Curr Microbiol (2017) 74:821–826
DOI 10.1007/s00284-017-1251-9

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

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Received: 18 October 2016 / Accepted: 11 April 2017 / Published online: 20 April 2017

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 ± 5 nm head and a contractile tail approximately 105 ± 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 *A. gracilis* gave a latent period of 16 min and rise period of 24 min and burst size of 60 PFU/cell. Genomic analysis of PB15 reveals a genome size of 37,333 bp 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.

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

Electronic supplementary material The online version of this article (doi:10.1007/s00284-017-1251-9) contains supplementary material, which is available to authorized users.

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Introduction

Bacteria of the genus *Alteromonas* represent one of the Gram-negative, strictly aerobic, heterotrophic marine bacteria [18]. Members of this genus are primarily marine with examples found in a wide range of oceanic ecosystems ranging from surface waters and sea ice to abyssal sediments. They are easy to isolate and grow *Alteromonas* in the laboratory [14]. Ecologically they are often associated with nutrient-rich environments including particulate material, marine snow, and marine animals [10, 13].

Viruses are the most ubiquitous and abundant organisms on Earth with an estimated total number of 10^{31} [22]. Most are bacteriophages that specifically infect bacteria and archaea [15, 22]. In marine ecosystem, bacteria are important drivers for biogeochemical cycle of carbon, other elements (N, P, Si, Fe, etc.) and energy production [22]. 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 [17, 22, 24, 26].

As far as we are aware, only one *Alteromonas gracilis* phage has been investigated previously. Phage PM2 was

first reported in 1968 by Espejo and Canelo [7]. The host cell was identified as an *Alteromonas espejiana* BAL-31. 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 [9]. In this study, we provide morphological and genomic information for a novel *A. 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 Fang Hong 2'* on August 2015 in the Yellow Sea, China. The sample was stored at 4 °C before analysis [19].

Bacterial Strain and Growth Condition

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

Phage Isolation

Seawater was filtered using 3 μm 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. [16]. Plaque picking was repeated five times, and the purified phage were stored in SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM TrisHCl) at pH 7.5 and 4 °C [6, 16].

Transmission Electron Microscopy

The purified phage were examined at 100 kV by transmission electron microscopy (JEOL-1200 EX, Japan) after negative staining with 2% w/v phosphotungstic acid at pH 7.2 [4, 20].

Determination of the Multiplicity of Infection (MOI)

The host strain B15 was grown to exponential growth phase, aliquoted into five vials each with a bacterial density of 1.00×10^8 per ml, and infected with different amounts of phage PB15. After 6 h of incubation, the samples were plated out and the optimal multiplicity of infection (MOI) was measured [23].

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 13,000 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 2 h. The phage titer was measured by the double-layer agar technique [16] in samples taken at 8 min intervals. The experiment was repeated three times [5, 12, 19]. The relative burst size was plotted against time to determine the latent and rise period.

Host Range Test

Host range tests were evaluated on a panel of *Pseudoalteromonas* strains, including *Pseudoalteromonas atlantica* T6c, *Pseudoalteromonas marina* Mano4 (T), *Pseudoalteromonas shioyasakiensis* SE3 (T), and *Pseudoxanthomonas kalamensis* JA40 by cross-infectivity test.

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 [3, 11]. The pH stability of the phage was investigated in SM buffer adjusted to different pH values across the range 2–12. 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 was 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 [2, 5, 19].

Genome Sequencing and Bioinformatic Analysis

Phage DNA was extracted according to the protocol of Veheust et al. [21]. 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 (https://blast.ncbi.nlm.nih.gov/). 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 ± 5 nm head diameter and a long non-contractile tail 105 ± 10 nm long and 8 ± 2 nm 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 min. Following this, there was a rapid increase in phage number during the rise period, and this lasted approximately 24 min (16–40 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 and 80 °C (Fig. 2b). Phage PB15 were stable for 2 h at pH values between 4 and 11. Almost no surviving infectious phages were observed at pH 2–3. These results suggested that extremes of pH might affect phage PB15 infectivity (Fig. 2c). PB15 was unaffected by chloroform. The results show that PB15 is not a lipid-containing phage.

