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Evidence for Production of Paralytic Shellfish Toxins by Bacteria Associated with *Alexandrium* spp. (Dinophyta) in Culture

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A substantial proportion of bacteria from five *Alexandrium* cultures originally isolated from various countries produced sodium channel blocking (SCB) toxins, as ascertained by mouse neuroblastoma assay. The quantities of SCB toxins produced by bacteria and dinoflagellates were noted, and the limitations in comparing the toxicities of these two organisms are discussed. The chemical nature of the SCB toxins in selected bacterial isolates was determined as paralytic shellfish toxins by pre- and postcolumn high-performance liquid chromatography, capillary electrophoresis-mass spectrometry, and enzyme immunoassay.

Paralytic shellfish toxins (PST) are potent neurotoxins which, if present in plankton, can be concentrated by filter feeding shellfish (27). This can lead to paralytic shellfish poisoning (PSP) in humans upon consumption of contaminated animals (27).

PST comprise saxitoxin (STX) and at least 20 other chemically related derivatives (40) which block sodium channels in mammalian nerve cells, thus preventing conductance of signals along the neuron (3, 27). The individual toxins vary in potency, with those containing a carbamate group (e.g., STX) being the most toxic and those with a sulfocarbamoyl group (e.g., C toxins) being the least toxic (50) (Fig. 1). The proportions and concentrations of these toxins, when present in organisms such as shellfish, are highly variable (50).

Although it is generally considered that PST are associated with dinoflagellates, such as *Alexandrium tamarense* (49), it has been suggested that heterotrophic bacteria are responsible for toxin synthesis in these organisms (30, 31, 45, 46). The original evidence to support these propositions was electron micrographs depicting bacteria inside dinoflagellate cells, the subsequent isolation of these bacteria, and determinations of their toxicities (31, 45, 46). However, other workers failed to detect intracellular bacteria in toxic dinoflagellates and were skeptical of claims that bacteria produced PST (37). Controversy has surrounded this issue, with discussions focusing on whether or not bacteria exist within the dinoflagellate cell and what, if any, could be their role in dinoflagellate toxicity. These topics have seemed to distract researchers from an important point, proving that bacteria are capable of autonomous production of these toxins. As a result, it was only recently that confirmation of the toxicity of the original PST-producing bacterium, isolated by Kodama's group in Japan, was reported (11, 30). However, little work has been done elsewhere. Therefore, this study was undertaken to determine if bacteria associated with dinoflagellates other than those originating from Japan were capable of autonomous production of PST. The number of bacteria capable of toxin production and the quantity of toxin produced by bacteria compared with that of dinoflagellates were also determined.

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MATERIALS AND METHODS

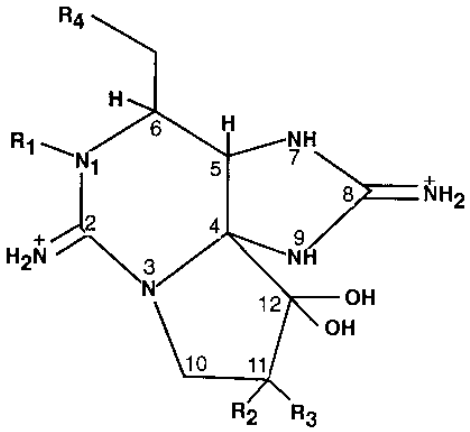
Source of dinoflagellate strains. The following dinoflagellate strains were used: *A. tamarense* UW2c and UW4 from germinated cysts, obtained from sediment off the west coast of Scotland (33–35) (Table 1); and *A. tamarense* NEPCC 407, *A. lusitanicum* NEPCC 253, and *A. affine* NEPCC 667, obtained from the North East Pacific Culture Collection (Table 1). All of these cultures have been reported as toxic (6–8, 17, 35, 36), with the exception of *A. affine* NEPCC 667 (1, 8). However, this strain is listed by the North East Pacific Culture Collection as toxic.

Culturing of dinoflagellate strains. Dinoflagellates were cultured for 28 days in 150 ml of f/2-enriched seawater (22) without copper and with added selenium (Na_2SeO_3) at a final concentration of 10^{-8} M. Cultures were exposed to a 12-h light-dark cycle at 15°C (ca. 125 μmol m^{-2} s^{-1} [irradiance]; cool white fluorescent tubes).

Cells obtained by centrifugation (2,000 × *g* for 20 min) of a 50-ml culture of each *Alexandrium* sp. were sonicated with 1 ml of 0.05 M acetic acid followed by centrifugation (10,000 × *g* for 10 min) to remove cell debris. The resultant supernatant was stored frozen at −20°C until used.

Isolation and growth of bacteria from dinoflagellate cultures. Tenfold dilutions of each *Alexandrium* culture were prepared in marine broth (Difco), and 0.1 ml of each was spread onto marine agar (Difco) plates. These plates were incubated at 20°C for 7 days, and the total viable count was calculated. Bacteria from the plates containing approximately 50 colonies were isolated and replated individually onto marine agar plates, followed by incubation at 20°C for 48 h. The resultant pure colonies were subsequently inoculated into 30 ml of marine broth. After incubation at 20°C for 24 h on a rotary incubator (120 oscillations per min), the supernatants were collected by centrifugation (10,000 × *g* for 20 min) and stored at −20°C until required. These samples were used in the initial screening for sodium channel blocking (SCB) activity of bacterial culture supernatants by mouse neuroblastoma (MNB) assay (16).

