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Screening of Bacteria with Antiviral Activity Against Infectious Hematopoietic Necrosis Virus (IHNV) from Estuarine and Marine Environments

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A total of 748 isolates from estuarine and marine environments were evaluated for antiviral activity against infectious hematopoietic necrosis virus (INHV) according to plaque reduction assay. Among isolates from estuarine environment, 267 (71%) of 376 strains exhibited a 50% plaque reduction of IHNV, including 58 strains (18%) able to reduce plaques more than 90%. On the other hand, 120 (32%) of 372 strains from marine environment showed a 50% plaque reduction, including 23 strains (6%) showing a 90% plaque reduction. The incidence of bacteria having antiviral activity in the estuarine isolates was approximately twice that of the marine isolates. Most of the bacteria which exhibited plaque reduction of more than 90% belonged to genera *Pseudomonas*, *Achromobacter*, and *Vibrio*.

A representative strain of those, *Achromobacter* sp. 51HW-25, produced antiviral agents heavily when incubated with shaking at 21°C for 60 h. The antiviral products appeared to be two kinds of substances, thermolabile macromolecule and thermostable low molecular compound of molecular weight of less than 1,000, when the bacterium was grown at 20°C. However, the bacterium grown at 25°C allowed production of only the low molecular compound.

Studies of viral survival and behavior in aquatic environments are of consequence to ecology as well as to virology and to public hygiene. It is also very important to know survival of fish viruses and interactional relationships with other microorganisms in aquacultural environments for prevention of fish viral diseases. It is reported that viral inactivation in environmental water is caused by bacteria present in the water.[1-5] Magnusson *et al.*[1] concluded that the causative bacteria of viral inactivation in sea water was *Vibrio marinus*. Toranzo *et al.*[4,5] demonstrated that cultural filtrates of *Pseudomonas* sp. or *Vibrio* sp. isolated from marine environment inactivated human enteroviruses. We previously found that antiviral substance producing bacteria were detected in high incidence (14%) in an aquatic environment such as the hatchery raceway; the detection rate was nearly the same between the water and sediment samples of the environment. Furthermore, it was known that most of the bacteria having antiviral activity were identified as *Pseudomonas* sp..[6]

In this report, we describe screening of antiviral substance producing bacteria from a total of 748 strains isolated from the water and sediment samples of estuarine and marine environments. Productivity of antiviral agents by a strain of *Achromobacter* sp. showing the most potent antiviral activity is also discussed.

Materials and Methods

Virus and Cell Cultures

INHV (strain ChAb), isolated originally in our laboratory from chum salmon *Oncorhynchus keta*, was used for this study. The stock virus was propagated at 15°C in rainbow trout *Salmo gardneri* gonad cells, RTG-2,[7] in a 75 cm^2-plastic flask (Falcon) containing 25 m*l* of MEM10-Tris, which was composed of Eagle's minimum essential medium (MEM, Gibco), 10% fetal bovine serum (M. A. Bioproduct), 0.075% $NaHCO_3$, 100 IU/m*l* penicillin (Sigma), 100 μg/m*l* streptomycin (Sigma), and 1.6% Tris-buffer (Tris (hydroxymethyl) aminomethane(Tris)-hydrochloride) (Sigma), pH 7.8. After the cytopathic effect developed and the cells desquamated completely from the bottom of the flask, the cells were centrifuged at 4,000 r.p.m. at 4°C for 20 min.

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The supernatants were filtered with a 0.45 μm pore-sized filter (Millex-HA, Millipore). The virus obtained was stocked at −80°C until used. Chinook salmon (*O. tschawytscha*) embryo cells, CHSE-214[8], were used for the plaque assay. The cells were grown in MEM10-Tris at 15°C and seeded in 1 m*l* of the growth medium per well in a 24-well (16 mm in diameter, Falcon) plate to give approximately 10^6 cells/m*l*/well. The 1-day-old confluent monolayers of these cells were subjected to plaque assay.

