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Isolation and characterization of a virulent bacteriophage *SPW* specific for *Staphylococcus aureus* isolated from bovine mastitis of lactating dairy cattle

Longping Li · Zhiying Zhang

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Abstract Mastitis in dairy cattle continues to be an economically important disease. However, control is complicated by a high prevalence of resistance to antibiotics. Phage therapy, therefore, is considered as an alternative way of controlling bacterial infections and contaminations. In this study, we have described isolation and characterization of a highly virulent phage *SPW* from wastewater of dairy farm, which possesses a strong lytic capability against mastitis-associated *Staphylococcus aureus,* the most important pathogen in bovine clinical and subclinical mastitis. The phage *SPW* produced large, round and clear plaques on bacterial culture plates. TEM showed phage *SPW* has an icosahedral head 62.5 nm in diameter and long tail of 106 nm, head and tail were held together by a connector of 18 ± 1.5 nm long and can be classified as a member of the *Myoviridae* family. Restriction analysis indicated that phage *SPW* was a dsDNA virus with an approximate genome size of 65–69 kb. One-step growth kinetics showed a short latency period of about 10–15 min and a rise period of 50 min and a relatively small burst size was 44 ± 3 phages particles/infected cell. Moreover, adsorption rates were not influenced by calcium ions and phage *SPW* was relatively stable in a wide range of temperature and pH values, and resistant to chloroform and isopropanol. The optimal multiplicity of infection (MOI) was 0.01. When phage *SPW* was used to infect five other clinically isolated pathogenic isolates, it showed relatively wide spectrum host range. Phage *SPW* was capable of eliciting efficient lysis of *S. aureus*, revealing it potentially as an effective approach to prophylaxis or treatment of *S. aureus*-associated mastitis in dairy cows.

Keywords *Staphylococcus aureus* · Bovine mastitis · Bacteriophage · Characterization

L. Li · Z. Zhang (✉)
Shaanxi Key Laboratory of Molecular Biology for Agriculture, College of Animal Science & Technology, Northwest A&F University, Yangling 712100, Shaanxi, People's Republic of China
e-mail: zhangzhy@nwsuaf.edu.cn

L. Li
e-mail: llp_315@163.com; llp315@nwsuaf.edu.cn

Introduction

Staphylococcus aureus is a gram-positive pathogen that causes a variety of animal diseases including mastitis in dairy cattle and brought substantial losses to dairy industry including reduced milk yield, milk quality and increased production costs. Currently, no effective treatment is available other than using antibiotics to control mastitis in dairy cows. However, the heavy use of antibiotics has enhanced the tolerance of bacterial pathogens and therefore inevitably increased the dosage that is required to produce the same result. More importantly, the so-called "super bug" antibiotic-resistant bacteria had evolved resulting from antibiotic abuse [1]. Thus, the growing concerns on the use of antibiotics have prompted researchers to seek alternative treatments. In this regard, phage therapy, which was invented and practiced during the early years of last century, has re-emerged to against *S. aureus*-associated mastitis infections [2–5]. Besides, phage may help us to understand the ecological behaviors and evolution of its host, which will eventually benefit the combat against bacterial infections. To date, several phage isolates and

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their derivatives such as phage K had been proven useful to treat *S. aureus* infections in chicken, mice, rabbit and dairy cattle [6–9]. Furthermore, some phages were successfully isolated against several lines of *S. aureus* derived from bovine mastitis [10–12].

Given the diversity of phages as well as their natural hosts that exist on earth, it would be not difficult to obtain a single solution (phage therapy) for bacterial infection among dairy cows. In the present study, we attempted to isolate a highly virulent phage that could efficiently kill *S. aureus* strain from local area dairy farm. We collected the cleaning wastewater from lactating dairy cattle udders before milking in a local dairy farm and obtained a virulent phage. The phage was characterized with respect to its morphology, stability and bacteriolytic activity in vitro assays. The phage isolate, which was named *SPW*, showed great therapeutic potentials to prophylaxis or treatment *S. aureus*-associated bovine mastitis in dairy cows.

Materials and methods

S. aureus isolation and characterization

Mastitis was diagnosed and milk samples were taken by veterinary practitioners and fresh milk samples were collected from a local dairy farm, and then 100 µl of milk sample was streak onto Columbia base agar containing 5 % sheep defibrinated blood agar and incubated for 24–36 h at 37 °C. Presumptive *S. aureus* colonies were identified on the basis of colony morphology and haemolysis on 5 % sheep blood agar. Identification was confirmed by conventional methods, including the catalase activity tests, a tube coagulase tests with rabbit plasma and the mannitol-fermenting tests. To further verify the identities of isolates, genomic DNA was extracted with phenol–chloroform, and then the highly variable regions of 16S rDNA gene [13] was examined by PCR and confirmation of the species level was carried out if sequences showed 99–100 % similarity to using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov). The finally identified catalase-positive, coagulase-positive, mannito-fermenting and PCR-positive *S. aureus* isolates were stored at −70 °C in LB containing 20 % (v/v) glycerol.

