Characterization and Comparative Genomic Analyses of *Pseudomonas aeruginosa* Phage PaoP5: New Members Assigned to PAK_P1-like Viruses

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As a potential alternative to antibiotics, phages can be used to treat multi-drug resistant bacteria. As such, the biological characteristics of phages should be investigated to utilize them as effective antimicrobial agents. In this study, phage PaoP5, a lytic virus that infects *Pseudomonas aeruginosa* PAO1, was isolated and genomically characterized. PaoP5 comprises an icosahedral head with an apex diameter of 69 nm and a contractile tail with a length of 120 nm. The PaoP5 genome is a linear dsDNA molecule containing 93,464 base pairs (bp) with 49.51% G+C content of 11 tRNA genes and a 1,200 bp terminal redundancy. A total of 176 protein-coding genes were predicted in the PaoP5 genome. Nine PaoP5 structural proteins were identified. Three hypothetical proteins were determined as structural. Comparative genomic analyses revealed that seven new *Pseudomonas* phages, namely, PaoP5, K8, C11, vB_PaeM_C2-10_Ab02, vB_PaeM_C2-10_Ab08, vB_PaeM_C2-10_Ab10, and vB_PaeM_C2-10_Ab15, were similar to PAK_P1-like viruses. Phylogenetic and pan-genome analyses suggested that the new phages should be assigned to PAK_P1-like viruses, which possess approximately 100 core genes and 150 accessory genes. This work presents a detailed and comparative analysis of PaoP5 to enhance our understanding of phage biology.

Bacteriophages or phages are abundant viruses that infect bacteria. The number of phages is approximately 10-fold higher than that of bacteria. Since their discovery in 1915, phages have influenced basic and applied biology. Since 1959, nearly 6,300 different phages have been examined through electron microscopy, including 6,196 bacterial and 88 archaeal phages. In October 2012, 759 phages, including 721 infecting bacteria and 38 infecting archaea, were completely sequenced. In February 2016, the number of completely sequenced phages reached 2,012, including 1,935 infecting bacteria and 77 infecting archaea, as revealed by the data from the National Center for Biotechnology Information (Bethesda, MA, USA). This number is lower than that of completely sequenced bacteria, which reached 5,020 in February 2016, although the genome size of phages is less than that of bacteria. Novel phages should be characterized and genomically analyzed to obtain additional valuable data regarding phages and help enhance our understanding of the evolutionary relationships between phages and bacteria.

As a Gram-negative opportunistic pathogen, *Pseudomonas aeruginosa* is the leading cause of local and systemic nosocomial infections; in some cases, its infection is life threatening. *P. aeruginosa* infections are difficult to treat with antibiotics because of its intrinsic multi-drug resistance. Thus, the biological characteristics of *P. aeruginosa* phages should be investigated to eradicate this notorious pathogen. *P. aeruginosa* phages are taxonomically diverse and genetically dissimilar; they have been widely considered for their application as therapeutic and typing agents. As of February 2016, 141 complete genome sequences of *Pseudomonas* phages mostly infecting *P. aeruginosa* have become available in GenBank. *P. aeruginosa* phages are classified into several distinct
35 kDa, including tail fiber, baseplate and major capsid, are important for phage PaoP5 morphogenesis. The major capsid protein (gp057, ~39 kDa) of PaoP5. The structural proteins with molecular weight higher than 15 kDa were identified. The results showed that the predominant band was predicted as the structural protein in the gel (Fig. 2A). Nine proteins with molecular weights ranging from 15 kDa to 76 kDa were identified. To identify the structural proteins of PaoP5, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was used to separate and visualize each structural protein in the gel (Fig. 2A). Nine proteins with molecular weights ranging from 15 kDa to 76 kDa were determined. Then, each protein band was excised for high-performance liquid chromatography (HPLC)–mass spectrometry (MS), permitting the allocation of nine protein bands to nine corresponding PaoP5 genes. The detailed parameters and results of mass spectrometry are shown in Fig. 2B. The lowest sequence coverage was 15.63% for gp078, but the MS search score was not the lowest. Notably, the MS search score of gp055 was 0.00, indicating that the score should be further verified. Three hypothetical proteins, including gp055, gp064, and gp075 (Fig. 2B), were separated by SDS–PAGE, suggesting that these are actually structural proteins. Thus, we updated the corresponding GenBank records of gp055, gp064, and gp075, and conferred them with the function “structural protein” instead of “hypothetical protein.” As expected, the predominant band was predicted as the major capsid protein (gp057, ~39 kDa) of PaoP5. The structural proteins with molecular weight higher than 35 kDa, including tail fiber, baseplate and major capsid, are important for phage PaoP5 morphogenesis.

New members (including PaoP5) were assigned to PAK_P1-like viruses. BlastN analysis revealed that the genome sequences of phages P1P1,8–10, KPP10-like11, and PB1-like viruses12. With the rapid development of genome sequencing, numerous novel P. aeruginosa phages have been identified. However, most of these phages have remained unclassified. Therefore, novel P. aeruginosa phages should be characterized and classified to facilitate the understanding of the interactions between P. aeruginosa and its phages and to help develop new approaches that combat this versatile pathogen.

Figure 1. Electron micrograph of PaoP5 phage particles. The sample was stained with phosphotungstate. The scale bar represents 100 nm. The black arrows indicate contracted tails.
tBlastX analysis of the 13 phage genomes revealed that these phages show striking similarities at the protein level (Fig. S5).

