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# Isolation and characterization of a bacteriophage and its potential to disrupt multi-drug resistant *Pseudomonas aeruginosa* biofilms

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ABSTRACT

A lytic *Pseudomonas aeruginosa* bacteriophage, vB_PaeM_LS1, was isolated and characterized herein. To examine the eligibility of bacteriophage vB_PaeM_LS1 as a therapeutic bacteriophage, we analysed its genome and compared it to similar bacteriophages. Genome of bacteriophage vB_PaeM_LS1 consisted of a linear, double-stranded DNA molecule 66,095 bp in length and with 55.7% G + C content. Neighbor-joining analysis of the large subunit terminase showed that bacteriophage vB_PaeM_LS1 had similarity to the *Pbunavirus* genus. The potential of the lytic bacteriophage to disrupt *Pseudomonas aeruginosa* biofilms was assessed by scanning electron microscopy and bacterial counts. This study revealed that the bacteriophage vB_PaeM_LS1 with its lytic effect showed a high potential impact on the inhibition of the growth of *Pseudomonas aeruginosa* biofilm formation.

## 1. Introduction

*Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen that can cause serious problems in clinical settings, particularly in patients with a genetic disorder in the cystic fibrosis transmembrane conductance regulator, and patients with burns or other wounds [1,2]. *Pseudomonas aeruginosa* is also one of the most common pathogens causing nosocomial infection, which can form biofilm adhesion on the surface of various materials and implants.

Biofilms are complex sessile microbial communities living on both biotic or abiotic surfaces that are embed in a matrix of extracellular polymeric substances (EPS) created by the microbial communities themselves [3]. EPS are comprised of polysaccharides, proteins, and lipids that surround the cells, forming a glycocalyx that can prevent the penetration of conventional antibiotics [4,5]. *Pseudomonas aeruginosa* biofilms are thought to be the underlying cause of many chronic and recurrent infectious diseases, making it difficult to treat infections caused by this bacterium. Antibiotics have been found to be ineffective in biofilm-growing bacteria due to inaccessibility of drug molecules permeating into the inner surface of the biofilms [6,7]. Moreover, *Pseudomonas aeruginosa* can form biofilms on every medical device surface. Multiple strategies have been applied to inhibit growth on medical surfaces and treat infections of *Pseudomonas aeruginosa* biofilms, such as quorum sensing disruption, enzymatic dispersal, chlorination, flushing, and UV disinfection. However, there are no effective strategies that can completely against biofilms. For this reason, there is an urgent need for the discovery and the development of antimicrobial agents that present a novel mechanism of action towards the infection [8]. Alternative agents and treatment strategies such as use of palmitic acid from *Synechococcus*, human placental extract, photodynamic therapy, reactive oxygen therapy and combination of antibiotics and peptides have been explored for treating biofilm-associated infections [9–12]. In addition, the use of bacteriophages as a novel antibacterial agent is also a promising therapeutic approach that may be helpful in fighting antibiotic-resistant strains of *Pseudomonas aeruginosa*.

Bacteriophage therapy is designed to treat bacterial infections using bacteriophages that infect bacteria, and has become a promising alternative to conventional antibiotics. Bacteriophages in many ways are advantageous over antibiotics, as they are self-replicating in the presence of host cells, have low inherent toxicity, are highly specific with minimal disruption to normal flora, and disappear with the absence of a host [13]. However, the main disadvantage is that bacteriophages only infect or lyse a limited number of bacterial strains. Therefore, isolation of novel bacteriophages and evaluation of their potential to lyse bacteria or disrupt bacterial biofilms is beneficial towards strengthening

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the antibacterial arsenal. Some bacteriophage therapies have been evaluated experimentally using animal models and clinical trials in recent years to determine their suitability to control *Pseudomonas aeruginosa* infections. These studies have demonstrated a potential therapeutic effect of specific bacteriophages in terms of reducing mortality and morbidity [14–20]. However, successful bacteriophage therapy requires a detailed understanding of bacteriophage-host interactions, in terms of bacteriophage dynamics in natural settings and of bacteriophage resistance in clinical settings.

Quantitative determination of viable bacteria and crystal violet assay are always used to determinate of biofilm characteristics. In this study we isolated a lytic bacteriophage from waste water samples collected from a local hospital; characterized its morphology and one-step growth curve, bacteriophage-host ranges, and bacteriophage-host interactions under surface-associated growth conditions (biofilm). This study thus provides the basis for the evaluation of bacteriophages with therapeutic potential to control biofilms of *Pseudomonas aeruginosa*.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

All bacterial strains used in this study are listed in Table 1. All experiments using strains of *Pseudomonas aeruginosa* were grown either in standard LB-medium (10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1000 mL water) or LB-agar (LB medium + 15 g/L agar).

