Antibacterial Efficacy of Phages against *Pseudomonas aeruginosa* Infections in Mice and *Drosophila melanogaster*  

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Phage therapy against *Pseudomonas aeruginosa* infections has received renewed attention owing to the increasing prevalence of antibiotic resistance in this bacterium. Here, we isolated and characterized two new potentially lytic bacteriophages (MPK1 and MPK6), which produced large and clear plaques on *P. aeruginosa* strain PAO1. Based on their morphology, MPK1 belongs to the *Myoviridae*, while MPK6 belongs to the *Podoviridae*. The group B polysaccharide of lipopolysaccharide was required for infection, suggesting that their host spectra are associated with the serotypes of *P. aeruginosa* strains. Intramuscular and intraperitoneal administration of MPK1 and, to a lesser extent, MPK6 significantly protected mice from mortality caused by PAO1-induced peritonitis-sepsis (*P < 0.01*). Mice treated with either phage also had lower bacterial burdens in their livers, lungs, and spleens. The antibacterial efficacy of MPK1 and MPK6 was also evaluated based on *Drosophila melanogaster* systemic infection caused by *P. aeruginosa*, for which phages were administered by feeding. Both phages significantly delayed the PAO1-induced killing of *D. melanogaster* (*P < 0.001*), although MPK1 persisted longer than MPK6 in uninfected *D. melanogaster* tissue samples. These results suggest that a mini-scale experiment using *D. melanogaster* infection is valid for evaluating the antibacterial efficacy of phage therapy against *P. aeruginosa* infections.

*Pseudomonas aeruginosa* is an opportunistic human pathogen that is ubiquitously found in various biotic and abiotic environments. It is frequently isolated from human patients afflicted with cystic fibrosis, otitis media, keratitis, and burn wound infections as an etiological agent of septicemia in immunocompromised individuals. This bacterium, generally from the environmental reservoir, can colonize large numbers of child patients before the age of 3 years (27) and adversely affect their pulmonary function (23). Furthermore, *P. aeruginosa* is also commonly found in peritonitis-sepsis cases secondary to ruptured appendices in otherwise healthy children (4). Peritonitis by *P. aeruginosa* is a serious threat also to the patients undergoing continuous ambulatory peritoneal dialysis (CAPD) (15), accounting for 10% of mortality cases associated with CAPD. The bacterial intoxication usually leads to high morbidity, CAPD failure, and late complications in those cases (14, 15, 17). Rodent models of *P. aeruginosa* peritonitis have been developed for understanding the pathophysiology implicated in peritonitis (32). The pathological consequences are generally bacteremia and infected livers, with interleukin-6 levels elevated within 6 h postinfection, and ultimately cause mortality within 48 h depending on *P. aeruginosa* strains and infection doses (5).

Although antibiotics are still widely used to control the bacterial infections, they are increasingly ineffective due to the emergence of antibiotic resistance. Selection and dissemination of intrinsic and acquired antibiotic resistance mechanisms increase the proclivity to resist the chemotherapy involving various antibiotics and promote the emergence of bacterial strains with multiple antibiotic resistances, which are associated with the mortality and morbidity in infected patients nowadays (22, 28). Hence, the development of new therapeutic and prophylactic strategies is compulsory for the control of bacterial infections.

An alternative and/or supplementary anti-infective modality for combating infections caused by antibiotic-resistant microorganisms which is currently being revisited in various countries is bacteriophages that are able to specifically target their host bacterial infections, and this modality is called phage therapy (29). Phage therapy is a method of harnessing phages as bioagents and was first introduced before the discovery of the first antibiotic, penicillin (30). Phages continue to be used in place of antibiotics for the treatment of bacterial infections in the former Soviet Union and Eastern Europe (25). Much more attention has recently been paid to phage therapy, as more and more bacteria have evolved antibiotic resistance. Thus, phage therapy may be a valuable alternative modality to antibiotics and has already been proven to be medically superior to antibiotics in certain cases (3, 19).