Preparation of bacterial samples for quantitative tissue culture and analytical analysis. Bacterial strains 667-2, 407-2, 253-19, UW4-1, and UW2c-6, obtained from their respective dinoflagellate cultures, were cultured for 18 h in 30 ml of marine broth (20°C; 120 oscillations min^{-1}). Ten milliliters of each of these cultures was subsequently inoculated into 500 ml of marine broth in a 2-liter flask and incubated for a further 24 h. After this period, a cell count was obtained from the application of 10-fold serial dilutions of each culture onto marine agar plates and incubation for 48 h at 20°C. Supernatants and cells were separated by centrifugation (10,000 × *g* for 20 min). This was repeated until a total of 5 liters of culture was obtained for each isolate. An aliquot (20 ml) of the supernatant for each isolate was stored at 20°C until processed by tissue culture. The cells harvested from a total of 5 liters of each isolate were weighed and resuspended in 0.05 M acetic acid (667-2, 36.5 g in 5 ml [equivalent to 1.4×10^{12} CFU/ml]; 407-2, 38.5 g in 40 ml [equivalent to 3.37×10^{11} CFU/ml]; UW4-1, 152 g in 100 ml [equivalent to 1.45×10^{11} CFU/ml]; and UW2c-6, 114 g in 80 ml [equivalent to 1.25×10^{11} CFU/ml]). However, only 1 liter of 253-19 was harvested; this gave 11.8 g (wet weight) of cells, which was resuspended in 10 ml of acetic acid (equivalent to 2.3×10^{11} CFU per ml). The resultant slurries were sonicated and



R_1	R_2	R_3	Carbamate Toxins	N- Sulfocarbamoyl Toxins	Decarbamoyl Toxins	Deoxydecarbamoyl Toxins
H	H	H	STX	B1	dc-STX	do-STX
OH	H	H	Neo-STX	B2	dc-Neo-STX	-
OH	H	$OSO3^-$	GTX 1	C3	dc-GTX 1	-
H	H	$OSO3^-$	GTX2	C1	dc-GTX2	do-GTX2
H	$OSO3^-$	H	GTX 3	C2	dc-GTX 3	do-GTX 3
OH	$OSO3^-$	H	GTX 4	C4	dc-GTX 4	-
			R_4: H_2N, O–, O	R_4: H, N, O–, $\bar{O}_3S$, O	R_4: HO-	R_4: H-

FIG. 1. Structures of PST (adapted from references 9 and 40). Abbreviations: STX, saxitoxin; neo-STX, neosaxitoxin; GTX 1 through 4, B1, and B2, gonyautoxins 1 through 6; dcSTX, decarbamoylsaxitoxin; dcGTX2 and 3, decarbamoylgonyautoxins 2 and 3; do-STX, 13-deoxydecarbamoylsaxitoxin; and do-GTX 2 and do-GTX 3, 13-deoxdecarbamoylgonyautoxins 2 and 3.

centrifuged (10,000 × *g* for 10 min), and the supernatant was collected and stored at −20°C.

MNB assay. The MNB assay for the detection of SCB toxins was performed by the method of Gallacher and Birkbeck (18). Briefly, ouabain and veratridine were added to MNB cells, leading to cell death due to sodium influx. However, in the presence of SCB toxins such as PST, cells survived and were quantified by the addition of the viable dye neutral red (18, 20). The resultant colorimetric response was measured by using an enzyme-linked immunosorbent assay (ELISA) plate reader. All samples were tested in triplicate, and controls consisted of (i) two sets of three wells containing cells with 200 μl of RPMI tissue culture medium (Gibco) and (ii) two sets of three wells containing ouabain (0.2 mM; Sigma) and veratridine (0.05 mM; Sigma) adjusted to a 200-μl volume by the addition of material equivalent to the sample but containing no toxins (i.e., for culture supernatant tested at 1/10 dilution in RPMI medium, marine broth at a 1/10 dilution in RPMI medium was added to the ouabain-veratridine controls). An STX dose-response curve (STX [Calbiochem] diluted in RPMI medium fresh daily) over the range from 0 to 100 nM was prepared for each assay. For initial screening of large numbers of bacterial supernatants, the samples were used in the assay at a 1/10 dilution. For quantitative evaluation of the bacterial supernatant, bacterial cells, and dinoflagellate cells, the samples were diluted in a twofold series in RPMI medium before addition to the assay. The coefficient of variation of each triplicate absorbance value was no more than 10%. The absorbance value obtained upon cell survival due to the presence of SCB toxins was compared to that of the STX dose-response curve, allowing the SCB activity to be defined as nanomolar STX equivalents and subsequently converted to STX equivalents expressed in nanograms per milliliter and picomoles per milliliter for calculation purposes.

In order to calculate the quantity of toxin excreted by bacteria into the surrounding medium on a per cell basis, the concentration of STX (expressed in picomoles per milliliter) was divided by the number of cells obtained from a 24-h culture (667-2, 1.4×10^9 CFU/ml; 407-2, 2.7×10^9 CFU/ml; 253-19, 2.3×10^9 CFU/ml; UW4-1, 2.9×10^9 CFU/ml; and UW2c-6, 2.0×10^9 CFU/ml).

To calculate the total quantity of toxins produced by bacteria, the number of cells from a 24-h culture (in CFU per milliliter) was multiplied by the volume of the culture (generally 5 liters) and divided by the volume of liquid used to

TABLE 1. Quantities of bacteria and proportions of SCB toxin-producing bacterial isolates from dinoflagellates[a]

Strain	Country of origin (reference)	Dinoflagellates (cells/ml)	Bacteria (CFU/ml)	Bacteria producing SCB toxins (%)[b]
A. affine NEPCC 667	Spain (13)	2.0×10^4	1.8×10^7	42
A. tamarense NEPCC 407	Canada (6)	1.8×10^4	6.9×10^7	60
A. lusitanicum NEPCC 253	Portugal (44)	5.6×10^4	4.3×10^7	60
A. tamarense UW4	United Kingdom (35)	2.1×10^4	7×10^6	54
A. tamarense UW2c	United Kingdom (35)	4.8×10^3	5.0×10^6	40

[a] Obtained from 28-day laboratory cultures of *Alexandrium* spp. from North America and Europe.
[b] As determined by the MNB assay of Gallacher and Birkbeck (18).

resuspend the cell pellet. The quantity of toxin (expressed in picomoles per milliliter) was subsequently divided by the total number of cells.

The concentrations of SCB toxins in dinoflagellate cell extracts were calculated in a manner similar to that used for bacterial cells, but the dinoflagellate cell numbers listed in Table 1 were used as the basis for determining the number of cells in 50 ml of dinoflagellate culture.

HPLC. Analysis of PST by high-performance liquid chromatography (HPLC) was carried out by both precolumn (12) and postcolumn (16) oxidation of toxins.

