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Effect of temperate bacteriophage vB_SauS_S1 on the adaptability and pathogenicity of *Staphylococcus aureus* ST398

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Abstract

Livestock-associated *Staphylococcus aureus* ST398 is a highly pathogenic species that causes infections in a wide variety of animals, including humans. The bacteriophage (phage) vB_SauS_S1 was isolated originally using a ST398 strain as its "isolating host", then the spot tests showed it was able to infect 73.33% (22/30) ST398 isolates. Phage S1 was assigned as a temperate phage based on genome analysis and phenotypic validation. Phylogenetic analysis showed that S1 was closely related to temperate phages tp310-2 and SA137ruMSSAST121PVL. Following infection of ST398 by phage S1, the lysogenic strain showed enhanced biofilm forming ability compared to the wildtype strain, and the invasion rate of MAC-T cells increased by 10.39%. The minimum inhibitory concentration showed that phage S1 did not change the antibiotic sensitivity of the lysogen strain, and the virulence of the lysogen strain did not change significantly in the injection models of *Galleria mellonella* (*G. mellonella*) and mice. The lysogen demonstrated superinfection immunity and reduced sensitivity to virulent phage infection. Thus, this study contributes to understanding the co-evolutionary relationships between temperate phages and the multi-host zoonotic pathogen *S. aureus* ST398.

Keywords ST398, Temperate phage, Lysogen, Adaptability, Pathogenicity

Introduction

Staphylococcus aureus is a highly pathogenic microorganism of substantial global concern to human and animal health. Infections with *S. aureus* cause various clinical symptoms (skin and soft-tissue infections, purulent inflammation, septicemia, and endocarditis) and foodborne illness (diarrhea and vomiting, etc.) [1]. In the United States, *S. aureus* causes approximately 241,000 foodborne outbreaks each year [2]. In China, 20–25% of foodborne bacterial contaminations are caused by *S. aureus* [3]. Most *S. aureus* isolates are host-specific, however, sequence type (ST) 398 is known to be a multi-host species that can cause animal infections in pigs, cattle, horses, rabbits, poultry and humans among others [4]. This lineage has been detected in Europe, North

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America, Singapore, South Korea, Japan and China, and is increasingly spreading worldwide [5].

Bacteriophages (phages) are predators of bacteria and are widespread in natural environments. They are known to play critical roles in bacterial mortality, biogeochemical cycling and horizontal gene transfer [6]. According to their life cycles, phages can be classified as either virulent or temperate. Virulent phages have attracted increased attention as a substitute for traditional antibiotics because of their significant therapeutic effects in treating multidrug-resistant infections [7]. The use of temperate phages in bacterial therapy has received less attention, even if there are reports of some successful engineering of lysogenic phages into lytic ones with relevant in vivo results, leading to meaningful clinical outcomes [8, 9]. Temperate phages are capable of both lytic and lysogenic cycles. Most temperate phages integrate their genome into the host chromosome after infection and remain lytically dormant (lysogenic cycle). Importantly, after entering the lysogenic cycle temperate phages typically protect the host from further infection by similar phages; this process is known as superinfection immunity [10]. Temperate phages can also participate in horizontal gene transfer by lysogenic conversion, which can lead to phenotypic changes in the host affecting many different traits, including virulence, motility, and interbacterial competition [11, 12].

Temperate phages are a double-edged sword due to their lytic and lysogenic cycles, which can either kill the bacteria or enhance the fitness and virulence of the host. Previous investigations have demonstrated that lysogenic conversion plays a significant role in *S. aureus* adaptability and virulence and contributes to the pathogenesis of staphylococcal infections [13, 14]. There are also studies indicating that temperate phage in combination with antibiotic synergy eradicates bacteria through depletion of lysogens [15]. Moreover, it has been shown that some temperate phages can be transformed into virulent phages by sodium pyrophosphate treatment, and show rapid and long-lasting host cell growth inhibition activity to efficiently control the growth of *S. aureus* in foods [16].

