Enhancement of Domoic Acid Production by Reintroducing Bacteria to Axenic Cultures of the Diatom *Pseudo-nitzschia multiseries*

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**ABSTRACT**

Axenic cultures of *Pseudo-nitzschia multiseries* (formerly *Pseudonitzschia pungens* f. *multiseries*) produce less domoic acid (DA) than the original bacteria-containing cultures. Bacterial strains isolated from two nonaxenic *P. multiseries* clones were reintroduced individually into cultures of three axenic *P. multiseries* strains. The bacteria did not substantially affect division rates or cell yields. However, they did cause a 2- to 95-fold enhancement of DA production (per cell basis) relative to the axenic culture, depending on the *P. multiseries* and bacterial strain used. Bacteria isolated from a nontoxic *Chaetoceros* sp. culture also enhanced DA per cell (by 115-fold), showing that it is not necessary for the bacteria to be isolated from a toxic culture in order to enhance toxin production. There was no evidence of intracellular bacteria in disrupted *P. multiseries* cells obtained from axenic cultures. Our results demonstrate an important, but nonessential, role of extracellular bacteria in DA production. Characterization of the bacterial strains using morphology, substrate utilization, and restriction fragment length polymorphism (RFLP) analyses clearly showed that we had isolated different species of bacteria from the various nonaxenic cultures. We conclude that not one but several bacterial species enhance DA production by *P. multiseries*.

**Key Words:** *Pseudo-nitzschia multiseries*, *Pseudo-nitzschia pungens*, Domoic acid, Bacteria, Neurotoxin, Batch culture, Restriction fragment length polymorphism

**INTRODUCTION**

The pennate diatom *Pseudo-nitzschia multiseries* (formerly *Pseudonitzschia pungens* f. *multiseries* [Hasle, 1995]) was the source of the neurotoxin domoic acid (DA) that contaminated blue mussels, *Mytilus edulis*, in Prince Edward Island, Canada, in 1987 [Bates et al., 1989] and caused human intoxications [Todd, 1993]. *Pseudo-nitzschia multiseries* produces DA in axenic culture [Douglas and Bates, 1992] as well as in nonaxenic culture [Subba Rao et al., 1988; Bates et al., 1991]. However, axenic cultures produce less DA than the original bacteria-containing cultures [Douglas et al., 1993]. It is possible that the antibiotic treatment used to render *P. multiseries* axenic [Douglas et al., 1993] may have altered the organism in such a way as to cause a decrease in DA production. Alternatively, interactions between the bacteria and the diatom may be responsible for the elevated production observed in nonaxenic cultures. These alternative hypotheses led us to study the influence of bacteria on DA production. An initial examination of these hypotheses is given in Bates et al. [1995].

Experiments were carried out to reintroduce bacteria isolated from nonaxenic cultures into axenic cultures, and to determine their effect on DA production. We also grew bacterial strains individually and examined them for autonomous production of DA. Finally, we characterized bacterial strains using both classical and modern microbial techniques.

The role of bacteria in DA production has only recently been investigated because DA intoxication is a relatively new problem. However, the involvement of bacteria in the production of paralytic shellfish poisoning (PSP) toxins has been debated for some time [Silva and Sousa, 1981; Doucette, 1995]. The findings range from no direct involvement of bacteria in PSP toxin production [Singh et al., 1982; Dimanlig and Taylor, 1985; Boczar et al., 1988], to the production of PSP by isolated intracellular [Kodama et al., 1990a; Doucette and Trick, 1995] and free-living [Tamplin, 1990; Kodama et al., 1990b] bacteria. Clearly, many questions remain about the role of bacteria in phycotoxin production. In this study we show that reintroducing bacteria into axenic cultures of *P. multiseries* enhances the production of DA.

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TABLE 1. Origin of Phytoplankton and Bacterial Strains*

<table>
<thead>
<tr>
<th>Species</th>
<th>Diatom strain</th>
<th>Origin</th>
<th>Bacterial strain</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudo-nitzschia multiseries</em></td>
<td>BRUD-A</td>
<td>Brudenell River, PEI (C. Léger, August 1989)</td>
<td>BD-1</td>
</tr>
<tr>
<td></td>
<td>POM</td>
<td>Pomquet Harbour, NS (M. Seguel, November 1989)</td>
<td>BD-2</td>
</tr>
<tr>
<td></td>
<td>KP-14</td>
<td>Cardigan River, PEI (K. Pauley, Fall 1990)</td>
<td>PM-1</td>
</tr>
<tr>
<td><em>Chaetoceros sp.</em></td>
<td>CHAET</td>
<td>Cardigan River, PEI (C. Léger, July 1990)</td>
<td>CH-1</td>
</tr>
</tbody>
</table>

*PEI, Prince Edward Island, Canada; NS, Nova Scotia, Canada.

