Isolation and characterization of two bacteriophages with strong in vitro antimicrobial activity against *Pseudomonas aeruginosa* isolated from dogs with ocular infections

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**Objective**—To isolate and characterize bacteriophages with strong in vitro lytic activity against various pathogenic *Pseudomonas aeruginosa* strains isolated from dogs with ocular infections.

**Sample**—26 genetically distinct *P aeruginosa* isolates.

**Procedures**—*P aeruginosa* strains were derived from dogs with naturally acquired ulcerative keratitis. From a large-scale screening for bacteriophages with potential therapeutic benefit against canine ocular infections, 2 bacteriophages (P2S2 and P5U5) were selected; host ranges were determined, and phage nucleic acid type and genetic profile were identified via enzymatic digestion. Electron microscopy was used to characterize bacteriophage ultrastructure. Bacteriophage temperature and pH stabilities were assessed by use of double-layer agar overlay titration. A cocultivation assay was used to evaluate the effect of the bacteriophages on bacterial host growth.

**Results**—P5U5 was active against all *P aeruginosa* isolates, whereas P2S2 formed lytic plaques on plates of 21 (80.8%) isolates. For each bacteriophage, the genomic nucleic acid was DNA; each was genetically distinct. Ultrastructurally, P2S2 and P5U5 appeared likely to belong to the *Podoviridae* and *Siphoviridae* families, respectively. The bacteriophages were stable within a pH range of 4 to 12; however, titers of both bacteriophages decreased following heating for 10 to 50 minutes at 45° or 60°C. Growth of each *P aeruginosa* isolate was significantly inhibited in coculture with P2S2 or P5U5; the dose response was related to the plaque-forming unit–to–CFU ratios.

**Conclusions and Clinical Relevance**—Bacteriophages P2S2 and P5U5 appear to be good candidates for phage treatment of infection caused by pathogenic *P aeruginosa* in dogs.
against a broad range of pathogenic *P. aeruginosa* strains isolated from dogs with ocular infections.

**Materials and Methods**

**Bacterial strains**—Twenty-six genetically distinct multidrug-resistant *P. aeruginosa* strains were derived from a collection of isolates obtained from dogs with opportunistic ocular infections (ie, naturally acquired ulcerative keratitis). Identification of the isolates was performed at the Cornell Animal Health Diagnostic Laboratory.

**Phage isolation, propagation, and titration**—For purposes of the study, a large-scale screening for bacteriophages with potential therapeutic benefit against ocular infections caused by *P. aeruginosa* in dogs was performed. Thirty different phage-host combinations were tested to select the bacteriophages with the broadest spectrum and strongest lytic activity across the host collection. For the isolation of bacteriophages P2S2 and P5U5, approximately 100 mL of each of 2 environmental samples (manure and uterine secretions of dairy cows from a commercial dairy farm near Ithaca, NY) was collected and centrifuged for 25 minutes at 3,000 X g at 4°C. The supernatants were collected and separately filtered-sterilized by use of a filter (pore size, 0.22 µm). Two *P. aeruginosa* isolates were selected from the aforementioned collection to serve as hosts for the 2 phages. Each host was separately cultured in LB broth until the OD660 value for each culture was 0.16 to 0.18 (approx 2.5 X 107 CFUs/mL). At this point, 8.8 mL of the filter-sterilized supernatant from the environmental samples was separately inoculated with 1 mL of 10X LB broth and 200 µL of 1 of the 2 hosts (approx 2.5 X 107 CFUs/mL); *P. aeruginosa* PA2 was inoculated in supernatant from manure, and *P. aeruginosa* PA5 was inoculated in supernatant from uterine secretions. The mixtures were incubated at 37°C for 18 hours with shaking; after incubation, each suspension was centrifuged at 10,000 X g for 10 minutes at 4°C, and the supernatant was sterilized by filtering through a filter (pore size, 0.22 µm). Each phage underwent single-plaque isolation twice and was propagated on its respective host strains. A high-titer stock in SM buffer (100mM NaCl, 8mM MgSO4, and 1M Tris-HCl; pH, 7.5) was prepared as described.

