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RESEARCH

Characteristics of a novel temperate bacteriophage against *Staphylococcus arlettae* (vB_SarS_BM31)

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Abstract

Background *Staphylococcus arlettae* is a rarely reported coagulase-negative staphylococcus (CoNS) isolated from infected humans and livestock. Observing phage-bacteria interaction could improve the understanding of bacterial pathogenetic mechanisms, providing foundational evidence for phage therapy or phage detection. Herein, we aimed to characterise and annotate a novel bacteriophage, vB_SarS_BM31 (BM31), specific to *S. arlettae*. This bacteriophage was isolated from a milk sample associated with bovine mastitis and collected in the Sichuan Province, China.

Results The BM31 genome comprised a linear double-stranded DNA of 42,271 base pair in length with a G + C content of 34.59%. A total of 65 open reading frames (ORFs) were assembled from phage DNA, of which 29 were functionally annotated. These functional genes were divided into four modules: the structural, DNA packing and replication, lysis, and lysogeny modules. Holin (ORF25), lysin (ORF26), and integrase (ORF28) were located closely in the entire BM31 genome and were important for lyse or lysogeny cycle of BM31. The phage was identified as a temperate phage according to whole genome analysis and life cycle assay, with basic biological characteristics such as small burst size, short latency period, and narrow host range, consistent with the characteristics of the family Siphoviridae, subcluster B14 of the *Staphylococcus* bacteriophage.

Conclusions The present isolation and characterisation of BM31 contributes to the *Staphylococcus* bacteriophage database and provides a theoretical foundation for its potential applications. To the best of our knowledge, BM31 is the only shared and completely reported phage against *S. arlettae* in the entire public database.

Keywords Mastitis · Endolysin · Integrase · Burst size · Lysogeny

Introduction

Staphylococci can directly or indirectly cause arthritis and comb necrosis in chickens, mastitis in dairy cattle, extraocular infections in horses, ovine staphylococcal dermatitis in sheep, and exudative epidermitis in pigs (Oduor et al. 2020). In clinical diagnosis, staphylococci are divided into two groups based on their capacity for coagulase production: coagulase-positive (CoPS) and coagulase-negative (CoNS) staphylococci (Hatoum-Aslan 2021). *Staphylococcus aureus* and *Staphylococcus epidermidis* have been studied extensively as typical CoPS and CoNS strains, respectively, while other types of CoNS strains remain poorly understood. *S. arlettae* is commonly regarded as a commensal species; however, it is associated with different types of infection in contexts of large-scale antibiotic use (Lavecchia et al. 2019). *S. arlettae* strains have been previously isolated from patients afflicted with human chronic prostatitis, otologic

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infection, rheumatic mitral stenosis, bovine mastitis, dairy goat intramammary infection, and pig exudative epidermidis (Bernier Gosselin et al. 2019; Dinakaran et al. 2012; Hou et al. 2000; Park et al. 2013; Xiao et al. 2021).

Bacteriophages (phages) are the most abundant biological entities on the planet, with a total estimated number of $> 10^{31}$ worldwide, outnumbering their bacterial hosts 10:1 (Brüssow and Hendrix 2002; Shareefdeen and Hynes 2021). They play a remarkable role in nearly all microbial ecosystems, including those found in the most extreme environments, such as soda lakes, hot springs, and hydrothermal vents, which are distant from us (van Zyl et al. 2015). Inside the human body, phages are found in the oral cavity, gut, and urogenital tract and are commonly associated with periodontal disease, gastrointestinal problems, and bacterial vaginosis, respectively (Ly et al. 2014; Ma et al. 2018; Miller-Ensminger et al. 2020; Nasioudis et al. 2017; Wagner et al. 2013; Wang et al. 2016). Temperate phages are present in a lysogenic cycle, where the infective phage integrates into the host genome (as a 'prophage') and is passively replicated when the host replicates without cell lysis until their eventual induction, resulting in a lytic life cycle. This phenomenon may be observed in the presence of antibiotics or host inflammation (Lawrence et al. 2019; Shareefdeen and Hynes 2021).

Emerging multidrug-resistant bacterial strains and limited efficacy of current antibiotics necessitate the development of novel antibiotics. Studies have shown that the use of antibiotics may cause dysbiosis, especially in the intestines or in places of secondary infections, and that excessive use of antibiotics destroys most of the natural intestinal flora in the human body (Langdon et al. 2016; Zurabov and Zhilenkov 2021). Phages have exhibited significant therapeutic effects in the treatment of multidrug-resistant infections in animal models and human clinical trials involving antibiotic agents that had been rendered ineffective (Beeton et al. 2015; Holguín et al. 2015; Merabishvili et al. 2009; Rhoads et al. 2009; Pincus et al. 2015; Rose et al. 2014; Seed and Dennis 2009; Semler et al. 2014; Wright et al. 2009). Although safety concerns remain due to the viral nature of phages, studies have shown that phages do not necessarily elicit an undesirable immune response and can kill *Escherichia coli* without altering the diversity of the normal human microbiota (Borysowski et al. 2017; Cepko et al. 2020; Łusiak-Szelachowska et al. 2017; Międzybrodzki et al. 2017). Other studies have shown that phages may be eliminated in the absence of a host bacterium without any evidence of residue formation (Rohde et al. 2018). Gene transfer in temperate phages means that virulent double-stranded DNA-tailed phages that carry out a strictly lytic infection cycle may have therapeutic applications and provide an alternative to antibiotics (Gordillo Altamirano and Barr 2019; Roach and Debarbieux 2017). Consistent study of temperate phages may be beneficial to assess the efficiency and safety of introducing phage-derived proteins, e.g. endolysin, or introducing genetic mutations that convert the phage to a virulent type, increasing phage lytic ability, and making it suitable for use in phage cocktails or in combination with antibiotics (Al-Anany et al. 2021; Dedrick et al. 2019; Hargreaves and Clokie 2014). Phages are also used as diagnostic tools, in food production, and in biotechnology (Dunne et al. 2018). For example, they can regulate the intestinal flora of livestock, prevent contamination of ready-meals by foodborne bacteria (*Salmonella*, *Listeria*), and may be used as bio-preservatives (Guenther et al. 2012; Guenther et al. 2009; Kazi and Annapure 2016; Kim et al. 2017). Phages and their derived proteins can be engineered to detect *S. aureus*, *Salmonella*, *Campylobacter jejuni*, and *Shigella flexneri* (Bhardwaj et al. 2017; Kittler et al. 2013; Lakshmanan et al. 2007; Singh et al. 2013).

Based on these potential applications, novel phages specific to different host bacteria are needed. In response to an appeal of the United States Food and Drug Administration, phages are not used in clinical treatment if any of their genes encode toxins or other proteins that may enhance bacterial virulence (Abedon et al. 2011). Therefore, all isolated phages must be fully characterised through complete genome sequencing and functional analysis before application. Detailed phage information is required when bacteriophage-derived proteins are used to detect or remove pathogenic bacteria in diagnostic or therapeutic products, or food (Schmelcher and Loessner 2014). This study aimed to characterise a temperate phage vB_SarS_BM31, which is first reported to against *S. arlettae*.

Materials and methods

Bacterial strains and growth conditions

The host strain *S. arlettae* was isolated from a milk sample obtained at a dairy farm in Sichuan, China. Other *Staphylococcus* spp. used in this study were also isolated from milk samples obtained from animals with clinical or subclinical mastitis and stored at − 80 °C with 50% glycerol in the laboratory. Partial bacterial 16S rRNA polymerase chain reaction (PCR) analysis was used to verify the strains (Table 1). All strains were grown in Luria–Bertani (LB) broth (pH 7.0 ± 0.1) at 37 °C.

