Phage JS02, a putative temperate phage, a novel biofilm-degrading agent for Staphylococcus aureus

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Significance and Impact of the Study: This study presents the phage vB_SauS_JS02, a temperate bacteriophage infecting multidrug-resistant isolates of S. aureus, which can effectively control S. aureus infection through inhibition of planktonic cell growth and biofilm formation. Bioinformatics analysis showed that this phage could be categorized as an unclassified species in the genus Triavirus, family Siphoviridae. Although temperate phages are not generally recommended for phage therapy, vB_SauS_JS02 showed high bacterial performance and interestingly no lysogenic appearance was observed.

Keywords
bacteriophage, biofilm, genome analysis, Staphylococcus aureus, temperate phage.

Abstract

Staphylococcus aureus is a biofilm-producing organism that is frequently isolated from various environments worldwide. Because of the natural resistance of S. aureus biofilm to antibiotics, bacteriophages are considered as a promising alternative for its removal. The bacteriophage vB_SauS_JS02 was isolated from livestock wastewater and showed activity against multidrug-resistant S. aureus. The phage vB_SauS_JS02 exhibited a broad host range and possessed a large burst size (52 PFU/CFU) as well as moderate pH stability (4–11) and appropriate thermal tolerance (40–50°C). Electron microscopy and genome sequence revealed that vB_SauS_JS02 belonged to Triavirus genus in Siphoviridae family. Genetic analysis of the 46 kb sequence of vB_SauS_JS02 revealed 66 ORFs. The predicted protein products of the ORFs were clustered functionally into five groups as follows: replication/regulation, DNA packaging, structure/morphogenesis, lysis and lysogeny. Although the phage vB_SauS_JS02 was a temperate phage, it exhibited a higher inhibiting and degrading activity against planktonic cells (80–90% reduction), even to S. aureus biofilm (~68% reduction in biofilm formation). Moreover, the removal activity of the phage vB_SauS_JS02 against both planktonic cells and S. aureus biofilms was even better than that of the antibiotic (ceftazidime). In summary, the present study introduced the phage vB_SauS_JS02 as a potential biocontrol agent against biofilm-producing S. aureus after making it virulent. It may be applicable for phage therapy.

Introduction

Methicillin-resistant Staphylococcus aureus is frequently detected in contaminated dairy farms (Riva et al. 2015; Schnitt and Tenhagen 2019). Therefore, it is important to control the occurrence of S. aureus in foods in order to ensure food safety. Because S. aureus has a high ability to form biofilms on a wide range of surfaces, alternative and safe approaches are required for its biocontrol in foods. Biofilms are aggregated structured communities of bacteria enclosed in a matrix (often referred to as extracellular polymeric substances), which is composed of proteins, DNA and polysaccharides. During growth in biofilms, bacteria may evade host defenses and become tolerant to antimicrobial agents that can inhibit free-floating, single-cell (planktonic) bacteria, thereby making biofilms particularly difficult to eradicate (Howlin et al. 2015).
Bacteriophages (phages) are viruses that infect only bacteria, and they replicate and propagate through host specificity and growth inhibition activity (Shahin et al. 2021; Zhang et al. 2021). Because antibiotics have been found to be ineffective against biofilms, there is a growing interest in the use of phages as a strategy for prevention and elimination of biofilm formation. On the basis of recent findings, bacteriophages are considered to play a crucial role in the prevention and elimination of biofilm-related infections (Lusiak-Szelachowska et al. 2020).

In general, the life cycle of a phage enters either of the following two stages after infection of the host strain: a lytic cycle or a lysogenic cycle (Skurnik and Strauch 2006). Although lytic phage seems to be an ideal candidate for controlling multidrug-resistant (MDR) S. aureus infections, more than 200 genome sequences of Staphylococcal bacteriophages are available in public genome databases, and most of these phages belong to the Siphoviridae family of temperate, tailed bacterial viruses (Goerke et al. 2009). Therefore, it is useful to obtain a virulent phage from a temperate one to control S. aureus. There are different ways to use temperate phage that is virulent or make it virulent, such as genetic engineering, isolation of natural virulent mutants that can infect a lysogenic host or phage-antibiotic synergy or stress activator. In a previous study, a temperate phage in Siphoviridae family was transformed into a virulent one by random gene mutation using sodium pyrophosphate, and this virulent mutant phage showed rapid and long-lasting host cell growth inhibition activity; this suggested that this phage may have the potential to serve as a novel biocontrol agent against S. aureus (Chang et al. 2019).