**Bacteriophage nucleic acid extraction**—Bacteriophage nucleic acid extraction from highly concentrated stocks was performed in small preparations from 0.25 mL of phage suspension. Contaminating bacterial nucleic acids were removed from concentrated phage solution by the addition of RNase A and DNase I to final concentrations of 20 and 5 µg/mL, respectively, followed by incubation for 30 minutes at 37°C. After nucleic digestion, 0.25 mL of Tris-SDS (0.3M Tris-HCl, 100mM EDTA, and 1.25% SDS; pH, 9.0) was added and the mixture was incubated at 65°C for 30 minutes. Subsequently, 0.25 mL of ice-cold 3M potassium acetate (pH, 4.8) was added, and the mixture was placed on ice for 5 minutes. Insoluble material was removed via centrifugation at 15,000 X g for 2 minutes at room temperature (approx 25°C), and the supernatant was transferred to a clean tube. Nucleic acid was precipitated by the addition of 0.7 volumes of isopropanol (approx 0.5 mL) to the supernatant; after a resting period of 2 minutes at room temperature, the nucleic acid was recovered via centrifugation at 15,000 X g for 1 minute. The nucleic acid pellet obtained was dissolved in 0.3 mL of Tris-EDTA (10mM Tris-HCl and 1mM EDTA; pH, 8.0), and 0.15 mL of 7.5M ammonium acetate was added to this solution; the nucleic acid was reprecipitated with 2 volumes (approx 0.9 mL) of cold ethanol. The mixture was centrifuged at 15,000 X g to spin down the nucleic acid, which was then washed with 0.5 mL of 70% ethanol. The nucleic acid pellet was dried at room temperature and then dissolved in Tris-EDTA. Nucleic acid concentration and purity were evaluated by determination of the optical density (at 230, 260, and 280 nm), and integrity was assessed via 0.8% (wt/vol) agarose gel electrophoresis.

**Nucleic acid type and restriction enzyme digestion**—The nucleic acid of each isolated phage was digested with DNase I and RNase A at room temperature and at 37°C, respectively, for 1 hour, according to the manufacturer’s instructions. The undigested nucleic acid of each phage was quantified as a control. Additionally, phages were concentrated from bacterial lysates by use of polyethylene glycol 8,000-70% NaCl centrifugation. High-titer stocks of phages were titrated by use of the standard soft agar overlay technique.

**Determination of phage host range (spot assay)**—The host range of each phage was determined by use of spot assays against each of the 26 *P. aeruginosa* isolates. Five microliters of concentrated phage lysate (> 10^10 PFUs/mL) was separately dropped onto plates containing LB agar overlaid with 0.75% LB agar mixed with one of the *P. aeruginosa* isolates; plates were then incubated for 18 hours at 37°C. Lyses were qualitatively measured by the presence of lytic zone and scored as clear (if complete lysis of the bacterial lawn was observed) or as turbid (if the lytic zone was not clear). Otherwise, hosts were considered to be phage insensitive. Percentage of lytic activity of each phage was calculated on the basis of formation of clear lytic zone of the bacterial lawn for each *P. aeruginosa* isolate. For the purpose of calculating the percentage of the lytic activity, a clear or turbid lytic zone was considered positive phage activity and absence of a lytic zone was considered negative activity. Phage lytic activity was defined as the total number of positive lytic zones divided by the total number of bacterial isolates tested.

**TEM images of P2S2 and P5U5 phages**—The morphological characteristics of P2S2 and P5U5 were determined via TEM. Polynvinyl formal resin-coated TEM grids were covered for 10 minutes with 7 µL of phage samples containing approximately 10^8 PFUs and immediately negatively stained for 5 seconds by use of 7 µL of 2% uranyl acetate (pH, 4.0). The grids were air-dried for 30 minutes and examined via TEM at 110,000 X to 140,000 X magnification. For each phage type, mean head diameter and length and mean tail di-
Thermostability and pH-stability tests—Phage thermostability was tested by incubation of 0.6 mL (containing approx 10^7 PFUs) of the isolated phages at 45° and 60°C. Aliquots (0.1 mL) were collected at 10-minute intervals for 50 minutes. The phage titer in each sample was determined by use of a double-layer agar plate method. Phage survival was expressed as a percentage ± SD of the phage titer prior to incubation (0 minutes). The tests were performed twice and in duplicates for both phages.