*P. aeruginosa* is a highly adaptable bacterium that could enhance its ecological fitness even in the presence of conventional antibiotic therapy. The rapid emergence of new *P. aeruginosa* strains as well as the persistence of the existing antibiotic-resistant clinical isolates has led to an urgent need to explore more sustainable alternative strategies such as phage therapy to manage *P. aeruginosa*-mediated infections. Recently, the therapeutic mixture of six lytic phages was shown to be effective in clinical ear infections caused by *P. aeruginosa* (26). The efficacy of phage therapy using a genetically modified filamentous phage (PESR) (10), lytic phage isolates, or phage cocktails has been also investigated against various experimen-
tal mouse infection models with _P. aeruginosa_ that include burn wound infection (20) and gut-derived sepsis (31). Because the pathophysiology caused by _P. aeruginosa_ infections is quite complicated, more and more relevant infection models need to be tested for the efficacy and relevancy of the antibacterial therapies.

In this study, we evaluated the therapeutic efficacy of newly isolated _Caudovirales_ phage strains, MPK1 and MPK6, against mouse peritonitis-sepsis caused by intraperitoneal (i.p.) infection with _P. aeruginosa_ strain PAO1 that may resemble the clinical pathophysiology of _P. aeruginosa_-induced peritonitis in humans. Based on the fact that _P. aeruginosa_ is able to infect hosts of multiple phylogenetic backgrounds, such as plants, worms, and insects, and the fact that a set of virulence factors identified from a _Drosophila melanogaster_ systemic infection-based screen are also required for mouse peritonitis (16), we here established a _D. melanogaster_ model to evaluate phage therapy and verified the antibacterial efficacy of both MPK1 and MPK6, using this nonmammalian infection model.

**MATERIALS AND METHODS**

- **Bacterial strains and culture conditions.** _Pseudomonas aeruginosa_ strain PAO1 was used as described elsewhere (11). The lipopolysaccharide (LPS) mutants (rmd, wbpM, and rmiC mutants) are gifts from Joe Lam (University of Guelph, Canada). Bacterial cells were grown in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, and 1% NaCl) with aeration or 2% Bacto agar (Difco) or cetrimide agar (Fluka) plates at 37°C.
- **Preparation of phage lysates.** Phage strains MPK1 and MPK6 are enriched by the plate lysate method, using _P. aeruginosa_ strain PAO1 as the host, as described elsewhere (12). The culture suspension was centrifuged at 8,000 × _g_ for 10 min at 4°C to remove the cell debris, and phage particles were precipitated from culture supernatant in the presence of 10% polyethylene glycol (average molecular weight, 8,000) and 1 M NaCl and then dissolved in 5 ml phage buffer (10 mM MgSO_4_, 10 mM Tris [pH 7.6], and 1 mM EDTA). Phage particles were concentrated by ultracentrifugation at 110,000 × _g_ for 3 h at 4°C and resuspended in phage buffer. The phage suspension was placed on top of a discontinuous CsCl gradient (1.45, 1.50, 1.70 g/ml) and centrifuged at 87,000 × _g_ for 2 h at 4°C. The phage band was collected and dialyzed.
- **TEM.** The virion morphology of phages MPK1 and MPK6 was determined by transmission electron microscopy (TEM) as described previously (12). Briefly, formvar-coated TEM grids were subjected to hydrophilic treatment (10 min) and floated with 1:100 diluted CsCl-purified phage samples (20 μl), and negative staining (5 s) using 20 μl H_/9262_ negatively stained with uranyl acetate, revealing their virion structure. Bar, 100 nm.

**Phage infection.** Phage infection is observed by either conventional plaque assay or spotting assay (11). For plaque assay, 10 μl of lysates that contain about 10^7 PFU phages was mixed with 10^7 CFU of _P. aeruginosa_ cells grown to the late logarithmic growth phase (i.e., optical density at 600 nm [OD_600_] of 0.7) and resuspended in 100 μl of phage buffer. After 10 min of incubation, 3 ml of top agar was added, and the mixture was plated. Plaques were visualized after 16 to 24 h of incubation at 37°C.

