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Bacillus horikoshii, a tetrodotoxin-producing bacterium isolated from the liver of puffer fish

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Abstract - A bacterium S184 isolated from the liver of puffer fish was found to produce tetrodotoxin (TTX). The toxin was purified from the cultures by activated charcoal column and C18 cartridge column after being cultured at 28 °C in broth medium. The results of toxicity mouse bioassay, liquid chromatography/fluorescent detection (LC/FLD), liquid chromatography/mass spectrometry (LC/MS), liquid chromatography/tandem mass spectrometry (LC/MS/MS) suggested that the strain could produce TTX during cultivation. The strain was identified as *Bacillus horikoshii* according to its physiological-biochemical characteristics and phylogenetic analysis based on 16S rDNA sequences.

Key words: *Bacillus horikoshii*; tetrodotoxin-producing bacterium; tetrodotoxin.

INTRODUCTION

Tetrodotoxin is one of the most potent neurotoxins known, which was initially found in *Tetrodontidae* and once thought to exist only in puffer fish. But subsequent studies suggest that TTX distributes widely in vertebrate species including puffer fish, goby, newt and frog, and invertebrate species inclusive of gastropod mollusk, crab, nemertean and starfish (Noguchi and Hashimoto, 1973; Hwang *et al.*, 1990; Daly *et al*., 1994; Hanifin *et al*., 2002; Tsai *et al*., 2002, 2004; Asakawa *et al.*, 2003). The wide distribution of TTX among the genetically unrelated animals makes the origin of TTX one of the most controversial and debatable topics for a long time. A postulation that TTX was produced originally by symbiotic microorganisms was put forward (Yasumoto *et al.*, 1986a).

TTX-producing bacteria have been isolated, such as *Pseudomonas* sp. from the alga (Yasumoto *et al.*, 1986b), *Vibrio* sp. from the Xanthid crab *Atergatis floridus* (Noguchi *et al.*, 1986), *Vibrio* strains, *Serratia marcescens*, *Microbacterium arabinogalactanolyticum*, *Nocardiopsis dassonvillei*, and *Bacillus* sp. from various species of puffer fish such as *Fugu vermicularis vermicularis*, *Fugu vermicularis radiatus*, *Fugu rubripes*, *Chelonodon patoca* (Noguchi *et al.*, 1987; Lee *et al.*, 2000; Yu *et al.*, 2004; Wu *et al.*, 2005a, 2005b; Yan *et al.*, 2005), *Vibrio alginolyticus* from chaetognatha (Thuesen and Kogure, 1989), *Alteromonas*, *Bacillus*, *Pseudomonas*, and *Vibrio* sp. from the blue-ringed cotopus *Octopus maculosus* (Hwang *et al.*, 1989), *Vibrio*, *Pseudomonas*, *Pasteurella*, *Aeromonas*, and *Plesiomonas* sp. from a gastropod *Niotha clathrata* (Cheng *et al.*, 1995), *Pseudoalteromonas* sp. from urchin (Ritchie *et al.*, 2000), *Vibrio* sp. from nemertean worms (Carroll *et al.*, 2003). Moreover, TTX-producing bacteria such as *Bacillus*, *Micrococcus*, *Acinetobacter*, *Aeromonas*, *Alteromonas*, *Moraxella*, *Vibrio*, *Streptomyces*, *Caulobacter*, *Alcaligenes*, *Flavobacterium* have also been isolated from marine and freshwater sediments (Do *et al.*, 1990, 1991, 1993). All these confirmed the microorganism origin of TTX.

TTX is a voltage-gated sodium channel blocker that provides effective tool for the research in neurobiology and neurophysiology. However, the yield of TTX from the ovaries and livers of puffer fish is limited. TTX-producing bacteria will make it possible not to destroy the puffer fish population. In the present study, a TTX-producing bacterium, *Bacillus horikoshii*, was isolated from the liver of puffer fish. It was identified by means of its physiological-biochemical characteristics and phylogenetic analysis based on 16S rDNA sequences.

