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Characterization and Genome Sequence of Marine Alteromonas gracilis phage PB15 Isolated from the Yellow Sea, China

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Characterization and Complete Genome Sequence of Marine *Alteromonas gracilis* phage PB15 Isolated from the Yellow Sea, China

**Abstract**

A novel marine *Alteromonas gracilis* siphovirus, phage PB15, was isolated from the surface water of the Yellow Sea in August 2015. It has a head diameter of $58 \pm 5$ nm head and a contractile tail approximately $105 \pm 10$ nm in length, and overall the morphology suggests that PB15 belongs to the family *Siphoviridae*. PB15 phage is stable at over the temperature range 0-60 °C. The best MOI of these phage was 0.1 and infectivity decreased above 60 °C. The results suggest that phage is stable at pH value ranging between 3.0 and 11.0. Chloroform test shows that PB15 is not a lipid-containing phage. A one-step growth curve with a strain of *Alteromonas gracilis* gave a latent period of 16 minutes and rise period of 24 minutes and burst size of 60 PFU/cell. Genomic analysis of PB15 reveals a genome size of 37,333bp with 45.52% G+C content, and 61 ORFs. ORF sequences accounted for 30.36% of the genome sequence. There is no obvious similarity between PB15 and other known phages by genomic comparison using the BLASTN tool in the NCBI database.

**Introduction**

Bacteria of the genus *Alteromonas* represent one of the oldest known genera of Gram-negative, strictly aerobic, heterotrophic marine bacteria [1]. The genus is primarily marine with examples found in a wide range of oceanic ecosystems ranging from surface waters and sea ice to abyssal sediments. It is very easy to isolate and grow *Alteromonas* in the laboratory [2] and isolates are available that have been isolated from the oceans all around the world. Ecologically they are often associated with nutrient-rich environments including particulate material, marine snow and marine animals [3, 4].

Viruses are the most ubiquitous and abundant organisms on Earth with an estimated total number of $10^{31}$ [5]. Most are bacteriophages that specifically infect bacteria and archaea [5, 6]. In marine ecosystem, bacteria are important drivers for biogeochemical cycle of carbon, other elements (N, P, Si, Fe etc.) and energy production [5]. Hence, as major agents for the mortality of prokaryotic cells, bacteriophage also play a key role in the global biogeochemical cycles and through structuring microbial communities, influencing the microbial food web processes in the ocean and mediating horizontal gene transfer between different microbes [5, 7, 8, 9].

As far as we are aware, few *Alteromonas gracilis* phage have been investigated previously. Phage PM2 was first reported in 1968 by Espejo and Canelo. The host cell identified as an *Alteromonas espejiana* BAL-31 strain. Phage PM2 is an icosahedral bacteriophage classified in the Corticoviridae. It is unique from other phages in that it contains lipid components in its virion structure [10]. Here we provide morphological and genomic information for a novel *Alteromonas gracilis* phage PB15 isolated from the Yellow Sea of China.
Materials and Methods

Location and Sampling

A seawater sample was collected at a depth of 3.0 m at during a cruise of R/V *Dong FangHong 2* in August 2015 in the Yellow Sea, China. The sample was stored at 4°C before analysis [11].

Bacterial Strain and Growth Condition

16S rRNA gene sequence of the host bacterial strain B15 had similarity of 100% to the type strain *Alteromonas gracilis* 9a2(T).

Phage Isolation

Seawater was filtered using 3μm pore-size filters (Whatman, England) to remove larger particles, followed by 0.2 μm pore-size low protein-binding PVDF filters (Millipore) to remove the remaining bacteria and phytoplankton. The detection and isolation of phage was performed using the standard double-layer agar method described by Middelboe et al. [12]. Plaque picking was repeated five times, and the purified phage were stored in SM buffer [100 mMNaCl, 8 mM MgSO₄, 50 mM TrisHCl] at pH 7.5 and 4°C [12, 13].

Transmission Electron Microscopy

The purified phage were examined at 100 kV using transmission electron microscopy (JEOL-1200 EX, Japan) after negative staining with 2% w/v phosphotungstic acid at pH 7.2 [14, 15].

Determination of the multiplicity of infection (MOI)

An *Alteromonas* culture was grown to exponential growth phase, aliquoted into five vials each with a bacterial density of $1.00 \times 10^8$ per ml and infected with different amounts of phage PB15. After 6 hours of incubation the samples were plated out and the optimal multiplicity of infection (MOI) was measured [16].

One-Step Growth Curve of PB15 phage

PB15 phage were added to a culture of the host bacterium B15 at an MOI of 0.1, and the mixture was incubated at 28°C for 1 min. Cells were then collected by centrifugation at 13000 rpm for 1 min and resuspended in 1 mL of fresh Luria-Bertani (LB) medium. This process was repeated twice to remove unadsorbed phage particles. The cell suspension was then added to 500 mL of LB broth and incubated with shaking at 28°C for 2h. The phage titer was measured by the double-layer agar technique [12] in samples taken at 8 min intervals. The experiment was repeated three times [11, 17, 18]. The relative burst size was plotted against time to determine the latent and rise period.

