Tetrodotoxin Occurrence in Ciliated Protozoa and Possible Bacterial Role in its Toxification

Hyung Ki DO, Masachika MAEDA*, Tamao NOGUCHI, Usio SIMIDU*** and Kazuhiro KOGURE*****

School of Bioscience and Food Technology, Handong University, Pohang 791-940, Korea
**Nansei Regional Fisheries Research Institute, 2-17-5, Maruishi, Ohno, Daiki-gun, Hiroshima 739-04, Japan*
***Faculty of Fisheries, Nagasaki University, 1-14, Bunkyo, Nagasaki 852, Japan*
****Research Laboratory No.3, Toyo Suisan Kaisha Ltd., 2-13-40, Kohnan, Minato-gu, Tokyo 108, Japan*
*****Ocean Research Institute, University of Tokyo, Minamidai, Nakano, Tokyo 164, Japan*

The occurrence of TTX in ciliated protozoa was investigated in order to clarify tetrodotoxin (TTX) accumulation mechanisms in marine organisms. Tissue culture bioassay, HPLC, and GC-MS analyses confirmed the occurrence of TTX in *Euplotes mutabilis* and also in bacteria isolated from the culture medium. Fluorescently labeled bacteria (FLB) were prepared with those bacteria, and predation by *E. mutabilis* was observed. The results indicated that TTX in bacteria can be transferred to higher trophic levels through the food chain.

Key words : Tetrodotoxin, *Euplotes mutabilis*, Tissue culture bioassay, sodium channel blocker

Inrtoduction

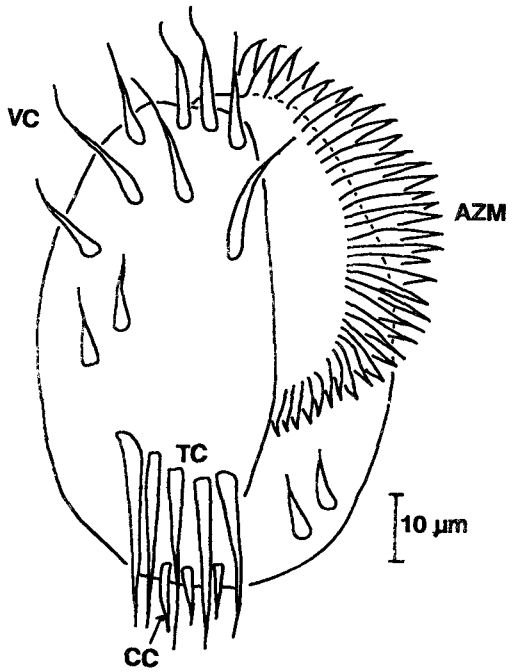
Tetrodotoxin (TTX), known as a puffer fish toxin, is a potent, non-protein neurotoxin which blocks sodium channels in excitable membranes (Evans, 1972; Kao, 1986). Since the finding of TTX in California newts (Mosher et al., 1964), many kinds of animals, especially marine invertebrates, were reported to possess this toxin (Mosher et al., 1984; Noguchi et al., 1983; Thuesen et al., 1988). Recently, various groups of bacteria including both type strains (Simidu et al., 1987) and marine isolates (Marita et al., 1987; Yotsu et al., 1987) have been found to produce TTX. In the previous papers, we reported the occurrence of TTX in marine sediment (Kogure et al., 1988a) and the occurrence of TTX-producing bacteria in the sediment (Do et al., 1990; Do et al., 1991). These facts led us to a hypothesis that bacteria are the major producers of TTX in nature, and TTX is accumulated to higher organisms through food webs.

To verify this hypothesis, it is necessary to confirm the occurrence of TTX in bacterivorous organisms. Among them, protozoa may play the most important role as direct consumers of bacteria (Anderson & Fenchel, 1985; Sherr et al., 1989). They are distributed widely in marine environments, and can be the link to higher trophic levels (Fenchel, 1982).

The purpose of this investigation was to clarify the occurrence of TTX in marine ciliated protozoa. The toxin was detected by using tissue culture bioassay and further chemically by HPLC and GC-MS. The ingestion of TTX-producing bacteria by the protozoa was also investigated.

