Vol. 67: 73–79, 2005 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published November 9

Experimental bath infection with *Flavobacterium psychrophilum*, inducing typical signs of rainbow trout *Oncorhynchus mykiss* fry syndrome

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ABSTRACT: *Flavobacterium psychrophilum* infection in salmonid fish, known as rainbow trout fry syndrome (RTFS) or bacterial coldwater disease (BCWD), is widespread in fish farms and natural waters. Despite many studies in which attempts at infection were made, an adequate method of infection has not yet been established. In this study, we evaluated a bath infection method in which we used bacteria at different stages of growth in the infection of rainbow trout *Oncorhynchus mykiss*. Rainbow trout with a mean body weight of 1.3 or 5.6 g, respectively, were infected by immersion in a bacterial suspension at different stages of growth (18 to 66 h shaking culture at 15°C). The fish immersed in a logarithmic phase culture showed higher mortality than those in other culture phases. Indeed, 1.3 and 5.6 g fish showed typical clinical signs including ulcerative tissue of the trunk and lack of caudal fin edge. *F. psychrophilum* was detected by immunohistochemistry (IHC) in these tissue samples. These results indicate that experimental bath infection using a logarithmic phase bacterial solution is the most appropriate method for studies of infectious mechanisms.

KEY WORDS: *Flavobacterium psychrophilum* · Logarithmic phase · Bath challenge · Virulence · Bacterial coldwater disease · Rainbow trout fry syndrome

INTRODUCTION

Flavobacterium psychrophilum has been known as the causative bacterium of a disease in juvenile salmonid fish since the 1940s (Borg 1960). The disease has been variously designated as a low-temperature disease (Borg 1960), saddleback disease (Borg 1960, Holt 1987), bacterial coldwater disease (Holt 1987), fry mortality syndrome (Lorenzen et al. 1991) and rainbow trout fry syndrome (Madsen & Dalsgaard 1998). The disease occurs in most areas of the world including the USA, Canada, Chile, Australia, Japan, Korea and several European countries (Holt 1987, Santos et al. 1992, Toranzo & Barja 1993, Wakabayashi et al. 1994, Bustos et al. 1995, Schmidtke & Carson 1995) causing serious mortalities and, hence, severe economic losses in hatcheries and farms. Naturally infected rainbow trout *Oncorhynchus mykiss* show external signs of ulcers on the body trunk and the caudal fin edge, exophthalmia and haemorrhage on the gills, and internal signs of anaemia, necrotic myositis, necrotic scleritis and cephalic osteochondritis (Holt et al. 1993, Ostland et al. 1997). The appearance of antibiotic-resistant *F. psychrophilum* (Kondo et al. 2001a) and the difficulty of treatment of fish stocks in natural waters has promoted vaccine study (Kondo et al. 2003, LaFrentz et al. 2004).

Several attempts have been made, using various challenge models, to study the pathogenesis of *Flavobacterium psychrophilum*. Rainbow trout *Oncorhynchus mykiss* and other salmonids have shown high mortality following intraperitoneal, intramuscular or subcutaneous injections used to investigate the virulence of the bacterium and to establish a challenge method for use in vaccine trials (Borg 1960, Holt 1987, Austin et al. 1992, Lorenzen 1994, Rangdale 1995, Madsen & Dalsgaard 1999, Garcia et al. 2000, Decostere et al. 2001). However,

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the direct delivery of bacteria into host internal tissue by injection is not a natural route of infection and bypasses the intrinsic defense mechanism of the mucus, skin, gills and gut of fish. Cohabitation, contact and immersion infection models have attempted to mimic the natural route of infection (Rangdale 1995, Madsen & Dalsgaard 1999, Decostere et al. 2000, Garcia et al. 2000, Madetoja et al. 2000, Liu et al. 2001, Busch et al. 2003). Some researchers have performed immersion experiments, most of them reporting successful but unreproducible results (Madsen & Dalsgaard 1999, Garcia et al. 2000). Reproducible bath infection for ayu *Plecoglossus altivelis*, using a logarithmic culture phase of *F. psychrophilum* and without stressing the fish, has previously been reported (Kondo et al. 2001b, 2003).