### 2.2. Bacteriophage isolation and purification

Bacteriophages were isolated from local hospital sewage (Affiliated Zhongshan Hospital of Dalian University) using strains of *Pseudomonas aeruginosa* DLG as previously described by Cao et al. [21] We previously isolated and characterized *Pseudomonas aeruginosa* DLG with a strong biofilm production capacity from lung samples of sacrificed minks. The strain was non-lysogenic bacteria, identified using mitomycin C induction. For purification of individual bacteriophages, individual plaques were picked and purified three times. In order to obtain high bacteriophage stocks, bacteriophage lysates were filtered using a 0.22 μm syringe filter (Millipore 33 mm Millex) and subjected to the treatment of polyethylene glycol (PEG) 8000 (10% wt/vol) and centrifuged at 12,000 × *g* for 10 min at 4 °C. The bacteriophage precipitates were re-suspended in SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris HCl (pH 7.5)) and stored at 4 °C.

### 2.3. Bacteriophage morphology by transmission electron microscopy (TEM)

Bacteriophage morphology was examined by transmission electron microscopy (TEM) [22]. Purified bacteriophage stock was applied onto carbon-coated copper acid grids, stained with 0.5% (w/v) uranyl acetate, and observed using a JEM-2000EX transmission electron microscope (JEOL Co, Tokyo, Japan).

### 2.4. One-step growth curve

A one-step growth curve was performed as described previously by Pajunen et al. [23], with the following modifications. *Pseudomonas aeruginosa* DLG was grown to a mid-exponential phase (OD = 0.6), harvested by centrifugation (10,000 × *g* for 5 min at 4 °C), and re-suspended in LB medium to adjust density (~$10^8$ cfu $mL^{-1}$). Bacteriophages were added to a multiplicity of infection (MOI) of 0.01 and incubated at 37 °C for 10 min to allow bacteriophages to adsorb to the host. Bacteria were pelleted (10,000 g for 5 min at 4 °C) and non-adsorbed bacteriophage particles were discarded. Bacterial pellets were suspended in 10 mL LB medium and incubated at 37 °C with orbital

**Table 1**
Bacterial strains used to determine the host range of phage vB_PaeM_LS1.

| Bacterial strains | Sources | Year | Susceptible to phage challenge |
|---|---|---|---|
| *Pseudomonas aeruginosa* 0212[a] | Mink with hemorrhagic pneumonia | 2002 | + |
| *Pseudomonas aeruginosa* 0205[a] | Mink with hemorrhagic pneumonia | 2002 | + |
| *Pseudomonas aeruginosa* 1-1[b] | Mink with hemorrhagic pneumonia | 2012 | + |
| *Pseudomonas aeruginosa* 5-1-1[b] | Mink with hemorrhagic pneumonia | 2012 | + |
| *Pseudomonas. aeruginosa* 5-2-1[b] | Mink with hemorrhagic pneumonia | 2012 | + |
| *Pseudomonas. aeruginosa* DLB1[b,e] | Mink with hemorrhagic pneumonia | 2015 | + |
| *Pseudomonas aeruginosa* DLG[b,e] | Mink with hemorrhagic pneumonia | 2015 | + |
| *Pseudomonas aeruginosa* PA01[c] | Human | 2016 | + |
| *Pseudomonas aeruginosa* DY-1[c,e] | Human | 2016 | + |
| *Pseudomonas aeruginosa* DY-2[c,e] | Human | 2016 | + |
| *Pseudomonas aeruginosa* DY-3[c,e] | Human | 2016 | – |
| *Pseudomonas aeruginosa* DY-4[c,e] | Human | 2016 | – |
| *Pseudomonas. aeruginosa* DY-5[c,e] | Human | 2016 | + |
| *Pseudomonas. aeruginosa DY-6* [c,e] | Human | 2017 | + |
| *Pseudomonas. aeruginosa DY-7* [c,e] | Human | 2017 | – |
| *Pseudomonas. aeruginosa DY-8* [c,e] | Human | 2017 | – |
| *Pseudomonas. aeruginosa DY-9* [c,e] | Human | 2017 | + |
| *Pseudomonas. aeruginosa DY-10* [c,e] | Human | 2017 | + |
| *Pseudomonas. aeruginosa DY-11* [c,e] | Human | 2017 | + |
| *Pseudomonas. aeruginosa DY-12* [c,e] | Human | 2017 | + |
| *Pseudomonas. aeruginosa DY-13* [c,e] | Human | 2017 | + |
| *Pseudomonas. aeruginosa DY-14* [c,e] | Human | 2017 | + |
| *Pseudomonas. aeruginosa DY-15* [c,e] | Human | 2017 | + |
| *Acinetobacter baumannii DY-13* [c,e] | Human | 2016 | – |
| ETEC *O157:H7* ATCC 351505[d] | The American Type Culture Collection | 2012 | – |
| *Staphylococcus aureus* ATCC 292135[d] | The American Type Culture Collection | 2012 | – |
| *Listeria Monocytogenes* ATCC 191115[d] | The American Type Culture Collection | 2012 | – |

+ +, susceptible; -, resistant.
[a] Kindly provided by institute of special animal and plant sciences of Chinese Academy of Agricultural Science.
[b] Isolated from lung samples of sacrificed minks.
[c] Isolated from clinical samples obtained from burn patients at the first hospital of Dalian Medical University.
[d] Purchased from the American Type Culture Collection.
[e] Multi-drug resistant *Pseudomonas aeruginosa*.

shaking (140 rpm). Samples were taken at 10 min intervals over a period of 2 h, immediately diluted, and plated for bacteriophage titration using plaque assays.