MATERIALS AND METHODS

Culture Conditions

Strains of *Pseudo-nitzschia multiseries* (Table I) were maintained and the experiments were carried out in medium f/2 [Guillard, 1975] at 18°C. Illumination was at a photon flux density of ca. 100 μE m⁻² s⁻¹, using cool-white fluorescent bulbs and a 12:12 hr light:dark cycle. Culture flasks were manually agitated and their positions were randomized daily. Axenic cultures were obtained by antibiotic treatment for 72 hr using 1.6:0.8 mg ml⁻¹ penicillin:streptomycin [Douglas et al., 1993]. These were tested for culturable bacteria by incubation in Bacto-peptone broth (Difco Laboratories, Detroit, MI; 1 g l⁻¹ seawater) and 2216 Marine Agar (Difco) at ca. 20°C for at least 20 days.

Isolation of Bacterial Strains

Strains of marine bacteria were isolated from cultures of *P. multiseries* and a nontoxic *Chaetoceros* sp. (Table I). The cultures were filtered (3.0-μm pore-size Nuclepore membrane, Pleasanton, CA) using sterile technique to remove the algal cells. Several drops of filtrate were then plated onto 2216 Marine Agar and the sample was incubated at ca. 20°C. Colonies were streaked 3-4 times until their purity was ensured. In the case of the bacteria isolated from *Chaetoceros* sp., a mixture of two strains persisted throughout the initial attempts at isolation. This was discovered after the first reintroduction experiment, when an additional isolation step yielded the two different strains. Aliquots of the isolated bacterial strains were cryopreserved at −80°C in 40% (V/V) glycerol.

Screening for Intracellular Bacteria

The possible presence of intracellular bacteria was investigated using axenic *P. multiseries* strain KP-14; samples of the corresponding nonaxenic cultures were used as controls. Aliquots from stationary phase cultures were transferred to sterile scintillation vials and were sonicated at 210 W (model VC-300 Vibra-Cell, Sonic & Materials, Inc., Danbury, CT) for 5, 10, 15, 20, and 30 sec with a sterile probe. Duplicate aliquots were withdrawn after each sonication period and placed into Bacto-peptone broth and incubated for up to 6 weeks. The sonicator probe was sterilized by autoclaving, and the procedure was carried out in a laminar-flow hood equipped with a High Efficiency Particulate Air (HEPA) filter.

Characterization of Bacterial Strains

To investigate the possibility that a specific bacterium is associated with toxic *P. multiseries* cultures, the bacterial strains isolated from the nonaxenic cultures were examined to determine whether they represented the same or different species. They were grown on 2216 Marine Agar at temperatures from 15–37°C, depending on the test procedure, and examined for cell and colony morphology. Carbon source utilization was examined using automated test kits (Biolog, Inc., Hayward, CA; bioMérieux Vitek, Inc., Hazelwood, MO). To further characterize these strains, whole cell fatty acid composition profiles were determined (Microbial ID, Inc., Newark, DE).

Restriction fragment length polymorphism (RFLP) analyses were performed on all the bacterial strains using the bacterial small subunit ribosomal RNA gene (SSU rDNA) following PCR amplification from total bacterial genomic DNA using GeneAmp PCR Core Reagents (Perkin Elmer Cetus, Norwalk, CT) and primers Eubac27F and 1492R [Lane, 1991]. Each PCR product was digested with individual restriction enzymes (total of 9, New England Biolabs, Beverly, MA), and the resulting fragment patterns were compared by an unpaired weighted mean average analysis of simple matching coefficients within the computer program NTSYS-pc [Rohlf, 1990].

