トゲモミジガイ(ヒトデ)から分離したVibrio alginolyticusによるテトロドトキシンの産生

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Vibrio alginolyticus, a TTX-producing Bacterium Isolated from the Starfish *Astropecten polyacanthus*

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Intestines of a starfish *Astropecten polyacanthus* were examined for microflora, as part of the studies on the mechanism of toxification of trumpet shell *Charonia sauliae* which contains mostly tetrodotoxin (TTX) and sporadically causes human poisonings. The starfish showed a somewhat unique microflora which was composed mainly of bacteria belonging to the two genera *Vibrio* and *Staphylococcus*. Four *V. alginolyticus* and six *V. damsela* strains were identified from among a total of 22 strains isolated.

The cell extract of each strain was analyzed for TTX by HPLC, UV spectrophotometry and GC-MS. TTX was identified in two *V. alginolyticus* strains by all the three methods. In addition, some *V. alginolyticus* and *V. damsela* strains, as well as some *Staphylococcus* strains, disclosed the presence of TTX when analyzed by GC-MS and/or HPLC.

It was concluded from these results that these strains, especially the two *V. alginolyticus* strains, could closely be involved in toxification of the starfish.

In December 1979, a food poisoning incident due to ingestion of a trumpet shell *Charonia sauliae* occurred at Shimizu, Shizuoka Prefecture. The responsible toxin was unexpectedly demonstrated to be tetrodotoxin (abbreviated TTX below) accumulated in the digestive gland, which came from the starfish *Astropecten polyacanthus*, one of trumpet shell's favorites.[1-3] Subsequent surveys showed that the toxicity of trumpet shell digestive gland was clearly higher in the bottom area than in other areas of Suruga Bay, in a rough agreement with the distribution of the starfish *A. polyacanthus*.[4] The true origin of the TTX, however, has remained to be elucidated.

In this connection, we have recently found a TTX-producing ability in *Vibrio* sp. which was isolated from the intestines of a xanthid crab *Atergatis floridus*.[5]

The present study was undertaken in these situations. The results showed that some *Vibrio* bacteria, especially *V. alginolyticus*, along with *Staphylococcus* bacteria, produced TTX, indicating their involvement in toxification of the starfish.

Materials and Methods

Materials

A live specimen (71 g in body weight) of the starfish *A. polyacanthus* was collected from Suruga Bay, Shizuoka Prefecture in October 1985 and immediately used. It showed a toxicity score of 32 MU/g, when assayed by the official method for TTX.[6]

Examination of Bacterial Flora

The intestinal contents of the specimen were examined for bacterial flora by the routine procedure using PYBG and 1/20 PYGB media. Main bacterial strains isolated were cultured in 1 *l* flasks containing a 1% NaCl-1% Phytone peptone medium at 25°C for 10 days and identified at the generic and/or species level according to the

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Bergey's Manual.[7]

Separation of "TTX Fraction" from Bacterial Culture

The bacterial strains were examined for TTX-producing ability as follows. The culture (1 *l*) of each bacterium was centrifugated at 3,000 rpm for 30 min. To the cells harvested was added 100 m*l* of 0.1% acetic acid, followed by 10 min-treatment with an ultrasonicator (Tomy Seiko). The mixture was heated in a boiling water bath for 20 min, cooled to room temperature, and filtered through a Toyo filter paper No. 5C. The filtrate was freeze-dried. The resulting solid was dissolved in a small amount of 0.03 M acetic acid, and applied to a Bio-Gel P-2 column (2.5×94 cm). A 0.03 M acetic acid was used as eluant, and 5-m*l* fractions collected. The fractions corresponding to those of authentic TTX were combined and freeze-dried. "TTX fraction" thus obtained was subjected to high performance liquid chromatography (HPLC), UV spectrophotometry, gas chromatography-mass spectrometry (GC-MS) as described below.

On the other hand, some of the culture broths separated were adjusted to pH 2.5 with acetic acid, and heated in a boiling water bath for 20 min. After cooling, the solution was adjusted to pH 5 with ammonia water, and treated with about 30 g of activated charcoal (Wako). After washing with water exhaustively, the charcoal was extracted with 1% acetic acid-20% ethanol, and freeze-dried. The solid obtained was dissolved with a minimum amount of 0.03 M acetic acid, followed by Bio-Gel P-2 column chromatography, and "TTX fraction" was obtained as with the bacterial cells.

