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Isolation and Complete Genome Sequence of a Novel *Marinobacter* Phage B23

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Abstract
We used the double-agar layer method to isolate a novel *Marinobacter* marina bacteriophage, B23, from the surface water sample of the Bohai sea of China. There is some work to better understand the phage. The result of transmission electron microscopy revealed that B23 belongs to the family Siphoviridae with a head of 80 nm in diameter and a tail of 230 nm. Microbiological characterization evidenced that phage B23 is stable at the temperatures from − 25 to 60 °C, and showed vigorous vitality at pH between 4.0 and 12.0. One-step growth experiment showed that it had a longer latent period and higher lysis efficiency. Furthermore, the complete genome of B23 was sequenced and analyzed, which consists of a 35132 bp DNA with a G + C content of 59.8% and 50 putative open reading frames. The genome was divided into five parts, consisting of DNA replication and regulation, phage packaging, phage structure, host lysis and hypothetical protein.

Introduction

Ocean is the cradle of life, viruses are the most abundant and genetically diverse life forms in the ocean, typically there are 10^6–10^9 VLPs/ml, place significant predation pressure on their hosts. They play a very important role in marine ecosystem. Marine viruses influence many biogeochemical and ecological process, they are the largest cycle in biogeochemical cycle [1, 2]. Viruses maybe the major vector for gene transfer in the ocean, they mediated transfers occur up to 10^{15} times per second [3, 4]. Not only are viruses abundant in oceans but, as is becoming clear, they also harbor enormous genetic and biological diversity. Of the large number, more than 50% coding sequences (CDSs) haven't been annotated [5].

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The genus *Marinobacter* was proposed by Gauthier et al. with the description of *Marinobacter hydrocarbonoclasticus* as the type species [6]. *Marinobacter* colonize a wide variety of marine ecosystems around the world ranging from psychrophilic to thermophilic environments with a high tolerance to salinity and pH [7]. In anoxic conditions, species of the genus *Marinobacter* were the dominant component in marine polycyclic aromatic hydrocarbon(PAH)-degrading communities [8]. Species of the genus *Marinobacter* might enhance the biodegradation of crude oil through the biosynthesis of glycolipids [9]. The 16S rRNA gene sequence of the bacterial host showed 99% homology to *Marinobacter salarius* strain R9SW1 [10]. Marine microorganisms, such as *Marinobacter*, derive energy and carbon from the degradation of petroleum hydrocarbons and drive the bioremediation process during anthropogenic oil spills. Such as the Deepwater Horizon (DWH) spill, *Marinobacter salarius* R9SW1 play an important role in degradation of *n*-hexadecane (HEX) [11].

Marinobacter has high stability in the presence of high salt concentration and low temperature, conditions characterizing the marine environment, the capability to disperse crude oil and the low ecotoxicity makes them important in combatting marine oil spills [12]. Some experimental results support the concept of a phage-driven microbial loop in the bioremediation of the marine oil spills [13]. Application of this concept in bioremediation of contaminated water has

the potential to increase the efficiency of processes [14]. So the study of *Marinobacter* phage is significant. To better understand *Marinobacter* phage diversity and phage-host infection mechanisms in the marine environment, we isolated and characterized the *Marinobacter* phage B23 from the Bohai Sea of China, and reported the complete sequence of the genome of bacteriophage B23 as well as a preliminary analysis of the functional features of the genes.

Materials and Methods

Sampling

The surface seawater sample was collected from a depth of 0.5 m in the Bohai Sea of China, and stored at 4 °C after collection [15, 16].

Bacterial Strains

We used the agar overlay method to isolate the host bacteria strain *Marinobacter*. The molecular identification of the isolate was obtained via 16S rRNA gene sequence analysis, then researched the homology of the 16S rRNA gene sequence by BLAST search [17]. The host strain was conserved in liquid Zobell medium [18].

Phage Isolation

The seawater sample was filtered through 0.22 m syringe filter membrane, then the phage was detected and isolated by the double-agar-layer method: 200 µl of seawater sample filtrate was transferred into a cryopreservation tube, 200 µl of the host suspension was added and mixed well, then adding 4.5 ml of the semi-solid medium, and poured over the surface of solid medium. Plaques were observed over the surface of the agar plate after 24 h. Following at least three packing for each plaque, and the purified phage was then stored in SM buffer at 4 °C until processing [19, 20].

Morphology Study by Transmission Electron Microscopy

Morphology of the purified phage was examined at 100 kV via transmission electron microscopy (JEOL-1200 EX, Japan) to reveal the structural features [21].