MATERIALS AND METHODS

Sample collection. Two specimens of puffer fish were collected from the Taiwan strait in October 2007. The samples were kept alive throughout transportation to our laboratory. Each sample was washed three times with sterile seawater, and then was aseptically dissected into the ovary, intestine, liver and gallbladder for immediate isolation of bacteria.

Culture medium and agar plates. Marine agar 2216 plates were used for isolation of bacteria. Marine broth 2216 was used

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for culturing bacteria. The medium was adjusted to pH 7.6, which contained (per litre) peptone, 5 g; yeast extract, 1 g; ferric citrate, 0.1 g; sodium chloride, 19.45 g; magnesium chloride, 5.9 g; magnesium sulphate, 3.24 g; calcium chloride, 1.8 g; potassium chloride, 0.55 g; sodium bicarbonate, 0.16 g; potassium bromide, 0.08 g; strontium chloride, 34 mg; boric acid, 22 mg; sodium silicate, 4 mg; sodium fluoride, 2.4 mg; ammonium nitrate, 1.6 mg; disodium phosphate, 8 mg.

Isolation of bacteria. Each type of organ of puffer fish was homogenized in a sterile mortar, and was suspended in sterile seawater and then serially diluted to three concentrations of 10^{-1}, 10^{-2} and 10^{-3}. A 150 µl of extract from each diluted sample was aseptically spread on marine agar 2216 plates. The plates were incubated at 25 °C. A mixture of colonies was formed on each plate, and each discrete colony was transferred to new plate until pure culture was obtained on the basis of colony morphology. The purified strains were stored at -80 °C in the presence of glycerol.

Culture and purification of TTX from bacterial cultures. Each isolated strain was inoculated into liquid medium and incubated at 28 °C with shaking at 200 rev/min for 2-3 d. The cultures were centrifuged at 8000 rev/min for 15 min. The supernatant was evaporated under reduced pressure at 40 °C after the pH had been adjusted to 4.5, and then was boiled at 100 °C for 10 min, cooled and centrifuged to remove debris. The supernatant was loaded into an activated charcoal column and the toxin absorbed in the charcoal was eluted with 1 % acetic acid in 20% ethanol. The eluate was evaporated under reduced pressure at 40 °C, and then was passed through a prepared cartridge column (LC-18 Sep-Pak cartridge, Supelco).The prepared cartridges were previously washed with 10 ml methanol, 10 ml 0.1% acetic acid and then water until the eluate was neutral before use. After applying sample into the cartridge, 30 ml 0.03% acetic acid was eluted. The eluate was lyophilized, and subjected to toxicity bioassay and detection of TTX by chemical analyses.

Assays of TTX.

Toxicity mouse bioassay. The extracts from bacterial cultures were injected into white mice intraperitoneally. The symptoms of mice after being injected with the extracts were observed and recorded. The death time was recorded at the last grasping breath of the mouse.

LC/FLD. The post column LC/FLD was performed using ion-paired HPLC on a ZORBAX SB-C_8 column (4.6 x 250 mm i.d., 5 µm, Agilent). The column was eluted with a mixture of 0.05 M Na_2HPO_4, 0.05M NaH_2PO_4 and 2.5 mM sodium 1-heptanesulfonic acid (pH 6.7) at a flow rate of 0.3 ml/min. The eluate was mixed with an equal volume of 4 M NaOH and heated in a reaction coil at 110 °C. For detecting fluorescence, the fluorescence detector (Waters 2475) was used, and the excitation and emission were set at 365 nm and 510 nm, respectively.

LC/MS. The Waters LC/MS system was used, which consisted of a 2767 Sample Manager, 2545 Binary Gradient Module, SFO System Fluidics Organizer, and 3100 Mass Detector. Data was collected in positive electrospray ionization mode. LC was performed on a Hypersil C_8 column (4.6 x 250 mm i.d., 5 µm) using 0.03% acetic acid as the mobile phase at a flow rate of 1 ml/min.

