Ecological Cycle of Thermostable Direct Hemolysin-Producing Strains of *Vibrio parahaemolyticus* in a Brackish-Water Area with Special Reference to Molluscs and Attached Microalgae

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ABSTRACT. Prevalences of thermostable direct hemolysin (TDH)-producing strains in communities of a gastropod mollusc, *Clithon retropictus*, and a bivalve mollusc, *Corbicula japonica*, and levels of the strains in attached microalgae and muddy sediments were investigated at a brackish-water area along Hashizu Creek and Togo Pond in Japan. *V. parahaemolyticus* was detected from attached microalgae at Hashizu Creek in summer months with the highest level of 1.4×10^5 cfu/g. Levels of the organism among 20 animals of *C. retropictus* and *C. japonica* at the area varied between non-detectable level and 10^3 per mollusc in summer months. TDH was detected from culture supernatants of 11–16% of strains isolated from the algae, sediments and *C. japonica* and 28% of those isolated from *C. retropictus* at Hashizu Creek. These evidences suggest that *C. retropictus* would get TDH-positive strains from the algae.—**KEY WORDS:** algae, TDH-producing strain, *Vibrio parahaemolyticus*.

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Vibrio parahaemolyticus is an estuarine organism which causes gastroenteritis after consumption of contaminated seafoods. Our previous survey at Hashizu estuary in Japan [6] showed that thermostable direct hemolysin (TDH)-producing strains of *V. parahaemolyticus* were detected at high levels from muddy sediments and *Clithon retropictus* (gastropod mollusc) but not from *Corbicula japonica* (bivalve mollusc). However, *C. retropictus* is an epibiose living on rocks and stones with few sediments while *C. japonica* is an endobiose living in the sediments. Although levels of *V. parahaemolyticus* in these molluscs were estimated for the pooled homogenates of 30 to 50 animals, the prevalences of the organism in the molluscan communities were not elucidated in our previous study [6].

C. retropictus ingests microalgae attached to rocks and stones in the estuary. As bacterial attachment to algal bodies has been observed by some investigators [1, 2, 5, 9, 10], TDH-producing strains were expected to attach to the estuarine algae and to be ingested by the gastropod. From these viewpoints, a survey was done to elucidate the prevalence of *V. parahaemolyticus* in the molluscan communities and to detect the organism from the microalgae at Hashizu estuary as described in the present paper.

MATERIALS AND METHODS

Monthly sampling was carried out from July 1984 to August 1985 at stations C and D in a brackish-water area along Hashizu Creek and Togo Pond in Tottori Prefecture, Japan, as shown in Fig. 1. Togo Pond is a lagoon connected with the Sea of Japan via Hashizu Creek as described previously [6]. Every 20 animals of *C. retropictus* and *C. japonica* were collected at each station and kept in small plastic containers individually. Microalgae attached to stones in water were scraped off with a knife. Samples of sediments were collected from the surfaces of the muddy sediments. All samples were collected under aseptic precautions and were brought to the laboratory in a cool box at 15°C or below for examination within 5 hr of collection.

V. parahaemolyticus was isolated on Bromothymol-blue-lactose agar plates (Nissui) supplemented with 5% NaCl and 1% sucrose at pH 7.4 (modified BTB agar) [6]. Molluscan tissue other than shell and muscle was homogenized in phosphate buffered saline (Na_2HPO_4 1.2g, KH_2PO_4 0.7 g, NaCl 6.8 g and KCl 0.2 g in 1,000 ml of distilled water, pH 7.4) (PBS). Viable counts of *V. parahaemolyticus* in the homogenized tissue were determined by filtration through membrane filters (Advantec A045A) of 0.45 μm pore size and 47 mm diameter which were

Fig. 1. Stations sampled at a brackish-water area along Hashizu Creek and Togo Pond in Tottori Prefecture, Japan.

then mounted on the modified BTB agar plates and incubated at 37°C for 18 hr. Viable counts of *V. parahaemolyticus* in sediments and algae were determined by streaking of the appropriate decimal dilutions of the homogenized samples on the modified BTB agar plates which were then incubated at 37°C for 18 hr. Any large green colonies at appropriate dilutions were purified by streaking for single colonies and identified as *V. parahaemolyticus* by the characters as described previously [6]. The viable counts were expressed as the number of colony forming units per gram (cfu/g) of the specimens.