Phage isolation, purification, and amplification

Phage BM31 was isolated from a milk sample (obtained from the Sichuan Province) contaminated with *S. arlettae* using the double-layer agar method described previously, with slight modifications. Briefly, 1 mL of milk sample

Table 1 Primers used in this study

Name	Primers	Size (bp)	Tm (°C)	Reference
16 S rRNA	27 F—5′ AGAGTTTGATCCTGGCTCAG 3′ 1492 R—5′ GGTTACCTTGTTACGACTT 3′	1465	55	Madurantakam Royam and Nachimuthu (2020)
CP[a]	F—5′ GGCAAACGTTCTATGGCGAG 3′ R—5′ GGATGTGATCCAGACGCAGT 3′	245	54	This study
CP1	F—5′ AACCCAGAGCTTGCTTCTCC 3′ R—5′ ACCTGTTGGGCTTGTGATGT 3′	366	55	This study
CP675	F—5′ GATAAGGCAAAAACTGCCGCT 3′ R—5′ AGCAAGCTCTGGGTTCTTAGC 3′	675	55	This study
PP[b]488	F—5′ GCTAACTGAAACGGGTGGCA 3′ R—5′ AAATGCCGATCTCGTTCGCT 3′	488	55	This study
TMP[c]81	F—5′ ATACGGGAAATCCGGTTGGG 3′ R—5′ TGAGCTAATCCACGTGGTGC 3′	81	55	This study

[a]*CP*, capsid protein; [b]*PP*, portal protein; [c]*TMP*, tape measure protein

was added to 5 mL of LB broth suspended with 500 µL of exponentially growing culture of *S. arlettae*, and incubated at 37 °C for 4 h and shaken at 225 rpm. Flocculent precipitates in milk were removed by centrifugation at 4000 rpm for 10 min at room temperature and filtered through a 0.45-µm syringe filter. Subsequently, 200 µL of exponentially growing culture of *S. arlettae* and 2 mL of filtrate were mixed with 2 mL of LB broth, incubated at 37 °C for 24 h and shaken. Cell debris was removed by centrifugation at 8000 rpm for 5 min at 4 °C and filtered through a 0.22-µm syringe filter. The suspected phage sample was obtained and stored at 4 °C in a dark room.

A mixture of 200 µL of *S. arlettae* and 200 µL of suspected phage sample, which were adsorbed over 25 min at room temperature, was mixed into 3 mL of warm LB soft agar (0.6% w/v, pH 7.0 ± 0.1) and poured onto LB agar plates (1.2% w/v), maintained stable for 30 min, and then incubated at 37 °C overnight. The presence of phages was confirmed by visualising the presence of clear or turbid plaques. The clear plaque was picked out using a sterile Pasteur pipette and dissolved into a chloride-magnesium sulphate (SM) buffer (5.8 g/L of NaCl, 2.0 g/L of $MgSO_4$, 50 mL/L of 1 M Tris, pH 7.5, and 5 mL/L of pre-sterilised 2% gelatine) in a 1.5-mL centrifugation tube, shaking for 2 h at 37 °C or stably standing overnight at 4 °C (Anand et al. 2015). The phage was tenfold serially diluted, and the double-layer agar method was performed to obtain new phage plaques. The process was repeated four or more times until the plaques were uniform.

When the plaque morphology was uniform, 5 mL of SM buffer was added to the plate with gentle shaking for 4 h at 37 °C. The liquid on the top of the plate was centrifuged at 5500 rcf for 5 min and filtered through a 0.22-µm syringe filter to obtain a high-titre phage.

Transmission electron microscopy

One microlitre of purified phage BM31, obtained using the method described above, was placed on top of a carbon-coated copper grid and stained with 2% phosphotungstic acid (pH = 7.0). The stained phage was observed using a transmission electron microscope (Lanzhou Veterinary Research Institute, China) at 80 kV 30,000 × magnification (Kyoung Min et al. 2018).

Chloroform and ether sensitivity

Phage sensitivity to chloroform and ether was used to determine whether a lipid substance was present in the capsid or tail fibre of phage BM31 (Espejo and Canelo 1968; Lu et al. 2017; Wei et al. 2020). For this process, 100 µL of phage (~ 10^8 plaque forming units [PFU]/mL) in SM buffer was mixed with 900 µL of chloroform and ether, shaken vigorously for 1 min, and incubated at room temperature for 1 h. The treated samples were immediately diluted in SM buffer and plated for phage titration using double-layer agar plates inoculated with *S. arlettae*; the original sample was used as a control group. The assay was performed in triplicates.

Thermal and pH stability

To evaluate the thermal stability of the phage, 1 mL of phage with SM buffer was incubated at 40, 50, 60, 70, and 80 °C. Samples were removed every 20 min for 1 h and phage titres were immediately determined at each time interval. To investigate the pH stability of the phage, 100 µL of phage was mixed with 900 µL of SM buffer, which was adjusted to pH 3, 5, 9, and 11 using HCl or NaOH, respectively. Phages in SM buffer at pH 7 served as controls. The phages

in solutions of various pH values were incubated for 1 h at room temperature, following which, the phage titres were determined. The assays were performed in triplicate.

Multiplicity of infection assay

To achieve the highest efficacy of the phage at the lowest cost and set a standard for further study, a multiplicity of infection (MOI) assay was established to determine the optimal ratio of PFU/colony forming units (CFU) (Feng et al. 2021). Briefly, the phage was diluted to $1 \times 10^{5-12}$ PFU/mL in an SM buffer and incubated with *S. arlettae* at a concentration of 1×10^{9}. The double-layer agar method was used to determine phage titration, and the assay was performed in triplicate.

One-step growth curve assay

The host strain *S. arlettae* (1×10^{7} CFU/mL) and phage BM31 (1×10^{4} PFU/mL) were first adsorbed at an MOI of 0.001 for 15 min at 37 °C, and subsequently centrifuged at 12,000 rcf for 1 min. The supernatant, which contained free phage, was discarded and washed twice in sterilised LB broth. The pellets that consisted of a bacteria-phage complex were resuspended using warm LB broth and immediately incubated at 37 °C and shaken for 150 min. The samples were removed and immediately stored at 4 °C for simultaneous phage titration at 0 min, at 5-min intervals within 30 min, 10-min intervals within the next 1 h, and 30-min intervals within the next 1 h. This assay was repeated three times. The latency period and burst size were calculated, representing the time required from phage invasion to progeny maturation and cell lysis, and the number of phage particles released from the bacterial cell, respectively (Madurantakam Royam and Nachimuthu 2020). The burst size was calculated using the formula: median number of plaques at $t_{\text{end of the replication cycle}}/t_{\text{start of the replication cycle}}$ (Eckstein et al. 2021).

Host range

In this study, 1×10^{9} PFU/mL of phage BM31 was prepared for the host range assay for all 13 *Staphylococcus* spp. strains (all isolated from bovine milk, Sichuan Province of China) involved in the host strain *S. arlettae*. Briefly, 100 µL of each strain was coated on LB agar plates and dried at room temperature for a few minutes until there were no water drops. Subsequently, 10 µL of BM31 and 10 µL of sterilised saline, which served as a control, were dropped on the coating plates and incubated overnight to observe whether there were any lytic plaques on each plate.