Phage enrichment and isolation

The enrichment method of Cerveny et al. [14] was adopted for the isolation of *S. aureus*-specific phages with some modifications. Briefly, twenty milliliter of waster water sample was cleared of debris and bacteria by centrifugation at 10,000×*g* for 10 min at 4 °C. The supernatant was transferred to a fresh tube and kept at 4 °C. To amplify phage, one milliliter overnight culture of *S. aureus* was mixed with 20 ml of fresh LB liquid medium (containing 10 mg/l $CaCl_2$) and 20 ml of water sample, and incubated for 10 h at 37 °C with shaking at 160 rpm, the culture media was centrifuged at 8,000×*g* for 10 min at 4 °C. The supernatant was filtered through Millipore filters (0.22-µm pore size). This amplification procedure was repeated three times. To examine the presence of phage, 0.1 ml final filtered culture media was mixed with 0.3 ml of *S. aureus* and incubated for 20 min at 37 °C. Then, the mixture was combined with 3 ml of preheated (47 °C) LB top agar (contain 0.4 % agar) and overlaid onto LB agar plate (1.5 %). The plate was incubated at 37 °C for 6 h or until lysis zones appeared.

Phage host range determination

The host range of the phage *SPW* was carried out as described previously by the spot tests method [15]. These assays were performed for a range of different bacteria, i.e., methicillin resistant *Staphylococcus aureus* (MRSA) 39, *S. aureus* SA456, *S. aureus* SA1, *S. aureus* SA17 and *Escherichia coli*. All these tested strains were clinical pathogen, obtained from raw milk samples and identified using homology comparison of the 16 *S rDNA* and *nuc* genes [13, 16].

Plaque purification, lysate preparation and phage tittering

Phage titer was determined by plaque-forming units (PFU) per ml using the double-layer agar plate method. In brief, after a series of dilution in sterile SM buffer (NaCl, 5.8 g; MgSO4·7H2O, 2 g; 1 M Tris·Cl pH 7.5, 50 ml; 2 % gelatin, 5 ml; add ddH_2O to 1,000 ml), 0.1 ml of phage and 0.2 ml of fresh *S. aureus* were added to a tube containing 3 ml of soft agar, which had been pre-warmed at 45 °C in a water bath. The mixture was then immediately overlaid onto a 1.5 % LB agar plate. Plates were incubated overnight at 37 °C until plaques appeared and to enumerate plaques on each plate.

Determination of optimal multiplicity of infection (MOI)

Multiplicity of infection was defined as the ratio of virus particles to potential host cells. The *S. aureus* cells obtained from their early logarithmic growth phase ($OD_{600} = 0.5$) infected with phage by using three different ratios (MOI = 0.01, 0.1 or 1). After incubation for 3.5 h at 37 °C, the phage lysate was centrifuged at 10,000×*g* for 3 min. The supernatant was filtered through a 0.22-um pore size syringe filter and used for determining the phage titer.

In all experiments, phage and *S. aureus*-free cultures were used as control. All assays were performed in duplicate. The highest value among phage titers was considered as the optimal MOI and used in subsequent large-scale phage production.

Large-scale amplification of phage

Large-scale amplification of phage was performed by inoculating 200 ml pre-warmed (37 °C) LB liquid medium with 1 % volume of overnight *S. aureus* before 1 ml of 10 mM $CaCl_2$ was added into the broth, and letting it grow to an early logarithmic phase. Then, *S. aureus* cells were infected with phage at a predetermined optimal MOI. The culture was continued at 37 °C with shaking at 160 rpm until complete lysis was observed (about 10 h). Ultimately, the culture was centrifuged at 8,000×*g* for 10 min and filtered through a 0.22 μm membrane.

Concentration and purification of phage lysates

Phage lysate was centrifuged at 8,000×*g* for 30 min and the supernatant filtered through a 0.22-μm pore size. The filtrate was treated with 0.5 ml of nuclease solution containing *DNAse* I and *RNAse* A (1 mg/ml, TaKaRa, China) at 37 °C for 2 h. Phages were then precipitated using modification of a method described by Lu Z et al. [17]. Polyethylene glycol 8000 and NaCl were added to final concentrations of 10 % (w/v) and 0.5 M, respectively. After gentle mixing, the phage preparation was incubated overnight at 4 °C. The phages were pelleted by centrifugation at 20,000×*g* for 30 min, then resuspended in 5 ml of 10 mM Tris-HCl buffer (pH 7.4). The phage preparation was overlaid on a CsCl (Sinopharm Chemical Reagent Co., Ltd, China) step gradient ($d = 1.7$, 1.5, 1.45, 1.32 g/ml, 1 ml each step) in 5 ml centrifuge tubes and centrifuged at 20,000×*g* for 6 h at 15 °C. The phage band (between $d = 1.7$ and $d = 1.5$) was drawn through the wall of the centrifuge tube using a syringe. The purified phage preparation was dialyzed against 10 mM Tris buffer for 24 h with three to four changes of buffer with a 6,000–8,000-Da pore size membrane.

Transmission electron microscope (TEM) imaging of phage

A CsCl-purified and polyethylene glycol 8000-NaCl-concentrated phage sample was negatively stained with 2 % (w/v) phosphotungstic acid (PTA) as following steps: 10 μl of phage suspension was spotted on the top of a collodion-carbon-coated copper grid, and allowed to adsorb for 30 min. The excessive amount of phage was removed by carefully touching the side of the copper grid with filter paper. Then, 5 μl of distilled water was spotted on the copper grid and removed shortly, followed by negative staining with PTA (2 %, w/v). 10 min later, excessive stain was removed, and the copper grid was allowed to air dry for 30 min. Finally, the copper grid was examined using a TEM (Japanese electronics JEM-100 SX; Electronics and Optics Laboratory, Xi'an Jiaotong University, Collage of Medicine, Xi an, China) operating at 75 kV.