**Mouse experiments.** Mouse infection was carried out using female ICR mice (aged 4 weeks), following the protocol approved by the Animal Care and Use Committee at Sogang University. Bacterial cells were grown to the stationary growth phase (OD_600_ of 3.0), harvested, washed twice with phosphate-buffered saline (PBS) buffer (2.7 mM KCl, 137 mM NaCl, 10 mM Na_2HPO_4_, and 2.7 mM KH_2PO_4_ [pH 7.0]), and then resuspended in PBS buffer at 2 × 10^7 CFU/ml. To induce peritonitis, mice were infected i.p. with 100 μl of the bacterial suspension (i.e., 2 × 10^7 CFU). For phage therapy, after 6 to 12 h postinfection, phage solution containing either 2 × 10^8 or 2 × 10^7 PFU in PBS buffer (100 μl) was administrated i.p. or intramuscularly (i.m.). For the enumeration of bacterial burden from mouse tissues, infected mice were anesthetized by inhalation of ether at the designated time points (0.5, 12, 24, 36, and 48 h postinfection). Lung, liver, and spleen samples were obtained aseptically and homogenized with a tissue homogenizer (Yamato Scientific Co., Ltd., Tokyo, Japan) in PBS buffer (1 ml). Portions of blood and homogenized tissue samples were plated onto cetrimide agar, which were incubated for 24 h at 37°C. For phage pharmacokinetics, phages were administered either i.p. or i.m. into the uninfected mice, from which the blood, lung, and liver samples were obtained and homogenized at the designated time points (0.5, 12, 24, 36, and 48 h). The tissue homogenates were subjected to filtration, and the filtrates were used for PFU measurement by plaque assay.

**Drosophila melanogaster experiments.** _Drosophila melanogaster_ Oregon R was grown at 25°C using the corn meal-dextrose medium (0.95% agar, 6.24% dry yeast, 4.08% corn meal, 8.62% dextrose, 0.1% methyl paraben, and 0.45% [vol/vol] propionic acid) as described elsewhere (18), with slight modification. Briefly, infection of flies was performed by prickin 5-day-old adult flies in the dorsal thorax with a 10-μm needle (Ernest F. Fullam, Inc.). The needle was dipped halfway into PBS-diluted bacterial suspension containing 10^7 CFU/ml from the stationary-growth-phase (OD_600_ of 3.0) cultures. At this dilution, we introduced 50 to 200 bacteria/animal. Infected flies were transferred to a new medium overlaid with 100 μl phage solution containing 5 × 10^7 PFU. Fly mortality was monitored for up to 48 h postinfection. Flies that died within 15 h under this condition (less than 5%) were not included in mortality determination. Mortality studies were repeated at least five times. For the enumeration of bacterial burdens, infected flies were anesthetized with CO_2_ and ground in LB medium at the designated time points. Homogenates of individual infected flies were plated onto cetrimide agar, which were incubated at 37°C for 24 h to measure the number of CFU per fly. For phage pharmacokinetics in _D. melanogaster_, flies were fed with phages for 12 h and then transferred to a new medium without phages. Flies were homogenized at the designated time points (0.5, 12, 24, 36, and 48 h); the tissue homogenates were filtered, and the filtrates were used for PFU measurement by plaque assay.

**Statistical analysis.** Kaplan-Meier log rank statistics was used to determine the statistical significance of the differences between the control and the treatment groups in mortality rates. The statistical significance in the numbers of viable bacteria recovered from blood and organs was verified by the Mann-Whitney U test.

