Bacteriophage therapy of infectious diseases in aquaculture

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Abstract

Bacteriophages may be candidates as therapeutic agents in bacterial infections. Here we describe the protective effects of phages against experimentally induced bacterial infections of cultured fish and discuss the potential for phage therapy in aquaculture. © 2002 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

Keywords: Bacteriophage; Phage therapy; Fish disease; Lactococcus garvieae; Pseudomonas plecoglossicida

1. Introduction

Two decades have elapsed since bacteriophages (phages) were reassessed scientifically as a therapeutic and prophylactic agent for bacterial infections. In the 1980s, following early enthusiastic but uncontrolled studies on the application of phages to prevention and treatment of human bacterial infections [1–3,12], epoch-making studies were carried out by Smith and colleagues [25–28]. They indicated, using Escherichia coli models with mice and farm animals, that phages could be used for both treatment and prophylaxis against bacterial infections. Independently of these studies, a series of successful clinical usages of phages for drug-resistant suppurative infections in humans were described by Polish and Soviet groups [1,24]. Thereafter, many successful results on phage therapies have been reported using various animal models [2,4,13,23,29–31]. Potential advantages of phage treatment over chemotherapy are: 1) the narrow host range of phages, indicating that the phages do not harm the normal intestinal microflora; and 2) the self-perpetuating nature of phages in the presence of susceptible bacteria, indicating the superfluousness of multiple administrations [3,25]. The latter lead to autonomous transfer of the administered phages between animals in a yard [4,28].

Cultured fish and shellfish, like other animals and humans, are constantly threatened by microbial attacks. Although chemotherapy is a rapid and effective method to treat or prevent bacterial infections, frequent use of chemotherapeutic agents has allowed drug-resistant strains of bacteria to develop. In particular, this problem in chemotherapy may be serious in Japan where 25 drugs are now licensed for fisheries use [10]. Needless to say, vaccination is an ideal method for preventing infectious diseases, but commercially available vaccines are still very limited in the aquaculture field. This is partly due to the fact that many different kinds of infectious diseases occur locally in a variety of fish and shellfish species. Studies on biological control such as probiotics have been sporadically reported in the field of fish pathology [6,18,34]; however, they involve substantial difficulties in scientific demonstration of the causal sequence, as mentioned in human use of probiotics [33]. In view of a scientific demonstration of phage treatment, the causal effect of phages in successful phage therapy can be definitively proven by confirming an increase in phage particles in the number or the presence of phages in the survivors, which is the result of the death of host bacterial cells. The feasibility of this demonstration distinguishes phage treatment from other biological controls, which fail to utilize scientific methodology in demonstrating causal relationships. Under these circumstances, phages, as specific pathogen killers, could be attractive agents for controlling fish bacterial infections. Phages of some fish pathogenic bacteria, such as Aeromonas salmonicida, A. hydrophila, Edwardsiella tarda and Yersinia ruckeri, have been reported. However, no studies on phages have been made with a view toward preventing bacterial infections in fish until our recent works [15,21].

In this paper, we briefly review our studies on phage effects against experimentally induced bacterial infections of cultured fish, focusing on Lactococcus garvieae infec-
tion of yellowtail Selenia quinqueradiata and Pseudomonas plecoglossicida infection of ayu Plecoglossus altivelis, and we discuss the potential for controlling bacterial infections in aquaculture by means of phages.

2. Phage therapy of Lactococcus garvieae infection

2.1. L. garvieae infection

The disease caused by L. garvieae, formerly Enterococcus seriolicida [11], has been responsible for the most serious economic damage to the yellowtail aquaculture industry in Japan since its first outbreak in 1974, mainly due to frequent occurrences in marketable-sized fish [14]. It is believed that L. garvieae is a typical opportunistic pathogen because the bacterium is ubiquitous in fish and their culture environments. Therefore, reducing stress factors such as poor water quality, overcrowding, overfeeding, and insufficient nutrition is generally important in controlling the disease. However, the difficulty in putting these methods into practice still results in heavy dependence on chemotherapeutics.

