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Associated Bacterial Flora, Growth, and Toxicity of Cultured Benthic Dinoflagellates *Ostreopsis lenticularis* and *Gambierdiscus toxicus*

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The growth, toxicity, and associated bacterial flora of 10 clonal cultures of the toxic benthic dinoflagellates *Ostreopsis lenticularis* and *Gambierdiscus toxicus* isolated from the coastal waters of southwest Puerto Rico have been examined. Clonal cultures of *O. lenticularis* grew more rapidly and at broader temperature ranges than those of *G. toxicus*. All five *Ostreopsis* clones were toxic, while only one of the five *Gambierdiscus* clones was poisonous. The degree of toxicity among poisonous clones was highly variable. The number of associated bacterial genera and their frequency of occurrence were quite variable among clones of both dinoflagellate genera. Bacterial isolates represented six genera (*Nocardia*, *Pseudomonas*, *Vibrio*, *Aeromonas*, *Flavobacterium*, and *Moraxella*) in addition to coryneform bacteria. Extracts of dinoflagellate-associated bacteria grown in pure culture were not toxic. *Gambierdiscus* clones were characterized by the frequent presence of *Pseudomonas* spp. (four of five clones) and the absence of coryneforms. In *O. lenticularis*, only one of five clones showed the presence of *Pseudomonas* spp., and *Moraxella* sp. was absent altogether. Detailed analyses of toxicity and associated microflora in a selected *Ostreopsis* clone, repeatedly cultivated (four times) over a period of 160 days, showed that peak cell toxicities developed in the late static and early negative culture growth phases. Peak *Ostreopsis* cell toxicities in the stationary phase of culture growth were correlated with significant increases in the percent total bacteria directly associated with these cells. Changes in the quantity of bacteria directly associated with microalgal cell surfaces and extracellular matrices during culture growth may be related to variability and degree of toxicity in these laboratory-cultured benthic dinoflagellates.

Toxins produced by marine dinoflagellates are among the most potent nonproteinaceous poisons known (4, 30, 31). Twenty-two dinoflagellate species are known to produce toxins (31, 34). These toxins include both water- and lipid-soluble moieties, which have hemolytic, neurotoxic, and gastrointestinal inflammatory activities. Toxins produced by *Gambierdiscus toxicus* Adachi and Fukuyo (2, 38, 39), *Ostreopsis lenticularis* Fukuyo (40), and other benthic dinoflagellates (32) have been proposed to be linked with ciguatera fish poisoning.

Fluctuations in natural populations of *G. toxicus* and its ecological associate, *O. lenticularis*, in Puerto Rico have been documented for several years (5, 5a). Both species form occasional blooms, with *O. lenticularis* reaching densities of greater than 100,000 cells per g (wet weight) of algal host tissue. Puerto Rican *G. toxicus* and *O. lenticularis* produce toxins that are lethal to mice. Recently reported toxicity in *O. lenticularis* is the first found for this species (34). While toxicity in *G. toxicus* has been recognized for some time, questions remain as to the number of toxins actually produced and their precise chemical nature. Chemical or pharmacological similarities between toxins produced by these sources have not been determined.

In nature, most microorganisms are found in heterogeneous aggregations with suspended detrital material or adhered to macroalgal, animal, and inanimate surfaces. These surface interactions are dynamic in nature and are important factors in microbial proliferation and survival. Aquatic algae in situ as well as in laboratory culture are often found to be associated with a variety of bacterial strains (6, 8, 17, 24, 25). The inter- and intraspecific interactions of marine microbial cells are frequently mediated by macromolecular surface components, suggesting the presence of specific receptor-ligand binding sites on interactive surfaces (16, 33, 35). Recently reported analyses of bacterial-microalgal interactions in a consortium of three bacteria associated with a chlorella-like green alga illustrate the complexity of these relationships (19). Bacteria are universally associated with algae in the ocean. The fact that many algae grow more slowly, if at all, in axenic rather than bacterized cultures suggests that the associations constitute a form of symbiosis.