For pH stability, samples of the isolated phages (0.1 mL of phage suspension containing approx 10^7 PFUs) were mixed in a series of tubes containing 0.9 mL of SM buffer, with adjusted pH values ranging from 4.0 to 11.0 and incubated for 1 hour at 37°C. Phage titers were determined by use of the double-layer agar plate method. Tests were performed twice and in duplicate for both phages.

Phage and P aeruginosa coculture assay—To evaluate the effect of each of the 2 phages on each of the 26 representative strains of P aeruginosa, bacterial growth in manually prepared 96-well microtiter plates containing the phages P2S2 and P5U5 at different MOIs was assessed. Each bacterial isolate was grown in LB broth and incubated at 37°C until an OD625 of 0.16 to 0.18 (approx 2.5 X 10^7 CFUs/mL) was reached. The cultures were diluted in LB broth to 10^8 CFUs/mL, and the tests were performed with MOIs ranging from 10^-4 to 10^0 PFUs/CFU in wells containing 10^5 CFUs of P aeruginosa. Optical densities of the cultures were periodically assessed spectrophotometrically in triplicate for 13 hours consecutively in a microplate reader. Both individual phages and the phage combination were tested. The assays were performed in duplicate, and 2 controls were used: a sterile control containing only LB broth and a positive control that contained the P aeruginosa isolate in LB broth without bacteriophages. Growth curves of the 26 bacterial isolates in the presence of each phage and in the presence of both phages combined were plotted by use of the mean OD625 value derived from the values in the duplicate assays.

Data management and statistical analysis—A general linear mixed model was used to analyze the effect of MOI on the growth curves of all 26 P aeruginosa isolates. The outcome variable was the OD625 value of the LB broth culture, which was modeled as a Gaussian (normally distributed data) variable. The assumption that the residuals were normally distributed was satisfied via visual evaluation of the distribution plot of the studentized residuals. The independent variables offered to the model were treatment (2 different phage preparations, 1 combined phage preparation, and control) and time (from 0 until 13 hours). The interaction of treatment and time was also included in the model. The data were longitudinally collected and therefore had a series of repeated measures of OD625 (a total of 13 values) throughout the study period. This implies that data points were correlated within each P aeruginosa isolate. To appropriately account for within-isolate correlation of the OD625, we modeled the error term by imposing a first-order autoregressive covariance structure. Variables and interactions were considered significant at a value of P < 0.05, and 95% CIs were estimated for all least squares means of OD625 by use of a general linear mixed-model equation.

Results

Isolation and host ranges of lytic P aeruginosa phages—Initial screening for new phages yielded 11 likely useful phages (data not shown). The 3 most important criteria used to narrow the selection of the candidates for further studies were host range, degree of lytic activity (estimated on the basis of the visual clarity of the spot assay), and genetic differences determined on the basis of the restriction enzyme digestion profile. Phages P2S2 and P5U5 were isolated from manure and uterine secretions of dairy cows, respectively, and were found to be genetically distinct; both had excellent lytic activity and were chosen for further analysis. The combined spectrum of lytic activity of both phages encompassed all P aeruginosa isolates tested (Table 1). Phage P5U5 was capable of causing complete lysis of the bacterial lawn of all P aeruginosa isolates tested. Phage P2S2 was active against 21 of the 26 (80.8%) isolates.

<table>
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<th>Bacterial isolates</th>
<th>P2S2</th>
<th>P5U5</th>
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<td>PA1</td>
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<td>PA2</td>
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The host range of each phage was determined by use of spot assays against each of the 26 P aeruginosa isolates. Lysis were qualitatively measured by the presence of lytic zone and scored as clear (if complete lysis of the bacterial lawn was observed (+)) or turbid (if the lytic zone was not clear (+/-)). Phage lytic activity was calculated as the total number of positive lytic zones divided by the total number of bacterial isolates tested.
Nucleic acid type and genetic diversity of *P aeruginosa* phages P2S2 and P5U5—The genomic nucleic acid of phages P2S2 and P5U5 could be digested with DNase I but not with RNase A, indicating that they consisted of DNA (Figure 1). The restriction analysis with EcoRI revealed different banding patterns of the fragments for P2S2 and P5U5, indicating that they are genetically distinct from each other. Additionally, it was inferred from the restriction enzyme digestion that the nucleic acid of both phages was double-stranded DNA because EcoRI is an endonuclease that specifically recognizes cleavage sites in double-stranded DNA substrates.