Identification of TTX and Related Substances

(1) High performance liquid chromatography

A Hitachi 638-50 HPLC system equipped with an AM-314 (YMC) column (0.6×30 cm) was used. A 0.05 M potassium phosphate buffer-1% methanol (pH 7.0) was used as eluant, and heptane-sulfonic acid as ion-pairing reagent. The eluate was mixed with 3 N NaOH at a 1:1 volume ratio, followed by heating at 100°C for 0.4 min, and the fluorophors formed were monitored by a Hitachi 650-10 fluorospectrometer (excitation at 381 nm, emission at 505 nm). The pattern thus obtained was compared to that for authentic TTX containing anhydroTTX (abbreviated anh-TTX below), which was prepared from the ovaries of a puffer *Fugu vermicularis porphyreus* as reported previously.[8]

(2) UV spectrophotometry

Small amounts of the freeze-dried "TTX fraction" was dissolved in 2 m*l* of 2 N NaOH, and heated in a boiling water bath for 45 min. After cooling, the solution was examined for the UV absorption spectrum characteristic to the C_9-base, 2-amino-6-hydroxymethyl-8-hydroxyquinazoline which should have been derived from TTX and/or related substances, if present.

(3) Gas chromatography-mass spectrometry

The above alkaline hydrolyzate was adjusted to pH 4 with 1 N HCl and extracted thrice with 5 m*l* each of 1-butanol. The extracts were combined, freeze-dried, trimethylsilylated by the procedure reported before,[1] and subjected to GC-MS on a Hitachi GC-mass spectrometer M-80. A column (0.3×200 cm) of Chromosorb W (AW DMCS) (60–80 mesh) coated with 1.5% OV 101 was used, and temperature raised from 165–200°C at a rate of 5°C/min. The ionizing voltage was kept at 70 eV and the ion source temperature at 200°C.

Results and Discussion

Bacterial Flora

The main bacterial strains isolated were found to belong to the two genera *Vibrio* and *Staphylococcus*. The starfish *A. polyacanthus* seems to be endowed with a somewhat unique microflora. Further attempts were made to identify 14 *Vibrio* strains isolated alive from the starfish specimen, by examining their biochemical and biological properties. As Table 1 shows, the 14 *Vibrio* strains were composed of four *V. alginolyticus*, six *V. damsela*, and four unidentified ones.

Production of TTX by Vibrio and Staphylococcus Bacteria

"TTX fraction" which was separated from the bacterial cells and/or culture broth of each of the 14 *Vibrio* and 4 *Staphylococcus* strains as described above, was subjected to instrumental analyses.

Fig. 1 shows an example of HPLC pattern of "TTX fraction" separated from the cells of *V. alginolyticus*. Two peaks were detected whose retention times agreed well with those of TTX and anh-TTX. Another peak which was supposed to be 4-epiTTX from the retention time (14.5 min), appeared in both *Vibrio* toxin and authentic toxin samples.

Fig. 2 shows an example of UV spectrum of the alkaline hydrolyzate of "TTX fraction" separated from the cells of *V. alginolyticus*. A shoulder appeared at around 270 nm, indicating the pre-

Table 1. Properties of 14 *Vibrio* strains isolated from the starfish intestines, along with their identification

	Strain No.				
	1, 3, 7, 8	5, 6, 9 10, 11, 13	2, 14	4	12
Growth on TCBS agar	Y*1	G*2	Y	G	Y
Oxidase	+	+	+	+	+
Catalase	+	±	+	+	+
Voges-Proskauer	+	+	−	−	−
Indole	+	−	+	+	+
Lysine decarboxylase	+	±	+	+	+
Ornithine decaboxylase	+	−	−	−	−
Arginine dihydrolase	−	+	−	−	−
Urease	−	+	−	−	−
H_2S (TSI)	−	−	−	−	−
Motility	+	+	+	+	+
Acid from:					
Glucose	+	+	+	+	+
Mannitol	+	−	+	−	+
Arabinose	−	−	−	−	−
Inositol	−	−	−	−	−
Rhamnose	−	−	−	−	−
Maltose	+	+	+	+	+
Sucrose	+	−	+	−	+
Lactose	−	−	−	−	−
ONPG	−	−	+	−	−
Growth in NaCl:					
0%	−	−	−	−	−
3%	+	+	+	+	+
6%	+	+	+	+	+
8%	+	−	−	−	−
10%	+	−	−	−	−
Identified as:	*V. alginolyticus*	*V. damsela*		(Unidentified)	

*1: Y=yellow, *2: G=green.

sence of the C_9-base specific to TTX or related substances.