Production of toxins by marine macrobiota and microalgal cells, associated with variable microbial consortia, poses important questions regarding the precise origin and mechanism of synthesis of the toxins in question. Thus, it has been suggested that palytoxin, one of the most potent marine toxins, may well be produced by a *Vibrio* bacterial symbiont of the zooanthid *Palythoa* sp., originally thought to be the sole source of this toxin (22, 23). The highly variable toxicity of *Palythoa* specimens taken at different geographical sites initially suggested the possible involvement of other factors in the elaboration of this toxin. Large variations in toxin production by strains of the dinoflagellate *Protogonyalaux tamarensis* (Lebour) Taylor from different geographical locations have also been reported (27). Four pure, distinct toxins were isolated from this dinoflagellate. Variability in clone toxin potency and the increasing numbers of dinoflagellate species associated with toxins suggest that dinoflagellate toxin production may be linked to symbiotic or contaminant bacteria (30, 31). Grown in bacterium-free (axenic) culture, *Ptychodiscus brevis* (Davis) Steidinger has been reported to retain toxicity, while *Gymnodinium veneficum* Ballantine lost toxicity (1, 26). Toxicity of *Prorocentrum minimum* Schiller and *Protogonyalaux tamarensis* has

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been proposed to be due to the presence of bacteria associated either with the medium or dinoflagellate endosymbionts (28). These investigators isolated a bacterial strain, a *Pseudomonas* sp., from cultures of *Protogonyalaux tamarensis* that induced toxicity when inoculated into cultures of a previously nontoxic *Gyrodinium* strain.

The work reported here was undertaken to identify the bacteria associated with benthic dinoflagellates *O. lenticularis* and *G. toxicus* originally isolated from coastal waters of southwest Puerto Rico and grown in clonal laboratory culture. Studies were conducted to ascertain the relationship of diversity, quantity, and direct microalgal cell association of these bacterial strains with dinoflagellate culture growth and toxicity.

MATERIALS AND METHODS

Dinoflagellate culture. Clonal cultures were obtained by the methods of Lewin (20) as adapted for benthic dinoflagellate isolation by Yasumoto et al. (39) and Ballantine et al. (5). Clonal isolates were obtained by transferring individual cells through three sterile seawater washes with drawn Pasteur pipettes. After the third wash, a single cell was similarly transferred into a test tube containing 10 ml of sterile f/2 medium (14, 21) with added germanium dioxide (5 mg/liter). Germanium dioxide is an inhibitor of silicon metabolism, and its presence in culture medium controls diatom contamination (20). Test tubes were then placed under reduced light conditions and were left undisturbed for approximately 1 month. In our experience, approximately 20% of the inoculated test tubes yielded viable clonal cultures.

Stock cultures of dinoflagellates were grown in standard f/2 medium prepared with artificial seawater. A water-soluble extract (0.1% by volume) of the red alga *Acanthophora spicifera* was added (Withers, personal communication), as it has been shown to increase the growth rate of both *G. toxicus* and *O. lenticularis* in culture. Optimal conditions for support of growth of *O. lenticularis* and *G. toxicus* with respect to light and temperature were evaluated with a cross-gradient culture apparatus (13). Experiments were conducted in stoppered 50-ml flasks containing initial concentrations of 75 dinoflagellate cells per ml. Light regimens of 20, 60, and 100 microeinsteins/m^2 per s combined with temperature regimens ranging from 20 to 30°C were tested. On the basis of these analyses, subsequent cultures were grown at 27°C in a light-to-dark regimen of 16:8 and at a light flux of 50 microeinsteins/m^2 per s.

