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PRODUCTION OF ANTI-INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS (IHNV) SUBSTANCES BY INTESTINAL BACTERIA

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Abstract

Three strains of Aeromonas spp. were isolated from intestinal contents of masu salmon could produce anti-infectious hematopoietic necrosis virus (IHNV) substance in growth medium containing extract from commercial fish food pellets. When these antiviral bacteria was mixed with fish food and fed to rainbow trout and masu salmon, Aeromonas spp. became dominant in the intestinal microflora and anti-IHNV activity was observed in homogenates of the intestinal contents of fish fed with Aeromonas spp. These rainbow trout and masu salmon fed Aeromonas spp. showed more resistance to artificial infection by IHNV.

Introduction

Previously, we had investigated fish pathogenic viruses in an aquatic environment in order to understand their survival and interactional relationships with bacteria for the prevention of fish viral disease in aquaculture (Yoshimizu et al. 1986).

In a series of studies on the microbial ecosystem, we reported that many bacteria producing antiviral substances could be isolated from aquatic environments such as hatchery water supply, river mouth water, and beach water (Yoshimizu et al 1986, Kamei et al 1987a,b,c, 1988a, Kimura et al 1989). A representative bacteria with antiviral activity was classified as Pseudomonas fluorescens biovar I (Kamei et al. 1988b). This bacteria produced an anti-viral substance designated 46NW-04A.

Productivity of the antiviral substance was increased by using the bioreactor system with bacterial cells immobilized on alginate beads (Yoshimizu et al. 1989). When IHNV added to fish rearing water was passed through the column of immobilized bacterial cells, IHNV in the rearing water was inactivated (Kimura and Yoshimizu 1988). This antiviral agent was identified as a peptide and antiviral activity was recognized against IHNV and OMV (Kimura et al. 1990).

In this study, we isolated bacteria producing an antiviral substance from the intestinal microflora of masu salmon (Oncorhynchus masou) and used this bacteria to control IHN in experimentally challenged rainbow trout (O. mykiss) and masu salmon. Anti IHNV activity was also demonstrated by plaque reduction in cell culture.

Materials and Method

Isolation of bacteria producing anti-IHNV substances from the intestinal microflora of salmonid fish

One year-old masu salmon cultured at 4 hatcheries in Hokkaido were sampled to determine intestinal microflora and counts of viable intestinal bacteria. One hundred and eight Aeromonas spp. strains were screened for antiviral activity against IHNV by the methods of Kamei et al. (1988a).

Production of anti-IHNV substance in the extract of commercial fish food pellets

The ability of the bacteria to produce an anti-IHNV substance when grown in extracts of commercial feed pellet was tested.

Production of anti-IHNV substance in the intestine of rainbow trout and masu salmon

Bacteria with antiviral properties were mixed with fish food and fed to rainbow trout and masu salmon for 3 weeks. Counts of viable bacteria and percent composition of Aeromonas spp. in the intestinal microflora of the fish were determined.

IHNV challenge of rainbow trout and masu salmon

Rainbow trout and masu salmon were used for experimental infection with IHNV. Fish were challenged by immersion in 100 $TCID_{50}$/ml for 60 min.

Molecular size of anti-IHNV substances produced by Aeromonas spp.

The culture fluids of 2 Aeromonas strains M-26 and 36 were centrifuged, and the anti-IHNV activity of supernatants were determined after ultrafiltration of 50,000, 20,000 and 10,000 molecular weight cut-off filter.

Production of anti-IHNV substance in the intestine of cultured masu salmon and rainbow trout

One year-old masu salmon and rainbow trout cultured at university's experimental station and fish farm near Hakodate were sampled and anti-IHNV activity was observed in homogenates of the intestinal contents.

