The isolation and characterization of Stenotrophomonas maltophilia T4-like bacteriophage DLP6

Danielle L. Peters¹, Paul Stothard², Jonathan J. Dennis¹*

¹ 6-065 Centennial Centre for Interdisciplinary Science, Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada, ² Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada

* jon.dennis@ualberta.ca

Abstract

Increasing isolation of the extremely antibiotic resistant bacterium Stenotrophomonas maltophilia has caused alarm worldwide due to the limited treatment options available. A potential treatment option for fighting this bacterium is ‘phage therapy’, the clinical application of bacteriophages to selectively kill bacteria. Bacteriophage DLP6 (vB_SmoM-DLP6) was isolated from a soil sample using clinical isolate S. maltophilia strain D1571 as host. Host range analysis of phage DLP6 against 27 clinical S. maltophilia isolates shows successful infection and lysis in 13 of the 27 isolates tested. Transmission electron microscopy of DLP6 indicates that it is a member of the Myoviridae family. Complete genome sequencing and analysis of DLP6 reveals its richly recombined evolutionary history, featuring a core of both T4-like and cyanophage genes, which suggests that it is a member of the T4-superfamily. Unlike other T4-superfamily phages however, DLP6 features a transposase and ends with 229 bp direct terminal repeats. The isolation of this bacteriophage is an exciting discovery due to the divergent nature of DLP6 in relation to the T4-superfamily of phages.

Introduction

The spread and increasing incidence of antibiotic resistance is imminent, with experts suggesting we will face a “post-antibiotic era” in the 21st century [1]. The situation has recently become dire, as a new mechanism of antibiotic resistance towards one of the globally-recognized ‘last-resort’ antibiotics colistin has evolved [2]. The extremely antibiotic resistant bacterium Stenotrophomonas maltophilia has been increasingly identified as a causative agent in both nosocomial and community-acquired infections [3]. Infections associated with S. maltophilia include, but are not limited to pneumonia, bacteremia, meningitis, endocarditis, catheter-related bacteremia/septicemia and acute exacerbations in patients with cystic fibrosis and chronic obstructive pulmonary disease [3, 4]. Due to the ubiquitous nature of S. maltophilia in the environment and the possibility of spreading this bacterium through cough-generated aerosols, infection prevention has proven to be difficult [3, 5]. Once infected with S. maltophilia, treatment options are limited due to its innate resistance to a broad array of antibiotics.
including trimethoprim/sulfamethoxazole, β-lactams, macrolides, cephalosporins, fluoroquinolones, aminoglycosides, carbapenems, chloramphenicol, tetracyclines, and polymyxins. Due to the limited treatment options, alternative strategies are being investigated in order to combat this extremely drug resistant bacterium.

The clinical application of bacteriophages to selectively kill infecting bacteria, known as "phage therapy", is a potential solution to extremely antibiotic resistant bacteria. During the Second World War, Soviet and German armies utilized phage therapy to treat dysentery of their soldiers, while the United States military conducted classified research on it [6]. Additionally, some clinicians worldwide continued to use phage therapy from the 1920s to the early 1950s [6]. Unfortunately, with the advent of broad-spectrum antibiotics and a limited understanding of phage biology, phage therapy was largely abandoned in the West. However, with the recent significant rise in antibiotic resistance in bacterial pathogens throughout the world, interest in the efficacious use of phage therapy has been renewed. Recent studies utilizing phage therapy to treat multi-drug resistant infections in animal models [7–16] and human clinical trials [17–20] have shown that phages can be a successful treatment option. To use phages in the clinical treatment of infections, the U.S. Food and Drug Administration requires characterization of the phages to prove they do not include moronic genes encoding toxins or other undesirable proteins which could enhance bacterial virulence [21]. Therefore, all phages isolated for use in clinical therapy must be fully characterized through complete genome sequencing and functional analysis. Towards that goal, the isolation and characterization of the novel S. maltophilia phage vB_SmoM-DLP6 (DLP6) is described herein. This phage is related to T4-superfamily of phages and exhibits an interesting combination of T4 and cyanobacteriophage genes.

Materials and methods

Bacterial strains and growth conditions

Five S. maltophilia strains were acquired from the Canadian Burkholderia cepacia complex Research and Referral Repository (CBCRRR; Vancouver, BC). The S. maltophilia strains used for isolation of phage from soil samples were D1585, D1571, D1614, D1576 and D1568. An additional 22 S. maltophilia strains were gifted from the Provincial Laboratory for Public Health—North (Microbiology), Alberta Health Services, for host range analysis. All strains were grown aerobically overnight at 30°C on half-strength Luria-Bertani (½ LB) solid medium or in ½ LB broth with shaking at 225 RPM.

Phage isolation, propagation, host range analysis and electron microscopy

DLP6 was isolated from planter soil located at the Kinsman Sports Center in Edmonton, Alberta, Canada using strain D1571 and a previously described extraction protocol [22]. Propagation of DLP6 was performed using soft agar overlays: 100 μl liquid culture and 100 μl phage stock were incubated 20 min at room temperature, mixed with 3 ml 0.7% ½ LB top agar, overlaid on a plate of ½ LB solid medium, and incubated at 30°C until plaque formation was complete. High titre stocks were made by overlaying plates with confluent lysis with 3 ml modified SM and the top agar was scraped into a sterile falcon tube. The top agar was pelleted by centrifugation for 5 min at 10,000 × g and the supernatant was removed and filter-sterilized using a Millex-HA 0.45 μm syringe-driven filter unit (Millipore, Billerica, MA), followed by storage at 4°C. Titres were obtained using serial dilutions of phage stock into SM, followed by the soft agar overlay technique described above and incubation at 30°C until plaque formation was complete.
Host range analysis was performed using a panel of 27 clinical *S. maltophilia* and 19 *P. aeruginosa* strains. Soft-agar overlays containing 100 μl liquid culture were allowed to solidify for 10 minutes at room temperature. Plates were spotted with 10 μl drops of DLP6 at multiple dilutions and assayed for clearing and/or plaque formation after incubation at 30˚C for 36 h.

For electron microscopy, phage stocks were prepared as described above with the following modifications: ½ LB agarose plates and ½ LB soft agarose were used for overlays, MilliQ-filtered water for phage recovery and a 0.22 μm filter was used for syringe-driven filtration. A carbon-coated copper grid was incubated with lysate for 2 min and stained with 4% uranyl acetate for 30 s. Transmission electron micrographs were captured using a Philips/FEI (Morgagni) transmission electron microscope with charge-coupled device camera at 80 kV (University of Alberta Department of Biological Sciences Advanced Microscopy Facility). The average capsid diameter, tail length and tail width were calculated using Microsoft Excel based on measurements from 10 individual virions.

### Phage DNA isolation, RFLP analysis and sequencing

DLP6 genomic DNA was isolated from bacteriophage lysate using the Wizard Lambda DNA purification system (Promega Corp., Madison, WI) with a modified protocol [23, 24]. A 10 ml aliquot of high-titre filter-sterilized phage lysate was treated with 10 μl DNase I (Thermo Scientific, Waltham, MA), 100 μl 100x DNase I buffer (1 M Tris–HCl, 0.25 M MgCl2, 10 mM CaCl2), and 6 μl RNase (Thermo Scientific) and incubated 1 h at 37˚C to degrade the bacterial nucleic acids. Following incubation, 400 μl of 0.5 M EDTA and 25 μl of 20 mg/ml proteinase K (Applied Biosystems, Carlsbad, CA) was added and incubated 1 h at 55˚C to inactivate DNase I. The lysate was cooled to room temperature and added to 8.4 g of guanidine thiocyanate, along with 1 ml of 37˚C resuspended Wizard DNA Clean-Up Resin (Promega Corporation, Madison, WI). This mixture was rocked for 10 min then pelleted by centrifugation for 10 min at 5,000 x g. The supernatant was drawn off until ~5 ml remained. Remaining mixture was resuspended by swirling, transferred to a syringe attached to a Wizard Minicolumn (Promega Corporation). The Wizard Minicolumn was attached to The Vac-Man Jr. Laboratory Vacuum Manifold (Promega Corporation) and placed under vacuum to remove the supernatant. The column was then washed with 2 ml 80% isopropanol and dried by centrifugation for 2 min at 10,000 x g. Phage DNA was eluted from the column following a 1 min incubation of 100 μl of 80˚C nuclease-free water (Integrated DNA Technologies, Coralville, IA) followed by centrifugation for 1 min at 10,000 x g. A NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA) was used to determine purity and concentration of eluted DNA.