Screening of Bacteria Having Antiviral Activity

A total of 748 bacterial isolates from the water and the sediment samples of estuarine and marine environments were collected seasonally from 1984 to 1985[9]: 376 isolates from the estuary of the Moheji river near Hakodate, Japan, and 372 isolates from the Nanaehama coast, also near Hakodate, Japan. All were screened for antiviral activity to IHNV. Bacteria were grown in 50 m*l* MCYG broth, which was composed of casamino acids (Difco), 5 g; yeast extract (Difco), 0.5 g; glucose, 1 g; 1,000 m*l* of 50% Herbst's artificial sea water and adjusted to pH 7.8, at 20°C for 48 h under shaking condition. The cultures were filtered with a Millex-HA filter and each 0.2 m*l* of the filtrates were reacted with an equal volume of diluted IHNV suspension (100–200 PFU/0.1 m*l*) at 15°C for 3 h. As a control, 0.2 m*l* of fresh MCYG medium was used. After reaction, the mixtures were assayed for infectious virus using the plaquing procedures previously reported.[10] Isolates causing a 50% plaque reduction compared with the control were considered to have antiviral activities.

Identification of Bacteria Having Antiviral Activities

Bacteria showing antiviral activities were identified to genus level by the method of Shewan *et al.*[11] A fermentative property of marine bacteria was examined by using the method of Leifson.[12]

Productivity of Antiviral Substances by a Representative Strain

A representative strain, *Achromobacter* sp. 51HW-25, which was isolated from the sea water collected at the Nanaehama coast in January, 1985, and which showed the most potent antiviral activity among the strains, was evaluated for productivity of antiviral substances. Incubation conditions for production of antiviral substances were first determined by testing incubation temperatures. One loopful of seed culture (3.7×10^8 CFU/m*l*) was incoculated to 15 m*l* of MCYG broth in L-form test tubes and incubated shaking at temperatures ranged from 10 to 33°C for 2 days with Temperature Gradient Bio-photorecorder (TN-112D, Toyo Kagaku Sangyo CO., LTD). After incubation, the cultural pH was measured, and the culture broth was examined for antiviral activity by plaque reduction assay. On the basis of the results, incubation periods were further determined at times of 12 h intervals for 7 days. The bacterium was grown in 400 m*l* MCYG broth in 500 m*l*-Sakaguchi flask and incubated shaking at 20°C for 7 days. At 12 h intervals 5 m*l*-aliquot of the culture was aseptically withdrawn, and the pH and optical density at 620 nm were measured. The antiviral activities of the cultural filtrates at each incubation temperature and time were expressed as the rate (%) of plaque reduction of IHNV to the control.

On the basis of these results, the bacterium was cultivated at 20 or 25°C for 3 days under shaking condition. After centrifugation, the cultural fluid was treated by ultrafiltration using Por Stirred Cell (Spectrum) with filter of 1,000 (molecularporous membrane disc, Spectrum), filters of 10,000 (molecut II GC, Millipore) and 30,000 (immersible CX-30, Millipore) molecular cut-off, and by dialysis against Dulbecco's phosphate buffered saline at 5°C overnight. Thermostability of culture filtrate dialysed was examined by heating at 50, 60, 100, and 121°C (autoclave) for 10 min. The same examination for culture filtrate cut off the components of molecular weight of more than 1,000 was also performed by heating at 40, 50, 60, 70, 80, 100, and 121°C (autoclave) for 10 min. These heated samples were cooled in ice just after the treatment. All the treated samples were filtered and estimated for comparative antiviral activity using the same method of plaque reduction assay described above.

Hydrolytic Activity of Achromobacter sp. 51HW-25 to Macromolecular Compounds

SA agar medium[13] was used as basal medium and supplemented with 0.5% arginate, 1% casein, 0.5% gelatin, 1% chitin, 0.5% starch, 5% egg yolk (lecithin), and 2% Tween 80, respectively. The Tween 80 medium was further supplemented with 0.01% $CaCl_2$. Commercial "DNA Agar" medium (Eiken) was used for test of DNA hydrolysis. Each medium was inoculated by streaking and incubated at 25°C for 7 days.

Results

Screening of Bacteria Having Antivirial Activity

A total of 748 strains isolated from estuarine and marine environments were screened for antiviral activity by using plaque reduction assay of IHNV. Among 376 strains isolated from the water and sediment samples of the estuary of the Moheji river, 166 (83%) of 199 strains isolated from the water sample reduced the plaques of IHNV to 50% compared with the control, including 46 (23%) showing a plaque reduction of more than 90%. On the other hand, 101 (57%) of 177 strains isolated from the sediment samples exhibited a 50% plaque reduction, including 13 strains (7%) showing a plaque reduction of more than 90%. The detection rate of bacteria with antiviral activity from the water samples was higher than that from the sediment samples (Table 1). The taxonomical genera of bacteria having antiviral activities are shown in Table 2. Most of the bacteria showing high plaque reduction of more than 90% from both the water and the sediment samples were identified as *Achromobacter* sp., *Pseudomonas* sp., and *Vibrio* sp.. *Pseudomonas* sp. was found to have the highest antiviral activity. Similar results were obtained in the bacteria isolated from the samples of the Nanaehama coast, although the detection rate was lower than that of the estuarine samples by about half (Table 3). Furthermore, such antiviral activity was more strongly detected in the bacteria from the water than from the sediment samples, similar to the results obtained with the estuarine samples. This tendency was not related to seasonal change. The bacterial genera showing antiviral activity are indicated in Table 4. *Achromobacter* sp., *Pseudomonas* sp., and *Vibrio* sp. exhibited similar results as in the estuarine environment, and were proved in relatively high incidence as bacteria having antiviral activity.