Study of laboratory experiments and natural populations strongly indicate that bacterium-temperate phage coevolution drives microbial communities' ecology and evolutionary processes, increasing the phenotypic and genetic diversity of microbial communities space [17]. Despite the reported benefits of lysogeny to the host in *S. aureus*, epidemiological data on the environmental prevalence of *S. aureus* temperate phages are limited [18], with most studies focusing on prophages induced from the bacteria. New insights could be gained on how to exploit novel antibacterial strategies by investigating and better understanding the influence of temperate phages on bacterial adaptability and pathogenicity. Hence, the isolation and analysis of new temperate phages is needed. In this study, we isolated a novel temperate *S. aureus* phage, vB_SauS_S1 (S1), from dairy farm sewage which can infect the multi-host *S. aureus* ST398 lineage, and compared biofilm forming ability, cell invasion, antibiotic sensitivity, virulence, and lytic phage sensitivity after phage reinfection between the wild type strain and the lysogen. This study contributes to the better understanding of the co-evolutionary relationships between temperate phages and *S. aureus* hosts.

Materials and methods

Bacterial strains and culture conditions

From 2021 to 2023, 135 *S. aureus* strains were recovered from raw milk samples in Jiangsu province, China, and they were subjected to whole genome sequencing (The sample collection was approved by the cow farm and met the requirements of the Animal Ethics Committee of Jiangsu Academy of Agricultural Sciences, and the collection of raw milk samples were based on published studies [19]). The isolates were routinely grown at 37 ℃ in liquid Luria-Bertani (LB), LB medium (1.5% agar) or LB soft agar overlays (0.7% agar) (Qingdao Hope Biotechnology Co., Ltd., Qingdao, China).

Isolation, purification and transmission electron microscopy of phage S1

S. aureus strains belonging to ST398 were used as the primary hosts to isolate phage. Isolation of phages was performed using the previously described method [20]. Thirty milliliter dairy farm sewage was centrifuged at 5000 rpm for 10 min to remove debris pellet and this was repeated 5 times. 3 mL of 1×10^8 CFU/mL ST398 isolate was mixed with 5 mL of the supernatant after centrifugation and incubated at 180 rpm (37℃) for 18 h, respectively. The mixture was centrifuged at 10,000 rpm for 5 min, and filtered with a 0.22 μm filter. Phage were detected by the conventional double-layered agar method. The phage S1 was initially isolated from plaques on a lawn of *S. aureus* ST398 isolate SA-22-53 (53), then was purified eight times from plaques using double-layer agar method as described elsewhere [21]. The purified phage stocks were stored at 4 °C for further analyses.

High titer phage stocks were obtained after ultracentrifugation through CsCl gradient [22]. The purified phages were deposited on a carbon-coated copper grid (Ted Pella Inc., USA) for 15 min, followed by staining using 2% phosphotungstic acid (PTA) and drying. Phage photos were taken using a Zeiss transmission electron microscope (TEM) EM902 (Zeiss, Oberkochen, Germany) at an accelerating voltage of 80 kV.

Thermal and pH stability

For thermostability testing, the phage was incubated at various temperatures (30 °C, 40 °C, 50 °C, 60 °C, 70 °C, and 80 °C) for 30 and 60 min in SM buffer (50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 8.1 mM $MgSO_4$, and 0.01% (w/v) gelatin) and then the phage titer was determined by the double-layer agar method. For pH stability testing, the phages were added to SM buffer, at different pH values ranging from 2 to 12 for 60 min at 37 °C, and then the phage titer was determined by the double-layer agar plate method.

One-step growth curve and adsorption of phage S1

One-step growth experiments were carried out in triplicate by a modification of methods described elsewhere [23]. Briefly, the early-exponential growth host strain 53 and phage S1 suspension were mixed at a multiplicity of infection (MOI) of 0.01 and allowed to adsorb for 15 min at 37 °C. Then, the mixture was centrifuged at 6000 rpm for 5 min at 4 °C to remove supernatant containing unadsorbed phages. The pellet was washed with preheated at 37 ℃ LB broth twice and the supernatant was discarded, followed by incubation at 37 ℃ with constant shaking of 180 rpm. Samples were collected every 10 min for a total of 150 min to measure phage titer by the double-layer agar method. The calculation formula for burst size was: Burst size = the number of liberated phage particles minus the number of unadsorbed phage particles divided by the number of initial bacteria [24]. The tests were repeated three times.

