Potential application of a newly isolated phage BUCT609 infecting Stenotrophomonas maltophilia

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Stenotrophomonas maltophilia (S. maltophilia) is widely distributed in nature and frequently causes nosocomial infections. In this work, the biological characteristics and genome of a new S. maltophilia phage BUCT609 isolated from hospital sewage with S. maltophilia strain No. 3015 as host was analyzed and its therapeutic effect in vivo was explored. It was observed by TEM that phage BUCT609 belongs to the Podoviridae with a 10 nm tail structure and a capsid with a diameter of about 50 nm. It has a short latent period (about 10 min) and its burst size is 382 PFU /cell when multiplicity of infection (MOI) is 0.01. Furthermore, it has a high survival rate in the environment with a pH range from 3 to 10 and temperature range from 4°C to 55°C. The complete genome of phage BUCT609 is linear double-stranded DNA of 43,145 bp in length, and the GC content is 58%. The genome sequence of phage BUCT609 shares <45% homology with other phages. No virulence genes and antibiotic resistance genes were found in bacteriophage BUCT609.

In vivo animal experiments showed that the survival rate of mice infected with S. maltophilia was significantly improved after the intranasal injection of phage BUCT609. Therefore, our study supports that phage BUCT609 could be used as a promising antimicrobial candidate for treating S. maltophilia infections.

KEYWORDS
Stenotrophomonas maltophilia, bacteriophage, podoviridae, genomic analysis, phage therapy

Introduction

Stenotrophomonas maltophilia is Gram-negative bacteria that widely exists in nature and also resides in the human respiratory and intestinal tract. The isolation rate of S. maltophilia was only less than Acinetobacter and Pseudomonas aeruginosa among non-fermented glycogram negative bacilli (An and Berg, 2018). As a conditioned pathogen, S. maltophilia is a major pathogenic bacterium for iatrogenic infection that can cause many diseases such as infections of the respiratory tract,
urinary tract, and wounds. In recent years, with the extensive use of broad-spectrum antibiotics, S. maltophilia has become one of the most important pathogens of nosocomial infection, which brings great difficulties to clinical treatment (Gokhan Gozel et al., 2015). Phages are specific for infection and lysis of host bacteria in the environment and organisms. During the recent years, many countries have made exciting achievements in phage therapy (Bao et al., 2020) which demonstrate the unique advantages of phage therapy and greatly promote further research of phage therapy, indicating that phage therapy has a very bright application prospect (Moelling et al., 2018).

In this study, phage BUCT609 was isolated from hospital sewage. The biological and genomic characteristics of phage BUCT609 were analyzed to evaluate the infectivity and animal experiments were conducted to explore its therapeutic effect on S. maltophilia in vivo. This study will increase the diversity of S. maltophilia phage and provide a potential candidate for phage-based therapy.

**Materials and methods**

**Bacterial strains and culture conditions**

S. maltophilia strain No. 3015 in our laboratory bacteria bank was used as the host bacterium of phage BUCT609, which is a clinical strain isolated from Shanghai Public Health Clinical Center. The bacteria were cultured 5 h to reach the exponential phase in Luria-Bertani (LB) medium at 37°C.

**Isolation and purification of phage BUCT609**

Phage BUCT609 was isolated from sewage in the China-Japanese Friendship Hospital of Beijing. Ten milliliters of sewage water were centrifuged at 12,000 × g for 5 min and filtered by membrane filtration with 0.22 μm pore diameter (Ding et al., 2020). The host bacteria were cultured to the exponential phase (OD_{600} = 0.7) at 37°C for phage infection. Then the filtered phage (100 μl) was mixed evenly with 500 μl of the host bacteria. The mixture was added into 5 ml LB liquid medium and incubated at 37°C for 5 h. Phage was diluted by phosphate-buffered saline (PBS) buffer and verified by the standard double-layer agar method. Eventually, a single plaque was picked up and amplified and purified three times (Chang et al., 2005).

To obtain a high titer of phage solution, 8 ml of 30% sucrose solution was slowly added to phage solution (32 ml). After centrifugation at 30,000 × g for 2 h at 4°C, the supernatant was discarded. The phage was re-suspended with 200 μl of PBS buffer.