Materials and Methods

1. Isolation and cultivation of protozoa



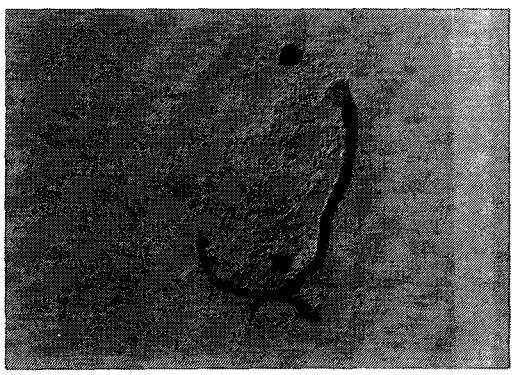


Fig. 1. Cellular structure of *Eupolotes mutabilis* (A) and its shape of nucleus (B). AZM : adoral zone of membranelles, TC : transverse cirri, CC : caudal cirri, VC : ventral cirri. Nucleus was stained by the method of Kosaka (1989).

The ciliated protozoa used was isolated from seawater in Seto Inlet, Japan, and assumed as clonal after sequential transfer for more than a year. The strain was cultured at 25℃ in sterilized seawater with a thin layer of agar medium (ZoBell's 2216E) at the bottom of the flask. The ZoBell's 2216E contains Bacto peptone (Difco) 5 g, Bacto yeast extract (Difco) 1 g, $FePO_4$ 0.1 g, Bacto agar 15 g, seawater 1,000 ml and pH 7.6~7.8. Judging from the cellular structure (Fig. 1A), the shape of nucleus stained by Kosaka method (1989, Fig. 1B), and the way of swimming, this isolate was identified as *Euplotes mutabilis*.

2. Extraction of toxin from protozoa

Cultured protozoa were cooled on ice, and centrifuged at 3000 rpm for 20 min.

The pellets were mixed with 0.1% acetic acid and ultrasonicated (Tomy UD-200, Ultrasonic Disruptor) to destroy the cells. After boiling for 20 min, the sample was cooled and filtered (Toyo Roshi No.1). The filtrates were passed through a charcoal column and eluted with 20% ethanol containing 1% acetic acid. The elutes were evaporated to dryness under vacuum pressure at below 40℃. After dissolving the residue with a small amount of distilled water, it was filtered through a SEP-PAK C_{18} Cartridge (Waters Associates), freeze-dried and then dissolved with a small amount of distilled water.

3. Isolation of bacteria from protozoa and toxin extraction

A portion of culture medium of the ciliate was serially diluted in sterilized seawater and plated on 1/10 ZoBll's 2216E agar plates. After incubation at 20℃ for 3 weeks, 20 strains were randomly isolated. Among them, two typical strains were selected and tested for the production of tetrodotoxin. One of the two was tentatively identified as *Acinetobacter* sp. according to the scheme by Simidu (Simidu, 1985). The other strain was a Gram negative rod, but the genus was not clearly identified. These two comprised a substantial fraction of total isolates. The isolates were cultured in L-medium (Do et al., 1991) with shaking at 25℃ for 2 days. Bacterial cells were harvested by centrifugation at 7,000 rpm for 15 min and washed twice with

0.3 M NaCl. After adding 0.1% acetic acid, the cells were ultrasonicated and boiled for 20 min. The sample was then treated as described above.

4. Analyses of the toxin

The occurrence of toxins in protozoa and bacterial strains was firstly investigated by the tissue culture assay method (Kogure et al., 1988b). This method enables the detection of sodium channel blocking (SCB) agents, including TTX, STX or related toxins. For identification of the SCB, the samples were analyzed using HPLC according to the procedures by Yasumoto and Michishita (1985) and further by gas chromatography-mass spectrometry (GC-MS). For the latter, the C_9 base (2-amino-6-hydroxymethyl-8-hydroxyquinazoline), prepared from *Euplotes* sp. by alkaline degradation was trimethylsilylated with a small amount of mixtures of *N, O*-bis (trimethylsilyl) acetamide, trimethylchlorosilane and pyridine (2 : 1 : 1) by the method of Narita et al. (1987). The prepared C_9 base derivative was analyzed on a Hitachi GC-Mass Spectrometer M-80 equipped with a column (0.3×200 cm) of Chromosorb W coated with 1.5% OV-101. The temperature was raised from 165℃ to 200℃ at a rate of 5℃/min. The ionizing voltage was 70eV and the ion source temperature was maintained at 200℃. Scanning was carried out in mass range of m/z 50~600 at 2 sec intervals.

5. Bacterivory of the ciliate

To examine whether the protozoa feed on bacteria or not, fluorescently labeled bacteria (FLB) were prepared according to the method of Sherr et al. (1987). The ingested FLB in *Euplotes* sp. were observed under a fluorescence microscope (Olympus, BH-2) using a mercury lamp. The B-B excitation filter and O-530 barrier filter were used.

