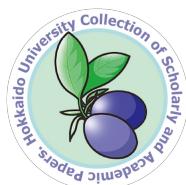




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Anti-Infectious Hematopoietic Necrosis Virus (IHNV) Substances Produced by Bacteria from Aquatic Environment

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Attempts were made to classify the antiviral properties of bacteria which has been isolated from various environmental waters and had the anti-infectious effects on infectious hematopoietic necrosis virus (IHNV). The majority of these bacterial strains were considered to produce the substances that cause direct inactivation of virus or inhibition of viral invasion into CHSE-214 cells. While a few of the isolates were considered to produce the substances that cause inhibition of viral replication. Among the four strains selected, two strains of *Pseudomonas* produced heat-stable and relatively low molecular weight substances; one strain of *Alteromonas* produced a high molecular weight, heat-labile substance; and one strain of *Pseudomonas* produced at least two kinds of substances unstable against heating.

In a series of studies on the microbial ecosystem of aquatic environment, it has been demonstrated that fish pathogenic viruses were inactivated in fresh-, brackish- and seawaters and their sediments, and the inactivation appeared to be caused by bacteria existing there (Kamei *et al.*, 1987b, 1988a). A large number of bacteria obtained from aquatic environment were screened for their antiviral activity, and 126 strains of them showed antiviral activity (Kamei *et al.*, 1987c, 1988b). Subsequently an antiviral substance produced by *Pseudomonas fluorescens* 46 NW-04, one of the 126 strains, was purified and identified as a peptide (Kamei *et al.*, 1988c, 1992; Kimura *et al.*, 1990).

In this study, we first attempted to classify antiviral properties of the substances produced by 63 strains selected from the 126 strains and secondly studied the thermostability and rough molecular size of the antiviral substances produced by 4 representative strains selected from the 63 strains.

Materials and Methods

Bacterial Strains

Of the 126 bacterial strains isolated previously (Kamei *et al.*, 1987c, 1988b), 125 strains were used for the determination of antiviral activity. One of the 126 bacterial strains lost its viability and failed to

be recovered. The strains had been stocked in TSB medium (BBL) with 20% glycerol at -80°C for about 5 years since 1987. Sixty-three of the 125 strains still maintained intense antiviral activity of more than 90% plaque reduction at the time this study started were used for the classification of antiviral properties.

To determine the characteristics of antiviral substances, four of the 63 strains, i.e., *Alteromonas* sp. 48HS-27 from sediment of Nanaehama coast near Hakodate; *Pseudomonas* sp. 51BBW-15 and 51BBW-29 from brackishwater and *Pseudomonas* sp. 51BBS-20 from sediment of Moheji River estuary near Hakodate, were selected as representative strains. These strains showed the strongest antiviral activity among the employed 63 strains by plaque assay. In the selection, strains showing cytotoxic effect against CHSE-214 cells were excluded.

Filtrate of Bacterial Culture

Before use, each strain was preincubated on FWA (Yoshimizu and Kimura, 1976) or SA (Yamamoto *et al.*, 1982) medium, then inoculated into CYG (Kamei *et al.*, 1988b) or MCYG (Kamei *et al.*, 1987c) broth and cultured with agitation at 25°C for 48 h. After incubation, culture fluids were filtrated through a $0.45\text{ }\mu\text{m}$ -pore size filter (Millex-HA, Millipore) and used for the classification of antiviral

properties.

Virus and Cell Cultures

Infectious hematopoietic necrosis virus (IHNV) strain ChAb, isolated from chum salmon *Oncorhynchus keta* (Yoshimizu *et al.*, 1989), was used for this study. The virus was propagated in RTG-2 cells. When cytopathic effect was complete, the culture fluid was filtrated through a 0.45 μ m-pore size filter and stored at -80°C until use. CHSE-214 cells were used for plaque assay according to Kamei *et al.* (1987a).

