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Bacteriophage control of *Pseudomonas plecoglossicida* infection in ayu *Plecoglossus altivelis*

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ABSTRACT: Two previously isolated phages were used to examine the therapeutic effects against *Pseudomonas plecoglossicida* infection in ayu *Plecoglossus altivelis*. Phage PPp-W4 (Podoviridae) inhibited the *in vitro* growth of *P. plecoglossicida* more effectively than Phage PPpW-3 (Myoviridae), and a mixture (PPpW-3/W-4) of the 2 phages exhibited the highest inhibitory activity. In phage therapy experiments, ayu were fed *P. plecoglossicida*-impregnated feed (10^7 CFU fish^{-1}) and then fed phage-impregnated feed (10^7 PFU fish^{-1}). Mortalities of fish receiving PPpW-3, PPpW-4, PPpW-3/W-4, and a control fish receiving no phages were 53.3, 40.0, 20.0 and 93.3%, respectively. Phage (PPpW-3/W-4)-receiving fish also showed high protection against water-borne infection with *P. plecoglossicida*. In a field trial, when phage (PPpW-3/W-4)-impregnated feed was administered to ayu in a pond where the disease occurred naturally, daily mortality of fish decreased at a constant level (5% d^{-1}) to one-third after a 2 wk period. The causal relationship of phages in this phenomenon was verified by the long-lasting appearance of administered phages in the kidneys of the fish, and a disappearance of *P. plecoglossicida* from apparently healthy fish. Neither phage-resistant organisms nor phage-neutralizing antibodies were detected in diseased fish or apparently healthy fish, respectively. These results indicate the potential for phage control of the disease.

KEY WORDS: Bacteriophage · Phage therapy · Fish disease · Biological control · *Pseudomonas plecoglossicida* · *Plecoglossus altivelis*

INTRODUCTION

An emerging bacterial disease, bacterial haemorrhagic ascites caused by *Pseudomonas plecoglossicida*, is posing a threat to cultured ayu *Plecoglossus altivelis* in Japan (Nakatsugawa & Iida 1996, Wakabayashi et al. 1996, Nishimori et al. 2000). The disease results in high mortality throughout almost the whole rearing period of ayu, particularly since there are no licensed chemotherapeutic agents available. There is a further complication in that application of florfenicol or sulfisozole as a chemotherapy for cold-water disease caused by *Flavobacterium psychrophilum* (another serious disease in cultured ayu: Wakabayashi et al. 1994, Iida & Mizokami 1996) is followed by the abrupt emergence of *P. plecoglossicida*, which results in heavy mortality.

Although bactriophages can theoretically act as therapeutic agents for bacterial infections, the term 'phage therapy' has long been abandoned since the early uncontrolled studies (reviewed by Barrow & Soothill 1997, Alisky et al. 1998, Sulakvelidze et al. 2001). However, since the 1980s, successful results on phage therapy have been reported using various animal models (Smith & Huggins 1982, 1983, Soothill 1994, Merril et al. 1996, Barrow et al. 1998) and in human drug-resistant suppurative infections (Slopek et al. 1987, Alisky et al. 1998). A recent study demonstrated that phage therapy frees bacteremic mice from vancomycin-resistant *Enterococcus faecium* (VRE) (Biswas et al. 2002). Several review papers have also been published (Lederberg 1996, Barrow & Soothill 1997, Alisky et al. 1998, Carlton 1999, Sulakvelidze et al. 2001, Summers 2001). The successful effect of phages in phage therapy has been proven by an increase in phage particles or the presence of phages in survivors, and the death of host bacterial cells. This distinguishes phage treatment from other biological controls such as

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probiotics (Reid 1999). Phages, as specific pathogen-killers, are attractive agents for controlling bacterial infections.

A previous report demonstrated that the oral administration of specific phages was effective in treating ayu experimentally infected with *Pseudomonas plecoglossicida* (Park et al. 2000). In order to examine the suitability of phage therapy for use in fish culture, further detailed therapeutic effects of *P. plecoglossicida* phages were examined against experimental and natural infections.

MATERIALS AND METHODS

Phages and bacteria. Two previously isolated strains of anti-*Pseudomonas plecoglossicida* phages (Park et al. 2000), small-plaque-forming PPpW-3 (Myoviridae) and large-plaque-forming PPpW-4 (Podoviridae), and a *P. plecoglossicida* PTH-9802 strain isolated from a diseased specimen of *Plecoglossus altivelis* were used in this study. All *Pseudomonas plecoglossicida* isolates from geographically and chronologically different sources exhibited similar sensitivity to phages of either type. The PTH-9802 is highly susceptible to either type of phage, and is highly virulent to ayu, the LD_{50} through intramuscular injection being about 10^1 CFU $fish^{-1}$. Phages were propagated using semisolid soybean casein digest agar (SCDA, Nissui) including PTH-9802 cells. Phage suspension (10^9 PFU ml^{-1}) was stored at 4°C until use. Bacteria were sub-cultured at 25°C on SCDA overnight prior to experiments.