Restriction fragment length polymorphism (RFLP) analysis was used with 18 FastDigest (Thermoscientific) restriction enzymes: BamHI, EcoRI, AcI, HpaII, XbaI, HindIII, KpnI, Smal, ApaI, SalI, PstI, SpHI, SacI, ClaI, NdeI, SpeI, XhoI and HaeIII. Restriction reactions were set up using 1 μl of FastDigest enzyme, 2 μl of FastDigest restriction buffer, 1 μg of phage DNA and topped up to 20 μl with nuclease free water. Reactions were incubated at 37˚C for 20 min and separated on a 1% (wt/vol) agarose gel in 1x TAE (pH 8.0). Sequencing of DLP6 was performed at The Applied Genomics Core at the University of Alberta. Purified DLP6 DNA was prepared for sequencing using a Nextera XT library prep kit, creating a library size of 223 bp. The library was used for paired-end sequencing on a MiSeq (Illumina, San Diego, CA) platform using a MiSeq v2 reagent kit. The Q30 for all reads was 92.5%.

### Lifecycle of DLP6

DLP6 resistant colonies of *S. maltophilia* D1571 were isolated by using the top agar overlay method using a phage stock at a titer of 1x10⁵ PFU/mL. Following overnight incubation at
30˚C, a 3 mL aliquot of SM was transferred to each plate, supernatant was collected and used for serial dilutions to obtain superinfection resistant single colonies on ½ LB plates. Individual colonies were picked, washed with SM and used to produce freezer stocks. Superinfection experiments were performed using overnight cultures of the potential lysogens and DLP6 at a 1x10^5 PFU/mL titer. Single colonies for each potential lysogen or pseudolysogen were used for colony PCR to detect the presence of DLP6. Identifying the temporary presence of DLP6 in the cell during pseudolysogeny was determined using specific sets of internal primers for DLP6.

**Pulse Field Gel Electrophoresis (PFGE)**

Overnight cultures of wild type D1571 and five DLP6 PCR positive single colony isolates were used to generate plugs following the protocol outline by Sueh et al. 2013[25]. A SpeI (New England Biolabs) or XbaI (Thermo Fisher Scientific) restriction digest was set up for all six samples and incubated for 45 min at 37˚C. A 1% agarose gel made with TAE was used for the PFGE. Switch time was 50–90 for 12 hrs at 6 V/cm. Ladders used were Saccharomyces cerevisiae and Lambda DNA/HindIII markers (Promega) for comparison of high and low molecular weight DNA. Gels were stained using SYBR Safe DNA Gel Stain (Thermo Fisher Scientific) and visualized with a ChemiDoc MP imaging system and the Image Lab software (Bio-Rad).

**Bioinformatics analysis**

A single contig was assembled using the CLC Genomics Workbench (Qiagen, Toronto, ON). Although no ambiguous regions in the contig were observed, PCR amplification and sequencing was used to confirm the assembly. All attempts by PCR amplification to identify a DNA segment between the direct repeats or to show genome circularization via direct repeat annealing were negative. Open reading frames (ORFs) were identified with the GLIMMER plugin [26] for Geneious [27] using the Bacteria and Archaea setting, as well as GeneMarkS (http://exon.gatech.edu/GeneMark/genemarks.cgi) for phage [28] and Prodigal [29]. Conserved domain searches were performed using CD-Search [30]. Pfam was used to identify functions for hypothetical protein hits from BLASTP [31]. The contig was annotated and confirmed with an Interactive Remote Invocation Service utilizing the RAST pipeline [32–34]. BLASTN and BLASTP (for full genomes and individual proteins, respectively) was used to gain more information for each RAST annotation, and to identify any potential related phages [35]. BLASTP hits above 1.00E^-3 were not recorded and the coding sequence (CDS) was annotated as hypothetical. Rho independent terminators were predicted using ARNold [36–38] searching both strands. Promoters were predicted using PHIRE [39] and plotted using WebLogo 3 [40]. tRNAs were identified using tRNAscan-SE using the general tRNA model [41]. Multiple sequence alignments were performed with the top 250 BLASTP hits for gp20 and from core T4 and cyanophage proteins [42] using the MUSCLE [43] plugin for Geneious [44]. The maximum number of iterations selected was 8, with the anchor optimization option selected. The trees from iterations 1 and 2 were not retained. The distance measure for iteration 1 was kmer6_6, and was pctid_kimura for subsequent iterations. The clustering method was UPGMB for all iterations. An unrooted tree was constructed from MUSCLE alignments with the FastTree 2.1.5 [45] plugin for Geneious [44]. The Jones-Taylor-Thornton model was used with rate category of sites set to 20. A PROmer comparison was conducted with DLP6 and ΦM12 with the following parameters: breaklen = 60, maxgap = 30, mincluster = 10, min-match = 3 [46].
Results and discussion
Isolation, host range and morphology
Phage DLP6 was isolated from a soil sample using clinical isolate S. maltophilia strain D1571 as the host. Propagation of DLP6 to high titre has proven difficult in liquid cultures, with liquid grown lysate concentrations remaining constant at $10^6$ PFU/ml despite attempts to increase progeny numbers. DLP6 exhibits a unique plaquing inhibition that was previously observed in Burkholderia cepacia complex phages KL1 and AH2 [47], as well as S. maltophilia phages DLP1 and DLP2 [22]. Although high titer stocks ($10^{10}$ plaque forming units [PFU]/ml) can easily be obtained using the top agar plating method, use of such high titre stock inhibits the plaque formation on a bacterial lawn. Plaquing of DLP6 is inhibited at titers above $10^6$ plaque forming units (PFU/ml). Plaque development occurs readily at 30˚C within 24 hr, forming diffuse plaques with irregular boarders and a mean size of $0.8 \pm 0.3$ mm. Host range analysis of DLP6 revealed a moderate host range within S. maltophilia clinical isolates, infecting 13 out of 27 clinical isolates (S1 Table). Whereas S. maltophilia phages DLP1 and DLP2 exhibited some cross-species infectivity [22], extended host range analysis of DLP6 using P. aeruginosa isolates did not yield successful infections. Initially, we produced evidence to suggest that DLP6 existed in S. maltophilia D1571 as a prophage. However, after extensive experimentation it was determined that DLP6 undergoes only pseudolysogeny. PFGE analysis using SpeI or XbaI separately showed no integration of the DLP6 genome into the S. maltophilia D1571 chromosome, and DLP6-specific PCR indicated the genome’s presence after 2–3 passages, but not after >5 passages. DLP6 is classified in the Myoviridae family of the Caudovirales order due to its icosahedral head and contractile tail (Fig 1). The average capsid height, tail length and width measurements for DLP6 are 99, 144, and 23 nm respectively.

