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<Short Communication>

Biological control of *Flavobacterium psychrophilum* infection in ayu (*Plecoglossus altivelis altivelis*) using a bacteriophage PFpW-3

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Abstract: The efficacy of using a bacteriophage (phage) to control *Flavobacterium psychrophilum* (*F. psychrophilum*) infection of ayu (*Plecoglossus altivelis altivelis*) was evaluated in this study. Intramuscular challenge failed to induce sufficient infection levels; therefore, a newly designed net-scratch challenge method was also used to induce bacterial infection. Administration of phage PFpW-3 in *F. psychrophilum*-infected ayu showed notable protective effects, increased survival rates and mean times to death. Additionally, the fate of inoculated bacteria and phage in ayu were investigated. Our results suggest that the phage PFpW-3 could be considered an alternative biocontrol agent against *F. psychrophilum* infections in ayu culture.

Keywords: *Flavobacterium psychrophilum*, PFpW-3, ayu, bacteriophages

Flavobacterium psychrophilum (*F. psychrophilum*) is the causative agent of coldwater disease (CWD) and has been recognized recently as being responsible for appreciable economic losses related to aquaculture worldwide [2]. In Korea and Japan, wild and farmed ayu (*Plecoglossus altivelis altivelis*) have been shown to be severely affected by *F. psychrophilum* [5, 11]. Infection of ayu occurs at different temperatures and exhibits different clinical signs than that of salmonid fishes [7]. To date, no commercial vaccine has been made available, and antibacterial chemotherapy tends to exert limited effects on CWD [14]. Moreover, with the emergence of antibiotic-resistant *F. psychrophilum* in fish cultures [10], alternative approaches to the treatment of CWD are urgently needed.

Recently, bacteriophages (phages) have been used as alternative therapeutic and prophylactic agents in aquaculture to control fish diseases and other infections in aqueous environments [8]. Several lytic phages infecting *F. psychrophilum* have been isolated and characterized [2, 3, 8, 14], and the application of phages as a protective measure against bacteria has been shown to reduce mortality in salmonids [2]. However, their effectiveness in preventing *F. psychrophilum* infection of ayu has not yet been evaluated. Therefore, the aim of this study was to evaluate the therapeutic potential of phages for *F. psychrophilum* infection of ayu using a novel experimental bacterial challenge method, and investigate the fate of the inoculated bacteria and phages.

F. psychrophilum strain N2-3 and the phage designated PFpW-3 [8] were used in this study. All bacterial culture and phage propagation procedures were carried out in accordance with a previous report [8], and a PCR assay [6] was used to confirm the presence of *F. psychrophilum* in fish of all experimental groups. For the animal experiments, four hundred healthy ayu (with an average weight of 48.9 g) obtained from the Tokushima Prefectural Fish Farming Center (Japan) were acclimatized in 300 L fiberglass tanks with a flow-through water supply at 18 ± 2°C. The ayu were kept at 18°C for one week, during which they received commercial dry feed pellets. The food supply was discontinued 24 h prior to bacterial challenge. All of the procedures described in this study involving animals and their care were performed in accordance with the guidelines for animal experiments of Hiroshima University, Japan.

In order to evaluate the control efficacy of phage PFpW-3 against *F. psychrophilum* infection in ayu, one hundred and ten fish were divided into two groups of equal size and maintained in 300 L fiberglass tanks with a flow-through water supply at 18 ± 2°C. They were then challenged with a 0.05-mL suspension of live *F. psychrophilum* N2-3 in phosphate-buffered saline (PBS) by intramuscular (IM) injection (6.2×10^5 CFU/fish; CFU, colony-forming unit). Subsequently, one group (hereinafter referred to as “IM exp”) was treated

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immediately with a phage suspension (at a final concentration of 3.4×10^6 PFU/mL; PFU, plaque-forming unit) by immersion in a bath for 30 min. The other group (hereinafter referred to as "IM cont") was treated with PBS only, as a control. A food supply was initiated on the following day at a rate of 0.5% of body weight, and the fish were kept for 14 days at 18°C.