***In vitro* anti-bacterial activity of phages.** Ten ml of soybean casein digest broth (SCDB, Eiken) was inoculated with PTH-9802 (4.8×10^2 CFU ml^{-1}) and serially 10-fold-diluted phages (10^6 to 10^{-2} PFU ml^{-1}), the phages being either PPpW-3, PPpW-4, or a mixture of PPpW-3 and PPpW-4 (PPpW-3/W-4), and then incubated with gentle agitation at 20°C. Every 12 h for up to 96 h incubation, the optical density (OD) was measured using a spectrophotometer. At the termination of each experiment, a loopful of each culture was inoculated onto SCDA and incubated at 25°C overnight; 10 separated colonies were picked from the plate and examined for sensitivity to each phage. The phage concentration in tubes inoculated with phages of 10^6, 10^{-1}, or 10^{-2} PFU ml^{-1} was also determined at 96 h post-incubation by using a double-agar-layer method described previously (Park et al. 2000).

Phage-neutralizing antibody production in fish. A total of 100 ayu weighing an average of 3.9 g were inoculated with phage mixture (PPpW-3/W-4) either orally or intramuscularly: 50 fish received phage-impregnated pellets orally for 7 successive days, while the other 50 fish were injected 4 times intramuscularly (IM) at 1 wk intervals. Phage doses in oral administration and IM injection were 7.6×10^7 PFU $fish^{-1}$ and 1.7×10^8 PFU $fish^{-1}$, respectively. Ten fish were bled without an anesthetic at 0, 1, 2, 3 and 4 wk after the first phage inoculation**,** and individual serum samples were examined for neutralizing antibodies against both phages inoculated using *Pseudomonas plecoglossicida* PTH-9802 strain as an indicator. Fifty microliters of serially 2-fold-diluted serum were mixed with 50 µl of phage suspension (10^3 PFU ml^{-1}), incubated at 25°C for 1 h, and subjected to a PFU assay by the double-agar-layer method. The virus-neutralizing titer of serum is shown as a reciprocal of the highest dilution of serum, which completely inhibited plaque formation.

Phage treatment of infected fish. Oral, water-borne and *in situ* challenges were carried out.

Oral challenge: Four groups of 30 ayu weighing an average of 2.7 g were fed pellets impregnated with live *Pseudomonas plecoglossicida* PTH-9802 cells (6.6×10^7 CFU $fish^{-1}$) at a feeding rate of 1.5%, based on body weight. Immediately after feeding with the bacteria-loaded pellets, 3 of the groups were fed pellets impregnated with either Phage PPpW-3 (3.5×10^7 PFU $fish^{-1}$), PPpW-4 (1.4×10^7 PFU $fish^{-1}$), or a mixture of PPpW-3/W-4 (2.4×10^7 PFU $fish^{-1}$). Group 4 received feed without phages and served as a control. Fish were observed daily in 40 l tanks with a flow-through system (20 ± 1°C) for 2 wk, and the kidneys of dead fish and survivors at the end of the experiment were subjected to bacterial isolation. The isolates were identified with the aid of rabbit anti-*P. plecoglossicida* serum.

Water-borne challenge: Four groups of 40 ayu (average weight 4.3 g) were used. Two tanks, 1 small (55 l) and 1 large (250 l), were connected with a water circulation system at 20 ± 1°C; 30 fish, the recipients of the bacteria, were first introduced into the large tank, and then 10 fish, the donors of the bacteria, which had been previously IM-injected with *Pseudomonas plecoglossicida* (6.0×10^3 or 1.6×10^4 CFU $fish^{-1}$), were placed in the small tank. Phage (PPpW-3/W-4)-impregnated feed was given to fish in the large tank (7.7×10^7 PFU $fish^{-1}$) 24 and 72 h after introduction of the donor fish. In another set of tanks (control group), fish in the small tank were similarly injected with the pathogen but fish in the large tank received phage-free feed at 24 and 72 h post-infection. The donor fish were removed from the tank within 24 h of death; 2 trials were conducted.

