Mode of the Intra-ovum Infection of *Flavobacterium psychrophilum* in Salmonid Eggs

Akira Kumagai^{1*} and Akatsuki Nawata²

¹Miyagi Prefecture Fisheries Technology Institute, Miyagi 986-2135, Japan

²Miyagi Prefecture Fisheries Technology Institute Freshwater
Fisheries Experimental Station, Miyagi 981-3625, Japan

(Received December 7, 2009)

ABSTRACT—The mode of intra-ovum infection by *Flavobacterium psychrophilum*, which causes bacterial cold-water disease and rainbow trout fry syndrome, was investigated in eggs of rainbow trout *Oncorhynchus mykiss*. When newly spawned unfertilized eggs were immersion-challenged with *F. psychrophilum* at concentrations of 10^7 CFU/mL or higher and subsequently inseminated and water-hardened, the bacteria were detected in the egg contents. The infection rate in eggs was related to the bacterial concentration in a dose-dependent manner. When mature female rainbow trout were intraperitoneally injected with *F. psychrophilum* (3.3×10^9 CFU/fish) 5–9 days prior to ovulation, intra-ovum infection was found in eggs spawned from the fish showing heavy contamination of the bacterium in the ovarian fluid ($> 10^6$ CFU/mL). These results indicate that *F. psychrophilum* can contaminate the surface of eggs in ovarian fluid and enter eggs during water-hardening. Live bacterial counts within experimentally infected eggs and immuno-staining observations on the eggs suggested that *F. psychrophilum* passively entered into the eggs (< 10 CFU/egg) and subsequently grew to over 10^7 CFU/egg in the perivitelline space by the eyed egg stage. Multiplication of the bacterium in the perivitelline space did not affect the survival of eggs.

Key words: intra-ovum infection, *Flavobacterium psychrophilum*, bacterial cold-water disease, rainbow trout fry syndrome, salmonid egg, *Oncorhynchus mykiss*

Flavobacterium psychrophilum is the causative agent of bacterial cold-water disease (BCWD) (Holt *et al.*, 1993) and rainbow trout fry syndrome (RTFS) (Bernardet and Kerouault, 1989), inducing severe economic loss in aquaculture worldwide (Nematollahi *et al.*, 2003). In Japan, outbreaks of BCWD have often occurred in coho salmon *Oncorhynchus kisutch* hatcheries and ayu *Plecoglossus altivelis* farms since the middle of the 1980s (Wakabayashi *et al.*, 1991, 1994). The disease still causes serious problems for aquaculture and resource enhancement of salmonid fish and ayu.

Generally, egg disinfection procedures using povidone-iodine have been recommended in order to prevent the transmission via egg except for the case of intra-ovum infection. However, it was noted that the iodophor treatment of eggs does not always prevent BCWD in subsequent fry (Holt *et al.*, 1993; Kumagai and Takahashi, 1997). Brown *et al.* (1997) and Cipriano (2005) showed evidence that *F. psychrophilum* intruded into salmonid eggs by cultivating naturally infected eggs. Thus, the ineffectiveness of the

povidone-iodine treatment against *F. psychrophilum* seems due to the invasion of the pathogen into the eggs.

The causative agent of bacterial kidney disease (BKD), *Renibacterium salmoninarum*, has been shown to be able to invade within eggs ovulated in heavily contaminated ovarian fluid in the female body cavity (Evelyn *et al.*, 1986) and there is evidence that the bacteria can enter eggs during oogenesis as well (Lee and Gordon, 1987; Lee and Evelyn, 1989). On the other hand, the mode of intra-ovum infection of *F. psychrophilum* is still unknown. *F. psychrophilum* has been detected in the ovarian fluid of sexually mature coho salmon (Holt *et al.*, 1993), chinook salmon *O. tshawytscha* (Holt *et al.*, 1993), rainbow trout *O. mykiss* (Rangdale *et al.*, 1996; Madetoja *et al.*, 2002; Madsen *et al.*, 2005), Atlantic salmon *Salmo salar* (Ekman *et al.*, 1999) and chum salmon *O. keta* (Misaka and Suzuki, 2007). Because eggs are in intimate contact with ovarian fluid after they have been released into the body cavity after ovulation, the surface of eggs obtained from ripe fish can be heavily contaminated with *F. psychrophilum*. Kumagai *et al.* (2000) reported that experimental intra-ovum infection with *F. psychrophilum*