However, due to the insufficient levels of infection observed in experimental studies employing *F. psychrophilum* in various fish species [4, 13], a newly modified bacterial challenge technique causing artificial skin abrasions, named the net-scratch (NS) method, was adopted to enhance infectivity. In an aquarium, 80 ayu were left in a net gyrating at 100–120 gyrations/min for 5 min until the entire skin surface was scratched. The fish were then directly challenged with suspensions of live *F. psychrophilum* N2-3 (at a final concentration of 6.2×10^5 CFU/mL) in PBS for 30 min by bath administration. After challenge, one group (hereinafter referred to as "NS exp") was treated immediately with a phage suspension (at a final concentration of 3.4×10^6 PFU/mL) for 30 min, also by bath administration. A second group (hereinafter referred to as "NS cont") was treated with PBS in place of phages, as a control. As above, food was supplied from the following day at a rate of 0.5% of body weight, and the fish were maintained at 18 ± 2°C for 14 days.

Separately, groups of ayu treated as above and kept under identical conditions were used to investigate the fate of the inoculated phages and bacteria. Kidneys (circa [ca.] 0.16 g each) from five randomly selected ayu per group were sampled at 0, 3, 24, 72, 120, and 168 h after bacterial challenge and phage administration. The zero hour samples were collected before challenge to be used as a control for all of the treatment groups. All samples were thoroughly homogenized in PBS. One half of each homogenate was used to estimate the concentration of bacterial CFUs, and the other, that of phage PFUs. Enumeration of *F. psychrophilum* in kidney samples was achieved using a standard spread plate technique on cytophaga agar [15]. To ascertain phage concentrations, kidney homogenate was inoculated into 1 ml cytophaga broth [1], which was then centrifuged for 10 min at 3,000 × g. The supernatant (250 µL) was subsequently subjected to a PFU assay based on the double-layer agar method, using *F. psychrophilum* N2-3 and cytophaga aga incubated for 48 h at 18°C.

F. psychrophilum is a component of the fish microbiota of the skin, mucus, and connective tissue of fins, gills and opercula, and is readily transmitted horizontally between fish by waterborne and direct-contact routes [12]. However, effective establishment of *F. psychrophilum* infection by oral challenge has not been documented thus far [4, 12]. Therefore, in order to evaluate the efficacy of phages in protecting against *F. psychrophilum* infection, IM and NS methods were simultaneously utilized in this study.

The protective effects of bath administration of phages against *F. psychrophilum* infection are demonstrated in

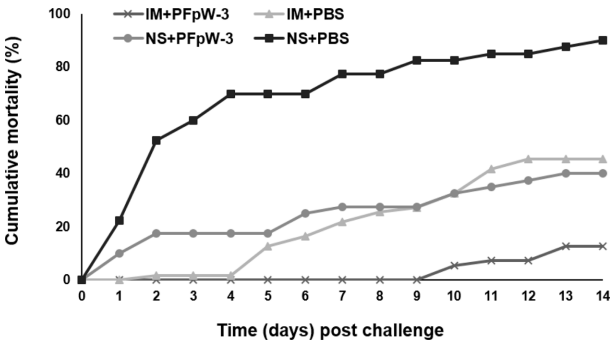

Fig. 1. The protective effects resulting from bath administration of phage PFpW-3 against *Flavobacterium psychrophilum* (*F. psychrophilum*) N2-3 challenge. IM+PFpW-3, IM-challenged and phage-administrated group; IM+PBS, IM-challenged group without phage administration; NS+PFpW-3, NS-challenged and phage-administrated group; NS+PBS, NS-challenged group without phage administration. IM, intramuscular injection; NS, net-scratch method.

Figure 1. The inoculated bacterium was identified in kidney samples from dead fish (n = 84) of all treatment groups, regardless of phage administration. Fish in the IM cont group began to die 2 days after bacterial challenge, and their cumulative mortality over the 2 weeks of the experiment was ca. 45.5% (with a mean time to death of 8.0 days). In contrast, those in the IM exp group began to die much later, with an average cumulative mortality of ca. 12.7% (and a mean time to death of 11.4 days). The mortality of phage-administered fish was therefore lower than that of those given PBS. However, IM administration in this study failed to induce high mortality and sufficient infectivity, necessitating a different approach to systemic bacterial challenge. It has previously been reported that abrasion of the skin and mucus enhances pathogen invasion of fish (including ayu) challenged by bath and cohabitation methods [12, 13]. Therefore, the newly designed NS infection technique was employed. Using this procedure, we observed increased infectivity and mortality compared to IM challenge. Fish in the NS cont group began to die 1 day after bacterial exposure, and cumulative mortality after 2 weeks was 90.0% (with a mean time to death of 3.8 days). In contrast, although NS exp fish also started dying at day 1, the average cumulative mortality in this group was 40.0% (with a mean time to death of 5.7 days). Interestingly, bacterial CFU/g values increased more rapidly in the NS cont group than the IM cont group, supporting the effectiveness of the NS method over IM challenge. Moreover, mortality in the phage-administered groups (IM exp and NS exp) was lower than that in the control groups (IM cont and NS cont), and the protective effects of phage treatment were more evident using the NS protocol than IM injection. These results suggest that phage PFpW-3 can be considered as an alternative biological control agent for *F. psychrophilum* infections in Korean and Japanese ayu cultures.