Natural challenge: Phage therapy was performed in a commercial fish culture pond where severe *Pseudomonas plecoglossicida* infection prevailed. At the beginning of the experiment, approximately 120 000 fish weighing an average of 20 g were reared in the pond (200 m^3) using ground water (water temperature

18°C). An automatic feeding machine was used to feed the fish. Phage (PPpW-3/W-4)-impregnated pellets (10^7 PFU fish^{-1}) were given to fish on Days 0, 1, and 8. To examine the phage-intake rate in the kidneys and intestines, 40 live fish were sacrificed 3 h after administration of phage-impregnated feed on Day 0. Thereafter, on Days 1 and 8, live fish samples were collected 1 h before the administration of phage-impregnated feed. Fish (live and dead, n = 40) and water samples on scheduled days (1, 2, 4, 6, 8 and 15) were subjected to isolation of bacteria and phages. To prevent outbreaks of *Flavobacterium psychrophilum* infection, an antibacterial drug (sulfisozole sodium) was administered for 8 d starting on Day 2 (110 mg kg^{-1} d^{-1}). *P. plecoglossicida* is not sensitive to this drug (Nakatsugawa & Iida 1996), and all isolates of *P. plecoglossicida* in the present study were resistant to the agent throughout the experimental period (MBC = 1000 µg ml^{-1} or higher). Serum samples (n = 20) were collected from live fish on Days –1 (i.e. 1 d before phage administration), 8, and 15 to examine for the presence of phage-neutralizing and *P. plecoglossicida*-agglutinating antibodies. Agglutination titers of serum were determined using formalin-killed cells of PTH-9802 in a 96-well microtiter plate. *P. plecoglossicida* isolates obtained from dead fish throughout the experimental period were also tested for their sensitivity to Phages PPpW-3 and PPpW-4.

Statistical analysis. Statistical analysis was performed using a chi-square test; $p < 0.01$ was considered significant.

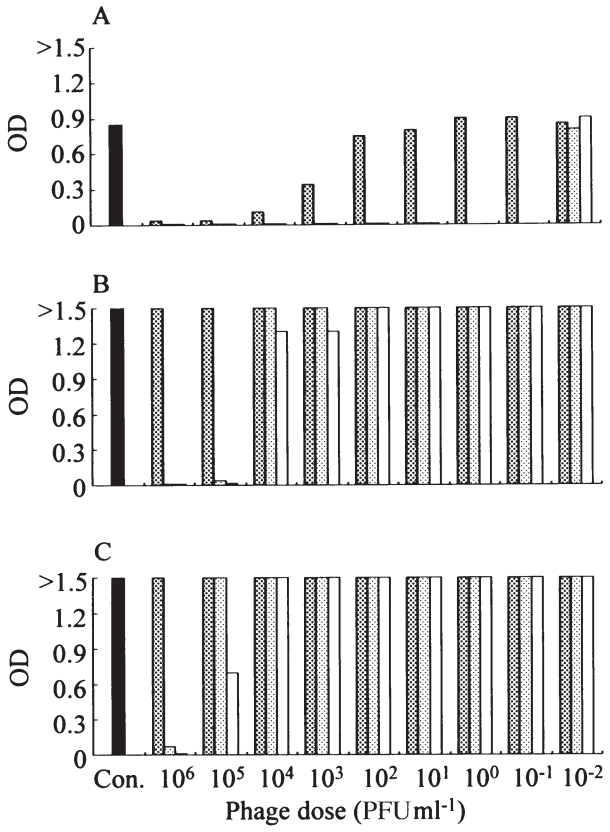

Fig. 1. *Pseudomonas plecoglossicida*. *In vitro* growth inhibition by phages. *P. plecoglossicida* was incubated with Phages PPpW-3 (■), PPpW-4 (▒), a mixture of PPpW-3 and PPpW-4 (□), or without phages (control: ■) for 24 h (A), 72 h (B), and 96 h (C). OD: optical density

RESULTS

In vitro phage activities

Bacterial growth was first observed at 24 h post-inoculation (p.i.) in a tube without phages (control), tubes inoculated at lower doses of PPpW-3, and in all tubes at a dose of 10^{-2} PFU ml^{-1} (theoretically phage-free). Thereafter, OD values reached 1.5 or higher at 36 h p.i. in the control, and within 72 h p.i. in tubes with PPpW-3 (Fig. 1). In the presence of phages, bacterial growth was inhibited in a dose-dependent manner against the number of inoculated phages. Irrespective of the dose of the inoculated phage, anti-bacterial activity of PPpW-3 was not recognized at incubation periods of 48 h or longer, while growth inhibitory activity of PPpW-4 was observed even after 96 h at a dose of 10^6 PFU ml^{-1}. The highest inhibition was obtained by inoculation of the mixed phages; in particular, when phages were inoculated at a dose of 10^6 PFU ml^{-1}, no increase in the OD was observed throughout the 96 h incubation period.

