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Characterization of a novel *Bacillus methylotrophicus* phage BM-P1

Ruirui Lv†, Ming Xu†, She Guo, Jingwei Yao, Hafiz Arbab Sakandar, Jing Guo, Can Zhang, and Xia Chen*,

Inner Mongolia Agricultural University, Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education; Key Laboratory of Dairy Products Processing, Ministry of Agriculture and Rural Affairs; Inner Mongolia Key Laboratory of Dairy Biotechnology and Engineering, Hohhot 010018, China

†These authors contributed equally to this work.
*Correspondence to: Xia Chen, Inner Mongolia Agricultural University, Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education; Key Laboratory of Dairy Products Processing, Ministry of Agriculture and Rural Affairs; Inner Mongolia Key Laboratory of Dairy Biotechnology and Engineering, Hohhot 010018, China. E-mail: chenxia8280@163.com

Abstract

Bacillus species have been widely used as probiotics in a number of food products. However, these are vulnerable to bacteriophage infections, which poses fermentation failure and eventually result in economic losses. Given this, we designed this study in which the bacteriophage of lysogenic *Bacillus methylotrophicus*, phage BM-P1, was induced successfully, and its biological characteristics and genome information were researched. The obtained results showed that phage BM-P1 belonged to *Myoviridae* family. The maximum number of infections for this phage was 10, with a burst size of 104.48±2.70 counts expressed per milliliter per infective center. Additionally, this phage was temperature- and pH-sensitive and divalent ions (Ca^{2+}, Mg^{2+}) and chloramphenicol did not have a significant influence on its adsorption capacity. Genomic analysis revealed that the genome size of phage BM-P1 was 153 087 bp with 41.94% GC content, including 258 coding sequences. Furthermore, 52 bacteriophages of *Bacillus* were classified into two clades by using phylogenetic analysis. Among them, phage BM-P1 with phage VB_BsuM-Goe3 and vB_BveM-Goe7 had the highest average nucleotide identity values, 95.23% and 95.28%, respectively. However, synteny analysis revealed transposition, deletion, and insertion in the genome of BM-P1. Considering this, it could be extrapolated that this phage is a new lysogenic phage. In conclusion, this study furthers the knowledge on the properties of *B. methylotrophicus* phages and provides seminal insights for designing effective antiphage strategies for fermentation industry.

Keywords: *Bacillus methylotrophicus* phage; morphology; resistance; adsorption; whole genome.

Introduction

Bacillus methylotrophicus is a Gram-positive bacterium that can utilize low carbon compounds such as methane, methanol, formaldehyde acid, methyl amine, etc. Since the discovery of *B. methylotrophicus* in 2010, researchers have carried out a slew of studies on its biological characteristics (Madhaiyan *et al.*, 2010). At present, its application as biological control in agriculture, especially in plant growth-promotion and food processing, has been reported (Mehta *et al.*, 2014; Seong *et al.*, 2019; Jemil *et al.*, 2020).

Bacteriophages are viruses that can infect and proliferate in bacterial cells. They are widely found in various environments such as seawater, sewage, soil, and the excreta of humans and animals (Dion *et al.*, 2020). They are considered one of the most important microorganisms in the ecosystem and can facilitate microbial evolution (Frost *et al.*, 2005). Lysogenic phages can integrate their genomes within the host cell. It is a well-established fact that under certain conditions such as antibiotic pressure, oxygen stress, organic reagent treatment, and ultraviolet irradiation, some prophages might be induced to undergo lytic growth and transformed into virulent ones (Penadés *et al.*, 2015). Due to the special characteristics of lysogenic phages, which might enter the lysogenic cycle without causing host cell lysis, their detection is difficult during fermentation. However, once lysogenic phages are induced into the lysis cycle, the growth of the strain could be affected, resulting in fermentation failure (Zhang *et al.*, 2015). As reported before, bacteriophages of *Bacillus* show great diversity in morphology, sequence length, sequence content and host range, reflecting the high degree of inter- and intraspecific variability of the species (Schofield and Westwater, 2009). To date (January 2022), 51 whole genomes of *Bacillus* phages have been published in the GenBank Database on NCBI (National Center for Biotechnology Information, USA; https://www.ncbi.nlm.nih.gov/), with genome sizes ranging from 45 to 165 kb; among them, *B. methylotrophicus* phage has not been reported separately. Therefore, it is imperative to further the understanding of the infection mechanism, biological

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attributes and genomic information of *B. methylotrophicus* bacteriophage.

The host strain *B. methylotrophicus* IMAU BM01, used in this experiment, was isolated from a traditional feed starter. When this starter is used in feed fermentation, abnormal fermentation phenomena (such as unqualified pH value) and fermentation failure often occur (Da Silva *et al.*, 2022; Muck *et al.*, 2018). Therefore, we designed this study to investigate the presence of the *Bacillus* phage and to evaluate the effect of physical and chemical conditions on phage viability and adsorption ability. Moreover, we deciphered the genomic characteristics of this phage. It might provide some theoretical basis for the subsequent study on the underlying mechanism of bacteriophages infection against host bacteria and could further our knowledge to fend off phage infections.

Materials and Methods

Bacterial strains and culturing conditions

The host strain *B. methylotrophicus* IMAU BM01, which was isolated from animal feed, was obtained from the Lactic Acid Bacteria Collection Center, Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, China. The host strain was grown at 36 °C in liquid nutrient broth (10 g/L soy peptone, 5 g/L NaCl, 3 g/L beef extract) for 18 h (Krasowska *et al.*, 2015).

Phage induction and enrichment

The above strain was grown in nutrient broth at 36 °C to the exponential phase (optical density at 600_{nm}≈0.5). Then, the cultures were transferred to sterile glass Petri plates and subjected to a UV-light source (254 nm) for 40 s, and samples were placed at a distance of 15 cm from the light source. These samples were pipetted back into culture tubes and incubated at 36 °C for 6 h. Afterwards, the samples were centrifuged at 5000×*g* for 10 min and supernatants were collected by filtering through a 0.22-μm filter. Subsequently, the phage particles were sedimented by adding 0.5 mmol/L NaCl and 10% (*m/v*) polyethylene glycol 8000 and incubated at 4 °C overnight (Colombet *et al.*, 2007; Fouladvand *et al*, 2020; Abdelsattar *et al.*, 2022). Finally, the precipitate was centrifuged at 12 000×*g* for 20 min and resuspended in Suspension Medium (SM) buffer (50 mmol/L Tris–HCl, 100 mmol/L NaCl, 10 mmol/L $MgSO_4$, 0.01% gelatin).

