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Identification of Deep-Sea-Sediment Bacteria Which Produce Tetrodotoxin

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Forty-nine bacterial strains were isolated from deep-sea sediments. Among them, 22 strains were shown by the tissue culture assay method to produce sodium channel blockers. For some strains, high-performance liquid chromatography analysis confirmed that the blocker was tetrodotoxin. Tetrodotoxin-producing bacteria seem to be widespread in marine sediment.

Tetrodotoxin (TTX) is a strong neurotoxin which blocks the sodium channel of neuronal cells. Recently, its wide distribution among diverse groups of organisms has been clarified (3, 7). Since bacterial production of this toxin was confirmed (4, 9), it seems reasonable to postulate that bacteria are the main producers of this toxin in nature. Although there is some knowledge of the production of TTX by type strains (6), the distribution of these producers in nature and their taxonomical information are still largely unknown.

We previously reported (1) that an unexpectedly high concentration of TTX is accumulated in marine sediments. Since there was no apparent difference between the concentrations in deep-sea samples (4,033 m) and near-shore samples (20 and 87 m), we suspect that bacteria are producing TTX in marine sediment regardless of the depth. The purpose of the present investigation was to examine the TTX productivity of bacteria isolated from deep-sea sediment and to clarify the taxonomical positions of these bacteria.

Sediment samples were taken, using a box corer as described previously (1). The bacteria in the sediment from Stn. C (4,033 m; 33°49.9′ N, 141°09.0′ E) were isolated, using ZoBell 2216E agar plates after 3 weeks of incubation at 20°C. All the experiments after sampling were done at atmospheric pressure. Therefore, these strains may not be representatives of the true barophilic bacteria. Forty-nine bacterial strains were randomly isolated, and the production of Na^+ channel blocker or TTX was investigated. Bacterial cells were cultured in 400 ml of L medium which contained the following: NaCl, 17.53 g; $MgSO_4 \cdot 7H_2O$, 2.46 g; K_2HPO_4, 1.0 g; Polypepton (Nihon Pharmaceutical Co., Ltd.), 5 g; and yeast extract (Difco Laboratories), 1.0 g in 1 liter of distilled water; the pH was adjusted to 7.5 to 7.6. After incubation at 25°C for 3 days with shaking, cells were harvested by centrifugation at 7,000 × *g* and washed twice more with 0.3 M NaCl solution. The cells were then transferred in 0.1% acetic acid solution and boiled for 20 min for the extraction of TTX or related toxins. After centrifugation at 25,000 × *g*, the supernatant was freeze-dried and reconstituted in a small amount of distilled water (ca. 200 μl). For high-performance liquid chromatography (HPLC) analysis, the sample was further purified by charcoal column, Bio-Gel (Bio-Rad Laboratories) P-2 column, and SEP-PAK C_{18} cartridges (Waters Associates). The sodium channel blocker was then assayed, using the tissue culture bioassay method (2). In brief, the mouse neuroblastoma cell line Neuro-2A (ATCC CCL131) was maintained in RPMI 1640 medium (GIBCO Laboratories). In the presence of ouabain, veratridine enhances sodium influx and subsequent cellular swelling and death. TTX or sodium channel blockers antagonize this effect, enabling the cells to continue their growth. The change in cellular morphology is observed for detecting the occurrence of sodium channel blockers under an inverted microscope. The minimum detectable level of TTX is ca. 200 pg. The conditions for HPLC analysis have been described elsewhere (8). The flow rate was changed to 0.7 ml/min. All bacterial strains were identified at the generic level by the method of Simidu (5). Among 49 bacterial strains investigated, 22 were found by the tissue culture bioassay method to produce sodium channel blockers. The extracts of some other strains caused damage to the neuroblastoma cells, so the presence of the toxin was not confirmed. Hence, there might have been some more TTX producers among the strains tested. Table 1 shows the results of identification. It is clear that TTX-producing bacteria are not restricted to certain taxonomical groups. Various groups of bacteria, both gram-positive and -negative, were found to possess the ability to produce the toxin. A preliminary investigation indicated that the toxin-producing bacteria listed in Table 1 do not always require sodium for their growth (data not shown). For the *Acinetobacter* sp., the TTX fraction was further analyzed, using HPLC (Fig. 1). A comparison with the authentic preparations shows that this strain is considered to produce TTX and its analog, anhydro-TTX. We

TABLE 1. Bacterial species isolated from deep-sea sediment which produce TTX or sodium channel blocker

Species	No. of isolates		Total no. of isolates
	Toxic	Nontoxic	
Bacillus	5	5	10
Micrococcus	4	3	7
Acinetobacter	3	3	6
Aeromonas	1	0	1
Alcaligenes	0	4	4
Alteromonas	5	4	9
Flavobacterium	0	1	1
Moraxella	2	1	3
Pseudomonas	0	2	2
Vibrio	1	0	1
Not identified	1	4	5

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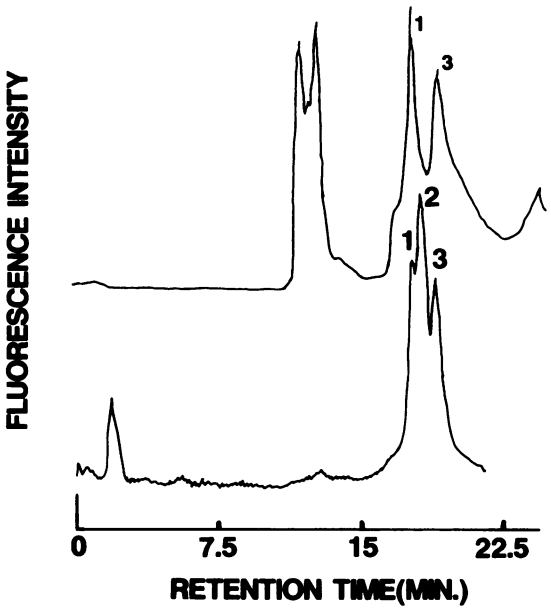

FIG. 1. HPLC chromatogram of a deep-sea-sediment bacterial strain (*Acinetobacter* sp., top) and authentic TTX and its analogs (bottom). Peaks: 1, TTX; 2, 4-epi-TTX; 3, anhydro-TTX.

confirmed the TTX production for four more strains, using HPLC.

We have not run HPLC analysis for other strains or for other sodium channel blockers, such as saxitoxin or gonyautoxin. Therefore, we cannot exclude the possibility that other sodium channel blockers were produced by the strains. However, in our experience so far, all the isolates which showed a positive result by the tissue culture method produced TTX by HPLC analysis. Therefore, we assume that most of the positive strains tested in the present study can actually produce TTX.

Although the strains investigated are still limited, the results indicate that TTX-producing bacteria are quite widespread among various bacterial groups in marine sediments. It seems reasonable to postulate that TTXs are synthesized solely by bacteria in the sediments and subsequently accumulated in the sediments by benthic organisms through the food web. The environmental conditions suitable for TTX production and the mechanism of TTX accumulation through the food web in the sediment community are now being investigated.

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