Results and Discussion

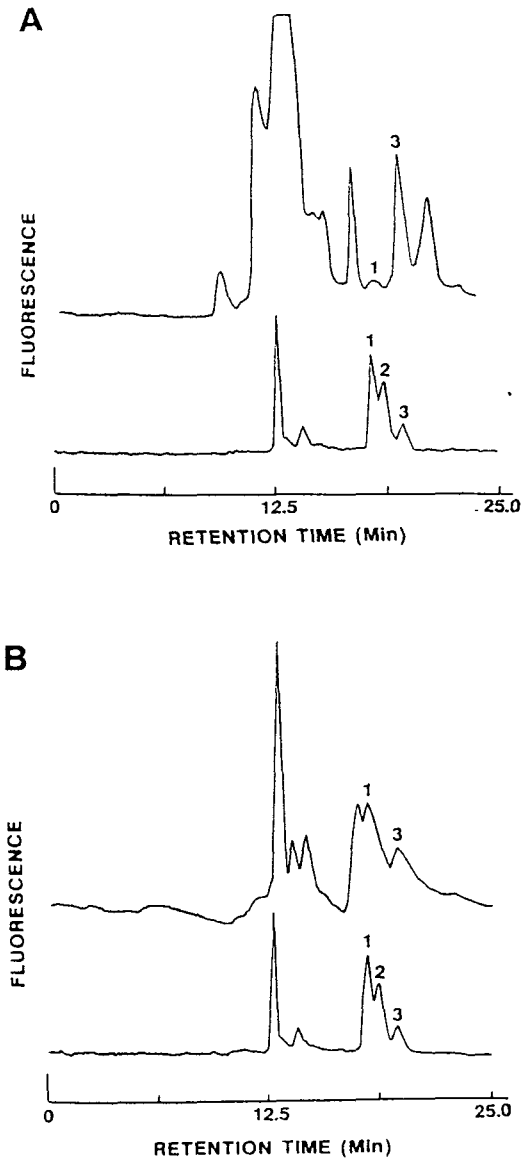


Fig. 2. Chromatogram of HPLC analyses. (A) extract of *Acinetobacter* sp., (B) extract of *Euplotes mutabilis*, Upper : sample, lower : standard, 1 : TTX, 2 : 4-epi-TTX, 3 : anhydro-TTX.

The occurrence of sodium channel blockers in the protozoa and two bacterial isolates was detected by the tissue culture assay (data not shown). The HPLC analysis indicated that the peak of these sodium channel blockers corresponded to TTX and its analogs

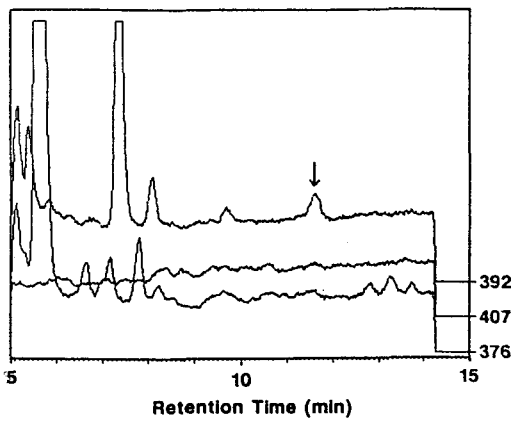


Fig. 3. Selected ion-monitored chromatogram of the TMS derivatives from the alkaline-degraded extract of *Euplotes mutabilis*. The arrow indicates the characteristic peak of C_9 base derivatives of TTX.

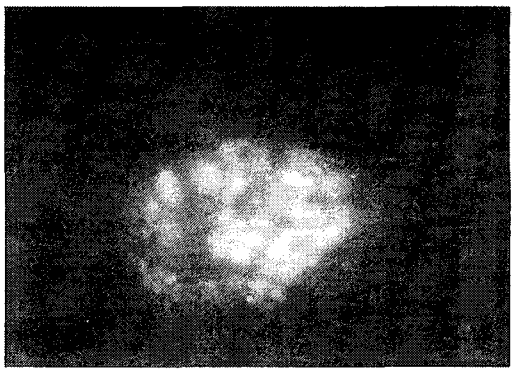


Fig. 4. Accumulation of fluorescently labeled bacteria (FLB) in *Euplotes mutabilis*.