LC/MS/MS. LC/MS/MS was performed on Agilent 1100 series LC/MSD Trap system coupled with a mass spectrometer. HPLC system was equipped with an Insertsil ODS-3 column (2.1 x 150 mm i.d., 5 µm, GL Science) at 30 °C. The injection volume was 10 µl. The mobile phase for TTX analysis was a solution containing 1 mM ammonium formate in 30 mM heptafluorobutyrate (pH 4.5) and methanol (99:1). The flow rate was 0.3 ml/min. The MS/MS measurements were based on a positive ion electrospray ionization interface using the following settings: nebulizer, N_2 (241 kPa); drying gas, N_2 (9.0 L/min, 350 °C); V-cap, 3500 V; fragmentor, 1.0 V.

Identification of the strain S184. The pH range for growth was determined in marine broth 2216 that was adjusted to various pH values, and the pH was adjusted prior to sterilization by addition of HCl or Na_2CO_3. The temperature range for growth was measured in marine broth 2216 at various temperatures. Growth at different NaCl concentrations was investigated in marine broth 2216. The ability of the strain to utilize substrates as sole carbon source was tested by supplementing minimal medium [0.2% $(NH_4)_2SO_4$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.05% $NaH_2PO_4 \cdot H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$, 0.05% K_2HPO_4] with 1% of the filter-sterilized carbon compound. Other characteristics including nitrate reduction, hydrolysis of starch, gelatin, and Tween 80 were performed as described by Gordon *et al.* (1973) and Claus and Berkeley (1986).

For phylogenetic analysis, the 16S rDNA was amplified from genomic DNA by PCR using the primers FC27 (5′-AGAGTTTGATCCTGGCTCAG-3′) and RC1492 (5′-ACGGCTACCTTGTTACGACT-3′) (Lane *et al.*, 1985). The amplified product of 16S rDNA was partially sequenced. The partial 16S rDNA sequence was compared with sequences in the GenBank database using BLAST at NCBI website (http://www.ncbi.nlm.nih.gov). Phylogenetic analysis was carried out using DNAMAN program. Phylogenetic tree was constructed by taking 1000 bootstrapping trials.

RESULTS

Toxin-screening of isolated bacteria strains

All 23 bacteria strains isolated on marine agar 2216 plates from the puffer fish were screened for TTX with toxicity mouse bioassay and LC/FLD. Among them, the strain S184 is found to produce TTX. In the mouse bioassay, mice injected with the extracted toxin show the typical symptoms of TTX intoxication: dyspnea and convulsion (Yasumoto *et al.*, 1986b). They were killed in 3 min.

Further confirmation of TTX from the strain S184

In order to confirm that the extract from S184 contained TTX truly, LC/MS was used. The mass spectrogram (Fig. 1) shows that the highest peak from S184 occurs at m/z 320, corresponding to the protonated molecular ion $(M + H)^+$ as TTX has a molecular mass of 319. There is also a minor peak at m/z 302, which is designated as anhydrotetrodotoxin.

But the molecular ion, $(M + H)^+$, for m/z 320 also represents isomer of TTX. So then LC/MS/MS was used to confirm that TTX rather than its isomer exists in the extract from S184 by analyzing the fragment ion spectra of the molecular ion (m/z 320). The TIC chromatograms (Fig. 2) indicate that the TTX peak of S184 is at the same retention time as the authentic TTX. The fragment ion spectra of the molecular ions, $(M + H)^+$, for TTX (m/z 320) of the authentic TTX and the extract from S184 obtained by the positive-ion MS/MS are shown in Fig. 3. The spectrum of TTX from S184 is almost identical to that obtained by the spectrum

