Isolation and Characterization of a Virulent Bacteriophage φPA-HF17 of *Pseudomonas aeruginosa*

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Abstract: *Pseudomonas aeruginosa, an important causative agent of nosocomial infection, is found throughout the hospital environment in moist reservoirs, and multidrug-resistant strains of P. aeruginosa have been increasingly reported worldwide. Bacteriophages are often considered potential therapeutic candidates in treating infectious diseases. In this study, a novel virulent bacteriophage φPA-HF17, specific infecting clinical isolates of P. aeruginosa, was isolated and characterized from environmental sewage. Transmission electron microscopy showed that phage φPA-HF17 had an icosahedral head with a very short tail, and exhibited characteristics typical of a podovirus. Restriction analysis indicated that phage φPA-HF17 was a double-stranded DNA virus, which might be digested by some restriction endonucleases. Phage φPA-HF17 was highly infectious with a rapid adsorption (>90% adsorbed in 4 min). In a one-step growth experiment, phage φPA-HF17 was shown having a latent period of 10 minute, with corresponding burst sizes of 200 PFU/cell. Furthermore, when φPA-HF17 alone was incubated at different pHs and different temperatures, the phage was stable over a wide pH range (4 to 10) and at extreme temperature (50℃). These results suggest that φPA-HF17 may be candidate therapeutic phage to control P. aeruginosa infection.*

Keywords: *Bacteriophage, Phage therapy, Pseudomonas aeruginosa.*

Introduction

Pseudomonas aeruginosa is a facultatively anaerobic, non-fermentative, gram-negative, rod-shaped bacterium very common in the hospital environment. This organism is an important causative agent of nosocomial and opportunistic infection in human, such as pneumonia, urinary tract infection, bacteremia, endocarditis, etc. [3, 21, 23]. Infections caused by *P. aeruginosa* are frequently life threatening in infants, elders and immunocompromised individuals [17]. In addition, *P. aeruginosa*, one of the most common multidrug-resistant (MDR) bacteria in hospitalized patients [13], routinely exhibits multiple mechanisms of drug-resistance, for instance, efflux pumps, antibiotic degrading or modifying enzymes, and limited membrane permeability [12, 19]. Therefore, the declining efficacy of antibiotic therapy triggers the development of novel therapeutics for *P. aeruginosa* infections.

Bacteriophages are viruses that specifically infect and kill bacteria. Bacteriophage (phage) therapy is one of several potential therapeutic approaches against bacterial infections. There are at least two aspects in supporting the application of phage therapy: (1) it has been used for decades in Eastern Europe [20]; (2) much more biological properties of phages have been achieved [11, 16]. Currently, several papers have been published on the use of phages as specific antimicrobial agents [1, 4, 5]. For therapeutic purposes, virulent or lytic bacteriophages are highly desirable due to their ability to kill target host cells.

In this study, clinical isolates of *P. aeruginosa* were collected and used as target cells to screen lytic phages from swage. A novel lytic phage (designated φPA-HF17) specifically infecting *P. aeruginosa* isolates was isolated and characterized. Its basic biological features including morphology, one-step growth, adsorption rate, pH and thermal stability were investigated.

Materials and methods

Bacterial strains, growth conditions and identification

Nine clinical isolates of *P. aeruginosa*, previously isolated at Department of Clinical Laboratory, the third affiliated hospital of Jilin University, were used for phage isolation and identification. The VITEK system (bioMérieux, Craponne, France) was employed to identify the bacterial isolates using the Gram-negative bacterial identification cards. Clinical isolates of *P. aeruginosa* were further confirmed by sequencing the 16S rRNA gene. Universal primers, 185f (5' TAG TTG GTG GGG TAA AGG C 3') and 705r (5' TTT CGC ACC TCA GTG TCA G 3'), were adopted to amplify the partial 16S rRNA gene. Sequences of partial 16S rRNA gene were deposited in GeneBank under accession number AF094715. All bacterial strains were grown on Nutrient agar and Luria-Bertani (LB) broth at 37°C. Bacterial growth was monitored turbidimetrically at OD_{600} of 0.4 (corresponded to 3×10^8 cells/ml).