No phages were detected in cultures inoculated with phages at 10^{-2} PFU ml^{-1} at 96 h p.i., while phages at high titers (10^8 to 10^{10} PFU ml^{-1}) were recovered from cultures initially inoculated with phages of 10^6 and 10^{-1} PFU ml^{-1} (Table 1). Colonies of dominant bacteria obtained from each culture at 96 h p.i. were tested for sensitivity against Phages PPpW-3 or PPpW-4. Some colonies obtained from cultures inoculated with PPpW-3 at 10^1 PFU ml^{-1} or higher doses were sensitive to PPpW-4 but not to PPpW-3, while all isolates from

Table 1. *Pseudomonas plecoglossicida*. Phage recovery from 96 h *in vitro* cultures of incubated with anti-*P. plecoglossicida* phages

Inoculation phage	Phage dose (PFU ml^{-1}) at Time 0		
	10^6	10^{-1}	10^{-2}
PPpW-3	1.6×10^9	3.6×10^9	0
PPpW-4	1.7×10^{10}	4.5×10^9	0
PPpW-3 + PPpW-4	2.8×10^8	6.2×10^9	0

other cultures were resistant to both phages, except those inoculated at a dose of 10^{-2} PFU ml^{-1} and controls.

Production of phage-neutralizing antibodies

No neutralizing antibodies against Phages PPpW-3 or PPpW-4 were detected in any serum sample obtained from fish inoculated orally or intramuscularly with phages (titer <1:2) throughout the experimental period.

Therapeutic effects of phages

Oral challenge

In the phage therapy experiments, in which *Plecoglossus altivelis* were challenged orally with *Pseudomonas plecoglossicida* and then received oral treatment with phages, the cumulative mortality of fish in the control group receiving phage-free feed was 93.3% after 2 wk. A significantly lower mortality was obtained in fish treated with PPpW-3 (53.3%), PPpW-4 (40.0%), and the phage mixture (20.0%) (Fig. 2). *P. plecoglossicida* was re-isolated from only 3 kidneys out of 58 survivors at 15 d p.i.

Water-borne challenge

Ayu fish injected intramuscularly with *Pseudomonas plecoglossicida* as donors (small tank) died 2 to 5 d after the injection. Significantly lower mortalities (26.7% in both trials) were produced in phage-treated groups, in contrast to those (90.0 and 100%) of phage-untreated controls (Fig. 3). *P. plecoglossicida* was re-isolated from only 2 kidneys out of 47 survivors at 15 d p.i.

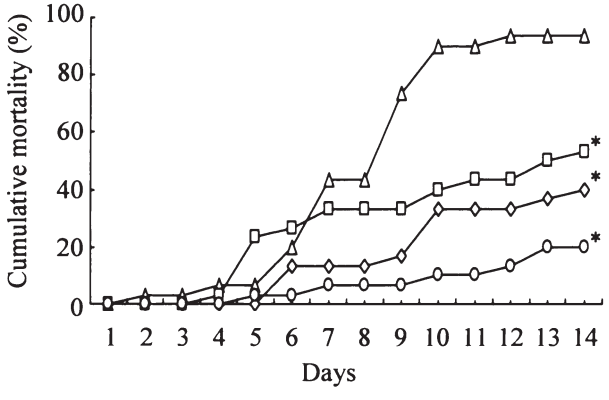

Fig. 2. *Plecoglossus altivelis.* Protective effects of phages against oral challenge with *Pseudomonas plecoglossicida* in ayu. Fish received feed loaded with Phages PPpW-3 (□), PPpW-4 (◇), a mixture of PPpW-3 and PPpW-4 (○), or phage-free feed (△) once immediately after oral administration of *P. plecoglossicida*-loaded feed. *$p < 0.01$

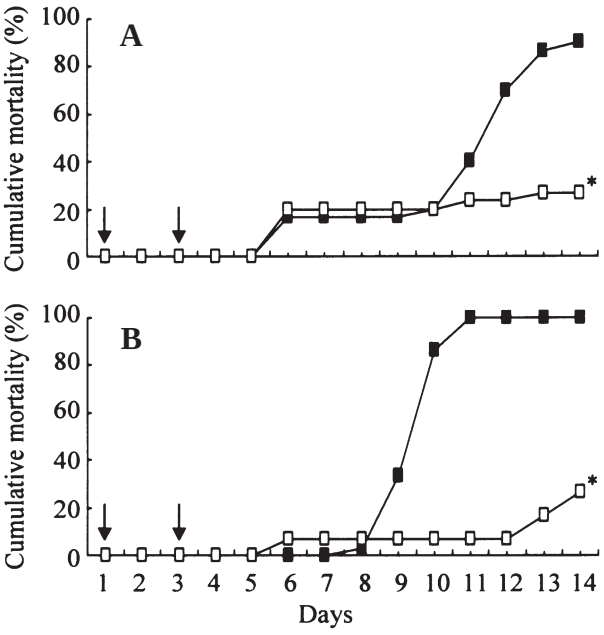

Fig. 3. *Plecoglossus altivelis.* Protective effects of phages against water-borne challenge with *Pseudomonas plecoglossicida* in ayu. Fish (n = 30) were exposed to water containing *P. plecoglossicida* shed from experimentally infected fish and received twice (arrows) feed loaded with a phage mixture of PPpW-3 and PPpW-4 (□) or phage-free feed (■). Two experiments were conducted separately (A, B). *$p < 0.01$