Phage S1 was added to an exponentially growing culture of *S. aureus* 53 at an MOI of 0.01 and incubated at 37 ℃. Aliquots (100 µL) were taken at different time points of 5, 10, 15, 20, 25, and 30 min, respectively and diluted in 0.9 mL of cold LB broth. Following centrifugation at 12,000 rpm for 5 min, then supernatants containing unadsorbed phages were determined by the double-layered agar assay.

Lytic assays and host range analysis of phage S1

The lytic activity of phage S1 on *S. aureus* 53 was assessed by an optical density growth curve assay. The host bacteria of *S. aureus* 53 cultures (1×10^8 CFU/mL) were mixed with the phage S1 at an MOI of 0.01. Two hundred microliters of the mixed cultures were incubated in a 96-well microplate at 37 °C, and the OD_{600} was measured every 1 h for 16 h at the multi-scan spectrum (Infinite® 200 PRO, TECAN). Bacterial growth without the addition of phage was also determined for a period of 16 h. All tests were repeated three times.

The different *S. aureus* isolates were inoculated in LB agar by the double agar overlay assay and 10 µL purified phage solution (1×10^9 PFU/mL) was dropped on the center of the double-layer agar medium. After overnight incubation at 37 ℃, strains in which clear plaques were observed were considered susceptible to phage infection. Each experiment was repeated three times.

Genome sequencing and analysis of phage S1

Phage genomic DNA was extracted using the Norgen Biotek Phage DNA Isolation Kit (Norgen Biotek Corp. Thorold, ON, Canada) according to the manufacturer's instructions. Phage genomic DNA was sequenced by Benagen Biotech Co., Ltd. (Wuhan, China) using Genome Sequencer Illumina NovaSeq platform. High quality reads and reads trimming were obtained by Trimmomatic [25] then, the sequences were assembled by CLC genomic workbench V.12 in a de novo assembly pipeline (https://digitalinsights.qiagen.com). Phage open reading frames (ORFs) were identified by GenemarkS (http://exon.gatech.edu/GeneMark/genemarks.cgi) [26]. All ORFs were aligned by BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Pfam (http://pfam-legacy.xfam.org/) and annotated manually. tRNAs were identified by tRNA Scan-SE (http://lowelab.ucsc.edu/tRNAscan-SE/). Phage schematic map was sketched with the CGView Server (https://proksee.ca/), and linear comparison figures of multiple genomic loci based on BLAST was created by Easyfg_2.2.5_win. Publicly available WGS datasets for *S. aureus* temperate phages were downloaded from GenBank and comparative genomic analysis was undertaken by Mash (v2.1) based on global mutation distances of the whole genome [19]. A subset of 15 genes associated with host cell lysis ($n = 3$), lysogeny ($n = 3$), and virulence ($n = 9$) amongst the 111 *S. aureus* temperate phages (phage S1 and 110 reference *S. aureus* phages) were assembled and aligned using BLASTx as described previously [27].

Generating SA-22-53-L (53^+) lysogens and genome sequencing

Phage S1 (1×10^8 PFU), was spotted on a lawn of 53 on LB medium plates and incubated overnight at 37 °C. Bacterial colonies within the lysis zone were isolated and purified, and tested for the sensitivity of host 53 infection by spot tests. The colonies with phenotypes of resistance to phage infection were PCR amplified using primers (F: 5′-TAAGCGTTGGCAATAAAGCTAAAGGTATT-3′, R: 5′-GTGTATAGGAATATTAACATCCATCCAAATAGTTATT-3′) designed according to phage lyase N-acetylmuramoyl-L-alanine amidase gene to confirm the presence of phage S1 genome in the bacterial chromosome. The primers were generated using SnapGene (version 4.3.6).