For purposes of establishing base-line information concerning clonal toxicity and associated bacterial flora, *G. toxicus* and *O. lenticularis* clones were grown in batch cultures (3 to 6 liters) under the culture conditions described above. Cells were harvested by screening (35-μm mesh) after appropriate periods of growth. One *Ostreopsis* clone (no. 116) was selected for analyses of growth and toxicity in a series of experiments each lasting from 28 to 49 days. Experiments were initiated at dinoflagellate concentrations of 75 cells per ml in 2.5-liter Fernbach flasks containing 1 liter of culture. Growth was evaluated microscopically by using a Sedgewick Rafter counting cell after 4 and 7 days and then at weekly intervals until the termination of the particular experiment. On each of these sampling days, a variable number of flasks (1 to 4) were selected for harvest.

Associated bacterial flora. Bacteria associated with all dinoflagellate clones were isolated in pure culture by repeated streaking on nutrient seawater agar, and isolates were identified to the generic level (18, 34). Classifications were made by using API 20E and other biochemical and antibiotic tests. Bacteria of the genus *Vibrio* were distinguished from nonfermenting *Pseudomonas* spp. by using 0/129 (2,4-diamino-6,7-diisopropyl-pteridine phosphate) disks at concentrations of 150 and 10 μg.

In *O. lenticularis* culture flasks selected for toxicity analyses, densities of the total bacterial populations relative to dinoflagellate cells (total bacterial cell/dinoflagellate cell ratio) and proportions of these bacteria directly associated with dinoflagellate surfaces and extracellular matrices were estimated by epifluorescence microscopy (15). The total bacterial cell/dinoflagellate cell ratio was taken to be equal to the sum of the unassociated free bacteria in the medium/dinoflagellate cell ratio and the bacteria closely associated with the dinoflagellates/dinoflagellate cell ratio. Thus, the total bacterial cell/dinoflagellate cell ratio (A) minus the bacteria recovered free in the dinoflagellate culture medium/dinoflagellate cell ratio (B) equalled C, the fraction of bacteria directly associated with dinoflagellate cell surface per dinoflagellate cell. Samples (10 ml) of culture suspensions were sonicated (Megason; Ultrasonic Instruments International, Inc.) to disrupt bacterium-dinoflagellate cell attachments. The resulting suspensions were stained with acridine orange and layered on filters (Nuclepore Corp.) for counting and subsequent determination of the ratios of the total bacterial cells to dinoflagellate cells (ratio A) in the respective cultures. In addition, unsonicated samples (50 to 100 ml) from these culture flasks were gently passed through filters (Gelman A-E, 1.0-μm pore size) which retained intact dinoflagellate cells while allowing unattached free bacterial cells to pass into the filtrate. Concentrations of these bacteria in the dinoflagellate-free filtrates were determined by using epifluorescence techniques, and ratios of these unassociated bacterial cells to dinoflagellate cells (ratio B) were subsequently determined. These ratios were used to calculate percentages of bacteria directly associated with *Ostreopsis* cells as follows: $\%C/A = [1 - (B/A)] \times 100$.

Toxicity analyses. Harvested dinoflagellate cells were briefly rinsed with distilled water and were sonicated in redistilled methanol. Extracts (final volumes, approximately 100 ml) were kept at laboratory temperatures (22°C) for 48 h. Extract suspensions were filtered (Whatman no. 1 filter paper), and filtrate solvent was removed by flash evaporation (Buchi, Rotavapor). The resulting residues were dried under nitrogen and were stored in a vacuum desiccator for subsequent toxicity analyses. These procedures and the methods on which they are based have been reported elsewhere (34). Similar extracts were prepared from pure cultures of the dinoflagellate-associated bacterial strains. Isolates were individually grown in suspension culture for 48 h at laboratory temperatures (22°C) in a modified Zobell 2216E medium (5.0 g of yeast extract–1.0 g of peptone–0.01 g of $FePO_4$ in 500 ml of artificial seawater and 500 ml of distilled water). Following incubation, the medium was centrifuged (1 h at 27,000 × g in a Sorvall RC2-B centrifuge at 4°C) and the pellet was suspended in redistilled methanol and sonicated for extraction as described above.