Natural challenge

The results of phage and bacterial isolation in the field trial of phage treatment using a commercial culture facility are shown in Table 2. For 2 wk prior to phage treatment, fish mortality in the pond was about 18 kg (ca. n = 900) d^{-1}.

When fish and rearing water were examined 1 and 2 d before phage administration, *Pseudomonas plecoglossicida* was isolated at high rates from both dead fish (82.5 and 97.5%) and apparently healthy fish (27.5 and 39.5%), and *P. plecoglossicida*-specific phages were not isolated from the kidney of the fish or the rearing water. In the phage-isolation trial performed on Day 0 to examine the intake rate of phages in fish, phages were detected in 90% (n = 40) of the kidneys at 3 h post-phage administration, while phages were detected in the intestines of all fish examined.

Phage detection rates in live (apparently healthy) and dead fish on Days 1 to 15 were 0 to 17.5% (average 7.1%, n = 240) and 7.5 to 92.5% (average 49.2%, n = 238), respectively. When phages were detected in

Table 2. Isolation of *Pseudomonas plecoglossicida* and phages from ayu *Plecoglossus altivelis* and rearing water during a field phage-therapy trial. First line: number of fish from which *P. plecoglossicida* or phages were isolated/fish examined; second line: concentration (range) of *P. plecoglossicida* (CFU g^{-1}) or phages (PFU g^{-1} or ml^{-1}); nt: not tested

Day	*P. plecoglossicida* isolation from kidneys of		Phage isolation from kidneys of		Water
	live fish	dead fish	live fish	dead fish	Phage
–2	11/40 (27.5%) 1.2×10^3 ~ > 1.5×10^4	33/40 (82.5%)	0/40 (0%)	0/40 (0%)	$<10^1$
–1	15/38 (39.5%) 7.3×10^2 ~ 1.1×10^4	39/40 (97.5%)	0/38 (0%)	0/40 (0%)	$<10^1$
0	6/40 (15.0%) 1.1×10^3 ~ > 1.5×10^4	nt	36/40 (90.0%) 5.0×10^1 ~ 4.1×10^3	nt	3.7×10^3
1	6/40 (15.0%) 4.0×10^2 ~ 5.5×10^3	29/40 (72.5%)	2/40(5.0%) 2.5×10^1 ~ 7.8×10^2	14/40 (35.0%) 5.0×10^1 ~ >1.0×10^4	5.3×10^2
2	4/40 (10.0%) 1.7×10^3 ~ >1.5×10^4	32/40 (80.0%)	7/40 (17.5%) 2.5×10^1 ~ 7.3×10^2	18/40 (45.0%) 2.5×10^1 ~ >1.0×10^4	1.2×10^3
4	7/40 (17.5%) 1.0×10^3 ~ >1.5×10^4	20/38 (52.6%)	0/40 (0%)	21/38 (55.3%) 2.5×10^1 ~ 5.6×10^3	$<10^1$
6	3/40 (7.5%) 1.6×10^3 ~ 4.5×10^3	30/40 (75.0%)	2/40 (5.0%) 5.0×10^1 ~ 7.5×10^1	37/40 (92.5%) 2.5×10^1 ~ >1.0×10^4	2.4×10^2
8	0/40 (0%)	34/40 (82.5%)	4/40 (10.0%) 1.2×10^3 ~ 8.0×10^3	25/40 (62.5%) 5.0×10^1 ~ >1.0×10^4	$<10^1$
15	0/40 (0%)	22/40 (55.0%)	2/40 (5.0%) 5.0×10^1 ~ 2.5×10^2	3/40 (7.5%) 2.5×10^1 ~ 5.0×10^1	2.0×10^1

live fish, the maximum concentration was 10^3 PFU g^{-1}, while that in dead fish was 10^4 PFU g^{-1} or higher. Phages were also isolated at a maximum 10^3 PFU ml^{-1} from rearing water after initiation of phage administration to fish. Detection rates of *Pseudomonas plecoglossicida* from live fish decreased gradually and became undetectable on Days 8 and 15. There were only 2 live fish in which the bacterium was detected at 10^4 CFU g^{-1} or higher (on Days 2 and 4). *P. plecoglossicida* was isolated from 52.5 to 82.5% of dead fish (average 70.2%, n = 238), indicating that factors other than *P. plecoglossicida* infection caused the mortalities. When 10 dead fish were examined quantitatively, the number of *P. plecoglossicida* in the kidneys ranged from 10^7 to 10^8 CFU g^{-1}. Out of 17 phage-carrying fish found on Days 1 to 15, only 2 had *P. plecoglossicida* in their kidneys.