TDH produced from the isolated strains was detected by enzyme immunoassay as follows. The strains were cultured in 1% Bacto-peptone (Difco) supplemented with 3% NaCl (pH 7.4) at 37°C for 24 hr. The cultures were centrifuged at 10,000 rpm for 20 min to obtain the supernatants, which were then boiled for 30 min and used as the antigens. Anti-TDH-mouse antibody prepared in our laboratory was used at a dilution of 1:1,000 in PBS supplemented with 1% Tween 80. A conjugate (peroxidase conjugated anti-mouse IgG goat IgG, Cappel) was used at a dilution of 1:4,000 in PBS supplemented with 1% Tween 80. Serially diluted antigen (50 μ l) in carbonate buffer (Na_2CO_3 6.36 g and NaHCO_3 5.04 g in 1,000 ml of distilled water, pH 9.5) was deposited in the wells of Microwell Plate 262170 (Nunc) and incubated at 4°C for 16 hr. After washing the wells three times with PBS supplemented with 0.05% Tween 20 (PBS-Tween solution), 50 μ l of 1% bovine serum albumin (Sigma) in PBS was added to each well. The wells

were incubated at 37°C for 2 hr and then washed three times with PBS-Tween solution. After the addition of the antibody (50 μ l), each well was incubated at 37°C for 45 min, and then washed with PBS-Tween solution. The conjugate (50 μ l) was added to each well and the wells were incubated at 37°C for 45 min. After washing the wells with PBS-Tween solution again, a substrate including 0.2 mM 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt and 0.04% H_2O_2 in citrate-phosphate buffer (0.1 M citric acid and 0.2 M Na_2HPO_4 , pH 4.7) was added. The plates were incubated at 37°C for 60 min and the absorbance was measured by EIA Reader EL-307 (Bio-Tek) at a wavelength of 405 nm. A value of nonspecific background absorbance plus 0.1 was estimated to be a cut-off point. A reciprocal of the highest dilution of the antigen showing an absorbance of over the cut-off point was estimated to be a titer of TDH.

RESULTS

Annual cycles of V. parahaemolyticus: Seasonal variations of *V. parahaemolyticus* in every 20 animals of *C. retropictus* and *C. japonica* collected at 2 stations are shown in Fig. 2. *V. parahaemolyticus* was detected from the molluscs at station C in May or June after non-detectable period in winter. Since then, the highest level of *V. parahaemolyticus* in 20 animals and rates of *V. parahaemolyticus*-positive animals increased in summer months. However, level of the organism varied between non-detectable level and 10^3 per animal even during the period from July to October. The highest level of the organism in 20 animals and the rates of *V. parahaemolyticus*-positive animals at station D were lower than those at station C. Weights of the tissue of each animal were 0.05–0.15 g for *C. retropictus* and 0.5–5.0 g for *C. japonica*.

As shown in Table 1, annual variations of *V. parahaemolyticus* in attached microalgae and sediments at 2 stations related to water temperature. The highest level of the organism detected in the algae, 1.4×10^5 cfu/g, was about 100 times higher than that detected in sediments, 1.5×10^3 cfu/g, at station C. The organism was still detected from the algae at 6.6°C in December, but not from the algae at station D.

Table 1 also shows annual variations of *V. parahaemolyticus* in two molluscs, in which viable units of the organism per gram of the molluscan

Fig. 2. Seasonal variations of *V. parahaemolyticus* in 20 animals of two molluscs at two stations. ●: *V. parahaemolyticus*-positive animal, ○: *V. parahaemolyticus*-negative animal.

tissue were calculated from the total count of the organism in 20 animals divided by the total weight of the tissue. At station C, the organism decreased to non-detectable level in *C. retropictus* at first, followed by *C. japonica* and sediments, and the algae in order from November 1984 to January 1985. The organism was first detected at 18°C in 1985 and increased remarkably at over 20°C. The highest level of the organism in microalgae, 1.4×10^5 cfu/g, was higher than that in sediments, 1.5×10^3 cfu/g. In molluscs, the highest level in *C. retropictus*, 3.1×10^3 cfu/g, was higher than that in *C. japonica*, 3.0×10^2 cfu/g. The level of the organism at station D was less than that at station C in any specimens.

Production of TDH from *V. parahaemolyticus* strains: A total of 550 strains of *V. parahaemolyticus* isolated in the present study was examined for production of TDH by enzyme immunoassay. The results are summarized in Table 2. TDH was detected from culture supernatants of 18 of 167 strains isolated from the algae, 14 of 111 strains from

muddy sediments, 34 of 120 strains from *C. retropictus* and 18 of 112 strains from *C. japonica* at station C. Levels of TDH in culture supernatants of TDH-producing isolates were 2048 to 8192 units, similar to those of clinical isolates, while those of TDH-non-producing isolates were less than 2 units.