* Corresponding author

E-mail: kumagai-ak557@pref.miyagi.jp

was induced by water-hardening the fertilized eggs of coho salmon in hypotonic suspensions of the bacteria at a concentration of 1.0×10^8 CFU/mL. These previous results suggested that ovarian fluid-derived bacteria on the egg surface enter eggs during the process of water-hardening, when the eggs are fertilized and water-hardened by standard hatchery practices without disinfection prior to fertilization.

Using rainbow trout eggs with surfaces contaminated with *F. psychrophilum*, the present study was undertaken to determine if the bacteria can enter eggs in the process of fertilization, to clarify the mode of intra-ovum infection of the bacterium.

Materials and Methods

Bacterium

F. psychrophilum strain FPC828 was used to artificially challenge eggs. This strain was isolated from the kidney of coho salmon fry in Miyagi, Japan in 1990 (Wakabayashi *et al.*, 1991). The bacterium were cultivated in 300 mL of a modified Anacker and Ordal's broth (tryptone 0.3%, yeast extract 0.2%, beef extract 0.02%, sodium acetate 0.02%, pH 7.2) at 15°C for 3 days. *F. psychrophilum* cells were collected by centrifugation ($1,660 \times g$ for 15 min at 4°C), rinsed 3 times with sterile phosphate-buffered saline (PBS), and then re-suspended in 30 mL of sterile PBS.

Intra-ovum infection trials by the immersion method

Eggs and milt were obtained from mature rainbow trout (3+ years old), which had been kept in Miyagi Prefectural Freshwater Fisheries Experimental Station. Pooled eggs collected from five females were rinsed three times with PBS, and were divided into twelve groups each consisting of 400 eggs. The cell suspension of *F. psychrophilum* was diluted by serial 10-fold dilutions with PBS. Two mL of the cell suspensions at six different concentrations (5.0×10^9 , 5.0×10^8 , 5.0×10^7 , 5.0×10^5 and 5.0×10^3 CFU/mL and PBS as a control) were separately added to each egg group in 180 mL conical flasks, and were gently agitated. Two egg groups were exposed to each bacterial concentration. Following the bacterial challenge to the egg surface, eggs were immediately dry-fertilized with milt pooled from five rainbow trout and were water-hardened for 45 min in 180 mL of *F. psychrophilum*-free well water. Just after the water-hardening, all of the egg groups were disinfected with 50 ppm povidone-iodine for 15 min. After the treatments, each duplicated group of eggs was incubated at $8 \pm 1^\circ\text{C}$ in a tray in 5 L aquarium with flow-through water at approximately 0.5 L/min flow rate. Dead eggs were removed from aquaria once a week in order to prevent fungal disease.

The challenged eggs were examined for intra-ovum

infection with *F. psychrophilum* 14 and 21 days after the challenge. Thirty live eggs were collected from one of two egg groups prepared for each bacterial concentration, and were disinfected again with 50 ppm povidone-iodine for 15 min to kill bacteria on the egg surface. Each egg was placed in a sterile tube and was then homogenized with a sterile rod. The homogenate was spread on an enriched Anacker and Ordal's agar plate (Lorenzen and Karas, 1992) with a cotton swab. The plates were incubated at 15°C for 7 days. Identification of the isolated *F. psychrophilum* was made by indirect fluorescent antibody technique (IFAT) using an antiserum against *F. psychrophilum* FPC828, according to the method described by Kumagai *et al.* (1998). The antiserum was provided from the University of Tokyo. For the control, 60 eggs were examined only after 23 days. The other group in the duplicated egg group was incubated without sampling and examined for the survival rate at the eyed-egg stage 30 days after the challenge.