**Transmission electron microscopy**

The morphological characteristics of phage BUCT609 were observed by transmission electron microscopy (TEM). As reported previously, 10 μl of purified phages were loaded onto a copper grid for 20 min and the phage particles were negatively stained with 10 μl of 2% (w/v) phosphotungstic acid (PTA) for 10 min. The samples were dried for 5 h at room temperature. Morphology of the phage was observed by a transmission electron microscope (JEM-1200EX, Japan) at 80 kV (Li et al., 2019).

**The optimal multiplicity of infection**

MOI refers to the ratio of the number of phages (PFU) to the host bacteria (CFU) and the optimal MOI is the multiplicity of infection when the phage can achieve the optimal growth state (Xing et al., 2017). Five hundred microliters of each purified phage and the host bacteria were mixed (MOI = 100, 10, 1, 0.1, and 0.01) evenly and incubated for 20 min. Then the mixture was added into fresh sterile LB liquid medium for 5 h at 37°C. After incubating, the mixture was centrifugated (12,000 × g, 5 min) and filtered to obtain the phage in the supernatant. The phage titer was determined by the standard double-layer agar method and the MOI with the highest titer is the optimal MOI.

**One-step growth curve**

To evaluate the infectivity of phage BUCT609 with the latent period and the burst size, it is necessary to study the one-step growth curve of the phage (Hagens and Loessner, 2010). The latent period is defined as the stage in which the phage proliferates in the bacterial cells. The burst size refers to the ratio of the ultimate titer of phage to the number of infected bacteria in the latent period. Phage BUCT609 was mixed with S. maltophilia strain No. 3015 (OD_{600} = 0.7) to the optimal MOI and allowed to adsorb at 37°C for 20 min. Then the mixture was centrifugated (12,000 × g, 1 min). The sediment containing phage was resuspended with 20 ml of LB liquid medium. The phage titer was determined by the soft agar overlay method every 10 min during the incubation at 37°C. Three replicates were performed and the results were averaged.

**Thermal stability and pH sensitivity**

To test the thermal stability of phage BUCT609, the purified phage was incubated at different temperatures (4, 37, 45, 55, 65, or 75°C) for an hour. Then, the phage titer was calculated by the method of soft agar overlay (Fan et al., 2012). Similarly, to explore the effect of pH on phage BUCT609, the phage suspensions were incubated at various pH values ranging from 1 to 12 at 37°C for an hour and the phage titer was calculated. Each experiment was...
performed three times and the initial phage concentration was about 10⁶ PFU/ml.

**Multilocus sequence typing and host range**

Multilocus sequence typing (MLST) is a bacterial typing method based on nucleic acid sequence determination (Larsen et al., 2012). In this experiment, multiple internal fragments of housekeeping genes were amplified by PCR and sequenced to analyze the variation of strains. Using housekeeping gene primers of *S. maltophilia* (atpD, gapA, guaA, mutM, nuoD, psaA, and recA) for PCR amplification (Supplementary Table 1) and the amplified products were sequenced and analyzed on the MLST database.

To determine phage BUCT609 host range, 13 strains of *S. maltophilia* were cultured to the exponential phase (OD₆₀₀ = 0.7). 500μl of each sample were added into 5 ml 0.7% LB agar medium. Then, the mixture was immediately poured into the solid medium. After solidification, 2μl phages were added to a double-layer plate, and PBS was added on the other side as a control. The formation of plaque was observed and recorded after 9 h.

**Antimicrobial susceptibility testing**

To quickly and effectively detect the sensitivity of pathogenic bacteria to various antibiotics and guide rational drug use in the clinic, the drug sensitivity test of *S. maltophilia* strain No. 3015 was carried out by disc diffusion method. The host bacteria were cultured to the exponential phase (OD₆₀₀ = 0.7). 500μl of each sample were added into 5 ml 0.7% LB agar medium. Then, the mixture was immediately poured into the solid medium. After solidification, the antibiotic sensitivity testing disc was placed on the plate for incubating at 37°C overnight. If the strain was sensitive to the drug, the formation of the inhibitory zone could be observed. This experiment was repeated three times.