One-Step Growth Curve Assay

The one-step growth curve calculates the latent period and burst size. The bacteriophage was added to mid-exponential phase culture of *Marinobacter* and allowed to adsorb at 25 °C for 15 min. Subsequently, the mixture was harvested by centrifugation (13,000 r for 30 s) to remove non-absorbed phage and then incubated at 25 °C with shaking. During a 2-h incubation, samples were collected every 8 min, then using the double-layer agar method to determine the phage titration [22].

PH Sensitivity and Thermal Stability

To investigate the effect of pH on phage infection, phage B23 were added to SM buffer, and the pH was adjusted from pH 2 to 13. After incubating for 2 h at 25 °C, the titer of each surviving phage was evaluated using the double-layer method. To research phage stability at different temperatures, phage B23 was added to SM buffer, and the mixtures were incubated at various temperatures: − 25 °C, 4 °C, 25 °C, 40 °C, 50 °C, 60 °C, and 70 °C. After 2 h of incubation, the survival rate of each treated sample was also determined by double-layer agar method [23, 24].

Host Range Test

The host range was detected by double-layer agar method: mixing with 4.5 ml 2216E broth, 200 µl of bacteria strain culture and 200 µl of phage culture, then poured onto a 2216E agar plate. The plate was incubated at 37 °C overnight. A clear zone in the plate indicated the presence of phage, and positive tests were confirmed by plaque assay [25]. *Marinobacter algicola* DG893 were used to test host range.

Genome Sequencing and Bioinformatic Analysis

We used the TIANamp Virus DNA Kit to extract the DNA of phage B23. Purified phage B23 genomic DNA was sequenced by the IlluminaMiseq 2 × 300 paired-end sequence methods. Gaps between remaining contigs were closed via the Gap Closer v1.12. ORFs were analyzed using RAST (http://rast.nmpdr.org/). The predicted functions of genes and the sequences of the amino acids were scanned using BLAST to search against the data set derived from all the complete genomes of viruses in NCBI [25, 26]. The complete sequence of bacteriophage B23 has been submitted to GenBank sequence library and assigned with the accession number KY939598.

Results

Identification of the Bacterial Strain

The 16S rRNA sequence shows similarity 99% to *Marinobacter salarius* R9SW1 deposited in GenBank.

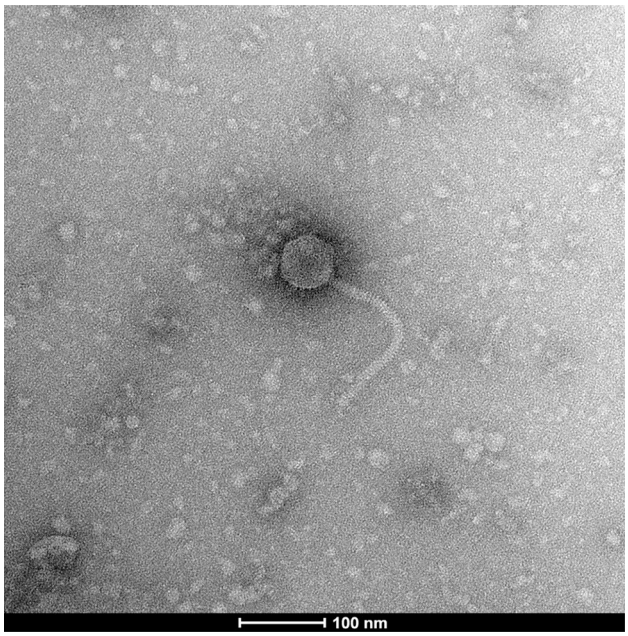

Fig. 1 Transmission electron microscope of PH101, the scale bar 100 nm

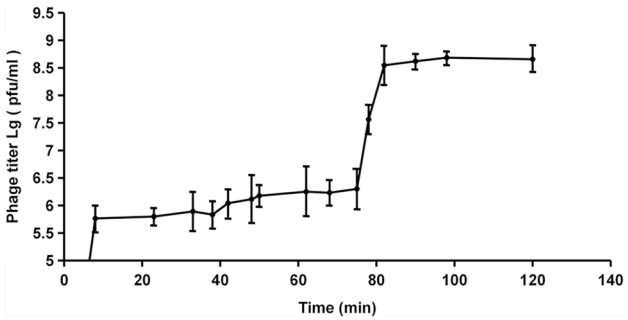

Fig. 2 One-step growth curve of phage B23

Morphology of Phage B23

The phage B23 was examined by transmission electron microscopy (Fig. 1), and it was classified as a member under the family Siphoviridae. It has been shown that the B23 has a head of ca. 80 nm in diameter and a tail of ca. 230 nm.