In the present study, we attempted a bath infection model for juvenile rainbow trout *Oncorhynchus mykiss* using *Flavobacterium psychrophilum* in the logarithmic, stationary and death culture phases, and obtained a high mortality and clinical signs similar to natural infection using the logarithmic culture phase. We suggest that this is a successful protocol for bath infection of rainbow trout.

MATERIALS AND METHODS

Bacterial strain and growth condition. *Flavobacterium psychrophilum* strain NCIMB1947 isolated in 1948 from the kidney of the coho salmon *Oncorhynchus kisutch* in the USA was used for a bath challenge. This strain represented the serotype Fp^T (O1) (Izumi & Wakabayashi 1994, Lorenzen & Olesen 1997). This bacterium was passed 5 times in rainbow trout *Oncorhynchus mykiss* before experiments. The bacterium was pre-cultured in 50 ml modified cytophaga broth (MCYT; 0.2% trypton, 0.05% yeast extract, 0.02% tuna extract [Kyokuto], 0.02% CH_3COONa, 0.02% $CaCl_2$) at 15°C for 48 h, and then 2.5 ml of the culture was inoculated into 1000 ml MCYT broth. The bacterium was cultured by shaking (100 rpm at 15°C). Growth phase was monitored by measuring the absorbance at 600 nm (0-2001, Hitachi), and the viable number of bacteria was cultured on MCYT agar using the agar spread method every 6 h after inoculation.

Experimental fish. Two month old rainbow trout *Oncorhynchus mykiss*, (mean weight 1.3 g), and 10 mo old rainbow trout (mean weight 5.6 g) were obtained from a local fish farm (Iwana-so, Ehime prefecture, Japan) with no previous recorded occurrence of infection with *Flavobacterium psychrophilum*. Ten fish were tested for the presence of *F. psychrophilum* before experiments. The fish were kept in a 3.3 m^3 concrete tank with continuously flowing well water at 17 to 18°C. The fish were acclimatized at 17°C in a circulating tank for at least 5 d and were fed dry commercial pellets (Nissui), corresponding to 3% of fish body weight, until the end of the experiment.

Bath challenge. The cultures for 18, 24, 48 and 66 h coincided with the middle logarithmic, late logarithmic, stationary and death phases for *Flavobacterium psychrophilum* (see Fig. 1). The concentration of bacterium at 18 h is approximately 10^7, at 24 h 10^8, at 48 h 10^9 and at 66 h 10^8 CFU ml^{-1}. Each of the 8 infection groups consisted of approximately 100 fish. Five thousand ml of the bacterial culture was harvested by centrifugation (1000 × *g* for 15 min at 4°C) and resuspended in 250 ml of the fresh MYCT broth and 4750 ml tap water. Bath infection was carried out by immersion in aerated well water (for 1 h at 17°C) in which both groups (1.3 g and 5.6 g) were immersed and cultured for 18 h, 24 h, 48 h and 66 h. These groups were classified as 18 h-1.3 g, 24 h-1.3 g, 48 h-11.3 g, 66 h-1.3 g, 18 h-5.6 g, 24 h-5.6 g, 48 h-5.6 g and 66 h-5.6 g (see Table 1). Fish from the control group were immersed for 1 h in sterile MYCT broth diluted 1:20 with well water. The final concentration of bacterium used ininfection is shown in Table 1. After immersion infection, fish were reared in circulating tanks at 17°C for 14 d.

Detection of *Flavobacterium psychrophilum* after experimental bath infection. Fish from both infected and control groups were killed by a blow to the head and tissues imprinted onto a glass slide. The presence of bacteria on the slide was confirmed by methylene blue staining. The presence of *F. psychrophilum* was confirmed by immunohistochemistry (IHC) using anti-*F. psychrophilum* NCIMB1947 strain rabbit serum. Rabbit serum directed against *F. psychrophilum* NCIMB1947 was used as a primary antibody (dilution 1:200). The binding of immunoglobulins by *F. psychrophilum* cells was shown using anti-goat immunoglobulins directed against rabbit antibody (Wako). The secondary antibody was conjugated with fluorescein isothiocyanate (FITC). FITC-labelled cells were observed under a fluorescence microscope.