**Phage DNA preparation**

Phage genomic DNA was extracted by the Proteinase K/SDS method (Pickard, 2009). 600μl of purified phage were incubated with DNase I and RNase A overnight at 37°C after which the enzymes were inactivated at 80°C for 15 min. 24μl of EDTA (20mM) with 1.5μl of proteinase K (50μg/ml) and 30μl of SDS (0.5%) were added to the mixture and were incubated at 56°C for 1 h. Phage DNA was extracted with an equal volume of extraction buffer (0.5%) were added to the mixture and were incubated at 56°C for 20 min. Phage DNA was extracted by the Proteinase K/SDS method (Pickard, 2009). 600μl of purified phage were incubated with DNase I and RNase A overnight at 37°C after which the enzymes were inactivated at 80°C for 15 min. 24μl of EDTA (20mM) with 1.5μl of proteinase K (50μg/ml) and 30μl of SDS (0.5%) were added to the mixture and were incubated at 56°C for 1 h. Phage DNA was extracted with an equal volume of extraction buffer (0.5%) were added to the mixture and were incubated at 56°C for 20 min.

Whole genome sequencing and bioinformatics analysis

The whole genome was sequenced using Illumina’s MiSeq sequencing platform (Thermo Fisher Scientific, United States; Koizich et al., 2013) and the low-quality sequences were filtered by Trimomatic (V0.32) program (Bolger et al., 2014). Then, the complete genomic sequence of BUCT609 was assembled by Newbler V3.0 software (Roche, Switzerland; Zhang et al., 2012) and CLC software (QIAGEN, Germany; Liu and Di, 2020). Using the online tools RAST (https://rast.nmpdr.org/; Aziz et al., 2008) to predict the ORFs and ORF Finder (https://www.bioinformatics.org/sms2/orf_find.html; Rombel et al., 2002) to annotate the DNA sequencing result. Sequence similarity analyses and comparisons were performed using the NCBI BLAST algorithm. A phylogenetic tree of phage BUCT609 was conducted by VICTOR (https://ggdc.dsmz.de/victor.php; Meier-Kolthoff and Göker, 2017) and average nucleotide identity (ANI) analysis was analyzed by another online tool VIRIDIC (http://rhea.icbm.uni-oldenburg.de/VIRIDIC/; Moraru et al., 2020).

**Antibacterial effect of BUCT609 in vivo**

Specific pathogen-free (SPF) Balb/c mice reared for 6–8 weeks, weighing 17-19 g, were chosen as the animal model in this study. The median lethal dose (LD50) refers to the minimum number of bacteria required to kill half of the animal population through a specified route of infection within a specified period of time (Welkos and Brien, 1994). Pick a single clone of *S. maltophilia* No.3015 strain on the agar plate and cultivate it in LB liquid medium to exponential phase (37°C, 220 rpm). Then, the bacteria were centrifuged at 10,000×g for 10 min and resuspended with PBS diluting to 8×10⁷ CFU/ml, 6×10⁶ CFU/ml, 4×10⁵ CFU/ml, and 2×10⁴ CFU/ml, respectively, for use. Forty mice were randomly divided into 5 groups (n = 8 in each group), of which 4 groups were used as the experimental group (injected with bacteria) and 1 group was used as the control group (injected with PBS). All mice were immunosuppressed by injection of cyclophosphamide (125 mg/kg) 4 days before infection and injected with cyclophosphamide (125 mg/kg) and dexamethasone (12.5 mg/kg) next day to stabilize the immunosuppression. After above pretreatment, each mouse was anesthetized by intraperitoneal injection of 50 mg/kg of 0.5% sodium pentobarbital. The experimental groups were injected intranasally with 40μl of No.3015 bacterial solution with titers of 8×10⁷ CFU/ml, 6×10⁶ CFU/ml, and 2×10⁵ CFU/ml, respectively, and the control group was injected with 40μl of sterile PBS solution. The activity status of mice was observed twice a day after infection. The changes in body weight and death situation were recorded.