Phage stability

Four temperatures (40, 50, 60 and 70 °C) used to study the thermal tolerance of phage *SPW* in LB broth. An aliquot of phage *SPW* was taken every 10 min for 1 h, and tittered by double-layer agar plate method. To test its pH stability, 100 μl of phage was mixed in a series of tubes containing 900 μl LB liquid medium with pH ranging from 2 to 10 (adjusted with 2 M NaOH or 18 % HCl), and incubated for 3 h at 37 °C. The titter was determined by double-layer agar plate method.

To further analyze phage *SPW* chemical stability, phage *SPW* was subjected to treatment with chloroform (5, 20, 50 or 100 %, v/v; Sinopharm Chemical Reagent Co., Ltd, China) for 24 h at 4 °C or isopropanol (10, 20, 50 or 100 %, v/v; Sinopharm Chemical Reagent Co., Ltd, China) for 2 h. In addition, phage *SPW* was also exposed to ultraviolet light (30 W, 35 cm wavelength; Ultraviolet analyzer WFH-204 B, Chen Du, China) treatment for 10, 20, 30, 40, 50 and 60 min, and then tittered immediately by double-layer agar plate method. The results were expressed as the percentages of survival phages from initial particles in each treatment group.

One-step growth curve assay

A previously described method [17] was modified and used to determine the one-step growth curve of phage *SPW*. In brief, phage was mixed with *S. aureus* cells from their early exponential growth phase at MOI of 0.01, and allowed to adsorb for 30 min at 37 °C, then centrifuged by 10,000×*g* for 30 s. The pellet containing infected cells was resuspended in 3 ml of pre-warmed LB liquid medium and incubated at 37 °C with shaking at 160 rpm. A 100 μl sample was taken at 10 min intervals up to 2 h and immediately tittered by the double-layer agar plate method. The experiments were carried out in triplicates. Latent period, burst time and burst size were calculated from the one-step growth curve as previously described [17].

Phage genome extraction and restriction analyses

Phage *SPW* was prepared in large scale as described above and then treated with 1 μg/ml of *DNase*I or *RNase*A

(Takara, China) at 37 °C for 1 h. NaCl crystal was added to a final concentration of 1 M and dissolved by swirling. Following incubation on ice for 1 h, the suspension was centrifuged at 10,000×*g* for 10 min at 4 °C. The supernatant was collected, and solid polyethylene glycol 8000 was added to a final concentration of 10 % (w/v). The mixture was stirred slowly at room temperature. After cooling and standing overnight on ice, the mixture was centrifuged at 10,000×*g* for 10 min at 4 °C. The phage pellet was then resuspended in 16 ml of SM buffer for each liter of original phage. The supernatant was collected by centrifuged at 10,000×*g* for 15 min and then added to a solution containing 20 mM EDTA (pH 8.0), 25 mg/ml proteinase K and 0.5 % sodium dodecyl sulfate (SDS). Following incubation at 56 °C for 1 h. Phage DNA was obtained by phenol–chloroform–isoamyl alcohol extraction and precipitated with 2 volumes of 95 % ethanol. The DNA was washed twice with 70 % cold ethanol (−20 °C). Finally, the pellet was air-dried and dissolved in 30 µl of ddH_2O for further restriction analysis. The quality of extracted DNA was examined by running 2 µl sample on a 0.8 % agarose gel, followed by staining with Gelview (1 µl/ml). Additionally, for restriction analyses, phage DNA was digested with restriction endonucleases as follows: 3 µl of extracted phage genome DNA (~500 ng/µl) was digested with restriction endonucleases *Eco*RI, *Hin*dIII, *Bam*HI, *Nde*I, *Eco*RV, *Sac*I, *Sal*I and *Pst*I according to manufacturer's recommendations (Takara, China) for overnight at 37 °C, the undigested nucleic acid of the Phage *SPW* was quantified as a control. The DNA fragments were examined by electrophoresis as above described.

Nucleic acid type

The nucleic acid of the phage *SPW* was digested with *DNase* I and *RNase* A (Takara, China) at 37 °C according to the manufacturer's instructions. Products of digested phage nucleic acid were separated by 0.8 % agarose gel electrophoresis. The undigested nucleic acid of phage *SPW* was quantified as a control by agarose gel electrophoresis.

Sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE)

The structural proteome of phage *SPW* was determined by separating a CsCl-purified phage preparation on a SDS-polyacrylamide gel. In briefly, an aliquot (30 µl) of CsCl-purified phage sample was mixed with 10 µl of buffer and 4 µl of reducing agent (1 M Tris-HCL, pH 6.8; 50 % glycerinum; 10 % SDS, bromophenol blue and β-mercaptoethanol). The mixture was heated in boiling water for 10 min and then subjected to electrophoresis on a 12 % SDS-PAGE gel at 78 V and 30 mA for 2 h. The protein bands were stained with Coomassie blue R-250 (Sigma, China), followed by destaining with a solution containing 50 % methanol and 10 % acetic acid. The reported molecular weight values were obtained using molecular weight standards (ProteinRuler® II Protein Ladders, Beijing TransGen Biotech Co., Ltd. Beijing, China).

Analysis of calcium ion effect on phage adsorption

The assay of calcium ion effect on phage adsorption was carried out as the previous study [18] with some modifications. The evaluation of calcium ion effect was made on the basis of the percentage of free phages at different time intervals of 0, 1, 2, 3, 4, 5, 6, 7 and 8 h. Then, the titers of un-adsorbed free phages in the supernatant were determined and the results were expressed as percentages of the initial phage counts.