Table 1. Number of bacterial strains showing anti-IHNV activities among 376 isolates from the estuary of the Moheji river

Source of isolates		50% plaque reduction	90% plaque reduction
Sample	Tested		
Water	199	166 (83%)*	46 (23%)
Sediment	177	101 (57%)	13 (7%)
Total	376	267 (71%)	59 (16%)

* rate of positive strains to tested strains.

Determination of Conditions for Antiviral Agent Production

The effect of incubation temperature on a marine bacterium, *Achromobacter* sp. 51HW-25, for antiviral agent production is shown in Fig. 1. The bacterium grew at temperatures ranging from 10 to 28°C with the highest antiviral activity at 21°C. During the incubation period, the cultural pH was almost 7 to 8. The time course of the antiviral agent production is illustrated in Fig. 2. Bacterial growth reached its maximum 60 h after incubation. Antiviral activity also reached its maximum simultaneously and maintained activity until the end of the tested incubation period. The cultural pH varied from 7.8 to 8.6 during the incubation period.

Comparative Antiviral Activities of the Cell-Free Culture Tested by Dialysis and Ultrafiltration

The bacterial culture after incubation was filtered and further dialysed and ultrafiltered for estimation of comparative antiviral activities. The results are displayed in Table 5. The cultures cut off compounds with molecular weight of more than 1,000 still exhibited potent antiviral activity (95% plaque reduction). The untreated culture and the dialysed culture also showed strong anti-

Table 2. Bacteria having anti-IHNV activities among 376 isolates from water and sediment samples of the estuary of the Moheji river

Genus	No. of isolates			
	50% plaque reduction		90% plaque reduction	
	Water	Sediment	Water	Sediment
Achromobacter	33 (8.7%)*	37 (9.8%)	13 (3.5%)	3 (0.8%)
Flavobacterium/Cytophaga	7 (1.9%)	10 (2.7%)	1 (0.3%)	0 (0%)
Pseudomonas	45 (12.0%)	36 (9.6%)	21 (5.6%)	6 (1.6%)
Vibrio	37 (9.8%)	16 (4.3%)	11 (2.9%)	4 (1.1%)
Not-identified	4 (1.1%)	4 (1.1%)	0 (0%)	0 (0%)

* rate of positive strains to tested strains.

viral activity (100% plaque reduction).

Thermostability of the Antiviral Agents in Cell-Free Cultures

It was suggested from Table 5 that at least two kinds of antiviral substances (one with a molecular weight of less than 1,000 and the other, a substance remaining in a dialysis membrane) were produced in the bacterial culture. Because of this evidence, thermostability of the antiviral substance with high molecular weight was investigated using the cultures grown at 20 and 25°C. These results are indicated in Table 6. When the bacterium grew at 25°C, little antiviral activity was observed in the dialysed culture while non-dialysed culture showed potent antiviral activity, suggesting no production of high molecular antiviral substance in the culture. However, the culture grown at 20°C showed antiviral activity for both the dialysed and non-dialysed cultures, suggesting that two kinds of both high molecular compound and low molecular compound with molecular weight of less than 1,000 were produced in the culture, and that the high molecular substance could be inactivated by heating at over 50°C. The thermostability of the low molecular substance in the culture fluid cut off the compounds with molecular weight of more than 1,000 is shown in Table 7. The antiviral activity of the substance was still

Table 3. Number of bacterial strains showing anti-IHNV activities among 372 isolates from the Nanaehama coast

Source of isolates		50% plaque reduction	90% plaque reduction
Sample	Tested		
Water	176	77 (44%)*	18 (10%)
Sediment	196	43 (22%)	5 (3%)
Total	372	120 (32%)	23 (6%)

* rate of positive strains to tested strains.