For precolumn HPLC, bacterial cell extracts were diluted 1/10 in 0.05 M acetic acid. Thirty microliters of UW4-1 and 15 μl of 667-2, 407-2, 253-19, and UW2c-6 were adjusted to a 60-μl total volume in water and used in precolumn HPLC by the method of Flynn and Flynn (12). The standards used were obtained from the National Research Council of Canada as a commercial kit (PSP-1) consisting of STX, neo-STX, and gonyautoxins 2 and 3 (GTX 2 and 3, respectively).

For postcolumn HPLC (16), bacterial cell extracts were diluted in 0.05 M acetic acid to a total volume of 1.5 ml in the following proportions: by 1/2 for 407-2, 253-19, and UW4-1; by 1/3 for UW2c-6; and by 1/5 for 667-2. Each 1.5-ml aliquot was divided into two 0.75-ml volumes. With one set, the total volume was readjusted to 1.5 ml by the addition of 0.6 ml of 0.05 M acetic acid and 0.15 ml of deionized water. The second set were processed further by the addition of 0.2 ml of 1 M HCl, followed by heating at 100°C for 15 min and the addition of 0.4 ml of 1 M sodium acetate and 0.2 ml of distilled water once the sample had cooled. The samples were analyzed by postcolumn HPLC by the method of Franco and Fernandez-Vila (16).

GTX standards were obtained from *Alexandrium minutum* ALIV cultures from the IEO collection (16) and quantified by A. Cembella (National Research Council of Canada). STX, decarbamoyl-STX (d-STX), and neo-STX were provided in an intercalibration exercise (BCR, Brussels, Belgium).

CE-MS. Capillary electrophoresis-mass spectrometry (CE-MS) was performed on a Beckman 2050 unit attached to a Finnigan MAT TSQ 700 mass spectrometer. The CE unit was fitted with a bare fused silica column (75 μm [inner diameter] by 85 cm). Samples were diluted in 0.05% formic acid and injected hydrodynamically for 5 s. Separations were performed by using 30 kV and an aqueous solution of 10% acetonitrile in 0.2% formic acid. The electrophoresis-fused silica column was directed into the electrospray ion source (Analytica of Bradford, Bradford, Mass.). A sheath flow of 60% isopropyl alcohol in 0.1% formic acid was used to establish electrical contact with the CE unit and increase the total volumetric flow to 3 μl/min. The electrospray source was operated in positive-ion mode, and the voltage was approximately −3,750 V. Nitrogen was used as the counter gas and was held at 170°C, as shown on the manifold, with a flow rate of 13 liters/min. Bacterial cell extracts previously filter sterilized (0.22-μm-pore-size nylon filter) and lyophilized were analyzed by selected ion monitoring of the protonated molecular ions corresponding to STX, neo-STX, C, and GTX toxins. No more than two ions were monitored during a single run. For sulfated toxins, voltages in the skimmer region of the electrospray ion source were set to avoid collisional fragmentation of the sulfate group. Standard toxins obtained from Sherwood Hall, Food and Drug Administration, Washington, D.C., were used to verify instrument operations.

ELISA. A series of twofold dilutions in buffer of bacterial cell extracts was used in a commercial enzyme immunoassay according to the protocol provided in the kit (Ridascreen; Digen, Oxford, United Kingdom). A color reaction indicated the presence of STX, dc-STX, GTX 2, GTX 3, B1, C1, or C2 either individually or in combination (51, 52).

RESULTS

Culture of dinoflagellate cells and their associated bacteria. Details of the *Alexandrium* species studied and their associated bacteria are listed in Table 1. Each dinoflagellate yielded a concentration of bacteria ranging from 5×10^6 to 6.9×10^7 CFU per ml of dinoflagellate culture, depending on the isolate. However, several of the smaller bacterial colonies from agar plates did not survive subsequent subculture. The majority of the bacteria tested were gram negative and largely unpigmented, with the exception of those isolated from cultures of *A. lusitanicum* NEPCC 253, from which 30% of bacterial isolates contained an orange pigment.

Screening and quantification of SCB toxin production by bacteria, as determined by MNB assay. Between 40 and 60% (equivalent to a maximum of 4.1×10^7 CFU of bacteria per ml of dinoflagellate culture) of a representative proportion of bacteria tested from each dinoflagellate culture produced SCB toxins in the bacterial culture supernatant, as determined by MNB assay (Table 1). Subsequently, five SCB toxin-producing bacterial strains, one from each dinoflagellate culture, were chosen for further study.

TABLE 2. Quantities of SCB toxins produced by bacteria and dinoflagellates[a]

Strain	Toxin production		
	Bacteria		Dinoflagellates
	Extracellular	Intracellular	
NEPCC 667	1.00 ± 0.05	0.0029 ± 0.0002	1.2 ± 0.03
NEPCC 407	0.76 ± 0.07	0.0066 ± 0.0003	5.9 ± 0.31
NEPCC 253	0.37 ± 0.03	0.0036 ± 0.0003	6.1 ± 0.12
UW4	1.0 ± 0.003	0.039 ± 0.0020	34.2 ± 0.34
UW2c	1.3 ± 0.008	0.13 ± 0.0028	10.5 ± 0.4

[a] The quantities of toxins produced (on a per cell basis) were calculated from the quantities of toxins determined by MNB assay and the cell numbers obtained from stationary-phase cultures of bacteria (24 h) and dinoflagellates (28 days). Data are expressed in 10^{-4} femtomoles of STX equivalents per cell, except for dinoflagellate (intracellular) data, which are expressed in femtomoles of STX equivalents per cell.

The quantity of SCB toxins detected in the supernatant of broth cultures from these five bacterial strains by MNB assay ranged from 84.8 to 294 nM STX equivalents, which equates to a maximum of 87.9 ng of STX equivalents produced per 1 ml of culture supernatant. A typical quantity of toxin excreted was 1×10^{-4} fmol of STX equivalents per bacterial cell, whereas the quantity of intracellular toxin was up to 1,000-fold less (Table 2).

Quantity of SCB toxins produced by dinoflagellate cultures, as measured by MNB assay. Cell extracts from *Alexandrium* cultures contained between 1.2 and 34.2 fmol of STX equivalents per cell, with *A. tamarense* UW4 being the most toxic and *A. affine* NEPCC 667 the least toxic (Table 2).