Morphological characterization of phages P2S2 and P5U5—Transmission electron microscopy (Figure 1) revealed that phage P2S2 was likely in the *Podoviridae* family (order Caudovirales), whose members have isometric heads and short, noncontractile tails. For P2S2, the mean head diameter and length were 61.6 and 65.4 nm, respectively, and mean tail diameter and length were 12.5 and 17.1 nm, respectively. In contrast, P5U5 had a long, flexible, noncontractile tail and icosahedral head. The morphological characteristics of P5U5 were similar to the virion structure of the *λ*-like phages of the *Siphoviridae* family (order Caudovirales), which have isometric heads and long, flexible, noncontractile tails. For P5U5, mean...
head diameter and length were 87.1 and 85.5 nm, respectively, and mean tail diameter and length were 10.6 and 275.0 nm, respectively.

In a sample collected 30 minutes after coculture of *P aeruginosa* PA5 with P5U5 in an MOI equal to $10^2$ PFUs/CFU, TEM was performed to evaluate phage activity in coculture (Figure 2). Bacterial debris and bacterial rupture with externalized cytoplasmic content were evident in TEM images.

Thermostability and pH stability of phages P2S2 and P5U5—The activity of phage P2S2 and P5U5 declined following heating for 10 to 50 minutes at both temperatures tested (45° and 60°C; Figure 3). Phage P2S2 was more stable at 45°C than at 60°C. However, P5U5 had a higher survival rate at 60°C than at 45°C.

The activity of each phage was relatively stable at all the pH ranges tested. No dramatic decrease in viability was observed in low or high pH conditions; for each pH condition, the mean titer was decreased by approximately 1.0 log$_{10}$ compared with the starting titer (Figure 4).

Coculture of phages and *P aeruginosa*—The ability of P2S2 and P5U5 to inhibit bacterial growth was tested in LB broth. The 2 phages were cultured separately and
together with each representative *P. aeruginosa* isolate at different MOIs. Analysis of coculture of all 26 isolates with P2S2 alone, P5U5 alone, or both phages (C) at different MOIs (10–2 [asterisk], 10–1 [cross], 100 [triangle], 101 [square], or 102 [diamond]). A control sample (containing the *P. aeruginosa* isolate in LB broth without bacteriophages [circle]) was included in each experiment.

All mean experimental OD₆₂₅ values were significantly ($P < 0.05$) lower than the control value (OD₆₂₅ = 0.53; 95% CI, 0.52 to 0.54). The dose response was related to the PFU:CFU ratio (Figure 5). Multiplicity of infection equal to $10^3$ completely inhibited growth of the 26 representative isolates cocultured with P5U5 or the phage combination. Mean OD₆₂₅ for MOIs ranging from $10^0$ to $10^2$ was < 0.4 for all phage treatments.

**Discussion**

In the study of this report, 2 phages that had strong lytic activity against a broad range of *P. aeruginosa* isolated from dogs with ocular infections were partially characterized. Phages appear to be truly ubiquitous in the natural environment and are among the most abundant inhabitants of the biosphere. Considering the concern about controlling bacterial pathogens that have emerged as resistant to some antimicrobials and, in certain cases, all clinically approved antimicrobials, renewed attention is turning to the therapeutic use of bacteriophages. In fact, phage therapy has been successfully used against a diversity of bacterial pathogens in animal trials.

Manure and uterine secretions of dairy cows were chosen as the bacteriophage sources because those biological materials have yielded a variety of phages in our previous studies. Initial screening for new phages prior to the present study yielded 11 likely useful phages (data not shown), of which P5U5 and P2S2 were the most promising for further investigation. Phage P5U5, by itself, was surprisingly capable of producing a clear lytic zone in the bacterial lawn of all *P. aeruginosa* isolates tested. We decided to also select P2S2 for the present study to enable us to prepare a combination of lytic phages for testing against growth of the *P. aeruginosa* isolates. Considering that the narrow host range, which is typical of most phages described, is one of the principal obstacles to phage therapy, these findings emphasized the considerable potential for in vivo use of the selected phages against *P. aeruginosa*.

