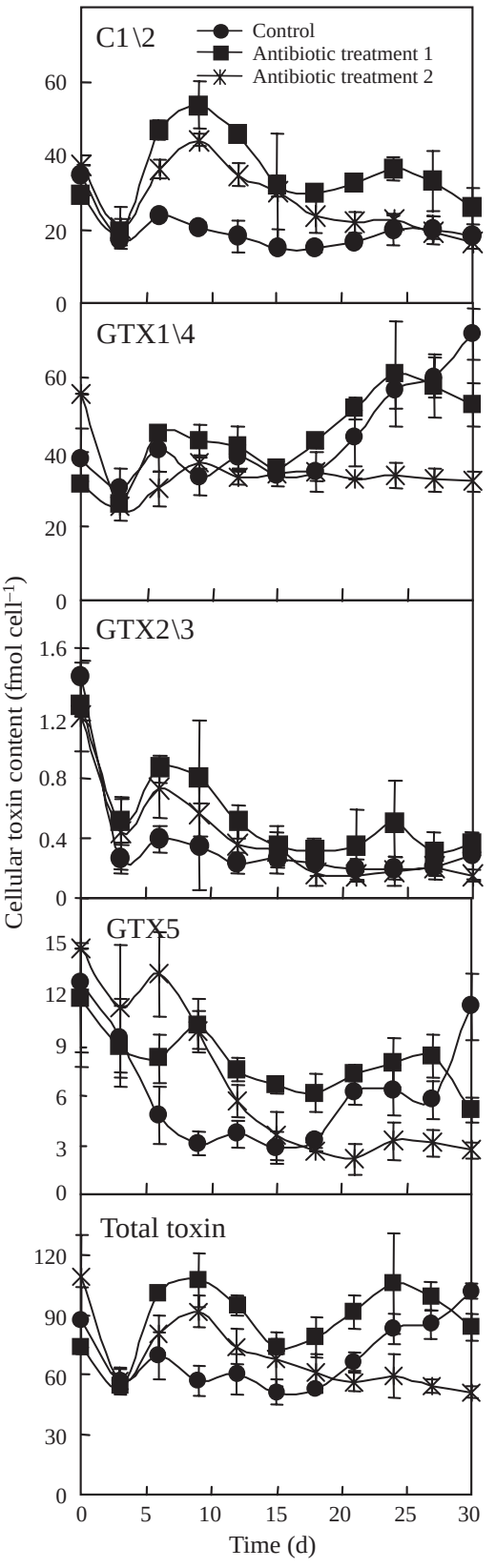

Fig. 6. Effects of antibiotic treatments on cellular toxin content in ATHK9301 cultures. Data are means ± SD of 4 to 5 replicates

growth resumed rapidly in aged ATCI01 and ATHK9301 cultures, especially in LSP. This rebounding of bacterial growth may have negative effects on either dinoflagellate growth or toxin production and, therefore, toxin yield and cellular toxin contents in cultures of ATCI01 and ATHK9301 after antibiotic treatment 1 dropped rapidly in LSP. The effects of these remaining bacteria on the toxicity of ATCI01 and ATHK9301 should be further investigated to obtain a clearer picture of the direct role of bacteria in algal toxin production. One residual bacterium isolated from the ATCI01 and HK9301 cultures exposed to antibiotic treatment 1 was capable of enhancing toxin production of the dinoflagellates with which it was associated (A. Y. T. Ho et al. unpubl.).