Intra-ovum infection trials by the spawners injection method

Fifteen ripe but preovulatory female rainbow trout were interperitoneally injected with 5 mL of the suspension of *F. psychrophilum* (6.5×10^8 CFU/mL) and were then held in a 1 ton tank until ovulation. Five and 9 days after the pathogen injection, ovarian fluid samples (approximately 0.5 mL/fish each) were aseptically collected from eight and three postovulatory females out of the challenged 15 fish with the method of Yoshimizu *et al.* (1985). The ovarian fluid samples were then serially 10-fold diluted with PBS and 100 μL of each diluted sample was spread on the enriched Anacker and Ordal's agar plate. The plates were incubated at 15°C for 7 days and then colonies of *F. psychrophilum*, identified with the IFAT, were counted.

After collecting ovarian fluid, eggs were squeezed into individual glass bowls from each fish. On the 5th day after the pathogen injection, approximately 200 eggs each from two females were pooled in a 250 mL conical flask. Thus, four lots each consisting of 400 eggs obtained from two of eight fish were generated. They were immediately dry-fertilized with milt pooled from five unchallenged rainbow trout. All egg lots were water-hardened and disinfected with povidone-iodine in the same manner as above. After the disinfection, each lot of eggs was discretely incubated. For the control, three lots each consisting of 400 eggs obtained from two of six unchallenged females were fertilized and incubated. On the 9th day after the pathogen injection, three lots of eggs, which were obtained from each fish of the three challenged postovulatory females, were fertilized and incubated. Three lots of eggs obtained from each fish of three unchallenged females were used as the controls. Twenty one and 25 days after fertiliza-

tion, the bacteriological examination was made in the same manner used for the immersion challenged eggs.

Live bacterial counts and IFAT observations

Four hundred eggs squeezed out from a rainbow trout were immersed in 2 mL of suspension of *F. psychrophilum* (1.0×10^9 CFU/mL), fertilized, and incubated as described above. Two hours and 1, 3, 5, 7, 14, 21, 28 and 42 days after incubation, ten live eggs were sampled at random and were disinfected with povidone-iodine (50 ppm, 15 min) to kill bacteria on the surface. The contents of the disinfected eggs were aseptically obtained using 2.5 mL syringes and 23 gauge needles at intervals until the 21st day. On the 28th and 42nd day, a small hole was bored in the eyed-stage eggs with a sterile needle, and their contents were sampled. The whole contents of the eggs sampled two hours and one day after incubation were individually spread without dilutions on the enriched Anacker and Ordal's agar plate. The contents of the eggs sampled from 3 to 42 days after incubation were serially 10-fold

diluted with PBS and each a 100 μ L was spread on the plate. The plates were incubated at 15°C for 7 days and then colonies of *F. psychrophilum*, identified with the IFAT, were counted.

After 28-day incubation, ten live eggs were sampled, and disinfected with immersion in 50 ppm povidone-iodine for 15 min. These eggs were embedded in O.C.T. compound (Miles Inc.) and sectioned at 7 μ m with a cryostat. Frozen sections were mounted on slides, dried in air for 30 min, fixed in acetone for 10 min, and dried again. The specimens were stained with the IFAT. Uninfected eggs were also examined as control.

Results

Intra-ovum infection trials by the immersion method

F. psychrophilum was detected from egg groups which were immersion-challenged with bacterial suspensions at concentrations higher than 10^7 CFU/mL. The higher the bacterial concentration, the higher was the

Table 1. Detection of *F. psychrophilum* from eggs challenged by the immersion method

Challenge dose (CFU/mL)	No. positive eggs/No. examined eggs			Eyed-egg rate (%)
	14*	21	23	
5.0×10^9	6/30	10/30		90
5.0×10^8	2/30	5/30		94
5.0×10^7	0/30	1/30		88
5.0×10^5	0/30	0/30		90
5.0×10^3	0/30	0/30		89
0			0/60	93

* Days after challenge

Table 2. Detection of *F. psychrophilum* from the content of eggs challenged by bacterial injection into preovulatory females