Electron microscope

Electron micrographs of phage were obtained as described by De-Antoni *et al.* (2010). Briefly, transmission electron micrographs (TEM) were taken using a Jeol H-7000 electron microscope (Jeol USA Inc., Peabody, MA, USA) operating at 75 kV. Phage morphology, capsid diameter, tail length and diameter were recorded. The specific steps are mentioned below: the phage stock with a titer of 10^8 plaque forming unit (PFU)/mL was used for electron microscope observation. A small amount of suspension drops of phage were added to the coated copper net, and 1 drop of 2% phosphotungstic acid (pH 7.0) was dropped. After the drops were left for 30 s, the wet dye was absorbed by filter paper and dried at room temperature for natural drying. Finally, the morphology of the phage was observed by TEM.

Determination of optimal multiplicity of infection

The host cells were infected with phage particles at different ratios (0.01, 0.1, 0.5, 1, 2, 10, and 100). After incubation at 36 °C for 4 h in a shaker at 160×*g*, 1 mL culture solution was taken and centrifuged for 10 min at 8000×*g*. The titer of phages was determined by double agar overlay assay (DLA) after gradient dilution. In brief, the bottom layer was prepared with nutrient agar containing 1.5% agar. Then, the top layer was poured containing the same medium with 0.6% agar that was mixed with the host bacterium and phage particles onto the bottom layer (Ács *et al.*, 2020). The plates were incubated at 37 °C for 24–26 h, and plaques observed and counted. The maximum number of infections (MOI) resulting in the highest phage titer (the highest PFU per milliliter) was considered as the optimal MOI (Lu *et al.*, 2003).

One-step growth curve

B. methylotrophicus was grown to the exponential phase (OD_{600}≈0.5), and then phage stock solution was added at the optimal MOI. After adsorption (15 min at 36 °C), cells were harvested by centrifugation (8000 r/min for 5 min). The resulting pellet was resuspended in nutrient broth and incubated at 36 °C. At regular intervals (15 min), 100 μL of each dilution was collected for bacteriophage enumeration (Capra *et al.*, 2010). The latent period, burst time, and burst size were calculated from one-step growth curve.

Factors influencing phage viability

Phages aliquots (10^8 PFU/mL) were suspended in 1-mL Eppendorf tubes containing nutrient broth and incubated at 0, 10, 20, 30, 36, 42, and 50 °C for 45 min. To evaluate the impact of pH on phage viability, the phages aliquots (10^8 PFU/mL) were suspended in nutrient broth at pH 2 to 11, and then incubated at 36 °C for 45 min. The viable phages were immediately counted by using the DLA method as described in the section of 'Determination of optimal multiplicity of infection'.

Factors influencing phage adsorption

The influence of temperature on the adsorption of phage was evaluated at 0, 10, 20, 30, 36, 42, and 50 °C. To perform this experiment, *B. methylotrophicus* cultures (at optimal MOI) were suspended in nutrient broth and incubated for 45 min. The influence of pH (from 2 to 11) on cell lysis was evaluated by inoculating *B. methylotrophicus* (at optimal MOI) in nutrient broth at 36 °C for 45 min.

The impact of divalent cations (Ca^{2+} and Mg^{2+}) on cell lysis was studied by incubating the infected cells (at optimal MOI) in nutrient broth with or without $CaCl_2$ (1.8 mmol/L) or $MgCl_2$ (0.8 mmol/L) at 36 °C. Samples were collected during incubation at various time points (0, 15, 30, and 45 min).

The minimum concentration of chloramphenicol needed to inhibit protein synthesis in the host strain was determined as described by Briggiler Marcó and colleagues (Briggiler Marcó *et al.*, 2010). Briefly, host cells were treated with chloramphenicol (20 or 100 μg/mL), and the chloramphenicol was removed after the inhibition of protein synthesis. Later, treated cells were infected with phage (at optimal MOI) and then incubated at 36 °C for 45 min.

For all the factors assessed, adsorption rates were always calculated as the ratio of initial phages. The corresponding dilution of the original phage suspension and non-adsorbed

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phages were measured in the supernatants after centrifugation (De Antoni *et al.*, 2010). The reduction in the number of phages due to adsorption was plotted against time (for the evaluation of the influence of divalent cations), temperature, pH value, and chloramphenicol.

DNA extraction, sequencing, and assembly

Bacteriophage DNA from culture with a titer >10^8 PFU/mL was extracted using the phenol-chloroform extraction method (Kim and Hwuii, 2018). *Bacillus* bacteriophage genome sequencing was performed by Anshan Biotechnology Co., Ltd. (Tianjin, China). The Sequel Binding Kit 2.0, Sequel Sequencing Kit 2.1, and Sequel SMRT Cell 1M v2 were employed to prepare DNA fragment library. Genomes were sequenced by PacBio SMRT (Pacific Biosciences, Menlo Park, CA, USA). The Flye (Version 2.8.3, https://github.com/fenderglass/Flye) program was used for assembly (Kolmogorov *et al.*, 2020). The data were first filtered to 50 Mb (random extraction) before assembly so that host sequence could not be assembled. After the assembly was completed, the Flye program provided information about the loops' formation. For small genomes like bacteriophages, Flye can generally be assembled at one time with only one contig.

Bioinformatics analysis

We used Glimmer (Gene Locator and Interpolated Markov ModelER; Version 3.02, http://ccb.jhu.edu/software/glimmer/index.shtml) to annotate the genome (Arthur *et al.*, 1999). tRNA in the phage genome was predicted by tRNAscan-SE (Version 1.3.1, http://lowelab.ucsc.edu/tRNAscan-SE/; Chan and Lowe, 2019). Subsequently, Clusters of Orthologous Groups (COG; http://www.ncbi.nlm.nih.gov/COG/) was used for annotating amino acid sequences (Kanehisa and Goto, 2000).

In this study, the whole genome sequences of 51 published *Bacillus* phages were collected by Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and downloaded for subsequent analysis. We constructed phylogenetic trees by the neighbor-joining method (Saitou and Nei, 1987). Average nucleotide identity (ANI) between two genomes was calculated using FastANI (Version 1.33, https://github.com/ParBLiSS/FastANI), with alignment fraction of at least 60% (Letunic and Bork, 2021). Additionally, the synteny of bacteriophages was analyzed by Mauve (http://darlinglab.org/mauve/mauve.html; Darling *et al.*, 2004).