Additionally, the fate of inoculated bacteria and phage in

Table 1. Fates of challenged *F. psychrophilum* N2-3 and administrated phage PFpW-3 in ayu

Time after treatment (h)	Fish sample nunmber	*F. psychrophilum* (CFU/g) in ayu kidney		PFpW-3 (PFU/g) in ayu kidney		*F. psychrophilum* (CFU/g) in ayu kidney		PFpW-3 (PFU/g) in ayu kidney	
		IM exp	IM cont	IM exp	IM cont	NS exp	NS cont	NS exp	NS cont
0*	0–1	–†	–†	–†	–†	–†	–†	–†	–†
	0–2	–	–	–	–	–	–	–	–
	0–3	–	–	–	–	–	–	–	–
	0–4	–	–	–	–	–	–	–	–
	0–5	–	–	–	–	–	–	–	–
3	3–1	–	–	3.8×10^2	–	–	3.5×10^2	2.0×10^2	–
	3–2	–	–	1.4×10^3	–	5.0×10^1	–	7.0×10^2	–
	3–3	1.0×10^2	1.8×10^2	2.3×10^2	–	–	–	2.3×10^2	–
	3–4	5.0×10^1	–	5.0×10^1	–	–	–	4.8×10^2	–
	3–5	–	–	1.5×10^2	–	–	–	3.5×10^2	–
24	24–1	–	5.0×10^1	6.0×10^2	–	–	2.8×10^2	2.3×10^4	–
	24–2	–	–	7.3×10^3	–	–	3.5×10^2	2.4×10^5	–
	24–3	1.5×10^2	–	4.0×10^2	–	–	6.3×10^2	1.4×10^5	–
	24–4	2.5×10^1	–	–	–	–	5.0×10^1	4.5×10^5	–
	24–5	6.8×10^2	5.0×10^1	5.0×10^1	–	7.5×10^1	–	2.8×10^5	–
72	72–1	–	–	–	–	1.5×10^2	3.5×10^2	9.7×10^3	–
	72–2	–	2.5×10^2	7.8×10^4	–	–	–	7.2×10^4	–
	72–3	–	1.8×10^4	–	–	–	–	2.3×10^3	–
	72–4	–	7.5×10^1	1.9×10^4	–	–	4.5×10^2	3.0×10^3	–
	72–5	–	–	–	–	5.0×10^1	–	5.4×10^4	–
120	120–1	–	–	–	–	–	–	–	–
	120–2	–	–	–	–	3.8×10^2	–	–	–
	120–3	–	–	–	–	–	–	–	–
	120–4	–	5.0×10^1	–	–	5.7×10^3	–	–	–
	120–5	–	–	–	–	–	–	–	–
168	168–1	–	–	–	–	–	–	–	–
	168–2	–	–	–	–	–	–	–	–
	168–3	–	–	–	–	–	–	–	–
	168–4	–	–	–	–	–	–	–	–
	168–5	–	–	–	–	–	–	–	–

*Zero hour samples were collected before bacterial challenges and phage administrations. †The quantities of phage and *F. psychrophilum* in kidney samples were lower than 2.5×10^1 PFU/g and 2.5×10^1 CFU/g, respectively. PFU, plaque-forming unit; CFU, colony-forming unit.

ayu were also investigated. *F. psychrophilum* was detected in samples collected from 3 to 120 h after challenge, and its concentration finally decreasing below the detection limit (2.5×10^1 CFU/g) by 168 h in all groups (Table 1). The kidney samples revealed notable differences in bacterial fate between the IM and NS groups (Fig. 2A). Regarding IM administration, *F. psychrophilum* CFU/g values in fish exposed to phages were higher than those of the controls until 24 h post-challenge. The protective effects of phage treatment were then observed 72 and 120 h after challenge in the IM exp group. However, an antibacterial impact was evident from 3 to 120 h in the NS exp group, samples from which exhibited low CFU/g values compared to those of the NS cont group. These findings further demonstrate that phage PFpW-3 is able to inhibit bacterial growth in ayu and that it could be used prophylactically to prevent horizontal transmission of *F. psychrophilum*.