2.2. L. garvieae phages

Phages specific to L. garvieae, designated as PLgY and PLgW, were isolated from diseased fish and sea water in fish culture cages, and the phage was identified as a member of the family Siphoviridae based on morphological and genomic features [19,20]. One-hundred-eleven clinical and environmental strains of L. garvieae were divided into 14 phage types (A to N), with the major phage type A, which contains 66% of strains examined; however, 90% or more strains of L. garvieae were sensitive to phage isolates such as PLgW-1 and PLgW-3. This uniformity of L. garvieae in phage sensitivity will be advantageous in phage treatment. The phages appeared extracellularly from infected L. garvieae cells after a latent period of 1 h, and then progeny increased until reaching the maximum number of 10^{10} PFU mL^{-1} after 5 h. L. garvieae grows well at 17 to 41°C, but lytic activity of the phage is observed at 29°C or lower.

Anti-L. garvieae phages survived in unsterilized natural seawater for at least 3 days and persisted well at various physicochemical (temperature: 5 to 37°C; salinity: distilled water to double-strength seawater; pH: 3.5 to 11.0) and biological conditions (feed, serum and alimentary tract extracts of yellowtail), except for acidity lower than pH 3.0 [15,19]. It seems that resistance to such low acidity is not a requisite for in vivo survival of phage, since the pH levels of digestive tracts of cultured yellowtails were higher than pH 3.4 even after feeding. This stability of phages with respect to environmental factors is of practical value for phage treatment. In vivo, the phage (PLgY-16) was detected in the spleens of yellowtails up to 24 h after intraperitoneal (i.p.) injection, and the phage was recovered from the intestine of yellowtails 3 h after the oral administration of phage-impregnated feed, but was undetectable 10 h later. Simultaneous administration of live L. garvieae and phage enhanced the survival time of the phage; the phage was recovered from the spleen 5 days after i.p. injection and from the intestine 24 h after oral administration [15]. The relatively long-term in vivo survival of phage is enough for the phage to encounter the host bacterium in infected fish.

2.3. Phage therapy

Protective effects of anti-L. garvieae phage were examined by i.p. or oral administration of phage against experimentally infected young yellowtails [15]. After i.p. challenge with L. garvieae, the survival rate (100%, n = 20) of fish receiving i.p. injection of the phage was much higher than that (10%, n = 20) of the control fish without phage injection. When fish were i.p.-injected with phages at different hours after L. garvieae challenge, a significantly higher protective effect (p < 0.01 or < 0.001 in a chi-square test) was demonstrated even in fish that received phage treatment 24 h later (Table 1). In other fish groups, to facilitate phage introduction into the fish organs, phage-infected bacterial cells as a source of phage were injected into fish after bacterial challenge. Interestingly, this use of bacterial cells as a protector or vehicle did not influence the curative effect of phage (Table 1).

Table 1 Phage treatment of yellowtails infected with L. garvieae

<table>
<thead>
<tr>
<th>Administration of</th>
<th>Time after L. garvieae infection when phage given</th>
<th>No. of fish: died/examined</th>
<th>Mortality (%)</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phage only</td>
<td>0 h</td>
<td>0/20</td>
<td>0</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>1 h</td>
<td>4/20</td>
<td>20</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>10/20</td>
<td>50</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>18/20</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Phage-infected</td>
<td>1 h</td>
<td>1/20</td>
<td>5</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>L. garvieae</td>
<td>24 h</td>
<td>11/20</td>
<td>55</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>20/20</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

a Reproduced from [15].

b Fish were i.p. injected with the phage (PLgY-16), immediately (0 h) or 1 h or 24 h after the L. garvieae challenge.

c Fish were i.p. injected with previously phage (PLgY-16)-infected L. garvieae as the source of phage, 1 h or 24 h after the L. garvieae challenge.
Protection was also obtained in yellowtails receiving phage-impregnated feed, and fish were challenged with an anal intubation with L. garvieae. Anal-intubated L. garvieae were detected constantly in the spleens of the control fish for 72 h or longer, while they were detected sporadically and disappeared from the phage-treated fish 48 h later. On the other hand, orally administered phages were detected in the intestines and spleens of the phage-treated fish 3 to 48 h later, with a maximum of 10^6 PFU g^{-1}. Phage-resistant mutants are fairly common in in vitro L. garvieae cultures, but all L. garvieae isolates from dead fish obtained during the in vivo experiments were still susceptible to the phage used. No neutralizing antibodies were detected in the sera of yellowtails that repeatedly received phage-impregnated feed.