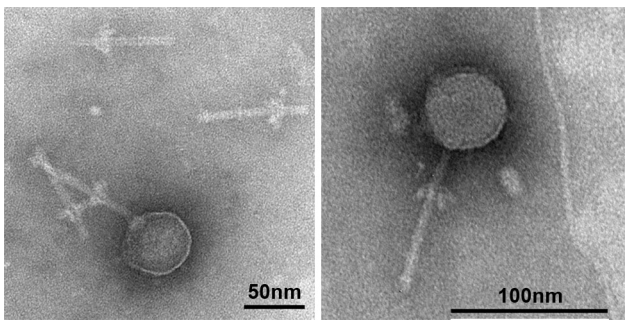

Fig. 1 Transmission electron microscope images of phage PB15

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 *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 11,335 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 $> 10^{-5}$). The phage PB15 showed no similarity to phage PM2 through BLASTP searches of the GenBank database. Overall these results indicate that PB15 is a novel phage.

Amongst the 26 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 S1). These unknown ORFs could be novel proteins whose hypothetical functions could possibly be deduced from their position in the genome. For example, the classical terminase consists of a large and a small subunit and involved in DNA translocation inside the capsid, but no small terminase subunit was detected in the phage PB15 genome. This result suggests that the hypothetical protein downstream from ORF20 may play a role as the small terminase subunit [25].

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 S1). 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

Table 1 Optimal multiplicity of infection (MOI) of phage PB15

No.	Bacterial titer (CFU/mL)	Plaque titer (PFU/mL)	MOI	Plaque titer (PFU/mL)
1	1.000×10^8	1.000×10^5	0.001	1.525×10^9
2	1.000×10^8	1.000×10^6	0.010	1.850×10^9
3	1.000×10^8	1.000×10^7	0.100	2.680×10^9
4	1.000×10^8	1.000×10^8	1.000	2.445×10^9
5	1.000×10^8	1.000×10^9	10.000	9.250×10^8

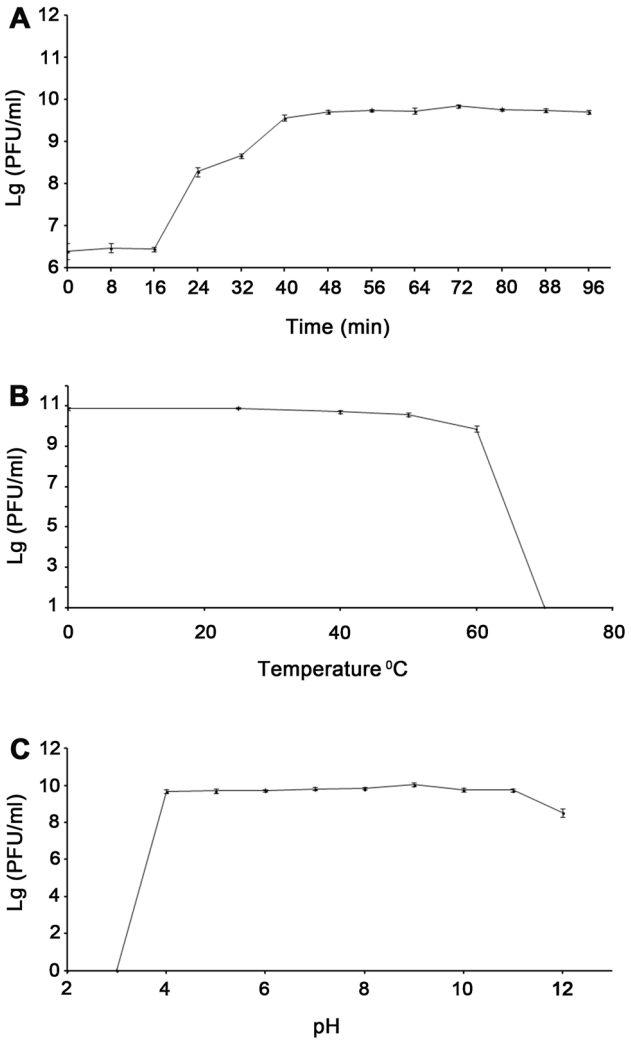

Fig. 2 The one-step growth curve for phage PB15 (**a**); thermal stability test of phage PB15 (**b**); pH stability test of phage PB15 (**c**)