### 2.5. Host range of the bacteriophage

The host range of bacteriophage vB_PaeM_LS1 was determined using a spot test assay as follows. Briefly, Tenfold serial dilutions ($10^1$-$10^8$) of phage lysate were dropped on LB lawns of agar containing a potential *Pseudomonas aeruginosa* host strain. Plates were incubated overnight at 37 °C and assessed for plaque formation. Clear plaque forming were scored as susceptible, no plaque forming as resistant. Details of bacterial strains used in this study were listed in Table 1.

### 2.6. Bacteriophage genome extraction, sequencing, and bioinformatics analysis

Bacteriophage genomes were extracted from purified bacteriophages as previously described [24]. Genomic DNA was sent to Biozeron (China) for sequencing using Illumina Hiseq paired-end platform with read quality evaluated by Trimmomatic-0.33 (http://ubuntu.osuosl.org/ubuntu/pool/universe/t/trimmomatic/). All sequence data was de novo assembled using SOAP denovov v2.04 (http://soap.genomics.org.cn/). Examination of rRNA and tRNA genes were done using RNAmmer-1.2 and tRNAscan-SE v1.3.1. Genes were predicted using GeneMarkS phage (http://topaz.gatech.edu/GeneMark/genemarks.cgi), Rapid Annotation using Subsystem Technology, RAST (http://rast.nmpdr.org/) and predicted ORFs were annotated for specific functions using BLASTP (BLAST 2.2.30 with the E-value lower than 1e-5) with Nr, swiss-Prot, COG, GO database. Bacteriophage virulence factor analysis was conducted using Virulence Factor Database (VFDB). Analyses of closely related bacteriophages was carried out by comparing amino acid sequences of the large subunit terminase using ClustalX v2.0, while construction of neighbor-joining trees were done using MEGA v.7.0.25.

### 2.7. Bacteriophage-biofilm interactions

The effect of bacteriophage vB_PaeM_LS1 on the formation of *Pseudomonas aeruginosa* biofilm was monitored for strain DLG. Biofilms were grown on coverslips placed over a 6-well plate with each well containing LB medium. Overnight culture of *Pseudomonas aeruginosa* DLG was diluted down to $10^6$ cfu $mL^{-1}$ in fresh LB broth. Bacterial dilution (200 μL) and LB medium (1.8 mL) was added to each well of a 6-well plate and incubated at 37 °C under static condition for 48 h. Fresh LB was renewed 24 h after the inoculation. Subsequently, each well was washed three times with 50 mL phosphate-buffered saline (PBS; pH 7.5) and filled with 500 μL bacteriophage suspensions ($1 \times 10^{10}$ pfu $mL^{-1}$) in LB. LB (500 μL) was added to both the positive (established biofilms) and negative controls, with the 6-well microplate being incubated at 37 °C for 4, 8, 12 h. Culture supernatant was removed, and each coverslip was washed three times with PBS.

Crystal violet assay and scanning electron microscopy were used to analyze bacteriophage-biofilm interactions. For crystal violet assay, coverslips were washed with PBS, and the biofilms were stained with 0.1% crystal violet for 15 min. Each coverslip was washed three times with PBS. For quantitation, the stained biofilm was solubilized by adding 2 mL of 33% (vol/vol) glacial acetic acid and the optical density (OD) was measured at 570 nm using a microplate reader (Multiskan Go, Thermo Scientific).

For scanning electron microscopy (SEM) [25], coverslips were fixed in 2.5% glutaraldehyde solution for 4 h and dehydrated using an ethanol alcohol series as follows: 10 min each in 50% and 70%, 15 min each in 80% and 90%, and 20 min in 100%. The samples were dried using critical point dryer (Bal-Tec CPD 030, Balzers, Liechtenstein) and examined using a scanning electron microscope (ZEISS EVO18, Germany) operating at 5 kV.

### 2.8. The live bacterial cells count of biofilm samples

The number of bacteria present on coverslips before and after infection of biofilms was enumerated in order to estimate the efficiency of the bacteriophage. Coverslips with biofilm were washed twice with PBS and placed in 50 mL tubes containing 5 mL of 0.9% NaCl solution. Tubes were vortexed (3 × 30 s) and serial dilutions were immediately performed in 0.9% NaCl solution for all live bacterial cells counts. All samples were immediately plated on LB-agar plates. Live bacterial cells on coverslips were counted at 4, 8, 12 and 24 h post bacteriophage infection. All samples were immediately processed and three replicates were performed.