Statistical analysis. Differences in cumulative mortalities were analyzed using the chi-square test (Excel, Microsoft).

RESULTS

Bath infection test conditions

Fig. 1 shows the growth curve obtained from the shaken culture of *Flavobacterium psychrophilum* NCIMB1947 strain in MCYT broth. This result shows that the bacterial solution reached a logarithmic phase at 6 to 30 h after inoculation when the viable counts were approximately $10^{4.0}$ to $10^{8.0}$ CFU ml^{-1}. For convenience, we divided the logarithmic phase into a middle- and a late

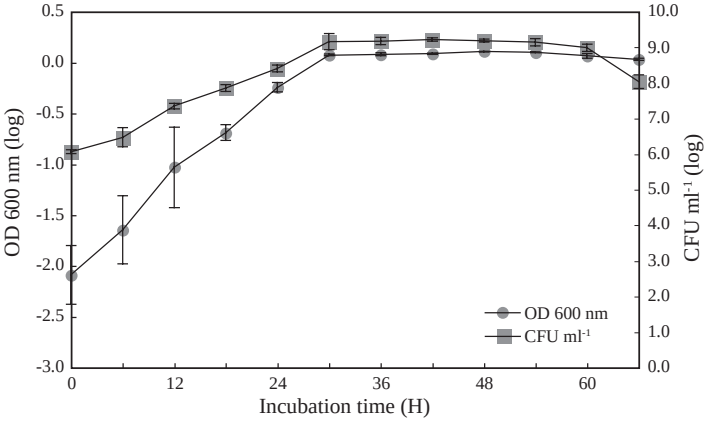

Fig. 1. *Flavobacterium psychrophilum*. Growth curve for bacteria cultured in modified cytophaga broth with shaking at 15°C. OD = optical density

logarithmic phase according to cell number. The culture reached a stationary phase at 30 h after inoculation, when the viable count was approximately $10^{9.0}$ CFU ml^{-1}. The bacterial solution at 66 h after inoculation showed a decrease in CFU (death phase). From this culture, bacterial solution at 18 h (middle logarithmic phase), 24 h (late logarithmic phase), 48 h (stationary phase) and 66 h (death phase) was used for the challenge.

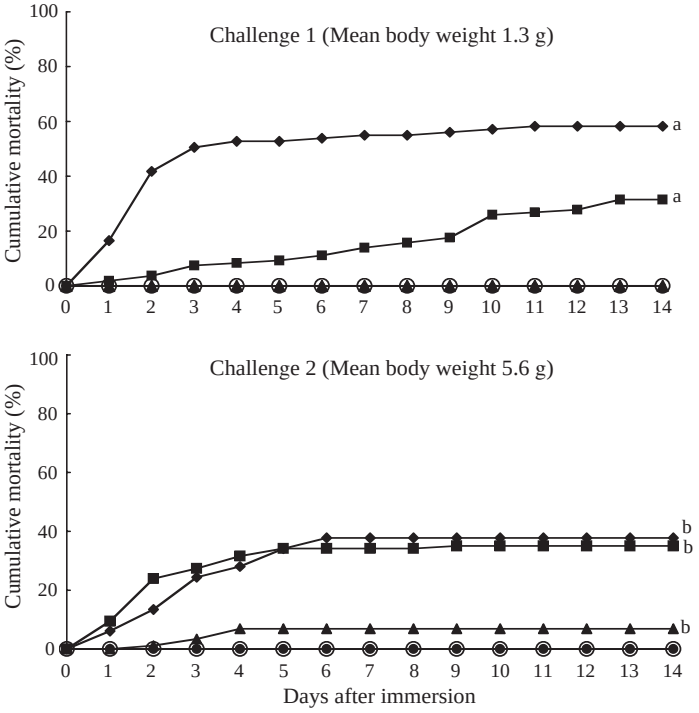

Fig. 2. *Flavobacterium psychrophilum*. Cumulative mortalities of rainbow trout by experimental bath infection: (■) challenge with 18 h cultured bacteria; (◆) challenge with 24 h cultured bacteria; (▲) challenge with 48 h cultured bacteria; (●) challenge with 66 h cultured bacteria; (○) control. The letters a and b indicate significant difference ($p < 0.01$) compared with the results for the control group