Thermal, pH and chloroform stability tests

Replicate 2 mL aliquots of phage suspension were incubated at temperatures of 0, 25, 40, 50, 60, 70 and 80°C for 2 h. Phage infectivity was then assayed by the spot test and the double-layer agar technique [19, 20]. The pH
stability of the phage was investigated in SM buffer adjusted to different pH values across the range 2 to 12. After incubating for 2 h at 28 °C, the surviving phage were diluted and enumerated using the double-layer agar method mentioned above. To test the effect of chloroform, 2 mL of a high titer phage suspension (60 PFU/cell) was put into a sterile tube and one drop of chloroform added. The solution was mixed gently, left for 30 min at room temperature and the bacteriophage titer was again assessed using the double-agar-layer technique [11, 17, 21].

**Genome Sequencing and Bioinformatic Analysis**

Phage DNA was extracted according to the protocol of Veheust et al [22]. Purified phage PB15 genomic DNA was sequenced at Sangon Biological Engineering (Shanghai) Co. Ltd. using an Illumina Miseq 2 × 300 paired-end sequence method. The sequencing was completed using an ABI 3730 automated DNA sequencer. Gaps between remaining contigs were closed using Gapcloser and GapFiller. Genome annotation was conducted using RAST (http://rast.nmpdr.org/). Sequence similarity searches were performed using the BLASTP algorithm against the SWISSPROT, NR, and TREMBL databases. Protein domain searches were performed using RPSBLAST against PFAM, CDD, and COG. To investigate the phylogeny of phage PB15, a phylogenetic analysis was performed using the major capsid protein sequence and MEGA 6 software.

**Results**

**Morphology of Phage PB15**

Morphological analysis of Phage PB15 using transmission electron microscopy indicated that this phage belongs to the family Siphoviridae. PB15 was found to have a 58 ± 5nm head diameter and a long non-contractile tail 105 ± 10nm long and 8 ± 2nm wide (Fig.1). 

**Optimal multiplicity of infection**

Host bacteria were infected with PB15 at different MOIs, with an MOI of 0.1 yielding the highest titer of phage (Table 1). Therefore, this was considered the optimal MOI and used for phage amplification in all subsequent experiments.

**One-Step Growth Curve**

The latent period was about 16 minutes. Following this there was a rapid increase in phage number during the rise period, and this lasted approximately 24 min (16 to 40 min post-infection) before the shift into a plateau period. The burst size of phage PB15 was about 60 PFU/cell (Fig. 2A).

**Thermal, pH and chloroform stability**

Phage PB15 retained plaque forming activity when incubated at temperatures of 50°C or less, indicating good thermal stability. However, at temperatures greater than 50°C, the phage titer declined. Phage incubated at 60°C
was viable, but the titer was significantly reduced. There were no viable phage at 70°C and 80°C (Fig. 2B). Phage PB15 were stable for 2 hours at pH values between 4 and 11. Almost no surviving infectious phage were observed at pH2-3. These results suggested that extremes of pH might affect phage PB15 infectivity (Fig. 2C). Phage PB15 was unaffected by chloroform. The results show that PB15 is not a lipid-containing phage.

Host Range Determination

Host range tests were evaluated on a panel of other strains by cross infectivity test. The results showed that the phage PB15 could not infect other Alteromonas and Pseudoalteromonas strains.

Genome Sequencing and Bioinformatic Analysis

The Phage PB15 genome is 37,333 bp and has a G+C content of 45.52%. A total of 61 ORFs were predicted in the phage genome without tRNA. Among the total 61 ORFs, 26 (42.62%) ORFs were predicted and assigned based on sequence similarity to other phage proteins (e-value < 10^{-5}) through BLASTP searches of the GenBank database. The minimum and maximum lengths are 135 and 2247 bp respectively. The total coding gene length is 11335 bp and the coding ratio is 30.36 %. The remaining 35 (57.38%) ORFs showed no significant evidence of homology with any other known phage proteins (e-value > 10^{-5}). Overall these results indicate that PB15 is a novel phage.

Amongst the latter group of ORFs mentioned above, seven (ORF2, ORF6, ORF13, ORF14, ORF18, ORF19, ORF60) have the highest similarity to ORFs from the Pseudoalteromonas phage Pq0 and four (ORF5, ORF7, ORF46, ORF61) are most similar to predicted ORFs from the Idiomarinaceae phage 1N2-2 (table 2). These unknown ORFs could be novel proteins whose hypothetical functions could possibly be deduced from their position in the genome.

BLASTP analysis of the complete genome sequence showed the main predicted functions modules of the phage PB15 ORFs to be: phage structure, phage packaging and binding, DNA replication and regulation, gene transfer protease (Fig. 3A) (Table 2). A phylogenetic tree was constructed with the protein sequences of the major capsid protein of some selected phages using neighbor-joining analysis. The results show that the siphovirus-type phage Alteromonas PB15 is closely related to Pseudoalteromonas phage Pq0 (Fig. 3B).

