Presence of Tetrodotoxin and Tetrodotoxin-Producing Bacteria in Freshwater Sediments

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The occurrence of tetrodotoxin (TTX) in sediment from Lake Suwa, Japan, was confirmed by a tissue culture assay, high-performance liquid chromatography, and gas chromatography-mass spectrometry. Altogether, 17 TTX-producing bacteria belonging to five genera were isolated from Lake Suwa and Pond Inokasira. Our results indicate that TTX and TTX-producing bacteria occur even in freshwater environments.

Tetrodotoxin (TTX) is a potent neurotoxin which blocks the sodium channel in excitable membranes (2, 5). Although it is well known as “pufferfish toxin,” TTX occurs in many kinds of marine organisms (1, 6, 10, 14, 16, 17, 21) and some terrestrial animals (6, 12). Since TTX-producing bacteria have been discovered (15, 22), it has been postulated that bacteria are responsible for the toxicity of the animals in which TTX occurs (9, 11).

Previously, we reported that TTX accumulated at high levels in marine sediments (7) and that many kinds of bacteria can produce TTX (3, 4). Some bacterial strains, such as Bacillus, Micrococcus, and actinomyce strains, are not typical marine bacteria. This led us to postulate that there may be TTX-producing bacteria and TTX in freshwater environments. The purpose of this work was to verify this hypothesis by examining two freshwater sediments.

Sediment samples were collected from Pond Inokasira, Tokyo, Japan, in May 1988 and from Lake Suwa, Nagano, Japan, in January 1989 and April 1992 by using an Ekman Birge sampler. To isolate bacteria, diluted samples were spread on plates containing modified ZoBell 2216E agar, which contained (per liter of distilled water) 0.5 g of Bacto Peptone (Difco), 0.1 g of yeast extract (Difco), 0.01 g of ferric citrate, 0.2 g of K2HPO4, and 15 g of Bacto agar (Difco); the pH of this medium was pH 7.5 to 7.6. After incubation at 20°C for 3 weeks, colonies were randomly isolated and used for toxin production analysis and further identification. The toxin in sediment samples was extracted as described previously (7) with a slight modification; prior to the filtration through a SEP-PAK C18 cartridge, the extract was treated with activated charcoal (Wako). The charcoal was washed with distilled water, and the toxin was extracted with 20% ethanol–1% acetic acid. The eluates were evaporated under vacuum pressure and filtered through SEP-PAK C18 cartridges (Waters Associates). The filtrates were freeze dried and dissolved in small amounts of distilled water.

To observe toxin production, bacterial isolates were cultured in 400 ml of L medium (4) lacking NaCl. After incubation at 25°C with shaking for 2 to 3 days, cell pellets were obtained by centrifugation and subsequent washing with distilled water. The pellets were suspended in 0.1% acetic acid, ultrasonicated, and boiled for 20 min. After cooling, each sample was centrifuged at 25,000 × g for 15 min. The resulting supernatant was filtered through a SEP-PAK C18 cartridge, freeze dried, and finally reconstituted in a small amount of distilled water. TTX-producing bacteria were identified at the generic level by using the scheme of Simidu (18).

The presence of sodium channel blockers was detected by the tissue culture assay method (8). For the positive samples, TTX and its analogs were identified by high-performance liquid chromatography (HPLC) (3). Final identification of TTX was accomplished by performing gas chromatography-mass spectrometry. Prior to the analysis, the extract was trimethylsilylated as described elsewhere (13). The C9 base obtained was then analyzed with a VG AutoSpecE mass spectrometer. Since TTX analogs were not detected. Washing of sodium channel blockers was detected by using the tissue culture assay method (8). The positive samples were identified as TTX and its analogs by high-performance liquid chromatography (HPLC) (3). The final identification of TTX was accomplished by performing gas chromatography-mass spectrometry. Prior to the analysis, the extract was trimethylsilylated as described elsewhere (13). The C9 base obtained was then analyzed with a VG AutoSpecE mass spectrometer. Since TTX analogs were not detected.

FIG. 1. HPLC chromatograms of extract of Lake Suwa sediment (line a) and of authentic TTX and its analogs (line b). Peak 1, TTX; peak 2, 4-epi-TTX; peak 3, anhydro-TTX.