In the present study, a novel Siphoviridae phage infecting MDR S. aureus, vB_SauS_JS02, was isolated and characterized to control MDR S. aureus. vB_SauS_JS02 is a temperate phage but has strong host inhibition activity. Moreover, the phage vB_SauS_JS02 showed a higher removal activity against planktonic cells of S. aureus than the antibiotic ceftazidine. The genome of this phage was sequenced and analyzed to determine the genes responsible for coding the capsid, tail, DNA modifier enzymes, virulence factor PVL, and lysogenic cycle mediators such as integrase. The phage vB_SauS_JS02 may be applicable for phage therapy as a potential biocontrol agent against biofilm-producing S. aureus after making it virulent.

**Results and discussion**

**Electron microscopy analysis of bacteriophage**

The isolated phage was found to form clear plaques on the host strain lawn culture. Transmission electron microscopy revealed a long hexagon head (80 ± 2 nm, n = 10) that was connected directly to a long non-contractile tail (300 ± 8 nm, n = 10) (Fig. 1a). The morphology analysis clearly indicated that the phage belongs to the order Caudovirales, family Siphoviridae. On the basis of the classification and nomenclature rules of viruses and according to the morphological evidence, the phage was named as vB_SauS_JS02. In the present study, we report a S. aureus temperate phage isolated from sewage effluent that showed an efficient activity which can control planktonic cell growth to prevent biofilm formation.

**One-step growth curve analysis and thermal and pH stability**

The one-step growth curve of the phage vB_SauS_JS02 showed a latent period of 20 min and a burst size of 52 PFU/infected cell when infecting S. aureus SF16 (Fig. 1b). The infectivity of phage was relatively stable at pH 6–9 and declined dramatically at lower or higher pH (Fig. 1c). In addition, the infectivity of the phage vB_SauS_JS02 remained intact when heated to 40 or 50°C and decreased rapidly above 60°C (Fig. 1d). The primary experimental evidences such as clear plaque formation, broad host range and well-stability in different pH values and temperatures encouraged us to consider JS02 as an advantageous candidate to control S. aureus infection.

**Phage host range and EOP analysis**

The hot spot assay revealed that most of the S. aureus strains (45 of 81 strains, 55%) were lysed by vB_SauS_JS02 (Table 1). The susceptible strains from the spot test were subjected to EOP analysis. A wide range of EOP (0.05 ± 0.02–0.94 ± 0.03) was obtained in the spot test (Table 1). Moreover, high (EOP ≥ 0.5) and medium (0.1 ≤ EOP < 0.5) production levels were observed in approximately 16 and 36% of the tested strains, respectively (Table 1).

**Genomic sequencing and comparative genomic analysis**

The digestion pattern of the phage genome showed that the genome was digested by Hind , EcoRI and EcoRV (Fig. S1). The phage vB_SauS_JS02 contained a long double-stranded linear DNA genome of 46,435 base pairs with a G+C content of 33.1% (Fig. 2). The open reading frame (ORF) analysis by GeneMarkS and Expasy revealed that the phage vB_SauS_JS02 genome contained 66 putative ORFs (Fig. 2). These 66 ORFs were similar to those of the genus deposited in the GenBank database with annotated functions. However, most of the annotated genes (39 of 66 ORFs) were hypothetical proteins (Table S1). Based on Canchaya et al. (2003) classification, the
sequences were assigned to five functional clusters as follows: replication/regulation, DNA packaging, structure/morphogenesis, lysis and lysogeny (Fig. 2).