Phage bacteriolytic activity in vitro experiment

Two milliliters of overnight *S. aureus* culture was inoculated into 200 ml of fresh LB liquid medium with 1 ml 10 mM $CaCl_2$ and incubated at 37 °C with shaking at 220 rpm until reached an early-exponential host bacteria culture ($OD_{600} = 0.5$). Phage *SPW* was added at MOI of 0.01 and kept at least 20 min at 37 °C. In parallel, an identical *S. aureus* culture without phage (MOI: 0) was used as control. The mixture was then grown at 37 °C for up to 60 h with shaking at 160 rpm. The phage bacteriolytic activity was assessed by monitored the cell absorbance of the culture solution (OD_{600}) at 30-min intervals for up to 10 h after phage infection, and this assay was performed in triplicate.

Results

Phage isolation and host specificity

In order to identify the most efficient phage for potential to treat or prevent bovine mastitis epidemics in local area, we decided to isolate the natural host strains of *S. aureus* in the vicinity places to our laboratory. To do so, fresh raw milk samples were collected from clinic mastitis dairy cows to detect the presence of *S. aureus* isolates. Using the wastewater of cleaning udder before milk, one virulent phage, specific for *S. aureus* and produced clear plaques at size of 2–3 mm in diameter, was identified. We designated the phage as *SPW*, which referred to a ***S****. aureus* specific **P**hage from **W**astewater cleaning udder. The infectivity of phage *SPW* was investigated with five other clinically isolated pathogenic bacteria of methicillin resistant

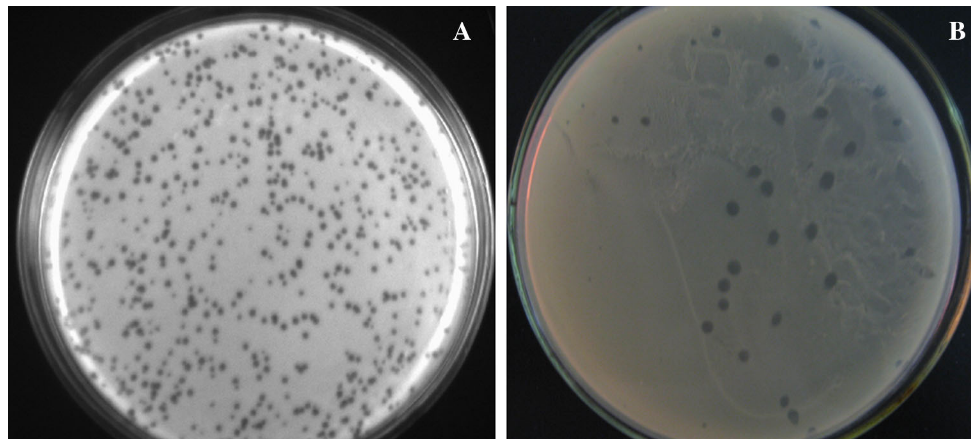

Fig. 1 Plaque formation by double layer agar plates showing polyethylene glycol precipitated and CsCl-purified phage *SPW*. Calculated PFU/ml was 10^9 and observed plaque size was 2.5–4 mm. Plate **a** shows lower dilution of viral titer and plate **b** shows higher dilution of viral titer

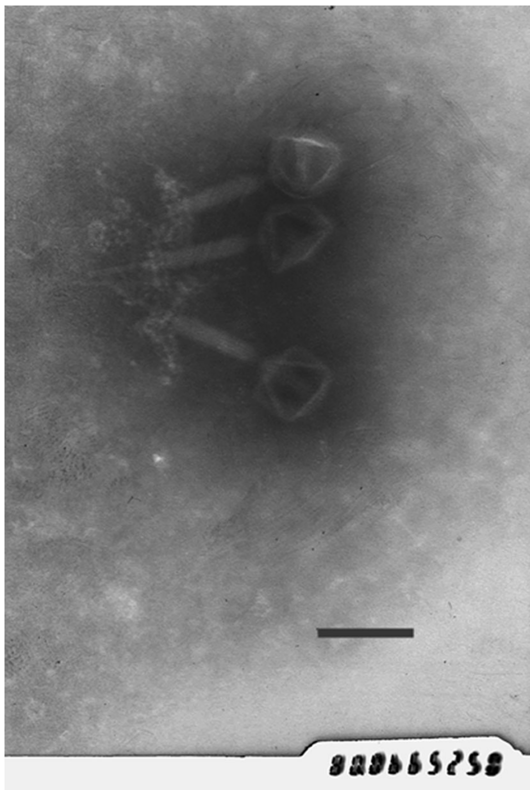

Fig. 2 Microphotograph obtained by TEM image of phage *SPW*. The *arrows* indicate contracted sheaths. The magnification 80,000×, the *bar* represents 100 nm

Staphylococcus aureus (MRSA) 39, *S. aureus* SA456, *S. aureus* SA1, *S. aureus* SA17 and *E. coli*. All phage host combinations were evaluated by spot test method in five independent experiments. Phage *SPW* showed growth inhibition in MRSA strain and *E. coli*, however, could infection *S. aureus* SA17, *S. aureus* SA456 and *S. aureus* SA1. These results indicated that phage *SPW* had a relatively wide host range for clinical *S. aureus*. Therefore, this lytic phage *SPW* was randomly chosen from the isolated phages for a detailed examination.

Tittering and morphological analysis by TEM

The plaques formed by CsCl-purified phage *SPW* were clear uniformed transparent round zones and their sizes were about 2–2.5 mm in diameter (Fig. 1). The titer of phage *SPW* from a large-scale preparation was determined as a little more than 10^9 PFU/ml. The phage *SPW* was further characterized with regard to its morphology. The ultrastructure was examined by using TEM. As shown in Fig. 2, the phage *SPW* had an isometric head of 62.5 nm in diameter and a regularly tail, which were 106 ± 1.5 nm long and 19 ± 1.5 nm wide. The head and tail were always held together by a connector named collar which was 18 ± 1.5 nm long. A base plate, which was approximately 81 ± 1.5 nm in diameter located on the tail also present. These morphological characteristics indicated that the phage *SWP* seems to be a member to the genus "Twort-like phages" of the family Myoviridae [19, 20].