Table 4. Bacteria having anti-IHNV activities among 372 isolates from water and sediment samples of the Nanaehama coast

Genus	No. of isolates			
	50% plaque reduction		90% plaque reduction	
	Water	Sediment	Water	Sediment
Achromobacter	32 (8.6%)*	20 (5.4%)	11 (3.0%)	1 (0.3%)
Flavobacterium/Cytophaga	9 (2.4%)	2 (0.5%)	0 (0%)	0 (0%)
Pseudomonas	25 (6.7%)	12 (3.2%)	7 (1.9%)	4 (1.1%)
Vibrio	9 (2.4%)	8 (2.2%)	1 (0.3%)	0 (0%)
Not-identified	2 (0.5%)	1 (0.3%)	0 (0%)	0 (0%)

* rate of positive strains to tested strains.

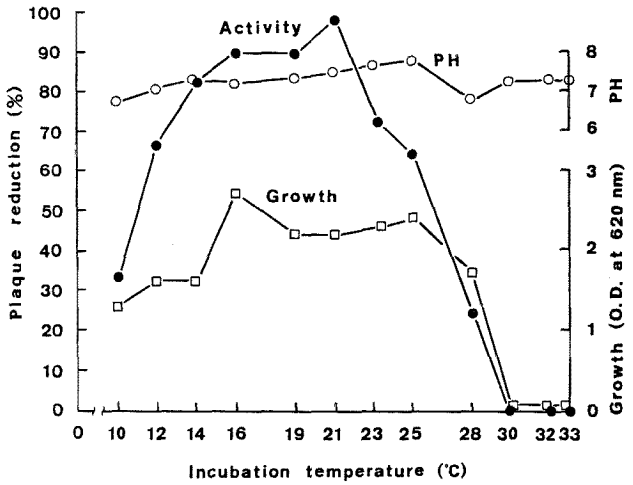

Fig. 1. Effect of incubation temperature of a marine bacterium, *Achromobacter* sp. 51HW-25, on production of anti-IHNV agent(s).

Table 5. Anti-IHNV activity of culture filtrate*1 of strain 51HW-25, which treated with dialysis or ultrafiltration

Sample	Plaque reduction
1,000 MW-cut*2	95%
10,000 MW-cut*2	91%
30,000 MW-cut*2	99%
dialysed*3	100%
untreated	100%

*1 The bacterium was cultivated in MCYG broth shaking at 20°C for 3 days.
*2 Culture fluid cut off the components with molecular weight of more than 1,000, 10,000, and 30,000.
*3 Culture fluid retained in the dialysis membrane.

quite stable after heat treatment, although a slight reduction in activity was caused by autoclaving.

Hydrolytic activity of various macromolecules by the bacterium also was tested for possibility of enzymatic inactivation of virus. The bacterium hydrolyzed casein, gelatin, starch, Tween 80, and DNA, but not alginate, chitin, and lecithin (Table 8).

Discussion

We previously reported that fish pathogenic viruses were inactivated in the raceway waters of

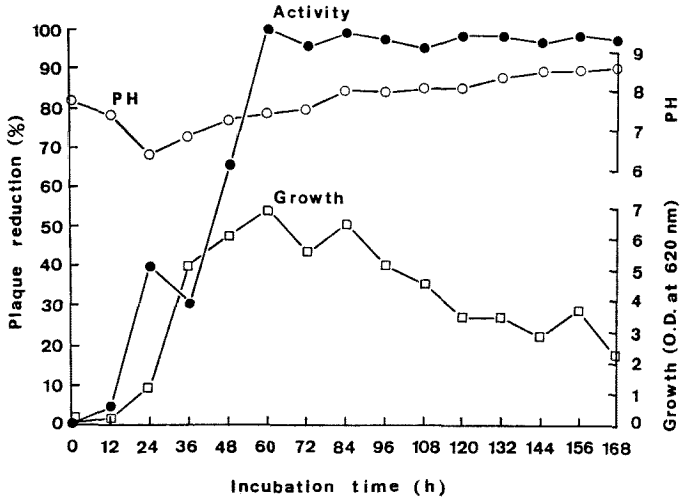

Fig. 2. Time course of anti-IHNV agent(s) production by a marine bacterium, *Achromobacter* sp. 51HW-25 at 20°C.