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spectrometer equipped with a model HP5890 gas chromatograph. A type DB-5 fused silica column (15 m by 0.25 mm [inside diameter]; film thickness, 0.25 μm) was used with helium as the carrier gas (25 ml/min; Hewlett-Packard). The oven temperature was increased from 160 to 250°C at a rate of 5°C/min. The electron ionization energy was 70 eV, and the ion source temperature was 200°C.

The tissue culture assay revealed the presence of sodium channel blockers in sediments from both Lake Suwa and Pond Inokasira. The concentrations seemed to be approximately 1 order of magnitude less than those in marine sediments (7). However, as the neuroblastoma cells appeared to be affected by concomitant unknown compounds, the exact concentrations were not determined. The HPLC analysis indicated that there was 4-epi-tetrodotoxin in Lake Suwa sediment (Fig. 1). In Pond Inokasira, however, the peaks were obscure, and the occurrence of TTX-related toxin was not confirmed. As the sediment in Pond Inokasira is often dredged, it cannot be considered a natural freshwater environment. On the other hand, Lake Suwa is a typical mesotrophic lake. For both sediments, it is possible that paralytic shellfish poison or other sodium channel blockers were also present.

For Lake Suwa sediment the occurrence of TTX or TTX-related compounds was further confirmed by gas chromatography-mass spectrometry. Figure 2 shows the typical pattern for a trimethylsilyl (TMS) derivative of C9 base, which is an alkaline degradation product of TTX and related compounds. Selected ion-monitored chromatograms (m/z 392, 407, and 376) indicated that a compound with a retention time identical to that of the authentic TMS derivative of C9 base was present in the TMS derivative from an alkaline-degraded sediment extract (Fig. 2A). The mass spectrum of this compound had ion peaks at m/z 392, 407, and 376, which are characteristic of the TMS derivative of C9 base (Fig. 2B).

Thus, all three analytical methods, including both biological and chemical methods, indicated that TTX occurred in freshwater sediments. Although our present observations are limited to two environments, it is very possible that freshwater sediments in various other areas also contain TTX or its analogs. It will be worth looking for TTX in

FIG. 2. Chromatograms from gas chromatography-mass spectrometry for a Lake Suwa sediment sample. (A and B) Total-ion chromatogram (TIC) and selected ion-monitored chromatograms of the TMS derivative from alkaline-degraded sediment extract (A) and of authentic TTX (B). (C and D) Mass spectra of the peak corresponding to authentic TMS C9 base in the TMS derivative from alkaline-degraded sediment extract (C) and from authentic TTX (D).
freshwater animals. We predict that some animals, especially benthic animals, also contain the toxin in their bodies.

We isolated 17 and 26 bacterial strains from Lake Suwa and Pond Inokasira, respectively. The numbers of TTX-producing strains belonging to different genera are shown in Table 1. We confirmed that 17 strains belonging to five genera produce TTX. Most of these strains were gram-positive organisms (either Bacillus or Micrococcus strains).

Figure 3 shows the HPLC chromatogram for Bacillus sp. strain b-11, which was isolated from the sediment of Pond Inokasira. The retention times of the sample peaks corresponded to those of standard TTX, 4-epi-TTX, and anhydro-TTX. On the other hand, the production of 4-epi-TTX was observed for Caulobacter sp. strain s-24 isolated from the sediment of Lake Suwa (Fig. 4).

Previous reports (3, 4, 19, 20) and this work clarified that bacteria belonging to the following genera are TTX producers: Bacillus, Micrococcus, Moraxella, Vibrio, Acinetobacter, Aeromonas, Alcaligenes, Alteromonas, Flavobacterium, Caulobacter, and Actinomycetes. The ability to synthesize TTX, therefore, is not restricted to a certain few genera. It is also noteworthy that we isolated at least one strain which produces gonyautoxin or saxitoxin from Pond Inokasira sediment (data not shown). There may be some bacteria which can synthesize several types of related toxins.

In conclusion, TTX is produced by freshwater bacteria and accumulates in sediments. Additional studies on the distribution of TTX in freshwater environments and the mechanism of toxin accumulation are now being undertaken.

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