Statistical analysis

All experiments were done in triplicate. Treatments using a single biocide with the same concentration were used as control. Means were compared using one-way analysis of variance (ANOVA) followed by IBM SPSS Statistics 20.0 software (IBM Corp, Armonk, NY, USA). Duncan's multiple range test was used to separate means. Graphs from all SPSS results were drawn by using Originpro software (Version 8.6, Originlab, Originlab Corp., Northampton, MA, USA).

Results and Discussion

Phage induction and phage morphology

The results showed that the OD_{600} value of the induction solution of *B. methyltrophic* IMAU BM01 fluctuated when irradiated for 10 s and was higher than that of the control group. When irradiated for 20 s, the OD_{600} value decreased and eventually stabilized. Eventually, after 30 s of irradiation, the OD_{600} value showed a decreasing trend, while a slight change was observed when irradiated for 40 s (Figure 1). DLA analysis showed that the plaques only appeared in the sample medium with 40 s of UV irradiation as it is a common tool for bacteriophage induction. In 2002, Woods *et al.* induced a phage from *Burkholderia thailandensis* strains with 254 nm UV lamp (25 cm spacing) irradiation for 20 s and showed clear phage spots on petri dishes (Woods *et al.*, 2002). However, different prophages require different UV irradiation time and distance, and the specific reasons are still obscure.

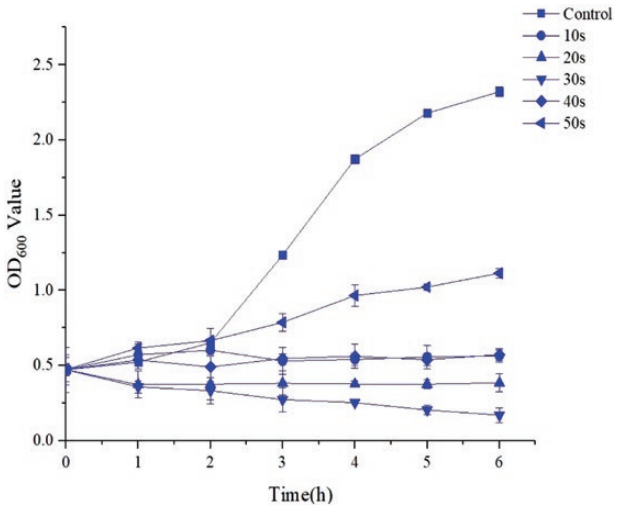

Figure 1. Changes in the OD_{600} values of *Bacillus methylotrophicus* after UV induction.

It is quite vivid in Figure 2A that bacteriophages are successfully induced. We purified this strain and evaluated its morphology in negatively stained preparations using TEM (Figure 2B). The results showed that phage BM-P1 had head diameter, non-contractile tail length and width of 136.86±2.01, 197.42±8.00, and 17.52±2.01 nm, respectively. According to the general classification criterion of viruses provided by the International Committee on Taxonomy of Viruses (ICTV) phage BM-P1 belongs to *Myoviridae* family (Mayo and Pringle, 1998; Hull and Rima, 2020). Moreover, its morphology was similar to the morphology of some *Bacillus* phages that belong to the Myoviridae family (Lee WJ *et al.*, 2011; Abosenna, 2017; Kuntal *et al.*, 2018), so we confirmed that this phage belongs to the Myoviridae family.

Determination of optimal multiplicity of infection

Multiplicity of infection is defined as the ratio of phage particles to host cells (Abedon, 2016). The results showed that the titer of phage produced at 10 MOI can reach the highest level (Table 1). Therefore, 10 was considered the optimal MOI for phage BM-P1. Ji and colleagues found the optimal MOI (0.1) for *Bacillus cereus* phage VMY22, which was isolated from Mingyong Glacier in China. Phage BM-P1 had a larger MOI than phage VMY22, indicating its weak infectivity (Ji *et al.*, 2015).

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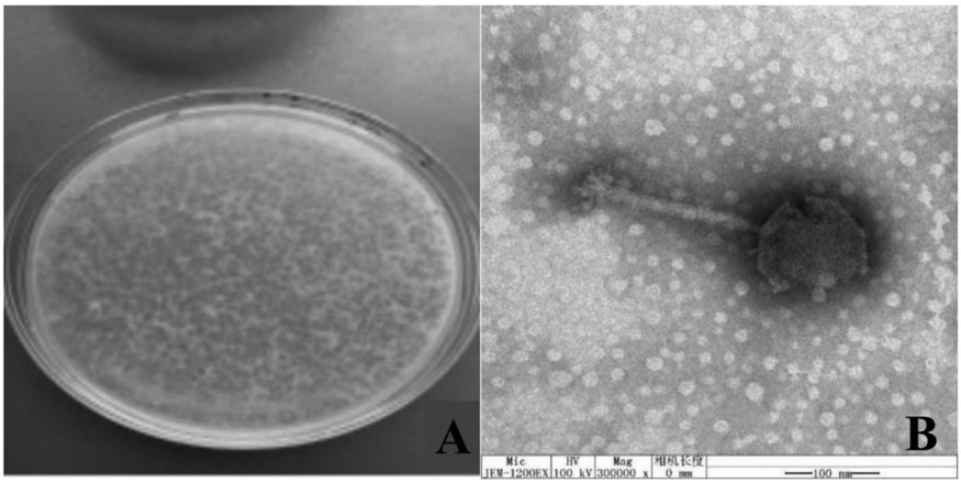

Figure 2. Morphology observation of the phage plaque and phage virions. (A) The plaques of phage BM-P1. (B) Virion morphology of phage BM-P1.

Table 1. The optimal multiplicity of infection of phage BM-P1

MOI	Number of bacteria (×10⁶ CFU/mL)	Number of phages (×10⁶ PFU/mL)	Titer at 4 h (×10⁶ PFU/mL)
0.01	100	1	6.46±0.37^{g}
0.1	100	10	14.5±0.62^{f}
0.5	200	100	117±2.94^{e}
1	100	100	100±3.74^{d}
2	50	100	162±2.35^{b}
10	10	100	813±2.16^{a}
100	1	100	132±1.63^{c}

Different lowercase letters represent significant differences between the two groups.