Bath challenge

Table 1 and Fig. 2 show the results of the bath challenge experiments. The Challenge 1 results show that the cumulative mortality rate of the 1.3 g fish in the 18 h and 24 h groups was significantly different from that of the 1.3 g fish in the 48 h group and the control group (chi-square test, $p < 0.01$).

The Challenge 2 results show that the mortality rate of the 5.6 g fish in the 18 h, 24 h and 48 h groups was significantly different from that of the 5.6 g control group (chi-square test, $p < 0.01$). The 5.6 g fish in the 18 h and 24 h groups were significantly different from those in the 48 h group (chi-square test, $p < 0.01$).

Table 1. *Flavobacterium psychrophilum*. Cumulative mortalities in the bath challenge test. Fish were immersed in aerated well water containing bacteria. See 'Materials and methods' for more details

Group	Mean body weight (g)	*F. psychrophilum* culture time (h)	Challenge dose (CFU ml^{-1})	Dead fish / challenged fish	Cumulative mortality 14 d post challenge (%)
Challenge 1					
18 h-1.3 g	1.4	18	2.0×10^7	34/108	31.5[a]
24 h-1.3 g	1.1	24	8.5×10^7	53/91	58.2[a]
48 h-1.3 g	1.4	48	3.4×10^8	0/101	0.0
66 h-1.3 g	1.3	66	2.0×10^6	0/105	0.0
Control-1.3 g	1.4	–	–	0/95	0.0
Challenge 2					
18 h-5.6 g	5.3	18	2.0×10^7	41/117	35.0[b]
24 h-5.6 g	6.4	24	8.5×10^7	31/82	37.8[b]
48 h-5.6 g	6.2	48	3.4×10^8	6/87	6.9[b]
66 h-5.6 g	5.6	66	2.0×10^6	0/107	0.0
Control-5.6 g	5.4	–	–	0/108	0.0

[a]Significant difference ($p < 0.01$) compared with the control-1.3 g group
[b]Significant difference ($p < 0.0$ 1) compared with the control-5.6 g group

Clinical signs

In both weight categories (1.3 and 5.6 g) the fish in the 24 h groups began to die on Day 1 post challenge and mortalities continued for the next 6 to 11 d. All dead fish in these groups showed the following typical signs of disease: ulcers on the body trunk and partial lack of caudal fin edge (Fig. 3). Infected 1.3 g fish from the 24 h group showed partial lack of the operculum (Fig. 3B). Internally, anaemia was observed in the form of pale gills, kidney, intestine, and liver (data not shown). Fish in both weight categories from the 18 h groups showed signs similar to those of the respective 24 h groups. However, in other culture phases and control groups, disease and internal signs were not observed.

Detection of *Flavobacterium psychrophilum* in tissue samples

In dead fish infected with logarithmic phase bacterial solutions, numerous bacteria were observed in ulcerative tissue, such as the surface of the trunk and caudal peduncle. *Flavobacterium psychrophilum* was detected by IHC in ulcerative tissue such as the surface of the trunk, caudal fin edge, spleen and kidney (Fig. 4). Numerous *F. psychrophilum* cells were observed on the body trunk and caudal fin edge. In the spleen and kidney, there were few bacteria. *F. psychrophilum* were not observed on any site of the control fish.