Genome characterization
Purified DLP6 gDNA was isolated and exposed to a panel of 18 restriction enzymes for RFLP analysis. Even though controls were performed to detect the presence of restriction inhibitors, the DLP6 genome was assembled into a linear scaffold of 168,489 bp with a GC content of 55.8% using 43,112 reads for a mean coverage of 57 reads and an overall Q30 score of 93.1%. The direct terminal repeats are covered by up to 45 reads (S1 Fig). The contig can be found in GenBank with the accession number KU682439.2. The genome is predicted to encode 241 coding DNA sequences and 30 tRNAs of 14 different specificities (Fig 2, S2 and S3 Tables). The genome is arranged in a semi-modular format, with DNA replication/repair genes (dark blue) primarily grouped together, whereas the regulatory genes (light blue) are mainly grouped within the same region as the DNA replication/repair genes. Auxiliary metabolism (black) genes are dispersed throughout the genome, occurring in small pairs rather than a large set. The phage morphogenesis (dark purple) genes are grouped in a cluster, with the exception of gp34 (AAY80_073; long tail fiber). The 30 tRNA genes are grouped together spanning the genome from 92,962–113,834 bp. There is no lysis (red) module in DLP6, rather four genes encoding typical lysis proteins are randomly located throughout the genome.

Three interesting phage-encoded proteins are ADP-ribosyltransferases (Alt: AAY80_209, ModA: AAY80_029, ADP-ribosyltransferase: AAY80_145) that were identified using the Pfam database. Two of these proteins are orthologs to T4 proteins Alt and ModA. It is known that phage T4 encodes three ADP-ribosyltransferases (Alt, ModA and ModB), each modifying specific groups of host proteins. The Alt protein is a component of the phage head and enters the host cell during the infection process with the phage DNA, ModA and ModB [48]. Following entry into the cell, Alt immediately ADP-ribosylates the host RNA polymerase (RNAP),
causing transcription of host genes to stop and transcription from the T4 “early” promoters to be carried out instead [49]. The ModA modification of the host RNAP prevents transcription from T4 early promoters and possibly primes the RNAP for T4-encoded auxiliary factors (gp55, gp33, gp45 and gp44/62) to transcribe middle and late genes [50]. Identification of the previously hypothetical proteins into the Alt and ModA families helps to provide insight into the possible role these proteins play in DLP6 phage infection and transcription initiation and regulation.

Phage promoter sequences lack the conserved structure observed in bacterial promoters with -10 and -35 regions; instead, they feature short consensus sequences that are specific to different phages [51]. These short consensus sequences were identified using the phage-specific program PHIRE, and visualized with WebLogo 3 (Fig 3). There are 25 phage promoters identified, with 22 of the phage promoters found repeating in groups of two in front of a gene cluster. A single phage promoter is located upstream of genes AAY80_058 (hypothetical protein) and AAY80_059 (peptidase protein). The next single phage promoter is located upstream of the gene cluster beginning with AAY80_139 (kinase protein) through to AAY80_150 (hypothetical protein). The final single phage promoter is located upstream of a small cluster of hypothetical proteins beginning at AAY80_217 through to AAY80_220. Two phage promoters are found upstream of AAY80_060 (hypothetical protein) in a gene cluster coding for 32 proteins, including DNA primase (AAY80_083) phage tail fiber (AAY80_073), ribonucleotide...
diphosphate reductase subunit alpha (AAY80_086) and beta (AAY80_089), and many hypothetical proteins (locus tags ending in 060, 063–067, 070–071, 074–077, 079–082, 084–085, 088 and 090). The next set of phage promoters is located upstream of AAY80_092 (hypothetical protein) to AAY80_112 (hypothetical protein). Annotated genes included in this gene cluster are AAY80_098 (RNase H), AAY80_104 (PhoH), AAY80_106 (exonuclease), AAY80_109 (SleB), AAY80_110 (dCMP deaminase) and AAY80_111 (deoxycytidylate deaminase). The next two sets of gene clusters, AAY80_113–120 and AAY80_121–130, encode hypothetical proteins only and both clusters are under control of two promoters each. Gene cluster AAY80_131–138 utilizes two promoter sequences and encodes mainly hypothetical proteins, but also a guanosine 3’,5’-bis(diphosphate) 3’-pyrophosphohydrolase (AAY80_131) and a...
transposase (AAY80_133). The adjacent gene cluster under control of two promoters is upstream of AAY80_151 (hypothetical protein) to AAY80_158 (hypothetical protein) and contains nine tRNAs. The remaining 21 tRNAs are under control of two promoters upstream of AAY80_159 (hypothetical protein) to AAY80_180 (hypothetical protein). Two promoters control the next gene cluster spanning from AAY80_181–202 that contains genes encoding many hypothetical proteins and proteins such as DNA ligase (AAY80_192), DNA helicase loader (AAY80_201) and ssDNA binding protein (AAY80_202). Two small gene clusters encoding a total of eight hypothetical proteins (AAY80_213–216 and AAY80_221–224) are both under control of two phage promoters. The last set of double phage promoters controls the gene cluster from AAY80_225 to AAY80_057 where the phage genome is circularized. This final gene cluster contains many proteins involved in DNA replication and homologous recombination.

Rho-independent transcription termination sites were discovered using ARNold, which utilizes two complementary programs, Erpin [36] and RNAmotif [37]. Of the 54 potential termination sites identified, only 19 were retained as authentic because they had a ΔG of -10 kcal/mol or less (Table 1). The 19 terminators were found downstream of AAY80_018 (precursor of major head subunit), AAY80_042 (triphosphate pyrophosphohydrolase), AAY80_057 (hypothetical protein), AAY80_090 (hypothetical protein), AAY80_091 (RuvC), AAY80_108 (hypothetical protein), AAY80_120 (hypothetical protein), AAY80_138 (hypothetical protein), AAY80_166 (hypothetical protein), AAY80_168 (hypothetical protein), AAY80_175 (endolysin), AAY80_180 (hypothetical protein), AAY80_199 and AAY80_200 (hypothetical proteins), AAY80_202 (ssDNA binding protein), AAY80_203 (lysozyme), AAY80_208 (endonuclease protein), AAY80_209 (hypothetical protein) and AAY80_212 (baseplate tail tube cap protein).

**Phylogeny using DLP6 gp20 protein**

A BLASTN search using DLP6 genome as a query revealed the closest hits are *Sinorhizobium* phages FN3, FM19, FM7 and FM12. All four phages have coverage of only 4% with a 72% identity. Although initial BLASTP and BLASTN searches indicated DLP6 was more closely related to members of the T4-superfamily of phages, more comparisons were required to classify DLP6 as a T4-superfamily phage. For a commonly used phylogenetic comparison, the protein sequence of portal protein gp20 was used in a BLASTP search to identify 250 of the most similar sequences [52–54]. The most significant hits came from cyanophages grouped into the T4-superfamily. A MUSCLE alignment was completed using the 250 BLASTP results compared to the DLP6 gp20 protein (AAY80_011). This alignment was then used to generate an
unrooted tree with the FastTree plugin for Geneious (Fig 4). The generated tree positions DLP6 in a clade with Sinorhizobium phages ΦM12, ΦN3 and Caulobacter phage Cr30 (Fig 4).