One-step growth curve

The results of one-step growth curve showed that the titer of phage BM-P1 had no significant change during the first 45 min, but a significant increase was observed at 105 min, which was gradually stabilized. Therefore, the latent period of phage BM-P1 was 45 min, the burst time was 45–105 min, and the burst size was 104.48±2.70 PFU/infective center (Figure 3).

One-step growth curve is a burst process from phage adsorption to the release of phage progeny, the duration and quantity of the burst greatly affect the function of phages. Mercanti *et al.* induced *Lactobacillus paracasei* ΦiLp84 and ΦiLp1308 phages by Mitomycin C. At 37 °C, the latency, burst time, and burst size of ΦiLp84 were 85 min, 131 min, and 46 PFU/infective center, respectively. While the latency, burst time, and burst size of ΦiLp84 were 85 min, 131 min, and 46 PFU/infective center, respectively (Mercanti *et al.*, 2015). El-Arabi and colleagues reported that the growth characteristics of phage Bc431v3 of *B. cereusstrain* LJH431 revealed a latent period of 85±5 min (El-Arabi *et al.*, 2013). Moreover, Peng and Yuan reported that the incubation period of *Bacillus cereus* bacteriophage vB_BceM-HSE3, lysis period and lysis volume were 60 min, 120 min, and 95 PFU/infective center, respectively (Peng and Yuan, 2018). In this study, the growth characteristics of phage BM-P1 were in line with other *Bacillus* phages after a long latent period, whereas these were not readily detected in the early stage of production.

Factors influencing phage viability

Influence of temperature on phage viability

The influence of temperature on phage viability is shown in Figure 4. From 0 to 36 °C, with an increase in temperature, the survival rate of phage was significantly increased. The highest survival rate (94%) was observed when the temperature reached to 36 °C. However, as the temperature increased, the survival rate of phage decreased. It was observed that the survival rate dropped to 75.09% at 50 °C. Notably, high temperature can inactivate phages by damaging their protein capsid and altering their DNA structure (Jończyk *et al.*, 2011). Peng and Yuan found that *Bacillus cereus* phage vB_BceM-HSE3 was stable at 37 °C; while, when the temperature was increased to 50 °C, its survival rate decreased to 1.2% (Peng and Yuan, 2018). Thus, temperature has a crucial impact on the stability of bacteriophages. Our results are in tandem with previous reports. Moreover, phage BM-P1 is more heat-tolerant than phage vB_BceM-HSE3.

Influence of pH on phage viability

Different pH ranges exhibited significant effect on the survival rate of phage BM-P1. As shown in Figure 5, more than 70% of the phages remained infective over pH values from 6 to 8. When the pH value increased to 9 or decreased to 5, its infectivity was decreased in either case. When the pH value was decreased to 3, it completely inactivated the phages. It has been reported that most phages are resistant to pH values ranging from 5 to 9 and almost all to pH 7 to 8 (Hazem, 2002). Moreover, when the pH value was decreased to 5, DNA was prone to depurination (that is, loss of purine bases in DNA). The breaking of phosphodiester bond causes the breakage of DNA base pair (Uhlenhopp and Krasna, 1969). This may be the main reason for phage inactivation when the pH value is decreased.

Environmental acidity and alkalinity are considered important factors that influence phage stability. Krasowska *et al.* evaluated the influence of pH (2–11) on the infectivity of four *Bacillus subtilis* phages (SIOΦ, SUBω, SPOσ, ARπ) and found that phages SIOΦ, SUBω, and SPOσ had 100% activity in the pH range from 6 to 8 (Krasowska *et al.*, 2015). However, decreases in phage viability at pH values below 6 or above 8 were quite apparent. Moreover, phage ARπ was

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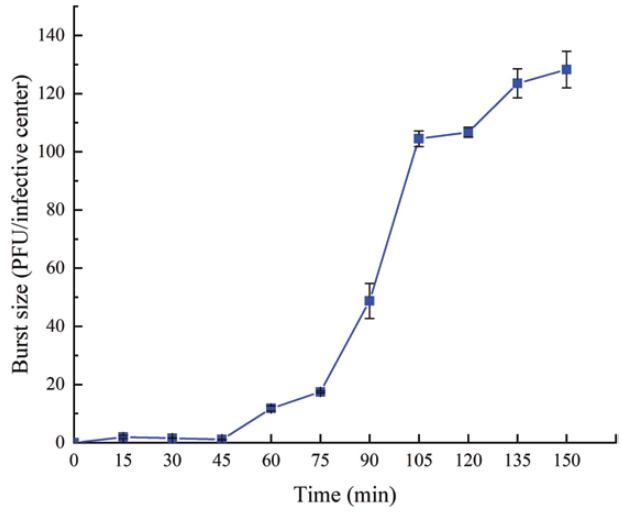

Figure 3. One-step growth curve of phage BM-P1. Error bars represent 95% CI.

one of the most sensitive phages to acid and alkaline con-

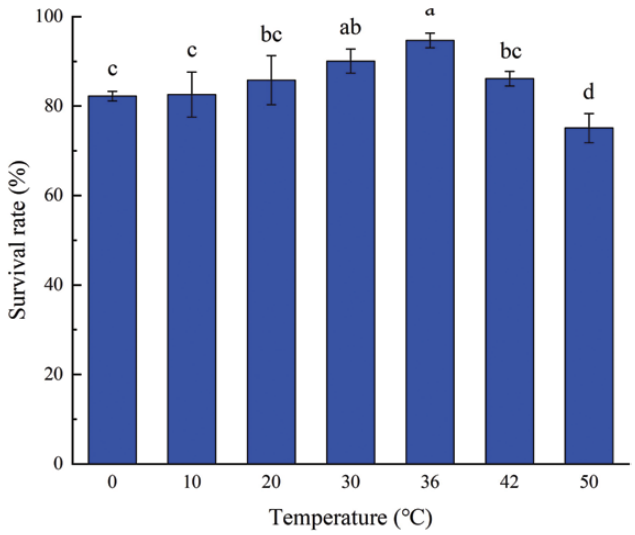

Figure 4. Influence of temperature on the viability of phage BM-P1. Different lowercase letters indicate significant differences in the survival rate of phage B1 under different temperature incubation conditions (P<0.05). Error bars represent 95% CI. (Notes: The same lowercase letter means there is no significance between the two groups. Different lowercase letters represent significant differences between the two groups.)

ditions. After 1 h incubation at pH 2, all tested phages lost infectivity (Krasowska *et al.*, 2015). Additionally, *Bacillus cereus* phage SWEP1 was stable between pH 5 and 10, but the titer dropped rapidly at pH 4 and 11 (Ruan *et al.*, 2021). Compared to phage BM-P1, phage SWEP1 was more tolerant to pH changes.