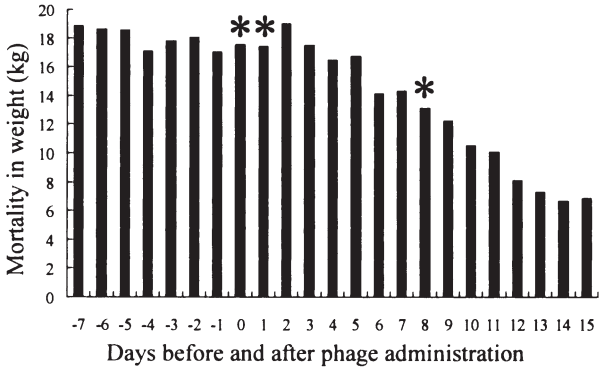

Fig. 4. *Plecoglossus altivelis*. Daily mortality of ayu after phage treatment against natural infection with *Pseudomonas plecoglossicida*. Fish in the *P. plecoglossicida* infection-prevailing culture pond received phage-impregnated feed on Days 0, 1, and 8 (*)

The daily mortality of fish decreased at a constant level (5.0%) from Days 3 to 15, thereafter reaching a steady state at about 6 kg mortality (ca. n = 300) d^{-1} (Fig. 4). A total of 90 *Pseudomonas plecoglossicida* isolates from dead fish on Days –2, –1, 0, 1, 2, 4, 6, 8 and 15 were all sensitive to both PPpW-3 and PPpW-4 phages. Serum samples (n = 60) obtained from live fish on Days –1, 8, and 15 were all negative for phage-neutralizing antibodies (titer <1:2), while agglutinating antibodies against *P. plecoglossicida* (PTH-9802) were detected in all serum samples (titers ranging from 1:2 to 1:16; Table 3).

DISCUSSION

Previously we reported that oral administration of phages (a mixture of PPpW-3 and PPpW-4) was effective in treating experimental infection of *Pseudomonas plecoglossicida* in ayu (Park et al. 2000), but it remains unclear as to which phage effectively contributed to this treatment, and as to the therapeutic effect of phages in natural infections. The present *in vitro* study proved that PPpW-4 inhibited the growth of *P. pleco-*

Table 3. Detection of phage-neutralizing and *Pseudomonas plecoglossicida*-agglutinating antibodies in ayu serum collected during a field phage therapy. Values are number of samples with antibodies

Day	Antibody titer against phages (PPpW-3/PPpW-4)			Antibody titer against *P. plecoglossicida* (PTH-9802)				
	<1:2	1:2	1:4	1:2	1:4	1:8	1:16	1:32
–1	20	0	0	11	6	3	0	0
8	20	0	0	0	7	12	1	0
15	20	0	0	0	2	15	3	0

glossicida more effectively than PPpW-3 (Fig. 1), and a mixture of the 2 phages induced the highest inhibitory activity. This higher effectiveness of PPpW-4 is partly explained by the fact that PPpW-4 was able to inhibit the growth of *P. plecoglossicida* cells resistant to PPpW-3, indicating that PPpW-4 has a wider host spectrum. Smith & Huggins (1983) indicated that phages with high activity *in vitro* are more active *in vivo*. In the present study, the protective effect of PPpW-4 against oral challenge was slightly higher than that of PPpW-3 (Fig. 2), and the highest protective effect against infection was obtained by administration of the mixed phages. The high protective effect of oral administration of phages (mixture of PPpW-3 and PPpW-4) was also demonstrated against water-borne challenge by bacteria shed from infected fish (Fig. 3).