Bioinformatics investigation by CLC genomics Workbench V12 and BLASTn analysis showed that the phage vB_SauS_JS02 genome was quite similar to that of the other members of Triavirus genus (Table S1). The ANIm value of the phage genome indicated that vB_SauS_JS02 shared 76–95.7% nucleotide sequence similarity to ΦSa2aw_st8, ΦSa2wa_st93, ΦSa2wa_st93mssa, Φ7401PVL, 47, 3A, 42e, ΦSauS-IPLA35, Φ12 and ΦSLT (Fig. S2). Furthermore, the relatedness of the phage vB_SauS_JS02 with the S. aureus phages (phage ΦSa2aw_st8, ΦSa2wa_st93, ΦSa2wa_st93mssa, Φ7401PVL, 47, 3A, 42e, ΦSauS-IPLA35, Φ12, and ΦSLT) based on nucleotide and amino acid sequences was confirmed by EasyFig software and Gepard (Fig. S3). The genome sections with similarities housed several gene groups, e.g., major capsid, tail protein and integrase (Figs S4 and S5). On the basis of the major capsid and the large subunit terminase protein sequences of the phage vB_SauS_JS02, it was confirmed that this phage is most related to phage ΦSa2aw_st8 (Fig. 3a,b). Thus, vB_SauS_JS02 was classified as a member of the family Siphoviridae, genus Triavirus. Gene-sharing network demonstrated the closer relationship of vB_SauS_JS02 to phages 3a, phi2968PVL, YMC, phi12, iPLA35, phi12 and 48 than phages vegas, Rani, 29, SA13, iPLAS, CNPx and so on (Fig. S6).

Most S. aureus isolates carry multiple bacteriophages in their genome (prophage), and based on our knowledge, all the known Staphylococcal temperate phages belong to the family Siphoviridae (Ingmer et al. 2019). On the basis of the morphological characteristics, the results indicated that the phage vB_SauS_JS02 belongs to the Siphoviridae family, which belonged to serogroup A, with a tail length of more than 200 nm and a head with a long hexagon head (Saito et al. 1981). According to the one-step growth curve analysis, the latent period for the phage JS02 was approximately 20 min, and the burst size was approximately 52 PFU/infected cell, which were similar to those observed for the drug-resistant S. aureus phage S1 that belonged to serogroup A (Saito et al. 1981).

Figure 1 Electron micrograph (a), one-step growth curve (b), pH stability (c) and thermostability (d) of the phage vB_SauS_JS02.
Table 1 Lytic activity of the phage vB_SauS_JS02 against Staphylococcus strains

| Bacterial strain | Infectivity (spot test) | Efficacy of plating (EOP) (mean ± SD) |
|------------------|-------------------------|---------------------------------------|
| ATCC 25923       | +                       | 0.34 ± 0.04                           |
| CMCC 26001       | +                       | 0.42 ± 0.02                           |
| CMCC 26003       | +                       | 0.44 ± 0.07                           |
| ATCC 29213       | +                       | 0.24 ± 0.03                           |
| A14              | +                       | 0.07 ± 0.03                           |
| A15              | +                       | 0.08 ± 0.02                           |
| A16              | +                       | 0.35 ± 0.05                           |
| J7               | +                       | 0.43 ± 0.04                           |
| J28              | +                       | 0.48 ± 0.06                           |
| M9               | +                       | 0.05 ± 0.02                           |
| MP00036          | −                       | 0                                     |
| MP00037          | −                       | 0                                     |
| MP00038          | −                       | 0                                     |
| MP00049          | −                       | 0                                     |
| MP00050          | −                       | 0                                     |
| MP00051          | −                       | 0                                     |
| MP00065          | −                       | 0                                     |
| MP00077          | −                       | 0                                     |
| MP00079          | −                       | 0                                     |
| MP00086          | −                       | 0                                     |
| MP00088          | −                       | 0                                     |
| MP00090          | −                       | 0                                     |
| MP00094          | −                       | 0                                     |
| MP00098          | −                       | 0                                     |
| MP00107          | −                       | 0                                     |
| S7               | +                       | 0.12 ± 0.02                           |
| SD5              | +                       | 0.42 ± 0.04                           |
| SD6              | +                       | 0.47 ± 0.04                           |
| SF15             | +                       | 0.77 ± 0.05                           |
| SF16             | +                       | 1.00                                  |
| SF22             | +                       | 0.33 ± 0.06                           |
| SF29             | +                       | 0.21 ± 0.03                           |
| SF30             | +                       | 0.16 ± 0.05                           |
| SF34             | +                       | 0.11 ± 0.03                           |
| SF36             | +                       | 0.74 ± 0.04                           |
| SF37             | +                       | 0.39 ± 0.05                           |
| SF38             | +                       | 0.75 ± 0.06                           |
| SF39             | +                       | 0.28 ± 0.03                           |
| SF46             | +                       | 0.73 ± 0.04                           |
| SF49             | +                       | 0.42 ± 0.06                           |
| SF50             | +                       | 0.28 ± 0.06                           |
| SF54             | +                       | 0.64 ± 0.04                           |
| SF55             | +                       | 0.47 ± 0.03                           |
| SF56             | +                       | 0.42 ± 0.02                           |
| SF60             | +                       | 0.44 ± 0.07                           |
| SM9              | +                       | 0.65 ± 0.02                           |
| TQ1              | +                       | 0.94 ± 0.03                           |
| XS-1             | +                       | 0.38 ± 0.06                           |
| XB1              | −                       | 0                                     |
| XG17             | +                       | 0.11 ± 0.05                           |
| XG19             | +                       | 0.18 ± 0.04                           |
| XG25             | +                       | 0.33 ± 0.03                           |