Isolation of bacteriophages

Procedure of phage isolation was conducted as previous description [10, 25]. Several specimens from raw sewage were collected and were immediately brought to the laboratory and centrifuged at 10,000 × g for 15 min at 4°C to remove debris. The supernatants were filtered through 0.45-μm-pore-size membranes (Millipore, Bedford, MA, USA). The filtrate was added to a fresh bacterial culture in LB. The mixture was incubated at 37°C for 8 h at 150 rpm, then centrifuged at 10,000 × g for 15 min and filtered through a 0.2-μm-pore-size membrane (Millipore). The filtrated supernatant was used to check the presence of lytic phages by the double-layer method [27] using Nutrient agar as the culture medium. The plate were incubated at 37°C and examined for the presence of plaques after 12 h. A pure phage stock was achieved through purified plaque using three consecutive round of single-plaque isolation. Phage stocks were stored at 4°C with 1% chloroform.

Preparation of phage for Transmission Electron Microscopy (TEM)

Morphology of purified phage particles was examined by transmission electron microscope of negatively stained preparations. Ten μl of phage particles (10^{10} PFU/ml) was spotted onto a 400-mesh-size formvar-carbon-coated copper grid, stained with 2% uranyl acetate and then examined by Hitachi TEM system with an accelerating voltage of 80 kV.

Extraction of phage DNA and restriction analysis

Phage particles were precipitated with polyethylene glycol (PEG), followed by 10,000 × g for 15 min. Pellets were resuspended in TE buffer and the procedure for extraction of phage DNA was carried out as previously described [27]. Phage genomic DNA was subjected to restriction digestion with nine restriction enzymes (*Nde* I, *Bgl* II, *Xho* I, *Xba* I, *Not* I, *Pst* I, *EcoR* V, *Hind* III, and *EcoR* I). The digested products were analyzed by agarose gel electrophoresis.

Adsorption rate and one-step growth assay

Procedures for adsorption rate were conducted by standard method [9]. Briefly, phages were mixed with 10 ml of mid-exponential host cells at multiplicity of infection (MOI) of 0.01. The mixture was incubated at 37°C at 150 rpm. At 1 min intervals for 10 min, 100 µl of sample was mixed with 450 µl SM buffer (50 mM Tris-Cl, pH 7.5, containing 0.58% NaCl and 0.2% $MgSO_4$) and 50 µl chloroform. The mixture was centrifuged at 10,000 × g for 6 min, and the percentage of unadsorbed phage in each sample was measured by the double-layer method.

For one-step growth experiments, 50 ml bacterial cells were incubated to mid-exponential phase and harvested by centrifugation. The pellet was resuspended in 20 ml of fresh LB medium. Phage solution was added to the bacterial suspension at a MOI of 0.01 and allowed to adsorb for 5 min. The mixture was continuously incubated at 37°C. Samples were taken at 10 min intervals and phage titer was determined by the double-layer method. Finally, one-step growth curve was deduced according to the constant phage titer.

Analysis of phage proteins

Phage solution was subjected to Amicon-100 filter, and the phage particles were washed three times with SM buffer. Purified phage particles were mixed with sample buffer and heated in a boiling water bath for 6 min. A volume of purified viral particles corresponding to 10^{12} PFU/ml was loaded directly onto a 12.5% SDS-PAGE for 2 h. Gels were stained with 0.1% Coomassie brilliant blue and documented using gel image system.

pH and thermal stability test

Resistance to different pH values at 37°C was carried out as previously documented [22]. Briefly, the pH of the LB was adjusted with either 1 M HCl or 0.5 M NaOH to obtain a pH ranging from 1 to 14. A total of 100 µl of bacteriophage suspension (5×10^8 PFU/ml) was innoculated into 5 ml of pH-adjusted medium. After incubation for 1 h at 37°C, the surviving phage particles were counted immediately using the double-layer method. Thermal stability of phage at different temperatures (50°C, 60°C, 70°C and 80°C) was determined by incubating the phage (10^7 PFU/ml) at the indicated temperature for 30 min and 60 min at pH 7.0 in nutrient broth; the surviving phages were then calculated.