In order to simulate phage therapeutic activity in natural infections, the effects of introducing phages into a diseased ayu culture pond was investigated, in which anti-*Pseudomonas plecoglossicida* phages were below detectable levels before the experiment. Phage administration lowered mortality to one-third during the 2 wk period, and approximately 30% of these mortalities were probably due to *Flavobacterium psychrophilum* infection, as indicated by the characteristic skin ulceration around the lower jaws. For technical reasons, this field trial did not have a control pond which lacked phage treatment. However, the sequence of phage treatment is indicated by the following results. Phages were isolated at a high rate (90%) from the kidneys soon (3 h) after phage administration, and were detected in live fish for a fairly long period (15 d) at an incidence of 7.1% (Table 2), while *P. plecoglossicida* disappeared from live fish by Day 8. The constant decrease (5.0%) in daily mortality from Days 3 to 15 (Fig. 4) was roughly parallel to the estimated phage-carrying rate (7.1%) of live fish mentioned above, indicating that the increased daily survival was phage-related. In our previous study (Park et al. 2000), orally administered anti-*P. plecoglossicida* phages disappeared from the kidneys of uninfected ayu within 12 h. The presence of phages at any given time in the internal organs results from *in vivo* phage-induced bacterial lysis because of the rapid elimination of phages from intact individuals (Geier et al. 1973, Merril et al. 1996). Therefore, the present long-lasting detection of phages in the fish and rearing water indicates continuous multiplication of phages in fish infected with *P. plecoglossicida*. Furthermore, although we had to use a drug (sulfisozole) to prevent mortality arising from *F. psychrophilum* infection in the present study, the fact that use of an antimicrobial agent invariably induces severe *P. plecoglossicida* infection suggests that the phage contributed to the marked reduction in fish mortality through *P. plecoglossicida* infection. Therefore, the current chemotherapy for *F. psychrophilum* in ayu culture is invaluable only when phage therapy for *P. plecoglossicida* is practised simultaneously. Phage therapy of *F. psychrophilum* infection may be an alternative solution.

Phages which do not encounter host cells are fated to disappear quickly from fish tissue (Nakai et al. 1999, Park et al. 2000). Therefore, fish from which phages were detected were those in which phages attacked host bacterial cells proliferating in the fish organs, and thus produced detectable levels of progeny. The high prevalence of phages in dead fish suggests that the phages provide little protection against naturally occurring infections when the fish are already in various stages of the disease process. Given that phages appeared in the fish prior to infection or at the terminal stage of infection, they may offer only limited therapeutic value. Based on the results that the bacterial concentration in live fish was 10^3 CFU g^{-1} or lower with a few exceptions and that phages were detected in the dead fish at high frequency (Table 2), it is possible that phages only offer protection at an early stage of infection. Higher therapeutic effects of phages found in experimental infections (compared with that in natural infections) possibly resulted from experimentally synchronized inoculation of bacteria and phages. Despite the self-perpetuating nature of phages in the presence of susceptible bacteria, multiple administrations of phages may be required to reduce bacterial populations in fish and water to safe levels during an outbreak of disease. Use of long-term *in vivo* survival phage mutants, which may be obtained by successive *in vivo* passages (Merril et al. 1996), may enhance phage duration in a fish population.

Previous research has noted a number of intrinsic obstacles in phage treatment (Lederberg 1996, Barrow & Soothill 1997, Alisky et al. 1998, Park et al. 2000, Sulakvelidze et al. 2001): the narrow host-specificity of phages, which causes complexity in preparation of

therapeutic phage strains; the quick appearance of phage-resistant organisms, as during chemotherapy; and the production of phage-neutralizing antibodies in fish, leading to ineffectiveness of repeated phage therapy. However, these were not real obstacles to phage therapy in the present study on *Pseudomonas plecoglossicida* infection for the following reasons: (1) *P. plecoglossicida* is composed of only a single phage type (Park et al. 2000); (2) phage-resistant *P. plecoglossicida* isolates induced in *in vitro* culture lack virulence to ayu (Park et al. 2000); (3) all *P. plecoglossicida* isolates from dead fish obtained in the present therapy experiments were still susceptible to phages used for treatment; (4) no neutralizing antibodies were detected in ayu that repeatedly received phage-impregnated feed or intramuscular injections of phages, or in fish in the field phage treatment. However, it is unclear if this arose from low immunogenicity of the phages to ayu, or was due to the low doses inoculated into fish (successive 7 d oral administration with 7.6×10^7 PFU fish^{-1} or 4 weekly IM-injections with 1.7×10^8 PFU fish^{-1}). Considering that the maximum phage concentration in the kidneys of apparently healthy fish was about 10^4 PFU g^{-1}, such low numbers of phages in the fish would be less immunogenic, indicating the possibility of repeated phage therapy.

Successful phage treatments by oral administration, particularly using an automatic feeding machine, may be of practical value as a route for therapeutic administration of phages to a large number of fish in aquaculture. In addition to successful phage therapy in *Pseudomonas plecoglossicida* infection of ayu and *Lactococcus garvieae* infection of the yellowtail *Seriola quinqueradiata* (Nakai et al. 1999), our preliminary study suggests that phage treatment is useful in controlling *Vibrio splendidus* infection (Sugumar et al. 1998) in cultured larvae of the Pacific oyster *Crassostrea gigas*. Thus, phage therapy may have many applications in the aquaculture field.