### 2.9. Evaluation of bacteriophage resistance by Pseudomonas aeruginosa biofilm cells

Biofilm cells that remained on the coverslip surfaces were analysed for resistance similarly to that of Sillankorva et al. [26]. Coverslips, after being treated with bacteriophages for 4, 8, 12 and 24 h, were put in tubes containing 1 mL of saline 0.9%. Dilutions were made and plated on LB-agar plates and incubated overnight. A total of 50 colonies from all the different plates were picked and tested for resistance using spot tests with the bacteriophage. These plates were again incubated overnight at 37 °C and checked for presence of bacteriophage plaques.

### 2.10. Statistical analysis

All the experiments were performed in triplicate with averages and standard deviations calculated for each time-point. Unpaired *t*-test was performed to analyze differences between the two groups of samples. All analyses were performed using Prism 5 (Graph Pad, CA, USA) and values of $p < 0.05$ were considered statistically significant.

## 3. Results

### 3.1. Bacteriophage isolation and morphology

One bacteriophage isolate obtained from the local hospital sewage was designated as vB_PaeM_LS1, which produced large and clear plaques in the plaque assays. TEM examination showed an isometric head structure with a diameter of 70 nm (Fig. 1A). The contractile tail, which consists of a neck, a contractile sheath, and a central tube, has a length of 120 nm. According to the International Committee on Taxonomy of Viruses (ICTV), bacteriophage vB_PaeM_LS1 was classified into *Myoviridae* family in the order *Caudovirales*.

### 3.2. One-step growth curves and host range of the bacteriophage

A one-step growth curve of bacteriophage vB_PaeM_LS1 with its host *Pseudomonas aeruginosa* DLG in LB broth revealed that the latent period was approximately 30 min. The average burst size was 98 phage particles per infected bacterial cell (Fig. 2). Host range assays showed that bacteriophage vB_PaeM_LS1 exhibited antibacterial activity against *Pseudomonas aeruginosa* (see Table 1). Bacteriophage vB_PaeM_LS1 could infect all the *Pseudomonas aeruginosa* strains isolated from mink and 75% (12/16) *Pseudomonas aeruginosa* strains isolated from human, suggesting that bacteriophage vB_PaeM_LS1 has a relative broad host-range.

### 3.3. Features of the vB_PaeM_LS1 genome

The bacteriophage vB_PaeM_LS1 genome is composed of 66,095 bp of linear double-stranded DNA, with 55.7% G + C content. The average gene density was 1.78 genes per 1000 bp. A total of 92 predicted open

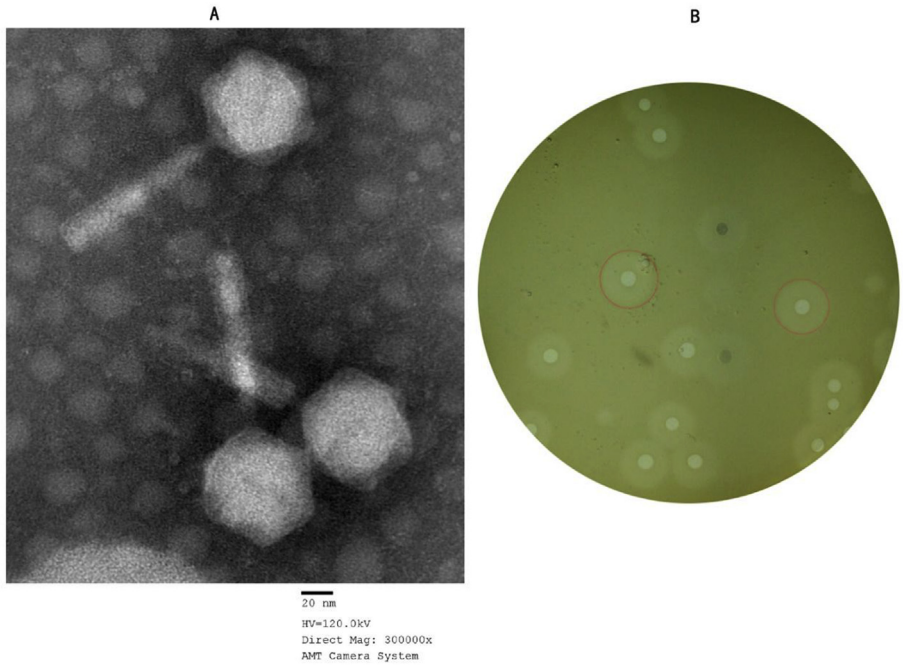


**Fig. 1.** Bacteriophage vB_PaeM_LS1. (A) Transmission electron micrographs of individual bacteriophage vB_PaeM_LS1. (B) Plaques of bacteriophage vB_PaeM_LS1 on host DLG.