Days post-injection* ¹	Lot	No. of females	No. of <i>Flavobacterium psychrophilum</i> cells in ovarian fluid (CFU/mL)		No. positive eggs/No. examined eggs	
					21* ²	25
5 days	Exp. 1	2	2.4×10^7	1.4×10^8	0/30	2/30
	Exp. 2	2	6.6×10^6	1.1×10^7	1/30	1/30
	Exp. 3	2	1.5×10^5	4.2×10^6	0/30	0/30
	Exp. 4	2	1.0×10^4	2.0×10^4	0/30	0/30
	Cont. 1	2		NE* ³	0/30	0/30
	Cont. 2	2		NE	0/30	0/30
	Cont. 3	2		NE	0/30	0/30
9 days	Exp. 1	1	1.2×10^6		1/30	0/30
	Exp. 2	1	1.5×10^5		0/30	0/30
	Exp. 3	1	2.0×10^5		0/30	0/30
	Cont. 1	1		NE	0/30	0/30
	Cont. 2	1		NE	0/30	0/30
	Cont. 3	1		NE	0/30	0/30

*¹ Gravid rainbow trout were intraperitoneally injected with *F. psychrophilum* suspensions (3.3×10^9 CFU/fish).

*² Days after challenge.

*³ Not examined.

prevalence of infection in eggs. Immersion-challenged eggs showed eyed-egg rates as high as that of unchallenged eggs (Table 1).

Intra-ovum infection trials by the spawner injection method

When preovulatory rainbow trout were interperitoneally injected with *F. psychrophilum* suspensions, the bacteria were detected from the ovarian fluid of all challenged fish, although bacterial concentrations varied largely from 1.0×10^4 to 1.4×10^8 CFU/mL (Table 2). Out of seven egg lots obtained from the challenged females, intra-ovum infection was detected only in three lots, all of which were obtained from fish showing heavy contamination in the ovarian fluid ($> 10^6$ CFU/mL). The prevalence of *F. psychrophilum* in the eggs was

1.7% (1/60) or 3.3% (2/60).

Live bacterial counts and IFAT detection within experimentally infected eggs

Live bacterial counts within experimentally infected eggs gradually increased until 14 days after the challenge, and reached to plateau at roughly 10^7 CFU/egg by 21 days after the challenge. Despite the gradual increase in the bacterial concentrations in the eggs, the detection rate of *F. psychrophilum* in the egg groups remained stable between 10% and 30% through the course of experimental period (Fig. 1).

Many IFAT-positive bacteria were observed in experimentally challenged eggs 28 days after incubation. Slender rods with specific fluorescence were mainly located inside the chorion, namely the perivitelline space (Fig. 2). On the other hand, no IFAT-positive bacteria were observed in the control eggs.

Fig. 1. The number of live *F. psychrophilum* cells recovered from eggs challenged by immersion in bacterial suspension. Rates of *F. psychrophilum*-positive eggs (open columns) and the mean numbers of live bacterial cells in the positive eggs (closed squares) are shown.

Discussion

In the present study, we demonstrated that intra-ovum infection of *F. psychrophilum* occurred in rainbow trout eggs, when the newly spawned eggs were artificially exposed to the bacteria at high concentrations ($> 10^7$ CFU/mL) prior to fertilization. Furthermore, the intra-ovum infection was found in eggs obtained from females showing heavy bacterial contamination in the ovarian fluid ($> 10^6$ CFU/mL). In cases of natural infection, salmonid spawners such as coho salmon, rainbow trout and masu salmon have often ovarian fluid containing the bacteria at 10^6 CFU/mL and higher (Kumagai *et al.*, unpublished data). From these results, it is sug-

Fig. 2. IFAT-positive bacteria in an egg experimentally infected with *F. psychrophilum*. IFAT-positive bacteria (arrows) are found in the perivitelline space. CH : chorion

gested that when female spawners are heavily infected with *F. psychrophilum*, the bacteria in the ovarian fluid are able to contaminate the egg surface and subsequently enter eggs during water-hardening.

According to Evelyn *et al.* (1984), the intra-ovum infection of BKD would occur via the micropyle since this is the only pore in the chorion large enough to permit the entry of *R. salmoninarum* (Groot and Alderdice, 1985). In the present study, live bacterial counts of *F. psychrophilum* within experimentally infected eggs gradually increased from less than 10 CFU/egg just after fertilization to over 10^7 CFU/egg at the eyed egg stage. In the process of water-hardening, *F. psychrophilum* at concentrations of below 10 CFU/egg might passively enter into the perivitelline space through the micropyle in the chorion, and subsequently the bacteria might be alive and multiply in the perivitelline space, as the eggs develop.