**RESULTS AND DISCUSSION**

Identification of the new _Caudovirales_ phage strains specific for _P. aeruginosa_. Phages specific to _P. aeruginosa_ strains were initially isolated from sewage samples, which had been obtained several times from Seoul and the suburban districts. Once isolated, phages were screened for their lytic activity on the basis of large and distinguishable plaque formation, a characteristic of lytic phages. We selected six potentially lytic phages for _P. aeruginosa_ strain PAO1 (named MPK1 to MPK6), based on the plaque size (>3-mm diameter after 18 h incubation at 37°C) (data not shown) and clarity (see Fig. 2). We determined the morphology of those six phages by using a transmission electron microscope. Based on their virion structure, with an icosahedral head of ~70 nm in diameter (data not shown) and contractile tails of ~110 nm in length with fibers (Fig. 1A), phages MPK1 to MPK5 belong to the _Myoviridae_ family (order _Caudovirales_). In contrast, MPK6 has a head

![FIG. 1. Transmission electron micrographs of MPK1 (A) and MPK6 (B) negatively stained with uranyl acetate, revealing their virion structure. Bar, 100 nm.](http://aac.asm.org/)
similar to that of MPK1 but has a stubby tail of <10 nm in length (Fig. 1B) and, thus, belongs to morphotype C, subdivision C1 of the Podoviridae family (order Caudovirales) (1, 2, 8). Conclusively, we have isolated six Caudovirales phages, which are most likely not temperate for P. aeruginosa. We selected MPK1 from the Myoviridae phages and MPK6 for the next experiments.

Receptor of phages MPK1 and MPK6. A number of surface receptors on P. aeruginosa cells have been implicated as bacteriophage receptors. Especially, the type IV pili (TFP) have been shown to function as the primary receptors for various P. aeruginosa phages, including filamentous phage Pf (6), single-stranded RNA phage PP7 (8), Bradley B type phage PO4 (7), and temperate transducing phages such as MP22 (12). We first determined whether TFP is required for the MPK1 and MPK6 infection and found that a pilA mutant of PAO1 was nonetheless sensitive to MPK1 and MPK6, which was further substantiated by the phage susceptibility of the twitching motility-defective clinical isolates in our culture collection (data not shown). The next candidate is LPS, although detailed molecular components in the LPS as the receptor remains elusive. Only the LPS core region is known to act as the phage receptor for phi PLS27 (13) and phi CTX (33). To determine whether LPS might act as the receptor for MPK1 and MPK6, we used a series of LPS mutants derived from strain PAO1: the rmd (deficient for group A polysaccharide), wbpM (deficient for group B polysaccharide), and rmlC (deficient for core polysaccharide and, hence, for group A and B polysaccharides as well) mutants. As shown in Fig. 2, while both phages could form clear plaques on the wild-type and rmd bacteria, no plaques were formed on rmlC bacteria. Interestingly, while MPK6 formed no plaque on wbpM mutant bacteria, MPK1 displayed tiny plaques on wbpM mutant bacteria, suggesting that the normal receptor might be affected by the group B polysaccharide mutation with alternative receptor(s) still present for MPK1 in wbpM mutant bacteria. Therefore, the LPS group B polysaccharide is most likely the primary receptor for both phage MPK1 and MPK6 entry into the PAO1 cells, although the molecular determinants for phage-receptor interactions seem to differ for both phages.

Lytic activity of phages MPK1 and MPK6 in vitro. The lytic activity of both MPK1 and MPK6 phages was examined based on the multistep bacterial killing curve in the phage life cycle. P. aeruginosa PAO1 cells (~10^8 CFU) were incubated in the medium containing 2 × 10^6 PFU of either MPK1 or MPK6 in 100 μl PBS. The number of viable bacteria gradually decreased up to ~10^2 CFU for MPK1 after 30 min of incubation versus ~10^3 CFU for MPK6 after 120 min (Fig. 3), demonstrating that both phages have potent lytic activity against P. aeruginosa PAO1 and that MPK1 is the more potent than is MPK6 for rapidly killing P. aeruginosa, at least under this growth condition. The capability of growth inhibition in vitro by these phages led us to further evaluate the antibacterial efficacy against experimental infections (in vivo) caused by P. aeruginosa.