Optimal multiplicity of infection (MOI) and one-step growth curve

The optimal MOI of phage *SPW* was determined to be 0.01. Based on the optimal MOI, we then established a one-step growth curve which showed the short latent period (defined as the time interval between the absorption and the beginning of the first burst) was about 10–15 min, the rise period was 50 min, and that the burst size was approximately 44 ± 3 PFU per infected cell (Fig. 3), which calculated as a ratio of the final count of liberated phage particles to the initial count of infected bacterial cells during the latent period.

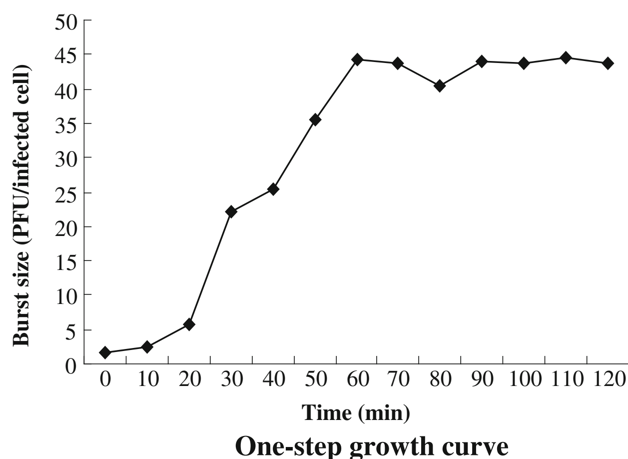

Fig. 3 One-step growth curve of phage *SPW*. The latent period was defined as the time interval between the adsorption and the beginning of the first burst. The burst size was calculated as ratio of the final count of liberated phage particles to the initial count of infected bacterial cells during the latent period

Phage stability

To evaluate the suitability of phage *SPW* for potential manufacture or therapeutic use in the future, its physical and chemical stabilities were examined. We found that the activity of phage *SPW* remained stable in a wide range of temperature up to 40 °C. Towards extreme conditions, it declined significantly after heating for 10–60 min at 60 or 70 °C, and was completely inactivated when heated to 70 °C for 40 min (Fig. 4a). In a test for variable pH conditions, phage *SPW* remained very active at pH ranging from 4 to 9, and retained its lytic capability when incubated at 37 °C for up to 1 h in pH conditions ranging from 2 to 10. The viability of phage *SPW* decreased slightly below pH 4 or above pH 10 (Fig. 4b). Furthermore, the viability of phage *SPW* was largely unaffected in the presence of 5 % chloroform and only slightly affected by 5 % isopropanol as shown in Fig. 4c and d, respectively. Fig. 4e showed that about 94 % of phage *SPW* survived from ultraviolet light (30w, 30 cm wave-length) treatment for up to 40 min, suggesting that ultraviolet light had little effect on the survival rate of phage *SPW*.

Analysis of calcium ion on phage *SPW* adsorption

Adsorption is often affected by the presence of divalent metal ions in the solution. Calcium ions were added in phage-*S. aureus* mixture. Phage *SPW* and its host cells were mixed in test tube in presence of 10 mM $CaCl_2$. Free phages were detected at different time intervals for 8 h. Result showed no significant difference between the two groups (Fig. 5). Calcium ions might stabilize phage adsorption process.

Phage genome and nucleic acid type characterization

As shown in Fig. 6a, the nucleic acid genome of phage *SPW* was completely only digested with *DNase* I, while it remained intact with *RNase* A treatment, which indicated that phage *SPW* was a double-stranded DNA. The size of its genome appeared to be more than 23 kb in comparison with *λ Hind*III DNA Marker (TaKaRa, China). Purified phage *SPW* genomic DNA was digested with a number of restriction enzymes including *Eco*RI, *Hind*III, *Nde*I, *Bam*HI, *Eco*RV, *Sac*I, *Sal*I and *Pst*I (TaKaRa, China). The resulting patterns from *Eco*RI, *Hind*III and *Nde*I restriction digestion were shown in Fig. 6b. However, the genome of phage *SPW* appeared resistant to digestion by *Bam*HI, *Eco*RV, *Sac*I, *Sal*I and *Pst*I restriction enzymes. Based on the digestion profiles, digested DNA fragments were summed up to calculate the size of phage genome. We estimated that the DNA genome of phage *SPW* isolated in this study was approximately 65–69 kb.

Proteomic analysis *SPW* phage protein

Polyethylene glycol (PEG 8000) precipitated and CsCl-purified phage *SPW* particles were subjected to SDS-PAGE analysis after washing with 0.1 M ammonium acetate three times in order to remove any residual bacterial protein. Proteomic patterns were obtained after Coomassie Blue G 250 staining and destaining steps. A total of three major and four minor protein bands were observed on the gel, with molecular weight ranging from 30 to 100 kDa for the three major protein with reference to ProteinRuler ® II Protein Ladders (12–100 kDa, Fig. 7).