Attempts to quantify the amount of toxin excreted by dinoflagellate cells into the surrounding medium were unsuccessful, as the dinoflagellate supernatant killed MNB cells. Since the media used to culture dinoflagellates did not adversely affect MNB cells, it was assumed that the cytotoxic effect was due to compounds released by dinoflagellates. The nature of this cytotoxic factor is unknown and could not be removed by heating (100°C, 20 min) or filtration (10 kDa; Millipore).

Comparison of the quantities of SCB toxins produced by dinoflagellates and bacteria in laboratory cultures. The number of bacterial cells obtained from a 24-h culture of each of the bacterial strains was, in most instances, approximately 2×10^9 CFU/ml (see Materials and Methods), whereas the number of dinoflagellate cells obtained from stationary-phase dinoflagellate cultures (28 days) was maximally 5.6×10^4 cells/ml (Table 1). Not surprisingly, this demonstrated that under laboratory conditions, the number of bacterial cells found in the stationary phase of their life cycle far exceeded the number of dinoflagellate cells at an equivalent point. Therefore, although bacteria yielded less toxin than did dinoflagellates on a per cell basis (Table 2), consideration of the total bacterial numbers in laboratory cultures indicated that bacteria produced, in some instances, substantially more toxin than did dinoflagellates in equivalent culture volumes (Table 3).

SCB toxin production by bacteria associated with dinoflagellate cultures. Bacterial toxicity is described above in terms of the number of bacteria obtained in pure culture; however, the number of culturable bacteria isolated from dinoflagellates was much lower, in the range of 10^6 to 10^7 CFU/ml of dinoflagellate culture, of which 40 to 60% produced SCB toxins (Table 1). On the basis of these figures and the quantity of toxin produced by each bacterial isolate's cells in

TABLE 3. Comparisons of the total toxin concentrations from equivalent volumes of 24-h bacterial cultures and 28-day dinoflagellate cultures

Strain	Total SCB toxicity (10^4 fmol of STX equivalents/ml of culture)			Bacterial toxicity[a]
	Dinoflagellates[b]	Bacteria		
		Intracellular	Extracellular	
NEPCC 667	2.4 ± 0.06	0.04 ± 0.003	14 ± 0.7	583
NEPCC 407	10 ± 0.53	0.02 ± 0.0008	21 ± 1.2	210
NEPCC 253	34 ± 0.71	0.08 ± 0.007	8.5 ± 0.6	25
UW4	48 ± 0.48	0.01 ± 0.0005	29 ± 0.9	60
UW2c	5 ± 0.2	0.03 ± 0.0006	26 ± 1.7	520

[a] As a percentage of dinoflagellate toxicity.
[b] Intracellular.

pure culture, a comparison of the bacterial contribution to the toxicity of each dinoflagellate culture was calculated. This indicated that culturable bacteria could account for a maximum of 3.1% (based on the figures for culture NEPCC 667) of the total toxicity of the dinoflagellate culture.

Detection of PST in bacterial cell extracts by ELISA. Bacterial cell extracts were exposed to anti-STX antibodies from a commercial competitive binding ELISA. All five bacterial cell extracts elicited a positive response; however, since the anti-STX antibody cross-reacts with neo-STX, dc-STX, GTX 1 through 4, B1, C1, and C2 (51, 52), it was not possible to determine which toxins were responsible for the reaction.

Detection of PST in bacterial cell extracts by HPLC. In efforts to confirm that the SCB activity of the cell extracts was due to PST, an initial evaluation was undertaken by precolumn HPLC (12). With this technique, several peaks which coeluted with PST standards were obtained. In order to confirm that the sample peaks corresponded with the exact retention times of commercially supplied toxins, the samples were spiked with these standards and the resultant retention times were observed. In all instances, the standard peaks overlaid the sample peaks, suggesting that they were equivalent (data not shown).

Subsequently, the samples were analyzed by the more time-consuming, two-solvent, postcolumn HPLC method (16), which has the advantages of greater sensitivity and better resolution. By using the first-solvent system, peaks which coeluted with STX and neo-STX alongside unresolved peaks representing the C and GTX toxins were detected in these five strains. The latter were separated into individual components by using the second-solvent system. This resulted in the identification of GTX 1 and GTX 4 in each bacterial strain and the detection of GTX 2 and GTX 3 in some of the strains at very low levels. Figure 2 shows typical profiles for three of the bacterial strains and GTX standards. Acid hydrolysis of bacterial cell extracts and the resultant increase in the equivalent GTX peaks (16) confirmed the presence of C toxins in strains 667-2, 407-2, and 253-19.

Overall, the results from the two different chromatographic systems supported the contention that the SCB toxins in bacterial extracts were PST.

Screening bacterial cell extracts for PST by CE-MS. Four of the bacterial cell extracts were analyzed further by selected ion monitoring by CE-MS at *m/z* 300 and 316, which indicated the presence of STX and neo-STX ions in all bacterial extracts. The presence of STX was further verified by an increase in the STX peak upon spiking with 0.89 μg of STX per ml (Fig. 3). A combination of ions having *m/z* values of 380, 396, 412, 476, and 492 was also detected, indicating that GTX and C toxins were also present. In most instances, the identifications of toxins by HPLC were supported by the presence of the corresponding ions by CE-MS (Table 4).

DISCUSSION

The MNB assay (18) was used extensively in this study for the initial screening of bacteria and the subsequent quantification of toxins. The data generated indicated that a high proportion of bacteria from the five *Alexandrium* cultures were capable of producing SCB toxins. The quantities of toxins obtained were similar to those previously described for bacteria isolated from seawater, fish, and shellfish (18, 21). However, although this assay is highly specific for the detection of SCB toxins (5, 20, 26) and more sensitive than the mouse bioassay (20, 26), it cannot distinguish PST from the other SCB toxin group, the tetrodotoxins (TTX). Further analysis by a variety of methods was therefore required to determine which, if any, of the PST group of toxins were present in bacterial cell extracts of a selected number of SCB-positive strains.