To explore the therapeutic effect of phage BUCT609 in vivo, 30 mice were equally divided into three groups (n = 10 in each
group), two of which were the experimental group (injected with both bacteria and phage) and one was the control group (injected with PBS only). Mice were immunosuppressed and anesthetized in the same manner as described above. 40 μl of No.3015 bacterial solution was injected into the nasal cavity of the experimental group and 40 μl of PBS solution was injected into the control group. After 2 h, 40 μl of phage BUCT609 was injected into one of the experimental groups, 40 μl sterile of PBS solution was injected into other groups. The activity status of mice was observed daily after infection and the changes in body weight and death situation were recorded.

Statistical analysis

Three replicates were performed for each experiment. Take the mean of the three sets of data and calculate the standard error. The data were subjected to one-way ANOVA test and Tukey test by GraphPad Prism 8.0.2 (GraphPad Software, Inc., La Jolla, United States). p < 0.05 means that the data of repeated groups are statistically significant.

Results

Morphology

A virulent phage BUCT609 was successfully isolated from untreated sewage in the hospital using S. maltophilia No. 3015 as the host bacterium. Phage BUCT609 can form transparent plaques with a diameter of 1 ~ 2 mm on a double-layer plate (Figure 1A). TEM images showed that the head diameter of phage BUCT609 was about 51.93 ± 1.68 nm, and the tail length was about 12.36 ± 1.37 nm, which can be inferred as a Podoviridae phage (Figure 1B).

The optimal multiplicity of infection and One-step growth curve

Phage and host bacteria were cocultured for 5 h at different MOI ratio as shown in Supplementary Table 2 and the average titer of each phage was measured, respectively. As can be seen from Supplementary Table 2, when MOI = 0.01, the phage titer was the highest with 3.1 x 10⁹ PFU/ml. Therefore, the optimal multiplicity of infection was defined as 0.01.

The one-step growth curve showed that the latent period was about 10 min (Figure 2A). In the following 60 min, the phage titer increased rapidly, where was the burst period. About 10 min later, the phage grew into the plateau phase. According to the calculation, phage BUCT609 had a large burst size (382 PFU/cell). Compared with the phage P24 (the latent period was 55 min and the burst size was 147 PFU/cell), phage BUCT609 had a shorter latent period and a larger burst size (Zhang et al., 2020). In conclusion, it was a lytic phage and had the potential to be used for phage therapy.

Thermal stability and pH sensitivity

The thermal stability test showed that phage BUCT609 was high-temperature sensitive (Figure 2B). There was no significant difference in the titer between 4 and 55°C for 1 h. At 65°C, phage titer decreased significantly (p < 0.0001) compared with that at 4°C and it was completely inactivated at 75°C. As for pH tolerance (Figure 2C), phage BUCT609 exhibited a strong tolerance from pH 3 to 10. At pH 2 or 11, the phage titer was significantly lower than at pH 7 (p < 0.0001). And at pH 1 or 12, the phage was almost completely inactivated. Compared with phage IME392 (temperature tolerance range: 30–50°C; pH tolerance range: 4–11; Hu et al., 2021), phage BUCT609 had a wider adaptation range of temperature and pH, indicating that it had a good temperature and pH tolerance and could be broadly applied for phage therapy in the future.

MLST and host range

The MLST results showed that 13 strains of S. maltophilia had different ST values, suggesting that they were 13 different species. The analysis of the host range results showed that phage BUCT609 could lyse not only S. maltophilia strain No. 3015 but also the other 4 S. maltophilia strains (No. 118, No. 548, No. 992, and No. 1207; Supplementary Table 3).

Antimicrobial susceptibility testing

The main reason S. maltophilia infection is difficult to treat is that the bacteria present low susceptibility to antibiotics (Gil-Gil et al., 2020). Drug susceptibility tests confirmed that the host S. maltophilia No. 3015 of phage BUCT609 was resistant to a variety of antibiotics but sensitive to only a few antibiotics such as ceftriaxone, tetracycline, trimethoprim, minocycline, and levofloxacin (Supplementary Table 4).