Pharmacokinetics of phages MPK1 and MPK6 in mice. Prior to evaluating the therapeutic efficacy, we examined the pharmacokinetics of both phages to determine which way is better to deliver phages in mice—the i.p. or i.m. route. Phages (5 × 10^7 PFU) were introduced via the i.p. or i.m. route into uninfected mice. Three mice from each group receiving phage samples i.p. or i.m. were euthanized at 0.5, 12, 24, 36, and 48 h postinfection. The number of phages was enumerated from organ (liver and lung) and blood samples (per milliliter) (Fig. 4). In each tissue sample examined, a consistent pattern of the relative PFU levels after administration of both phages by the different routes was observed: PFU levels after i.m. administration were higher than those after i.p. administration (i.m. > i.p.), which is consistent with the previous study using a phage cocktail containing 10^8 PFU against burn wound infections by P. aeruginosa (20). Under all conditions, MPK1 displayed better persistence than does MPK6, although similar numbers were observed right after (i.e., 30 min) phage administration.
MPK6 was hardly recovered from blood, especially when MPK6 was delivered by the i.m. route (Fig. 4D). These results suggest that both \textit{P. aeruginosa} phages have similar pharmacokinetics in mice, but MPK1 displayed better persistence than did MPK6 in animal tissues.

**Therapeutic efficacy of phages MPK1 and MPK6 against mouse peritoneal infection.** Phages MPK1 and MPK6 were injected by either the i.m. or i.p. route with different doses (2$ \times $ 10$^6$ PFU and 2$ \times $ 10$^7$ PFU in 100$ \mu $l PBS) of \textit{P. aeruginosa} strain PAO1 6 h postinfection. The 2$ \times $ 10$^7$ PFU dose of both phages significantly protected the infected mice compared to the untreated mice (Fig. 5A and B). No significant protection was observed with the 2$ \times $ 10$^6$ PFU dose of MPK6 administered by the i.m. route (Fig. 5A) ($P = 0.316$). Despite the better pharmacokinetics of the i.m. administration, as shown in Fig. 4, the i.p. administration displayed better efficacy than the i.m. administration for both MPK1 and MPK6. It is most likely that the i.p. administration can deliver the phages more directly or effectively to the infection site, since we used the peritonitis model.

To verify whether the administration of phages could inhibit the bacterial proliferation, we determined the bacterial loads in mouse organs, including spleen, lung, and liver, since peritonitis is concomitant with bacterial infiltration at the liver of infected animals (5). The organ samples were obtained from live animals at 24 h postinfection. As shown in Fig. 5C, treatment with both phages could significantly reduce the bacterial loads; MPK1 treatment could reduce the bacterial burdens by about 2 to 3 logs in lung, spleen, and liver of live mice at 24 h postinfection, whereas slightly less reduction (by about 2 logs) was observed in the MPK6-treated live mice. These results suggest that both phages are highly effective in controlling \textit{P. aeruginosa}-induced peritonitis by inhibiting bacterial proliferation in vivo, for which MPK1 displays better efficacy than MPK6 does.

**Pharmacokinetics and therapeutic efficacy of phages MPK1 and MPK6 in D. melanogaster.** The \textit{D. melanogaster} systemic infection model is well established for studying the bacterial virulence mechanisms and needs a very small number of \textit{P. aeruginosa} cells (50 to 200 CFU) to infect, compared to the...
murine peritonitis model (10^7 CFU). It causes death within 48 h as a result of systemic infection by bacterial proliferation of up to ~10^7 CFU under our optimized experimental conditions (16, 18). To establish the D. melanogaster model to evaluate phage therapy against P. aeruginosa, we exploited phage feeding on a group of flies (n = 50) in a fly vial by overlaying the phage samples containing 5 × 10^7 PFU onto a fly vial containing 2.5 ml of corn meal medium.