Results

Identification of P. aeruginosa clinical strains

As described in Material and Methods, DNA fragment containing 16S rRNA gene from each clinical isolates was amplified by PCR and sequenced. PCR products were subjected to electrophoretic analysis, and the size of amplified DNA was 521 bp approximately (not shown). The sequences of PCR products were deposited to GeneBank and aligned to search for the most similar sequences. The results showed that the sequence of nine clinical isolates

was consistent with that of *P. aeruginosa* ATCC15692. Therefore, nine clinical isolates were validated to be *P. aeruginosa*.

Morphology of bacteriophages φPA-HF17 revealed by TEM

Using the described enrichment method, a lytic bacteriophage, designated as φPA-HF17, was isolated from raw sewage. This phage formed clear plaques of approximately 2-4 mm in diameter on *P. aeruginosa* lawns. Images of bacteriophage φPA-HF17 were developed using TEM. As shown in Fig. 1, the phage φPA-HF17 had an icosahedral head, about 50 nm in diameter, and a short non-contractile tail. Thus, phage φPA-HF17 was morphological similar to phages belonging to the *Podoviridae* family, *Caudovirales* order.

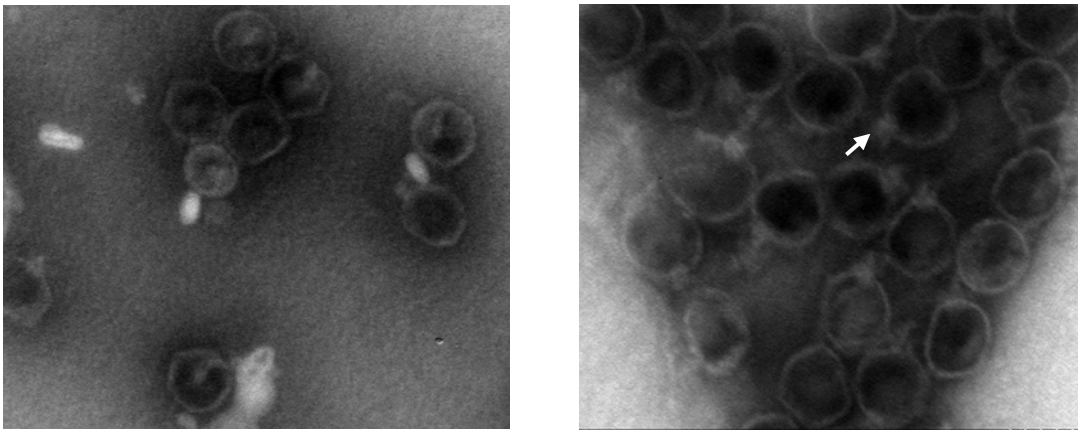

Fig. 1 Transmission electron micrograph of phage φPA-HF17
(an arrow indicates the short non-contract tail of phage, *Bar* corresponds to 100 nm)

Restriction analysis of phage φPA-HF17 genome

After enrichment of phage φPA-HF17, phage DNA was extracted and purified as described. Genomic DNA was cut by 9 restriction endonucleases. The results showed that the φPA-HF17 DNA was cut by *Nde* I, *Xho* I, *Xba* I, *Pst* I, *Hind* III and *EcoR* I (Fig. 2). The restriction analysis also demonstrated that phage φPA-HF17 was a dsDNA virus. Determination of the phage genome sequence is also underway.

Adsorption rate and one-step growth curve of phage φPA-HF17

The phage exhibited rapid adsorption onto the host cells as shown in Fig. 3. More than 90% of the phage particles were adsorbed in 4 min. A one-step growth experiment was carried out to determine the latent period and burst size of phage φPA-HF17. The latent period (defined as the time interval between the adsorption and the beginning of the first burst) was 10 min, and the burst size was 200 PFU/infected cell (Fig. 4).