The PCR positive strain was identified as lysogen strain SA-22-53-L (53^+). The genomic DNA from the wildtype and the lysogen strains was extracted using a Bacterial DNA Extraction Kit (Magen, Guangzhou, China) according to the manufacturer's instructions. Oxford Nanopore sequencing (ONT) in combination with Illumina

sequencing was used to generate the complete genome sequence of the wildtype and lysogen strains. Sequence reads were assembled and combined using Unicycler (version 0.4.8). Comparative genomic analysis between wildtype and lysogen strains was performed using BLAST Ring Image Generator (BRIG) software [28], genome sequences of wildtype and lysogen strains were annotated by RAST, and phage S1 insertion site was further analysed by SnapGene.

Comparing antimicrobial susceptibility, biofilm formation, and cell invasion assay of wildtype and lysogen strains

The minimum inhibitory concentration (MIC) of each antimicrobial against wildtype and lysogen strains were determined using the broth microdilution method according to that previously described [19], including tetracycline (TET), penicillin (PEN), oxacillin (OXA), ampicillin (AMP), vancomycin (VAN), gentamicin (GEN), ciprofloxacin (CIP), amikacin (AMI), clindamycin (CLI), erythromycin (ERY), chloramphenicol (CHL), moxifloxacin (MOX), streptomycin (STR), sulfamethoxazole (SUL), kanamycin (KAN), and rifampicin (TIF). *S. aureus* ATCC 29,213 was used as a control strain for antimicrobial susceptibility testing.

The quantitative analysis of biofilm formation using crystal violet primary staining method has been described elsewhere previously [29]. Briefly, 20 µl of log phase cultures of wildtype and lysogen strains were added to 1 mL Trypticase Soy Broth (TSB) in 24-well flat-bottom microtiter plates, and incubated at 37 °C for 24 h, respectively. Subsequently, wells were washed with PBS, and 200 µl of 95% (v/v) ethanol was added to each well to fix the biofilms for 30 min. Then 200 µl of 1% (w/v) crystal violet were added to the well for staining for 20 min. Finally, the bound crystal violet was dissolved with 200 µl of 33% (w/v) acetic acid for 15 min and the absorbance measured at 570 nm. All experiments were repeated three times.

Mammary epithelial cells (MAC-T) were used to analyze the invasion ability of wildtype and lysogen strains as previously described [30]. MAC-T cells were cultured overnight in sterilized 24-well plates at the density of 2×10^5 cells in DMEM (containing 10% fetal bovine serum) at 37 °C in a 5% (v/v) CO_2 incubator. Then, the cells were infected with 10 µl of log phase culture wildtype and lysogen strains (2×10^8) and co-incubated for 2 h at 37 °C. Subsequently, nonadherent bacteria were aspirated and discarded, the cells were subsequently incubated with gentamicin (200 µg/mL) and lysostaphin (10 µg/mL) medium for 2 h to kill the adherent bacteria. After washing, MAC-T cells were lysed with 0.5% (v/v) Triton X-100 for 5 min. Bacteria were enumerated from 10-fold serial dilutions of cell lysates using the plate colony-counting method.

Comparing virulence of wildtype and lysogen strains

Overnight cultures of wildtype and lysogen strains were adjusted to 1×10^8 CFU/mL. Groups of 10 *G. mellonella* larvae were injected in the right last proleg inoculated with 10 µl of PBS-washed strains, then incubated at 37 °C. The control group was injected with 10 µl of sterile PBS. The fatalities were recorded every 1 d interval for a total of 7 d.

Overnight cultures of wildtype and lysogen strains were adjusted to 1×10^9 CFU/mL. Groups of 13 BALB/C 6-week-old male mice including 3 mice were used for the bacterial load count, were intraperitoneally injected with 100 µl of PBS-washed strains, then incubated at room temperature. The control group was injected with 100 µl of sterile PBS. The fatalities were recorded every day. Three mice were euthanized after two days of infection. To give maximum welfare to the experimental animals, we chose to anesthetize the experimental mice with sodium pentobarbital at a dose of 0.1 mg/g within 30 min and euthanized them through cervical dislocation. The heart, liver, spleen, and lung were collected and homogenized, and *S. aureus* colonies were counted on chromogenic medium *S. aureus* agar (CHROMagar, Paris, France) after plating 100 µl of the appropriate serial dilutions.