Discussion

According to the overall genomic organization and sequence similarities revealed here and the morphological features presented in a previous study [23], phage PB15 appears to be a member of the Siphoviridae family and closely related to Pseudoalteromonas phage Pq0. All of the typical stages in the multiplication of bacteriophage were seen in the one-step growth curve (Fig. 3A). Phage PB15 proliferates efficiently, with a short latent period (16 min), a large burst size (60 PFU/cell), and a high adsorption rate. Highly acidic pH values of 2 to 3 were
lethal to phage PB15, whilst the pH range 4-11 favored maximum infectivity (Fig. 3C). It is also notable that phage activity was also observed at pH 12.0. Phage PB15 has good thermal stability between 0 and 60 °C but activity was completely lost at 70 °C (Fig. 3B). Chloroform test shows that PB15 is not a lipid-containing phage.

The bioinformatics analyses extend our knowledge of bacteriophage [22]. The functional module for tail structural components and assembly is proposed to cover ORF2, ORF5, ORF7 and ORF8. ORF5 and ORF7 were found to exhibit significant similarity (53% and 39% overall identity) to the tail protein of Idiomarinaceae phage 1N2-2, and the minor tail proteins of various other phage. The protein specified by ORF2 shares 32% sequence identity with the tape-measure protein (TMP) of Pseudoalteromonas phage Pq0. PB15 is a long tailed phage and based on the observed similarities, ORF2 may also function as a tail length TMP in PB15. In almost all phage, the genes located between the major tail and head proteins are involved in the formation and connection of the head and tail structures and DNA packaging [23]. This is consistent with the position of ORF8, which is located between the tail and head proteins of PB15, and the shared 31% resemblance with the protein from Marinomonas phage P12026. The module for the capsid protein of the PB15 phage involves ORF13 and ORF18. ORF13 was predicted to encode the major capsid protein and ORF18 was identified as a minor capsid protein based on sequence similarity with that of Pseudoalteromonas phage Pq0 (64% and 49%, respectively) [24].

The DNA replication and regulation module includes ORF10, ORF14, ORF19, ORF40 and ORF45. ORF10, ORF14 and ORF19 were determined as gene transfer agent proteins, while ORF10 showed homology (27% overall identity) with Hyphomicrobium sulfonivorans. ORF14 and ORF19 showed considerable homology (61% and 46% overall identity) with Pseudoalteromonas phage Pq0. They help the phage DNA to penetrate and enter the host cells. ORF40 showed 53% similarity to an ATPase of Escherichia phage and ORF45 was found to share 66% homology with a helicase of Salicola phage CGphi29. ORF12, ORF39 and ORF42 have roles in DNA binding and the structure and expression patterns imply it might be a DNA binding transcription regulator [18]. All of the above proteins have diverse physiological roles in replication, recombination, repair, and packaging of phage DNA.

The terminase is a component of the molecular motor that translocates genomic DNA into empty capsids during DNA packaging [23]. In the PB15 genome, the terminases comprise large and small subunits encoded by ORF20 and ORF21 respectively. ORF20 is 66% homologous with Neisseria sp. KH1503 and ORF21 showed 34% similarity with Clostridium phage phiCP39-O.

A hypothetical protein is a protein whose existence has been predicted, but where there is a lack of experimental evidence that it is expressed in vivo. BLASTP analysis of the complete genome sequence showed that of the 61 predicted ORFs in phage PB15. 35 (57.38 %) had no match with putative functions or conserved
domains in the BLASTP database. This is probably due to the absence of similar integrase or recombinase genes in the sequence databases. When PB15 was compared with related phages with respect to phylogenetic position, no significant similarity was observed in the genome sequence with other phages at the genomic level. These results provide additional evidence that *Alteromonas* phage PB15 is a novel bacteriophage.

In conclusion, we analyzed the morphological properties and the genome sequence of the phage PB15. Previous studies of *Pseudoalteromonas* phage are relatively common, whereas, as far as we can ascertain, few *Alteromonas gracilis* phage have been investigated previously. With the development of marine virology, researchers have recognized that marine phage play important roles in promoting microbial evolution, accelerating microbial food loop dynamics and regulating microbial communities. In addition, the majority of phage gene functions are still unknown and need to be better understood. Our results add to the growing body of data for the research field and open the way for future studies.

**Genome Sequence Accession Number**

The complete genome sequence of phage PB15 was submitted to NCBI using Sequin under Accession Number KX982260.

**Acknowledgments**

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**References**


5. Weitz J S, Wilhelm S W (2012). Ocean viruses and their effects on microbial communities and
biogeochemical cycles. F1000 Biology Reports 4:17


**Fig. 1.** Transmission electron microscope images of phage PB15.
Fig. 2. The one-step growth curve for phage PB15 (A); thermal stability test of phage PB15 (B); pH stability test of phage PB15 (C).
Fig. 3. Cycle graph of the signed genome of phage PB15 (A); Neighbour-joining tree for selected phages constructed from their helicase protein sequences. Bootstrap values > 50 are shown on the nodes. The bar represents the 5% sequence change estimated (B).
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