Bacteriolytic activity test in vitro experiment

In order to determine phage *SPW* bacteriolytic activity, we co-cultured phage *SPW* with *S. aureus* by adding phage *SPW* to an early logarithmic phase of *S. aureus* ($OD_{600,}$ 0.5) culture at MOI of 0.01, the absorbance (OD_{600}) continued to increase during the incubation. However, the growth of host cell was strongly prohibited after phage infection 3 h. During about 6 h, the OD_{600} value of phage and *S. aureus* co-culture mixture decreased to 0.025, and remained nearly zero without a sign of recovery up to 60 h, while the absorbance ($OD6_{00}$) of the *S. aureus* culture was not infected by phage *SPW* continued to increase during the incubation (Fig. 8). The kinetics of killing of host cells by phage *SPW*, which was quantified by measuring cell density at 30 min intervals for up to 10 h after infection, suggested that phage *SPW* was highly effective against *S. aureus* (Fig. 8). Those assays were performed as least more than three repeats and for up to 60 h.

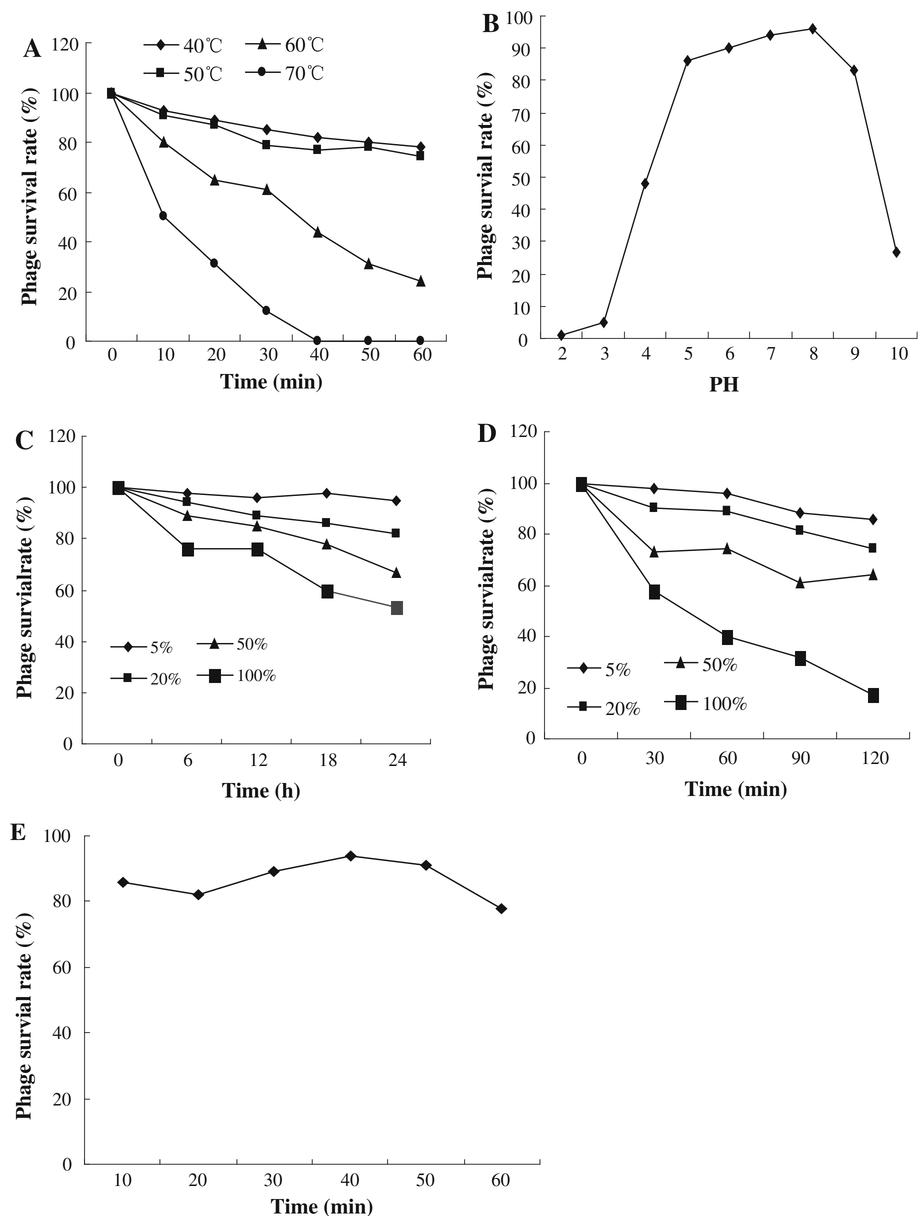

Fig. 4 Resistance tests of the phage *SPW*. **a** Thermo stability. Phage *SPW* was incubated at various temperatures as indicated. Samples were collected at 10 min intervals for 60 min. **b** pH stability. Phage *SPW* was incubated under different pH conditions for 3 h. **c** Chloroform treatment. **d** Isopropanol treatment. **e** Ultraviolet light treatment. In **a**–**e**, the results were expressed as the percentile of survival rate of initial particles in each treatment group, and tittered immediately by double-layer agar plate method

Discussion

Bovine mastitis is a significantly costly disease for dairy industry worldwide. Although a wide variety of pathogens have been isolated as causative agents of this disease, *S. aureus* is considered to be the most important pathogen in bovine clinical and subclinical mastitis. Mastitis caused by *S. aureus* is a major concern to the dairy industry due to its resistance to antibiotic treatment and its propensity to recur chronically. This organism easily develops resistance to commonly used antibiotics. These problems suggest that the need of an alternative therapy for this bacterial disease. Scientists and clinicians alike are looking retrospectively to find a promising alternative treatment in the form of phage therapy [11, 12, 21]. Phage therapy against *E. coli* had significantly progressed over the past decade [22–24]. However, little is known about *S. aureus*-specific phages and the one that has therapeutic value for *S. aureus*-associated bovine mastitis in China. Previous studies, a number of phages have been isolated and described mainly aiming *S. aureus*-associated diseases other than bovine mastitis [6, 22–24]. Recently, the therapeutic value of phage against bovine mastitis [2, 10] and significant results had been made in these studies.