Analysis of phage proteins by SDS-PAGE

SDS-PAGE analysis of phage φPA-HF17 revealed that three major proteins and at least 8 minor proteins were observed on the gel, with molecular weight ranging from10 to 170 kilodalton (Fig. 5). In the Fig. 5 Lane 1, PageRuler Prestained Protein Ladder #26617 (Thermo); Lane 2 and Lane 3, Phage φPA-HF17 (10^{12} PFU/ml). Three major structural proteins, with molecular weight ranging from 35 to 40 kilodalton, were found.

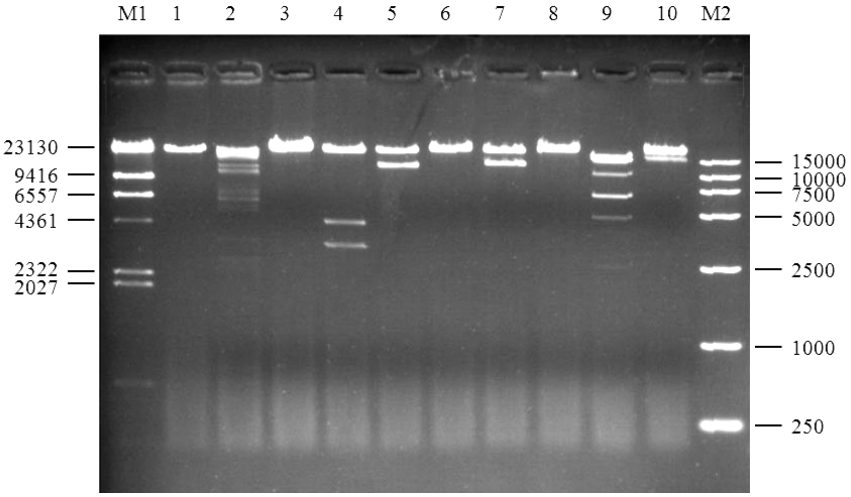

Fig. 2 Restriction analysis of phage φPA-HF17 genome
M: DNA marker; Lane 1: DNA genome; Lane 2: Digestion of DNA with *Nde* I; Lane 3: Digestion of DNA with *Bgl* II; Lane 4: Digestion of DNA with *Xho* I; Lane 5: Digestion of DNA with *Xba* I; Lane 6: Digestion of DNA with *Not* I; Lane 7: Digestion of DNA with *Pst* I; Lane 8: Digestion of DNA with *EcoR* V; Lane 9: Digestion of DNA with *Hind* III; Lane 10: Digestion of DNA with *EcoR* I