Factors influencing phage adsorption

Influence of temperature on phage adsorption

From Figure 6, we can see that temperatures from 0 to 42 °C did not influence phage adsorption, and the adsorption rate was more than 90% after 45 min. The highest adsorption rate

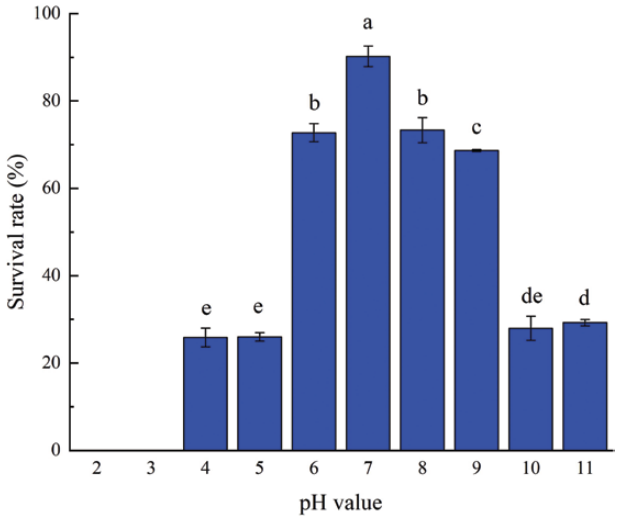

Figure 5. Influence of pH on the viability of BM-P1. Different lowercase letters indicate significant differences in the survival rate of phage BM-P1 under different pH culture conditions (P<0.05). Error bars represent 95% CI. (Notes: The same/containing the same lowercase letter means there is no significance between the two groups. Different lowercase letters represent significant differences between the two groups)

could be achieved at 36 °C (94.78%). When incubated at 50 °C, unabsorbed phage particles were increased to 15.1%.

The first step in phage proliferation is adsorption on the host cell surface. The impact of temperature on phage propagation could be due to temperature-dependent changes in the bacterial cell wall affecting the adsorption ability of the phages (Caso *et al.*, 1995). Contrary to our results, the amount of adsorbed DLc1 on *Bacillus cereus* 1582-3B decreased with decreasing temperature, demonstrating a temperature-dependent adsorbing capacity (Li *et al.*, 2020). For other reports, the maximum adsorption rate of phage QF12 was observed in the temperature range of 20–40 °C (approximately 97%) but was reduced to 86% at 0 and 10 °C. However, no significant impact of temperature (0–40 °C) was observed on the adsorption rates of QP4, QF9 and CHD phages. It could be inferred that the heat-sensitivity of different phages was different due to different receptors (Suárez *et al.*, 2010).

Influence of pH on phage adsorption

Adsorption rates for phage BM-P1 at different pH values (pH 4–11) on the host strain are shown in Figure 7. It could be observed that from pH 5 to pH 9, the adsorption rate did not show significant differences (P<0.05). At pH 4, 10, and 11, the adsorption rate of phage BM-P1 was decreased to 81.18%, 84.53%, and 83.81%, respectively.

When the pH was increased to 11, its adsorption rate was still 83.81%. Similar results have been reported for the influence of pH value on the adsorption of phage BM-P1. For example, the highest adsorption value (>90%) of *Lactobacillus plantarum* phage P1 was observed in a wider pH range (from 5 to 9; Chen *et al.*, 2019). Krasowska *et al.* found that *B. cereus* bacteriophages SIOφ and SPOσ were alkali-tolerant (pH 9–10; Krasowska *et al.*, 2015), and these results were in tandem with our results.

The influence of pH on the survival and adsorption efficiency of phage is due to the change in pH, which leads to the adsorption

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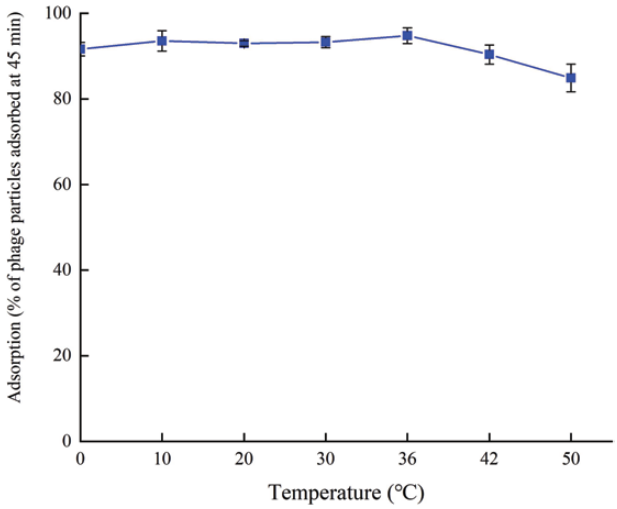

Figure 6. Influence of temperature on the adsorption of phage BM-P1. Error bars represent 95% CI.

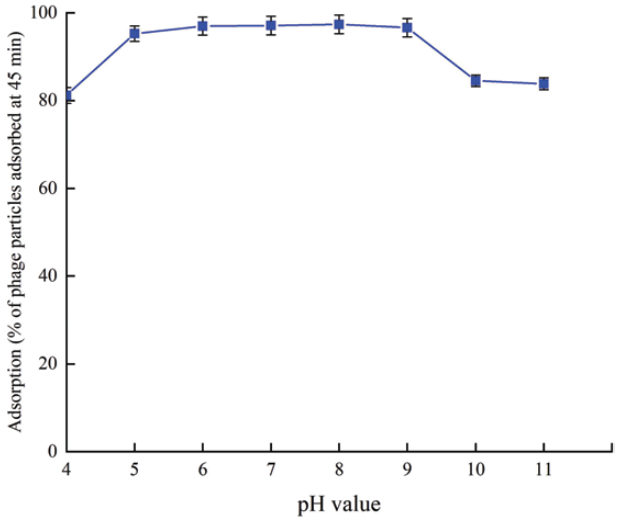

Figure 7. Influence of pH on the adsorption of phage BM-P1. Error bars represent 95% CI.

of phages on the surface of bacteria. The change in charge on the site or receptor affects the effect of electrostatic interaction between them. Electrostatic adsorption in the first stage of phage adsorption (that is, the reversible non-specific adsorption stage) plays a crucial role (Cormier and Janes, 2014). Therefore, the pH inevitably affects the adsorption efficiency of bacteriophage, and then affects its infection activity. Different phages possess different optimal adsorption pH values. However, once phages encounter their host cells, irreversible adsorption occurs if receptors are available. Broadly, there are two kinds of receptors located on the cell wall of Gram-positive bacteria, including proteins and polysaccharides. Previous reports indicated that *Bacillus cereus* phage vB_BceP-DLc 1 was stable between pH 5 and 11, but under acidic condition the carbohydrate structure (such as teichoic acid) on the cell surface of *B. cereus* 1582-3B was destroyed, resulting in the failure of phage adsorption to the bacterial surface (Li *et al.*, 2020). However, the real causes of this phenomenon are obscure.