Classification of Antiviral Properties

The antiviral properties of substances produced by the 63 strains were determined by the following three methods: (1) CHSE-214 cells were propagated in 24 well plate for 48 h with each well containing 1 ml of MEM10-Tris medium (Eagle's minimum essential medium, Gibco). After incubation, 0.1 ml of the medium from each well was removed and replaced with an equal volume of the bacterial culture filtrate in duplicate wells for each of the bacterial strains and was left to react for 24 h. The cells were then washed three times with 1 ml of Hanks' BSS (Gibco) and subsequently 100 PFU/0.2 ml of IHNV was inoculated into each well and allowed to adsorb to the cells for 1 h at 15°C and 0.8 ml of methylcellulose overlay medium (Kamei *et al.*, 1987a) was added. The antiviral substances which reduce the plaque number compared with controls using fresh CYG or MCYB broth were considered to inhibit viral adsorption of CHSE-214 cells or make the cells resistant to IHNV (Kitamura, 1976). (2) An IHNV suspension of 0.2 ml (approximately 200 PFU/0.1 ml) was mixed with equal volume of the bacterial culture filtrate and left to react for 3 h at 15°C . After the cell culture medium of 72 h-old CHSE-214 cells in 24 well plate was removed, a 0.2 ml aliquot of the mixture of IHNV suspension and the bacterial culture filtrate was inoculated into 2 wells of 24 well plate, and allowed the virus to adsorb to the cells for 1 h at 15°C . Then the cells were washed three times with 1 ml of Hanks' BSS and added 1 ml of the overlay medium. The antiviral substances which reduce the plaque number compared with controls using fresh CYG or MCYB broth were considered to inactivate the virus directly or inhibit the ability of the virus to invade the cells. (3) The cell culture medium of 72 h-old CHSE-214 cells in 24 well plate

was removed and each well was inoculated with 100 PFU/0.2 ml of IHNV and allowed the virus to adsorb to the cells for 1 h and washed three times with 1 ml of Hanks' BSS. A mixture of 9:1 of overlay medium and each bacterial culture filtrate was prepared and 1 ml of this mixture was overlayed into each well. The antiviral substances which reduce the plaque number compared with controls using fresh CYG or MCYB broth were considered to inhibit the viral replication in the cells.

The antiviral substances produced by the strains showing antiviral activity in tests (1), (2) and (3) were described as the substance showing antiviral properties of Type I, II, and III, respectively.

Determination of Thermostability and Rough Molecular Size of Antiviral Substances Produced by the 4 Representative Strains

The four representative strains were incubated in CYG or MCYB broth at 25°C for 48 h and filtrated through Millex-HA filter. The filtrates were heated for 15 minutes from 30 to 100°C at intervals of 10°C , and at 121°C , then immediately cooled down with crushed ice, and the antiviral activity was determined by the plaque assay of Kamei *et al.* (1987b, 1988a). The filtrates from the four strains stored at 0°C without heating were used as controls.

The culture fluids of the four representative strains were centrifuged at $6,000 \times g$ at 4°C for 20 min, and the antiviral activity of supernatants were determined after ultrafiltration of 10,000, 20,000 and 50,000 molecular weight cut-off filters (Centricut Mini V-10, V-20 and V-50; KURABOU).

Results and Discussion

Classification of Antiviral Properties

There was no strain produced antiviral substance of Type I. Fifty-two (82.5%) of the 63 strains produced the antiviral substances of Type II, 10 strains (15.9%) of Type III and 1 strain (1.6%) of both Type II and Type III. Twenty-five strains (40%) of the 63 strains were identified as genus *Pseudomonas*. And 11 strains (55%) of the 20 strains from seawater environment were genus *Moraxella* and produced Type II substances (Table 1).

There are several reports on the antiviral mechanisms of substances produced by microorganisms in aquatic environment (Herrmann and Cliver, 1973;

Table 1. Generic composition of the isolates with anti-IHNV activity from fresh-, brackish- and seawater environment.

Genus* ¹	Freshwater		Brackishwater			Seawater	
	II* ²	III* ²	II	III	II+III* ²	II	III
<i>Pseudomonas</i>	5	2	6	8	1	3	0
<i>Vibrio</i>	1	0	3	0	0	0	0
<i>Aeromonas</i>	0	0	3	0	0	1	0
<i>Moraxella</i>	—	—	8	0	0	11	0
<i>Alteromonas</i>	—	—	5	0	0	4	0
<i>Flavobacterium</i>	0	0	0	0	0	1	0
Unidentified	1	0	0	0	0	0	0
Total (63)	7	2	25	8	1	20	0

*¹ Bacteria isolated by FWA medium were identified by the method of Shewan *et al.* (1960) and the isolates by SA medium were by the method of Ezura *et al.* (1988).

*² Type II, III; see Materials and Methods in text.