3. Phage therapy of Pseudomonas plecoglossicida infection

3.1. P. plecoglossicida infection

Ayu is the most popular freshwater fish for culture and sports fishing in Japan. Bacterial hemorrhagic ascites caused by P. plecoglossicida [17] has been one of the most devastating diseases in the ayu culture industry in Japan since the early 1990s. The disease occurs in fish at any developmental stage throughout the culture period. Some antimicrobial agents, such as florfenicol and sulfisoxazole, are used to treat coldwater disease caused by Flavobacterium psychrophilum [32], another serious disease for cultured ayu. After such treatment, particularly when it is coupled with overfeeding, P. plecoglossicida infection abruptly emerges and results in heavy mortality. This is a typical example of microorganism substitution in fish disease. Thus, the causative P. plecoglossicida was believed to be an opportunistic pathogen, though an infection experiment by intramuscular injection revealed that the bacterium is highly virulent to ayu with a LD50 of 10^{1.2} CFU fish^{-1}. P. plecoglossicida survives and proliferates well in ayu-rearing freshwater, indicating that the bacterium may be ubiquitous in ayu culture environments and will cause rapid horizontal transmission of the disease, though the precise infection mechanisms of the disease remain unsolved. At present, there are no licensed chemotherapeutics effective against the disease, and no procedures to control the disease other than reducing predisposing factors such as overcrowding and overfeeding.

3.2. P. plecoglossicida and phages

P. plecoglossicida strains are homogeneous with respect to biochemical characteristics, and all isolates obtained from geographically and chronologically different sources are members of a single serotype and a single phage type [16,21,35]. However, in two previous papers the authors described conflicting results for motility of the bacterium and the presence of bloody ascites in affected fish; both of these characteristics were positive in one study [35], and both were negative in the other study [16]. The relationship between the motility of the bacterium and different clinical conditions (bloody ascites) in affected fish remains unclear. Both motile and nonmotile strains are equally virulent to ayu.

Two types of bacteriophage specific to P. plecoglossicida were isolated from diseased ayu and the rearing pond water. One type of phage (PPpW-3), forming small plaques, was tentatively classified as Myoviridae, and another type (PPpW-4), forming large plaques, was classified as Podoviridae. All examined P. plecoglossicida strains, either motile or nonmotile, which were isolated from diseased ayu of geographically different areas from 1991 to 1999, exhibited quite similar sensitivity to phages of either type [21]. In in vitro conditions, PPpW-4 inhibited the growth of P. plecoglossicida more effectively than PPpW-3, but the mixture of two phages exhibited the highest inhibition. The lytic activities of phages were observed at temperatures from 10 to 30°C or less, which covers the entire range of rearing water temperature in ayu culture. Interestingly, phage-sensitive strains of P. plecoglossicida were highly virulent to ayu, while phage-resistant variants of the strain were less virulent (LD50: higher than 10^8 CFU fish^{-1}). Ultraviolet irradiation or mytomycin C induced no temperate phages from any of the strains examined.