Even when intra-ovum infection occurred in approximately 30% of eggs in the group challenged by a high concentration of *F. psychrophilum*, the ratios of eyed-eggs in these groups remained high (Table 1). It is interesting that the eggs can still survive when *F. psychrophilum* multiplied in the perivitelline space at concentrations of more than 10^7 CFU/egg (Fig. 1). Cipriano (2005) also reported that *F. psychrophilum*, was isolated at concentrations more than 10^8 CFU/g from live Atlantic salmon eggs at the eyed egg stage. These results indicate that the multiplication of the bacteria in the perivitelline space does not affect the survival of eggs. If the eggs in which the intra-ovum infection occurred died by the eyed egg stage, the spread of BCWD via contaminated eggs could be prevented by removing all the dead eggs before introducing the eyed-eggs into hatcheries. As the pathogen-contaminated eggs could survive, however, it is difficult to prevent the delivery and transportation of contaminated eggs. On the other hand, Cipriano *et al.* (1995) reported *F. psychrophilum* involvement in the mortality among Atlantic salmon eggs at the eyed stage. Epizootiological case studies on mass mortalities of eggs in hatcheries are needed in order to clarify the affection of *F. psychrophilum* present within eggs to survival of eyed eggs.

In the case of BKD, eggs have been shown to acquire intra-ovum infections following ovulation when surrounded by heavily contaminated ovarian fluid (Evelyn *et al.*, 1986) and during oogenesis (Lee and Evelyn, 1989). Since the eggs released into the body cavity following ovulation come into contact with the pathogen in BCWD as in BKD, it is possible that *F. psychrophilum* has also already entered the eggs in the female body cavity, but few studies have been carried out to clarify the intra-ovum infection of the newly spawned unfertilized eggs derived from spawners with heavily contaminated ovarian fluid. Further research is

needed to determine if the occurrence of intra-ovum infection can occur prior to fertilization.

The fact that bacteria originated from the ovarian fluid could enter into eggs during water-hardening suggests the possibility that disinfection with povidone-iodine prior to fertilization could effectively prevent the intra-ovum infection of *F. psychrophilum*. Further work to evaluate the efficacy of disinfection with povidone-iodine of eggs just after spawning should be undertaken to establish effective methods to prevent vertical transmission of *F. psychrophilum*.

Acknowledgements

We are thankful to Professor H. Fukuda (Tokyo University of Marine Science and Technology) for helping preparing frozen sections and to Dr. T. Yoshinaga (The University of Tokyo) for reviewing the manuscript. This study was supported by a grant from the Ministry of Agriculture, Forestry, and Fisheries of Japan and Miyagi Prefectural Government.

References

- Bernardet, J. F. and B. Kerouault (1989): Phenotypic and genomic studies of '*Cytophaga psychrophila*' isolated from diseased rainbow trout (*Oncorhynchus mykiss*) in France. *Appl. Environ. Microbiol.*, **55**, 1796–1800.
- Brown, L. L., W. T. Cox and R. P. Levine (1997): Evidence that the causal agent of bacterial cold-water disease *Flavobacterium psychrophilum* is transmitted within salmonid eggs. *Dis. Aquat. Org.*, **29**, 213–218.
- Cipriano, R. C. (2005): Intraovum infection caused by *Flavobacterium psychrophilum* among eggs from captive Atlantic salmon broodfish. *J. Aquat. Anim. Health*, **17**, 275–283.
- Cipriano, R. C., L. A. Ford and J. D. Teska (1995): Association of *Cytophaga psychrophila*, with mortality among eyed eggs of Atlantic salmon (*Salmo salar*). *J. Wildl. Dis.*, **31**, 166–171.
- Ekman, E., H. Borjeson and N. Johansson (1999): *Flavobacterium psychrophilum* in Baltic salmon *Salmo salar* brood fish and their offspring. *Dis. Aquat. Org.*, **37**, 159–163.
- Evelyn, T. P. T., J. E. Ketcheson and L. Prosperi-Porta (1984): Further evidence for the presence of *Renibacterium salmoninarum* in salmonid eggs and for the failure of povidone-iodine to reduce the intra-ovum infection rate in water-hardened eggs. *J. Fish Dis.*, **7**, 173–182.
- Evelyn, T. P. T., L. Prosperi-Porta and J. E. Ketcheson (1986): Experimental intra-ovum infection of salmonid eggs with *Renibacterium salmoninarum* and vertical transmission of the pathogen with such eggs despite their treatment with erythromycin. *Dis. Aquat. Org.*, **1**, 197–202.
- Groot, E. P. and D. F. Alderdice (1985): Fine structure of the external egg membrane of five species of Pacific salmon and steelhead trout. *Can. J. Zool.*, **63**, 552–566.
- Holt, R. A., J. S. Rohovec and J. L. Fryer (1993): Bacterial cold-water disease. In "Bacterial diseases of fish" (ed. by V. Inglis, R. J. Roberts and N. R. Bromage). Blackwell Sci. Publ., London, pp. 3–22.
- Kumagai, A. and K. Takahashi (1997): Imported eggs responsible for the outbreaks of cold-water disease among cultured coho salmon in Japan. *Fish Pathol.*, **32**, 231–232.