Results and Discussion

Isolation of bacteria producing anti-IHNV substances from the intestinal microflora of salmonid fish

Masu salmon cultured in Hokkaido were sampled to determine intestinal microflora and counts of viable bacteria. Counts ranged from 10^3 to 10^7CFU/g. Bacteria of the genus Aeromonas and family Enterobacteriaceae were the dominant normal intestinal microflora observed in salmonid fish from a fresh water environment (Yoshimizu et al. 1976). The percent composition of Aeromonas spp. in the intestinal microflora ranged from 16 % to 59 %, and 108 different strains of Aeromonas spp. were isolated. One hundred and eight Aeromonas spp. strains were screened for antiviral

Table 1. Number of bacteria showing anti-IHNV activity among 108 Aeromonas spp. isolated from the intestinal contents of masu salmon

Source	PFU reduction	
	>60 %	>90 %
Nanae (Univ.)	0 (0%)	0 (0%)
Nanae (Private H.)	0 (0%)	0 (0%)
Mori H.	10 (13%)	3 (3%)
Chitose H.	0 (0%)	0 (0%)
Total	10 (13%)	3 (3%)

activity. Three strains designated M-26, M-36, and M-38 isolated from Mori Hatchery, showed strong antiviral activity (Table 1).

Production of anti-IHNV substance in the extract of commercial fish food pellets

Aeromonas sp. strain M-38 produced an anti-IHNV substance in growth medium containing extract from commercial fish food pellets (Table 2). Strain M-26 also produced an anti-IHNV substance (data not shown). These results suggest that bacteria producing anti-IHNV substances could be easily incorporated into the diets of fish.

Table 2. Anti-IHNV activity of an extract of commercial food pellets homogenized, sterilized, and seeded with Aeromonas sp. strain M-38

(Pellet:Water)		PFU reduction	
		Filter*	Autoclaved
1 : 2	15 °C, 5 days	93.1	73.4
	25 °C, 2 days	88.1	74.5
1 : 4	15 °C, 5 days	81.5	77.9
	25 °C, 2 days	68.5	60.0
1 : 8	15 °C, 5 days	69.6	64.5
	25 °C, 2 days	39.3	51.0

*: Filter sterilization using 0.45 um filter

Production of anti-IHNV substance in the intestine of rainbow trout

After 3 weeks, Aeromonas spp. were the dominant bacteria in the intestinal microflora of both fish fed bacteria in their diet (test group) and not fed (control group), and counts of viable bacteria reached 10^7CFU/g (Table 3).

Table 3. Counts of viable bacteria and percent composition of intestinal microflora of rainbow trout fed Aeromonas sp. strain M-26 in their diet

Group	Fish No.	Viable bacteria /g	Microflora (%)		
			Aeromonas	Pseudomonas	Others
Test*1	1	4.3×10^8	100	0	0
	3	4.2×10^8	87	7	6
	5	8.5×10^7	93	0	7
Control*2	1	1.2×10^8	93	0	7
	3	9.8×10^7	100	0	0
	5	2.1×10^8	97	0	3

*1: Fish fed Aeromonas sp. M-26
*2: Fish not fed Aeromonas sp. M-26

Anti-IHNV activity was observed in homogenates of the intestinal contents of rainbow trout fed Aeromonas sp. M-26 in commercial feed (test group) and in homogenates of fish fed untreated commercial feed (controls). However, a difference in the level of antiviral activity was observed (Table 4). The level of antiviral activity observed in homogenates of the intestinal contents of the test group was nearly as high as that observed in the bioreactor system (Yoshimizu et al 1989).

Table 4. Anti-IHNV activity of the intestinal contents of rainbow trout fed Aeromonas sp. strain M-26 in their diet

Group*1	Anti-IHNV activity PFU reduction	Max. dilution showing 90 % PFU reduction
Test-1	100	x 20
Test-2	100	x 40
Control-1	100	x 10
Control-2	100	x 20

*1: Intestinal contents of 5 fish were pooled

IHNV challenge of rainbow trout

Fish fed Aeromonas sp. M-26 then challenged with IHNV had a 9.6 % mortality, during 1 month, while the fish not fed the bacteria had a 29.2 % mortality. Fish fed but not challenged had a 3.3 % mortality (Table 5). This experiment shows that fish fed Aeromonas spp. prior to an I challenged had greater resistance to infection.