3.3. Phage therapy

Oral administration of phage-impregnated feed to ayu increased resistance to experimental infection with P. plecoglossicida [21]. In the first trial, fish were orally challenged with live P. plecoglossicida-loaded feed and immediately received phage (PPpW-3/PPpW-4 mixture)-impregnated feed. Mortality in the control fish groups receiving feed without phage was initiated at 7 d after the bacterial challenge, and the cumulative mortality in 2 weeks was 65.0%, while fish receiving phage-impregnated feed immediately after bacterial challenge survived to live longer, and there was only 22.5% cumulative mortality (Table 2). Such protective effects of phage treatment, significantly (p < 0.001) decreasing mortalities, were demonstrated in fish receiving phage 1 h or 24 h after bacterial challenge. Inoculated P. plecoglossicida was isolated from all the kidneys of dead fish irrespective of phage treatment and from some survivors in control groups, but not from any of the fish that had received phages and survived. In addition, the bacteria isolated from fish that had received phage treatment and died were still susceptible to both phages used. Although PPpW-4 produced higher protection than did PPpW-3 in the second trial with single use of each phage, the mixture of both phages exhibited the highest protective effect (Table 2). When phage therapy was evaluated under cohabitation conditions with fish which had been previously infected with P. plecoglossicida, phage (PPpW-3/PPpW-4)-receiving fish showed sig-
Fish were fed *P. plecoglossicida* feed. Fish were challenged by cohabitation with previously infected fish with intramuscular-injection of *P. plecoglossicida* phage-impregnated feed or phage-free feed (control).

**Table 3**

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Phage used</th>
<th>Time after <em>P. plecoglossicida</em> infection when phage given</th>
<th>No. of fish: died/examined</th>
<th>Mortality (%)</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1$^b$</td>
<td>PPpW-3 + PPpW-4</td>
<td>0 h</td>
<td>8/40</td>
<td>22.5</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>0 h</td>
<td>26/40</td>
<td>65.0</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td></td>
<td>PPpW-3 + PPpW-4</td>
<td>1 h</td>
<td>0/50</td>
<td>0.0</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>1 h</td>
<td>39/50</td>
<td>78.0</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td></td>
<td>PPpW-3 + PPpW-4</td>
<td>24 h</td>
<td>5/40</td>
<td>12.5</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>24 h</td>
<td>32/40</td>
<td>80.0</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td>2$^b$</td>
<td>PPpW-3</td>
<td>0 h</td>
<td>16/30</td>
<td>53.3</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td></td>
<td>PPpW-4</td>
<td>24 h</td>
<td>12/30</td>
<td>40.4</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>0 h</td>
<td>6/30</td>
<td>20.2</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td></td>
<td>PPpW-3 + PPpW-4</td>
<td>24 h</td>
<td>28/30</td>
<td>93.3</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td>3$^c$</td>
<td>PPpW-3 + PPpW-4</td>
<td>24 h &amp; 72 h</td>
<td>8/30</td>
<td>26.7</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>24 h &amp; 72 h</td>
<td>30/30</td>
<td>100</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td>4$^c$</td>
<td>PPpW-3 + PPpW-4</td>
<td>24 h &amp; 72 h</td>
<td>8/30</td>
<td>26.7</td>
<td>$p &lt; 0.001$</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>24 h &amp; 72 h</td>
<td>27/30</td>
<td>90.0</td>
<td>$p &lt; 0.001$</td>
</tr>
</tbody>
</table>

$^a$ Reproduced from [21].

$^b$ Fish were challenged by oral administration of *P. plecoglossicida*-impregnated feed and immediately (0 h) or 1 h or 24 h later received phage-impregnated feed.

$^c$ Fish were challenged by cohabitation with previously infected fish with intramuscular-injection of *P. plecoglossicida*, and 24 h and 72 h later fish received phage-impregnated feed or phage-free feed (control).

Table 3

<table>
<thead>
<tr>
<th>Time after inoculation (h)</th>
<th><em>P. plecoglossicida</em> ($\log_{10}$ CFU g$^{-1}$) in fish fed:</th>
<th>Phage count ($\log_{10}$ PFU g$^{-1}$) in fish fed:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>bacteria alone</td>
<td>bacteria and phage</td>
</tr>
<tr>
<td>0</td>
<td>$&lt; 2$</td>
<td>$&lt; 2$</td>
</tr>
<tr>
<td>3</td>
<td>3.5</td>
<td>4.8</td>
</tr>
<tr>
<td>12</td>
<td>4.7</td>
<td>$&lt; 2$</td>
</tr>
<tr>
<td>24</td>
<td>5.1</td>
<td>$&lt; 2$</td>
</tr>
<tr>
<td>48</td>
<td>4.6</td>
<td>$&lt; 2$</td>
</tr>
<tr>
<td>72</td>
<td>4.2</td>
<td>$&lt; 2$</td>
</tr>
</tbody>
</table>

$^a$ Modified from [21].