Initial screening, utilizing antibodies to STX (51, 52) and precolumn HPLC (12), indicated that PST were indeed present in bacteria. Subsequent detailed analysis by postcolumn HPLC (16) demonstrated that GTX 1 and GTX 4 were common to all five bacterial cell extracts. STX, neo-STX, GTX 2, GTX 3, and the C toxins were also detected in some of the strains, but at much lower levels. These toxin profiles are similar to those recently reported for the original PST-producing

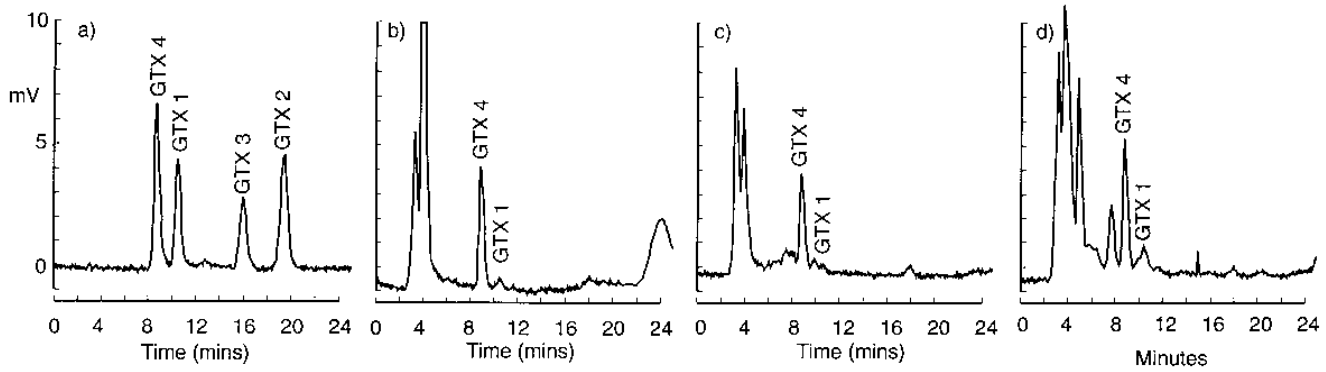


FIG. 2. Profiles of bacterial cell extracts obtained by postcolumn HPLC, operated under the solvent system specific for the detection of GTX toxins. (a) GTX 1, GTX 2, GTX 3, and GTX 4 standards; (b through d) GTX profiles of 407-2, 253-19, and UW4-1 bacterial cell extracts, respectively.

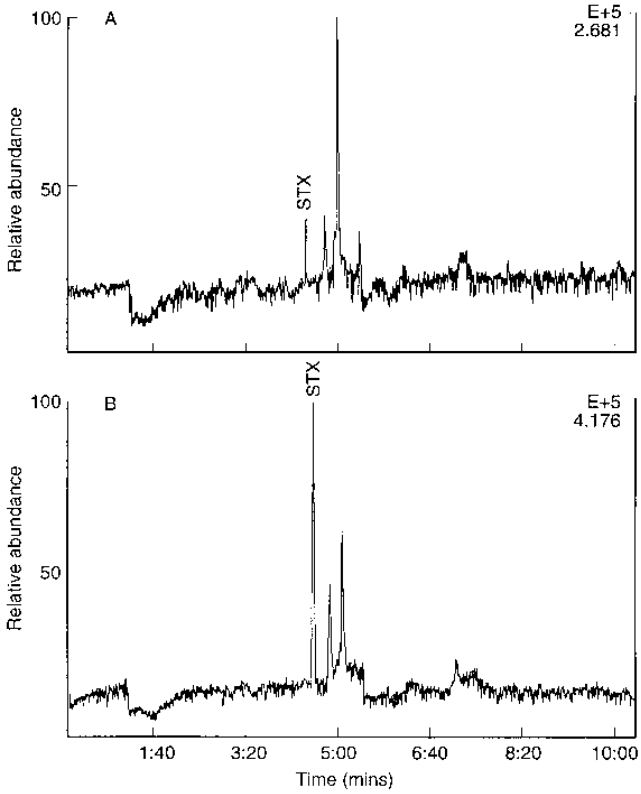


FIG. 3. Ion monitoring electrophoretogram of UW4-1 bacterial cell extract. (A) Selected ion monitoring electrophoretogram of UW4-1 bacterial cell extract, using *m/z* 300, the protonated molecular ion for STX; (B) selected ion monitoring electrophoretogram of an aliquot of UW4-1 bacterial cell extract spiked with standard STX (0.89 μg/ml), using *m/z* 300 to monitor the protonated molecular ion of STX. The *y* axis measures relative abundance, which normalizes to the height of the largest peak; hence, in panel A, the scale is normalized to the unknown peak adjacent to the STX peak. However, upon spiking of the cell extract with STX, the STX peak becomes the higher of the two; thus, in panel B, the scale is normalized to the STX peak and the unknown peak appears to be smaller.

bacterium, a *Moraxella* sp. (11), and for the dinoflagellates of origin (6, 7, 17, 36).

The data obtained by HPLC were supported by CE-MS, although in some instances the identities of a few toxins were not confirmed in the latter. This may be attributed to the lyophilization step incorporated into the CE-MS methodology and to differences in the detection limits for particular toxins. CE-MS also indicated that TTX may have been present in some of the bacterial extracts; however, further work is required to clarify this.

Kodama's group has reported that SCB toxin-producing bacteria can be isolated from a range of dinoflagellates obtained from Japanese waters (39). The work reported here demonstrated that SCB toxin-producing bacteria were associated with *A. affine*, *A. tamarense*, and *A. lusitanicum*, which were originally isolated from five separate sources of wide geographic separation, thereby extending Kodama's original observations to dinoflagellates obtained from North America and Europe. In addition, the isolation of PST-producing bacteria from *A. lusitanicum* NEPCC 253 supports recent reports from another independent source (14, 15) of the association of toxic bacterial strains with this dinoflagellate.

This study also presents data which allow a comparison of the quantities of PST produced by bacteria and dinoflagellates. Since it is generally accepted that the toxins remain in the dinoflagellate cell (2), although to our knowledge this has not been fully investigated, the dinoflagellate cell toxicity was compared to the total bacterial toxicity (i.e., intracellular and extracellular).