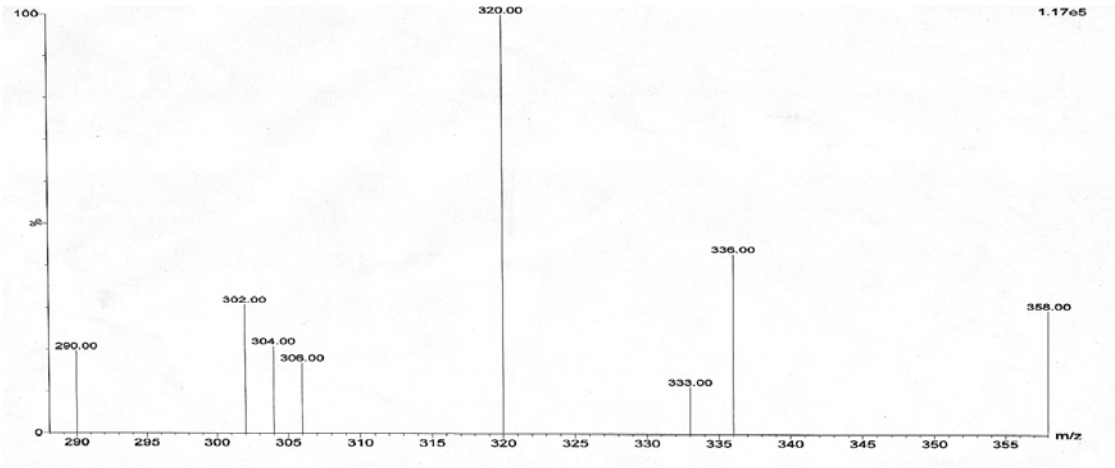

FIG. 1 - Mass spectrogram of the extract from S184.

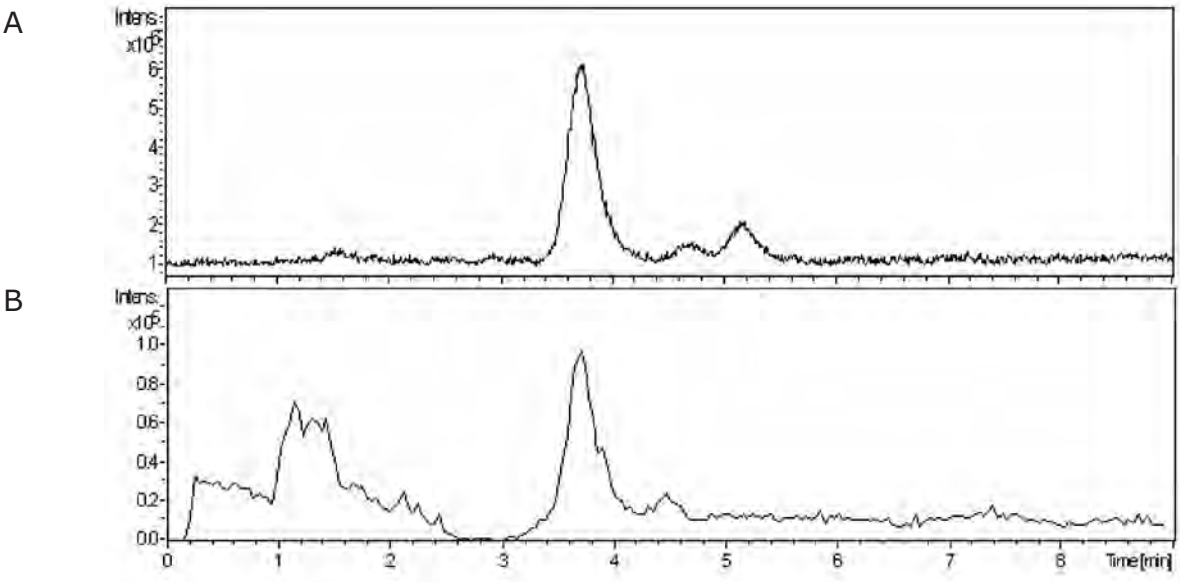

FIG. 2 - TIC chromatograms of (A) the authentic TTX and (B) the extract from S184.