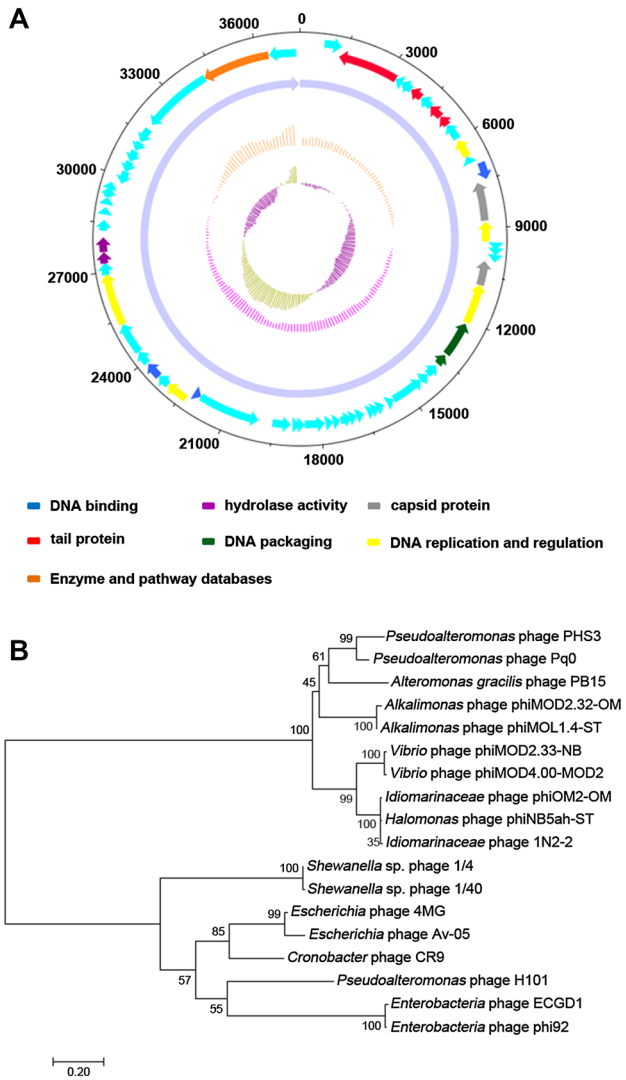

Fig. 3 Cycle graph of the signed genome of phage PB15 (**a**); neighbor-joining tree for selected phages constructed from their helicase protein sequences. Bootstrap values >50 are shown on the nodes. The *bar* represents the 5% sequence change estimated (**b**)

Alteromonas PB15 is closely related to *Pseudoalteromonas* phage Pq0 (46.65% similarity) (Fig. 3b).

Discussion

According to the overall genomic organization and sequence similarities revealed here and the morphological features presented in a previous study [8], phage PB15 appears to be a member of the *Siphoviridae* family and closely related to *Pseudoalteromonas* phage Pq0. All 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 2–3 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 [21]. 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 phages. 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 [8]. 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) [1].

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 [12]. All 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 [8]. In the PB15 genome, ORF20 encodes large terminase subunit and shows 66% homologous with *Mannheimia* phage vB_MhS_535AP2.

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.

Previous studies of *Pseudoalteromonas* phage are relatively common, whereas, as far as we can ascertain, only one *A. 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 is 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.

Acknowledgements We greatly appreciate the Officers, Crew, and scientific staff onboard the research vessel Dong Fang Hong 2 for facilitating the collection of the seawater samples. This work was supported by the National Natural Science Foundation of China (NSFC Grants 41076088, 31500339, 41676178), the National Key Basic Research Program of China (973 Program, grant number 2013CB429704), China Postdoctoral Science Foundation (Grants 2015M570612 and 2016T90649), and Fundamental Research Funds for the Central University of Ocean University of China (Grant Numbers 201564010 and 201512008).

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