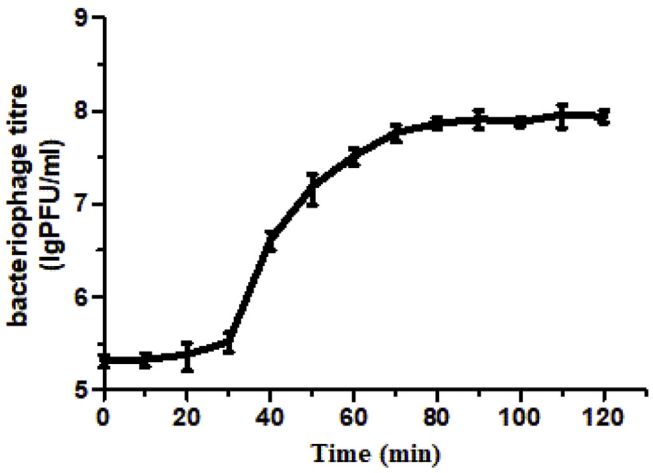


**Fig. 2.** One-step growth curves of vB_PaeM_LS1 bacteriophages in the presence of DLG host.

reading frames (ORFs) and 0 tRNA gene were present. Among the predicted ORFs, only 36 were predicted to have specific functions, with approximately 60% of the predicted proteins being unknown in function. The vB_PaeM_LS1 genome annotation and features are summarized in Fig. 3. Functional ORFs were categorized in several functional modules, including structure protein, DNA replication and regulation, and lysis modules.

The genome of vB_PaeM_LS1 appears to be highly homologous to genomes Pbunavirus genus. The annotated genomes of the bacteriophage showed that the overall ORF organization is very similar to proteins encoded by the Pbunavirus bacteriophages. Neighbor joining analyses of the large subunit terminase of all related *P. myoviruses* reveal that vB_PaeM_LS1 has a high similarity with Pbunavirus bacteriophages (Fig. 4). These results confirm that vB_PaeM_LS1 belongs to a new member of Pbunavirus genus. The genome sequence of bacteriophage vB_PaeM_LS1 has been deposited in the GenBank database, under accession number MG897799.

### *3.4. Bacteriophage vB_PaeM_LS1 disrupts Pseudomonas aeruginosa strain DLG biofilms*

Results from SEM micrographs showed uniformity of densely associated bacteria in the control biofilm; in contrast, infection with bacteriophage vB_PaeM_LS1 disrupted these biofilms, leaving cell debris on the coverslips (Fig. 5).

The antibiofilm activity of bacteriophage vB_PaeM_LS1 was observed by using crystal violet assay. OD570 measurement results of the resolubilized crystal violet suggested that the maximum biofilm reduction occurred when treated with bacteriophage for 8 h (Fig. 6). Further confirming the above results, bacterial counts from coverslips

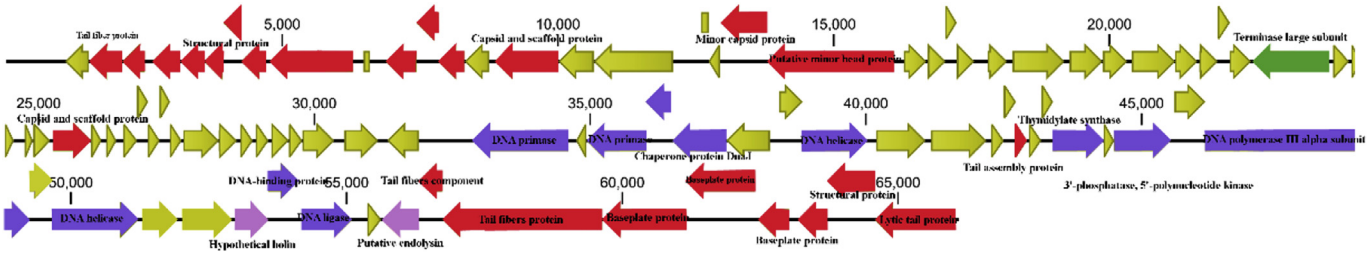


**Fig. 3.** Genomic map of bacteriophage vB_PaeM_LS1 with the genome shown in linear form. Red arrows indicate structure protein, blue arrows indicate DNA replication and regulation, green arrows indicate DNA packing protein, purple arrows indicate cell lysis protein.