(continued)

Bioinformatics analysis of the phage genome showed that this phage could be categorized as an unclassified species within Triavirus genus (Table S1). The JS02 genome contained 46,435 bp with a G+C content of 33.1% (Fig. 2). Genome analysis of the phage revealed 66 predicted ORFs; however, most of the annotated genes (39/66 ORFs) were hypothetical proteins (Table S1). The module for lysis included amidase and holin. ORF63 and ORF64 encoded proteins that were nearly identical to amidase (N-acetylmuramidase), and ORF62 encoded a protein identical to holin, which is responsible for degrading peptidoglycan and bacterial biofilms (Amankwah et al. 2021). In the lysogeny modules, the predicted proteins of ORF02 and ORF03 were identified as integrase and showed highest similarity to that of S. aureus ΦSa2aw_st8 (Coombs et al. 2020). Finally, the predicted proteins of ORF65 and ORF66 which responsible for coding Panton-Valentine leucocidin (PVL) group showed 100% identity with S. aureus Φ7401PVL (Pincus et al. 2015) that responsible for
coding Panton-Valentine leucocidin (PVL) group of genes.

Antibacterial activity of the phage vB_SauS_JS02 against planktonic cells and biofilm

To study the effect of phage exposure on biofilm formation, we first followed bacterial growth in the planktonic phase in the presence of increasing MOIs (0, 1, 10 and 100) of the phage vB_SauS_JS02 and ceftazidime (16 µg ml\(^{-1}\), sub-MIC) within 7 h. At all the MOIs tested, bacterial growth in the planktonic phase was equal to that of the non-treated control until 4 h post-treatment (Fig. 4a). Growth of the bacteria infected with vB_SauS_JS02 at all the MOIs showed a significant reduction (\(P < 0.001\)) compared to that of the bacterial control from 5 to 7 h post-infection (Fig. 4a). Additionally, the growth of the bacterial samples infected with vB_SauS_JS02 at all the MOIs was significantly reduced compared to that after ceftazidime treatment (sub-MIC, 16 µg ml\(^{-1}\)) at 7 h time point (\(P < 0.05\), Fig. 4a). At different MOIs (0, 1, 10 and 100), the phage vB_SauS_JS02 caused an 80–90% reduction of S. aureus population at 7 h when compared with the ceftazidime treatment (without phage) in which 60% reduction was observed. The results of the bacterial reduction test indicated that the growth of bacterial cells was inhibited in the presence of vB_SauS_JS02 at different MOIs in the early phase culture of S. aureus. Moreover, the results of the assay of JS02 activity against planktonic cells showed a significant reduction in absorbance measured at OD\(_{570}\) as compared to that noted for the ceftazidime (16 µg ml\(^{-1}\), sub-MIC) treatment group.