Characterization of phage BUCT609 genome

The complete sequence of phage BUCT609 was 43,145 bp in length and the CG content of the genome was 58% that had been submitted to the NCBI database with the accession number MW960043. The online comparison tool BlastN showed that it had 42% homology with S. maltophilia phage vB_SmeS_BUCT703 (GenBank: OM735688.1) at nucleotide level. The results of RAST online annotation showed that BUCT609 had 56 open reading frames (ORFs), of which 25 had known functions and the rest
were annotated as hypothetical proteins. The majority of ORFs presented an ATG start codon (87.5%) while 2 of them started with TTG and 5 with GTG. There is no tRNA in the whole genome. The 25 functional proteins are shown in different colors on the whole genome map (Figure 3).

**Functional ORF analysis**

Same as most dsDNA phage, phage BUCT609 has the same modular genomic structure such as DNA replication, regulation, phage packaging, structural, and host lytic proteins (Table 1). The structural proteins of phage BUCT609 are mainly distributed at the front of the genomic sequence. According to previous data analysis, ORF6, ORF8, ORF9, ORF13, and ORF14 were predicted to be tail related proteins. ORF14 encodes tail tubular protein A (TTPA) that is usually responsible for adhering the phage to host cells (Pyra et al., 2017), having 62.98% homology to the protein of *S. maltophilia* phage Ponderosa at amino acid level (the following homology values are all at amino acid level in this section). Besides, the major capsid protein was encoded by ORF16 and had 75.62% homology with that of *S. maltophilia* phage Ponderosa.

Terminase is a key protein in phage DNA packaging that includes large subunit and small subunit. Generally, terminase large subunit and terminase small subunit are adjacent and both involved in the splicing and packaging process of phage DNA. The large subunit is responsible for ATP-driven DNA translocations while the small subunit interacts with terminase large subunit and initiate packaging through binding and cleaving specifically near the initial packaging site (Weiditch et al., 2019, Gao et al., 2020). Terminase small subunit specifically recognizes viral DNA, while the terminase large subunit plays an important role in ATP recognition and hydrolysis (Loredo-Varela et al., 2013). As is shown from the chart, ORF3 from phage BUCT609 encodes the terminase large subunit that has 69.26% homology with that of *S. maltophilia* phage Ponderosa. The terminase small subunit is the product of ORF14 and exhibited 39% identity to that of *Xanthomonas* phage Xaa_vb_phi31. The portal control protein is encoded by ORF18 and forms a channel at the phage tail attachment site through where the phage can inject its genome into the host cell (Prevelige Jr and Cortines, 2018).

Replication is a complex process involving a variety of proteins and enzymes. In the initiation phase, the DNA helicase encoded by ORF33 can unlock double-stranded DNA by hydrolyzing ATP for energy, with 63.53% homology to that of *S. maltophilia* phage Ponderosa. DNA primer enzyme encoded by ORF35 has 63.44% homology with that of *Xanthomonas* phage Xaa_vb_phi31, which can catalyze the synthesis of RNA primer. Different DNA polymerases play different roles in DNA replication (Hoitsma et al., 2020). Exhibiting 69.95% identity to DNA polymerase of *S. maltophilia* phage Ponderosa, the DNA polymerase encoded by ORF30 is mainly used for DNA replication and repair. Furthermore, exonuclease is a class of enzymes that degrade nucleotides one by one from the end of a polynucleotide chain (Dean et al., 2001). In phage BUCT609, ORF26, ORF27, and ORF28 encode three different exonuclease enzymes, with 44.4% homology to that of *Xylella* phage Cota, 65% homology to that of *Xanthomonas* phage Xaj24 and 65.12% homology to that of *Xanthomonas* phage XAA VB phi31, respectively. The DNA ligase encoded by ORF23 plays an important role in the process of DNA replication and repair, catalyzing the reaction that two adjacent bases connect consuming ATP and has 42.42% homology to the protein of *S. maltophilia* phage Ponderosa by comparison (Shi et al., 2018). ORF21 encodes RNA polymerase whose main function is to synthesize RNA using DNA or RNA as templates and triphosphate ribonucleoside as substrates. Due to that RNA is involved in the transcription of genetic information about genes within a cell, it is also called a transcriptase (Griesenbeck...
et al., 2017). The RNA polymerase ORF21 has 100% coverage but only 72% identity with that of *S. maltophilia* phage Ponderosa.