Bacterial Reintroduction Experiments

To provide comparable inocula of axenic and nonaxenic *P. multiseries* cultures, 500-ml flasks, each containing 300 ml of f/2 medium, were inoculated with axenic or nonaxenic strains POM and KP-14 to an initial cell density of ca. 5,000 cells ml⁻¹. A second flask of each axenic *P. multiseries* strain was used to produce bacterial reintroduction inoculum cultures. These cultures received bacteria from a 5-day-old 2216 Marine Agar plate ca. 4 hr after inoculation with axenic *P. multiseries*. A bacterial colony and the associated agar plug were transferred into the inoculation flask using the wide end of a sterile Pasteur pipette. A sterile agar plug was
added to the other set of flasks containing axenic *P. multiseries* to serve as a control. Seven days later, at the end of the exponential phase and prior to production of DA [Bates et al., 1991], the axenic, nonaxenic, and bacterial reintroduction inoculum cultures were used to inoculate duplicate experimental flasks to an initial cell density of ca. 5,000 cells ml$^{-1}$. Cell growth was monitored by optical density (OD) measurements at 800 nm with a spectrophotometer (Spectronic 20, Bausch and Lomb, Rochester, NY). Cell number was estimated from the regression of OD vs. cell number, determined separately for each *P. multiseries* strain.

**Domoic Acid Analyses**

DA was determined in phytoplankton “whole cultures” (i.e., cells plus medium) [Bates et al., 1989] using a high-sensitivity (0.1 ng ml$^{-1}$) FMOC derivatization method [Pocklington et al., 1990]. DA per cell was calculated by dividing the concentration of DA in the whole culture by the total number of cells.

**RESULTS**

**Bacterial Reintroduction Experiments**

Figures 1–3 show examples of three of the five experiments carried out. The introduction of bacteria into axenic cultures of *P. multiseries* usually did not affect the division rate or stationary phase cell yield (Figs. 1–3, top). An exception is experiment 3 (Fig. 2A), where one of the cultures that had bacteria reintroduced showed a slower growth rate and a lower cell density at stationary phase, and the axenic culture also exhibited a reduction in final cell yield; we have no explanation for this. Although there was variation in the production of DA within the different axenic cultures, two patterns were apparent: 1) lower production of DA in axenic cultures, and 2) enhanced DA production in axenic cultures to which bacteria were reintroduced, expressed as either DA per ml (Figs. 1B, 2B, and 3B) or DA per cell (Figs. 1C, 2C, and 3C).

Table II summarizes the results of all the experiments. Axenic cultures of *P. multiseries* produced from 8–38 times less DA than did nonaxenic cultures, depending on the strain tested. Reintroduction of bacteria into axenic cultures resulted in a 2–115-fold enhancement in DA production on a per cell basis, depending on the *P. multiseries* and the bacterial strains used. The least enhancement occurred with *P. multiseries* strain KP-14, and the most with strain POM. The axenic cultures that received bacteria produced 0.2–14 times the DA of the original nonaxenic cultures.

The extent of enhancement was more closely a function of the strain of *P. multiseries* used than of the strain of bacterium added. For example, the enhancement was low when bacterial strain BD-1 was inoculated into *P. multiseries* strains BRUD-A and KP-14, but high when the same bacterium was inoculated into strain POM. Likewise, the origin of the bacterial strain was not a critical factor influencing the amount of DA produced. For example, bacterial strains BD-1 and BD-2 were isolated from *P. multiseries* BRUD-A (Table I), but they enhanced DA production more in strain POM.
than in BRUD-A or KP-14 (Table II). Bacterial strain PM-1, originating from *P. multiseries* POM, enhanced DA produc-

The bacteria isolated from a nontoxic culture of *Chaetoceros* sp. also enhanced DA production. This effect was
TABLE II. Summary of Results for Enhancement of Domoic Acid Production as a Result of Reintroducing Bacteria to Axenic Cultures of Pseudo-nitzschia multiseries