Biofilm formation in the presence of increasing MOIs (0–100) of the phage vB_SauS_JS02 and ceftazidime (16 µg ml\(^{-1}\), sub-MIC) at 24 h time point was also monitored by the standard crystal violet (CV) assay. Similar results were obtained in the groups infected with different MOIs (Fig. 4b). Pretreatment with phage (MOI: 0–1) significantly inhibited biofilm formation (\(P < 0.001\), Fig. 4b). At MOIs of 0, 1, 10 and 100, the phage JS02 removed 62, 70, 69 and 69% of the biofilm’s biomass after 24 h, respectively. Cefazidime at the concentration of 16 µg ml\(^{-1}\) reduced the biofilm’s biomass by 43% after...
The SEM technique was used to confirm the results obtained by the CV assay (Fig. 4c–e). The results of SEM confirmed the above-mentioned observations. Biofilm formation was suppressed in both the phage pre-treated (MOI: 0·1) and the antibiotic (ceftazidime, 16 μg ml⁻¹, sub-MIC) pre-treated plates (Fig. 4d,e). Moreover, the effect of the phage treatment was better than that achieved after the ceftazidime treatment (Fig. 4b,d,e). Bacteriophage vB_SauS_JS02 exhibited a good preventive effect on biofilm formation. In addition, pre-treatment with the phage JS02 remarkably inhibited biofilm formation of the SF16 strain. The S. aureus SF16 strain exhibited a biofilm-forming capacity and was identified as a high-risk pathogen based on its resistance to various antibiotics such as tetracycline, ciprofloxacin, penicillin, ampicillin, clindamycin and azithromycin. Biofilm formation and development is a significant virulence factor. Biofilm-forming bacteria were found to inhibit the effectiveness of antibiotic treatment by reducing its penetration to bacteria and exhibiting the ability to evade from host responses. Phage-bacteria interaction is highly dependent on both the particular bacterial strain and the phage.

Figure 3 Phylogenetic relationship of the phage vB_SauS_JS02 was investigated using the UPGMA method with 2000 bootstrap replications based on amino acid sequences of major capsid (a) and terminase (large subunit, b) proteins. The sequences of these proteins in phages vB_SPuM_SP116 (Bao et al. 2020) and vB_SflS-ISF001 (Shahin et al. 2019b) were used as outgroups.
For instance, similar to planktonic cells, the response of the biofilm to the phage was strain-dependent; the temperate phage ΦPan70 produced a reduction of 99.9% to ~999% depending on the strain (Holguin et al. 2015).

Although the mechanism of phage-biofilm interaction is still unclear, it is believed that phage-derived enzymes degrade the major exopolysaccharide protective layer of the biofilm matrix, and consequently, phages can reach and kill bacterial cells in the biofilm (Milho et al. 2019). Furthermore, phages can diffuse through the pores and channels in the biofilm to reach different layers of the biofilm (Sadekuzzaman et al. 2017). The effectiveness of the phage to destroy established biofilms needs further research.

It is widely known that virulent phages are suitable to be developed as biocontrol agents. Although the JS02 phage showed very efficient S. aureus growth inhibition activity, the existence of lysogeny associated genes makes it a little risky to introduce as a suitable biocontrol agent for some reasons. For instance, the insertion of phage genome into host cell chromosome makes these cells potentially resistant to the closed phages. In addition, some undesirable genes such as genes encoding toxins and virulence factors are transferred by lysogenic phages. On the other hand, while the existence of well-known mechanisms of resistance superinfection immunity and superinfection exclusion against the proposed phage (Fogg et al. 2010; Cumby et al. 2012; Bondy-Denomy et al. 2016) had not been performed precisely, there were also no evidence to assume that resistance mechanisms were at play, since colonies were observed in the middle of plaques or the OD increased in broth culture based experiments. Moreover, regarding the fact that resistance to antibacterial agents would be possible even in the case of the most powerful drugs and agents, resistance to phages either lytic or temperate would not be impossible. Thus, the efforts in phage therapy should be focused on minimizing the resistance rate in parallel to busting the antibacterial potential by combining phages with other antibacterial agents such as antibiotics, as suggested by Al-Anany et al. (2021).

For vB_SauS_JS02, the following aspects indicated its high performance to control S. aureus infection: absolutely clear plaque formation, high titre progeny, broad host range and adequate inhibitory effect on planktonic cells and biofilms of S. aureus. A previous study reported that mutations induced in the lysogeny module by using sodium pyrophosphate led to the loss of lysogenic capability, which enabled the selection of lytic variants (Gutierrez et al. 2018). Another study showed that the
temperate phage SA13, when transformed into a virulent phage by gene mutations, acted as an antimicrobial agent against *S. aureus* (Chang et al. 2019).