G. mellonella larvae and BALB/C male mice were obtained from Yangzhou University. All experiments were approved by the Chinese guidelines for animal welfare, and the animal protocol was approved by the Animal Welfare Committee of Jiangsu Academy of Agricultural Sciences (Clinical trial number: IACUC-RE-2024-06-007).

Sensitivity test of wildtype and lysogen strains to virulent phages

S. aureus virulent phages JS25 and SD6 were diluted 10-fold serially diluted at the initial concentration of 1×10^8 PFU/mL, 10 µl was spotted on a lawn of wildtype and lysogen strains on LB medium plates and incubated overnight at 37 °C, respectively. The plaques were observed after overnight incubation to evaluate the sensitivity of virulent phages to wildtype and lysogen strains.

In addition, we also observed the growth curve of bacteria in mixed cultures of phages to reflect the effect of virulent phages on wildtype and lysogen strains. Briefly, the phage S1 was mixed with wildtype and lysogen strains at an MOI of 10, respectively. Two hundred microliters of mixed cultures were incubated in 96-well microplate and the microplate was placed in the multiscan spectrum (Infinite® 200 PRO, TECAN) at 37 °C, and OD_{600} was measured every 1 h to reflect the sensitivity of wildtype and lysogen strains to virulent phages for a total of 15 h. All tests were repeated three times.

Table 1 Primers used in this study

Primer	Sequence (5′–3′)
gyrB-RT-F	ACATTACAGCAGCGTATTAG
gyrB-RT-R	CTCATAGTGATAGGAGTCTTCT
icaA-RT-F	GTTGGTATCCGACAGTATA
icaA-RT-R	CACCTTTCTTACGTTTTAATG
icaR-RT-F	TGCTTTCAAATACCAACTTTCAAGA
icaR-RT-R	ACGTTCAATTATCTAATACGCCTGA
clfA-RT-F	CAGCGATTCAGAATCAGA
clfA-RT-R	GGCGGAACTACATTATTG
clfB-RT-F	CTGAGTCACTGTCTGAATC
clfB-RT-R	CTCAGACAGCGATTCAGA
fnbA-RT-F	TTCCTTAACTACCTCTTCT
fnbA-RT-R	CAATCATATAACGCAACAG
fnbB-RT-F	GCGAAGTTTCTACTTTTG
fnbB-RT-R	CAACCATCACAATCAACA

RNA extraction and adhesion gene expression of wildtype and lysogen strains

Total RNA was extracted from both wild-type and lysogenic strains using the HiPure Bacterial RNA Kit (Magen, Guangzhou, China) according to the manufacturer's instructions. Then, 300 ng total RNA was reverse transcribed into cDNA using HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, China). The expression levels of six genes (*icaA*, *icaR*, *clfA*, *clfB*, *fnbA*, and *fnbB*) associated with adhesion were determined by RT-qPCR. The primers have been described previously [31, 32] and are presented in Table 1, with *gyrB* as the endogenous gene. Relative gene expression levels were analyzed using the formula $2^{-(\Delta\Delta Ct)}$.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (Version 9.01; GraphPad, San Diego, CA, USA). $P < 0.05$ indicates the determination of a significant difference. The significance of the experimental data was evaluated by multiple t-tests.

Results

Phage S1 morphology and onestep growth curve analysis

The plaque diameter of bacteriophage S1 on the double-layer plate was about 0.4 mm, and the plaque was translucid with a clear boundary and no halo (Fig. 1A). Morphological analysis using Transmission Electron Microscopy (TEM) demonstrated that phage S1 had a prolate head of about 80 ± 2 nm that was connected to a long noncontractile tail of about 220 nm (Fig. 1B). The overall morphology indicated that phage S1 was a Siphovirus of the "A" type [33].

The one-step growth curve of the phage S1 at an MOI of 0.01 showed that the latent period was 30 min and the rise phase 60 min. The average burst size was 41 ± 2 plaque-forming units (PFU) per host cell (Fig. 2A).

The stability of phage S1 in response to temperature and pH

The activity of phage S1 showed no change after incubation at 30 ℃, 40 ℃, and 50 ℃. However, the phage titer decreased by 4.5 $\log_{10}$ PFU after incubation at 60 ℃ for 30 min and was completely inactivated at 60 ℃ for 60 min (Fig. 2B).