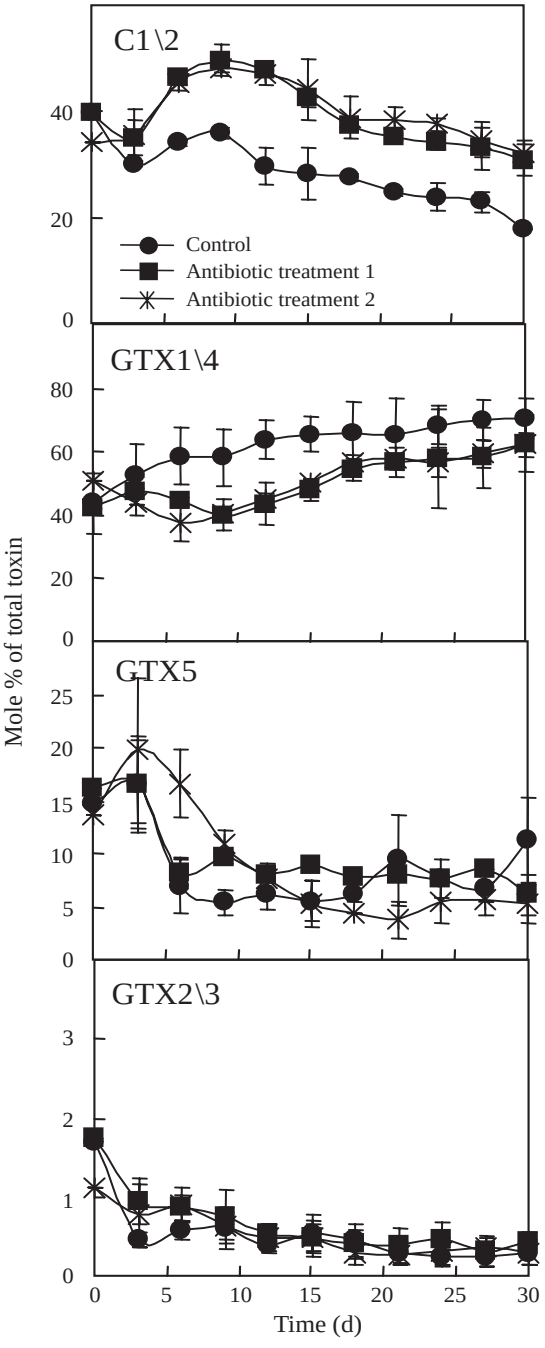

Fig. 7. Effects of antibiotic treatments on toxin composition in ATHK9301 cultures. Data are means ± SD of 4 to 5 replicates

The total toxin production and cellular toxin content of ATCI01 cultures after antibiotic treatment 2, however, were dramatically reduced even though an enhancement of cell growth was observed as in antibiotic treatment 1. Streptomycin and gentamicin are aminoglycosides that not only disable bacterial ribosomes, but also affect the small ribosomes in the mitochondria of eukaryotic cells (McKane & Kandel 1996). Enhancement of dinoflagellate growth was also demonstrated in the axenic dinoflagellate cultures that were inoculated into fresh media after the antibiotics had been washed away. Thus, the reduction of toxin production by the ATCI01 cells could be due to the killing of bacteria in the dinoflagellate culture rather than the poisoning effect of previous antibiotic treatments. These antibiotic-killed bacteria might include toxin-producing bacteria or bacteria that induce dinoflagellate toxin production. Toxin production in the axenic ATHK9301 cultures was higher during MEP and MSP, but was lower in LSP than that of the control cultures. The enhancing effect of the antibiotic treatment on toxin production by the ATHK9301 strain in the early growth stages might have been due to the higher nutrient availability as suggested above. It may also be due to the increased growth rates during the early growth stages in the axenic ATHK9301 culture (Table 2). Lower toxin production in the LSP could be due to the killing of bacteria that are involved in toxin production or due to a slow down or change in algal metabolism.

The complete loss of toxicity by *Alexandrium lusitanicum* has recently been reported by Martins et al. (2004), perhaps due to the elimination of associated bacteria essential for toxin synthesis after prolonged antibiotic treatments. In the present study, however, both the ATCI01 and ATHK9301 strains retained their ability to produce toxins in the axenic cultures (after antibiotic treatment 2), which suggests that the eliminated bacteria are not essential to toxin production in ATCI01 and ATHK9301 cultures; toxin production by these 2 strains may be a hereditary activity of the toxigenic algae, as suggested by Sako et al. (1992). However, both the total toxin yield and cellular toxin content of the axenic ATCI01 and ATHK9301 cultures throughout the growth phases were different from that of control cultures as described previously. These results are different from those of Kim et al. (1993) and Dantzer & Levin (1997), who recorded similar levels of toxin production in the axenic and wild type cultures.

Table 4. ANOVA and Tukey's HSD of pooled data showing effects of antibiotic treatments and changes of time (different growth phases) on toxin composition of ATHK9301 strain. Tukey's HSD: values arranged from left to right in ascending order; those not significantly different (p = 0.05) are connected by a line. MEP: mid-exponential phase (Day 6); ESP: early stationary phase (Day 12); MSP: mid-stationary phase (Day 21); LSP: late stationary phase (Day 30); C: control culture; T1: antibiotic treatment 1; T2: antibiotic treatment 2