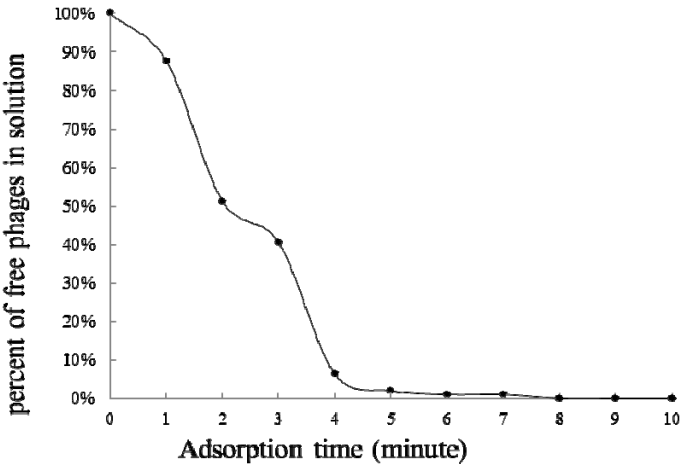

Fig. 3 Adsorption of phage φPA-HF17 to *P. aeruginosa*

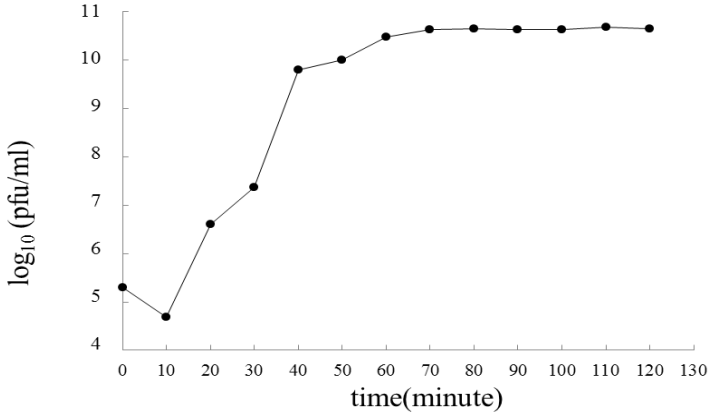

Fig. 4 One-step growth curve of phage φPA-HF17 on *P. aeruginosa*

Stability investigation

As shown in Fig. 6, no obvious effect on φPA-HF17 activity was observed after 1 hour of incubation at pH levels ranging from 5 to 10. However, the phage φPA-HF17 completely lost its activity at pH 11 or higher and pH 3 or lower. When incubation at pH 4 for 1 h, a titer of 1.37×10^4 PFU/ml of active phage φPA-HF17 was detected at the end of the incubation. The maximum stability of the phage was observed at a pH 5, 6, 7, 8, and 9. Heat resistant capability of phage φPA-HF17 at pH7.0 was performed. The results showed that φPA-HF17 was extremely heat stable (Fig. 7); nearly 100% phage particles (10^7 PFU/ml) remained alive after 30 min and 60 min at 50°C, however, the number of viable phages decreased from 10^7 PFU/ml to both10^6 PFU/ml after 30 min and 4.5×10^6 PFU/ml after 60 min at 60°C, respectively.

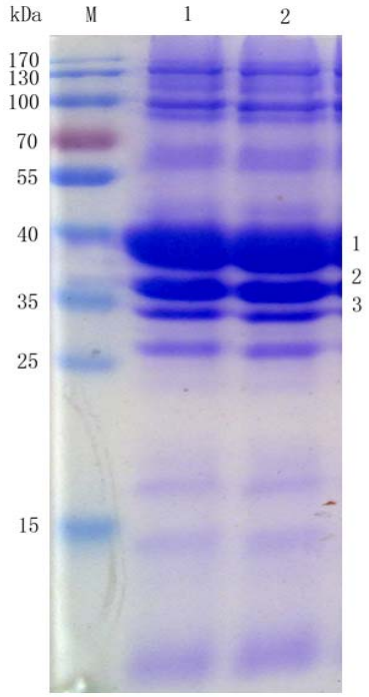

Fig. 5 SDS-PAGE analysis of phage φPA-HF17 proteins.

