APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2001, p. 2393–2394 Vol. 67, No. 5
0099-2240/01/$04.00+0 DOI: 10.1128/AEM.8.3.2393–2394.2001

Letter to the Editor

No Ability To Produce Tetrodotoxin in Bacteria

Bacterial production of tetrodotoxin (TTX) was first reported by Yasumoto et al. (8). Detection of bacterially produced TTX has been primarily by high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS). In September 1995, *Applied and Environmental Microbiology* published a paper indicating that both the HPLC and GC-MS methods lacked specificity for TTX (3). In that paper, I pointed out that the previously reported production of TTX by bacteria should be revised or reexamined.

In April 2000, *Applied and Environmental Microbiology* published data of Lee et al. (2) reporting that *Vibrio* strains isolated from the intestines of puffer fish produce TTX and its derivatives. Among the detection methods used by Lee et al. (2), thin-layer chromatography and electrophoresis had been commonly used through the 1970s. These methods have been little used since the 1980s because the separation of TTX is better accomplished by HPLC and GC-MS. Also, while Lee et al. also used HPLC and GC-MS, the detection of TTX by all four of these methods relies on alkali hydrolysis (2). Therefore, the analytical methods used by Lee et al. are nonspecific for TTX.

In the article of Lee et al. (2), a large TTX peak was shown in Fig. 1 while no toxicity was reported in Table 1. It is, however, well known that a good correlation is observed between TTX concentrations calculated by mouse bioassay and HPLC when TTXs from puffer fish and newt are used (7). Also, the apparent lower limit for TTX detection by HPLC is 10 ng 10 μl^{-1} (= 1,000 ng ml^{-1}) (7) and that by mouse bioassay is 220 ng ml^{-1} (1), indicating that TTX detected by HPLC can be easily detected by mouse bioassay. Therefore, the results of Lee et al.'s Fig. 1 and Table 1 may indicate false-positive results by the analytical methods. This possibility had been pointed out in the 1995 paper (3).

There has been no description of the determination of the structures of the toxins obtained from bacteria, although a large amount of TTX should easily be obtained by their cultivation. The chemical structures of TTXs from puffer fish, newt, and blue-ringed octopus have already been reported (6). It has, therefore, been thought even by Japanese scientists that the chemical structures of the substances obtained from bacteria were TTX, although no one has shown these structures. Hence, a structural determination should not be a hard task and is indispensable for confirmation of production of TTX by bacteria.

Production of TTX by bacteria has been presented to support the food chain origin of TTX in puffer fish (8). The food chain origin of TTX was first hypothesized from the observation that cultured puffer fish have no TTX, which suggested that puffer fish have no ability to produce TTX. It has, however, been demonstrated recently that cultured puffer fish have detectable TTX (4) and that these fish can produce TTX (5). The progress in TTX research, including the 1995 paper (3), is not referenced in the article of Lee et al. (2).

As the methods used to detect TTX lack specificity, I cannot agree with the conclusions of Lee et al. (2) that *Vibrio* strains produce TTX.

REFERENCES

1. **Kawabata, T.** 1978. Assay method for tetrodotoxin, p. 232–240. *In* Food hygiene examination manual, vol. 2. Japan Hygiene Association, Tokyo, Japan.
2. **Lee, M. J., D. Y. Jeong, W. S. Kim, H. D. Kim, C. H. Kim, W. W. Park, Y. H. Park, Y. S. Kim, H. M. Kim, and D. S. Kim.** 2000. A tetrodotoxin-producing *Vibrio* strain, LM-1, from the puffer fish *Fugu vermicularis radiatus*. Appl. Environ. Microbiol. **66:**1698–1701.
3. **Matsumura, K.** 1995. Reexamination of tetrodotoxin production by bacteria. Appl. Environ. Microbiol. **61:**3468–3470.
4. **Matsumura, K.** 1996. Tetrodotoxin concentrations in cultured puffer fish, *Fugu rubripes*. J. Agric. Food Chem. **44:**1–2.
5. **Matsumura, K.** 1998. Production of tetrodotoxin in puffer fish embryos. Environ. Toxicol. Pharmacol. **6:**217–219.
6. **Mosher, H. S.** 1986. The chemistry of tetrodotoxin. Ann. N. Y. Acad. Sci. **479:**32–43.
7. **Yasumoto, T., and T. Michishita.** 1985. Fluorometric determination of tetrodotoxin by high performance liquid chromatography. Agric. Biol. Chem. **50:** 793–795.
8. **Yasumoto, T., D. Yasumura, M. Yotsu, T. Michishita, A. Endo, and T. Kotaki.** 1986. Bacterial production of tetrodotoxin and anhydrotetrodotoxin. Agric. Biol. Chem. **50:**793–795.