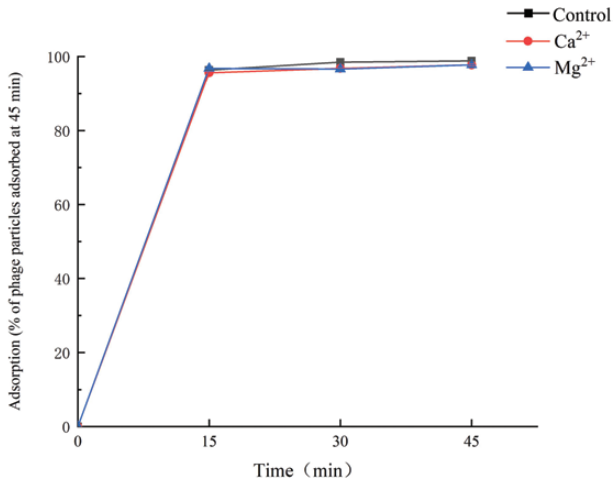

Figure 8. Influence of divalent cations on the adsorption of phage BM-P1. Error bars represent 95% CI.

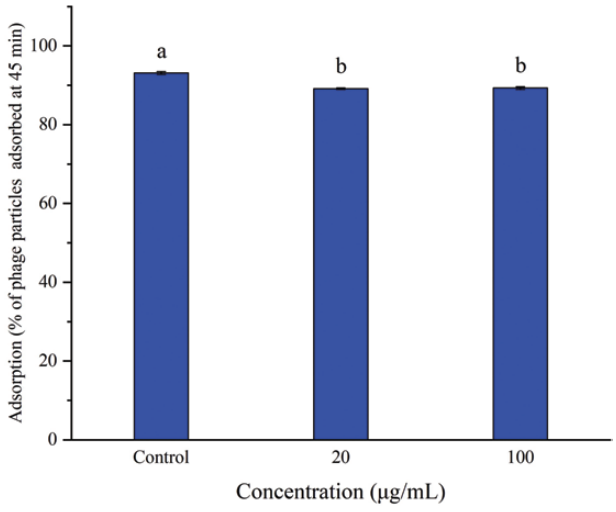

Figure 9. Influence of cell protein synthesis inhibitors on the adsorption of phage BM-P1. Different lowercase letters indicate significant differences in phage BM-P1 adsorption rates under different protein synthesis inhibitors concentration conditions (P<0.05). Error bars represent 95% CI. The same lowercase letter above the bars means there is no significance between the two groups. Different lowercase letters represent significant differences between the two groups.

Influence of divalent cations on phage adsorption

The adsorption kinetics in the presence and absence of Ca^{2+} or Mg^{2+} for the phage BM-P1 are shown in Figure 8. For phage BM-P1, cell lysis in nutrient broth was achieved even without the addition of Ca^{2+} or Mg^{2+}. Calcium ions did not have a significant (P<0.05) influence on phage adsorption kinetics. After incubation for 45 min, 97% of phage particles were adsorbed in nutrient broth with or without Ca^{2+} or Mg^{2+}. Therefore, divalent cations had little influence on the adsorption of phage BM-P1.

The presence of divalent cations in the medium promotes phage attachment by reducing repulsion and neutralizing the charge between the host cell surface polymer and the phage surface (Chhibber *et al.*, 2015). Quiberoni *et al.* reported that no influence of Ca^{2+} on *Lactobacillus delbrueckii* phage BYM and LL-H adsorption kinetics was observed (Quiberoni *et al.*, 2011). Cell lysis in MRS broth was achieved even without Ca^{2+}, but

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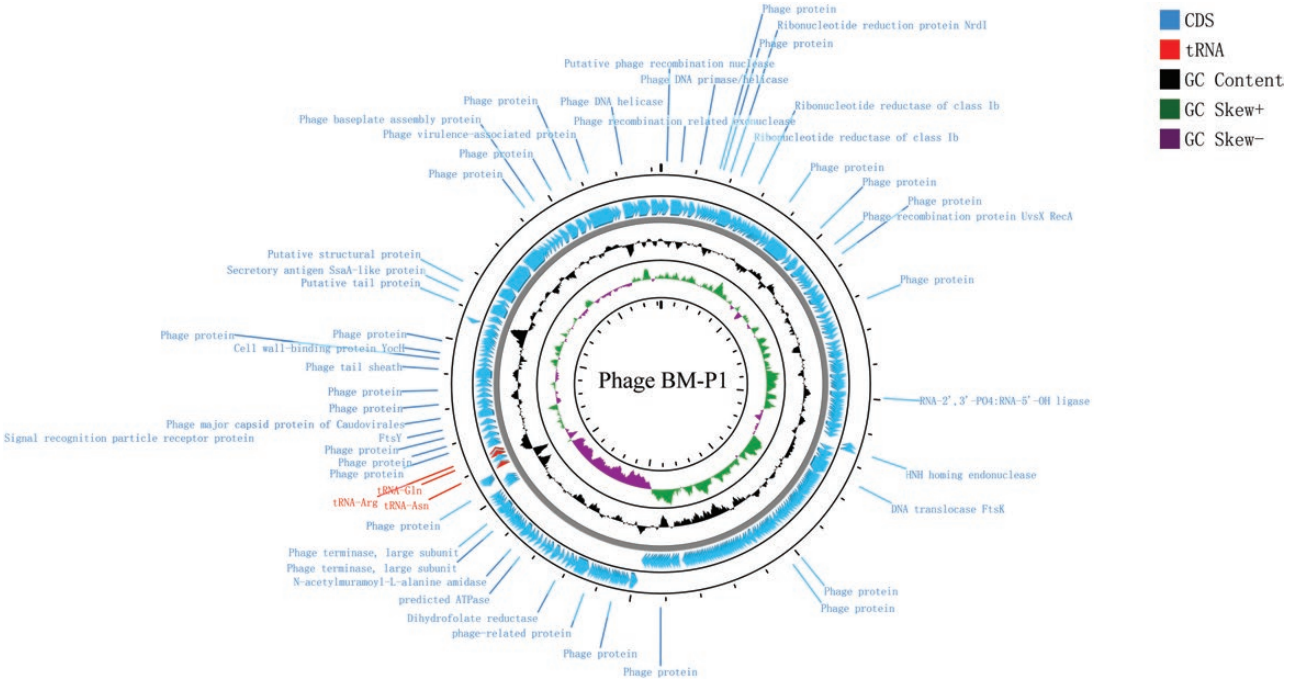

