Phosphate-Dependent Anti-*Vibrio parahaemolyticus* Activity in Neritid Gastropod Molluscs

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Clithon retropictus, an estuarine neritid gastropod mollusc, is an important reservoir of thermostable direct hemolysin (TDH)-producing strains of *Vibrio parahaemolyticus* [3]. *V. parahaemolyticus* and *Escherichia coli* strains were found to survive in the gastropod for at least 21 days, in which irregular fluctuations were observed on viable counts of *V. parahaemolyticus* strains, but not those of *E. coli* strain [4]. When serial dilutions of *C. retropictus* homogenates contaminated with *V. parahaemolyticus* in phosphate buffered saline (PBS) were streaked directly on Bromothymol-Blue Lactose Agar (Nissui) supplemented with 3% NaCl (modified BTB agar), far fewer colonies of *V. parahaemolyticus* than those expected appeared on the agar in our early studies. Though membrane filter-cultivation technic was found to increase the viable counts in the homogenates, the irregular fluctuation remained [4]. Recently, the irregular fluctuation was found to be diminished when the molluscan homogenates were diluted in artificial seawater. From our preliminary evidences that PBS was not toxic to *V. parahaemolyticus* strains and that the irregular fluctuation was not observed on Trypticase Soy Agar (BBL) or Heart Infusion Agar (Nissui), the molluscan homogenate was suspected to have an activity to inhibit growth of *V. parahaemolyticus* on the modified BTB agar in the presence of phosphate ion. Prevalence of the activity in some molluscs and some properties of the activity in *C. retropictus* are described in the present paper.

V. parahaemolyticus D-3 and H-45 (TDH-producing strains), *V. parahaemolyticus* R-13 and N-18 (TDH-non-producing strains), *Vibrio cholerae* Y-1 and *E. coli* K-12 were used in this study. *V. parahaemolyticus* strains were grown in Heart Infusion Broth (Nissui) supplemented with NaCl at a final concentration of 2.5% at 37°C for 8 hr with shaking. Other organisms were grown in Heart Infusion Broth at 37°C for 24 hr. The organisms were washed three times and suspended in PBS (0.12 M NaCl, 0.009 M Na₂HPO₄, 0.005 M KH₂PO₄, pH 7.4) and artificial seawater [4] with a salinity of 35 permil (‰) for *V. parahaemolyticus* strains of 5‰ for *V. cholerae* and *E. coli* strains at a density of 4×10³ colony forming units per ml. Bovine serum albumin (Sigma) was added to PBS and artificial seawater at a concentration of 0.4% to avoid aggregations of the organisms.

Molluscs tested in this study were 6 gastropods (*C. retropictus*, *Heminerita japonica*, *Nerita albicilla*, *Turbo cornutus*, *Haliotis discus* and *Pugilina ternaiana*), 2

bivalves (*Crassostrea gigas* and *Anadara broughtonii*) and 5 cephalopods (*Octopus vulgaris*, *Paroctopus dofleini*, *Todarodes pacificus*, *Euprymna morsei* and *Abraliopsis scintillans*). The molluscan soft tissues were removed from the shells, weighed, added in 4 volumes of PBS or artificial seawater and homogenized using Polytron 10ST (Kinematica). After centrifugation at 27,000 × g for 20 min, the supernatants were filtered through membrane filter, 0.45 µm pore size, to remove particulate materials.

One ml aliquot of binary dilutions of the molluscan homogenates was mixed with one ml of *V. parahaemolyticus* suspensions in PBS or artificial seawater, incubated at 25°C for 90 min and then the viable counts of the organisms were estimated on the modified BTB agar plates. Viable counts of other organisms were estimated on Bromothymol-Blue Lactose Agar (Nissui) plates. Viable counts of the organisms incubated in the absence of the molluscan homogenates were regarded as control counts of the organisms. A reciprocal of the sample dilution corresponding to 50% survival of the control count was regarded as a titer of the antibacterial activity in unit per gram wet weight of the molluscan soft tissue (unit/g) tentatively.

Activities of the molluscan homogenates to inhibit growth of the organisms are shown in Table 1. In PBS, the activities of *C. retropictus* and *H. japonica* homogenates to inhibit *V. parahaemolyticus* strains were 77-140 and 320-860 units/g, respectively. The activities of the other molluscan homogenates to inhibit *V. parahaemolyticus* strains were low or non-detectable. *V. cholerae* Y-1 and *E. coli* K-12 were not inhibited by the homogenates. In artificial seawater, on the other hand, antibacterial activity was not detected in any homogenates.