DISCUSSION

V. parahaemolyticus was detected from attached microalgae at station C with the highest level of 1.4×10^5 cfu/g but not from those at station D. Eighteen of 167 strains isolated from the algae and 34 of 120 strains from *C. retropictus* at station C produced TDH. Levels of TDH in culture supernatants of the isolated strains were similar each other and to those of clinical strains. These evidences suggest that *C. retropictus* at station C would get TDH-producing strains from the attached algae at the feeding and that the gastropod at station D would have few opportunities to get the strains from

Table 1. Number of *V. parahaemolyticus* in relation to water temperature at two stations

Date of sampling	Water temperature (°C)	Number of organism per gram			
		<i>Clithon retropictus</i>	<i>Corbicula japonica</i>	Muddy Sediment	Microalgae
Station C					
1984					
July	28.8	ND ^{a)}	12	210	ND
August	29.3	240	20	620	1600
September	23.9	830	300	590	42000
October	18.5	440	32	1500	1100
November	13.8		2.1	290	130
December	6.6	0	0	0	67
1985					
January	5.5	0	0	0	0
February	4.4	0	0	0	0
March	8.0	0	0	0	0
April	15.7	0	0	0	0
May	18.6	0	0.1	0	3.3
June	20.1	20	4.6	120	13
July	29.0	490	88	170	1200
August	29.3	3100	ND	ND	140000
Station D					
1984					
July	29.8	ND	0	6	ND
August	30.8	0	0.4	0	0
September	26.8	0	6.2	73	0
October	17.8	0	3.9	190	0
November	13.6	0	0.3	26	0
December	7.3	0	0	0	0
1985					
January	4.7	0	0	0	0
February	4.5	0	0	0	0
March	9.5	0	0	0	0
April	16.1	0	0	0	0
May	21.4	0	0	0	0
June	22.3	0	0	0	0
July	31.0	490	0	0	0
August	31.5	7.2	ND	ND	0

a) Not done.

Table 2. Seasonal variations of thermostable direct hemolysin-producing *V. parahaemolyticus* as determined by enzyme immunoassay

Source of bacterial strain	Date of sampling							
	1984 Aug.	Sep.	Oct.	Nov.	1985 May	June	July	Aug.
Station C								
Microalgae	3/13 ^{a)}	5/26	0/12	2/13	0/3	0/3	3/58	5/39
Muddy sediment	2/17	3/22	4/19	2/18	— ^{b)}	0/21	3/14	ND ^{c)}
<i>C. retropictus</i>	14/25	1/22	4/18	—	—	1/17	2/18	12/20
<i>C. japonica</i>	6/12	6/35	1/13	3/21	—	0/14	2/17	ND
Station D								
Muddy sediment	—	0/11	0/9	0/3	—	—	—	ND
<i>C. retropictus</i>	—	—	—	—	—	—	0/3	4/4
<i>C. japonica</i>	1/1	0/9	—	—	—	—	—	ND

a) Number positive/number examined.

b) No strain was isolated.

c) Not done.

the algae.

Rate of TDH-producing strains isolated from *C. retropictus* was significantly higher than that from the other specimens, which is consistent with our previous evidences [6]. Our previous study [8] showed that TDH-producing strain D-3 survived in *C. retropictus* maintained in artificial seawater at 25°C at the highest level of 10^4 cfu/g for 21 days after oral inoculation, that TDH-non-producing strain R-13 was rather unstable in the gastropod and decreased to 10^2 cfu/g or less for 14 days and that both strains were cleared within 3 days from *Nerita albicilla*, a marine member of family Neritidae including *C. retropictus*. From these evidences, *C. retropictus* is supposed to have a supporting system for selective survival of TDH-producing strains though it doesn't refuse survival of TDH-non-producing strains.

TDH-producing strains were detected from *C. japonica* in 1984–85 (Table 2) but not in 1982–83 [6]. As TDH-producing strains are prevalent in the estuary, any animals inhabiting the area could be contaminated with these strains even if they might be estranged from the ecological cycle of the strains in ordinary conditions. Our previous study [7] showed that TDH-producing strain D-3 survived in *C. japonica* at the highest level of 10^2 cfu/g for at least 21 days regardless of the coexistence of TDH-non-producing strain R-13. TDH-producing strains might overcome the ecological barrier to be ingested by the bivalve in 1984–85.

The organism was detected at a higher level of 10^4 cfu/g from only one animal of *C. japonica* compared with the level less than 10^3 cfu/g in the other 19 animals at station C in September 1984 (Fig. 2). Five of 31 strains isolated from the animal produced TDH. Rate of TDH-producing strains among *V. parahaemolyticus* detected from the animal was similar to that from the bivalve community at station C (18/112). These evidences suggest that *V. parahaemolyticus* can survive at unusual levels in the bivalve and that the bivalve would not have a supporting system for selective survival of TDH-producing strains.

The level of *V. parahaemolyticus* in the molluscs varied among every 20 animals at each station, which would reflect, at least in part, the feeding and excreting activities of each animal and the variegated prevalences of *V. parahaemolyticus* at the estuary.

V. parahaemolyticus was detected from copepods, zooplankton, in Chesapeake Bay in the U.S.A. [3]. *V. parahaemolyticus* was observed to attach to the surface structure, chitin, of the copepods [4]. The organism attached to the copepods seems to be hardly ingested by the benthic algivore, *C. retropictus*, while the filter-feeder, *C. japonica*, might ingest the organism with detritus derived from zoo- and phyto-planktons and occasionally from sediment particles suspended at the disturbances of stream.

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