Phages can be isolated from a wide variety of sources such as sea water, sewage water/sludge ponds etc. They mainly "feed" on the organisms that are present in their natural habitat. They are host specific and evolve along

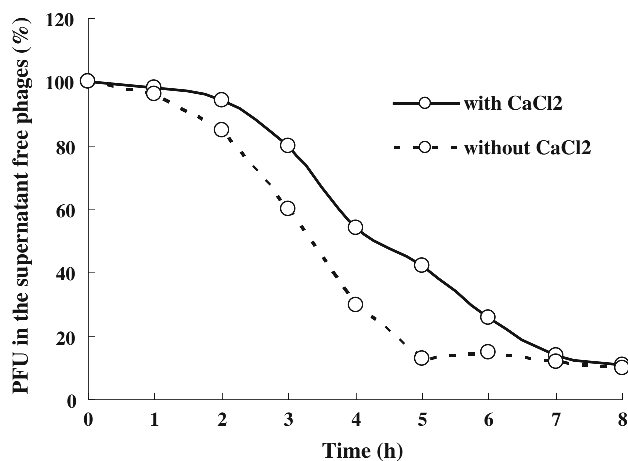

Fig. 5 Adsorption rate of phage *SPW* at different time (0, 10, 20, 30, 40, 50 and 60 min) intervals. Samples were taken from the supernatants to measure free phage particles. Divalent metal ions effect on adsorption rate was analyzed by adding 10 mM $CaCl_2$ to the mixture of phage *SPW* and *S. aureus* cells. At intervals, tubes were removed and centrifuged (10,000×*g* for 5 min) to sediment the phage-adsorbed bacteria. Then, titres of un-adsorbed free phages in the supernatant were assayed using the double-layer plate technique, and the results expressed as percentages of the initial phage counts

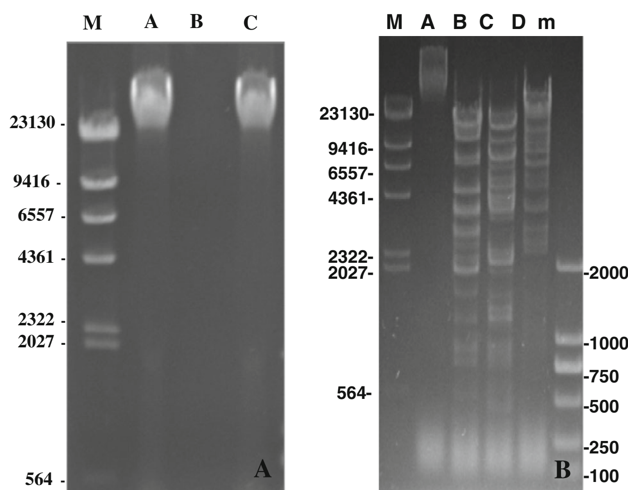

Fig. 6 Phage *SPW* nucleic acid type and DNA characterization. **a** Phage genome was non-treated (*lane A*), or treated with *DNase* I (*lane B*) or *RNase* A (*lane C*) and resolved on 0.8 % agarose gel. *Lane M*, λ *Hind* III-digested DNA Marker (3403, TaKaRa). **b** The genomic DNA of phage *SPW* was non-digested (*lane A*) or digested with restriction enzymes *Eco*RI (*lane B*), *Hind*III (*lane C*), *Nde*I (*lane D*). *Lane M*, λ *Hind*III-digested DNA Marker (3403, TaKaRa). *Lane m*, DL2000 DNA Marker (3427A, TaKaRa)

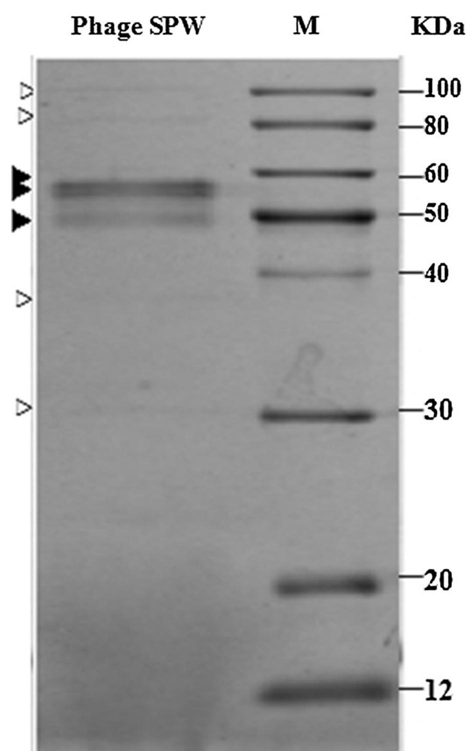

Fig. 7 12 % SDS-PAGE of polyethylene glycol precipitated and CsCl-purified phage *SPW*. *Black arrows* indicate the major proteins bands, *hollow arrows* show the minor proteins bands and M, protein marker