Some properties of the activity in *C. retropictus* are shown in Table 2. The activity was labile to heat treatment and stable to acid and alkaline treatments. It was salted out in 80% saturated ammonium sulfate but not filtered through Diaflo membrane YM-100 (Amicon). From these evidences, it seems to be associated with proteins or their attachments.

Phosphate-dependent anti-*V. parahaemolyticus* activity was detected in two neritid molluscs, *C. retropictus* and *H. japonica*. The activity was low or non-detectable in the other molluscs. The activity seems to be not common in neritid molluscs because it was not detected in *N. albicilla* homogenate. Bactericidal pigment, fulvoplumierin, was detected in *N. albicilla* [8], though it seems to be different from the present activity because no activity was detected in *N. albicilla* in the present study.

Table 1. Antibacterial activity of the molluscan homogenates

Mollusc	Bacterial strain tested					
	<i>V. parahaemolyticus</i>				<i>V. cholerae</i>	<i>E. coli</i>
	D-3	H-45	R-13	N-18	Y-1	K-12
Gastropod						
<i>C. retropictus</i>	77(<10) ^{a)}	87(<10)	140(<10)	78(<10)	<10(<10)	<10(<10)
<i>H. japonica</i>	860(<10)	500(<10)	700(<10)	320(<10)	<10(<10)	<10(<10)
<i>N. albicilla</i>	<10(<10)	<10(<10)	<10(<10)	<10(<10)	<10(<10)	<10(<10)
<i>T. cornutus</i>	<10(<10)	47(<10)	<10(<10)	34(<10)	<10(<10)	<10(<10)
<i>H. discus</i>	<10(<10)	35(<10)	<10(<10)	<10(<10)	<10(<10)	<10(<10)
<i>P. ternatana</i>	<10(<10)	<10(<10)	<10(<10)	21(<10)	<10(<10)	<10(<10)
Bivalve						
<i>C. gigas</i>	<10(<10)	<10(<10)	<10(<10)	<10(<10)	<10(<10)	<10(<10)
<i>A. broughtonii</i>	<10(<10)	<10(<10)	<10(<10)	<10(<10)	<10(<10)	<10(<10)
Cephalopod						
<i>O. vulgaris</i>	<10(<10)	23(<10)	<10(<10)	24(<10)	<10(<10)	<10(<10)
<i>P. dofleini</i>	<10(<10)	55(<10)	46(<10)	43(<10)	<10(<10)	<10(<10)
<i>T. pacificus</i>	<10(<10)	38(<10)	70(<10)	31(<10)	<10(<10)	<10(<10)
<i>E. morsei</i>	<10(<10)	<10(<10)	<10(<10)	<10(<10)	<10(<10)	<10(<10)
<i>A. scintillans</i>	<10(<10)	<10(<10)	<10(<10)	<10(<10)	<10(<10)	<10(<10)

a) Number indicates antibacterial activity in PBS (unit/g). Number in parenthesis indicates the activity in artificial seawater (unit/g).

Table 2. Some properties of anti-vibrio activity in homogenates of *C. retropictus*

Treatment	Anti-vibrio activity
Heat treatment	
50°C 10 min	61 ^{a)}
60°C 10 min	28
70°C 10 min	<10
0.1N NaOH at 25°C for 30 min	130
0.1N HCl at 25°C for 30 min	110
Filtrate through	
Diaflo membrane YM-100	<10
Control	77

a) Activity was measured using *V. parahaemolyticus* D-3.

Antibacterial factors have been detected in many molluscs [1, 2, 5, 6, 8, 9], while phosphate-dependency of the factors has not been reported. Sodium hypophosphite was shown recently to inhibit growth of *V. parahaemolyticus* [7]. However, the molluscan homogenates *per se* are not probable to change phosphate ion to hypophosphite ion because the activity was not detected on Trypticase Soy Agar or Heart Infusion Agar (data not shown). In addition, growth of *V. parahaemolyticus* D-3 on Heart Infusion Agar was not suppressed by the pretreatment of the organism at 25°C for 90 min in 0.014 M-sodium

hypophosphite, the highest level presumably generated from phosphate ion in PBS. Thus hypophosphite ion was not probable to participate in the activity even if it would be generated in our experimental system. Conclusively, the activity seems to be carried by unknown factor(s) generated from the molluscan homogenates in the presence of phosphate ion and the modified BTB agar.

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