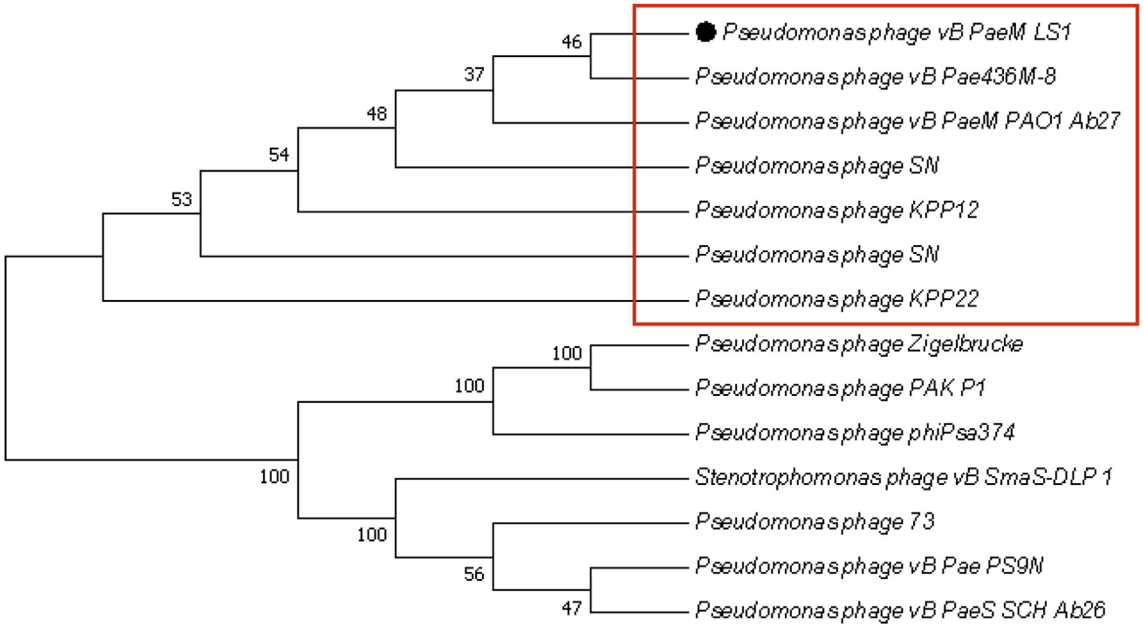


**Fig. 4.** Neighbor-joining tree of terminase large subunits from bacteriophage vB_PaeM_LS1 and the related or similar Pseudomonas myoviruses. Red box represents the Pbunavirus genus. The numbers at the nodes represent the bootstrap probabilities.

before and after bacteriophage addition were measured (Fig. 7). Counting of bacterial colonies indicated the biofilm cells about 93.5% (from 7.2 to 6 log10 CFU $mL^{-1}$) reduction in treated with bacteriophage for 4 h, but the most effective biofilm reduction (99.7%, from 7.3 to 4.8 log10 CFU $mL^{-1}$) was observed in samples taken 8 h after bacteriophage addition. The experimental data within 12 h showed that the bacterial colonies were significantly lower in bacteriophage-mediated groups compared to the control groups. At 24 h, regrowth of biofilm cells was observed, suggesting that certain cells acquired bacteriophage resistance. Results of our subsequent bacteriophage resistance studies indicated that this is indeed the case.