PFpW-3 was detected from 3 h post-administration in samples from both groups treated with this phage (Fig. 2B and Table 1), indicating that it permeated tissues and reached the kidneys of fish in each group equally, regardless of skin damage. Thus, as both groups were treated in the same manner (bath administration), phages were unlikely to have entered through the skin. Moreover, the PFU/g values of the phage-administered groups increased 24 and 72 h post-treatment, suggesting that phages had successfully propagated and lysed

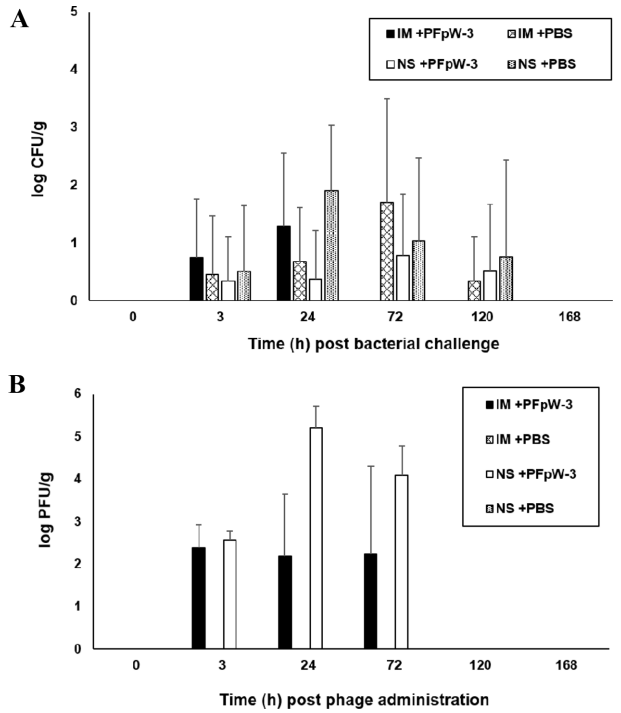

Fig. 2. Fates of phage PFpW-3 and *F. psychrophilum* N2-3 in ayu kidney samples from IM-challenged and phage-administrated group (IM+PFpW-3), IM-challenged group without phage administration (IM+PBS), NS-challenged and phage-administrated group (NS+PFpW-3) and NS-challenged group without phage administration (NS+PBS), respectively. Bars indicate SD. *No phage (PFpW-3) and *F. psychrophilum* (N2-3) was detected in the group (Detection limit: $< 2.5 \times 10^1$ PFU/g and $< 2.5 \times 10^1$ CFU/g). The zero hour samples were collected before treatment and used as control of phage-administrated group and bacterial-challenged group.

F. psychrophilum in ayu kidneys. However, the extent and timing of such increases differed between the two groups. Phage PFU/g values in the NS exp group had increased by 24 h post-administration, whereas those in the IM exp group were similar at 3 and 24 h, eventually increasing at 72 h. Based on these results, we speculate that the early increase in phage levels inhibited fatal bacterial growth, resulting in retained mean time to death and reduced mortality in the NS exp group compared to the IM exp group.

Interestingly, fluctuations in the concentration of phages between 72 and 120 h post-administration did not correspond to changes in bacterial CFU/g values. Furthermore, despite the presence of *F. psychrophilum* in ayu kidneys at 120 h, phage levels were dramatically decreased, to below the detection limit (2.5×10^1 PFU/g). Similar results were obtained in a previous study involving *Aeromonas salmonicida* [9], in which phages were found to be undetectable in the kidneys of rainbow trout (*Oncorhynchus mykiss*) 200 h after inoculation. Thus, it may be hypothesized that the phages, having permeated the fish and propagated in their bacterial host in the kidney, maintain their infectivity for a limited time before finally decreasing in number due to an unknown response in the ayu. According to previous phage stability tests [8], no other environmental conditions can cause such a rapid decrease in phage PFU levels. Thus, the sudden reduction in phage concentrations in the kidney observed in the present work reveals that ayu may also develop humoral immunity to phages administered during biocontrol efforts.

Recent investigations concerning the application of phages in aquaculture have mainly concentrated on their interactions with host bacteria, whereas the immune responses of fish to such phages have not been well studied. Therefore, the relationship between phages and recipient fish should also be considered as an important factor affecting their successful use in aquaculture biocontrol.

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