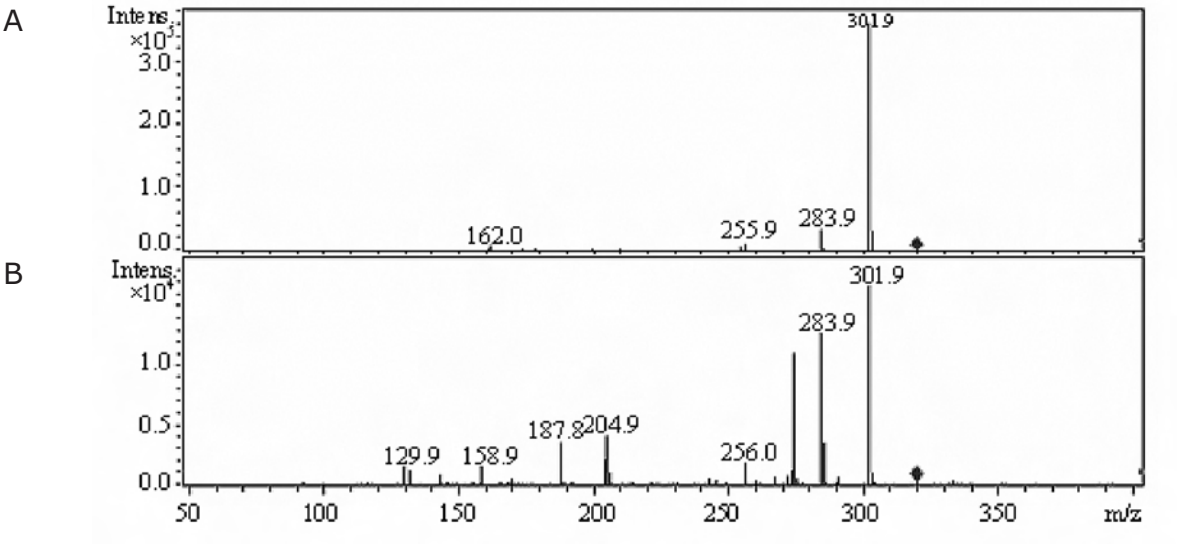

FIG. 3 - MS/MS spectra of m/z 320 of (A) the authentic TTX and (B) the extract from S184.

TABLE 1 - Physiological-biochemical characteristics of the strain S184 and related strain

Characteristic	Strain S184	*B. horikoshii* (DSM 8719)	Characteristic	Strain S184	*B. horikoshii* (DSM 8719)
Growth at			Nitrate reduction	-	-
10 °C	+	+	Hydrolysis of		
20 °C	+	+	Starch	+	+
30 °C	+	+	Gelatin	+	+
37 °C	+	+	Tween 80	+	-
40 °C	+	+	Growth on		
50 °C	-	-	L-Arabinose	-	-
Growth in NaCl (w/v)			Xylose	-	-
0%	+	ND	D-Galactose	-	-
3%	+	ND	L-Rhamnose	-	-
5%	+	+	D-Sorbitol	-	-
7%	+	+	D-Fructose	-	+
8%	+	+	α-Lactose	-	-
9%	+	+	D-Glucose	-	+
10%	+	-	Sucrose	-	+
Growth at pH			Glycerol	-	+
5.0	+	ND	Starch	+	+
6.0	+	-	Inositol	-	-
7.0	+	+	Maltose	+	+
8.0	+	+			
9.0	+	-			

+: positive; -: negative; ND: no data. Data for *Bacillus horikoshii* are from Nielsen *et al.* (1995), Li *et al.* (2002) and Logan *et al.* (2002).

of the authentic TTX. The fragment ions of TTX from S184 are similar to the characteristic fragment ions of the authentic TTX. Their similarity is higher than 900. So we consider that the strain S184 can produce TTX indeed.