As of February 3, 2016, 1,718 complete genomes of Caudovirales, including 463 Myoviridae, 319 Podoviridae, 913 Siphoviridae, and 23 unclassified Caudovirales were released in GenBank9. Among the 1,718 Caudovirales, 134 members infect Pseudomonas species (mostly P. aeruginosa). We conducted a phylogenetic analysis of 149 phages, including 134 members of Caudovirales that infect Pseudomonas species and 15 related members of Caudovirales that infect bacteria of other genera. The result indicated that the phages of the same genus are clustered into one clade, making the phylogenetic tree divide into several clades (Fig. 3). As expected, PaoP5 was clustered into PAK_P1-like viruses. In the year 2015, PAK_P1-like viruses was reported to have six members, including PaP1, JG004, vB_PaeM_C2-10_Ab1, PAK_P1, PAK_P2, and PAK_P410. Herein, we proposed that seven new members, including PaoP5, K8, C11, vB_PaeM_C2-10_Ab01, vB_PaeM_C2-10_Ab02, vB_PaeM_C2-10_Ab08, vB_PaeM_C2-10_Ab10, and vB_PaeM_C2-10_Ab15, should be assigned to PAK_P1-like viruses (Fig. 3). Although distributed in different clades, phages VCM, phiPsa374, and KPP10-like viruses are closely related to PAK_P1-like viruses (Fig. 3). Thus, all of these phages should be grouped into the subfamily of the Myoviridae named Felixounavirinae as proposed previously10.

Given the 13 complete genomes of PAK_P1-like viruses, we performed a pan-genome analysis. Results showed that the pan-genome of PAK_P1-like viruses comprises approximately 100 core genes and 150 accessory genes (Fig. S6). Hence, new additional members of PAK_P1-like viruses are predicted to be characterized and sequenced in the near future. The core genome of this phage genus is expected to contain less than 100 genes, but infinitely close to a certain amount, which constitutes the minimal genome23 of these phages, thus providing useful clues for synthetic biology analysis24.

Materials and Methods

Bacterial strains and culture condition. P. aeruginosa PAO118,21 was used as the host bacterium of phage PaoP5. As for host spectrum assay of PaoP5, the tested 95 P. aeruginosa strains were isolated from the Department of Burn of the first affiliated hospital of the Third Military Medical University (Southwest Hospital,
Chongqing, China) and cultivated in our laboratory. Bacteria were grown in Luria–Bertani (LB) liquid medium or plated onto solid LB medium containing 1.5% (w/v) agar and cultured at 37 °C with aeration.

**Transmission electron microscopy (TEM).** Filtered phage lysates (~10¹¹ PFU/mL) were placed on copper grids to allow adsorption for 10 min, then negatively stained with 2% phosphotungstic acid (PT-A, pH 4.5) for 1 min and subsequently air dried. Phage particles were observed using TECNAI 10 electron microscope (Philips, The Netherlands) at a voltage of 80 kV and with a magnification of 130,000. Images were acquired digitally with a camera (gatan Model 785) inside the microscope. Brightness and contrast were adjusted with Adobe Photoshop CS5.

**SDS–PAGE and HPLC–MS of the PaoP5 structural proteins.** Structural protein analysis was performed as described previously⁴. Briefly, the purified phage particles were heat-denaturized and loaded onto a 15% (w/v) polyacrylamide gel to visualize PaoP5 structural proteins. SDS–PAGE (12% [w/v] and 10% [w/v]) was also performed to better separate proteins with different molecular weights. Proteins were stained with Coomassie Brilliant Blue R250 dye and washed with methanol–acetic acid–H₂O. Then, protein bands were excised from the gel for HPLC–MS analysis. The data from HPLC–MS analysis were processed by Agilent Spectrum Mill proteomics software to allocate each band to the corresponding gene.

**Comparative genomic analysis.** Thirteen complete phage genome sequences were subjected to BlastN comparisons by using blast 2.2.29+(ftp://ftp.ncbi.nlm.nih.gov/blast/)²⁵,²⁶ and visualized by BRIG (http://brig.sourceforge.net/)²⁷ with a 80% identity cut-off. The PaoP5 genome sequence was used as reference. Phage genome sequences were subjected to tBlastX analysis by using EasyFig (http://mjsull.github.io/Easyfig/)²⁸ with a 33% identity cut-off. Major capsid protein sequences of phages (belonging to *Caudovirales*) infecting *P. aeruginosa*...
were downloaded from GenBank. The multiple sequence alignments of major capsid protein sequences were conducted using ClustalW with default parameters, and the phylogenetic tree was constructed and displayed by MEGA 6.06 (http://www.megasoftware.net/) with the neighbor-joining method. We then constructed a Venn diagram using an online tool for calculating and drawing custom Venn diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn). Pan-genome analyses were performed using CoreGenes 3.5 Batch Submission Tool (http://binf.gmu.edu:8080/CoreGenes3.5/BatchCoreGenes.html) and Panseq (https://lfz.corefacility.ca/panseq/ analyses#userPan) with default parameters, and the results of CoreGenes and Panseq were combined to better present the pan-genome of PAK_P1-like viruses.

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Author Contributions
The author(s) have made the following declarations about their contributions: S.Lu, F.H. and M.S. conceived and designed the experiments; M.S., S.Le, S.Lu, G.L., M.L., X.Z., Y.T. and W.S. performed the experiments; M.S., F.H., X.R., X.J., Y.Y., S.Li and J.W. analyzed the data; G.L., J.W., X.Z. and H.Z. contributed reagents/materials/analysis tools; M.S., F.H. and S.Lu wrote the paper.

Additional Information
Accession code: The complete genome sequence and annotations of PaoP5 have been deposited in GenBank under the accession number KU297675.

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