(Fig. 2A and B). Further, the selected ion-monitored chromatogram of the trimethylsilyl derivative of the toxin was obtained. Mass fragment ions at m/z 376, 392 and 407 which are characteristic of the C_9 base derivative appeared at Rt. 11.7 min (Fig. 3). From these results, it was concluded that the sodium channel blocker in *E. mutabilis* was TTX or one of its analogs. Both bacterial strains tested also showed the production of TTX by the methods described above.

The number of *E. mutabilis* reached to ca. 5×10^2 cells/ml. Using the FLB, the ingestion of TTX- producing bacteria was clearly shown (Fig 4). Although the quantitative measurement was not performed, numerous FLB were observed in the body under the fluorescence microscope.

The mechanism of toxification of various types of marine animals has not yet been fully understood. Although it seems reasonable to assume that bacteria are the sole or major producers of TTX in nature, it is prerequisite to confirm the transfer of this toxin to bacterivorous organisms. The present result supports this idea and indicates the possibility of TTX accumulation through trophic levels.

Microscopic observations clarified that bacterial populations in aquatic ecosystems are maintained at rather constant level, ca. 10^6/ml (Hobbie et al., 1977; Simon et al., 1992). This is explained by the observation that bacterial production is balanced with protozoan grazing (Anderson & Fenchel, 1985; Berniger et al., 1991). The recent general concept is that about one third of primary production in aquatic environments will go through bacterial populations (Cole et al., 1988). Therefore, TTX in marine bacterial populations may be constantly transferred to protozoa in the sea. The ciliate used in the present work was identified as *Euplotes mutabilis*. *Euplotes* is quite widely distributed in marine environments, especially in coastal shallow water (Sleigh, 1989), and is assumed to play an important role in the microbial loop of these environments.

It is noteworthy that photosynthetic *Dinophysis* that is responsible for diarrhoeic shellfish poisoning (DSP) were reported recently to contain food vaculoes in the cells (Jacobson & Anderson, 1994). It was suggested that ciliates could be important as their prey. It was also clarified that some heterotrophic *Dinophysis* species feed solely on ciliates (Hansen, 1991). If the ciliates contain toxins such as DSP, paralytic shellfish poisons (PSP), or TTX, those chemicals could be transferred to the predator and subsequently accumulated in their bodies.

Some precautions, however, will be necessary to interpret the present data. It is still possible that TTX was synthesized by the protozoa itself of by its sym-

biotic bacteria. In the present investigation, the culture medium was not completely bacteria-free and the quantitative analysis of TTX transfer from bacteria to protozoa was not accomplished. If protozoa also produce TTX, however, this leads to another question; for what purpose do they synthesize TTX? The same question can be also extended to all toxin-containing animals, if we assume their own production. At present, it is very difficult to answer this question, and we think it more reasonable to assume that TTX is produced by bacteria in nature and transferred to higher trophic levels through the food web. The present result strongly supports this idea. As for symbiotic bacteria, there was no indication of their presence in the ciliated used, although more careful examination is still required. In any case, TTX and its analogs can be widely distributed among various marine animals.

In conclusion, the occurrence of TTX and its analogs in ciliated protozoa and the ingestion of TTX-producing bacteria were confirmed. To our knowledge, this is the first report on the occurrence of TTX in protozoa. Further quantitative and extensive investigation to clarify the toxification mechanisms of various marine organisms will be required.

References

Anderson, P and T. Fenchel. 1985. Bacterivory by microheterotrophic flagellates in seawater samples. Limnol. Oceanogr., 30, 198~202

Berninger, U. G., B, J. Finlay and P. Kuuppo-Leinikki. 1991. Protozoan control of bacterial abundances in freshwater. Limnol. Oceanogr., 36, 139~147.

Cole, J. J., S. Findlay and M. L. Pace. 1988. Bacterial production in fresh and saltwater ecosystems : a cross-system overview. Mar. Ecol. Prog. Ser., 43, 1~10.

Do., H. K., K. Kogure and U. Simidu. 1990. Identification of deep-sea sediment bacteria which produce tetrodotoxin. Appl. Environ. Microbiol., 56, 1162~1163.

Do, H. K., K. Kogure, C. Imada, T. Noguchi, K, Ohwada and U. Simidu. 1991. Tetrodotoxin production of actinomycetes isolated form marine sediment. J. Appl. Bacteriol., 70, 464~468.

Evans, M. H. 1972. Tetrodotoxin, saxitoxin and related substances : their applications in neurobiology. Inter. Rev. Neurobiol., 15, 83~166.