Fish were fed *P. plecoglossicida*-impregnated feed, feed impregnated with phages (PPpW-3 + PPpW-4), or phage-impregnated feed after bacterial challenge.

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**4. Potential for phage control in aquaculture**

As stated above, rediscovery of phage therapy began with Smith and his colleagues’ works using various animal models of cattle and human diseases. Our phage therapy studies for aquatic animals are fairly well under way but its efficacy has been demonstrated against experimentally induced infections [15,21]. In our very recent field trials of phage therapy, we succeeded in markedly reducing the mortality of ayu due to *P. plecoglossus* infection. When phage-impregnated feed was administered to ayu in a diseased pond (200 m$^3$, fish no. = 120,000), daily mortality decreased at a constant level (5% a day) and phage therapy lowered mortality to one-third during the two-week period. Results obtained so far indicate the potential for phage control of bacterial diseases in aquaculture. In particular, successful phage treatments by oral administration, as demonstrated in both fish models, are

significantly lower ($p < 0.001$) mortality than untreated control fish (Table 2).

*P. plecoglossicida* in fish receiving bacteria-loaded feed first appeared in the kidney 3 h after feeding, and then were detected at levels of $10^6.3$ and $10^3.9$ CFU g$^{-1}$ from all kidneys examined 72 h or later. In fish receiving phage-loaded feed, inoculated phage emerged at concentrations of $10^{5.2}$ and $10^{3.5}$ PFU g$^{-1}$ in kidneys 3 and 12 h later respectively, but disappeared 24 h later. On the other hand, when fish received phage-impregnated feed after bacterial challenge, *P. plecoglossicida* were not detected in kidneys of fish at 12 h or later, after a slight appearance at 3 h postchallenge, and phage was detected from the kidneys at 3, 12 and 24 h postchallenge (Table 3). These growth dynamics of administered bacteria and phages in fish explain that phage killing of bacteria in the internal organ caused survival of fish.
of practical value as a route for therapeutic administration of phages to a large number of fish. Acid sensitivity of phages is not a determinant for phage treatment due to relatively high pH levels in fish digestive tracts as shown in yellowtail. Such a high pH of the stomach and abomasum contents was also noticed in very young animals, and the abomasum at this pH level has little harmful effect on orally administered phages [28]. This route of phage administration will be directly advantageous for cases in which the oral route is the major route for pathogen transmission, as are L. garvieae infection of yellowtail and P. plecoglossicida infection of ayu. Interestingly, orally administered phages quickly appeared in the kidneys of fish, without host bacterial cells as a transport vehicle. Easy movement of phage from the alimentary tract to the blood circulation system was also observed in humans and rats [8,24]. It has already been noted that phages are not as rapidly inactivated in animal tissue fluids and blood as previously thought [2,25]. A technical problem which remains is how to select or find the most aggressive strain to enhance the therapeutic effect. Phages with high activity in vitro are more active in vivo [25], as was our P. plecoglossicida phage. Successful in vivo passages will be an alternate method to obtain long-term in vivo survival phage mutants [13].

There is also an indication that phages can invade the fish body through skin and/or gills, since phages are easily demonstrable in the kidney after dipping fish in phage solution. Bath administration of phages will be effective for those in which infection is initiated by bacterial colonization on the skin and gills. Cold water disease caused by F. psychrophilum is the typical example of this infection type. Yet there are no reports on phages of this world-important pathogen. In addition to these diseases, other bacterial infections of fish will be suitable for phage therapy. Furthermore, our preliminary studies suggest that phage treatment is useful for controlling Vibrio infection of Pacific oyster Crassostrea gigas larvae. Thus, phage therapy may have many applications in the aquaculture field.

