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BIOLOGICAL CONTROL OF INFECTIOUS HEMATOPOIETIC NECROSIS BY ANTIVIRAL SUBSTANCE PRODUCING BACTERIA

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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 anti-viral 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 masu salmon and rainbow trout (*O. mykiss*). Anti IHNV activity was also demonstrated by plaque reduction in cell culture.

Isolation of bacteria producing antiviral 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. Counts ranged from 10^3 to 10^7 CFU/g. Bacteria of the genus *Aeromonas* and family Enterobacteriaceae were the dominant normal intes-

tinal 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 activity against IHNV by the methods of Kamei et al (1988a). Three strains designated M-26, M-36, and M-38 isolated from Mori Hatchery, showed strong antiviral activity (Table 1).

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

Source	PFU reduction	
	>50 %	>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%)

Production of antiviral 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. *Aeromonas* sp. strain M-38 produced an anti-IHNV substance in growth medium containing extract from commercial fish food pellets (Table 2).

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 μm filter

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.

Production of antiviral substance in the intestine of rainbow trout

Bacteria with interval properties were mixed with fish food and fed to 1.0-1.5 g rainbow trout for 3 weeks. Counts of viable bacteria and percent composition of *Aeromonas* spp. in the intestinal microflora of the fish were determined. After 3 weeks, *Aeromonas* spp. was 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 10^7 CFU/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

Experiment	Fish No.	Viable bacteria Per gram	Microflora (%)		
			Aeromonas	*Pseudomonas*	Others
Test*1	1	1.6×10^7	100	0	0
	3	1.8×10^7	87	7	6
	5	6.7×10^7	93	0	7
Control*2	1	2.1×10^7	97	0	3
	3	8.5×10^6	100	0	0
	5	7.7×10^6	97	0	3

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

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

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).

IHNV challenge of rainbow trout

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

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	3.8×10^6	13	87	0

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

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 not fed and challenged by IHNV was 1.0 % and fish not fed and not challenged was 0.5 %. These fish had a dominant intestinal microflora of *Aeromonas* spp. 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).

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.

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-A	Challenged	5-7	200	1.0
Control-A	Control	5-7	200	0.5
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

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 antiviral substances showed more resistance to infection by IHNV. Anti-IHNV activity was also demonstrated in vitro using homogenates of the intestinal contents of fish predominantly colonized by *Aeromonas* spp. Large-scale field experiments are in progress to control IHN by feeding diets containing live *Aeromonas* spp. producing antiviral substances.

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