One-Step Growth Curve Assay

One-step growth curve shows that phage B23 had a considerably longer latent period of 90 min followed by a quick rise in the phage titer, and a burst size of about 19.3 virions per cell (Fig. 2).

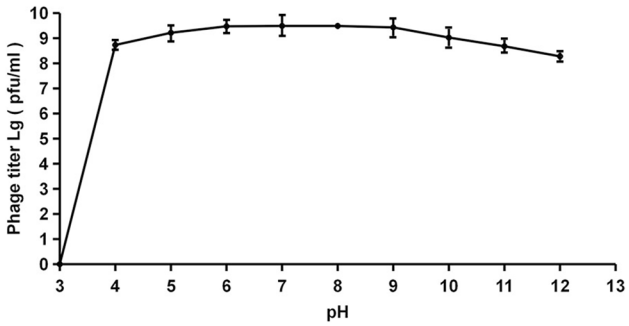

Fig. 3 PH stability of phage B23

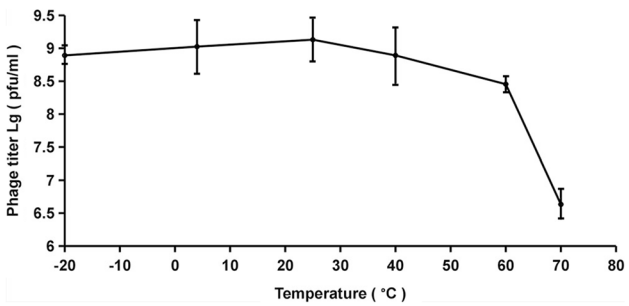

Fig. 4 Temperature stability of phage B23

pH and Temperature Stability

The stability of the phage was investigated under different thermal and pH conditions based on input and residual plaque forming unit (pfu) numbers [27]. Phage B23 showed best stability at pH 8, and good stability from pH 4 to 12. While, at pH 3, no actively infectious phage was detected. The results showed that the phage might be unstable at low pH, while the number of plaques was found to increase with increasing pH, reaching the highest number at pH 8 (Fig. 3). In addition, the results of thermal stability tests show B23 was relatively heat stable from 4 to 5 °C, and no significant loss in phage activity was observed, but decreased sharply with increasing temperature at above 60 °C and above (Fig. 4).

Host Range of Phage B23

Host range tests showed that phage B23 did not infect other bacterial strains in the cross infectivity studies.

Genome Sequencing and Bioinformatic Analysis

The B23 genome has a total length of 35,132 bp and a G+C content of 59.8%. The NCBI nucleotide blast analysis of the complete genome sequence indicated that B23 shares

extremely limited similarities with other known phage nucleotide sequences, which confirmed its status as a novel *Marinobacter* phage species.

Marinobacter phage genomes were annotated using RAST, and manually inspected for alternative start codons. 50 ORFs were detected and of these, 47 in the positive strand and 3 in the negative strand. And the average length is 606 bp, the minimum length is 171 bp, and the maximum length is 2094 bp. Most of ORFs (52 ORFs) had an ATG start codon, but there were also incidences of alternative start codons GTG (8 ORFs).

The functions of ORFs were searched by BLASTP. Among the 50 potential ORFs identified, 29 ORFs were annotated as known genes. In terms of genome organization, the predicted ORFs were classified into five groups, including DNA replication, regulation and nucleotide metabolism, host lysis, phage packaging, phage structure and hypothetical protein. The genome map was drawn by DNA Master (Fig. 5). The majority of proteins related to the DNA replication, regulation and nucleotide metabolism is located in the left arm of the B23, such as DNA-binding protein (ORF2). In the mid-range and the right arm of the genome, the phage structure genes such as head subunit protein (ORF32) and tail proteins (ORF38, ORF41, ORF44, ORF47, ORF48) are found.

BLASTP analysis showed that the proteins encoded by the B23 genome had the closest hits to proteins of 7 bacteriophages (Table 1). Eight ORFs of phage B23 had the highest similarity to predicted ORFs from the *Vibrio* phage martha 12B12 (Table 1). A phylogenetic tree based on the amino acid sequence of the terminase large subunit, constructed using the neighbor-joining method, showed a clustering of phage B23 with *Thiobacimonas* phage vB ThpS-P1, *Rhodovulum* phage vB RhkS P1 and *Pelagibaca* phage vB PeaS-P1 (Fig. 6). Further conserved protein domain analysis of predicted ORFs showed that 26 conserved domains were detected (Table 2).