DLP6 contains features from T4-superfamily enteric bacteriophages and T4-superfamily cyanophages

Genomic organization of DLP6 is similar to the Sinorhizobium transducing phage ΦM12, which has been classified into a new T4-superfamily fusing features of cyanophages and phages of enteric bacteria [42] (Fig 5). A set of “core universal” and “nearly universal” proteins has been determined for T4-superfamily phages [55, 56]. DLP6 contains all of the core and nearly universal proteins common to all T4-superfamily phages (Table 2). A MUSCLE comparison was used to align the T4-superfamily phage core proteins against their respective orthologs from 18 T4-superfamily phages, and their percent identity to each ortholog was determined (Table 2). Similar to ΦM12, DLP6 contains a second copy of gp19 tail tube monomer that was not found in the 17 other phages studied (21% similarity). The order of the gene products presented in Table 2 corresponds to the order they are found within the DLP6 genome. This order differs from the T4-superfamily phages, which start with gp41 (DNA primase-helicase). Results from the MUSCLE alignment reveal that DLP6 core proteins are most similar to their orthologs from cyanophages, with the exception of the UvsX protein, which is most similar to T4-superfamily phages of enteric bacteria. The alignment also shows DLP6 has the highest percent identity to ΦM12 core proteins. The ortholog with the highest similarity to a DLP6 protein was ΦM12 gp23, with 65% similarity. Overall, the most highly conserved proteins between DLP6 and the T4-superfamily cyanophages are RegA (early transcriptional regulator) and gp23 (major capsid protein), with similarity rates averaging 59 and 57% respectively. This suggests that DLP6 is divergent from the T4-superfamily phages. DLP6 does share additional

| Start | Program | Strand | Sequence | -ΔG |
|-------|---------|--------|----------|------|
| 15290 | both    | +      | CAATAAGAAGCGCGCCCAAGCCGCTTTTTT | 16.80 |
| 32949 | both    | +      | AAGCCAGAAGCGCGCCCAAGCCGCTTTTTT | 17.50 |
| 43682 | both    | +      | TCAACGACTTAGACGGCGTCCTCCGGCTTTTTT | 16.10 |
| 72171 | both    | +      | CTAATTTAAGGCGCTTCCGGGCTTTTTT | 13.40 |
| 72816 | Rnamotif | +      | GACTTAAGAAGCGCGCCCAAGCCGCTTTTTT | 13.50 |
| 80627 | both    | +      | TTATTTGAAGGCGCTTCCGGCTTTTTT | 13.46 |
| 85964 | both    | +      | GACTTAAGAAGCGCGCCCAAGCCGCTTTTTT | 14.80 |
| 93801 | both    | +      | CGCGCCCTAGAAGGCGCCCTTTTTT | 13.32 |
| 112696 | both    | +      | GTAATCTCAAAGCGCTTGGTGTCACAGAT | 15.70 |
| 113321 | Rnamotif | +      | CTAATTTAAGGCGCTTCCGGCCTTTTTT | 16.80 |
| 117336 | Rnamotif | -      | ATCAATCTCTTGAGAAGCGCTTTTTT | 11.70 |
| 119975 | both    | +      | TCAACGACTTAGACGGCGTCCTCCGGCTTTTTT | 17.10 |
| 128844 | both    | +      | CTTGAGAAGGCGCTTGGTGTCACAGAT | 17.60 |
| 129411 | Erpin   | +      | CTGGACGCAAAGCGCGCTTT | 14.90 |
| 131176 | both    | -      | GCAAAGAAGGCGCGCTTTTTT | 18.20 |
| 131178 | both    | +      | GTAATTTAAGGCGCTTCCGGCTTTTTT | 17.80 |
| 136291 | both    | -      | GATACAGAAGGCGCTTCCGGCGGCTTTTTT | 15.00 |
| 136293 | both    | +      | TGAAGAAGAAGGCGCTTCCGGCGGCTTTTTT | 17.80 |
| 139414 | Rnamotif | +      | GACTTAAGAAGCGCGCCCAAGCCGCTTTTTT | 17.60 |

Table 1. Predicted Rho-independent terminators in DLP6. Rho-independent terminators were identified using the ARNold [36, 37] program and putative terminators with a ΔG value of -10 kcal/mol or less were retained. DNA that is predicted to form the loop in the RNA is in emboldened, whereas DNA that is predicted to encode an RNA stem is underlined.
proteins that are found within the T4-superfamily cyanophages or the T4-superfamily enteric phages.

All sequenced T4-superfamily cyanophages feature an accessory core set of 25 gene clusters (T4-GCs) that are not found within enteric bacteria T4-superfamily phages [55]. Of this accessory core, DLP6 encodes six of the core proteins: CobS (porphyrin biosynthetic protein), PhoH (P-starvation inducible protein), T4-CG 313 (hypothetical protein), T4-GC 321 (hypothetical protein), VlrC (predicted structural protein) and MazG (pyrophosphatase) (Table 3).
Although DLP6 does contain these T4-superfamily cyanophage accessory core proteins, the DLP6 proteins are again divergent, with the maximum similarity found with the PhoH (P-starvation inducible protein) at 45% similar to P-HM1 cyanophage.

DLP6 was found to contain nine out of the designated 30 non-cyanophage core T4-superfamily proteins. A MUSCLE alignment of these nine proteins indicates that although this phage does contain the proteins, they do not share high amino acid similarities (Table 4). The protein sharing the highest similarity was the RnaseH protein, which had a maximum similarity of 32.6% with the RB43 phage protein. Again, these results demonstrate that DLP6,
Table 2. MUSCLE alignment percent identity score of full-length protein sequences of DLP6 against universal core and nearly universal core proteins of 18 T4-superfamily phages. Numbers indicate percent similarity to the related DLP6 protein.