Extracts were assayed for their toxicity in 20-g white Swiss CF1 mice. Known quantities of dried extracts to be tested were suspended in 0.15 M phosphate buffer solution (pH 7.4) containing 5% Tween 80 (34). Inocula of between 0.2 and 0.5 ml were administered by intraperitoneal injection. Control animals received injections of equal volumes of Tween–phosphate-buffered saline medium. Mice were observed for 48 h, and 50% lethal dose values were calculated by standard methods (42). Dinoflagellate extracts were ex-

amined at concentrations of 0.01 to 5 mg per animal (0.5 to 250 mg per kg of body weight). Each extract was tested at from four to eight concentrations, decreasing geometrically from the highest levels tested. Three or four mice were inoculated at each extract concentration in a given experiment. Toxicities of the extracts reported here are expressed in terms of "mouse units" (MU) per cell extracted. An MU was defined as the amount (in milligrams) of toxic extract inoculated into a single 20-g mouse that resulted in the death of 50% of the mice injected with this quantity of material. The total MU in an extract (i.e., milligrams of toxic extract per MU) was calculated and toxicity was expressed in terms of MU per cell (total MU per total number of cells extracted).

RESULTS AND DISCUSSION

Laboratory-cultured dinoflagellates. *O. lenticularis* demonstrated optimal growth (shortest mean generation time, 1.03 days) at 29°C and light fluxes of 60 and 100 microeinsteins/m^2 per s. In general, at temperatures less than 27°C, the mean generation time was shorter at light fluxes of 20 microeinsteins/m^2 per s than at 100 microeinsteins/m^2 per s. The reverse was true at temperatures greater than 29°C. Rapid growth could nevertheless be elicited at both high and low light levels by changes in temperature. For *Gambierdiscus* cultures, temperatures below 23°C and above 30°C were not suitable for growth. The shortest mean generation time obtained with *G. toxicus* was 4.40 days. Thus, cultures of *O. lenticularis* were capable of considerably more rapid growth at broader temperature ranges than those of *G. toxicus*.

Dinoflagellate-associated bacterial flora. A total of 41 bacterial isolates were recovered from the dinoflagellate clones examined in this study and were classified by genera. The 10 dinoflagellate clones examined had associated bacteria of six genera as follows: *Nocardia*, 20%; *Pseudomonas*, 50%; *Vibrio*, 30%; *Aeromonas*, 20%; *Flavobacterium*, 20%; and *Moraxella*, 10%. Coryneform bacteria were associated with 10% of the clones. *Pseudomonas* was the most frequently present genus. On the basis of the methods employed here, this genus may have included bacteria of the genus *Alteromonas*. An endocellular *Pseudomonas* species has been reported to play a role in determining toxicity in other dinoflagellates (28).

Dinoflagellate toxicity and associated bacteria. Thirty toxicity assays of dinoflagellate clone extracts were conducted. The results are summarized in Table 1. Of five isolates of Puerto Rican *G. toxicus*, only one clone proved to be toxic, while all five clones of Puerto Rican *O. lenticularis* were poisonous. Toxicity of *Gambierdiscus* clone 105 (162 MU/10^6 cells) was within the range of *Ostreopsis* clone toxicities (16 to 651 MU/10^6 cells) determined. *Ostreopsis* clones showed considerable variability in their toxicity. Assays of methanolic extracts made of pure cultures of the dinoflagellate-associated bacterial genera indicated that none of them were toxic.

Bacterial genera and their frequency of occurrence were quite variable among clones of both dinoflagellate genera. *Gambierdiscus* clones were characterized by the frequent presence of *Pseudomonas* spp. (four of five clones) and the absence of coryneforms. Only one of five *Ostreopsis* clones showed the presence of *Pseudomonas* spp., and *Moraxella* spp. were absent altogether. In toxic *Gambierdiscus* clone 105, the only bacterial genus found was *Pseudomonas*. It is of interest that the most toxic *Ostreopsis* clone (116-5) was the only clone of this dinoflagellate genus tested that had an associated *Pseudomonas* species. All *Ostreopsis* clones were toxic, and three of five clones had only one associated bacterial strain. No bacteria were found associated with *Ostreopsis* clone 117.