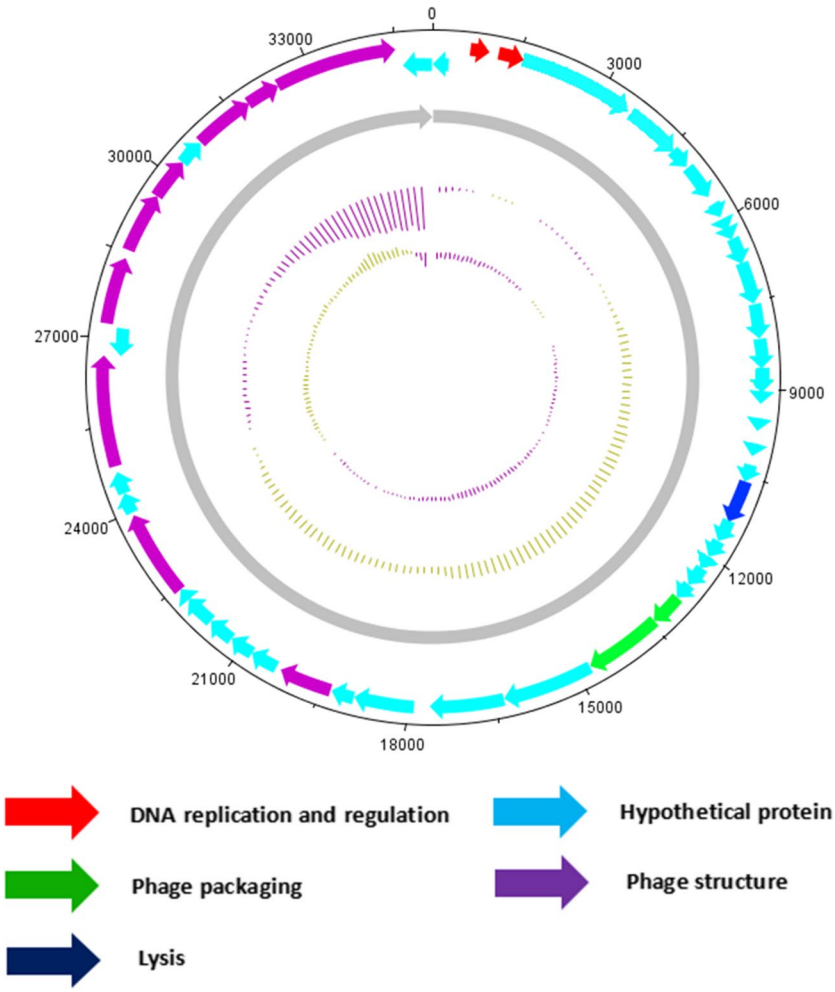

Fig. 5 Cycle graph of the signed genomes phage B23

Table 1 Phages with common genes as phage B23 by BLASTP

Phage name	Phage family	Genome length (bp)	GenBank accession number	Common ORFs with B23
Vibrio phage martha 12B12	*Myoviridae*	33,277	NC_021070.1	8
Escherichia virus Mu				4
Vibrio phage VBpm10	Unclassified	33,314	JF974302	3
Pseudomonas phage JBD25	*Siphoviridae*	39,552	NC_027992	3
Enterobacteria phage SfV	*Myoviridae*	37,074	NC_003444	2
Salmonella phage 118970_sal3	Unclassified	77,375	NC_031940	2
Pseudomonas phage *vB*_PaeS_PM105	*Siphoviridae*	39,593	NC_028667	2

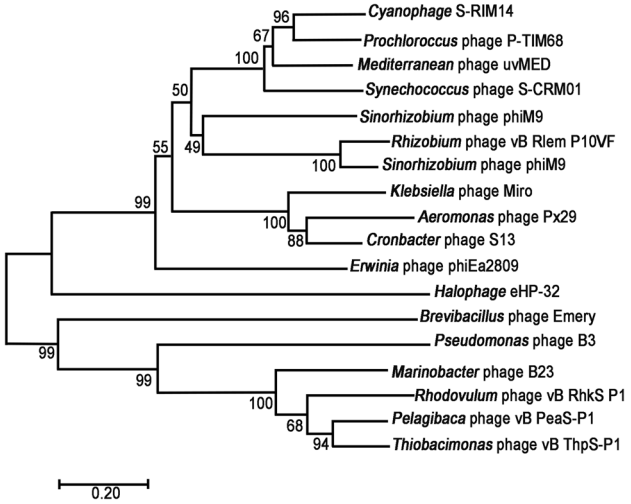

Fig. 6 Phylogenetic tree for selected phages constructed from the protein sequence of their terminase large subunit

Discussion

In the current study, a Siphoviridae phage, named B23, was isolated from water samples of the Bohai Sea. B23 was able to infect strain *Marinobacter* in this study and formed clear plaques. One-step growth analysis revealed that the phage has a long latent period, indicating that B23 has high lytic activity and robust propagation. Further investigation of its ability to inhibit bacterial growth under various conditions, the B23 showed high stability to temperatures and pH [28].