Fenchel, T. 1982. Ecology of heterotrophic microflagellates. 4. Quantitative occurrence and importance as bacterial consumers. Mar. Ecol. Prog. Ser., 9, 35~42.

Hansen, P. J. 1991. *Dinophysis*-a planktonic dinoflagellated genus which can act both as a prey and a predator of a ciliated. Mar. Ecol. Prog. Ser., 69, 201~204.

Hobbie, J. E., R. J. Daley and S. Jasper. 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol., 33, 1225~1228.

Jacobson, D. M. and P. A. Andersen. 1994. The discovery of mixotrophy in photoxynthetic species of *Dinphysis* (Dinophyceas) : light and electron microscopical observations of food vacuoles in *Dinophysis acuminata, D. norvegica* and two heterotrophic dinophysoid dinoflagellates. Phycologia, 33, 97~110.

Kao, C. Y. 1986. Structure-activity relations of tetrodotoxin, saxitoxin, and analogues. Annals of New York Academy of Sciences, 479, 52~67.

Kogure, K., H. K. Do, E. V. Thuesen, K. Nanba, K. Ohwada and U. Simidu. 1988a. Accumulation of tetrodotoxin in marine sediment. Mar. Ecol. Prog. Ser., 45, 303~305.

Kogure, K., M. L. Tamplin, U. Simidu and R. R. Colwell. 1988b. A tissue culture assay for tetrodotoxin, saxitoxin and related toxins. Toxicon, 26, 191~197.

Kosaka, T. 1989. Technical Manual In 8th International Congress of Protozoology Workshop.

pp. 12~13.

Mosher, H. S., F. A. Fuhrman, H. D. Buchwald and H. G. Fisher. 1964. Tarichatoxin-Tetrodotoxin : A potent neurotoxin. Science, 144, 1100~1110.

Mosher, H. S. and F. A. Fuhrman. 1984. Occurrence and origin of tetrodotoxin. In *Seafood toxins*. American Chemical Society, Washington D. C., pp. 333~344.

Narita, H., S. Matsubara, N. Miwa, S. Akahane, M. Murakami, T. Goto, M. Nara, T. Noguchi, T. Saito, Y. Shida and k. Hashimoto. 1987. *Vibrio alginolyticus*, a TTX-producing isolated from the starfish, *Astropecten polyacanthus*. Nippon Suisan Gakkaishi, 53, 617~621.

Noguchi, T., A. Uzu, K. Koyama, J. Maruyama, Y. Nagashima and K. Hashimoto. 1983. Occurrence of tetrodotoxin as the major toxin in a xanthid crab *Atergatis floridus*. Nippon Suisan Gakkaishi, 49, 1887~1892.

Sherr, B. F., E. B. Sherr and R. D. Fallon. 1987. Use of monodisperses, fluorescently labeled bacteria to estimate in-situ protozoan bacterivory. Appl. Environ. Microbiol., 53, 958~965.

Sherr, B. F., E. B. Sherr and C. Pedros-Alio. 1989. Simultaneous measurement of bacterioplankton production and protozoan bacterivory in estuarine water. Mar. Ecol. Prog. Ser., 54, 209~219.

Simidu, U. 1985. Identification of marine bacteria. In *Methods in marine microbiology*. Gakkai Shuppan Center, Tokyo, pp. 228~233.

Simidu, U., T. Noguchi, D. F. Hwang, Y. Shida and K. Hashimoto. 1987. Marine bacteria which produce tetrodotoxin. Appl. Environ. Microbiol., 53, 1714~1715.

Simon, M., B. C. Cho and F. Azam. 1992. Significance of bacterial biomass in lakes and the ocean comparison to phytoplankton biomass and biogeochemical implications. Mar. Ecol. Prog. Ser., 86, 103~110.

Sleigh, M. 1989, Protozoa and other protists. Cambidge Univ. Press, Cambridge, UK.

Thuesen, E. V., K. Kogure, K. Hashimoto and T. Nemoto. 1988. Poison arrowworms : a tetrodotoxin venom in the marine phylum Caetognatha. J. Exp. Mar. Biol. Ecol. 116, 249~256.

Yasumoto, T. and T. Michishita. 1985. Fluorometric determination of tetrodotoxin by high performance liquid chromatography. Agric. Biol. Chem., 49, 3077~3080.

Yotsu. M., T. Yamazaki, Y. Meguro. A. Endo, M. Murata, H. Naoki and T. Yasumoto. 1987, Production of tetrodotoxin and its derivatives by *Pseudomonas* sp. isolated from the skin of a puffer fish. Toxicon. 25, 225~228.

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