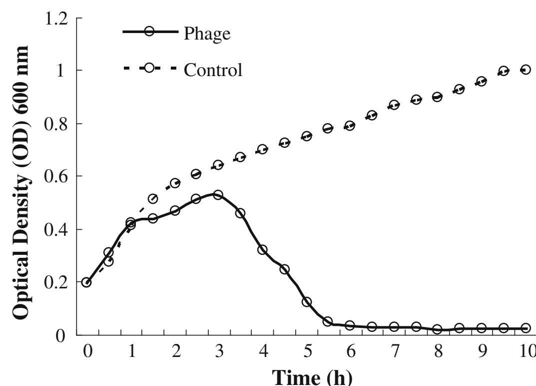

Fig. 8 The bacteriolytic activity of the phage *SPW*. *S. aureus* was non-infected or infected with phage *SPW* and co-cultured until cell lysis occurred. The optical density (OD_{600}) of *S. aureus* non-infected or infected with phage *SPW* was measured

with their host. To date, the study of systematic screening *S. aureus*-specific phage similar to ours had not been conducted in China. In the present study, *S. aureus* isolates were recovered from milk samples collected from lactating cows with clinical mastitis and identified according to their colonies morphology, biochemical properties and molecular method. The double-layer agar plate method was employed to screen the *S. aureus* specific-phages from wastewater from a local dairy farm. The isolated phage *SPW* showed broad lytic activities with the tested *S. aureus* strains. Phage *SPW* was specific for *S. aureus*, but no plaque production was observed in the examined *E. coli*

and MRSA39. Furthermore, the phage *SPW* was characterized morphologically by using TEM as Fig. 2, its therapeutic effect was examined in in vitro experiments (Fig. 8). Morphological characteristics confirmed that phage *SPW* seems to be a member to the genus "Twort-like phages" of the family Myoviridae. In this family, a phage is non-enveloped, and consists of an isometrically head, a tubular contractile tail and the head were always separated from the tail sheath by a collar, and the genome is a linear, double-stranded DNA. Similar to other phage genomes consisting of dsDNA, the genomic DNA of phage *SPW* was completely digested by *DNase* I but not *RNase* A (Fig. 6). A representative species of this family is T4 phage [19, 20].

Based on the one-step growth curve, we analyzed the multiplication parameters of phage *SPW*. This is the first report on the latent time and burst time of a phage against *S. aureus* associated with bovine mastitis in our local dairy farm of lactating dairy cattle. It also indicated that phage *SPW* was highly active in infected *S. aureus* cells. Taken together, these results had demonstrated that phage *SPW* was capable of infecting and killing bovine mastitis-associated *S. aureus*, which underlined great potentials of its use in animals. Furthermore, as shown in Fig. 3, the phage *SPW* showed a relatively large burst size with a short latent period, which is further suggestive of the lytic nature of the phage.

A number of factors, i.e., ions (e.g., Mg^{2+} and Ca^{2+}), pH, temperature, and the growth medium can influence phage adsorption rate and its infection. Previously, it has been reported that calcium and magnesium ions have positive effect on phage-bacterium attachment. These ions have an electrostatic bonding effect on the interactions of phage bacterium systems [25]. Calcium ions stabilize the interaction of virion with receptors. Different concentrations of calciumions have shown maximum effects on infectivity of different phages. In our research, the phage *SPW* showed significantly more infectivity with a 10 mM calcium chloride concentration and calcium ions might stabilize phage *SPW* adsorption process (Fig. 5). Furthermore, phage was often quite sensitive to protein denaturation in an acidic environment, the ability to survive within a wide range of pH values is a feature requested for therapeutic applications. Therefore, phage *SPW* could be used by oral administration to cows or for udder medicated bath as a proprietary liniment before and after milking in dairy farms as a bovine mastitis prophylaxis agent. Considering the possible loss of viability during transportation from laboratory to dairy farms in future animal experiments, it is important to seek a better condition to maintain the activities of phage *SPW*. In this regard, the studies on its resistance to heat and chemical agents not only explored effective control measures to minimize the loss of phage activities but also helped to understand the viability of *SPW* when exposed to different environmental conditions. In the present study, phage *SPW* has shown a very broad range of thermal stability, it remained active for 3 months at 4 °C and viable at 40 or 50 °C for 1 h incubation and became completely inactive at 70 °C for 40 min, while maximum infectivity was observed at 37 °C (Fig. 4a); however, it was quite resistant to chloroform, isopropanol and ultraviolet light treatment (Fig. 4b–d). We proposed that during a short period of transportation or storage at room temperature or the present of 5 % (v/v) chloroform might give a protection to phage *SPW*.

The bacteriolytic activity of phage *SPW* was investigated in an in vitro co-culture assay. When *S. aureus* was not infected by phage *SPW*, the OD_{600} continued to increase after the incubation. In contrast, when *S. aureus* was infected with phage *SPW* at MOI of 1 and 0.01, bacterial growth was apparently retarded (Fig. 8). The OD_{600} was increased approximately 2–3 h after infection (about 3 h), but started to decrease after phage infection. These results demonstrate that phage *SPW* possesses strong bacteriolytic activity, which is important for use in phage therapy.

In conclusion, a highly virulent phage was isolated and characterized from a local dairy farm, which had therapeutic potentials to treatment or prophylaxis bovine mastitis caused by *S. aureus*. Based on morphology and the genomic analysis, phage *SPW* resembled a previously reported Myoviridae phage of *Staphylococcus*. Phage *SPW* had strong a lytic activity against *S. aureus* at MOI of 0.01 in a bacteriolytic activity test. Thus, the isolated phage will be effectively used to treatment or/and prophylaxis mastitis caused by *S. aureus* infection. However, further study about antibacterial activity of this phage in additional strains or in vivo host cells will be required to expand phage application.

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Conflict of interest The authors declare that they have no competing interests.

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