Table 5. Results of IHNV challenge of rainbow trout reared fed Aeromonas sp. strain M-26 in their diet

Group	Water Temp. (°C)	Number of fish	Cumulative mortality(%)
Test*1	13-15	30	9.6
Control-1*2	13-15	30	29.2
Control-2*3	13-15	30	3.3

*1: Fish fed Aeromonas sp. M-26 then challenged with 100 $TCID_{50}$ for 60 min
*2: Fish not fed Aeromonas, challenged with 100 $TCID_{50}$ for 60 min
*3: Fish not fed Aeromonas but not challenged with IHNV

Production of anti-IHNV substance in the intestine of masu salmon

In the second part of this study, masu salmon were fed Aeromonas sp. strain M-38. To select the control fish, intestinal microflora of masu salmon cultured in our laboratory was observed and

Table. 6. Counts of viable bacteria and percent composition of intestinal microflora of masu salmon fed Aeromonas sp. strain M-38 in their diet

Group	Fish No.	Viable bacteria (g)	Microflora (%)		
			Aeromonas	Pseudomonas	Others
Test	1	4.3×10^8	100	0	0
	3	4.2×10^8	97	0	3
	5	8.5×10^7	100	0	0
Control -A	11	1.2×10^8	97	0	3
	13	9.8×10^7	100	0	0
	15	2.1×10^8	97	0	3
Control -B	21	2.3×10^4	0	100	0
	23	6.7×10^4	27	73	0
	25	3.8×10^8	13	87	0

antiviral activity of intestinal contents and isolates from intestinal contents were tested. Fish with a natural population of predominant Aeromonas spp. and Pseudomonas spp. in the intestinal contents could be found (Table 6).

Table 7. Anti-IHNV activity of the intestinal contents of masu salmon fed Aeromonas sp. strain M-38 in their diet

Group*1	Dominant species of bacteria in intestine	Anti-IHNV activity of intestinal contents PFU reduction (*2)
Test-1	Aeromonas sp.	100 (x 20)
Test-2	Aeromonas sp.	100 (x 20)
Control-A-1	Aeromonas sp.	100 (x 10)
Control-A-2	Aeromonas sp.	100 (x 20)
Control-B-1	Pseudomonas sp.	66
Control-B-2	Pseudomonas sp.	85

*1: Intestinal contents of 5 fish were pooled
*2: Maximum dilution showing at least a 90 % PFU reduction

The intestinal contents both groups of masu salmon, Aeromonas sp. M-38 in their diet and those not fed the bacteria, showed anti-IHNV activity by the plaque reduction method. Intestinal contents from fish that had predominantly Pseudomonas spp. did not show any anti-IHNV activity (Table 7).

IHNV challenge test of masu salmon

Masu salmon, body weight 0.5 g, fed Aeromonas sp. M-38 were challenged with IHNV (100 $TCID_{50}$/ml, 60 min). The cumulative mortality of masu salmon fed Aeromonas sp. M-38 then challenged with IHNV was 0.5 %; fish fed but not challenged was 0 %. Fish with Pseudomonas spp. as the dominant intestinal bacteria challenged with IHNV had a 72 % mortality, while unchallenged fish had a 6.0 % mortality (Table 8).

Table 8. Results of IHNV challenge test*1 for masu salmon fed Aeromonas sp. strain M-38 in their diet

Group	Treatment	Water Temp. (°C)	Number of fish	Cumulative mortality (%)
Test	Challenged	5-7	200	0.5
Test	Control	5-7	200	0.0
Control-B	Challenged	5-8	100	72.0
Control-B	Control	5-8	100	6.0

*1: IHNV strain ChAb was used; challenge dose was 100 $TCID_{50}$, 60 min

While the cumulative mortality of masu salmon fed Aeromonas sp. M-38 and fish not fed, and then challenged with IHNV (10^4 $TCID_{50}$/ml) was 90 and 91 %, respectively (Data was not shown).

Molecular size of anti-IHNV substances produced by Aeromonas spp.

The anti-IHNV activities of unfiltrated and filtrated culture fluids were shown in Table 9. Aeromonas spp. strain M-26 and 36 showed the anti-IHNV activity after filtration through the 10,000 MW cut-off filter.

Production of anti-IHNV substance in the intestine of cultured masu salmon and rainbow trout

The dominant microflora of 1-year-old masu salmon and rainbow trout cultured at university's experimental station and fish farm near Hakodate was Aeromonas spp. and the level of antiviral activity observed in homogenates of the intestinal contents was varied from 1:20 to 1:160, and was

Table 9. Molecular size of anti-IHNV substances produced by Aeromonas sp. M-26 and M-36.