Identification and extraction of nucleic acid

Following the manufacturer's instructions, DNAse I, RNAse A, and mung bean nuclease (Takara Biomedical Technology Co., Ltd., Beijing, China) were used to confirm the type of nucleic acid in the phage (Kumar et al. 2021).

Phage DNA was obtained from the purified phage suspension using a viral RNA/DNA extraction kit (Takara Biomedical Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions.

Genome assembly and annotation

Phage genomic DNA was sequenced by Personalbio (Personalbio Technology Co., Ltd., Shanghai) using next-generation sequencing on an Illumina NovaSeq platform. Quality control analysis was performed using FastQC v0.11.7. A5-MiSeq v20160825 and SPAdes v3.12.0 were used for genome sequence assembly.

ORFs were predicted using the GeneMarkS v4.32 (Besemer et al. 2001). Homology searching was performed with BLASTp against the NCBI nonredundant database, and sequences with an E-value of $< 10^{-5}$ were considered homologues. HHpred against the Protein Data Bank and Pfam databases were used to predict more distant homologues (Söding et al. 2005). The genome was analysed for tRNA genes using the tRNAscan-SE 2.0 (Lowe and Chan 2016). The Comprehensive Antibiotic Resistance Database (https://card.mcmaster.ca/) and virulence factors of pathogenic bacteria (http://www.mgc.ac.cn/VFs/main.htm) were used to detect the antibiotic resistance and virulence genes, respectively. PHASTER (http://phaster.ca/) was used to identify the most closely related phages and the presence of prophages (Dakheel et al. 2019). The replication method and life cycle of BM31 were predicted using the phage classification prediction program (http://www.phantome.org/PHACTS/index.php) (Lu et al. 2017). A genomic map was generated using CGView (CGView server) (Grant and Stothard 2008).

Life cycle

The lifecycle of BM31 was determined based on whether the specific structural proteins harboured in phage BM31 were present in *S. arlettae* (host strain) before and after lysis with BM31 (Lu et al. 2017). Briefly, 5 mL of LB broth was added to the double agar plate lawned with *S. arlettae*, which was lysed by BM31 and rocked gently at room temperature for 2 h. The broth at the top of the agar was collected in microcentrifuge tubes and centrifuged at 5500 rcf for 5 min. The supernatant was discarded, and 1 mL of fresh LB broth was added to resuspend the pellets, followed by centrifugation. The contaminating phage was removed by repeating the washing step thrice. After the final centrifugation step

and removal of the supernatant, the bacteria pellets and the original isolated *S. arlettae*, which was stored at − 80 °C, were both used to streak for isolation on LB agar plates and incubated overnight at 37 °C. Single colonies were randomly collected for PCR using primers specific to BM31 (Table 1) designed according to phage genome annotation, targeting ORF4 (portal protein), ORF6 (capsid protein), and ORF15 (tape measure protein). All these primers were generated using SnapGene (version 4.3.6). Visualisation of specific bands under transilluminator suggests that BM31 can integrate its own genome into the chromosome of host bacteria, as this ability belongs to lysogeny phages. In contrast, it suggests that the phage possesses a lytic life cycle.

Taxonomic analysis

The whole genome and major capsid protein sequences in phage BM31 were compared phylogenetically with those in other similar phages (compared using BLASTN) extracted from the GenBank database (http://www.ncbi.nih.gov/) using MEGA 7 software (version 7.0.26). ClustalW was used to align the inferred amino acid sequences using default parameters. Based on multiple sequence alignment, the Tamura-Nei model was selected, and a maximum likelihood tree was constructed with 1000 bootstrap replicates.

ANI values between BM31 and other *Staphylococcus* phages were generated using the ANI Calculator (ANI Calculator | Ezbiocloud.net). A heat map was created using TBtools v1. 0,986,853. Genome comparison between BM31 and its closest similar phage was performed using Geneious Prime (Mauve Plugin version 1.1.3).

Statistical analysis

Data were collected and analysed using GraphPad Prism version 7 software. The *t*-test was used to analyse the differences between two groups, and ANOVA was used for more than two groups. Error bars in the figures represent the standard deviation of the mean of three replicate experiments. Differences were considered statistically significant at p values of < 0.05.

Results and discussion

Phage morphology

The isolated phage formed large and clear plaques of 1.98 ± 0.02 mm diameter with turbid halos after overnight incubation on its indicator host (Fig. 1a). Such halos may indicate the presence of a phage-encoded depolymerase that could eliminate exopolysaccharides in the bacterial cell wall (Chmielewska-Jeznach et al. 2020).

Transmission electron microscopy (TEM) classified BM31 as a Siphoviridae phage with a B1 morphotype based on an isometric head averaging 63 ± 7 nm in diameter and a long, non-contractile tail with an average tail length and width of 168 ± 7 nm and 14 ± 7, respectively (Ackermann and Eisenstark 1974). A baseplate was observed at the end of the tail (Fig. 1b).

Basic characteristics

No phage particles survived in chloroform or ether, whereas the phage titre in the control group (SM buffer) remained approximately 2×10^8 PFU/mL (Fig. 2a). This result suggests that phage BM31 is susceptible to both chloroform and ether, implying the presence of lipids in the capsid or a surrounding lipid layer in the phage (Wang and Li 2018). Therefore, chloroform or ether should not be used in further studies on BM31.

In the pH stability assay, BM31 showed the highest titre at an average of 2×10^7 PFU/mL at pH 7, surviving incubation at pH 9 for 1 h with an average titre of 1.4×10^7 PFU/

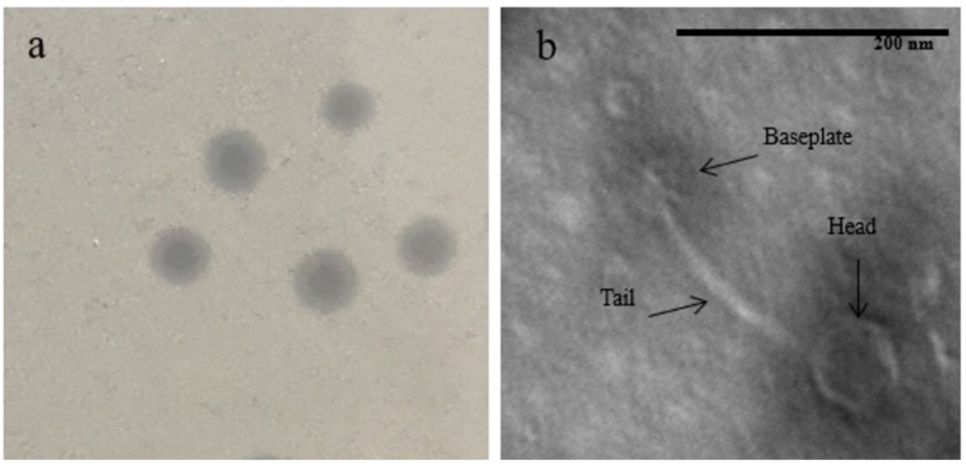

Fig. 1 Morphological characteristics of phage BM31. **a** Plaques on the plate formed by BM31. **b** Transmission electron microscopy image of BM31, scale bars indicate 200 nm