- Kumagai, A., K. Takahashi, S. Yamaoka and H. Wakabayashi (1998): Ineffectiveness of iodophore treatment in disinfecting salmonid eggs carrying *Cytophaga psychrophila*. *Fish Pathol.*, **33**, 123–128.
- Kumagai, A., S. Yamaoka, K. Takahashi, H. Fukuda and H. Wakabayashi (2000): Waterborne transmission of *Flavobacterium psychrophilum* in coho salmon eggs. *Fish Pathol.*, **35**, 25–28.
- Lee, E. G. H. and T. P. T. Evelyn (1989): Effect of *Renibacterium salmoninarum* levels in the ovarian fluid of spawning chinook salmon on the prevalence of the pathogen in their eggs and progeny. *Dis. Aquat. Org.*, **7**, 179–184.
- Lee, E. G. H. and M. R. Gordon (1987): Immunofluorescence screening of *Renibacterium salmoninarum* in the tissues and eggs of farmed chinook salmon spawners. *Aquaculture*, **65**, 7–14.
- Lorenzen, E. and N. Karas (1992): Detection of *Flexibacter psychrophilus* by immunofluorescence in fish suffering from fry mortality syndrome: a rapid diagnostic method. *Dis. Aquat. Org.*, **13**, 231–234.
- Madetoja, J., I. Dalsgaard and T. Wiklund (2002): Occurrence of *Flavobacterium psychrophilum* in fish-farming environments. *Dis. Aquat. Org.*, **52**, 109–118.
- Madsen, L., J. D. Møller and I. Dalsgaard (2005): *Flavobacterium psychrophilum* in rainbow trout, *Oncorhynchus mykiss* (Walbaum), hatcheries: studies on broodstock, eggs, fry and environment. *J. Fish Dis.*, **28**, 39–47.
- Misaka, N. and K. Suzuki (2007): Detection of *Flavobacterium psychrophilum* in chum salmon *Oncorhynchus keta* and virulence of isolated strains to salmonid fishes. *Fish Pathol.*, **42**, 201–209.
- Nematollahi, A., A. Decostere, F. Pasmans and F. Haesebrouck (2003): *Flavobacterium psychrophilum* infections in salmonid fish. *J. Fish Dis.*, **26**, 563–574.
- Rangdale, R. E., R. H. Richards and D. J. Alderman (1996): Isolation of *Cytophaga psychrophila*, causal agent of rainbow trout fry syndrome (RTFS) from reproductive fluids and egg surfaces of rainbow trout (*Oncorhynchus mykiss*). *Bull. Eur. Ass. Fish Pathol.*, **16**, 63–67.
- Wakabayashi, H., M. Horiuchi, T. Bunya and G. Hoshiai (1991): Outbreaks of cold-water disease in coho salmon in Japan. *Fish Pathol.*, **26**, 211–212.
- Wakabayashi, H., T. Toyama and T. Iida (1994): A study on serotyping of *Cytophaga psychrophila*, isolated from fishes in Japan. *Fish Pathol.*, **29**, 101–104.
- Yoshimizu, M., T. Kimura and J. R. Winton (1985): An improved technique for collecting reproductive fluid samples from salmonid fishes. *Prog. Fish-Cult.*, **47**, 199–200.