Kendo Matsumura
Yamaguchi Prefectural Research Institute of Public Health
2-5-67 Aoi
Yamaguchi 753-0821, Japan

Phone: 083-922-7630
Fax: 083-922-7632
E-mail: aat43203@par.odn.ne.jp

Author's Reply

In the letter of Dr. K. Matusmura in response to our recent paper (1), he pointed out that contaminants from a bacterial culture medium may confound the identification of tetrodotoxin (TTX), which puffer fish produce. The contamination issue was possibly based on the results of his previous study of TTX production by bacteria (2).

Our conclusive response to his criticism is that bacterial production of TTX is still a valid proposition and that exogeneous production should be regarded as a theory based on this production of the toxin. Our conclusive and persuasive claims are based on the following reasons. (i) Our *Vibrio* sp. strain was isolated as a single colony, and the single colony was separately cultured in medium. This indicates that although high-performance liquid chromatography (HPLC) or gas chromatography-mass spectrometry might detect the same peaks as those obtained from the bacterial medium, positive toxicity in a mouse bioassay would not be detected. However, our TTX and its derivatives purified from the bacterial culture cells showed a strong toxicity by mouse bioassay. (ii) Only one *Vibrio* sp. strain from three *Vibrio* species tested produced TTX and its derivatives during bacterial cultures. Therefore, efforts to examine whether intestinal *Vibrio* species are responsible for the production of TTX or not should be carefully performed. Only a few *Vibrio* sp. strains produce TTX, and thus isolation and characterization of bacterial strains may be key points. It is, therefore, assumed that Dr. Matsumura's strain may not be a TTX-producing *Vibrio*, although he cultured *Vibrio* species. Please note that there are many kinds of *Vibrio* species in *Fugu*'s intestinal tissues. (iii) Our study used both preparative

thin-layer chromatography and electrophoretic resolution for isolation of TTX from bacterial cells. (iv) How could a single transfer of a bacterial colony be contaminated by so much TTX before it was used in the next analytical procedure? (v) When *Fugu* fish were experimentally cultivated in an artificial aquarium, the toxicity (MU of TTX) of the *Fugu* fish was severely reduced with a reduction in intestinal bacterial flora.

Dr. Matsumura's criticism may be an insignificant result derived from the difficulties described above. Especially with HPLC data, similar peaks are sometimes confused, and his claims may be the results of technical problems during HPLC. Furthermore, one more possibility for his obtaining opposing results is that he may have cultured *Vibrio* spp. which do not produce any TTX. I would like to suggest that he isolate a TTX-positive bacterium, further characterize it, and then discuss his results with us.

Interestingly, the criticism made by Dr. Matsumura was also subjected to evaluation in a Japanese journal named *Chemistry and Biology* (3), which is published monthly by the Japan Society for Bioscience, Biotechnology, and Agrochemistry (formerly the Agricultural Chemical Society of Japan). It was concluded that bacterial production of TTX is still a valid supposition, and thus his conclusion was ignored by Dr. T. Noguchi, Nagasaki University Faculty of Fisheries, Nagasaki, Japan, and Dr. T. Yasumoto, Tohoku University Faculty of Agriculture, Sendai, Japan. We immediately contacted Dr. Noguchi again to discuss his conclusions more carefully, resulting in a conclusive determination that TTX is produced by marine bacteria.

In our scientific and principal elucidation of the exogenous and indogenous production of TTX in bacteria, we had not cited Dr. Matsumura's paper (2), in which he insists that bacteria do not have the ability to produce TTX, that contaminants from the bacterial culture medium may confound the identification of the TTX, and that puffer fish do produce the toxin. I would like to respectfully ask him to consider carefully reexamining TTX production by bacteria. As we mentioned in our paper, a final resolution of this contradiction would be obtained by an elucidation of genes for TTX synthesis at the molecular level.

REFERENCES

1. **Lee, M.-J., D.-Y. Jeong, W.-S. Kim, H.-D. Kim, C.-H. Kim, W.-W. Park, Y.-A. Park, K.-S. Kim, H.-M. Kim, and D.-S. Kim.** 2000. A tetrodotoxin-producing *Vibrio* strain, LM-1, from the puffer fish *Fugu verimcularis radiatus*. Appl. Environ. Microbiol. **66:**1698–1701.
2. **Matsumura, K.** 1995. Reexamination of tetrodotoxin production by bacteria. Appl. Environ. Microbiol. **61:**3468–3470.
3. **Yasumoto, T.** 1996. There are still bacteria which produce tetrodotoxin in Fugu. Chem. Biol. (Kagak to Seibutsu) **34:**837–839. (In Japanese.)

Dong-Soo Kim
Department of Food Science and Technology
Kyungsung University
Nam-ku
Pusan 608-736, Korea

Cheorl-Ho Kim*
Department of Biochemistry and Molecular Biology
College of Oriental Medicine
Research Center for Intractable Diseases
Dongguk University
Kyung-Ju
Kyungpook 780-714, Korea

Phone: 82-54-770-2663
Fax: 82-54-770-2281
E-mail: chkimbio@dongguk.ac.kr