We first examined the pharmacokinetics of both phages which could be orally administered to D. melanogaster, in order to assess their potential toxicity as well as their persistence in fly tissues. Uninfected flies were fed with either phage (5 × 10^7 PFU) and transferred to a new medium without phage feeding. As shown in Fig. 6A, phages were recovered from fed flies, indicating that phages might be successfully administered by the overlay on top of a medium. About 10^6 PFU of both phages were transferred by only 12 h of feeding. Also, the MPK1 level was maintained for up to 48 h after transfer to a new medium, although the PFU gradually decreased by about 3 logs at 48 h. Interestingly, MPK6 was poorly recovered and disappeared before 12 h without bacterial infection after transfer to a new medium. Furthermore, both phages were not toxic at all by 12 h of feeding. No fly died even with phage feeding for more than 72 h (data not shown). These results suggest that phage feeding can be exploited to administer phages for phage therapy for D. melanogaster infection.

Next, the antibacterial efficacy of both phages was evaluated, based on their protective effects from PAO1-induced mortality and proliferation in the D. melanogaster infection model. Phages MPK1 and MPK6 were administered by overlaying 5 × 10^7 PFU on a fly vial to accommodate 50 PAO1-infected flies (i.e., feeding 10^6 phage per fly). Both phages significantly protected the infected flies, compared to no phage feeding (Fig. 6B). Similar levels of efficacy were observed for both phages (P = 0.637), although a better pharmacokinetics of MPK1 was observed in fly tissues.

We examined the protective effect on the bacterial proliferation in fly tissues by phage feeding. As shown in Fig. 6C, MPK6 and, to the larger extent, MPK1 inhibited the bacterial proliferation under this infection condition. Since P. aeruginosa levels reach 10^7 CFU (i.e., by 5 log increase) when the infected flies died (18), >2 log inhibition could be sufficient to resist PAO1-induced killing when flies started to die. These results suggest that both phages are highly effective in controlling P. aeruginosa-induced D. melanogaster killing by inhibiting bacterial proliferation in vivo.

Conclusion. In this study, we have isolated two new P. aeruginosa phages (MPK1 and MPK6), whose primary receptor is the group B polysaccharide of P. aeruginosa LPS, and evaluated their antibacterial efficacy based on mouse and Drosophila melanogaster infection models. Several cases of phage therapy against the experimental infections by P. aeruginosa in mice were reported, which include gut-derived sepsis and burn wound infections (20, 31). A recent study of pyocin therapy was based on a mouse peritonitis model (24). All those experimental infection models appear relevant, considering that the complicated pathophysiology was caused by P. aeruginosa infections. More importantly, P. aeruginosa is a multihost pathogen that can intoxicate phylogenetically diverse hosts, including mammals, insects, worms, and plants, involving a distinct set of virulence factors. Thus, various infection models should be exploited to evaluate and confirm the antibacterial efficacy of antimicrobial agents.

One of the meaningful aspects of the present study is that we established a new therapeutic animal model to evaluate the antibacterial activity in vivo using a nonmammalian model host, D. melanogaster. We are currently investigating how phages are administered and where they are specifically localized within fly tissues to optimize this model for further studies. In recent studies, a nonmammalian live-animal infection model using a nematode (Caenorhabditis elegans) infection model has been successfully exploited for high-throughput screens to isolate new antimicrobial compounds against Enterococcus faecalis or Candida albicans (9, 21). A major advantage of using nonmammalian model hosts is that the research costs (mammalian animals and space, etc.) and, more importantly, the experimental scale can be significantly reduced, since the small size of the nonmammalian model animals requires far less space and smaller amounts of pathogens and antimicrobials for the experimental settings, which in turn
enables the high-throughput analysis from the chemical libraries as well. Another great advantage of such live-animal infection models is that new antimicrobial compounds can be isolated, which do not inhibit the growth of the target microorganisms but do attenuate the virulence pathways and/or enhance the host immune response. Therefore, a further optimization of the D. melanogaster model to evaluate the antibacterial efficacy of various phages against P. aeruginosa infection will enhance our insights into antimicrobial therapy and provide a platform to specifically modulate the interface between bacterial virulence and host immunity.

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