For the lytic mechanism, the phage lysed the host bacteria under the combined action of holin, lyase, and spanin. Holin encoded by OFR5 plays a crucial role in the penetration of phages after attaching to the host. Holin forms pores in the cell membrane and begins the process of osmosis and dissolution (Palmer et al., 2021). By the BlastN comparison, the holin of phage BUCT609 has 59.68% homology with *S. maltophilia* phage Ponderosa. Most phages produce a two-component protein complex consisting of outer membrane lipoprotein (o-spanin) and inner membrane protein (i-spanin) which are necessary for the host outer membrane destruction (Cahill and Young, 2019). Phage BUCT609 has an o-spanin ORF54 with 57.14% homology to the protein of *S. maltophilia* phage Ponderosa and i-spanin ORF55 with 30.85% homology to the protein of *Xylella* phage Paz. The endolysin transcribed by ORF56, which showed 65.06% identity to that of *S. maltophilia* phage Ponderosa, was synthesized by host bacteria and can result in bacteria death through inducing the lysis of bacteria cell walls specifically and effectively (Ghose and Euler, 2020).

**Phylogenetic analysis**

A total of 21 phages in NCBI had homology (>0%) with BUCT609. To study the evolution of phage BUCT609 and its relationship with other phages, a phylogenetic tree of complete genome sequences of above 22 phages including BUCT609 was constructed. According to VICTOR's classification of family, genus, and species, 21 strains of phages homologous to phage BUCT609 belonged to the same genus. From NCBI records, all of these strains were presumed to be *Autographiviridae* family and *Pradovirus* genus. However, at the species level, there were 16 different clusters, among which BUCT609 independently represented a species.
To determine whether phage BUCT609 could form an independent clade as a new genus, average nucleotide identity (ANI) analysis with other 21 phages was analyzed. The results showed that BUCT609 had a maximum similarity with Stenotrophomonas phage vB_SmaS_P15 (57.3%), which was sufficient for classification at the level of a new genus (Figure 4B).

Antibacterial effect of BUCT609 in vivo

We conducted animal experiments to further explore the possibility of phage BUCT609 for clinical treatment. In mouse models, there was no significant change before and after No.3015 infection (fluctuation within 1 gram). Remarkably, the hair of the mice before bacterial infection was white and shiny while sparse and coarse after infection. Besides, they appeared weaker and clustered together, suggesting that S. maltophilia infections could have adverse effects on the organism. The results of infection in mice with different doses were shown in Figure 5A. No mice died in the control group. The survival rate of mice gradually decreased with the increase of injected bacterial titer. When the titer of No.3015 was $4 \times 10^7$ CFU/ml, the survival rate of mice was 50%, which was defined as the value of LD50.