TABLE 1. Associated bacteria and dinoflagellate toxicity

Dinoflagellate clone	Associated bacterial genus or type[a]	Dinoflagellate toxicity (MU/10^6 cells)
G. toxicus		
94-13	N, V, A	0
101-16	P, M	0
105-13	P	162
106-13	P, V	0
107-24	P, F	0
O. lenticularis		
95-49	N, A	42
116-5	P, F	651
117-5	None found	45
119-4	V	53
120-2	c	16

[a] N, *Nocardia*; V, *Vibrio*; A, *Aeromonas*; P, *Pseudomonas*; M, *Moraxella*; F, *Flavobacterium*; c, coryneform bacteria.

Dinoflagellate culture growth, toxicity, and bacterial flora. *O. lenticularis* clone 116 was transferred 48 times between the first and fourth experiments. With increasing numbers of transfers, there was a reduction in the observed toxicity. Peak toxicity in the first experiment was 5,785 MU/10^6 cells and was only 49 MU/10^6 cells in the final experiment of the series, at the end of 1 year during which these experiments were conducted. These results differ from those of Durand-Clement (12), who reported little change in the toxicity of cultured *G. toxicus* over a period of 3 years. While peak cell densities did not show systematic change, the time required to achieve maximum cell densities increased from 14 (experiments 1 and 2) to 28 days (experiments 3 and 4). Despite these changes in toxicity and growth rate, *Ostreopsis* cells showed maximum toxicity on the same day of culture growth in all four experiments. The average relative growth rate of the cultures in all four experiments (the relative growth rate in each expressed as the ratio of the growth rate [reciprocal of the mean generation time $\times 10^2$] for any given interval of culture growth to that of the growth rate over the initial 4-day period of that experiment) is summarized in Fig. 1. In each experiment, cultures grew most rapidly during the initial 4-day period. The average relative toxicities of cells (expressed as the ratio of toxicity of the cells sampled at a given time to the toxicity of the cells used to initially inoculate the cultures in each experiment) showed peak values after 28 days of culture growth (Fig. 1). Peak cell toxicities were found in the late static and early negative growth phases of these cultures. Maximum cell toxicities have been reported elsewhere for cultures in exponential growth phase (9) as well as during stationary phase and the initial stages of negative growth (3, 7, 28, 29, 31, 40).

The genera of bacteria found with *Ostreopsis* clone 116 did not change during the course of these studies, suggesting a selectivity in the dinoflagellate-bacterial association similar to that reported in other algal-bacterial systems (11, 19). Figure 2 shows the changes in total bacterial cell/dinoflagellate cell ratio and fraction (percentage) of those bacteria closely associated with *Ostreopsis* clone 116 cell surfaces during culture growth. The total bacterial cell/dinoflagellate cell ratio remained essentially constant through the initial 28

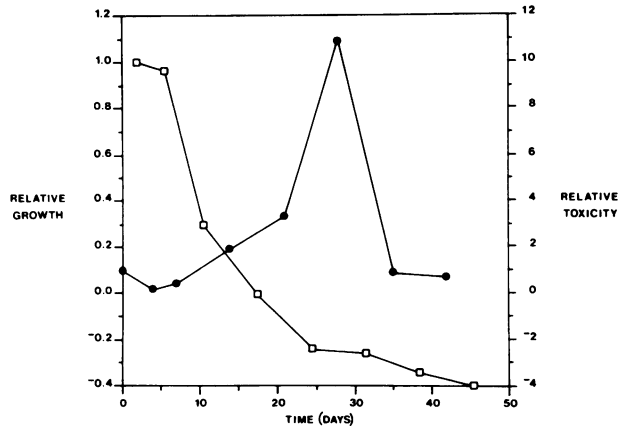

FIG. 1. Relative growth of *O. lenticularis* (□), expressed as the ratio of the growth rate (reciprocal of the generation time) for a given period to the growth rate of the culture during the first 4 days of culture growth, and the relative toxicity of the *Ostreopsis* cells (●), expressed as the ratio of the toxicity of the dinoflagellate cells (MU/10^6 cells) during a given period of culture growth to the toxicity of the cells used to initiate the cultures in this study, versus time of culture growth.