Table 2. Thermostability of anti-IHNV substances produced by *Pseudomonas* spp. 51BBW-29, 51BBW-15, 51BBS-20 and *Alteromonas* sp. 48HS-27.

Temp. (°C)* ¹	Plaque reduction (%)			
	<i>Pseudomonas</i> spp.			<i>Alteromonas</i> sp.
	51BBW-29 (III* ²)	51BBW-15 (III* ²)	51BBS-20 (II+III* ²)	48HS-27 (II* ²)
Unheated	100	100	100	100
30	100	100	100	98
40	100	100	100	97
50	100	100	100	84
60	100	100	100	33
70	100	100	100	8
80	100	100	16	7
90	100	92	17	2
100	89	90	4	0
121	17	43	0	0

*¹ Heated for 15 minutes.

*² Type II, III; see Materials and Methods in text.

Table 3. Anti-IHNV activity of culture filtrates of *Pseudomonas* spp. 51BBW-29, 51BBW-15, 51BBS-20 and *Alteromonas* sp. 48HS-27 through the ultrafilters.

Sample	Plaque reduction (%)			
	<i>Pseudomonas</i> spp.			<i>Alteromonas</i> sp.
	51BBW-29	51BBW-15	51BBS-20	48HS-27
Unfiltered	100	92	92	98
50,000 MW-cut*	99	100	95	23
20,000 MW-cut*	99	94	65	8
10,000 MW-cut*	100	97	67	2

* The ultrafilters were used to cut off the components of culture fluid with molecular weight; 50,000, 20,000 and 10,000.

Ward and Ashley, 1976, 1977; Richards *et al.*, 1978; Toranzo *et al.*, 1983; Kamei *et al.*, 1992). In a brief summary of these reports, the antiviral mechanisms are divided into the two types: (1) blockage of viral binding sites to a target-cell receptor, which is known with polysaccharides produced by marine algae (Richards *et al.*, 1978) and a peptide produced by *Pseudomonas* sp. 46NW-04 (Kamei *et al.*, 1992), and (2) disruption of viral proteins or nucleic acids by enzymes such as proteolytic enzymes and/or ribonuclease (Ward and Ashley, 1976; Toranzo *et al.*, 1983). These antiviral substances in the previous reports are considered to have the same antiviral property with type II in our classification.

Thermostability and Rough Molecular Size of Antiviral Substances Produced by the 4 Representative Strains

The thermostability of antiviral substances produced by the 4 strains were shown in Table 2. All unheated bacterial culture filtrates shown 100% plaque reduction, and heated culture filtrates of *Pseudomonas* spp. 51BBW-29, 51BBW-15, 51BBS-20 and *Alteromonas* sp. 48HS-27 showed more than 90% plaque reduction at 90, 100, 70 and 40°C, respectively.

Antiviral activities of unfiltered and ultrafiltered culture fluids were shown in Table 3. *Pseudomonas* spp. 51BBW-29 and 51BBW-15 showed more than 90% plaque reduction after filtration through the 10,000 MW cut-off filter. These results suggest that *Pseudomonas* spp. 51BBW-29 and 51BBW-15 produced heat-stable and comparatively low molecular weights antiviral substances belonging to Type III. As heat-stable antiviral substances of low molecular weight, ammonia against poliovirus (Ward and Ashley, 1977) and a peptide against IHNV and *Oncorhynchus masou* virus (OMV) (Kamei *et al.*, 1992) have been reported.

Antiviral activity of the culture fluid of *Alteromonas* sp. 48HS-27 was almost lost after filtration through the 50,000 MW filter. The substance of Type II which produced by *Alteromonas* sp. 48HS-27 was regarded as comparatively high in molecular weight and unstable against heating. As heat-labile antiviral substances of high molecular weight, proteolytic enzymes and/or ribonucleases have been reported (Herrmann and Cliver, 1973; Toranzo *et al.*, 1983).

Pseudomonas sp. 51BBS-20, which produced anti-

viral substances of both Type II and III, showed 65 and 67% reduction of antiviral activity after filtration by the 20,000 and 10,000 MW cut off filters, respectively. This suggests that this strain might produce at least two kinds of antiviral substances different in molecular weight of comparatively unstable against heating.

In conclusion, each of the bacteria from aquatic environment seems to produce antiviral substances specific for it, but further study is needed to determine the kind of antiviral substances produced by the representative strains.

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