| Gene product: function | Cyanophages | Enteric phages |
|------------------------|-------------|----------------|
|                        | ΦM12  | S-SM2  | P-SSM4 | P-Syn1 | HTVCC008M | S-Syn9 | S-Syn19 | S-ShM2 | P-Syn33 | S-SM1 | P-HM1 | S-CRM01 | T4 | Aeh | 44 | RR | 40 | RB43 | ΦW-14 |
| gp1: neck protein      | 53.2  | 35.5  | 34.2  | 34.3  | 39.1  | 36.1  | 35.7  | 33.5  | 35.7  | 37.2  | 36.1  | 22.9  | 21.7  | 19.4 | 20.2 | 23.1 | 25.2 |
| AAY80_004              |        |       |       |       |       |       |       |       |       |       |       |       |       |      |      |      |      |      |
| gp12: neck protein     | 43.9  | 17    | 28    | 28.8  | 26.3  | 24.4  | 27.2  | 23.2  | 22.8  | 28.6  | 18.3  | 20.4  | 24.7  | 26.7 | 24.8 | 27.7 | 24.5 | 24.8 |
| AAY80_005              |        |       |       |       |       |       |       |       |       |       |       |       |       |      |      |      |      |      |
| gp15: proximal tail sheath stabilization | 40 | 31.9  | 27.9  | 32    | 31.5  | 33.2  | 27.3  | 34.8  | 34.1  | 33.3  | 27    | 30.9  | 24.3  | 26.8 | 23.5 | 16.6 | 23.6 | 20.8 |
| AAY80_006              |        |       |       |       |       |       |       |       |       |       |       |       |       |      |      |      |      |      |
| gp16: small terminase  | 30.7  | 27.6  | 25.9  | 25.5  | 21.4  | 29.2  | 27.5  | 31.1  | 26.7  | 30.4  | 30.4  | 30.1  | 21.7  | 18.6 | 16.7 | 17.6 | 23.2 | 15.2 |
| AAY80_007              |        |       |       |       |       |       |       |       |       |       |       |       |       |      |      |      |      |      |
| gp17: large terminase  | 42.5  | 47.2  | 48.1  | 47.2  | 46.9  | 48.5  | 48.7  | 48.9  | 49    | 48.8  | 46.1  | 42.8  | 27.8  | 28.5 | 27.7 | 29.8 | 29.8 | 32   |
| AAY80_008              |        |       |       |       |       |       |       |       |       |       |       |       |       |      |      |      |      |      |
| gp18: tail sheath monomer | 48.4  | 33.3  | 37.3  | 35    | 43.7  | 35.7  | 38.1  | 13.5  | 37.6  | 38.8  | 34.4  | 32.8  | 31.7  | 31.8 | 30.9 | 31.5 | 31.8 | 21.9 |
| AAY80_009              |        |       |       |       |       |       |       |       |       |       |       |       |       |      |      |      |      |      |
| gp19: large terminase  | 46.8  | 40.1  | 33.5  | 34.2  | 32.6  | 33.5  | 35.3  | 28.2  | 33.7  | 34    | 30.1  | 40.7  | 33.3  | 30.6 | 31.8 | 36.8 | 34.9 | 13.8 |
| AAY80_010              |        |       |       |       |       |       |       |       |       |       |       |       |       |      |      |      |      |      |
| gp20: tail tube monomer | 46.1  | 46.7  | 47.7  | 46.6  | 43.2  | 49.1  | 49.1  | 46.7  | 46.7  | 49.6  | 46.2  | 40.7  | 33.4  | 32   | 33.7 | 36   | 32.7 | 31.2 |
| AAY80_011              |        |       |       |       |       |       |       |       |       |       |       |       |       |      |      |      |      |      |
| gp21: prohead core scaffold/protease | 41.3  | 49.3  | 47.7  | 54.1  | 51.6  | 49.1  | 50    | 49.1  | 47.7  | 49.1  | 49.5  | 51.1  | 32.1  | 32.5 | 32.8 | 34.6 | 32.4 | 30   |
| AAY80_016              |        |       |       |       |       |       |       |       |       |       |       |       |       |      |      |      |      |      |
| gp22: scaffold prohead core protein | 22.3  | 29.1  | 26.5  | 29.9  | 30.1  | 26.5  | 26.5  | 26.6  | 27    | 25.9  | 27.8  | 25.9  | 19.3  | 20.2 | 22.1 | 17.8 | 20.6 | 11.6 |
| AAY80_017              |        |       |       |       |       |       |       |       |       |       |       |       |       |      |      |      |      |      |
| gp23: precusor of major head subunit | 65.4  | 59.6  | 55.7  | 52.5  | 60.5  | 57.1  | 58    | 56.3  | 58    | 55.8  | 55.9  | 53.8  | 34.4  | 33.3 | 34.8 | 35   | 34.3 | 44.8 |
| AAY80_018              |        |       |       |       |       |       |       |       |       |       |       |       |       |      |      |      |      |      |
| DexA: exonuclease      | 32.5  | 32.1  | 35.7  | 30.5  | 33.3  | 35    | 34.2  | 32.9  | 29.3  | 22.2  | 12.2  | 13.7  | 11.2  | 8.2   | 14.6 | 11.4 |
| AAY80_019              |        |       |       |       |       |       |       |       |       |       |       |       |       |      |      |      |      |      |
| gp3: head proximal tip of tail tube | 27.3  | 31.5  | 29.1  | 31.4  | 18.5  | 29.3  | 27.6  | 28.3  | 29.2  | 30.9  | 29.4  | 27.2  | 20    | 18.5 | 17.8 | 17.5 | 20.3 | 14.8 |
| AAY80_020              |        |       |       |       |       |       |       |       |       |       |       |       |       |      |      |      |      |      |

(Continued)
Table 2. (Continued)

| Gene product: function | Cyanophages | Enteric phages |
|------------------------|-------------|----------------|
|                        | ΦM12 | S-SM2 | P-SSM4 | P-Syn1 | HTVC008M | S-Syn9 | S-Syn19 | S-ShM2 | P-Syn33 | S-SM1 | P-HM1 | S-CRM01 | T4 | Aeh | 44 RR | KVP 40 | RB43 | ΦW-14 |
| gp46: recombination endonuclease subunit | 45.7 | 42.9 | 43.4 | 43.2 | 44.3 | 44.3 | 45.9 | 45 | 44.9 | 45.2 | 44.2 | 37.8 | 31.2 | 25.5 | 29.2 | 28 | 31.3 | 20.5 |
| gp45: sliding clamp accessory protein | 34.2 | 43.8 | 40.1 | 38.6 | 42.2 | 33.5 | 40.8 | 35.9 | 38.8 | 39.6 | 40.6 | 39 | 28 | 27 | 26.9 | 36.9 | 26.1 | 25.8 |
| gp44: clamp loader subunit | 46.8 | 55.7 | 54.1 | 48.9 | 52.9 | 56.2 | 54.3 | 55.3 | 54.6 | 55.3 | 52.4 | 48.1 | 34.4 | 36.6 | 35.3 | 35.7 | 32.5 | 32.8 |
| gp62: clamp loader subunit | 37.1 | 31.8 | 29.6 | 32.1 | 35.6 | 33.1 | 31.6 | 42.5 | 33.8 | 31.6 | 37.2 | 31.8 | 21.1 | 18.1 | 19.5 | 27.3 | 21 | 25.5 |
| RegA: early gene translational repressor | 52.2 | 60 | 60 | 59.2 | 59.2 | 60 | 62.3 | 60.8 | 60 | 60.8 | 60 | 56.2 | 49.6 | 48.4 | 48.4 | 48.4 | 41.6 | 33.1 |
| gp43: DNA polymerase | 39.7 | 42.2 | 41.1 | 40 | 38 | 42.6 | 41.1 | 40.9 | 41 | 42.6 | 41.9 | 39.6 | 28.7 | 31.3 | 18.8 | 31.3 | 29.8 | 26.4 |
| UsvX: recombination protein | 47.9 | 23.9 | 25.3 | 26.4 | 25.1 | 25.3 | 25.3 | 24.5 | 24.7 | 26.1 | 25.8 | 26.2 | 49.9 | 52.7 | 47.8 | 46.1 | 35.1 |
| gp41: DNA primase-helicase | 50.5 | 57 | 58.8 | 53.7 | 54.6 | 53.5 | 53.7 | 56.2 | 53.5 | 54.3 | 53.8 | 51.7 | 37.4 | 40.9 | 39.7 | 38.6 | 38.7 | 31 |