The present study demonstrates the vital role of phage genome sequencing of the candidate agents to be used as alternative therapy. While vB_SauS_JS02 showed almost all lytic phage characteristics and seemed to be an ideal candidate for controlling *S. aureus* infection, it also harbored lysogenic-mediated genes and could be introduced as a temperate phage based on genome analysis. To our knowledge temperate phages might be avoided as antibacterial agents because of their numerous demerits. However, mutant temperate phages could be incapable of both entering the lysogeny cycle and effectively killing host cells that have been lysogenized by related temperate phages (Krylov et al. 2012). Isolation or engineering of mutants is an additional step for the development of a therapeutic phage that would be necessary if obligatory lytic phages are selected. Sometimes antibiotics act as a stress factor and can induce a lysogenic cycle to lytic. Al-Anany et al. (2021) eradicated *Escherichia coli* through the synergistic effects of a temperate phage and signals that awaken it. The synergy effect between vB_SauS_JS02 and antibiotics will be further studied.

In summary, this study presents the phage vB_SauS_JS02 as a temperate bacteriophage infecting multidrug-resistant isolates of *S. aureus* that can effectively control *S. aureus* infection through inhibition of planktonic cell growth and biofilm formation. Bioinformatics analysis showed that this phage could be categorized as an unclassified species in the genus *Triavirus*, family Siphoviridae. Although temperate phages are not recommended for phage therapy, selecting gene mutations or engineering of genes coding for PVL and integrase as recommended for phage therapy, selecting gene mutations will be an additional step to develop temperate phages for phage therapy.

**Materials and methods**

**Host strains**

From 2010 to 2019, hundreds *S. aureus* strains were recovered from several sewage samples, Jiangsu province, China. The isolates were kept in Luria-Bertani (LB) broth supplemented with 30% glycerol at −80°C. All the *S. aureus* isolates were treated by 0.5 μg ml⁻¹ of mitomycin C (Sigma-Aldrich, Missouri) and only prophage-free isolates were used for further experiments (Kropinski & Clokie 2009). Non-plaque formers were initially screened by a spot assay, and then they were approved as a non-lysogenic *S. aureus* after further measurement through the double-layer plaque assay. In addition to 81 prophage-free wild, four reference strains, namely, *S. aureus* CMCC 26001, CMCC 26003, ATCC 29213, and ATCC 25923 were also used as host in some steps. All bacteria were routinely cultured in tryptic soy broth (TSB, Qingdao Hope Bio-Technology Co. Ltd, Qingdao, China) or tryptic soy agar (TSA).

**Bacteriophages isolation**

*Staphylococcus aureus* SF16 (nonlysogenic strain) was used as the primary host to isolate bacteriophage in the present study. Phage isolation was done based on the conventional protocol as described previously with slight modification (Zhang et al. 2015). Shortly, the exponential phase culture of the *S. aureus* strain was inoculated with sewage effluent collected from a local livestock industry in Jiangsu province, China before incubation at 37°C for 24 h. Then, the mixture was centrifuged for 20 min at 10 000 g and filtered through a 0.22-μm pore size membrane filter. The presence of specific phages in sample was determined by the spotting assay on lawn culture of the host bacteria (Han et al. 2013). Pure phage was obtained by the three repetitions of the single-plaque isolation method as described elsewhere (Shahin et al. 2019a; Yazdi et al. 2020). At the end, to obtain the high-purified phage particle, the filtered phage solution was subjected to ultracentrifugation through CsCl gradient (Zhang et al. 2015). The purified phage solution was stored at 4°C till further experiments.

**Transmission electron microscopy**

The purified phage lysate was transferred onto a carbon-coated copper grid (Ted Pella Inc., Redding, CA, USA) and then, negatively stained using 2% phosphotungstic acid (PTA) as described previously (Bao et al. 2019). The phage particle was observed using a Zeiss transmission electron microscope (TEM) EM902 (Zeiss, Oberkochen, Germany) at an accelerating voltage of 100 kV.