BLASTP analysis of the complete genome sequence showed 50 ORFs in phage B23. In total, 30 of the 50 predicted ORFs were not found to have any matches of putative functions in the BLASTP database. In all of these ORFs, 26 conserved domains were detected.

Based on the similarity of its sequence and organization to those of other phages to analyze the knowledge about phage B23. Structural gene region, the genes encoding the structural proteins are located from ORF32 to ORF49. ORF32 encodes phage major head subunit gpT, shares high levels of amino acid identity with *Pseudomonas* phage LPB1. It is typical for the head protein genes in phage genomes to be clustered together and to precede the tail protein genes [29]. This suggests that the hypothetical protein downstream from ORF32 may be involved in viral head structural formation despite their products lacking identity to any known bacteriophage structural proteins. The downstream of the phage head morphogenesis-encoding region is a putative tail morphogenesis region. By BLASTP analysis, ORF38, ORF39, ORF41, ORF44, ORF47 and ORF48 encode tail protein.

For the phage packaging module of phage B23, composed of the small terminase subunit and the large subunit. ORF27 encodes large terminase subunit and shows highly similar to the putative large terminase from the *Rhodovulum* phage vB RhkS P1. Typically, the genes encoding the small terminase are located immediately upstream of those encoding the large terminase subunit and transcribed in the same direction, which is consistent with the analysis of BLASTP, ORF26 encodes the small terminase subunit of B23 [30].

By the BLASTP analysis, there are only four ORFs about the DNA replication, regulation, and nucleotide. ORF2 encodes DNA-binding protein containing HTH_35 domain, and shows homology to DNA-binding protein in *Haemophilus* phage SuMu. ORF3 also encodes DNA-binding protein. ORF43 encodes DNA circulation protein. Phage lysis modules typically consist of lysozyme and holin genes that together are responsible for bacterial lysis and release of phage progeny [31]. In the B23 genome we could identify only the gene of Phage lysin (ORF20), with no ORF displaying identity to any known holin protein. But the hypothetical protein (ORF19) contain Holin_2–3 domain (pfam13272). It suggests that this might be a new holin genes of phages [32].

In this study, we analyzed the morphological properties and the genome sequence of the phage B23. Present work would provide basic data to further understand the complex phage–host interactions and could be used as basic knowledge for future work.

Table 2 Genomic annotation of B23 and conserved domains detected

ORF	Strand	Start	Stop	Function	Conserved domains accession
2	+	652	975	DNA-binding protein	pfam13693
3	+	1133	1567	gp13	
4	+	1564	3534	Hypothetical protein	pfam02914
5	+	3612	4568	Hypothetical protein	COG2842
15	+	8646	9050	Hypothetical protein	pfam08765
19	+	10,293	10,595	Hypothetical protein	pfam13272
20	+	10,598	11,320	Transglycosylase SLT domain protein	cd00254
23	+	12,011	12,235	Hypothetical protein	COG1734
26	+	12,888	13,442	Terminase small subunit	pfam11985
27	+	13,435	14,772	Terminase large subunit	COG4373
29	+	16,340	17,614	F protein	COG2369
30	+	17,888	18,910	I protein	pfam10123
32	+	19,323	20,243	Head subunit protein	pfam10124
34	+	20,817	21,233	Hypothetical protein	pfam07030
35	+	21,230	21,694	Hypothetical protein	pfam05069
36	+	21,691	22,227	Hypothetical protein	pfam08874
37	+	22,232	22,435	Hypothetical protein	pfam10948
38	+	22,439	23,929	Tail sheath protein	COG4386
39	+	23,977	24,330	Hypothetical protein	pfam10618
40	+	24,330	24,710	Hypothetical protein	COG4518
41	+	24,818	26,713	Phage tail tape protein	TIGR01760
43	+	27,248	28,411	Phage tail protein	COG4228
44	+	28,554	29,594	Tail protein	COG4379
45	+	29,585	30,313	Putative baseplate assembly protein	pfam06890
46	+	30,310	30,759	Hypothetical protein	pfam07409
47	+	30,760	31,821	Tail protein	pfam04865
48	+	31,809	32,402	Tail protein	COG3778
49	+	32,405	34,498	Tail protein	

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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