It is known that some structural parts of bacteria such as the flagellum, pilus, capsule, teichoic acid, lipopolysaccharide, and outer membrane porins could have or act as specific receptors for phage adsorption. The differences observed in susceptibility of the bacterial host to the phages may also be due to variation in surface receptor molecules (adsorption blocking), restriction modification systems in the hosts, and other phage-resistant mechanisms such as abortive infection or acquired resistance mechanisms such as the recently reported clustered regularly interspaced short palindromic repeats (CRISPR) system.

Both phages used in the present study likely belong to the order Caudovirales. Many isolated *P. aeruginosa* phages are members of the viral order Caudovirales, including the families *Siphoviridae*, *Podoviridae*, and *Myoviridae*. The appearance of the phages in TEM images along with the Universal Virus Database of the International Committee on Taxonomy of Viruses Index of Viruses criteria indicated that phage P5U5 likely belongs in the *Podoviridae* family, whose members have isometric heads and short, noncontractile tails. In contrast, P5U5 has a long, flexible, noncontractile tail and...
sahedral head; the appearance of P5U5 is similar to the virion structure of the Φ-like phages of the Siphoviridae family, which have characteristic isometric heads and long, flexible, noncontractile tails.

In the present study, the survival rate of phages P2S2 and P5U5 decreased following heating, but phage activity was still observed for as long as 30 minutes during incubation. Different survival rates have been observed for other phages. The activity of the phage SMP, which is capable of infecting Streptococcus suis type 2 strains, decreased significantly following heating for 10 to 50 minutes at 40° or 45°C and ceased entirely following heating for 10 minutes at 50°C. The activities of P2S2 and P5U5 in the present study were relatively stable at all the pH conditions tested. Compared with the starting viability level, no dramatic decrease in viability of either phage was observed in low or high pH conditions. Similar results were observed for bacteriophage particles used as a DNA vaccine delivery system. In that study, diluted suspensions of the phage were essentially stable in a broad pH range, with no significant decrease in titer for 24 hours. Results of another in vitro study indicated that the anti-Salmonella phages st104a and Felix 01 were capable of survival (survival rate, 10% and 30%, respectively) during exposure to porcine gastric juice at a pH of 2.5 for up to 120 minutes, unlike the phage st104b, for which activity disappeared completely after 2 hours of similar exposure. The phage SMP had relatively stable activity in conditions of pH 5 to 9, but had dramatically decreased activity under lower or higher pH conditions.

Phage P2S2, phage P5U5, and the combined phage preparation were capable of notable bacterial growth inhibition in the present study, and the dose response was related to the PFU:CFU ratio. The inhibition effect was considered to be due to the lytic activity of phages on the host. Additionally, in cocultures with P5U5 or the phage combination, an MOI equal to 10^2 completely inhibited growth of the 26 representative isolates. Santos et al tested the effect of a phage combination preparation and identified efficient antimicrobial activity against multidrug-resistant E. coli isolated from uterine secretions of Holstein dairy cows. The phage combination significantly inhibited bacterial growth at all MOIs tested, compared with control findings; an MOI as low as 10^4 affected the growth of 1.25% of the E. coli isolates. The MOIs for the phage combination that inhibited at least 50% and at least 90% of the isolates were 10^3 and > 10^2 PFUs/CFU, respectively. In a similar experiment, the potent lytic capabilities of 2 probable novel anti-S aureus phages, ΦSA039 and ΦSA012, against 7 representative hemolysin-positive S aureus isolates obtained from mastitic cow milk were identified. Remarkably, ΦSA012-resistant bacteria were not observed in 3 of 7 isolates tested after a 65-hour period of incubation following phage addition.

Although phages are known to interact specifically with host bacteria, the chances of successful treatment via phage therapy might be greatly increased by use of various phages that have strong lytic activities against a wide range of hosts in combinations that allow the activity spectrum of any one phage to be complemented by those of other phages. Therefore, the isolation of potential novel phages (identified through screenings of environmental sources) on different hosts is essential. Our study was limited to an in vitro analysis of the lytic effect of phages (isolated from environmental samples) on representative multidrug-resistant P aeruginosa isolated from dogs with ocular infections. Although the combination of phages P2S2 and P5U5 might be a potential alternative to conventional treatment of ocular infection in animals, further purification of the phage particles should be performed before animal trial studies are conducted.

References