The trimethylsilyl derivative of the alkali degradation product was subjected to GC-MS. Mass fragment ions at *m/z* 407 (parent peak), 392 (base peak) and 376 which are specific to the trimethylsilylated C_9-base, appeared at almost the same retention time irrespective of the strain. An example of the chromatogram is shown in Fig. 3. Each of the trimethylsilyl derivatives showed peaks at *m/z* 407, 392 and 376, evidencing the presence of the C_9-base in "TTX fraction" tested. In Fig. 4 is shown an example of the mass spectrum.

All the data thus obtained for the bacterial cell extracts are summarized in Table 2.

In terms of GC-MS, TTX was detected in eight out of the 14 *Vibrio* strains: four strains of *V. alginolyticus*, two strains of *V. damsela*, and two unidentified strains. TTX was not detected in some of the 8 strains when analyzed by HPLC and/or UV spectrophotometry, probably because of their relatively lower sensitivity than that of GC-MS. It was noted that all the three methods clearly detected TTX in two *V. alginolyticus* strains, No. 3 and 7. On the other hand, three out of the four staphylococcal strains showed the presence of the C_9-base when analyzed by GC-MS.

Bacterial cell extracts were used in all the above instrumental analyses. Analyses of culture broths from *Vibrio* strains No. 2–4 gave the same results as for their cell extracts (data not shown).

In the next place, *V. alginolyticus* strain No. 7, one of the two typical TTX-producers, was cultured in several 500-m*l* flasks containing a 1% NaCl-1% Phytone peptone medium at 25°C. At 24 h-intervals, the bacterial cells were harvested and extracted as described above, and determined for TTX toxicity by the official assay method.[6]

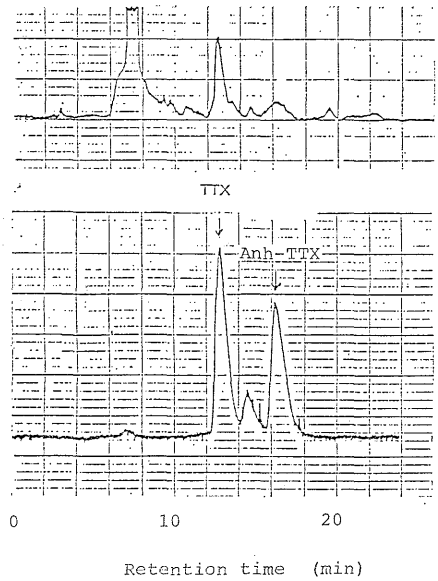

Fig. 1. HPLC of *V. alginolyticus* (strain No. 3) toxin (upper) and authentic TTXs (lower).

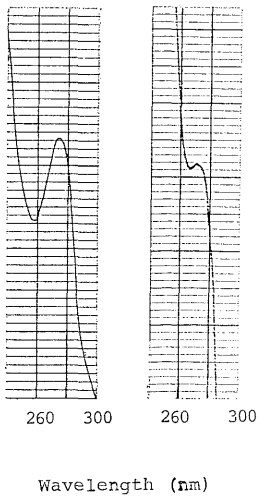

Fig. 2. UV absorption spectra of the alkaline hydrolyzates of *V. alginolyticus* (strain No. 7) toxin (right) and authentic TTXs (left).

Results showed that no toxicity was detected in 24 h- and 48 h-culture, but as high a toxicity as 213 MU per flask rather suddenly appeared in 72 h-culture.

All these data seem to indicate that *Vibrio* and *Staphylococcus* bacteria, especially *V. alginolyticus*, are closely involved in toxification of the starfish *A. polyacanthus* which, in turn, toxifies the trumpet shell *C. sauliae* by the food chain.[2]

TTX was long believed to be contained exclusively in the pufferfish belonging to the family Tetraodontidae until 1964 when this toxin was also found in the California newt *Taricha torosa*.[6]

Since then, TTX was detected one after another, in a goby *Gobius criniger*, atelopid frogs, and the blue-ringed octopus *Octopus maculosus*.[9] TTX and/or related substances have also been found in several gastropod mollusks including *C. sauliae*, three starfishes including *A. polyacanthus*, a xanthid crab *A. floridus*, and two types of flatworms.[10-12]

The present results, along with recent findings of *Vibrio* sp. from the xanthid crab[5] and *Pseudomonas* sp. from a calcareous alga[13] as TTX-producing bacteria, may provide a useful clue to elucidate the mechanism of toxification of TTX-containing animals.