Figure 10. Genome atlas of phage BM-P1. The innermost circle indicates the GC skew on the positive and negative strands (green and purple). The second circle indicates the GC content (black). The outer circle is coding sequences (blue) and tRNA (red).

the process was faster in its presence (Quiberoni *et al.*, 2011). Therefore, the requirements for Ca^{2+} and Mg^{2+} are variable for adsorption or completion of the lytic cycle of different phages.

Influence of cell protein synthesis on phage adsorption

Chloramphenicol is a typical broad-spectrum antibiotic. It can interact with the 50 S ribosomal subunit to inhibit bacterial protein synthesis, thereby affecting the adsorption of bacteriophages to host bacteria (Li *et al.*, 2020).

The chloramphenicol concentrations used in this study were 20 μg/mL and 100 μg/mL. Under that condition, after 45 min of incubation, more than 80% of initial phage particles were adsorbed with or without chloramphenicol (Figure 9). This result was in agreement with the reports of Chen *et al.* (2016). In general, protein synthesis is not obligatory for the adsorption process.

Genome information of *Bacillus* phage BM-P1 genome

The assembled genome sequence of phage BM-P1 (accession number: OK349510) was uploaded to the GenBank database on NCBI on 30 September 2021. The genome length of phage BM-P1 was 153 087 bp and GC content was 41.94%. According to the gene function prediction, 5 tRNA genes and 258 coding sequences were predicted in the genome (Figure 10).

Functional annotation of phage BM-P1 protein

To understand the genomic information of phage BM-P1, we annotated its protein function using the COG database. We annotated 14 protein functions (Table 2). DNA repair endonuclease and ribonucleotide reductase can hydrolyze host nucleic acid into ribonucleotide and further transform into deoxynucleotide, and these two provide materials for the synthesis of phage genome (Kropinski *et al.*, 2013). In this process, DNA polymerase and DNA primer enzyme catalyze

Table 2. Gene function annotation information

Gene_ID	Function
Gene 12	Protein involved in ribonucleotide reduction
Gene 13	Ribonucleotide reductase alpha subunit
Gene 157	Serine protease, subtilisin family
Gene 16	Ribonucleotide reductase beta subunit, ferritin-like domain
Gene 17	Thiol-disulfide isomerase or thioredoxin
Gene 175	Dihydrofolate reductase
Gene 177	Thymidylate synthase
Gene 2	DNA repair exonuclease SbcCD nuclease subunit
Gene 28	Exonuclease
Gene 31	Recombination protein RecA
Gene 5	DNA primase
Gene 58	DNA-directed RNA polymerase specialized sigma subunit
Gene 81	DNA segregation ATPase FtsK/SpoIIIE and related proteins
Gene 27	DNA-binding protein

phage DNA synthesis. RNA polymerase σ factor can modify host RNA polymerase and recognize the promoter region of phage genome to achieve phage gene expression (O'Flaherty *et al.*, 2004). These proteins are involved in the replication process of phages. In this study, dihydrofolate reductase (COG 0262) was present in the genome of phage BM-P1. Lee and colleagues predicted that dihydrofolate reductase, thymidylate synthase and thymidylate kinase could synthesize folic acid and free radical *S*-adenosine methionine (SAM), thus enhancing the metabolism of phage infection (Lee JH *et al.*, 2011). The DNA binding protein was annotated in phage BM-P1. Schuch *et al.* believed that DNA binding protein

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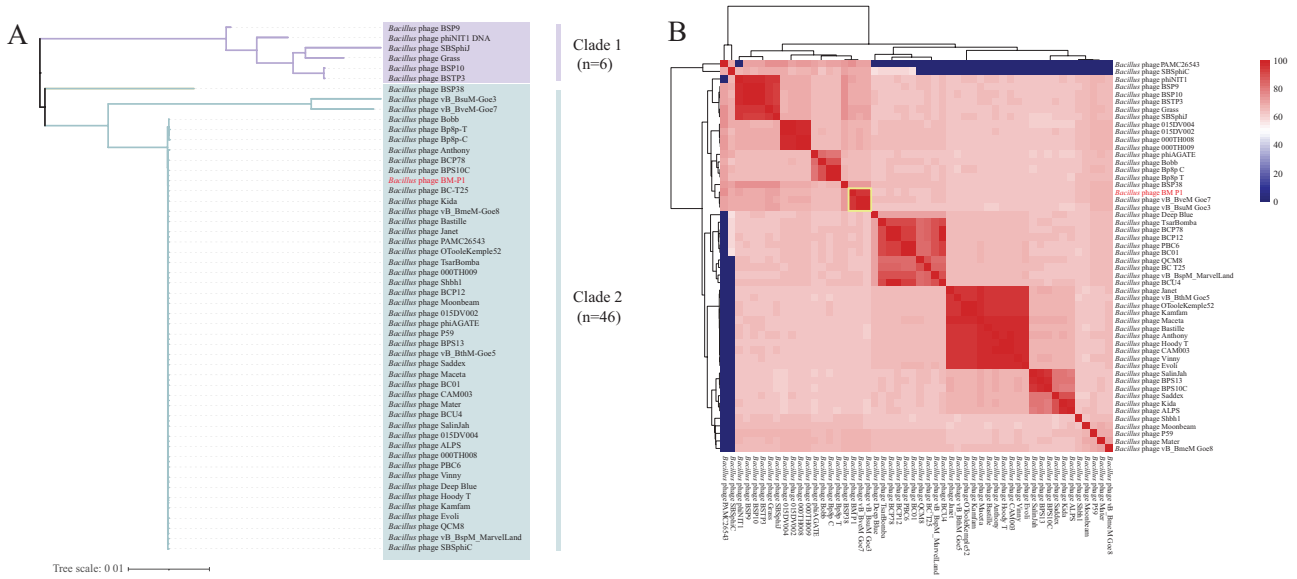

Figure 11. Phylogenetic relationships of 52 bacteriophage strains of *Bacillus*. (A) Phylogenetic tree. (B) Average nucleic acid identity.