As the dinoflagellate cells were not axenic, the comparison is more realistically described as one between pure cultures of bacteria and dinoflagellate cells with their associated microflora. Therefore, several factors must be borne in mind. The first is that the number of bacteria obtained from dinoflagellate cultures (Table 1) represents the number of culturable bacteria under the conditions used. Since reports that less than 1% of marine bacteria are currently culturable in standard laboratory media exist (43), this count may not be an accurate representation of the true number of associated bacteria. In addition, the proportion of the dinoflagellate microflora which consists of the isolates described in Table 2 is unknown. The final point is that the culture conditions used for bacteria do not reflect their in vivo environment, where they are likely to adhere to or be under the dinoflagellate theca (14, 41) and are exposed to dinoflagellate exudate. Bacterial adhesion can lead to changes in phenotypic expression which, in some instances, are shown by increased production of cellular products (10); it is not known if similar effects occur in this system.

Despite these points, some information can be obtained by a comparison of the toxicity data. The results in Table 2 demonstrate that although dinoflagellates were harvested at the stationary phase of their life cycle, when toxin production may not have been optimum (2, 4), dinoflagellates produced substantially more toxin than did cultured bacteria on a per cell basis. However, it is apparent from the literature that dinoflagellates have biovolume values which are up to 20,000 times larger than those of bacteria (23, 24, 29, 32, 47). Estimating the quantity of toxin produced on this basis indicates that in some instances, bacteria can produce quantities of PST equivalent to those produced by dinoflagellates. In addition, the smaller bacterial size is negated when the number of each

TABLE 4. PST detected in bacterial cell extracts by HPLC and CE-MS

Toxin(s)	Detection in bacterial cell extracts of[a]:							
	667-2		407-2		UW4-1		UW2c-6	
	HPLC	CE-MS	HPLC	CE-MS	HPLC	CE-MS	HPLC	CE-MS
STX	+	+	+	+	+	+	+	+
Neo-STX	+	+	+	+	+	+	+	+
GTX 2 and 3, B2	ND	+	+	+	ND	ND	ND	ND
GTX 1 and 4	+	ND	+	+	+	?	+	ND
C toxins	+	+	+	+	+	+	ND	+

[a] +, toxin detected; ND, not detected; ?, result ambiguous due to proximity to the detection limit.

organism present in culture is considered (i.e., bacteria, approximately 10^9 cells/ml in culture; dinoflagellates, 10^4/ml). This difference in cell numbers means that the lower quantity of toxin produced by bacterial cells is balanced by their increased numbers in pure culture (Table 3). Obviously, further work is required to refine this observation, with more in-depth studies of the effects of different stages of both organisms' life cycles on biovolume and toxin production.

The data were also used to calculate that the contribution of culturable bacteria to a dinoflagellate culture's toxicity was approximately 3%. However, this value suffers greatly from the variables explained above. In addition, it is not known if bacterial cells associated with dinoflagellates excrete PST in the same manner as those grown in batch culture do. Therefore, a realistic conclusion is that bacteria can contribute to the toxicity of a dinoflagellate culture, but the significance to dinoflagellate toxicity requires further elucidation.

Indeed, much further work is required to explain the role of bacteria in dinoflagellate toxicity. Initial steps to meet this objective include molecularly based studies to define the bacterial population of dinoflagellate cultures and to determine if the bacteria isolated in this study are representative of the total population. In addition, experiments which mimic more closely the conditions found in vivo (i.e., taking into account the possible effects of adhesion and dinoflagellate exudate) are being designed. Ultimately, this work will be advanced considerably by obtaining axenic dinoflagellate cultures. Although several authors have reported that such cultures exist, the true bacterium-free status of those currently available (4, 25, 28, 48) is open to question, primarily due to the methods used to determine the extent of bacterial contamination. An alternative approach is that adopted by Ishida's group (25, 42), who, through cross-hybridization experiments with dinoflagellates, have claimed to show that toxin production is encoded by the dinoflagellate chromosome; it could be argued, though, that those reports do not demonstrate toxin synthesis but only the inheritance of toxin profiles.

Aside from dinoflagellates, it should be realized that bacterial production of PST is of interest, independent of the topic of PSP, especially as bacteria also produce the other SCB toxins, the TTX group (38, 53). Questions as to what the possible function, if any, these SCB toxins may have in bacteria can be raised. Previous work has indicated that the TTX group are secondary metabolites in some bacterial species (19). Are they merely by-products of cell metabolism? Alternatively, do they have a purpose, perhaps in sodium regulation? This is an area which is worthy of further investigation.

Overall, this study provides strong evidence that a range of bacterial species isolated from dinoflagellate cultures are capable of autonomous production of PST. However, there is much scope for further investigation into the precise relationship between bacteria and dinoflagellates in toxin production and PSP events, particularly in determining which genetic elements and environmental factors are involved.