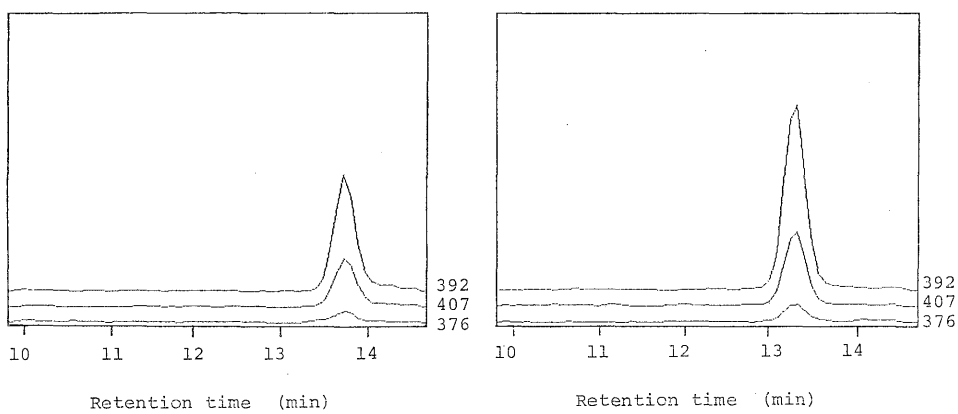

Fig. 3. Selected ion-monitored chromatograms of the TMS derivatives from the alkali-hydrolyzed *V. alginolyticus* (strain No. 3) toxin (right) and authentic TTXs (left).

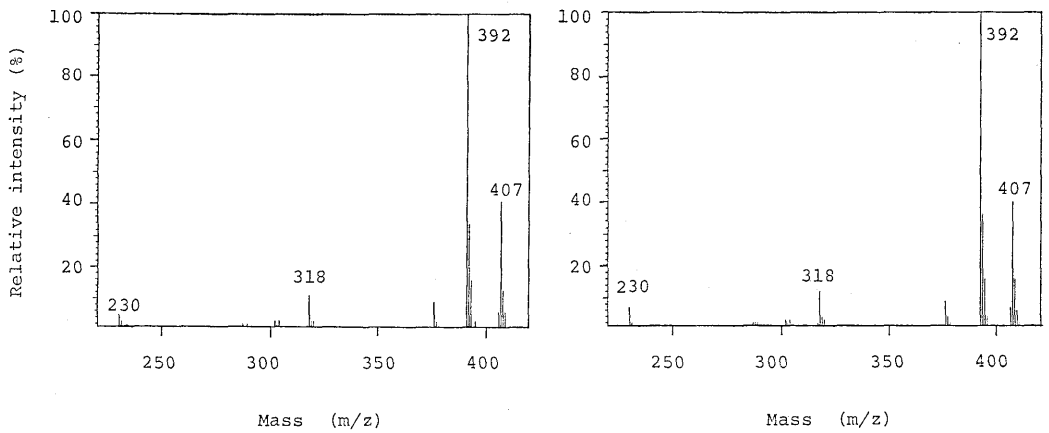

Fig. 4. Mass spectra of the C_9-base-$(TMS)_3$ derivative from the alkali-hydrolyzed *V. alginolyticus* (strain No. 3) toxin (right) and authentic TTXs (left).

Table 2. Instrumental analyses for TTX or the C_9-base in "TTX fraction" from *Vibrio* and *Staphylococcus* strains isolated

Serial No. of strain	Species	HPLC (TTX)	UV (C_9-base)	GC-MS (C_9-base)
1	*Vibrio alginolyticus*	−*1	±*1	+*1
2	*Vibrio* sp.	−	±	+
3	*Vibrio alginolyticus*	+	+	+
4	*Vibrio* sp.	−	−	+
5	*Vibrio damsela*	+	−	+
6	*Vibrio damsela*	−	−	−
7	*Vibrio alginolyticus*	+	+	+
8	*Vibrio alginolyticus*	±	−	+
9	*Vibrio damsela*	−	−	+
10	*Vibrio damsela*	−	−	±
11	*Vibrio damsela*	−	−	−
12	*Vibrio* sp.	→*2	−	±
13	*Vibrio damsela*	→	−	−
14	*Vibrio* sp.	→	−	±
15	*Staphylococcus*	→	−	−
16	*Staphylococcus*	→	−	−
17	*Staphylococcus*	→	−	+
18	*Staphylococcus*	→	−	+

*1 +, Detected; ±, judgement difficult; −, not detected.
*2 Not assayed.

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