The effect of phage BUCT609 in lysing host bacteria in vivo was studied by injecting phage BUCT609 to mice infused with LD50. The vital signs of all mice stabilized after 3dpi after the treatment (Figure 5B). After injection of phage BUCT609, the survival rate of mice increased to 80%, which was 30% higher than that of the other group of mice that did not receive treatment. There was no significant change in the weight of
| ORFs | Start | Stop | Strand | ORFs | Start | Stop | Strand | ORFs | Start | Stop | Strand | ORFs | Start | Stop | Strand | Predicted Function | Best-match BLASTp Results | Accession number | E-values | Cover | Identity |
|------|-------|------|--------|------|-------|------|--------|------|-------|------|--------|------|-------|------|--------|------------------|--------------------------|----------------|----------|-------|----------|
| ORF1 | 897   | 130  | R      | hypothetical protein | Xylella phage Paz | YP_008858922.1 | 1.00E-68 | 96% | 53.36% |
| ORF2 | 1366  | 911  | R      | hypothetical protein | Xanthomonas phage Xaa_vB_phi31 | QO169551.1 | 1.00E-40 | 99% | 50.65% |
| ORF3 | 3165  | 1375 | R      | terminase large subunit | Stenotrophomonas phage Ponderosa | QEG09767.1 | 0 | 99% | 69.26% |
| ORF4 | 3448  | 3149 | R      | terminase small subunit | Xanthomonas phage Xaa_vB_phi31 | QO169549.1 | 3.00E-09 | 100% | 39.00% |
| ORF5 | 3626  | 3438 | R      | holin class II | Stenotrophomonas phage Ponderosa | QEG09765.1 | 1.00E-16 | 98% | 59.68% |
| ORF6 | 4206  | 3628 | R      | tail fiber protein | phage Titan-X | QGH45075.1 | 1.00E-36 | 99% | 38.78% |
| ORF7 | 5216  | 4203 | R      | hypothetical protein | Xanthomonas phage Xaa_vB_phi31 | QGH45074.1 | 8.00E-58 | 100% | 38.76% |
| ORF8 | 6424  | 5213 | R      | tail fiber protein | Stenotrophomonas phage Ponderosa | QEG09762.1 | 1.00E-134 | 70% | 66.67% |
| ORF9 | 7540  | 6425 | R      | tail fiber protein | Stenotrophomonas phage Ponderosa | QEG09761.1 | 0 | 100% | 80.65% |
| ORF10 | 11683 | 7601 | R      | transglycosylase | Stenotrophomonas phage Ponderosa | QEG09760.1 | 0 | 100% | 69.15% |
| ORF11 | 14140 | 11696 | R | internal virion protein | Stenotrophomonas phage Ponderosa | QEG09759.1 | 0 | 97% | 49.69% |
| ORF12 | 14979 | 14149 | R | internal virion protein | Stenotrophomonas phage Ponderosa | QEG09758.1 | 7.00E-94 | 99% | 51.99% |
| ORF13 | 17519 | 14979 | R | tail tubular protein B | Stenotrophomonas phage Ponderosa | QEG09757.1 | 0 | 100% | 73.32% |
| ORF14 | 18149 | 17529 | R | tail tubular protein A | Stenotrophomonas phage Ponderosa | QEG09756.1 | 7.00E-91 | 99% | 62.98% |
| ORF15 | 18418 | 18203 | R | hypothetical protein | Xanthomonas phage Xaa_vB_phi31 | QO169538.1 | 4.00E-14 | 100% | 55.56% |
| ORF16 | 19464 | 18463 | R | major capsid protein | Stenotrophomonas phage Ponderosa | QEG09754.1 | 2.00E-169 | 99% | 72.62% |
| ORF17 | 20248 | 19493 | R | scaffold protein | Xylella phage Paz | YP_008858906.1 | 2.00E-54 | 96% | 44.94% |
| ORF18 | 21762 | 20245 | R | portal protein | Stenotrophomonas phage Ponderosa | QEG09752.1 | 0 | 98% | 71.20% |
| ORF19 | 22094 | 21759 | R | hypothetical protein | Stenotrophomonas phage Penderosa | QEG09751.1 | 4.00E-34 | 91% | 72.53% |
| ORF20 | 22384 | 22205 | R | hypothetical protein | Xylella phage Paz | YP_008858903.1 | 6.00E-15 | 100% | 57.63% |
| ORF21 | 24947 | 22548 | R | RNA polymerase | Stenotrophomonas phage Ponderosa | QEG09749.1 | 0 | 100% | 71.62% |
| ORF22 | 25189 | 24953 | R | hypothetical protein | not hits | | | |
| ORF23 | 26232 | 25186 | R | DNA ligase | Stenotrophomonas phage Ponderosa | QEG09747.1 | 1.00E-75 | 99% | 42.42% |
| ORF24 | 26592 | 26323 | R | hypothetical protein | Stenotrophomonas phage Ponderosa | QEG09745.1 | 4.00E-15 | 96% | 50.00% |
| ORF25 | 27173 | 26589 | R | hypothetical protein | Xanthomonas phage Xaa_vB_phi31 | QO169527.1 | 1.00E-46 | 100% | 41.59% |
| ORF26 | 28025 | 27183 | R | Phage exonuclease | Xylella phage Cota | CAB1282933.1 | 6.00E-83 | 98% | 44.40% |
| ORF27 | 28264 | 28022 | R | DNA exonuclease | Xanthomonas phage XAJ24 | YP_009785928.1 | 2.00E-27 | 100% | 65.00% |
| ORF28 | 29363 | 28413 | R | exonuclease | Xanthomonas phage Xaa_vB_phi31 | QO169524.1 | 4.00E-139 | 95% | 65.12% |

(Continued)
the mice before and after phage treatment (fluctuation within 1 gram). Even though the survival rate of the mice improved after the treatment, the coarseness of the hair was not cured. However, the data were sufficient to demonstrate that phage BUCT609 was bacteriostatic in vivo and could be used as a potential clinical antimicrobial agent.