DISCUSSION

In order to develop a practical vaccine against RTFS, setting up a reproducible experimental bath infection is important because injecting bacteria into the host's body is not a natural route of *Flavobacterium psychrophilum* infection. The bath infection method, however, resembles a natural infection and thus seems to be the best infection method. Previous researchers have performed immersion experiments, most of them reporting that experimental fish die but that these results are not reproducible (Madsen & Dalsgaard 1999, Garcia et al. 2000). Kondo et al. (2003) showed that reproducible bath infection using the logarithmic phase of *F. psychrophilum* strain G3724 in ayu *Plecoglossus altivelis* is possible. In the present study, we investigated the same infection method in rainbow trout fry. *F. psychrophilum* strain NCIMB1947 has been well characterized (Secades et al. 2001), and therefore, this strain is a practical and useful strain in the study of pathogenic mechanisms. However, this strain belongs to Fp^T. Lorenzen & Olesen (1997) suggested that isolates belonging to the serotype Fp^T were less virulent than other isolates. We reported that loga-

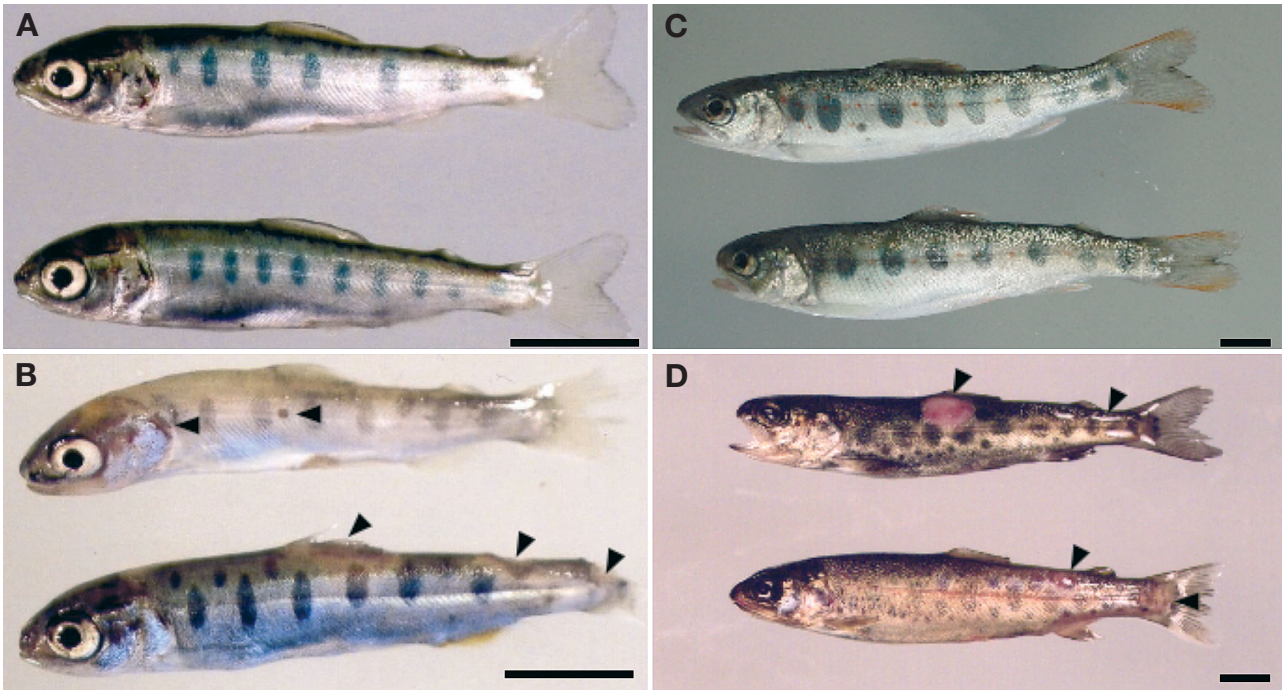

Fig. 3. Typical signs of rainbow trout fry syndrome. (A) Control for 1.3 g group fish; (B) infected 1.3 g group fish; (C) control for 5.6 g group fish; (D) infected 5.6 g group fish. Arrowheads indicate partial lack of operculum, ulcers on the trunk and dorsal side and lack of fin edge. Scale bar = 10 mm