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REFERENCES

1. **Anderson, D. M., J. J. Sullivan, and B. Reguera.** 1989. Paralytic shellfish poisoning in Northwest Spain: the toxicity of the dinoflagellate *Gymnodinium catenatum*. Toxicon **27:**665–674.
2. **Anderson, D. M., D. M. Kullis, J. J. Sullivan, S. Hall, and C. Lee.** 1990. Dynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp. Mar. Biol. **104:**511–524.
3. **Baden, D. G., and V. L. Trainer.** 1993. Mode of action of toxins of seafood poisoning, p. 49–74. *In* I. R. Falconer (ed.), Algal toxins in seafood and drinking water. Academic Press, London, United Kingdom.
4. **Boczar, B. A., M. K. Beitler, J. Liston, J. J. Sullivan, and R. A. Cattolico.** 1988. Paralytic shellfish toxins in *Protogonyaulax tamarensis* and *Protogonyaulax catenella* in axenic culture. Plant Physiol. **88:**1285–1290.
5. **Catterall, W. A., and M. Nirenberg.** 1973. Sodium uptake associated with activation of action potential ionophores of cultured neuroblastoma and muscle cells. Proc. Natl. Acad. Sci. USA **70:**3759–3763.
6. **Cembella, A. D., J. J. Sullivan, G. L. Boyer, F. J. R. Taylor, and R. J. Andersen.** 1987. Variation on paralytic shellfish toxin composition within the *Protogonyaulax tamarensis*/*catenella* species complex: red tide dinoflagellates. Biochem. Syst. Ecol. **15:**171–186.
7. **Cembella, A. D., J. C. Therriault, and P. Beland.** 1988. Toxicity of cultured isolates and natural populations of *Protogonyaulax tamarensis* from the St. Lawrence estuary. J. Shellfish Res. **7:**611–621.
8. **Cembella, A. D., and G. Lamoureux.** 1993. A competitive inhibition enzyme linked immunoassay for the detection of paralytic shellfish toxins in marine phytoplankton, p. 857–862. *In* T. J. Smayda and Y. Shimizu (ed.), Toxic phytoplankton blooms in the sea. Elsevier, Amsterdam, The Netherlands.
9. **Cembella, A. D., S. E. Shumway, and R. Larocque.** 1994. Sequestering and putative biotranformation of paralytic shellfish toxins by the sea scallop *Placopecten magellanicus*: seasonal and spatial scales in natural populations. J. Exp. Mar. Biol. Ecol. **180:**1–22.
10. **Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott.** 1995. Microbial biofilms. Annu. Rev. Microbiol. **49:**711–745.
11. **Doucette, G. J., and C. G. Trick.** 1995. Characterisation of bacteria associated with different isolates of *Alexandrium tamarense*, p. 33–38. *In* P. Lassus, G. Arzul, E. Erard-LeDenn, P. Gentien, and C. Marcaillou-Le Baut (ed.), Harmful marine algal blooms. Lavoisier, Paris, France.
12. **Flynn, K. J., and K. Flynn.** 1996. An automated HPLC method for the rapid analysis of paralytic shellfish toxins from dinoflagellates and bacteria using low temperature precolumn oxidation. J. Exp. Mar. Biol. Ecol. **197:**145–157.
13. **Fraga, S., D. M. Anderson, I. Bravo, B. Reguera, K. A. Steidinger, and C. M. Yentsh.** 1988. Influence of upwelling relaxation on dinoflagellates and shellfish toxicity in Ria de Vigo, Spain. Estuarine Coastal Shelf Sci. **27:**349–361.
14. **Franca, S., S. Viegas, V. Mascarenhas, L. Pinto, and G. J. Doucette.** 1995. Prokaryotes in association with a toxic *Alexandrium lusitanicum* in culture, p. 45–51. *In* P. Lassus, G. Arzul, E. Erad-LeDenn, P. Gentien, and C. Marcaillou-Le Baut (ed.), Harmful marine algal blooms. Lavoisier, Paris, France.
15. **Franca, S., L. Pinto, P. Alvito, I. Sousa, and V. Vasconcelos.** 1996. Studies on prokaryotes associated with PSP producing dinoflagellates. *In* T. Yasumoto, Y. Oshima, and Y. Fukugo (ed.), Harmful and toxic algal blooms. Intergovernmental Oceanographic Commission of UNESCO.
16. **Franco, J. M., and P. Fernandez-Vila.** 1993. Separation of PSP toxins by reverse phase HPLC, with postcolumn reaction and fluorimetric detection. Chromatography **35:**613–620.
17. **Franco, J. M., S. Fraga, M. Zapata, I. Bravo, P. Fernandez, and I. Ramilo.** 1995. Comparison between different strains of genus *Alexandrium* spp. of the *minutum* group, p. 53–58. *In* P. Lassus, G. Arzul, E. Erad-LeDenn, P. Gentien, and C. Marcaillou-Le Baut (ed.), Harmful marine algal blooms. Lavoisier, Paris, France.
18. **Gallacher, S., and T. H. Birkbeck.** 1992. A tissue culture assay for the direct detection of sodium channel blocking toxins in bacterial culture supernates. FEMS Microbiol. Lett. **92:**101–108.
19. **Gallacher, S., and T. H. Birkbeck.** 1993. Effect of phosphate concentration on production of tetrodotoxin by *Alteromonas tetraodonis*. Appl. Environ. Microbiol. **59:**3981–3983.
20. **Gallacher, S., K. Evans, M. Blackburn, M. Waldock, and T. H. Birkbeck.** 1993. Tissue culture assay for paralytic shellfish poisons, p. 203–210. *In* R. G. Kroll, A. Gilmour, and M. Sussman (ed.), New techniques in food and beverage microbiology. Blackwell Scientific Publications, Oxford, United Kingdom.
21. **Gallacher, S., and T. H. Birkbeck.** 1995. Isolation of marine bacteria producing sodium channel blocking toxins and the seasonal variation in their frequency in seawater, p. 445–450. *In* P. Lassus, G. Arzul, E. Erad-LeDenn, P. Gentien, and C. Marcaillou-Le Baut (ed.), Harmful marine algal blooms. Lavoisier, Paris, France.
22. **Guillard, R. R. L.** 1975. Culture of phytoplankton for feeding marine invertebrates, p. 29–60. *In* W. L. Smith and M. H. Chanley (ed.), Culture of marine invertebrate animals. Plenum Publishing Corp., New York, N.Y.
23. **Heldal, M., S. Norland, G. Bratbak, and B. Riemann.** 1994. Determination of bacterial cell number and cell volume by means of flow cytometry, transmission electron microscopy, and epifluorescence microscopy. J. Microbiol. Methods **20:**255–263.