(Continued)
| Td: thymidylate synthase  | gp61: DNA primase  | NrdA: ribonucleotide reductase subunit A  | NrdB: ribonucleotide reductase subunit B  | gp33: late promoter transcription factor  | NrdC: glutaredoxin  | gp58: DNA helicase loader  | gp32: ssDNA binding protein  | gp51: baseplate hub assembly catalyst  | gp26: baseplate hub  |
|---------------------------|---------------------|------------------------------------------|------------------------------------------|------------------------------------------|---------------------|----------------------------|----------------------------------|------------------------------------------|----------------|
|                           | AAY80_062           | AAY80_083                                 | AAY80_086                                | AAY80_112                                 | AAY80_173           | AAY80_201                   | AAY80_202                         | AAY80_205                                 | AAY80_206                  |
|                           | 11.4 12.5 11.3 12.5 | 44.6 34 35.8 37.8 37 | 46.8 46.3 47.4 45.5 47.7 47.3 46.5 48.1 47.9 47.9 48.1 43.6 29.9 17 | 23.3 22.7 27.4 21.3 23.8 24.4 22.6 22.4 26.2 29.9 26.7 22.7 19 | 39 29.5 29.5 30.9 32.2 25.4 30.8 29.5 28.2 28.2 25.6 29.1 36.8 27.3 | 34.6 32.5 35.5 | 29.1 34.1 35.2 28.4 35.5 35 31.2 30.1 | 34.8 46.7 44.5 44.7 46.5 47.1 | 48 49.5 48.5 47.3 46.7 34.2 30.2 30.6 33.1 25.3 | 39.6 49.1 37.3 34.9 28.9 33.3 38.6 48.3 31.8 35.6 39.6 31 7.1 | 7.1 13.1 19.1 22.7 5.5 9.7 |
|                           |                     |                                          |                                          |                                          |                     |                            |                                  |                                          |                            |                            |                            | 11.4 12.5 11.3 12.5 10.9 11.6 11.9 11.4 10.6 11.4 9.3 10.8 7.4 12.1 8.8 |
| gp5: baseplate hub and tail lysozyme | gp25: baseplate wedge | gp6: baseplate wedge | gp7: baseplate wedge initiator | gp8: baseplate wedge |
|-------------------------------------|-----------------------|----------------------|-------------------------------|---------------------|
| AAY80_232                           | AAY80_233             | AAY80_234            | AAY80_235                     | AAY80_236           |
| **gp5**                             | **gp25**              | **gp6**              | **gp7**                       | **gp8**             |
| 11.2                                | 44                    | 33.1                 | 11.3                          | 20.1                |
| 9.4                                 | 27.8                  | 26.7                 | 5.3                           | 16.4                |
| 9.6                                 | 35.1                  | 24.3                 | 4                            | 19.6                |
| 5.8                                 | 33.8                  | 30.5                 | 6.3                           | 14.4                |
| 22.2                                | 36.6                  | 31.6                 | 7.4                           | 18.3                |
| 8.7                                 | 30.8                  | 24.7                 | 4.7                           | 19.3                |
| 8.7                                 | 29.1                  | 24.7                 | 4.5                           | 19.7                |
| 9.2                                 | 28.4                  | 23.1                 | 4.4                           | 19.1                |
| 9                                  | 29.9                  | 25.1                 | 4.7                           | 20.3                |
| 8.6                                | 26.1                  | 23.5                 | 4.8                           | 17.4                |
| 7.2                                 | 27.1                  | 29.6                 | 4.4                           | 18.4                |
| 8.7                                 | 30.1                  | 27.3                 | 11.1                          | 10.5                |
| 16.7                                | 25.8                  | 21.5                 | 10.8                          | 12.1                |
| 15.9                                | 27.9                  | 21.6                 | 10.5                          | 11                  |
| 15.7                                | 25.6                  | 23.6                 | 10.1                          | 10.9                |
| 22.3                                | 27.3                  | 21.8                 | 11.1                          | 11.5                |
| 13.8                                | 28.8                  | 22.7                 |                               |                     |
| 10.3                                | 25.2                  | 22.6                 |                               |                     |

https://doi.org/10.1371/journal.pone.0173341.t002
although classified within the T4-superfamily, it is more divergent than the other accepted members.

Differences between the T4-superfamily superfamily members discussed in this paper as compared to DLP6 are interesting, given that DLP6 contains the complete set of T4-superfamily core group of proteins, six accessory core cyanophage proteins, and nine non-cyanophage accessory core proteins. Moreover, the ends of DLP6 genome feature 229 bp direct terminal repeats, unlike the genomes of other members of the T4-superfamily of phages which are cyclically permuted. This finding is unusual, and suggests that DNA circularization in the host cell occurs at cohesive sites. The direct terminal repeats are located in a region of DNA devoid of ORFs, or homology to known DNA sequences. The average number of tRNAs encoded by the T4-superfamily phages is ten, with the exception of S-CRM01, a freshwater cyanophage that encodes 33 tRNAs [57]. The DLP6 genome contains 30 tRNAs, which is in the high range for the T4-superfamily. DLP6 also encodes a transposase (AAY80_133) (Fig 2, S2 Table).

Conclusions

*S. maltophilia* bacteriophage DLP6 was isolated from a soil sample using *S. maltophilia* strain D1571 as the host bacterium. Phage DLP6 exhibits a moderate host range, infecting 13 out of 27 clinical *S. maltophilia* strains. A phylogenetic comparison of gp20 portal protein against the

### Table 3. MUSCLE alignment percent identity score of DLP6 amino acid sequences against T4-superfamily cyanophage accessory core proteins.

DLP6 contains six of the 25 T4-superfamily cyanophage core proteins. Numbers indicate percent similarity to the related DLP6 protein.

| Gene product: function | Cyanophages |
|------------------------|-------------|
|                        | ΦM12 | S-SM2 | P-SSM4 | Syn1 | HTVC008M | Syn9 | Syn19 | S-ShM2 | Syn33 | S-SM1 | P-HM1 | S-CRM01 |
| CobS: porphyrin biosynthetic protein | 41.6 | 41.9 | 43.6 | 40.3 | 42.2 | 41.2 | 41.9 | 41.5 | 41.1 | 42.7 | 40.7 |
| PhoH: P-starvation inducible protein | 33.0 | 42.5 | 44.4 | 44.7 | 42.8 | 44.0 | 43.8 | 42.2 | 43.0 | 42.6 | 44.8 | 39.2 |
| ΦM12: hypothetical protein | 33.5 | 30.6 | 31.2 | 36.0 | 33.1 | 30.5 | 31.9 | 31.4 | 30.5 | 31.9 | 26.4 | 28.1 |
| T4 Gc 313: hypothetical protein | 39.2 | 25.3 | 17.7 | 20.7 | 30.4 | 24.1 | 25.3 | 25.3 | 17.7 | 24.1 | 21.0 | 14.3 |
| T4 Gc 321: hypothetical protein | 19.3 | 14.7 | 16.3 | 13.0 | 17.5 | 16.2 | 16.4 | 15.5 | 16.3 | 15.8 | 14.9 | 13.7 |
| MazG: pyrophosphatase | 16.9 | 16.4 | 20.6 | 16.3 | 19.4 | 16.9 | 16.3 | 18.1 | 16.9 | 16.3 |

https://doi.org/10.1371/journal.pone.0173341.t003

### Table 4. MUSCLE alignment percent identity score of DLP6 amino acid sequences against T4-superfamily non-cyanophage core proteins.

This table includes 10 of the 32 non-cyanophage core proteins. Numbers indicate percent similarity to the related DLP6 protein.

| Gene product: function | Enteric phages |
|------------------------|---------------|
|                        | T4 | Aeh | 44 RR | KVP 40 | RB43 | ΦW-14 |
| RnaseH AAY80_098 | 28.9 | 32.5 | 28.9 | 32.2 | 32.6 | 8.8 |
| gp1: dNMP kinase AAY80_139 | 12.9 | 10.8 | 10 | 12.3 | 8.9 | 9.2 |
| dCMP deaminase AAY80_110 | 24.5 | 24.6 | 22.8 | 26.6 | 18 | 16 |
| DsbA: ssDNA binding protein AAY80_035 | 28.9 | 20.5 | 20.5 | 22.9 | 25.3 |
| gp34: long tail fiber AAY80_073 | 11.9 | 11.6 | 12.1 | 11.6 | 11.9 |
| gp30: dna ligase AAY80_192 | 24.7 | 23.5 | 21.9 | 29.7 | 24.5 | 32.3 |
| Tk: thymidine kinase AAY80_139 | 13.5 | 13.5 | 12 | 11.4 | 13.5 | 8.4 |
| GroES: head assembly chaperone with GroEL AAY80_164 | 16.7 | 12.8 | 14 | 20 | 7.6 |
| gp59: loader of gp41 DNA helicase AAY80_201 | 23.4 | 24.8 | 19.3 | 21.7 | 20.5 |

https://doi.org/10.1371/journal.pone.0173341.t004
top 250 BLASTP results places DLP6 in a clade with *Sinorhizobium* phages ΦM12, ΦN3 and *Caulobacter* phage Cr30. Although DLP6 does encode all of the T4-superfamily core and nearly universal core orthologs, the similarity between these proteins and their nearest neighbors is typically less than 60%. The DLP6 genome also encodes six T4-superfamily cyanophage core proteins, but again, the nearest neighbor similarity is below 40%. There are nine T4-superfamily non-cyanophage core proteins found within the DLP6 genome, though the similarity between the DLP6 proteins and the T4-superfamily enteric phage orthologs averaged less than 30% similarity. Unlike other T4-superfamily phages, DLP6 possesses 229 bp direct terminal repeats at the ends of its genome instead of circular permutation. Although DLP6 also encodes a transposon, experimental investigation has shown it does not form a stable prophage in *S. maltophilia* strain D1571. The results presented in this paper suggest DLP6 is a divergent T4-superfamily phage, exhibiting characteristics not yet identified in other T4-superfamily phages.