**One-step growth curve analysis**

To determine the latent period and phage burst size, one-step growth curve analysis was carried out as described elsewhere with some modifications (Chang et al. 2015). Briefly, the early-exponential growth phase of host cells (*S. aureus* SF16) was harvested by centrifugation (8 000 g, 10 min) and resuspended in fresh TSB. The phage was added at a multiplicity of infection (MOI) of 1 and incubated at 37°C for 10 min. Then, the mixture was centrifuged at 6000 g for 10 min, and the pellet was suspended in TSB, followed by incubation at 37°C with constant shaking of 100 rpm. Samples were taken at 10-min intervals (up to 90 min) and phage titres were
immediately determined by the double-layer agar plate method (DLA). This experiment was done triplicate.

Thermal and pH stability
For thermo-stability, the phage was incubated at 40, 50, 60 min for phage titration using the DLA method. For 

Host range analysis
Host range of the phage was assessed against reference strains and wild isolates using the spotting assay (Yazdi et al. 2019). Briefly, 10 μl of phage suspension (10² PFU per ml) was spotted individually onto the surface of bacterial lawn culture plates and incubated at 37°C. The plates were checked for clear plaque formation after 18–24 h. The appearance of clear plaque was considered as positive result.

Efficiency of plating (EOP)
The efficiency of plating (EOP) was defined as the ratio of phage titre on the target host to phage titre on the reference host bacteria (S. aureus SF16) and also was used to determine the effectiveness of phage to cause productive infection in different S. aureus isolates. EOPs were only calculated for the Staphylococcus isolates that showed a clear zone in the spotting test. The experiment was performed triplicate, and EOP of each phage/bacterial strain combination was classified as high (EOP ≥ 0.5), medium (0.1 ≤ EOP < 0.5), low (0.001 < EOP < 0.1), or no (EOP ≤ 0.001) production level, according to the mean ± SD score of EOP (Shahin et al. 2019c).

Bacteriophage DNA extraction
Bacteriophage genome was extracted based on the method of Chang et al. (2015). The phage DNA was digested using the restriction enzymes HindⅢ, EcoR and EcoRV (TaKaRa, Tokyo, Japan), according to the manufacturer’s instructions. After digestion, the fragments were visualized by electrophoresis in 0.8% agarose containing ethidium bromide (0.5 μg ml⁻¹).

Genome sequencing and genomic analysis
The preparation of the gene library and the whole-genome sequencing were performed by Benagen Biotech Co., Ltd (Wuhan, China) using the Genome Sequencer Illumina NovaSeq System. The open reading frames (ORFs) were predicted using Glimmer 3.02 (Bardina et al. 2016), and nucleotide and protein sequences were scanned for homologs with the alignment search tools (BLASTP, BLASTX and BLASTN search) available at the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Protein motif search was conducted using HMMER (https://www.ebi.ac.uk/Tools/hmmer/search/phmmer), and annotations were made by referring to published genomes of other phages with at least 98% similarity. The genome was screened for tRNA-encoding genes by using tRNAscan-SE (Schattner et al. 2005). The average nucleotide identity distance matrix (ANIm) value was calculated for classifying and identifying the phage genome (Yoon et al. 2017). EasyFig (ver. 3.2) was used to compare the whole genome with related phage genomes showing the highest similarity at nucleotide and amino acid levels (Darling et al. 2004; Sullivan et al. 2011). Phylogenetic trees were constructed using MEGA 7.0 to investigate the evolutionary relationship of the phage. The complete genome sequence of the S. aureus phage was deposited in GenBank under accession number LC541428. Pangengome analysis of the isolated phage was performed by CLC genomics workbench 12 (Qiagen, Aarhus, Denmark) and compared with all phages belong to Triaviruses and unclassified Triavirus genus using the neighbor joining construction method with Kimura 80 as nucleotide distance measure. Bootstrap analysis was performed using 1000 replicates. The complete coverage and identity similarity of the isolated bacteriophage against all phages in Triaviruses and unclassified Triavirus genus was constructed using the default parameter of the database (Darzentas 2010). Moreover, the gene-sharing network of vB_SauS_JS02 was visualized with vContact2 0.9.5 (Bin Jang et al. 2019). Taxonomic relationship of vB_SauS_JS02 was compared with NCBI bacterial and archaeal viral refSeq V85 with ICTV+NCBI taxonomy as references and protein-protein similarity method conducted by BlastP. The vContact2 output network file was visualized by Cytoscape (ver. 3.8.0) (Shannon et al. 2003) using edge-weighted spring-embedded model. The selected node (vB_SauS_JS02), attributed nodes and edges were visualized in phage genus.