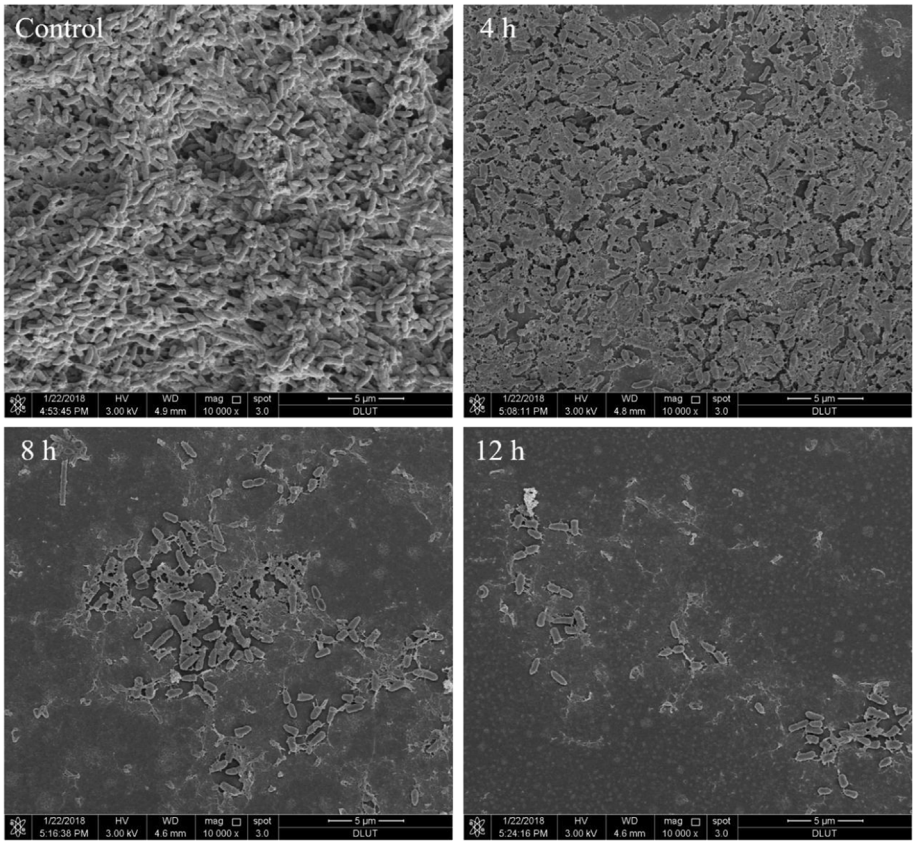


**Fig. 5.** Scanning electron micrograph (SEM) of *Pseudomonas aeruginosa* biofilm under the treatment of lytic bacteriophage vB_PaeM_LS1.

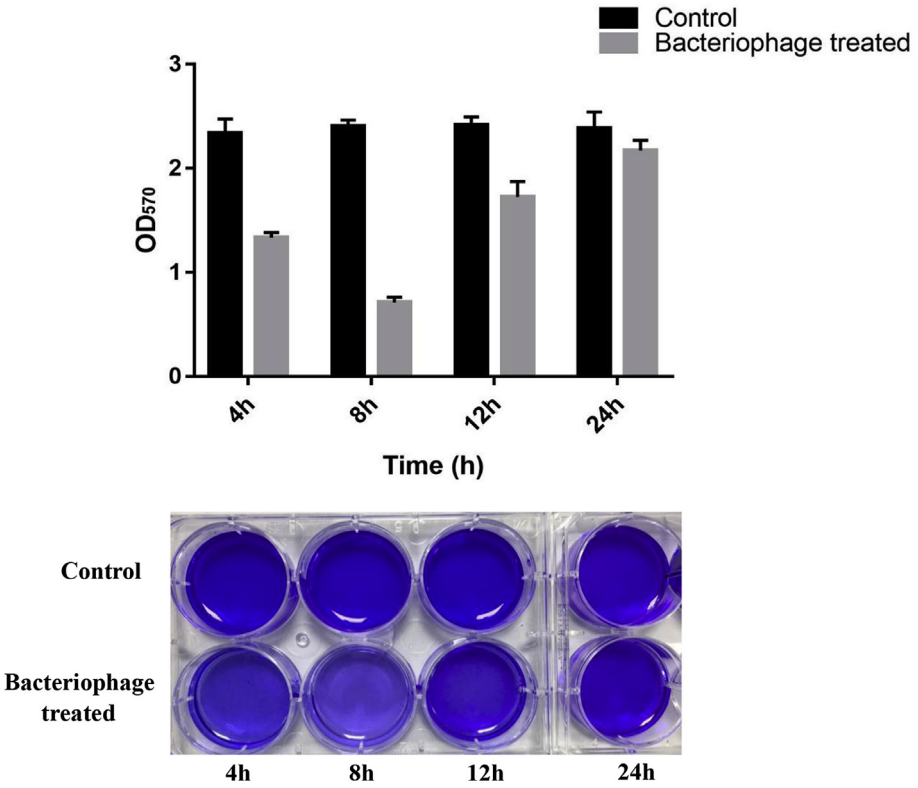


**Fig. 6.** Crystal violet for quantitation of biofilms treated with or without lytic bacteriophage vB_PaeM_LS1.

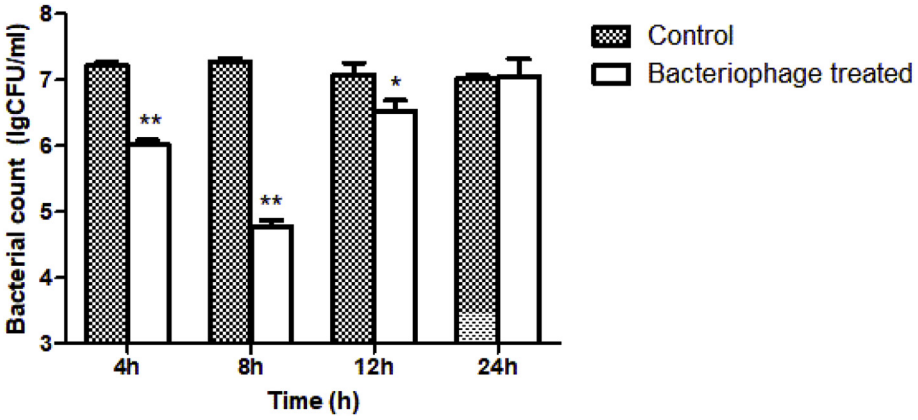


**Fig. 7.** Number of *Pseudomonas aeruginosa* biofilm (48 h age) live cells before and after exposure to bacteriophage vB_PaeM_LS1. Error bars represented the standard deviation **p < 0.01, *p < 0.05 (unpaired *t*-test).