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LITERATURE CITED

Alisky J, Iczkowski K, Rapoport A, Troitsky N (1998) Bacteriophages show promise as antimicrobial agents. J Infect 36:5–15

Barrow PA, Soothill JS (1997) Bacteriophage therapy and prophylaxis; rediscovery and renewed assessment of potential. Trends Microbiol 5:268–271

Barrow P, Lovell M, Berchieri A Jr (1998) Use of lytic bacteriophage for control of experimental *Escherichia coli* septicemia and meningitis in chickens and calves. Clin Diagn Lab Immunol 5:294–298

Biswas B, Adhya S, Washart P, Paul B, Trostel AN, Powell B, Carlton R, Merril CR (2002) Bacteriophage therapy rescues mice bacteremic from a clinical isolate of vancomycin-resistant *Enterococcus faecium*. Infect Immun 70:204–210

Carlton RM (1999) Phage therapy: past history and future prospects. Arch Immunol Ther Exp 47:267–274

Geier MR, Trigg ME, Merril CR (1973) Fate of bacteriophage lambda in non-immune germ-free mice. Nature 246: 221–223

Iida Y, Mizokami A (1996) Outbreaks of coldwater disease in wild ayu and pale chub. Fish Pathol 31:157–164

Lederberg J (1996) Smaller fleas … ad infinitum: therapeutic bacteriophage redux. Proc Natl Acad Sci USA 93: 3176–3168

Merril CR, Biswas B, Carlton R, Jensen NC, Creed GJ, Zullo S, Adhya S (1996) Long-circulating bacteriophage as antibacterial agents. Proc Natl Acad Sci USA 93:3188–3192

Nakai T, Sugimoto R, Park KH, Matsuoka S, Mori K, Nishioka T, Maruyama K (1999) Protective effects of bacteriophage on experimental *Lactococcus garvieae* infection in yellowtail. Dis Aquat Org 37:33–41

Nakatsugawa T, Iida Y (1996) *Pseudomonas* sp. isolated from diseased ayu, *Plecoglossus altivelis*. Fish Pathol 31: 221–227 (in Japanese)

Nishimori E, Kita-Tsukamoto K, Wakabayashi H (2000) *Pseudomonas plecoglossicida* sp. nov., the causative agent of bacterial hemorrhagic ascites of ayu, *Plecoglossus altivelis*. Int J Syst Evol Microbiol 50:83–89

Park SC, Shimamura I, Fukunaga M, Mori K, Nakai T (2000) Isolation of bacteriophages specific to fish pathogen, *Pseudomonas plecoglossicida*, as a candidate for disease control. Appl Environ Microbiol 66:1416–1422

Reid G (1999) Testing the efficacy of probiotics. In: Tannock GW (ed) Probiotics, a critical review. Horizon Scientific Press, Wymondham, p 129–140

Slopek S, Weber-Dabrowska B, Dabrowski M, Kucharewicz-Krukowska A (1987) Results of bacteriophage treatment of suppurative bacterial infections in the years 1981–1986. Arch Immunol Ther Exp 35:569–583

Smith HW, Huggins MB (1982) Successful treatment of experimental *Escherichia coli* infections in mice using phage; its general superiority over antibiotics. J Gen Microbiol 128: 307–318

Smith HW, Huggins MB (1983) Effectiveness of phages in treating experimental *Escherichia coli* diarrhoea in calves by means of bacteriophages. J Gen Microbiol 129: 2659–2675

Soothill JS (1994) Bacteriophages prevents destruction of skin grafts by *Pseudomonas aeruginosa*. Burns 20:209–211

Sugumar G, Nakai T, Hirata Y, Matsubara D, Muroga K (1998) *Vibrio splendidus* biovar II as the causative agent of bacillary necrosis of Japanese oyster *Crassostrea gigas* larvae. Dis Aquat Org 33:111–118

Sulakvelidze A, Alavidze Z, Morris JG Jr (2001) Bacteriophage therapy. Antimicrob Agents Chemother 45:649–659

Summers WC (2001) Bacteriophage therapy. Annu Rev Microbiol 55:437–451

Wakabayashi H, Toyama T, Iida T (1994) A study on serotyping of *Cytophaga psychrophila* isolated from fishes in Japan. Fish Pathol 29:101–104

Wakabayashi H, Sawada K, Ninomiya K, Nishimori E (1996) Bacterial hemorrhagic ascites of ayu caused by *Pseudomonas* sp. Fish Pathol 31:239–240 (in Japanese)

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