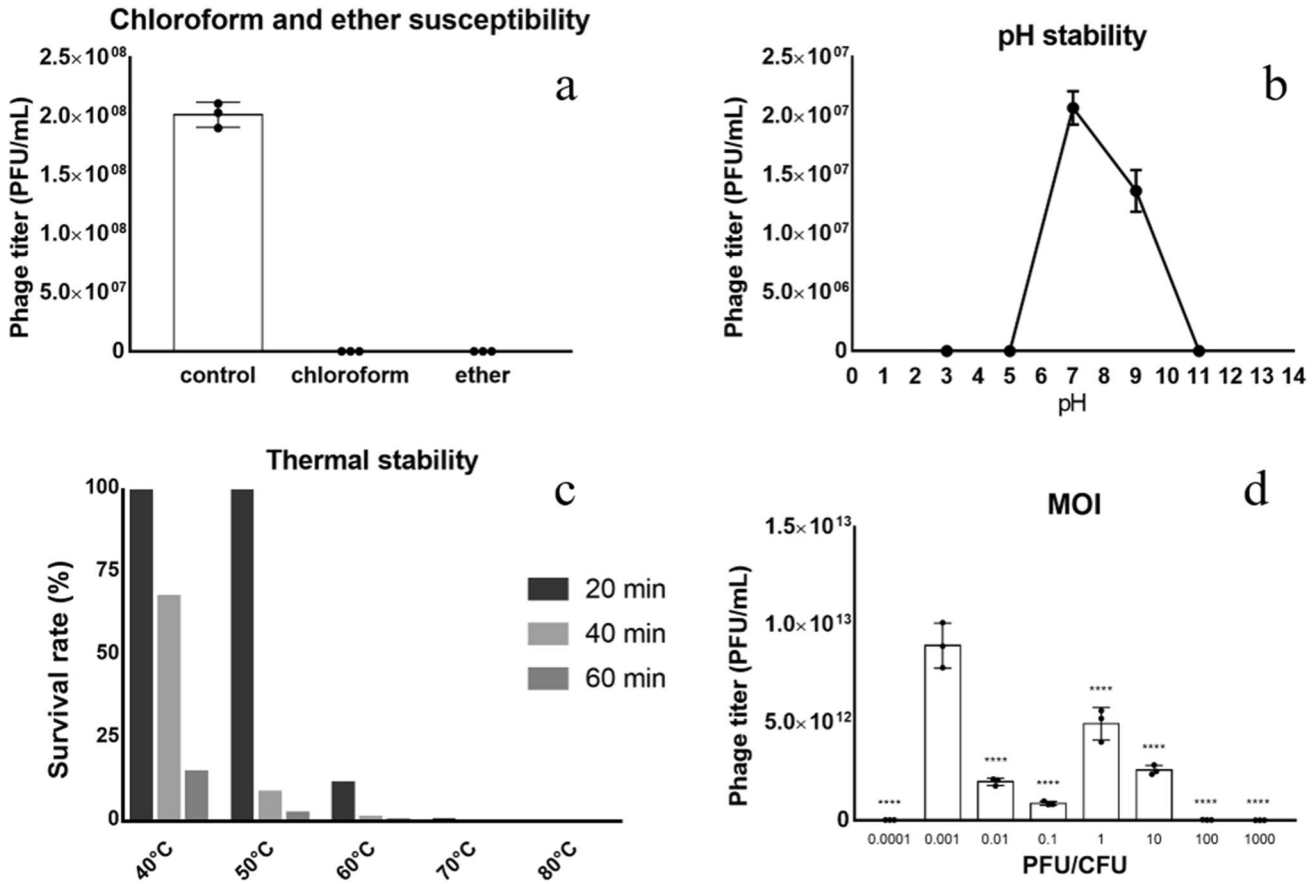

Fig. 2 Basic characteristics of phage BM31. **a** Chloroform and ether assays, control group, chloride-magnesium sulphate (SM) buffer. **b** Phage stability from pH3 to pH11. **c** Thermal stability of BM31. **d** Multiplicity of infection (MOI). ****, $p < 0.0001$

mL (70% survival rate); no phage particle survived in other pH groups (Fig. 2b). This finding indicates that BM31 could stand a weakly alkaline environment, but the optimal survival environment may be neutral.

The thermal stability assay indicated that the tolerable temperature for BM31 was 40–60 °C (Fig. 2c), and this phage demonstrated good thermal stability. The results showed that after 60 min of incubation at 40 °C, the phage titre was 1.07×10^{12} (14.50% survivability) when the original titre was 7.40×10^{12}. After 60 min of incubation at 50 °C and 60 °C, the phage titres were determined to be 1.70×10^{11} (2.29% survivability) and 2.32×10^{10} (0.31% survivability), respectively. BM31 could not survive at a temperature of 70 °C for more than 20 min, and at 80 °C, the survival duration was less than 20 min.

The results of the MOI assay showed that BM31 exhibited the best capacity for host lysis and self-replication when the MOI value reached 0.001. The optimal MOI value was associated with relatively low costs and yielded a phage titre significantly higher than that of other groups (Fig. 2d). Specifically, the viability of BM31 is stable, which is related to its structure since the Siphoviridae family is reportedly the most stable phage (Wintachai et al. 2020).

One-step growth curve

For phage BM31, the latency period was 20 min with a burst size of 49 phage particles per infected cell. Compared to other phages, BM31 had a shorter latency period and smaller burst size. The burst size is correlated with the rates of synthesis and assembly of phage components, latency period, metabolic activity, living environment, and protein synthesis machinery of host bacteria, rather than the cell size or DNA composition of the phage (Pan et al. 2021). The one-step curve for BM31 is represented in Fig. 3.

Host range

In all 13 staphylococcal strains tested for host range (Table 2), BM31 could only lyse the host strain of *S. arlettae*, suggesting that it is specific to its host with an extremely narrow host range.

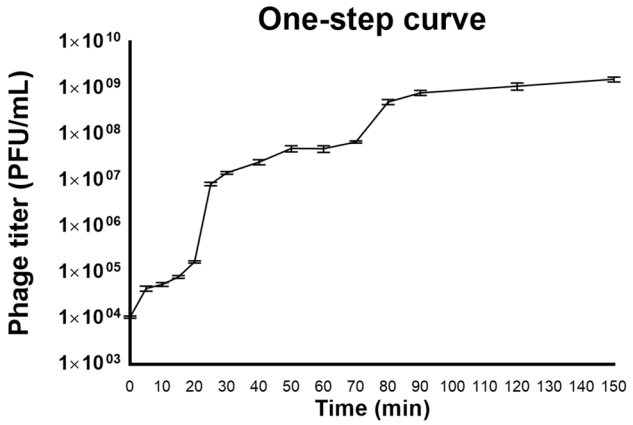

Fig. 3 One-step curve of BM31

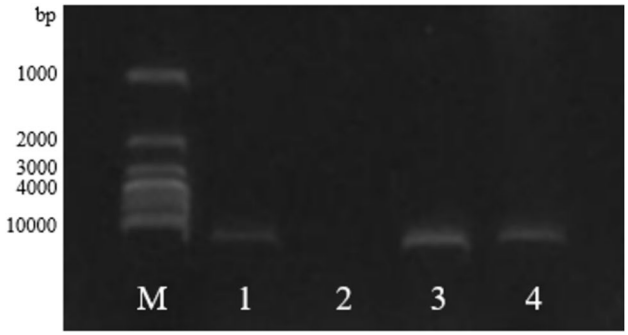

Fig. 4 Nucleic acid identification of BM31. M, 1-kb DNA ladder (COOLABER SCIENCE & TECHNOLOGY Co., LTD., Beijing, China); 1, BM31 treated with mung bean nuclease; 2, BM31 treated with DNAse I; 3, BM31 treated with RNAse A; N, original BM31 genome as control. Original gel is presented in Supplementary Fig. 1