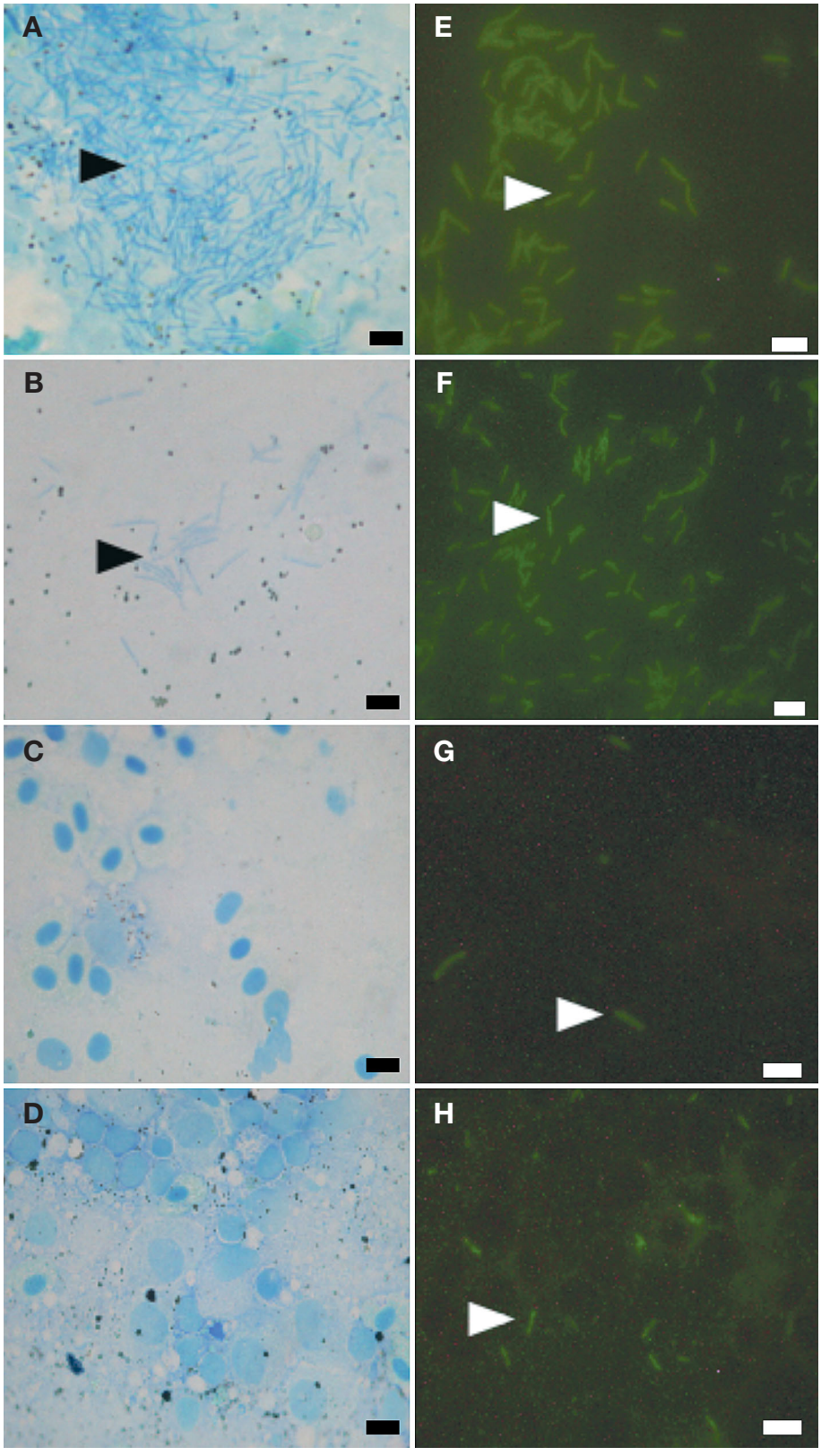

Fig. 4. Tissue imprint of rainbow trout after bath challenge. (A) and (E): trunk lesion; (B) and (F): caudal fin; (C) and (G): spleen; (D) and (H): kidney. Black arrowheads show rod-shaped bacteria (methylene blue stain). White arrowheads show *Flavobacterium psychrophilum* stained with anti-*F. psychrophilum* 1947 strain rabbit serum. Magnification ×1000 (scale bar = 2 µm)