Susceptibility of planktonic cells
The S. aureus solution (OD₆₀₀ = 0.1) was seeded onto a 96-well sterile polystyrene microtitre plate. To determine the effect of the phage and the antibiotic (ceftazidime) on planktonic culture simultaneously, the bacterial culture was mixed with the phage at different ratios (MOIs of 0.1, 1, 10 and 100) and with ceftazidime at the sub-
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minimum inhibitory concentration (MIC) of 16 µg ml⁻¹. Following incubation, the absorbance of the different samples was measured at OD₅₇₀ at appropriate time points (1–7 h). Wells containing bacteria without phage and antibiotic were used as controls. This experiment was repeated thrice (Jamal et al. 2015).

Prevention of biofilm formation

To establish the potential of the isolated phage to prevent biofilm formation, *S. aureus* cells were treated with different concentration of the phage to obtain MOIs of 0.1, 1, 10 and 100 with or without antibiotic (16 µg ml⁻¹, sub-MIC value) in a 96-well sterile polystyrene microtitre plate. After incubation at 37°C for 24 h, the content of the wells was removed, and the wells were gently washed twice with 0.9% NaCl, dried in the inverted position before stained with 1% crystal violet for 10 min. The plates were washed again using distilled water and 200 µl of 0.9% NaCl solution was added to each well. The optimal absorbance at 570 nm was measured in an ELISA plate reader. This experiment was repeated three times, and the percentage of biofilm removal was calculated and compared to that of the well containing only bacteria (control) (Kwiatek et al. 2017). The percentage reduction in a particular treatment was calculated by the following formula:

\[
\text{Percentage reduction} = (1 - T/U) \times 100,
\]

where \(T\) = bacterial load in the treated sample and \(U\) = bacterial load in the untreated sample.

Examination of biofilm by scanning electron microscopy

The effect of phage vB_SauS_JS02 on the formation of *S. aureus* biofilm was monitored for strain SF16. In the pre-treatment experiment, TSB broth containing phage vB_SauS_JS02 (MOI = 0.1) or the ceftazidime (16 µg ml⁻¹, sub-MIC) was added on coverslips placed over a 24-well plate with each well along with the bacterial culture and incubated the plate at 37 ± 1°C for 24 h. *S. aureus* biofilms were studied by scanning electron microscopy (SEM). For SEM, coverslips were fixed in 2% gluteraldehyde solution for 4 h and dehydrated using a serial concentration of ethanol as follows: 10 min in 50%, 10 min in 70%, 15 min each in 80%, 15 min in 90% and 20 min in absolute ethanol. The samples were dried before being examined using a scanning electron microscope (ZEISS EVO-LS10, Germany) operating at 5 kV.

Statistical analysis

The obtain data were analyzed by one-way or two-way analysis of variance using GraphPad PRISM software (ver. 5.02). A P-value of <0.05 was considered to indicate a statistically significant difference.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author contributions

LZ, KS and RW planned the experiments; KS and AS contributed to genome analysis; LZ, KS, HD, HW and LS performed the experiments; LZ wrote the manuscript; KS and RW edited the manuscript for intellectual content. All authors read and approved the final manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Comparison of the basic characteristics of the phage vB_SauS_JS02 and other similar phages.

Figure S1. Genomic DNA fingerprinting of phage gDNA.

Figure S2. Average nucleotide identity (ANI) distance matrix of vB_SauS_JS02 and the other phages of the genus Triavirus.

Figure S3. Comparison of the genome of the phage vB_SauS_JS02 with those of the closest related Staphylococcus aureus phages (phage Sa2aw_st8, ΦSa2wa_st93, ΦSa2wa_st93mssa, Φ7401PVL, 47, 3a, 42e, IPLA35, Φ12, and ΦSLT) at the nucleotide (a) and amino acid levels (b).

Figure S4. Dot plot analysis of the phage vB_SauS_JS02 and all related Staphylococcus phages.

Figure S5. Similarity (a) and identity (b) of the phage vB_SauS_JS02 with the other associated Triavirus phages are presented as heat map.

Figure S6. Gene-sharing network of vB_SauS_JS02, vB_SauS_JS02, and similar phages showed as colored circles.