Identification of the strain S184

The physiological-biochemical characteristics of the strain S184 are listed in Table 1. The strain grows between 10 and 40 °C. Growth is observed from pH 5.0 to pH 9.0, and the optimum is about pH 8.0. Growth is detected in the presence of 10% NaCl. The strain can hydrolyze starch, gelatin and Tween 80, and does not reduce nitrate to nitrite. The carbohydrate utilization profiles exhibit no growth on L-arabinose, xylose, D-galactose, L-rhamnose, D-sorbitol, D-fructose, α-lactose, D-glucose, sucrose, glycerol, inositol. As shown, the strain is closest to *Bacillus horikoshii*.

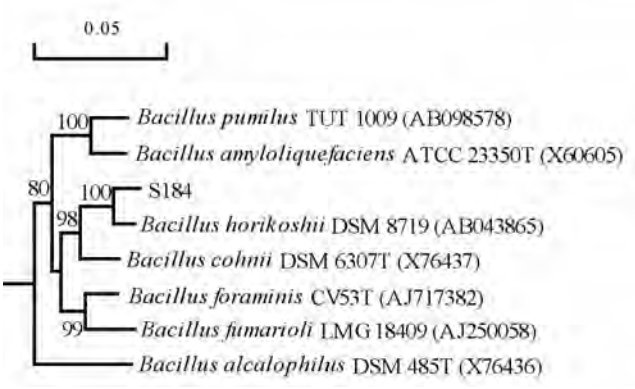

FIG. 4 - Phylogenetic tree showing the phylogenetic position of S184 based on 16S rDNA sequence analysis. Bootstrap values (expressed as percentages of 1000 replications) greater than 80 % are shown at branch points.

The phylogenetic analysis based on the partial 16S rDNA sequence data indicates that S184 is most closely related to the genus *Bacillus* (Fig. 4). The sequence about 800 bp of 16S rDNA was submitted to GenBank. Using the online program BLAST, the related sequence is searched from the databank. It is found that S184 is closely related to *Bacillus horikoshii* DSM 8719, and the sequence similarity is 100%.

Based on the results presented above, the strain S184 is identified as *Bacillus horikoshii*.

DISCUSSION

Various TTX-producing bacteria have been isolated from different TTX-bearing organisms and environmental habitats. The number of bacteria species is increasing continuously. The discovery of TTX-producing bacteria supports the theory that TTX is originated from the symbiotic microorganisms of the TTX-bearing organisms.

In the present study, TTX and its analogues have been detected in the strains isolated from the puffer fish. Moreover, the fragment ions of TTX were applicable to LC/MS/MS to identify TTX in the extract from S184. LC/MS/MS is a new useful method for further identification. The strain S184 was identified based on the physiological-biochemical characteristics and phylogenetic analysis based on 16S rDNA sequences. It was found that the strain was closely related to *Bacillus horikoshii*.

TTX is a potent neurotoxin selectively affecting the sodium ion gated channel, which is currently under development as a potential pharmacological drug. But the yield of TTX is limited by the yield of puffer fishes as TTX is obtained by the extraction from the organs of puffer fishes. Biosynthesis of TTX directly from TTX-bearing bacteria in vitro is considered much more efficient and economical than the traditional method.

However, the cultivation conditions for the bacteria in the laboratory are different from their natural habitat. So many microorganisms in the natural environment can not be cultivated in the laboratory, and the amount of TTX production by bacteria is very small. This suggested that, based on the present findings, the optimal culture conditions for maximum toxin yield as that in the puffer fish should be studied, such as the optimum medium composition, temperature, pH, salinity, dissolved oxygen level, and light intensity, and that the research on the mechanism of biosynthesis of TTX should be carried out to be helpful in bulk production of TTX directly from TTX-bearing bacteria in vitro in future.

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