**Discussion**

Phage BUCT609 was successfully isolated from hospital sewage using *S. maltophilia* No. 3015 as the host bacterium. The host range test showed that 5 of 13 strains of *S. maltophilia* could be lysed by phage BUCT609. Though it does not have a broad host range as
phage DLP3, one of the most widespread S. maltophilia phages infecting 22 out of 29 S. maltophilia strains (Peters et al., 2020), BUCT609 still have a relatively wide host range compared with phage AXL3 that can lyse 5 out of 29 S. maltophilia strains (McCutcheon et al., 2020). In previous studies, phage SM1 was found to be effective in animal S. maltophilia infection model with 184 PFU/cell burst size (Zhang and Li, 2013). However, the burst size of phage BUCT609 is 382 PFU/cell, which was much higher than that of SM1. Meanwhile, given its temperature stability and wide pH tolerance, BUCT609 shows promising potential to use against multi-drug resistant S. maltophilia infections. In addition, phylogenetic tree and ANI results showed that phage BUCT609 is an independent new clade that can be classified in subsequent phage discoveries.

For Autographiviridae phages that can lyse Gram-negative bacteria, there are two systems for lysing bacteria: the holin-endolysin and pinholin-SAR endolysin pathways (Cahill and Young, 2019). In the former, holin first forms micron-scale pores in the inner membrane, releasing active endolysin into the periplasm to degrade peptidoglycan, thus completing the first step of lysing bacteria. According to genome analysis, the cleavage mechanism of phage BUCT609 was mainly mediated by holin protein encoded by ORF5. Endolysin ORF56 could promote the lysis of host bacteria as well. At present, there are many studies on holin protein, showing that holin protein can be used as a new antibacterial agent. HolGH15, the holin protein in Staphylococcus aureus phage GH15, can cause changes in the structural properties of Listeria monocytogenes leading to shrinkage, resulting in the release and removal of cellular contents and ultimately leading to the host death. HolGH15 (the final concentration: 240μg/ml) can reduce L. monocytogenes (the initial concentration: 10^6 CFU/ml) to undetectable levels at 4°C (Song et al., 2021), which shows that the holin protein of phage can potentially become a new type of antibacterial drug by spraying or soaking. BUCT609 can provide a new antibacterial agent in the field of antibacterial as well.

However, phage therapy has many challenges such as safety, ethics, intellectual property rights, and stability (Skurnik et al., 2007). Although this study initially explored the therapeutic effect of phage BUCT609 in mice, the stability of its treatment was not explored. In vivo experiments demonstrated that phages can reduce the bacterial load and weight of abscesses (Capparelli et al., 2007). At the same time, increasing evidence suggests that phages may have a major impact on the immune system by interacting with macrophages, neutrophils, and T-cell polarization (Chechushkov et al., 2021). Phage therapy as a potential regimen against drug-resistant bacteria still requires adequate clinical trials.

**Conclusion**

Phage therapy has become a new possibility to treat clinically drug-resistant bacteria. The diversity of phages may contribute to the development of phage-based therapies. In vivo animal studies...
demonstrated that phage BUCT609 as a new member to the S. maltophilia bacteriophage provided empirical data for phage cocktail therapy in combination with antibiotics against multi-drug resistant bacterial infections.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The animal study was reviewed and approved by the Seventh Medical Center of the PLA General Hospital.

Author contributions

KH: data curation, writing – original draft, investigation, and validation. YD: data curation, writing – original draft, investigation and validation. LS: supervision and writing – review and editing. ML: supervision, validation, writing – review and editing. HF: conceptualization, methodology, supervision, validation, and writing – review and editing. YT: conceptualization, supervision, validation, and writing – review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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