Toxin	Growth phase	ANOVA results MS effect	MS error	$F_{2, 12}$	p	Tukey's HSD culture		
C1/C2	MEP	229.136	2.630	87.117	<0.001	C	T2	T1
	ESP	528.751	7.046	75.048	<0.001	C	T2	T1
	MSP	255.123	5.329	47.874	<0.001	C	T1	T2
	LSP	306.955	6.384	48.085	<0.001	C	T1	T2
GTX1/4	MEP	564.307	7.577	74.474	<0.001	T2	T1	C
	ESP	644.101	9.949	64.741	<0.001	T1	T2	C
	MSP	124.036	7.268	17.067	<0.001	T1	T2	C
	LSP	107.570	9.024	11.920	0.001	T1	T2	C
GTX5	MEP	138.197	2.973	46.481	<0.001	C	T1	T2
	ESP	5.072	1.219	4.162	0.042	C	T2	T1
	MSP	42.826	0.691	61.954	<0.001	T2	T1	C
	LSP	52.740	1.381	38.185	<0.001	T2	T1	C
GTX2/3	MEP	0.164	0.026	6.390	0.013	C	T1	T2
	ESP	0.026	0.009	2.991	0.088	C	T2	T1
	MSP	0.021	0.021	0.999	0.397	T2	C	T1
	LSP	0.035	0.008	4.263	0.040	C	T2	T1

Toxin	Treatment	MS effect	MS error	$F_{2,12}$	p	Growth phase			
C1/C2	C	237.913	4.834	49.219	<0.001	LSP	MSP	ESP	MEP
	T1	350.231	4.475	78.259	<0.001	LSP	MSP	ESP	MEP
	T2	229.914	6.732	34.151	<0.001	LSP	MSP	ESP	MEP
GTX1/4	C	128.619	10.148	12.675	<0.001	MEP	ESP	MSP	LSP
	T1	431.374	6.377	67.642	<0.001	ESP	MEP	MSP	LSP
	T2	658.648	8.839	74.518	<0.001	MEP	ESP	MSP	LSP
GTX5	C	27.562	2.888	9.543	<0.001	MEP	ESP	MSP	LSP
	T1	4.912	0.562	8.738	0.001	LSP	ESP	MSP	MEP
	T2	165.206	1.248	132.400	<0.001	MSP	LSP	ESP	MEP
GTX2/3	C	0.092	0.011	8.376	0.001	LSP	MSP	ESP	MEP
	T1	0.236	0.019	12.590	<0.001	MSP	LSP	ESP	MEP
	T2	0.435	0.018	24.264	<0.001	MSP	LSP	ESP	MEP

Our observations match those of Gonzalez et al. (1992) and Hold et al. (2001) and suggest that dinoflagellate-associated bacteria can somehow indirectly affect toxin production as suggested by Doucette et al. (1998) and Green et al. (2004). Bacterial competition for nutrients with algae may be a major reason for the change in toxin production in the 2 dinoflagellate cultures. However, this does not explain why the toxin production of ATCI01 cultures and the cellular toxin contents in MSP and LSP of ATHK9301 cultures were reduced in the absence of bacteria. The presence of bacteria that are involved in toxin production of ATCI01 and ATHK9301 may also affect dinoflagellate toxicity in the ATCI01 and ATHK9301 cultures. However, strong evidence to prove this suggestion is not provided by this study.

Despite the dramatic change in toxin production in the ATCI01 cultures after antibiotic treatments, toxin composition (mol %) of ATCI01 did not change with the destruction of bacteria in the dinoflagellate cultures. The toxin profiles of the ATHK9301 cultures, however, changed with different growth stages and also changed when the bacteria were destroyed in dinoflagellate culture by antibiotic treatments. The molar percentages of C1/C2, GTX5 and GTX2/3 in all dinoflagellate cultures decreased gradually, while that of GTX1/4 increased throughout the growth stages. The results are similar to that of Boczar et al. (1988),

who observed changes in the toxin composition of the isolates of *Alexandrium tamarense* and *A. catenella* at various growth stages in axenic cultures. In the antibiotic treatments, the molar percentage of GTX1/4 decreased while that of C1/C2 increased when the growth rates in the dinoflagellate cultures increased during the exponential growth phase (Fig. 7). The cellular production of C1/C2 increased while that of GTX1/4 remained similar or lower than that of the control cultures in the antibiotic treatments (Fig. 6). This indicates that higher growth rates favour the cellular production of C1/C2, but not the production of GTX1/4. A trace amount of GTX2/3 (~1 mol % of total toxin) was detected in all the ATHK9301 cultures in this study. The 2 toxins were not detected in the ATHK9301 cultures by Wang et al. (2005). This change in toxin composition could be due to the fact that the dinoflagellate cultures were maintained under laboratory conditions with high nutrient availability and frequent subculturing.