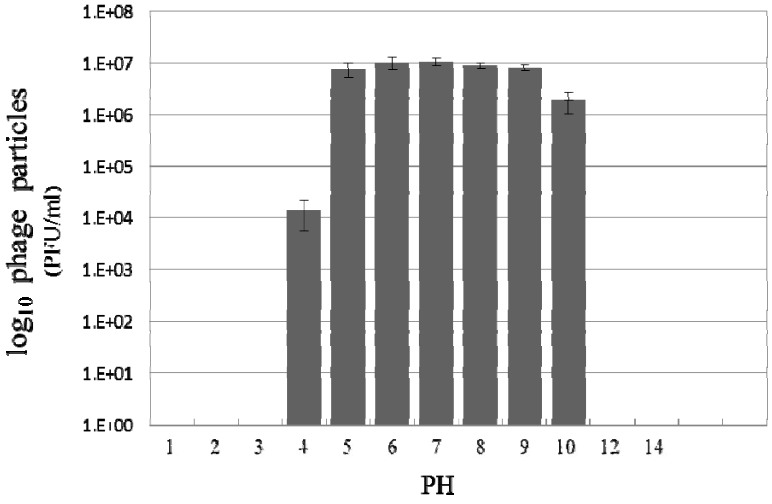

Fig. 6 pH stability test of phage φPA-HF17

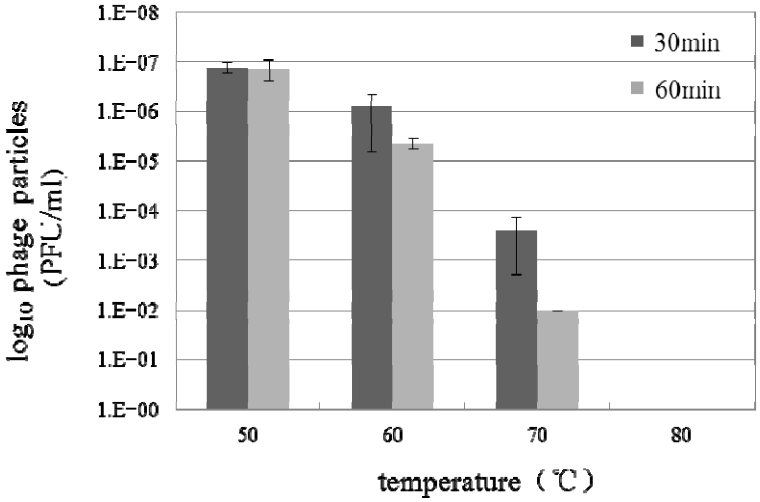

Fig. 7 Heat stability test of phage φPA-HF17

Discussion

The search for potential therapeutic phages against *P. aeruginosa* has regained much attention because of the increasing prevalence of metallo-β-lactamase and 16S rRNA methylase coproducing *P. aeruginosa* strains associated with human infections [7]. Bacteriophages are thought to be the most abundant microorganisms on the earth [8, 24], whose total count is estimated to be more than 10^{30} [18]. As ubiquitous, they can be found in varies types of water and soils as well as in gastrointestinal tract of animals. Recently, a few publications have documented the efficacy of bacteriophage agents in eliminating clinical *P. aeruginosa* isolates both *in vitro* and *in vivo* [6, 15, 26]. In this study, phage φPA-HF17 specific to *P. aeruginosa* was isolated and characterized from swage.

Bacteriophages of *P. aeruginosa,* belonging to tailed virus with double stranded DNA genome, are classified into three families of the order of *Caudovirales,* including *Myoviridae, Podoviridae,* and *Siphoviridae* [14]. Among the tailed phages of the genus *Pseudomonas*, there are about 36% from the family *Myoviridae*, the same percentage from the family *Siphoviridae* and 28% belonging *Podoviridae* [2]. Our results showed that phage φPA-HF17 had an icosahedral head with a very short tail, and the size of its head was 50 nm in diameter, approximately. Based on its morphological features, phage φPA-HF17 was tentatively classified as a member of *Podoviridae* family. To the best of our knowledge, other researchers had reported isolation of the *P. aeruginosa* specific phages belonging to families *Podoviridae* and *Siphoviridae* [10]. Purified virion DNA was digested with several restriction endonuclease and subsequently subjected to electrophoresis analysis. The results showed that the φPA-HF17 DNA was cut by 6 of the 9 enzymes, including *Nde* I, *Xho* I, *Xba* I, *Pst* I, *Hind* III and *EcoR* I. With respect to one-step growth assay, latent period and burst size are the parameter used to measure phage infectivity. In this study, phage φPA-HF17 proliferates efficiently with a relatively short latent period (10 min) and a large burst size (200 PFU/cell). Furthermore, φPA-HF17 adsorbs more rapidly (> 90% adsorbed in 4 min). Hence, φPA-HF17 could be considered as a candidate for phage therapy, as it has rapid adsorption rate and efficient proliferation at 37°C. In addition, pH stability showed that no reduction of phage φPA-HF17 was observed when incubation at pH 5, 6, 7, 8, 9 and 10. Therefore, φPA-HF17 was stable over a wide pH range (5 to 10). All of these characteristics have implications for the use of this phage as a stable antimicrobial agent for the treatment of *P. aeruginosa* infections.

Conclusion

In this study, a lytic phage of *P. aeruginosa*, φPA-HF17, was characterized, combined with its efficient in lysing *P. aeruginosa* isolates and its pH stability, it may be a good candidate to be used as an alternative agent against *P. aeruginosa* infections.

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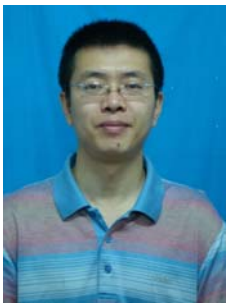

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