Genomic overview

Treatment of the nucleic acid in BM31 with DNAse I resulted in digestion of its genome, whereas treatment with RNAse A and mung bean nuclease showed no sensitivity, thereby revealing that BM31 is a dsDNA virus (Fig. 4). The complete dsDNA genome of phage BM31 was 42,271 bp in size, with 34.59% of G + C (GenBank accession no. MZ488273). Functional prediction of the 65 ORFs using BLASTp analysis produced significant matches for 53 proteins. Of the 53 proteins with hits, only 26 were assigned to putative functions (Table S1). The remaining 12 proteins could not be matched with any of the proteins in the National Centre for Biotechnology Information (NCBI) database and were annotated as hypothetical. ORFs with distant homology to BM31, identified via HHpred analysis, are listed in Table 3. Although these hypothetical proteins were distributed throughout the BM31 genome, a clear modular organisation was evident, consisting of genes involved in virion morphogenesis (blue), DNA packing and replication (green), and lysis (purple) (Fig. 5). The most known functional genes were on the positive strand, except the integrase (ORF28) and transcriptional regulator (ORF32), which were in the middle of the gene cycle. No tRNA genes, antibiotic-resistance genes, or prophages were detected. However, a virulence factor named *pemK* and *mazF*-like toxin (ORF30, pfam02452), which is an endoribonuclease toxin of the type II toxin-antitoxin system, were observed (Oliveira et al. 2019).

Structural module

According to the function of annotated genes, the whole genome of BM31 was divided into four modules: structural,

Table 2 List of staphylococci used for the host range test

Species	Origin	Host	Accession	Per. Ident	Susceptibility
S. arlettae (host strain)	Sichuan	Bovine	MN851073.1	99.65%	+
S. arlettae			JX188021.1	99.45%	–
S. aureus			MK809241.1	99.58%	–
S. aureus			MK809239.1	99.65%	–
S. equorum			KX832694.1	99.30%	–
S. haemolyticus			MH542264.1	99.44%	–
S. haemolyticus			JF784022.1	99.44%	–
S. cohnii			MW767053.1	99.65%	–
S. chromogenes			CP046028.1	99.51%	–
S. lentus			KM010146.1	99.44%	–
S. caprae			CP031271.1	99.45%	–
S. saprophyticus			KJ009395.1	99.79%	–
S. epidermidis			MT604781.1	99.58%	–
Total	13				

Table 3 ORFs with distant homology to BM31 identified using HHpred analysis

ORF	Pfam ID[a]	Strand	E-value	Putative protein encoded
30	PF02452	–	1.00E – 07	PemK-like, MazF-like toxin of type II toxin-antitoxin system
33	PF12844	+	3.80E – 12	Helix-turn-helix (HTH) domain
62	PF06116	+	4.90E – 10	transcriptional activator RinB

[a]Pfam ID data can be found at https://pfam.xfam.org/

lysis, lysogeny, and DNA packing and replication genes. Proteins involved in capsid assembly and packing included the terminase small subunit (TerS) (ORF1), terminase large subunit (TerL) (ORF2), portal protein (ORF4), prohead protease (ORF5), and capsid protein (ORF6). The small and large subunits of phage terminase are key enzymes involved in DNA translocation and head filling (Mitchell and Rao 2006). The prohead protease is observed in some bacteria, likely as a result of horizontal transfer. The portal protein is a crucial element of the packing motor, which pumps the phage genome into the capsid with the assistance of a large subunit terminase (ATPase activity) (Kyrkou et al. 2020). Seven proteins were identified as structural proteins involved in tail morphogenesis and phage assembly: a head–tail adaptor (ORF9), head–tail joining protein (ORF10), major tail protein (ORF12), tail length tape measure protein (ORF15), tail fibre protein (ORF16, ORF17), and minor structural protein (ORF19). ORF15 was the longest sequence in the complete genome of BM31 and was annotated as a tail length tape measure protein (TMP), a tail-associated protein. TMP is not only present in tailed phages, but also in tail-less phages, determining the tail length and DNA transition into the host cell during infection (Gao et al. 2012; Mahony et al. 2016; Zhang et al. 2020). For most phages, tail fibres are the first proteins to recognise receptors on the bacterial membrane and initiate an infection (Geng et al. 2020). The location of capsid assembly genes upstream of the tail assembly genes and the presence of the longest TMP revealed that this structural module corresponds to the typical Siphoviridae morphogenesis module (Hatfull 2008).

Lysis module

The lytic cassette is composed of holin (ORF25) and lysin (ORF26). The lysin (ORF26) component consisted of an N-terminal CHAP endopeptidase domain (pfam 05,257,

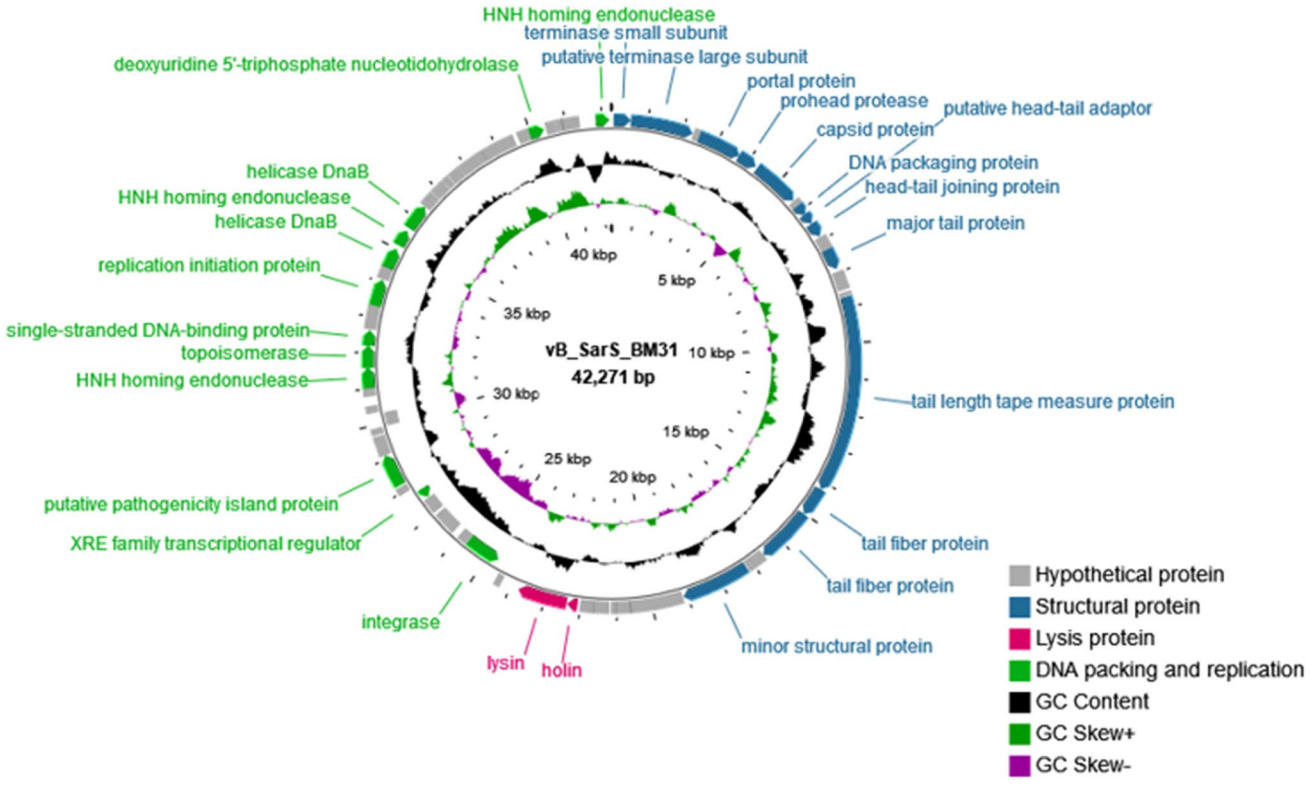

Fig. 5 Circularised genomic map of BM31. Assigned putative functions for each of the 65 predicted ORFs are as follows: structural (blue), lysis (purple), DNA packing and replication (green), and hypothetical (grey). No tRNA was identified. BM31 has a GC content of 34.59%. Image created using CGView