<table>
<thead>
<tr>
<th>Experiment</th>
<th>P. multiseries strain</th>
<th>Condition</th>
<th>Bacterial isolate</th>
<th>Domoic acid (pg·cell⁻¹)</th>
<th>Enhancement factor, relative to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nonaxenic</td>
<td>Axenic</td>
</tr>
<tr>
<td>1</td>
<td>BRUD-A</td>
<td>Reintroduced</td>
<td>BD-1</td>
<td>0.34</td>
<td>0.7</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.52</td>
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<td></td>
<td></td>
<td></td>
<td>0.03</td>
<td></td>
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<tr>
<td>2</td>
<td>POM</td>
<td>Reintroduced</td>
<td>BD-1</td>
<td>8.86</td>
<td>2.7</td>
</tr>
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<td></td>
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<td>9.31</td>
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<td>3.27</td>
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<td></td>
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<td></td>
<td>0.17</td>
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<td>3</td>
<td>POM</td>
<td>Reintroduced</td>
<td>BD-1</td>
<td>3.80</td>
<td>12</td>
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<td>4</td>
<td>POM</td>
<td>Reintroduced</td>
<td>BD-1</td>
<td>0.50</td>
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<td>0.38</td>
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<td></td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>KP-14</td>
<td>Reintroduced</td>
<td>BD-1</td>
<td>2.96</td>
<td>0.4</td>
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<td></td>
<td></td>
<td>1.91</td>
<td>0.2</td>
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<td></td>
<td></td>
<td>2.43</td>
<td>0.3</td>
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<td></td>
<td></td>
<td>8.23</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.88</td>
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</tbody>
</table>

*Measured on last day of experiment as indicated.

most dramatic in the case of strain POM (Fig. 2, Table II), but it also occurred with strain KP-14 (Fig. 3, Table II). It is therefore not necessary that bacteria be isolated from a DA-producing diatom in order to enhance DA production.

Tests for Intracellular Bacteria

To date, our attempts to isolate intracellular bacteria have shown only negative results. A sonication time of 20 sec disrupted >98% of the P. multiseries cells, yet no bacteria grew in the nutrient broth when inoculated with the cell extract. Disruption of the control nonaxenic P. multiseries culture showed bacterial growth within 1 day after inoculation, even after being sonicated for 30 sec. This strongly suggests that had intracellular bacteria been present, they would have survived the sonication.

Characterization of Bacterial Strains

The appearance of each of the bacterial stains growing on agar plates was slightly different, although each had a glossy surface. Strains BD-1 and BD-2 were creamy beige; strain PM-1 was a lighter beige than strains BD-1 and BD-2; strain CH-1 was creamy white; and strain CH-2 was yellowish.

Microscopic examination of the strains isolated from toxic cultures of P. multiseries showed that the bacterial cells were all Gram-negative rods 0.5–2.0 μm long. Use of modern taxonomic detection methods based on carbon substrate utilization and on whole cell fatty acid composition did not provide close matches with any species contained in the taxonomic data bases available. In fact, the “best guess” at taxonomic identification provided by each of the three tests was different for each isolate. The use of these techniques did, however, enable us to establish that BD-1 and BD-2 were identical (having been isolated from the same colony), and that in the other cases we had isolated different bacteria from the other nonaxenic cultures of P. multiseries and Chaetoceros sp.

RFLP analyses performed on the bacterial SSU rDNA also clearly showed that all the strains, except BD-1 and BD-2, were different (Fig. 4). The findings that strains BD-1 and BD-2 were inseparable is consistent with the results noted above. Strain PM-1 was 61% similar to BD-1 and BD-2, and thus is clearly distinguishable at the species level. Strain CH-1 was only 43% similar to the other strains, indicating little relatedness.

DISCUSSION

Interactions between phytoplankton and bacteria have been studied for many years [Cole, 1982]. These include competition for available organic matter, provision of extracellular materials by the phytoplankton which are of benefit to the bacteria and vice versa, and production of toxic substances by the phytoplankton which inhibit the growth of bacteria and vice versa [Jolley and Jones, 1977; Rausch de Traubenberg and Soyer-Gobillard, 1990].

There is, however, considerable controversy surrounding the role of bacteria in the production of phycotoxins [Doucette, 1995]. At least three lines of evidence implicate marine bacteria in the production of PSP toxins by dinoflagellates. First, numerous clones of a single dinoflagellate species and...
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Relative Similarity

Fig. 4. Dendogram of relative similarity values, based on restriction fragment length polymorphism (RFLP) analyses of bacterial SSU rDNA, for bacteria isolated from nonaxenic strains of Pseudo-nitzschia multiseries and Chaetoceros sp.