Contrary to previous reports that the toxin profiles of PST-producing dinoflagellates remained constant under a wide variety of growth stages and environmental conditions (Boyer et al. 1987, Ogata et al. 1987, Kim et al. 1993, Oshima et al. 1993, Sako et al. 1995, Parkhill & Cembella 1999), our data showed that the toxin composition of ATHK9301 varied. Similarly, the dinoflagellate toxin composition changed with growth rate and culture conditions (Boczar et al. 1988, Anderson et al. 1990a, Wang et al. 2005). In addition to these reports, the present study suggests that the toxin profiles in dinoflagellates could also change with growth stages and the presence or absence of bacteria. The susceptibly of toxin profiles of dinoflagellates to different environmental conditions and growth stages could be strain-, isolate- and/or species-specific. Our findings may also explain the dramatic changes in toxicity of the same harmful algal bloom species/strain isolated from natural marine environments where the diversity and abundance of bacteria coexisting with harmful algal bloom species were spatially and temporally different (Wang et al. 2005). Bacterial influences, similar to those demonstrated in our laboratory cultures, on the toxicity of different harmful algal bloom species are expected to occur in natural environments.

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LITERATURE CITED

Anderson DM, Kulis DM, Sullivan JJ, Hall S (1990a) Toxin composition variations in one isolate of the dinoflagellate *Alexandrium fundyense*. Toxicon 28:885–893

Anderson DM, Kulis DM, Sullivan JJ, Hall S, Lee C (1990b) Dynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp. Mar Biol 104:511–524

Anderson DM, Kulis DM, Qi YZ, Zheng L, Lu S, Lin YT (1996) Paralytic shellfish poisoning in southern China. Toxicon 34:579–590

Bechemin C, Grzebyk D, Hachame F, Hummert C, Maestrini SY (1999) Effect of different nitrogen/phosphorus nutrient ratios on the toxin content in *Alexandrium minutum*. Aquat Microb Ecol 20:157–165

Boczar BA, Beitler MK, Liston J, Sullivan JJ, Cattolico RA (1988) Paralytic shellfish toxins in *Protogonyaulax tamarensis* and *Protogonyaulax catenella* in axenic culture. Plant Physiol 88:1285–1290

Boyer GL, Sullivan JJ, Andersen RJ, Harrison PJ, Taylor FJR (1987) Effects of nutrient limitation on toxin production and composition in the marine dinoflagellate *Protogonyaulax tamarensis*. Mar Biol 96:123–128

Dantzer WR, Levin RE (1997) Bacterial influence on the production of paralytic shellfish toxins by dinoflagellated algae. J Appl Microbiol 83:464–469

Doucette GJ, Powell CL (1998) Algal–bacterial interactions: can they determine the PSP-related toxicity of dinoflagellates. In: Reguera B, Blanco J, Fernandez ML, Wyatt T (eds) Harmful algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, Grafisant, p 406–409

Doucette GJ, Kodama M, Franca S, Gallacher S (1998) Bacterial interactions with harmful algal bloom species: bloom ecology, toxigenesis, and cytology. In: Anderson DM, Cembella AD, Hallegraeff GM (eds) Physiological ecology of harmful algal blooms, Vol G41. Springer-Verlag, Berlin, p 619–647

Flynn K, Franco J, Fernandez P, Reguera B, Zapata M, Wood G, Flynn KJ (1994) Changes in toxin content, biomass and pigments of the dinoflagellate *Alexandrium minutum* during nitrogen refeeding and growth into nitrogen and phosphorus stress. Mar Ecol Prog Ser 111:99–109

Gallacher S, Smith EA (1999) Bacteria and paralytic shellfish toxins. Protist 150:245–255

Gallacher S, Flynn KJ, Franco JM, Brueggemann EE, Hines HB (1997) Evidence for production of paralytic shellfish toxins by bacteria associated with *Alexandrium* spp. (Dinophyta) in culture. Appl Environ Microbiol 63: 239–245

Gonzalez I, Tosteson CG, Hensley V, Tosteson TR (1992) Role of associated bacteria in growth and toxicity of cultured benthic dinoflagellates. Bull Soc Pathol Exot 85:457–459; available at: http://www.pathexo.fr/pages/english/journal.html

Green DH, Llewellyn LE, Negri AP, Blackburn SI, Bolch CJS (2004) Phylogenetic and functional diversity of the cultivable bacterial community associated with the paralytic shellfish poisoning dinoflagellate *Gymnodinium catenatum*. FEMS Microbiol Ecol 47:345–357