days of culture growth. Following this period, there was a steady, significant increase in the total bacterial cell/dinoflagellate cell ratio through 49 days of culture growth. The percent total bacteria directly associated with the dinoflagellate cells was high (above 70%) in the inocula used to initiate the dinoflagellate cultures in this study. This percentage decreased significantly (to values below 10%) during the first 7 days, followed by sharp increases (60 to 80%) at 21 to 35 days of culture growth. Chrost reported a general decline in bacterial populations during blooms of mixed phytoplankton, which he attributed to the production of allelopathic substances (10).

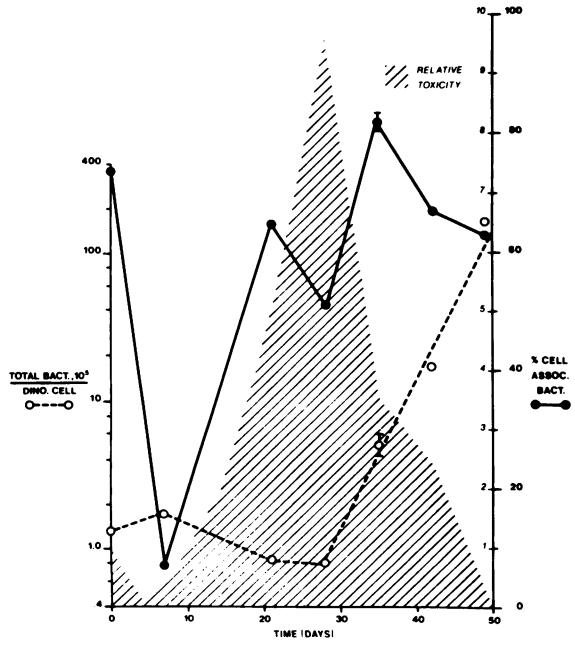

FIG. 2. The total bacterial cell/*O. lenticularis* cell ratio and the percent bacterial cells directly associated with these dinoflagellate cells, versus time of culture growth. Shaded area indicates the changes in the relative toxicity of *Ostreopsis* cells during the period of culture growth.

The dinoflagellate cells appeared to exercise some control on both the total density and distribution of the bacterial populations present in the respective culture flasks. Peak dinoflagellate culture growth rates (first 4 to 7 days of culture, Fig. 1) were associated with reduced numbers of bacteria directly associated with the dinoflagellate cells (Fig. 2), while peak relative dinoflagellate cell toxicity (Fig. 2, shaded area) was associated with a significantly increased fraction of closely associated bacteria. Later stages of culture growth (35 to 49 days) were marked by reductions in dinoflagellate cell toxicity and relatively uncontrolled increases in the total bacterial cell/dinoflagellate cell ratio. Increases in bacterial population densities associated with the decline of phytoplankton blooms have been reported elsewhere (10, 36).

Results presented here show that bacterial genera associated with *O. lenticularis* grown in clonal laboratory culture are not toxic when grown individually in pure culture. Marked increases in the proportion of these bacteria directly associated with the surfaces or extracellular matrices of these microalgal cells were correlated with the development of peak dinoflagellate toxicity during the static phase of their culture growth. Subsequent declines in dinoflagellate culture density and toxicity corresponded to uncontrolled increases in the total bacterial cell/dinoflagellate cell ratio and decreasing proportions of bacteria directly associated with *Ostreopsis* cells. These results suggest that associated bacterial flora may play a role in the phasic development of toxicity in laboratory growth cycles of these algal-bacterial consortia.

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