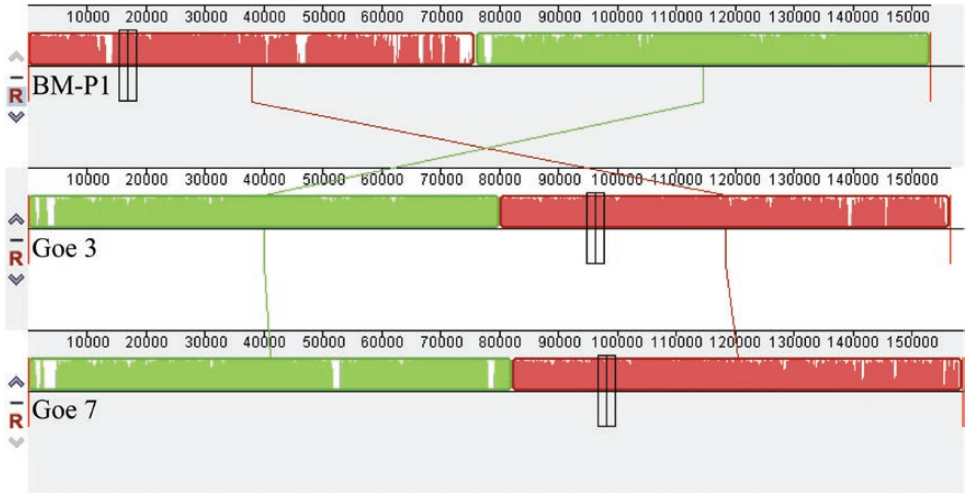

Figure 12. Synteny analysis of *Bacillus* phage BM-P1 with VB_BsuM-Goe3 and vB_BveM-Goe7 (green and red represent similar fragments, white represents special fragments).

was related to sporulation of *B. subtilis*, so the existence of DNA binding protein BC01_036 might change the adaptive behavior of bacteria, such as sporulation (Schuch *et al.*, 2014). In this study, DNA binding protein was detected in the BM-P1 genome of the phage. However, further studies should be undertaken to explore whether the DNA binding protein gene in the phage BM-P1 genome can be integrated into the host bacteria and lead to metabolic changes in the host. It was further revealed that no gene encoding RNA polymerase was found in the phage BM-P1 genome, which may indicate that the phage needs to rely on the host transcription system.

Phylogenetic analysis

Along with BM-P1, we selected the whole genome of 51 previously published bacteriophages of *Bacillus* spp. and constructed a phylogenetic tree using the maximum likelihood method to explore the evolutionary relationship of *Bacillus* bacteriophages (Figure 11A). The results showed that 52 bacteriophages of *Bacillus* could be divided into two main branches. Clade 1 included 6 phage genomes, while Clade 2 contained 46 phage genomes including BM-P1. The ANI value was calculated to further understand the differences among the *Bacillus* bacteriophages genomes (Figure 11B). The results showed that intra- and inter-group ANI values were 90.18%–100% and 75.10%–87.55%, respectively. Among them, the ANI between BM-P1 and other phages ranged from 62.15% to 95.28%. It was reported that although the virus particle structure of the protein has a strong similarity and conservation, the phage at the nucleotide level might exhibit substantial diversity. Evolutionarily, phages possess unique contact with each other, which may respond to the selective pressure of the host and a variety of gene exchange events occur, to contribute to their diversity. Notably,

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the ANI values between BM-P1 and phage VB_BsuM-Goe3 or vB_BveM-Goe7 were the highest (95.23% and 95.28%, respectively). Previous studies showed that the length of VB_BsuM-Goe3 genome was 156 430 bp, and the GC content was 41.93%, with 5 tRNA and 246 coding sequences (Willms *et al.*, 2017). Furrer and colleagues determined the genome of vB_BveM-Goe7 and found that the length of vB_BveM-Goe7 genome was 158 674 bp and the GC content 41.84%, with 5 tRNA and 251 coding sequences (Furrer *et al.*, 2020). Another study found that the nucleic acid matching degree of vB_BveM-Goe7 and VB_BsuM-Goe3 reached 98.18%, but they belong to the subfamilies Bastillevirinae and Spounavirinae, respectively (Willms *et al.*, 2017).

Synteny analysis

To further observe the relationship among the genome of these three phages, collinear comparison of genomes was conducted. It was observed that the overall gene arrangement of phage BM-P1 was different, and the transposition, deletion, and insertion occurred in DNA fragments (Figure 12). Moreover, phage BM-P1 contained approximately 7400 bp specific gene fragments. Therefore, phage BM-P1 could be considered a new phage.

Conclusions

In this study, a novel *Bacillus methylotrophicus* phage, BM-P1, belonging to the Myoviridae family, was induced successfully. Its latent period was 45 min, and the burst size was 104.48±2.70 PFU/infective center. Our results showed that this phage was sensitive to temperature and pH. Divalent cations (Ca^{2+}, Mg^{2+}) and chloramphenicol had minor impact on its adsorption. Moreover, its genome size was 153 087 bp and its GC content was 41.94%, including 258 coding sequences. Phenotypic characteristic analysis and genomic analysis showed that it can be considered as a novel bacteriophage. This study expands the knowledge on the genetic diversity of *Bacillus* bacteriophages and provides a theoretical foundation for the prevention and control of bacteriophage in fermentation plants.

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Author Contributions

Xia Chen conceived and designed the experiment, and provided constructive comments on the manuscript. Ruirui Lv, Ming Xu, Jingwei Yao, She Guo, Can Zhang, and Jing Guo conducted the experiments; Ming Xu and Ruirui Lv analyzed the data; and Ming Xu, Ruirui Lv, and Hafiz Arbab Sakandar drafted, edited, and revised the manuscript.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Ethical Approval

This article does not contain any studies involving humans or animals.

Data Availability

The genome data of phage BM-P1 have been uploaded to GenBank with accession number OK349510 (https://www.ncbi.nlm.nih.gov/). The genomic information of other phages downloaded from NCBI (https://www.ncbi.nlm.nih.gov/) is presented in supplement file 1.

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