Although many previous reviews have pointed out and addressed a number of intrinsic obstacles to phage treatment and prevention for terrestrial animals [1,3,12], there still remain some issues to be addressed for phage application in aquaculture. The supposed obstacles are as follows:

First, the narrow host specificity of phages is a disadvantage for phage therapy. It is strain-specific rather than species-specific, which leads to difficulty when preparing phages of highly diverse bacterial variants. Our aquatic animal models, however, do not indicate that this is always an essential weak point of phage therapy. There may exist a major phage type in L. garvieae. Some phage isolates are so broad in their infectivity that they can be lytic for 90% or more strains of the organism [19]. For other bacteria such as P. plecoglossicida, only a single phage type is known [21]. A specific cell surface substance as a virulence factor, such as the capsule of E. coli or of L. garvieae, may determine this low diversity of the organism [2]. Such a surface substance, however, has not been identified in P. plecoglossicida.

Second, rapid appearance of phage-resistant bacteria is a problem for treatment, as in chemotherapy. Phage-resistant mutants are fairly common in L. garvieae and P. plecoglossicida cultures. However, all L. garvieae and P. plecoglossicida isolates from dead fish obtained during therapy experiments were still susceptible to phages used for treatment. Furthermore, phage-resistant variants of P. plecoglossicida, which were induced in vitro, lacked virulence for ayu [21]. In successful phage control against a systemic infection of mice with E. coli (O18:K1:H7 ColV+) or diarrhea in calves by enteropathogenic E. coli (O9:K30,99 and O20:K101,987P), only the less virulent K− types emerged as phage-resistant organisms [25,26]. A surface component associated with bacterial virulence also seemed to be the receptor for phage attachment, and consequently phage-resistant variants of a virulent organism would not be pathogenic as stated in previous papers [2,3,21].

Third, phage-neutralizing antibodies produced as the result of phage administration, either by the oral or parenteral route, will be an obstacle for phage therapy against recurrent infections. Enteropathogenic E. coli phage-neutralizing antibodies were found in the sera of human beings, cattle and pigs, and in bovine colostrum. The neutralizing antibodies were induced by orally administered phage or its parenteral inoculation at much higher levels of antibody [28]. However, no such neutralizing antibodies were detected from yellowtail and ayu that repeatedly (successive seven days) received phage-impregnated feed (about 108 PFU fish−1) or even from ayu after receiving intramuscular injections (4 times, weekly) of phage solution at a dosage of 106 PFU fish−1. Conversely, this low immunogenicity of phage to fish might provide an advantage for phage therapy in fish.

Lastly, the risk that phages might mediate genetic exchange among bacteria, i.e. transduction or phage conversion, may be the final problem raised. It is well known that some temperate phages contribute to bacterial virulence. Temperate phages with broad infectivity over species would strongly support antiphage therapy views [9]. However, this possibility is probably unlikely for our therapeutic phages because of their extremely narrow host specificity.

5. A hypothetical role of phages in nature

The high abundance of viruses in aquatic environments indicates that phage infection is an important factor in the ecological control of planktonic microorganisms [5,22] and many other biogeochemical processes [7]. Through phage therapy studies using fish models, however, we postulate that naturally occurring phages might contribute not only to the killing of such planktonic bacteria but also to the killing of pathogens proliferating in the internal body of fish where there exists a range of defense factors against invading microorganisms. This idea was derived from the follow-
ing facts: 1) lytic phages are frequently isolated from rear-
ing water where a specific bacterial infection prevails among
fish; 2) phages appear in the internal organs (e.g. kidney) im-
mediately after oral or water-borne administration of phages
to fish, and can survive for a relatively long time in situ; and
3) phage-neutralizing antibodies are not easily produced by
fish under either experimental or natural conditions. In or-
der to demonstrate this hypothesis, we examined live fish
in ayu culture ponds in which P. plecoglossicida infection
prevailed. P. plecoglossicida phages at high concentrations
(maximum: 10^3 PFU g⁻¹) were detected in the kidneys of
apparently healthy fish at an incidence of 2.8% (n = 534).
There is no doubt that the presence of phages at high num-
bers in live fish results from in vivo bacterial killing of
phage. Therefore, naturally occurring phages must at least
partly contribute to the survival of fish after otherwise fatal
bacterial infection.

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