S1 was relatively stable at pH 3–10 and only declined slightly (0.8 $\log_{10}$ PFU) at pH 11. The phage titer

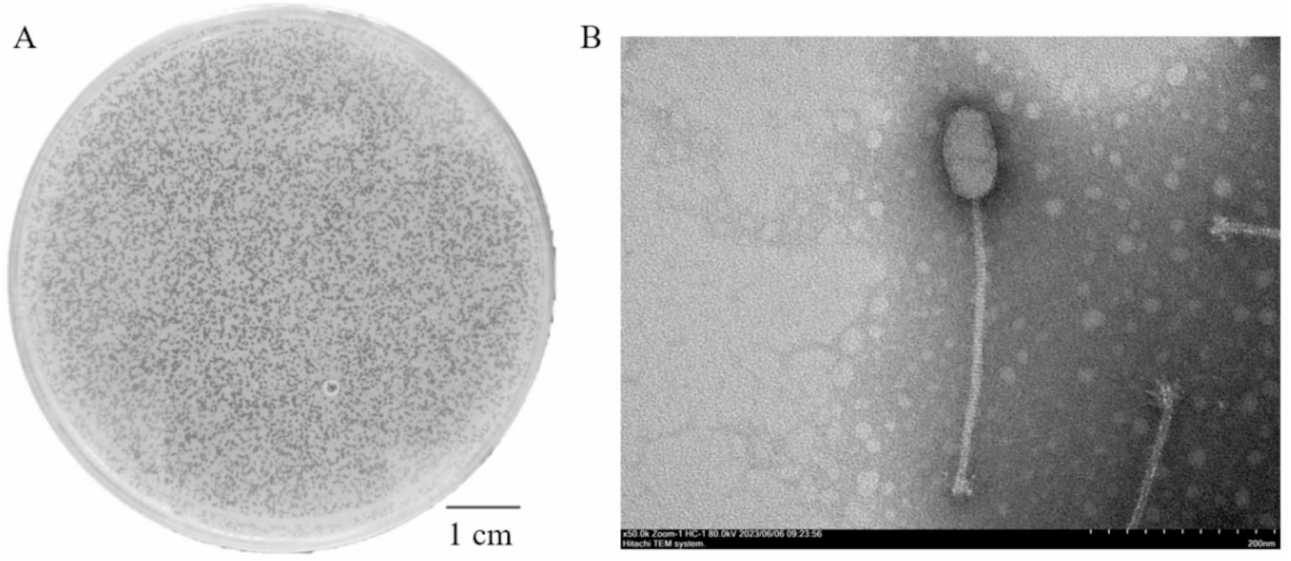

Fig. 1 Phage S1 on double layer plate and transmission electron microscopy. (**A**). Plaque of phage S1 on a double layer plate. (**B**)The morphology of phage S1 as revealed by transmission electron microscopy