24. **Hitchcock, G. L.** 1982. A comparative study of the size-dependent organic composition of marine diatoms and dinoflagellates. J. Plant Res. **4:**363–377.
25. **Ishida, Y., C. H. Kim, Y. Sako, N. Hirooka, and A. Uchida.** 1993. PSP toxin is chromosome dependent in *Alexandrium* spp., p. 881–887. *In* T. J. Smayda and Y. Shimizu (ed.), Toxic phytoplankton blooms in the sea. Elsevier, Amsterdam, The Netherlands.
26. **Jellet, J. F., L. J. Marks, J. E. Stewart, M. L. Dorey, W. Watson-Wright, and J. F. Lawrence.** 1993. Paralytic shellfish poison bioassays: automated endpoint determination and standardization of the *in vitro* tissue culture bioassay, and comparison with the standard mouse bioassay. Toxicon **30:**1143–1156.
27. **Kao, C. Y.** 1993. Paralytic shellfish poisons, p. 75–86. *In* I. R. Falconer (ed.), Algal toxins in seafood and drinking water. Academic Press, London, United Kingdom.
28. **Kim, C. H., Y. Sako, and Y. Ishida.** 1993. Variation of toxin production and composition in axenic cultures of *Alexandrium catenella* and *A. tamarense*. Nippon Suisan Gakkaishi **59:**633–639.
29. **Kim, G. H., J. S. Park, S. G. Lee, and K. H. An.** 1993. Population cell volume and carbon content in monospecific dinoflagellate blooms, p. 769–773. *In* T. J. Smayda and Y. Shimizu (ed.), Toxic phytoplankton blooms in the sea. Elsevier, Amsterdam, The Netherlands.
30. **Kodama, M., T. Ogata, and S. Sato.** 1988. Bacterial production of saxitoxin. Agric. Biol. Chem. **52:**1075–1077.
31. **Kodama, M., T. Ogata, S. Sakamoto, S. Sato, T. Honda, and T. Miwatani.** 1990. Production of paralytic shellfish toxins by a bacterium *Moraxella* sp. isolated from *Protogonyaulax tamarensis*. Toxicon **28:**707–714.
32. **Kroer, N.** 1994. Relationship between biovolume and carbon and nitrogen content of bacterioplankton. FEMS Microbiol. Ecol. **13:**217–224.
33. **Lewis, J.** 1990. The cyst-theca relationship of *Oblea rotunda* (Diplopsalidaceae, Dinophyceae). Br. Phycol. J. **25:**339–351.
34. **Lewis, J.** 1991. Cyst-theca relationships in *Scrippsiella* (Dinophyceae) and related orthoperidinioid genera. Bot. Mar. **34:**91–106.
35. **Lewis, J., S. Gallacher, and W. A. Higman.** Unpublished data.
36. **Mascarenhas, V., P. Alvito, S. Franca, I. Sousa, A. G. Martinez, and J. A. Rodriguez-Vazquez.** 1995. The dinoflagellate *Alexandrium lusitanicum* isolated from the coast of Portugal: observations on toxicity and ultrastructure during growth phases, p. 71–76. *In* P. Lassus, G. Arzul, E. Erad-LeDenn, P. Gentien, and C. Marcaillou-Le Baut (ed.), Harmful marine algal blooms. Lavoisier, Paris, France.
37. **Nelinda, M., V. Dimanlig, and F. J. R. Taylor.** 1985. Extracellular bacteria and toxin production in *Protogonyaulax* species, p. 103–108. *In* D. M. Anderson, A. W. White, and D. G. Baden (ed.), Toxic dinoflagellates. Elsevier, Amsterdam, The Netherlands.
38. **Noguchi, T., J. K. Jeon, O. Arakawa, H. Sugita, Y. Deguchi, Y. Shida, and K. Hashimoto.** 1986. Occurrence of tetrodotoxin and anhydrotetrodotoxin in *Vibrio* sp. isolated from the intestines of a xanthid crab, *Atergatis floridus*. J. Biochem. **99:**311–314.
39. **Ogata, T., M. Kodama, K. Komaru, S. Sakamoto, S. Sato, and U. Simidu.** 1990. Production of paralytic shellfish toxins by bacteria isolated from toxic dinoflagellates, p. 311–315. *In* E. Graneli, B. Sundstrom, L. Edler, and D. M. Anderson (ed.), Toxic marine phytoplankton. Elsevier, Amsterdam, The Netherlands.
40. **Oshima, Y., S. I. Blackburn, and G. M. Hallegraeff.** 1993. Comparative study on paralytic shellfish toxin profiles of the dinoflagellate *Gymnodinium catenatum* from three different countries. Mar. Biol. **116:**471–476.
41. **Rausch de Traubenberg, C., and M. O. Soyer-Gobillard.** 1990. Bacteria associated with a photosynthetic dinoflagellate in culture. Symbiosis **8:**117–133.
42. **Sako, Y., C. H. Kim, and Y. Ishida.** 1992. Medelian inheritance of paralytic shellfish poisoning toxin in the marine dinoflagellate *Alexandrium catenella*. Biosci. Biotechnol. Biochem. **56:**692–694.
43. **Schut, F., E. J. de Vries, J. C. Gottschal, B. R. Robertson, W. Harder, R. A. Prins, and D. K. Button.** 1993. Isolation of typical marine bacteria by dilution culture: growth, maintenance, and characteristics of isolates under laboratory conditions. Appl. Environ. Microbiol. **59:**2150–2160.
44. **Silva, E. S.** 1962. Some observations on marine dinoflagellate cultures. Notas Estud. Inst. Biol. Marit. **26:**1–11.
45. **Silva, E. S.** 1982. Relationship between dinoflagellates and intracellular bacteria. Mar. Al. Pharmacol. Sci. **2:**269–288.
46. **Silva, E. S.** 1990. Intracellular bacteria: the origin of dinoflagellate toxicity. J. Pathol. Toxicol. Onol. **19:**124–128.
47. **Sime-Ngando, T., G. Bourdier, C. Amblard, and B. Pinel-Alloul.** 1991. Short-term variations in specific biovolumes of different forms in aquatic ecosystems. Microb. Ecol. **21:**211–226.
48. **Singh, H. T., Y. Oshima, and T. Yasumoto.** 1982. Growth and toxicity of *Protogonyaulax tamarensis* in axenic culture. Bull. Jpn. Soc. Sci. Fish. **48:**1341–1343.
49. **Steidinger, K. A.** 1993. Some taxonomic and biological aspects of toxic dinoflagellates, p. 1–28. *In* I. R. Falconer (ed.), Algal toxins in seafood and drinking water. Academic Press, London, United Kingdom.
50. **Sullivan, J. J.** 1993. Methods of analysis for algal toxins: dinoflagellates and diatom toxins, p. 29–48. *In* I. R. Falconer (ed.), Algal toxins in seafood and drinking water. Academic Press, London, United Kingdom.
51. **Uselber, E., R. Dietrich, E. Martlbauer, and G. Terplan.** 1994. Effect of heterologous paralytic shellfish poisoning toxin-enzyme conjugates on the cross-reactivity of a saxitoxin enzyme immunoassay. Lett. Appl. Microbiol. **18:**337–339.
52. **Uselber, E., and E. Martlbauer.** Unpublished data.
53. **Yasumoto, T., D. Yasumura, M. Yotsu, T. Michishita, A. Endo, and Y. Kotaki.** 1986. Bacterial production of tetrodotoxin and anhydrotetrodotoxin. Agric. Biol. Chem. **50:**793–795.