subclones of one clonal culture grown under the same conditions, irrespective of physiological condition, have a widely different toxin content, suggesting that the variability is acquired, not inherited [Ogata et al., 1987]. Second, scallops became toxic in the absence of known PSP-producing dinoflagellates, but in the presence of bacteria-size particles [Ogata et al., 1982; Kodama, 1989; Kodama et al., 1990b]. Third, intracellular bacteria were found in dinoflagellates and were hypothesized to play a role in the production of PSP toxins [Silva, 1982, 1990]. An intracellular bacterium, Moraxella sp., was later isolated from Protogonyaulax tamarensis (= Alexandrium tamarense) and was shown to produce saxitoxin and gonyautoxins [Kodama and Ogata, 1988; Kodama, 1990; Kodama et al., 1990a; Doucette and Trick, 1995]. Kodama et al. [1993] suggested that intracellular bacteria in A. tamarense also produce tetrodotoxin. Silva and Sousa [1981] found that Prorocentrum minimum became toxic after being inoculated with a bacterium, Pseudomonas sp., isolated from toxic Gonyaulax tamarensis (= A. tamarense), later reidentified as A. lusitanicum (S. Franca, personal communication).

On the other hand, evidence to the contrary has also been reported. Axenic cultures of P. tamarensis (= A. tamarense) produced normal levels of PSP toxins [Singh et al., 1982; Boczar et al., 1988], suggesting no direct involvement of bacteria. Dimanlig and Taylor [1985] found no association between bacterial composition and PSP toxin production in P. tamarensis (= A. tamarense) cultures. Furthermore, they were unable to demonstrate the transfer of toxicity using extracellular bacteria, and no intracellular bacteria were found. Franca et al. [1993] likewise found no intracellular bacteria in toxic Gymnodinium catenatum and suggested that bacteria are not required for toxin production. Aikman et al. [1993] showed that bacteria isolated from Prorocentrum hoffmannianum were nontoxic, and Kim et al. [1993] reported that the ability to synthesize PSP toxins is a genetic trait.

Our results with three strains of P. multiseries clearly show that axenic cultures produce substantially less DA than nonaxenic cultures, confirming previous results [Douglas et al., 1993]. Thus far we have found no evidence that extracellular bacteria isolated from these nonaxenic P. multiseries strains are capable of DA production. Since axenic cultures still produce low amounts of DA, it might be argued that intracellular bacteria were present and responsible for this production. However, we found no evidence of viable intracellular bacteria in disrupted cells of axenic strain KP-14. Likewise, electron microscopy by MacPhee et al. [1992] found no intracellular bacteria in natural populations of P. multiseries.

The enhancement in DA production after reintroducing bacteria into axenic cultures confirms that these cultures retained their ability to synthesize toxins at the higher levels observed in the original nonaxenic cultures. This demonstrates that the toxin production mechanism was not adversely affected by the antibiotic treatment used to render the cultures axenic. The broad range in response to bacterial addition (2–115-fold enhancement) suggests a complex interaction between bacteria and P. multiseries with respect to DA production. Indeed, it is common for different clones, and even for the same clone, of P. multiseries to produce different amounts of DA during different experiments, even when growth conditions remain the same [cf. Ogata et al., 1987].

On a time scale of days, it is likely that there is a relationship between the number of bacteria and the amount of DA produced in batch culture. This could explain the large increase in DA production after the beginning of the stationary phase of P. multiseries [Bates et al., 1991], when bacterial numbers also increase due to the availability of organic exudates [cf. Rausch de Traubenberg and Soyer-Gobillard, 1990]. However, because axenic cultures also exhibit an increase in DA production during stationary phase [Douglas and Bates, 1992], factors other than bacteria must trigger this elevated toxin synthesis.

Each of the bacterial strains tested enhanced DA production, although to varying degrees. Moreover, the bacterial strains isolated from the different P. multiseries cultures were only distantly related to each other (Fig. 4). The results indicate that, rather than a single bacterial species, many strains of bacteria may enhance DA production. This is
supported by the finding that bacteria isolated from a nontoxic Chaetoceros sp. also markedly enhanced DA production by P. multiseris.

Our results indicate an important but nonessential role of extracellular bacteria in DA production. We are currently investigating the mechanism(s) by which bacteria enhance DA production. Bacteria may produce precursors that are used directly in DA synthesis, regenerate nutrients, provide essential dissolved organic matter (e.g., elicitors), or increase the carbon dioxide content of the growth medium, thus providing carbon for DA synthesis. Finally, it is possible that certain bacteria produce DA autonomously, as has been indicated for PSP toxin production by Moraxella sp. [Kodama et al., 199a; Doucette and Trick, 1995]. This is currently under investigation.

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REFERENCES