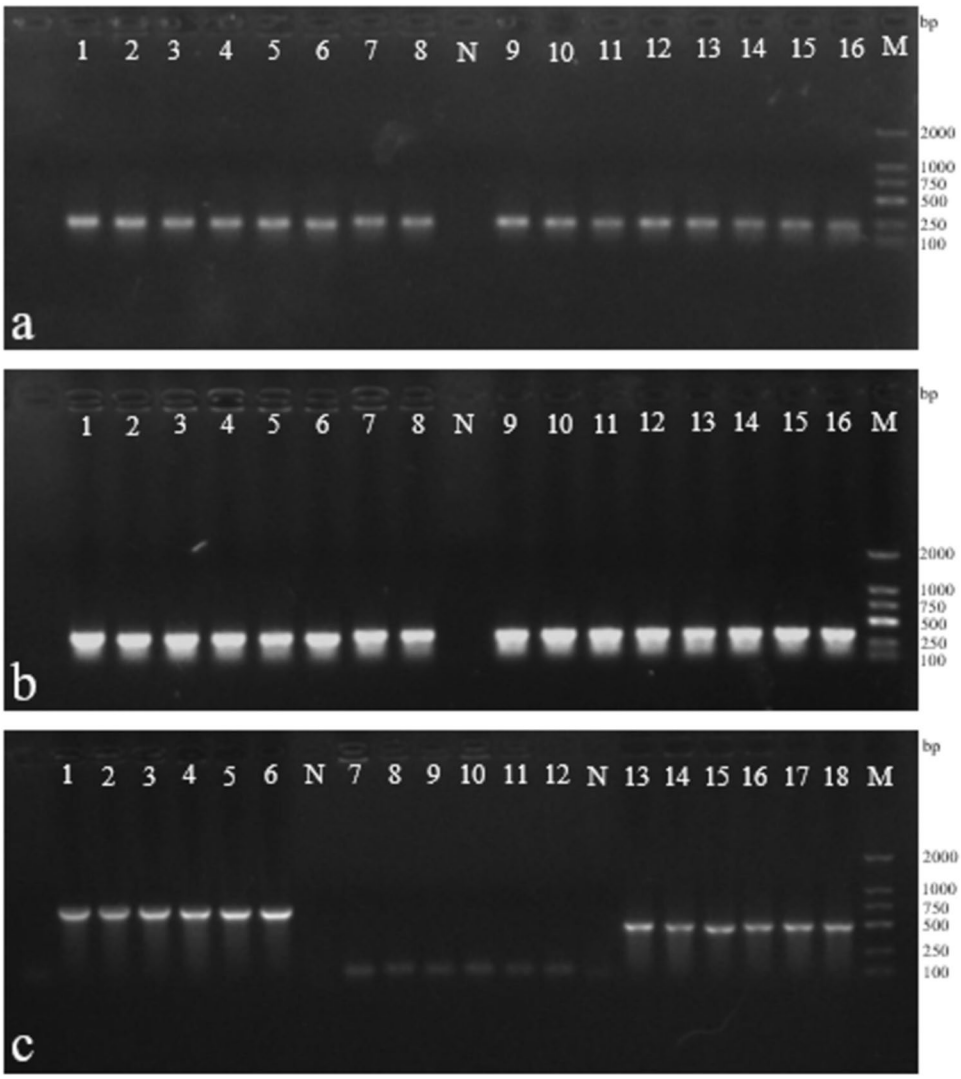

Fig. 6 Electrophoresis map showing specific structural protein of phage BM31 in *S. arlettae*. Lane M, DL 2000 DNA marker; lane N, negative control: PCR product without bacterial template. **a** Lanes 1–16, random clone of *S. arlettae* using primer CP, and original gel is presented in Supplementary Fig. 2. **b** Lanes 1–16, random clone of *S. arlettae* revealed using primer CP1, and original gel is presented in Supplementary Fig. 3. **c** Lanes 1–6, 7–12, and 13–18, random clone of *S. arlettae* revealed using primers CP675, TMP81, and PP488, respectively; and original gel is presented in Supplementary Fig. 4

amino acids 27–115) and an N-acetylmuramoyl-L-alanine-amidase domain (MurNAc-LAA, cd02696, amino acids 173–355), which was the same as the domain components of the lysin in phage Ph28 (Muharram et al. 2020). In addition, the lysin component of BM31 contained a SH3 peptidoglycan-binding domain (amino acids 377–438) in the C-terminus, which confirms that modularity is prevalent in staphylococcal lysins (Oliveira et al. 2013; Pertics et al. 2020). The holin (ORF25) component belongs to the SPP1 family (PF04688), which corresponds to 90.59% similarity to an uncultured Caudovirales phage from South Africa (van Zyl et al. 2018). Consecutive use of lysin and holin could lead most tailed phages to lyse and control the length of the infection cycle (Lu et al. 2017).

Lysogeny module

The lysogeny cassette contains integrase (ORF28) but lacks a CI-type repressor, which is normally present in other staphylococcal phages (García et al. 2009). However, the lambda repressor-like, Cro/C1-type helix-turn-helix (HTH) domain (ORF33), which is a DNA-binding domain and transcriptional regulator, was predicted downstream of the XRE family transcriptional regulator (ORF32) (Hargreaves et al. 2014). The HTH domain may indicate the presence of a repressor that cannot be annotated beyond unknown function proteins or hypothetical proteins in the BM31 complete genome. The phage_integrase domain (PF00589.24) of BM31 belongs to pfam00589, indicating that the integrase is a tyrosine recombinase (Y-Int), which possesses tyrosine as a catalytic residue (Oliveira et al. 2019). Preliminary evidence suggests that BM31 possesses two lyse systems and tends to obey the lysogenic cycle. Exception may be conditions that involve cellular stress, wherein anti-repressor proteins in the phage interfere with the function of the repressor protein and force the phage to adopt the lytic cycle in a process called a lytic-lysogenic switch (Das et al. 2020). The transcriptional activator RinB (ORF62) is associated with