**Supporting information**

S1 Fig. Illumina pair-end sequencing reads aligning to start (1A) and end (1B) of DLP6 contig. Geneious was used to map R1 and R2 to DLP6 reference contig. Sensitivity was set to medium sensitivity/fast. Fine-tuning was iterated up to five times. Reads were not trimmed. Coverage at ends was between one and 45.

(PDF)

S1 Table. Host range determination of DLP6.

(DOCX)

S2 Table. Bacteriophage DLP6 coding sequences.

(DOCX)

S3 Table. DLP6 tRNA annotations.

(DOCX)

**Acknowledgments**

The authors would like to thank David Speert of the Canadian *Burkholderia cepacia* complex Research and Referral Repository (CBCCRRR) for the kind gift of numerous *S. maltophilia* strains. The authors would also like to thank the Provincial Laboratory for Public Health—North (Microbiology), Alberta Health Services, for access to *S. maltophilia* clinical isolates.

**Author Contributions**

Conceptualization: DLP JJD.

Data curation: DLP PS.

Formal analysis: DLP PS JJD.

Funding acquisition: JJD.

Investigation: DLP.

Methodology: DLP PS JJD.

Project administration: JJD.

Resources: PS JJD.
Software: PS.
Supervision: JJD.
Validation: JJD.
Visualization: DLP PS JJD.
Writing – original draft: DLP.
Writing – review & editing: JJD.

References
1. World Health Organization. Antimicrobial resistance: global report on surveillance. World Health Organization. Geneva, Switzerland. 2014.
2. Olaitan AO, Morand S, Rolain J-M. Emergence of colistin-resistant bacteria in humans without colistin usage: a new worry and cause for vigilance. Int. J. Antimicrob. Agents. 2016; 47:1–3. https://doi.org/10.1016/j.ija.2015.11.009 PMID: 26712133
3. Brooke JS. *Stenotrophomonas maltophilia*: an emerging global opportunistic pathogen. Clin Microbiol Rev. 2012; 25:2–41. https://doi.org/10.1128/CMR.00019-11 PMID: 22232370
4. Waters V, Atanau EG, Lu A, Yau Y, Tuillie E, Ratjen F. Chronic *Stenotrophomonas maltophilia* infection and mortality or lung transplantation in cystic fibrosis patients. J Cyst Fibros. 2013; 12(5):482–6. https://doi.org/10.1016/j.jcf.2012.12.006 PMID: 23294530
5. Wainwright CE, France MW, O'Rourke P, Anuj S, Kidd TJ, Nissen MD, et al. Cough-generated aerosols of *Pseudomonas aeruginosa* and other Gram-negative bacteria from patients with cystic fibrosis. Thorax. 2009; 64(11):926–31. https://doi.org/10.1136/thx.2008.112466 PMID: 19574243
6. Hermoso JA, Garcia J, Garcia P. Taking aim on bacterial pathogens: from phage therapy to enzybiotics. Curr Opin Microbiol. 2007; 10(5);461–72. https://doi.org/10.1016/j.mib.2007.08.002 PMID: 17904412
7. Seed KD, Dennis JJ. Experimental bacteriophage therapy increases survival of *Galleria mellonella* larvae infected with clinically relevant strains of the *Burkholderia cepacia* complex. Antimicrob Agents Chemother. 2009; 53(5):2205–8. https://doi.org/10.1128/AAC.01166-08 PMID: 19223640
8. Hagens S, Habel A, von Ahsen U, von Gabain A, Blasi U. Therapy of experimental *Pseudomonas* infections with a nonreplicating genetically modified phage. Antimicrob Agents Chemother. 2004; 48 (10):3817–22. https://doi.org/10.1128/AAC.48.10.3817-3822.2004 PMID: 15386440
9. Morello E, Saussereau E, Maura D, Huere M, Touqui L, Debarbeyroux L. Pulmonary bacteriophage therapy on *Pseudomonas aeruginosa* cystic fibrosis strains: First steps towards treatment and prevention. PLoS One 2011; 6(2):e16983. https://doi.org/10.1371/journal.pone.0016983 PMID: 21347240
10. Kumari S, Harjai K, Chhibber S. Bacteriophage versus antimicrobial agents for the treatment of murine burn wound infection caused by *Klebsiella pneumoniae* B5055. J Med Microbiol. 2011; 60:205–10. https://doi.org/10.1099/jmm.0.018580-0 PMID: 20965914
11. Waseh S, Hanifi-Moghadam P, Coleman R, Masotti M, Ryan S, Foss M, et al. Orally administered P22 phage tailspike protein reduces *Salmonella* colonization in chickens: Prospects of a novel therapy against bacterial infections. PLoS One. 2010; 5(11):e13904. https://doi.org/10.1371/journal.pone.0013904 PMID: 21124920
12. Golshahi L, Lynch KH, Dennis JJ, Finlay WH. In vitro lung delivery of bacteriophages KS4-M and ФКЗ using dry powder inhalers for treatment of *Burkholderia cepacia* complex and *Pseudomonas aeruginosa* infections in cystic fibrosis. J Appl Microbiol. 2011; 110(1):106–17. https://doi.org/10.1111/j.1365-2672.2010.04863.x PMID: 20875034
13. Semler DD, Goudie AD, Finlay WH, Dennis JJ. Aerosol phage therapy efficacy in *Burkholderia cepacia* complex respiratory infections. Antimicrob Agents Chemother. 2014; 58(7):4005–13. https://doi.org/10.1128/AAC.02388-13 PMID: 24798268
14. Holguín AV, Rangel G, Clavijo V, Prada C, Mantilla M, Gomez MC, et al. Phage Pan70, a putative temperate phage, controls *Pseudomonas aeruginosa* in planktonic, biofilm and burn mouse model assays. 2015; 7(8):4602–23.
15. Beeton ML, Alves DR, Enright MC, Jenkins ATA. Assessing phage therapy against *Pseudomonas aeruginosa* using a *Galleria mellonella* infection model. Int J Antimicrob Agents. 2015; 46(2):196–200. https://doi.org/10.1016/j.ija.2015.04.005 PMID: 26100212
16. Pincus NB, Reckhow JD, Salem D, Jammeh ML, Datta SK, Myles IA. Strain specific phage treatment for Staphylococcus aureus infection is influenced by host immunity and site of infection. PLoS One. 2015; 10(4):e0124280. https://doi.org/10.1371/journal.pone.0124280 PMID: 25909449

17. Wright A, Hawkins CH, Anggård EE, Harper DR. A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant Pseudomonas aeruginosa; a preliminary report of efficacy. Clin Otolaryngol. 2009; 34(4):349–57. https://doi.org/10.1111/j.1446-8107.2009.01973.x PMID: 19673983