### 3.5. Development of bacteriophage resistance by PA biofilm cells

To determine whether *Pseudomonas aeruginosa* DLG biofilm cells developed resistance to bacteriophage vB_PaeM_LS1, cells from the remaining biofilms were isolated and tested for resistance to bacteriophage vB_PaeM_LS1. None of selected 50 clones were found to have bacteriophage-resistant bacteria at 4, 8, and 12 h after bacteriophage addition. However, 12 out of 50 clones isolated at 24 h were found to develop bacteriophage resistance (data not show).

## 4. Discussion

In this study, *Pseudomonas aeruginosa* bacteriophage vB_PaeM_LS1 was isolated from hospital sewage and was shown capable of forming a clear transparent area with halos on the lawn of bacteria (Fig. 1B). In addition, the halo of the plaques became larger after a few days of incubation, which may link to the lysis function of endolysin [27]. Furthermore, potential lysis of bacteriophages tested on mink and human bacterial strains showed that the bacteriophage could efficiently lyse *Pseudomonas aeruginosa* strains (Table 1). Probably bacteriophage vB_PaeM_LS1 have a vigorous host receptor binding protein. This is also a possibility that there is a high similarity of *pseudomonas aeruginosa* in mink farming and hospitals in Dalian, China. However, characterization and exploration of the bacteriophage vB_PaeM_LS1 host range need to be further investigated. In addition, We should pay more attention to the problem of drug-resistant *pseudomonas aeruginosa* in Chinese mink farms.

Host species specificity is desirable for potent bacteriophage application. However, some narrow-host-range bacteriophages are strain specific. This may be due to the highly specific tailspike protein in the bacteriophages [28,31]. To overcome this limitation, as well as strain resistance, polyvalent bacteriophages (bacteriophages with broad host

ranges) or cocktails containing different bacteriophages would be a good strategy, especially in multiple microbial infections [32].

Based on the morphology and genomic analyses, we suggest that vB_PaeM_LS1 should be a member of the *Pbunavirus* subgroup due to the fact that; (i) it displayed typical *Myoviridae* morphology; (ii) all the structural proteins were found to share high similarity with structural proteins from *Pbunavirus* genus bacteriophages; and (iii) phylogenetic analyses of large subunit terminase of all the related *pseudomonas myoviruses* indicated that vB_PaeM_LS1 is homologous to other Pbunavirus bacteriophages. It is worthwhile to mention that construction of phylogenetic trees for bacteriophages remains a challenge due to the non-existence of marker genes present in all species [33].

Another concern related to bacteriophage therapy is the possibility of transduction, where bacterial virulence genes may be transferred to other bacteria by the bacteriophages, resulting in the transfer of pathogenicity islands (PAIs) and virulence factors leading to the development of a new pathogen or an even more resistant bacterial [34–36]. The genome annotation results showed that there is no gene associated with integrase, suggesting that this bacteriophage is a lytic bacteriophage. Furthermore, a virulence factor analysis of the bacteriophage genome did not reveal the presence of human virulence genes, suggesting that this bacteriophage may be suitable for further therapeutic applications.

Assessing the efficacy of vB_PaeM_LS1 to reduce *Pseudomonas aeruginosa* biofilms, our results indicated the potential of a lytic bacteriophage for preventing and dispersing *Pseudomonas aeruginosa* biofilms formed under static conditions for 48 h. Bacteriophage vB_PaeM_LS1 could greatly reduce the number of biofilm cells present on coverslips within 12 h of infection experiment, with the reduction even more substantial after 8 h of bacteriophage application. The bacteriophage vB_PaeM_LS1 is an interesting biological agent as it has a great ability of lysing biofilm cells in a rapid time.

It was suspected that the bacteria became resistant to the bacteriophages over time. This was evident as biofilm cells became resistant to bacteriophages after long bacteriophage exposure. Our experiments showed that bacteriophage vB_PaeM_LS1 is more effective in the reduction of biofilm over a short period of time, and may not be useful for a longer run due to the emergence of bacteriophage-resistant clones. Further studies are needed to confirm this phenomenon. So some researchers have suggested other new strategies for treating bacterial infections, such as bacteriophages cocktail, combination of bacteriophages and antibiotics, polyvalent bacteriophages or genetically-modified bacteriophages [32,37–40].

Successful application of bacteriophage therapy in the treatment of *Pseudomonas aeruginosa* mediated biofilm infections requires detailed knowledge of the bacteriophage-host interactions and the regulation of anti-bacteriophage resistance in its corresponding bacterial hosts. Our results herein added to the suite of the basis for the evaluation of bacteriophages with therapeutic potential to control *Pseudomonas aeruginosa* biofilms and its potential use in therapeutic applications.

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## Compliance with ethical standards

The authors declare there is no conflict of interest and this article also does not contain any studies with human participants or animals performed by any of the authors.

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