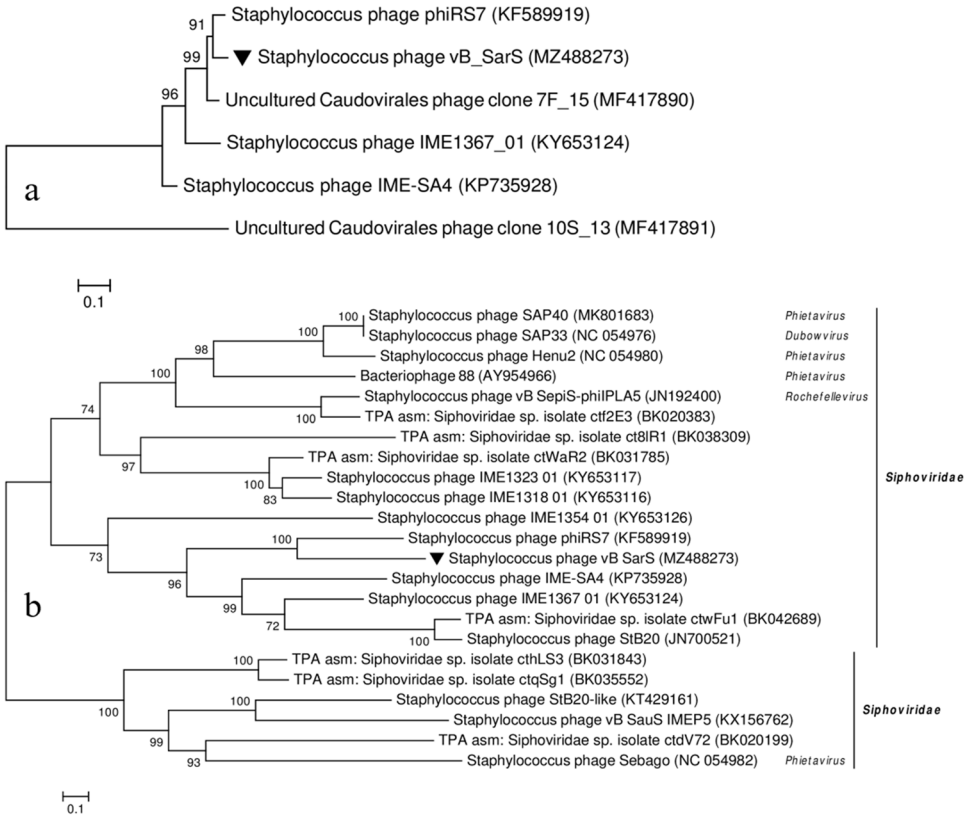

Fig. 7 Phylogenetic tree of BM31 and other related phages obtained from GenBank. **a** Phylogenetic tree based on capsid protein; **b** phylogenetic tree based on complete genome. Black inverted triangle, phage BM31 in this study. The number on the branch point represents credibility (values closer to 100 represent stronger credibility). The length of the branches represents the genetic distance; the shorter the ruler, the closer the relationship

integrative recombination of phages. For the staphylococcal phage phi11, rinA and rinB are required for active expression of the *int* gene, which is the sole viral gene responsible for integrative recombination.

DNA packing and replication module

The DNA packing and replication module consists of a single-stranded DNA-binding protein (ORF42), a replication initiation protein (ORF44), helicase DnaB (ORF46, ORF48), and others. All known temperate staphylococcal phages belong to the family Siphoviridae, suggesting that phage BM31 may be classified as a lysogenic phage (Ingmer et al. 2019); specifically, when integrase (ORF28), pathogenicity island protein (ORF34), and dUTPase (ORF60) are annotated in BM31. The pathogenicity island may be a type of phage-related chromosomal island, such as *S. aureus* pathogenicity islands (SaPIs), which are highly mobile and superantigen-encoding genetic elements closely associated with temperate phages (Novick et al. 2010). SaPIs carry genes encoding the toxic shock syndrome toxin, staphylococcal enterotoxin B, and other important virulence factors (Lindsay et al. 1998; Moller et al. 2019; Novick et al. 2010). SaPIs reside stably in prophages and can only achieve transduction by hijacking capsids of helper phages, rather than by themselves. The *stl* gene inside SaPI is a master repressor and can be depressed by dUTPase, which is expressed by the helper phage, so that the SaPI lytic cycle is activated. SaPI DNA was packed into these phage capsids after TerS recognised and cleaved the SaPI *pac* sites, resulting in the release of mature progeny phages (decreased in number), and SaPI particles (with smaller capsids than progeny phages) were released after bacterial lysis (Moller et al. 2019; Novick et al. 2010). HNH homing endonuclease (ORF40, ORF47, ORF65) genes are highly specialised selfish genetic components commonly present in phage genomes (Kyrkou et al. 2019, 2020). The HNH endonuclease may facilitate its own and relevant genes' mobility, even excluding other competing phages by cleaving their DNA (Goodrich-Blair and Shub 1996; Mohamed et al. 2012). It may also interfere with the expression of genes surrounding its insertion position and disrupt the active ORFs (Pan et al. 2021).

Life cycle

All phage proteins used in this study (capsid protein, portal protein, and tape measure protein) were detected in *S. arlettae* colonies which were randomly selected, irrespective of whether the strain was invaded by BM31 (Fig. 6). The results suggest that BM31 has a lysogeny-like life cycle.

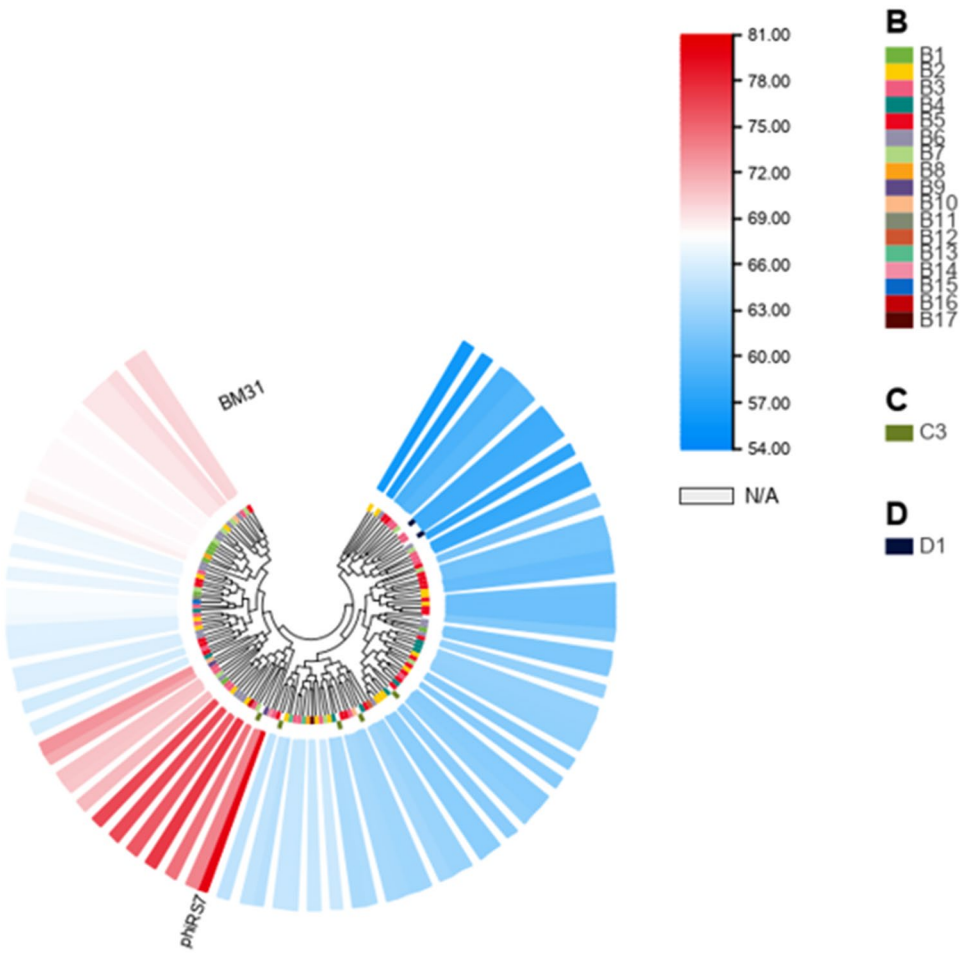

Fig. 8 Heat map of ANI. Values on legend, ANI (%). Row contains all 139 *Staphylococcus* phages (subcluster B1-17, C3, and D1); each subcluster was marked by a different colour. Only the phage with the highest ANI value was expressed in the form of text