rithmic phase culture of *F. psychrophilum* strain G3724 has high virulence (Kondo et al. 2002, 2003); therefore, we examined whether the logarithmic phase of *F. psychrophilum* strain NCIMB1947 also has high virulence. At the beginning of this study, we determined the logarithmic, stationary and death phases of *F. psychrophilum* strain NCIMB1947. The growth curve of this strain showed logarithmic (6 to 30 h), stationary (30 to 60 h) and death phases (60 to 66 h).

In this study, we demonstrated that *Flavobacterium psychrophilum* in the logarithmic culture phase caused higher mortality than the stationary and death phases (0 to 6.9% mortality) of rainbow trout fry *Oncorhynchus mykiss* (2 and 10 mo old). In a previous study we showed that cumulative mortality by bath infection achieved a maximum of 70% using logarithmic phase *F. psychrophilum* strain G3724 in ayu *Plecoglossus altivelis* (Kondo et al. 2003). Our findings conclude that *F. psychrophilum* strains G3724 and NCIMB1947 in the logarithmic phase are highly virulent in both ayu (Kondo et al. 2003) and rainbow trout. In this study, we attempted to evaluate in detail the virulence of *F. psychrophilum* in the logarithmic, middle and late logarithmic phases. Our results indicated that the late logarithmic phase (24 h) has a higher virulence (58.2% mortality) than the middle logarithmic phase (18 h; 31.5% mortality) in the mean body weight of 1.3 g group. However, the bacterial number in the late logarithmic phase differed from the middle logarithmic phase concentration. Therefore, our results did not reflect the virulence of these 2 phases, indicating that further study of the virulence of middle and late logarithmic phases is needed. Additionally, the mortality rate in the mean body weight of 5.6 g group was significantly different from the control group.

Rainbow trout *Oncorhynchus mykiss* naturally infected in hatcheries usually show clinical signs such as trunk ulcers, lack of caudal fin edge, anaemia, exophthalmia and haemorrhage of the gills, necrotic myositis, necrotic scleritis and cephalic osteochondritis (Holt et al. 1993, Ostland et al. 1997). In the present study, trunk ulcers, lack of caudal fin edge, anaemia in the form of pale gills, kidney, intestine and liver were found in the mean body weight of 1.3 and 5.6 g groups. Numerous bacteria were identified in the affected parts of the body surface. Therefore, our bath infection mimicked natural infection of RTFS. The mean body weight of 1.3 g group showed more severe signs than the mean body weight of 5.6 g group. Madetoja et al. (2000) found that size differences affect the level of immunocompetence in rainbow trout. In this study, the mortality rate for the mean body weight of 1.3 g group of low-level immunocompetence was higher than for the mean body weight of 5.6 g group, thus reflecting the findings of studies which determined that RTFS almostly always occurs in small rainbow trout (0.2 to 2.0 g) (Santos et al. 1992, Nematollahi et al. 2003).

In conclusion, we believe this is the first study to succeed in infecting fish using a bath infection method with logarithmic phase bacteria and avoiding the stress of injection. Logarithmic phase culture showed a high mortality of fish, which demonstrated typical signs of disease and numerous bacteria on body surface tissues. The experimental bath infection described here using a logarithmic phase bacterial solution may be a successful method in evaluating the pathogenesis of fish disease and the development of a candidate vaccine.

Acknowledgements. This research was supported by a Regional Science Promotion Program grant from the Japan Science and Technology Agency (JST), and a New Energy and Industrial Technology Development Organization (NEDO) grant. We thank Y. Nakatuka for his technical assistance and advice.

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Editorial responsibility: David Bruno, Aberdeen, UK

Submitted: September 13, 2004; Accepted: May 9, 2005
Proofs received from author(s): October 14, 2005