18. Merabishvili M, Pirnay JP, Verbeken G, Chanishvili N, Lashkhi N, et al. Quality-controlled small-scale production of a well-defined bacteriophage cocktail for use in human clinical trials. PLoS One. 2009; 4(3):e4944. https://doi.org/10.1371/journal.pone.0004944 PMID: 19300511

19. Rhoads DD, Wolcott RD, Kuskowski MA, Wolcott BM, Ward LS, Sulakvelidze A et al. Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial. J Wound Care. 2009; 18:237–8, 240–3. https://doi.org/10.12968/jowc.2009.18.6.42801 PMID: 19661847

20. Rose T, Verbeken G, Vos DD, Merabishvili M, Vanechoutte M, Lavigne R, et al. Experimental phage therapy of burn wound infection: difficult first steps. Int J Burns Trauma. 2014; 4(2):66–73. PMID: 25356373

21. Abedon ST, Kuhl SJ, Blasdel BG, Kutter EM. Phage treatment of human infections. Bacteriophage. 2021.

22. Lynch KH, Abdu AH, Schobert M, Dennis JJ. Genomic characterization of JG068, a novel virulent podolytic bacteriophage of Burkholderia cenocepacia. BMC Genomics. 2013; 14:574. https://doi.org/10.1186/1471-2164-14-574 PMID: 23978260

23. Delcher AL, Bratke KA, Powers EC, Salzberg SL. Identifying bacterial genes and endosymbiont DNA with Glimmer. Bioinformatics. 2007; 23(6):673–9. https://doi.org/10.1093/bioinformatics/btm009 PMID: 17237039

24. Lynch KH, Abdu AH, Schober M, Dennis JJ. Genomic characterization of JG068, a novel virulent podovirus active against Burkholderia cenocepacia. BMC Genomics. 2013; 14:574. https://doi.org/10.1186/1471-2164-14-574 PMID: 23978260

25. Shueh CS, Neela V, Hussin S, Hamat RA. Simple, time saving pulsed-field gel electrophoresis protocol for the typing of Stenotrophomonas maltophilia. J Microbiol Methods. 2013; 94(2):141–3. https://doi.org/10.1016/j.mimet.2013.06.001 PMID: 23756145

26. Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbishe MK, DeWeese-Scott C, et al. The CDD: a Conserved Domain Database for the functional annotation of proteins. Nucleic Acids Res. 2011; 39:D225–30. https://doi.org/10.1093/nar/gkq1189 PMID: 21109532

27. Finn RD, Bateman A, Clements J, Coggill P, Eberhardt RY, Eddy SR, et al. Pfam: The protein families database. Nucleic Acids Research. 2014; 42:222–30.

28. Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, et al. CDD: a Conserved Domain Database for the functional annotation of proteins. Nucleic Acids Res. 2011; 39:D225–30. https://doi.org/10.1093/nar/gkq1189 PMID: 21109532

29. Hyatt D, Chen GL, Locascio PF, Land ML, Larimer FW, Hauser LJ. Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics. 2010; 11:119. https://doi.org/10.1186/1471-2105-11-119 PMID: 20211023

30. Aziz RK, Bartels D, Best AA, DeJongh M, Dins T, Edwards RA, et al. The RAST Server: Rapid Annotations using Subsystems technology. Nucleic Acids Research. 2008; 36:92–9. https://doi.org/10.1093/nar/gko369 PMID: 18080260

31. Finn RD, Bateman A, Clements J, Coggill P, Eberhardt RY, Eddy SR, et al. Pfam: The protein families database. Nucleic Acids Research. 2014; 42:222–30.

32. Finn RD, Bateman A, Clements J, Coggill P, Eberhardt RY, Eddy SR, et al. Pfam: The protein families database. Nucleic Acids Research. 2014; 42:222–30.

33. Hyatt D, Chen GL, Locascio PF, Land ML, Larimer FW, Hauser LJ. Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics. 2010; 11:119. https://doi.org/10.1186/1471-2105-11-119 PMID: 20211023
36. Gautheret D, Lambert A. Direct RNA motif definition and identification from multiple sequence alignments using secondary structure profiles. J Mol Biol. 2001; 313(5):1003–11. https://doi.org/10.1006/jmbi.2001.5102 PMID: 11700055

37. Macke TJ, Ecker DJ, Gutell RR, Gautheret D, Case DA, Sampath R. RNAMotif, an RNA secondary structure definition and search algorithm. Nucleic Acids Res. 2001; 29(22):4724–35. PMID: 11713323

38. Lesnik EA, Sampath R, Levene HB, Henderson TJ, McNeil JA, Ecker DJ. Prediction of Rho-independent transcriptional terminators in *Escherichia coli*. Nucleic Acids Res. 2001; 29:3583–94. PMID: 11522828

39. Lavigne R, Sun WD Volckaert G. PHIRE, a deterministic approach to reveal regulatory elements in bacteriophage genomes. Bioinformatics. 2004; 20:629–35. https://doi.org/10.1093/bioinformatics/btg456 PMID: 15038369

40. Crooks GE, Hon G, Chandonia J-M, Brenner SE. WebLogo: a sequence logo generator. Genome Res. 2004; 14:1188–90. https://doi.org/10.1101/gr.849004 PMID: 15034147

41. Delcher AL, Phillippy A, Carlton J, Salzberg SL. Fast algorithms for large-scale genome alignment and comparison. Nucleic Acids Res. 2002; 30(11):2478–83. PMID: 12034836

42. Lynch KH, Stothard P, Dennis JJ. Comparative analysis of two phenotypically-similar but genomically-distinct *Burkholderia cenocepacia*-specific bacteriophages. BMC Genomics. 2012; 13:223. https://doi.org/10.1186/1471-2164-13-223 PMID: 22676492

43. Miller ES, Kutter E, Mosig G, Arisaka F, Kunisawa T, Rüger W. Bacteriophage T4 genome. Microbiol Mol Biol Rev. 2003; 67:86–156. https://doi.org/10.1128/MMBR.67.1.86-156.2003 PMID: 12626685

44. Koch T, Raudonikiene A, Wilkens K, Rüger W. Overexpression, purification, and characterization of the ADP-ribosyltransferase (gpAlt) of bacteriophage T4: ADP-ribosylation of *E. coli* RNA polymerase modulates T4 "early" transcription. Gene Expr. 1995; 4(4–5):253–64. PMID: 7787417

45. Sullivan MB, Coleman ML, Quinlivan V, Rosenkranz JE, Defrancesco AS, Tan G, et al. Portal protein diversity and phage ecology. Environ Microbiol. 2008; 10:2810–23. https://doi.org/10.1111/j.1462-2900.2008.01702.x PMID: 18673386

46. Sullivan MB, Huang KH, Ignacio-Espinoza JC, Berlin AM, Kelly L, Weigele PR, et al. Genomic analysis of oceanic cyanobacterial myoviruses compared with T4-like myoviruses from diverse hosts and environments. Environ Microbiol. 2010; 12:3035–56. https://doi.org/10.1111/j.1462-2900.2010.01280.x PMID: 20662890
56. Petrov VM, Ratnayaka S, Nolan JM, Miller ES, Karam JD. Genomes of the T4-related bacteriophages as windows on microbial genome evolution. Virol J. 2010; 7:292. https://doi.org/10.1186/1743-422X-7-292 PMID: 21029436

57. Dreher TW, Brown N, Bozarth CS, Schwartz AD, Riscoe E, Thrash C, et al. A freshwater cyanophage whose genome indicates close relationships to photosynthetic marine cyanomyophages. Environ Microbiol. 2011; 13:1858–74. https://doi.org/10.1111/j.1462-2920.2011.02502.x PMID: 21605306