Table 6. Thermostability of dialysed culture filtrate* of strain 51HW-25 incubated at different temperatures

Incubation temperature	Plaque reduction of IHNV					
	Dialysed Heat treatment (°C)					Non-dialysed Non-heated (25°C)
	25	50	60	100	121	
25°C	16%	0%	6%	0%	13%	93%
20°C	100%	0%	10%	0%	0%	99%

* Culture fluid retained in the dialysis membrane and filtered.

Table 7. Thermostability of a low molecular weight (<1,000) of anti-IHNV substance, fractionated from a culture filtrate of strain 51HW-25

Untreated*	Plaque reduction of IHNV Heated (°C)						
	40	50	60	70	80	100	121
99%	99%	100%	99%	100%	99%	99%	94%

* Culture fluid which was not treated by ultrafiltration and kept at 25°C.

Table 8. Hydrolysis of macromolecular compounds by *Achromobacter* sp. 51HW-25

Substrate	
Alginate	−
Casein	+
Chitin	−
DNA	+
Gelatin	+
Lecithin	−
Starch	+
Tween 80	+

salmonid hatcheries, and that this phenomenon appeared to be attributed to the exoproducts from *Pseudomonas* sp.[14] Furthermore, our recent study demonstrated that such antiviral activity could be found in relatively high incidence in the bacterial isolates from aquacultural environments.[6] In the present study, a total of 748 isolates from the estuarine and marine environments were screened for antiviral activity and the bacteria exhibiting antiviral activities occurred more in these environments than in freshwater environments. Furthermore, such antiviral activity was found more in the bacteria from an estuarine environment than from a marine environment (possibly due to differences in eutrophic conditions for microorganisms). Up to date, comparative occurrence of these bacteria having antiviral activity has not been estimated between estuarine and marine environments, though there are some reports for antiviral activity by epiphytic bacteria from intertidal seaweeds of estuarine environments.[4,15] There are several reports about antibiotic production by marine bacteria[16-19] with some having been isolated and characterized as high molecular weight antibiotic.[20] Other studies on antiviral activity of some antibiotic-producing marine bacteria have been reported, as well, but the isolation and characterization of the antiviral substances have not been performed.[4,5] In addition, it is reported that *Actinomycetes* from littoral sediments produce antibiotics to bacteria,[21] but no reports for antiviral antibiotic-producing bacteria from the sediments of marine environments have been published. In this study, we demonstrated that bacteria having antiviral activity was detected in both water and sediment samples of the estuarine and marine environments though the occurrence in the sediment samples was lower than in the water samples. Thus, it was found that many bacteria having antiviral activity would be present in the estuarine and marine environments and play an antiviral action in these environment ecosystems. Such antiviral activity was found in various bacterial genera and especially, *Achromobacter*, *Pseudomonas*, and *Vibrio* showed potent antiviral activity. These findings are coincident with the report of Toranzo *et al.*[4]

In the experiments using a representative strain showing potent antiviral activity, a marine bacterium, *Achromobacter* sp. 51HW-25, most strongly produced the antiviral substances at 21°C though growth behavior was nearly the same at incubation temperatures ranging from 16 to 25°C, suggesting that the bacterium is psychrophilic. The culture's antiviral activity seemed to be regardless of pH in the range from 6 to 8. Antiviral substances released in culture when incubated at 20°C for 3 days could be presumed to be composed of at least two kinds of compounds; one a low molecular compound with a molecular weight of less than 1,000 and another a high molecular compound failing to pass through the dialysis membrane. The high molecular compound was heat-labile, suggesting the presence of enzymes or other proteins. Furthermore, since the bacterium hydrolyzed protein such as casein and gelatin and also nucleic acid such as DNA, it was suggested that this viral inactivation probably occurred by enzymatic cleavage of viral structure.

Ballester *et al.*[20] have reported that the high molecular weight antibiotic is produced by a marine bacterium, though not identified whether or not it is protein. On the other hand, Wratten *et al.*[22] isolate an antibiotic from a marine pseudomonad and also determine the structure. Thus, antibiotic production by marine bacteria are known, but most of the reports concern antibacterial, not antiviral effects. Recently, Ward *et al.*[23] describe inactivation of enteric viruses by the proteolytic bacterial enzyme released in fresh water. Our results also suggested that the fish pathogenic viruses were inactivated by marine bacterial metabolites which included a thermolabile, high molecular compound. Furthermore, we demonstrated that numerous bacteria inciting antiviral activity are inhabiting the estuarine and marine environments, and provided some considerations that they appear to play a significant ecological role among microorganisms in the aquatic ecosystem.

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