	Percentage of anti-IHNV activity after filtration of MW cut-off	
	M-26	M-36
Un-filtrated	100	100
50,000 MW-cut	110	100
20,000 MW-cut	92	58
10,000 MW-cut	98	81

nearly as high as that observed in the intestinal contents of rainbow trout and masu salmon fed Aeromonas spp. (Table 10)

Table 10. Anti-IHNV activity of the intestinal contents of masu salmon and rainbow trout culture at Nanae

Fish No.	Species	Anti-IHNV activity of intestinal contents PFU reduction*
1	masu salmon	100 (x 40)
2	masu salmon	100 (x 80)
3	masu salmon	100 (x 80)
4	masu salmon	100 (x 80)
5	masu salmon	100 (x 80)
6	rainbow trout	100 (x160)
7	rainbow trout	100 (x 20)

*: Maximum dilution showing at least a 90 % PFU reduction

Our results show the relationship between the normal bacterial flora in the fish intestine and the susceptibility of that fish to infection by IHNV. Fish with either a naturally occurring or an artificially induced (fed in the diet) intestinal microflora composed predominantly of Aeromonas spp. producing anti-IHNV substances showed more resistance to infection by IHNV. Anti-IHNV activity was also demonstrated in vitro using homogenates of the intestinal contents of cultured fish predominantly colonized by Aeromonas spp.

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References

Kamei, Y., M. Yoshimizu, Y. Ezura and T. Kimura. 1987a. Effects of estuarine and marine waters on the infectivities of infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV). Bull.Fac.Fish.Hokkaido. Univ., 38: 271-285.

Kamei, Y., M. Yoshimizu, Y. Ezura and T. Kimura. 1987b. Effect of environmental water on infectivities of infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV). J. Appl. Ichology, 4: 37-47.

Kamei, Y., M. Yoshimizu, Y. Ezura and T. Kimura. 1987c. Screening of bacteria with antiviral activity against infectious hematopoietic necrosis virus (IHNV) from estuarine and marine environments. Nippon Suisan Gakkaishi., 53: 2179-2185.

Kamei, Y., M. Yoshimizu, Y. Ezura and T. Kimura. 1988a. Screening of bacteria with antiviral activity from the fresh water salmonid hatcheries. Microbial. Immunol., 32: 67-73.

Kamei, Y., M. Yoshimizu, Y. Ezura and T. Kimura. 1988b. Identification of Pseudomonas sp. 46NW-04 which produces antiviral agent against fish viruses. Bull.Fac.Fish.Hokkaido Univ., 39: 124-132.

Kimura, T. and M. Yoshimizu. 1988. Application of the anti-IHNV substance produced by bacteria isolated from environmental water of salmonid fish for control of the IHN-II. Anti-IHNV reaction of the Pseudomonas sp. 46NW-04 coated beads. Ann.Rep.Fish.Dis.Control(FIDIC), pp.148-150

Kimura, T., M. Yoshimizu, Y. Ezura and Y. Kamei. 1989. Interaction between fish pathogenic viruses and microorganisms in environmental water of fish. In Hattori, T., Y. Ishida, Y. Maruyama, R.Y. Morita and A. Uchida (eds.), Recent Advances in Microbial Ecology. Japan. Sci.Soc.Press, Tokyo, pp. 587-591.

Kimura, T., M. Yoshimizu, Y. Ezura and Y. Kamei. 1990. An antiviral agent (46NW-04A) produced by Pseudomonas sp. and its activity against fish viruses. J.Aquat.Animal.Health., 2: 12-20.

Yoshimizu, M. and T. Kimura. 1976. Study on the intestinal microflora of salmonids. Fish Pathology, 10: 243-259.

Yoshimizu, M., H. Takizawa, Y. Kamei and T. Kimura. 1986. Interaction between fish pathogenic viruses and microorganisms in fish rearing water : Survival and inactivation of infectious pancreatic necrosis virus, infectious hematopoietic necrosis virus and Oncorhynchus masou virus in rearing water. Fish Pathology, 21: 223-231.

Yoshimizu, M., N. Myouga, Y. Kamei, Y. Ezura and T. Kimura. 1989. Production of antiviral substances by bacterial cells immobilized alginate beads. In Miyachi S, Karube I, Ishida Y (eds.). Current Topics in Marine Biotechnology. The Japanese Society for Marine Biotechnology, Tokyo, pp. 77-80.