APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1995, p. 3468–3470 Vol. 61, No. 9
0099-2240/95/$04.00+0

Reexamination of Tetrodotoxin Production by Bacteria

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Received 10 January 1995/Accepted 19 June 1995

Vibrio alginolyticus **has been reported as a good producer of tetrodotoxin (TTX), but the toxin extracted from this bacterium did not react to the monoclonal antibody against TTX. Surprisingly, chromatographic analyses detected high TTX peaks for polypeptone and yeast extracts used as medium materials, which were, as expected, all negative by the mouse bioassay. These results may require us to revise the bacterial production of TTX.**

Tetrodotoxin (TTX) is one of the best known marine toxins that is isolated predominantly from many species of puffer fish (2, 9). This toxin is, however, produced only by various species of bacteria (7, 10, 13, 14, 18). The bacterial production of TTX was first reported by Yasumoto et al. (17), who demonstrated TTX in the culture broth of a *Pseudomonas* sp. containing 3% NaCl and 1% polypeptone. Noguchi et al. (11) also detected TTX in *Vibrio* sp. cells. High-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) have been used primarily for the detection of TTX (15, 16). The purpose of my study was to reexamine whether bacteria have an ability to produce TTX, because HPLC and GC-MS analyses detected high TTX peaks for polypeptone and yeast extracts used as medium materials, suggesting that these chromatographic analyses lack specificity for TTX.

To examine the bacterial production of TTX, bacteria were isolated and identified from the intestinal contents of *Fugu niphobles* by the method of Kungsuwan et al. (4), in which *Vibrio alginolyticus*, randomly selected as a good producer of TTX (7), was cultured in 5 liters of L medium with the same components as those reported by Do et al. (1) for 7 days at room temperature. The cells were harvested by centrifugation at 7,000 × *g* for 15 min and washed twice with 0.15 M NaCl. These cells were transferred to a 0.1% acetic acid solution. The toxin was then extracted from these cells by the method of Narita et al. (8), and the extracts were dissolved in 2.5 ml of distilled water. The medium materials used for cultivation were also examined as backgrounds. Twenty-five grams of polypeptone (Nihon Pharmaceutical Co., Ltd.), 5 g of yeast extract (Difco Laboratories), and 25 g of soluble starch (Katayama Chemical) were each dissolved in 5 liters of distilled water. These solutions were treated by the TTX extraction method of Yasumoto et al. (16), and the extract was dissolved in 2.5 ml of distilled water. The conditions for HPLC and GC-MS (Hewlett-Packard series II and 5791 series, respectively) analyses of these extracts were described elsewhere (6, 15). The mouse bioassay using ddY mice weighing 18 g was performed by the official method (3). The simple and rapid neutralization test for TTX (5) was carried out as follows. To 1 ml of toxin solution from bacterial samples containing less than 3 mouse units (MU) of TTX, 1 ml of immunoglobulin G solution (250 μg/ml) from monoclonal antibody against TTX (5) that was recently produced in our laboratory was added, mixed, and incubated 30 min at room temperature. The mixture was then administered intraperitoneally to the animals. In a previously reported experiment (5), 3 MU of TTX extracted from puffer fish was neutralized completely by 250 μg of monoclonal antibody per ml; i.e., all mice survived. Also, TTX was extracted from the ovary of the puffer fish, *F. niphobles* (8), and used as standard TTX throughout the present study.

The results of this experiment are shown in Fig. 1 and Table 1. TTX from two strains of *V. alginolyticus* (strains 33 and 87) was detected by both HPLC and GC-MS. Surprisingly, both HPLC and GC-MS detected high TTX peaks and fragment ions (*m/z*, 392, 407, and 376) in polypeptone and yeast extracts, although it is unlikely that TTX was present in the medium materials. Indeed, these samples were all negative, as expected, by the mouse bioassay. This suggests that the chromatographic analyses falsely detected substances contained in the medium materials as TTX, tempting us to speculate that the chromatographic analyses are not specific for TTX. Also, the fact that TTX peaks were not observed for all bacterial strains may reflect their different nutrient requirements, if the medium is the source. The mouse bioassay was then carried out with

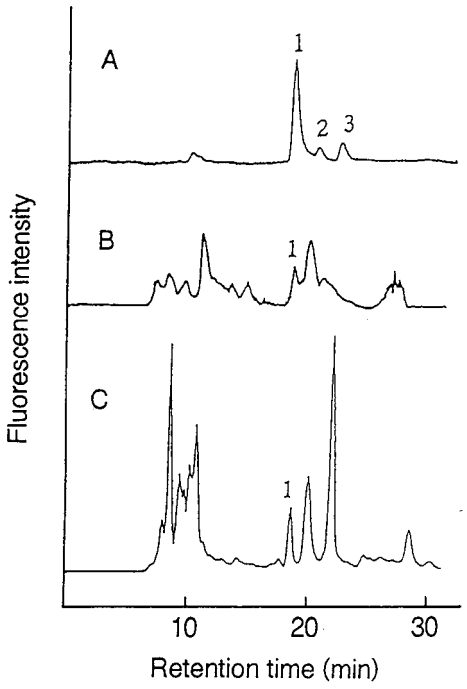

FIG. 1. Typical HPLC chromatogram of TTX and its derivatives. (A) Authentic TTX and its derivatives; (B) extract from *V. alginolyticus*; (C) extract from polypeptone. Peaks: 1, TTX; 2, 4-*epi*-TTX; 3, anhydro-TTX.