All randomly selected colonies had several phage genes encoding phage structural proteins, both before and after lysis by phage BM31. Phage genes detected in the host strain genes may be the result of their ability to integrate its own genome into the host chromosome and achieve self-replication by bacterial replication, suggesting that the examined phage is a temperate phage. The phage genes were also observed in the colony that was never treated with BM31, likely because both *S. arlettae* and BM31 were isolated from one milk sample. As we first isolated the host strain, it is likely that the phage had already undergone the lysogenic cycle and integrated into the host strain chromosome. Findings from both genome annotation and the PHACTS online software analyses suggested that BM31 may be a temperate phage with a *pac* site.

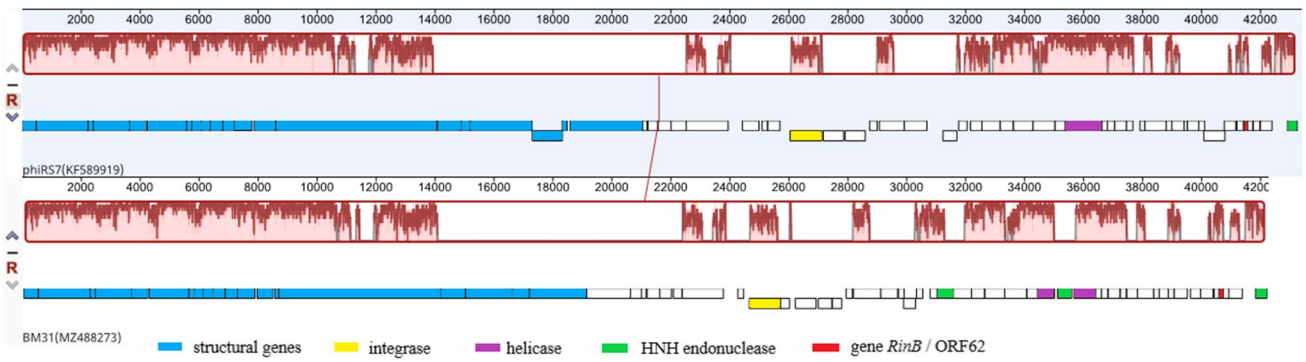

Fig. 9 Genome comparison of BM31 and phiRS7 by Geneious Prime. The scale at the top of genomes is in base pairs. Each rectangular square represents an ORF; the upper ORFs are in positive strand, and the lower ORFs are in negative strand

Taxonomic analysis

BM31 showed the closest sequence identity to phage phiRS7 (KF589919), with 36% query coverage and 83.82% nucleotide identity. To investigate the relationship between phage BM31 and other related phages stored in GenBank, two maximum likelihood phylogenetic trees were constructed based on the amino acid sequences of the phage capsid protein (ORF6) and complete genome sequences. Both trees revealed a close relationship between BM31 and *Staphylococcus* phage phiRS7, which belongs to the family Siphoviridae (Fig. 7). This result is consistent with that of the PHASTER online analysis software. Combined with the morphology characteristics determined by TEM, phage BM31 was defined as a member of family Siphoviridae.

All 205 *Staphylococcus* phages can be divided into four clusters (A, B, C, and D) (Oliveira et al. 2019). Following this standard, we aimed to classify phage BM31 into one of them. Three conserved pfams of BM31 were annotated; pfam1520 (ORF26) was the same as subcluster C3; pfam692 (ORF60) and pfam1844 (ORF65) were the same as subcluster D1, also presenting with temperate behaviour and long tail morphology, which were specific to cluster B. Therefore, 132 phages of cluster B, 5 phages of subcluster C3, and 2 phages of subcluster D1 were compared to BM31 through average nucleotide identity (ANI) analysis (Supplementary Dataset File). The results showed that BM31 contained the highest ANI of 79.83% with phiRS7 (KF589919); therefore, we classified BM31 into the same subcluster as phiRS7 in B14, which is a subcluster under cluster B (Fig. 8).

To obtain a more nuanced understanding of the genomic structural differences between BM31 and other phages, BM31 was comparatively analysed with the genome of the most homologous phiRS7 phage (Fig. 9). The results showed high degree of similarity and conservation between the genomes of the two phages, especially in the structural genes. Insertion and deletion variations in parts of the genes are mainly reflected in integrase, helicase, and HNH endonuclease. The results also prove the possible existence of a RinB gene in the BM31 genome that was originally annotated as a hypothetical protein (ORF62).

While we aim to use BM31 as a therapy/detection application, one problem is the virulence of the phage itself. The presence of the lysogenic cycle and *pemK* and *mazF*-like toxin may determine the rare possibility of phage BM31 being an antibiotic agent. Under these circumstances, whether the endolysin protein derived from BM31 may be a safe sole agent or used in coordination with antibiotics requires further study. Research has shown that the phage particle, the endolysin derived from the phage, and the cell wall binding domain derived from endolysin are all valid detection agents. The extremely narrow host range for BM31 indicates the high specificity of the phage acting as a detector. To achieve simultaneous multiple bacterial detection, the phage requires a wide host spectrum. Whether the expression of endolysin protein will broaden the host range also needs to be verified in subsequent experiments.

Conclusion

BM31 is a novel and rare phage that is highly specific to *S. arlettae*. The present findings supplement the phage database of CoNS, elucidating the mechanisms underlying bacteria-phage interactions, which may translate into clinical applications. BM31 survived in both normal and stable environments and exhibited a small burst size and short latency period. The small burst size may be associated with its lysogenic life cycle, and shorter latencies may suggest a rapid and high efficacy when the phage is applied. Although *S. arlettae* is not a major pathogenic bacterium, it was isolated from a bovine mastitis milk sample in this study, and it may contaminate dairy products. BM31 could not be used as a therapeutic agent due to lysogenic replication; however, phage-derived proteins such as lysin may be used to detect and control *S. arlettae* in further studies.

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Author contribution All authors contributed to the study conception and design. Conceptualisation: Guangli Han and Xueping Yao; methodology: Zidan Luo; investigation: Biao Lu and Jieru Zhang; resources: Pengfei Zhang; data curation: Guangli Han; writing, original draft preparation: Guangli Han; review and supervision: Kang Yong, Yin Wang, Yan Luo, Zexiao Yang, Meishen Ren, Xueping Yao, and Suizhong Cao. All authors have read and approved the final manuscript.

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Data availability All data generated or analysed during this study are included in this article and supplementary information files. The complete genome sequence of the novel phage BM31 has been deposited in GenBank under accession number MZ488273.

Declarations

Ethics approval and consent to participate All experimental protocols in this study were approved by experimental ethics committee of Sichuan Agricultural University. All methods were carried out in accordance with the 1964 Helsinki declaration and its later amendments ethical standards. The collection of milk samples had been permitted by dairy farm.

Competing interests The authors declare no competing interests.

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