Guisande C, Frangopulos M, Maneiro I, Vergara AR, Riveiro I (2002) Ecological advantages of toxin production by the dinoflagellate *Alexandrium minutum* under phosphorus limitation. Mar Ecol Prog Ser 225:169–176

Gurung TB, Urabe J, Nakanishi M (1999) Regulation of the relationship between phytoplankton *Scenedesmus acutus* and heterotrophic bacteria by the balance of light and nutrients. Aquat Microb Ecol 17:27–35

Hold GL, Smith EA, Birkbeck TH, Gallacher S (2001) Comparison of paralytic shellfish toxin (PSTs) production by the dinoflagellates *Alxandrium lusitanicum* NEPCC 253 and *Alexandrium tamarense* NEPCC 407 in the presence

and absence of bacteria. FEMS Microbiol Ecol 36:223–234
John EH, Flynn KJ (2000) Growth dynamics and toxicity of *Alexandrium fundyense* (Dinophyceae): the effect of changing N:P supply ratios on internal toxin and nutrient levels. Eur J Phycol 35:11–23
Keller MD, Selvin RC, Claus W, Guillard RRL (1987) Media for the culture of oceanic ultraphytoplankon. J Phycol 23: 633–638
Kim CH, Sako Y, Ishida Y (1993) Variation of toxin production and composition of axenic cultures of *Alexandrium catenella* and *Alexandrium tamarense.* Nippon Suisan Gakkaishi 59:633–639
Kodama M, Ogata T, Sato S (1988) Bacterial production of saxitoxin. Agric Biol Chem 52:1075–1077
Lu YH, Chai TJ, Hwang DF (2000) Isolation of bacteria from toxic dinoflagellate *Alexandrium minutum* and their effects on algae toxicity. J Nat Toxins 9(4):409–417
Martins CA, Kulis D, Franca S, Anderson DM (2004) The loss of PSP toxin production in a formerly toxic *Alexandrium lusitanicum* clone. Toxicon 43:195–205
McKane L, Kandel J (1996) Microbiology: essentials and applications, 2nd edn. McGraw-Hill, New York, p 375–406
Ogata T, Ishimaru T, Kodama M (1987) Effect of water temperature and light intensity on growth rate and toxicity change in *Protogonyaulax tamarensis*. Mar Biol 95:217–220
Oshima Y (1995) Postcolumn derivatization liquid chromatographic method for paralytic shellfish toxins. J AOAC Int 78:528–532
Oshima Y, Blackburn SI, Hallegraeff GM (1993) Comparative study on paralytic shellfish toxin profiles of the dinoflagellate *Gymnodinium catenatum* from three different countries. Mar Biol 116:471–476
Parkhill JP, Cembella AD (1999) Effects of salinity, light and inorganic nitrogen on growth and toxigenicity of the marine dinoflagellate *Alexandrium tamarense* from northeastern Canada. J Plankton Res 21:939–955
Sako Y, Kim CH, Ishida Y (1992) Mendelian inheritance of paralytic shellfish poisoning toxin in the marine dinoflagellate *Alexandrium catenella*. Biosci Biotechnol Biochem 56(4):692–694
Sako Y, Naya N, Yoshida T, Kim CH, Uchida A, Ishida Y (1995) Studies on stability and heredity of PSP toxin composition in the toxic dinoflagellate *Alexandrium*. In: Lassus P, Arzul G, Denn EEL, Gentien P, Baut CML (eds) Harmful marine algal blooms. Proceedings of the 6th International Conference on Toxic Marine Phytoplankton. Lavoisier Publishing, Paris, p 345–350
Wang CH, Ho AYT, Qian PY, Wong PK, Hsieh DPH (2004) Antibiotic treatment enhances C2 toxin production by *Alexandrium tamarense* in batch cultures. Harmful Algae 3:21–28
Wang DZ, Hsieh DPH (2005) Growth and toxin production in batch cultures of a marine dinoflagellate *Alexandrium tamarense* HK9301 isolated from the South China Sea. Harmful Algae 4:401–410
Wang DZ, Ho AYT, Hsieh DPH (2002) Production of C2 toxin by *Alexandrium tamarense* CI01 using different culture methods. J Appl Phycol 14(6):461–468
Wang DZ, Jiang TJ, Hsieh DPH (2005) Toxin composition variations in cultures of *Alexandrium* species isolated from the coastal waters of southern China. Harmful Algae 4: 109–121

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