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TABLE 1. Detection of TTX in *V. alginolyticus* and the medium materials by HPLC, GC-MS, mouse bioassay, and neutralization test

Sample	TTX detected by[a]:			
	HPLC (MU/ml)	GC-MS	Mouse bioassay (MU/ml)	Neutralization test
V. alginolyticus				
19	−	−	−	ND
33	+ (27.2)	+	−	ND
87	+ (11.4)	+	+ (1.05)	−
98	−	±	+ (<1.0)	−
107	−	±	−	ND
161	−	−	−	ND
Medium				
Polypeptone	+ (26.9)	+	−	ND
Yeast extract	+ (14.7)	+	−	ND
Soluble starch	±	±	−	ND
TTX	+	+	+	+

[a] Symbols: +, clearly detected or positive; ±, difficult to detect; −, not detected or negative. One mouse unit is defined as the amount of TTX which kills a mouse in 30 min. TTX used as a positive control is from puffer fish ovary. In HPLC, the mouse units were calculated from the peak height of standard TTX, while in the mouse bioassay, the value was calculated from the time of death. The neutralization test was not done (ND) when the mice survived in the mouse bioassay.

samples from two strains (strains 87 and 98), with killing of mice, although there were no mice with typical signs of TTX poisoning. Also, the neutralization tests of these samples were, as expected, all negative. Although strain 33 had a high TTX level that was equivalent to 27.2 MU/ml, all mice treated with this strain survived in the mouse bioassay. The TTX concentrations were calculated from the times of death of the mice and compared with the concentrations obtained from the peak heights of HPLC; no correlation was observed between them. On the other hand, TTX (3 MU) that was extracted from a puffer fish ovary and used as a positive control was detected by all tests. These results strongly suggest that HPLC and GC-MS methods have their limits in detecting TTX.

The HPLC system for TTX was developed by Yasumoto and Michishita (16), who described that this method was specific for TTX and had a good correlation to the official mouse bioassay (3). The GC-MS method was applied to autopsy materials containing TTX by Suenaga and Kotoku (15). The present results, however, suggest that these HPLC and GC-MS methods may lack specificity for TTX. This explanation is confirmed by the following findings. (i) Both HPLC and GC-MS analyses detected TTX in polypeptone and yeast extracts. (ii) The TTX detected in the bacterial samples had no reaction to the monoclonal antibody. (iii) There was no correlation between the TTX concentrations from HPLC and those from the mouse bioassay. Furthermore, there have been no data describing TTX concentrations in samples of bacteria or culture broths, although the concentrations can be calculated easily from peak heights in the HPLC analysis (see references 1, 4, 7, 10, 11, 14, 17, and 18). In a preliminary experiment of the neutralization test, the toxins extracted from Japanese newts, *Triturus pyrrhogaster* (2), and puffer fish reacted to the monoclonal antibody, while toxin from the starfish *Astropecten polyacanthus* (kindly supplied from H. Narita) that has been identified as TTX (12) did not, probably indicating that at least the starfish toxin is not TTX, even though it may be a TTX-related substance (unpublished data). These observations strongly support the findings obtained here by the neutralization test. In the HPLC system (16), TTX is mixed with 4 N NaOH and heated at 110°C after being separated on a Develosi octyldecyl silane column. Fluorescent product is then monitored. On the other hand, in the GC-MS (15), 0.1 ml of TTX solution is mixed with 0.5 ml of 1.5 N NaOH and heated at 80°C for 45 min in a water bath. TTX is extracted from the mixture with *n*-butanol and finally trimethylsilated for GC. That is, TTX must be converted to 2-amino-6-hydroxymethyl-8-hydroxyquinazoline (a C_9 base) by alkaline degradation in both systems, and the fluorescence intensity is then measured in the HPLC system, increasing the possibility that a false-positive result may be obtained when an unknown substance with the same retention time as that of TTX is present in the samples. This may be a reason why high TTX peaks were detected even in the medium materials. Also, there has been no description of the structural determination of the toxins obtained from the bacteria or the culture broths. The present results may, therefore, require us to revise or reexamine the TTX productivity of bacteria.

I thank R. Endo and S. Miyamura for valuable discussions throughout the present study.

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