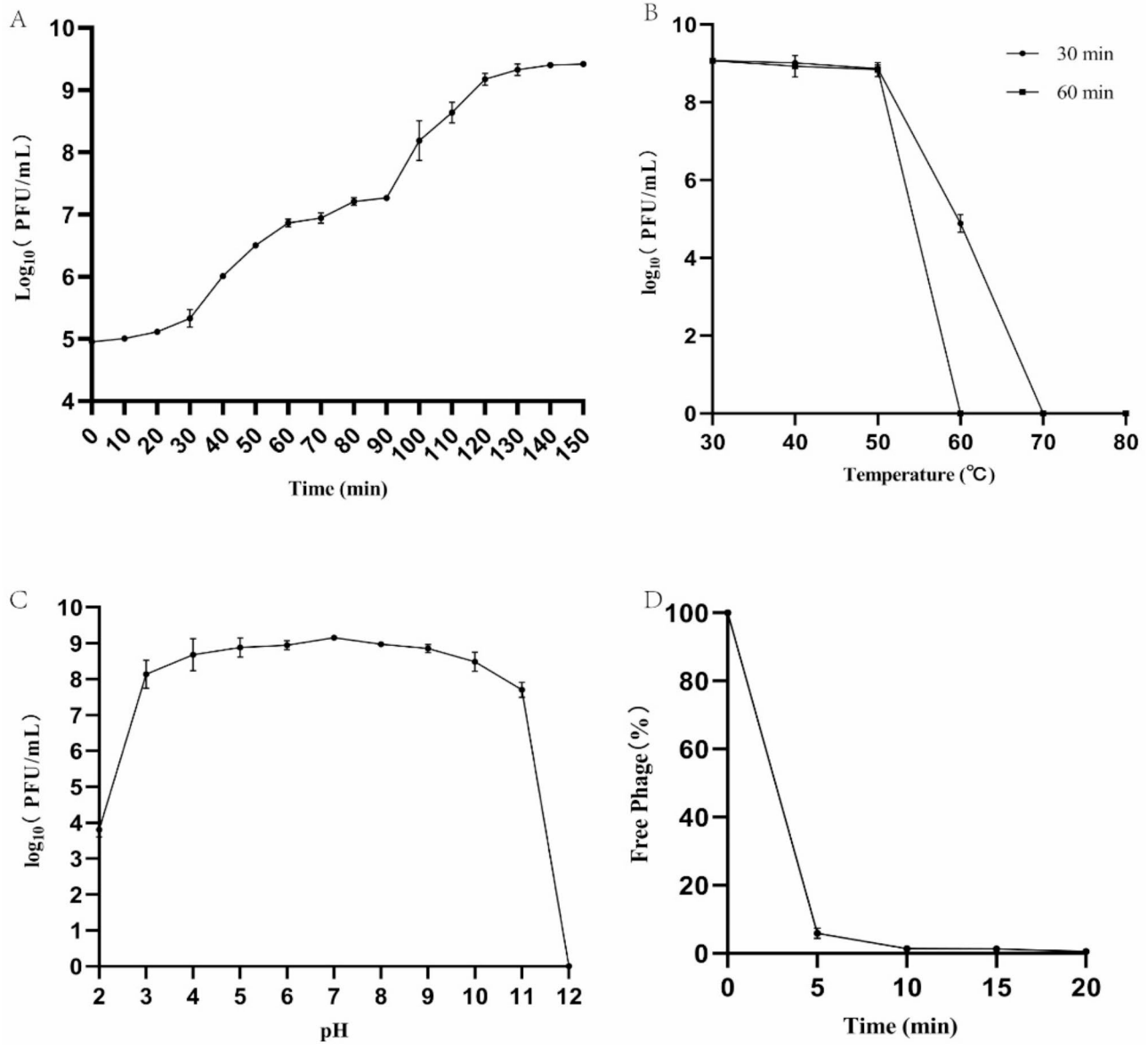

Fig. 2 Phenotypical characterization of phage S1. (**A**) The one-step growth curve. (**B**) Thermal stability. (**C**) pH stability. (**D**) Adsorption of phage S1

decreased dramatically (4.3 $\log_{10}$ PFU) at pH 2, and no viable phages were detected at pH 12 (Fig. 2C).

The adsorption and lytic activity of phage S1

Cell adsorption measurements showed that the percentage of free phages decreased to 5% at 5 min, and less than 1.5% at 10 min, indicating that 95% of S1 phages adsorb to host cells after 5 min (time of adsorption) (Fig. 2D).

The in vitro lysis assay for phage S1 on the host was determined at an MOI of 0.01. The control culture without any phage showed a typical log shaped growth, entering stationary phase at about 6 h post inoculation. The culture with phage S1 showed no growth of *S. aureus* 53 for 7 h, then the OD_{600} value started to increase (Fig. 3).

Phage S1 host range

Phage S1 formed plaques on 52/134 (38.8%) of the *S. aureus* isolates tested (Fig. 4). Phage S1 was active against most ST398 and ST50 strains, and was somewhat active against ST1, ST97, and ST8086 strains, with limited activity against other ST's (although fewer of these isolates were tested) (Fig. 4).

Phage S1 comparative genomic analysis

Genomic characterization of phage is a crucial step in understanding phage biology; hence, the isolated phage genome was analyzed in detail. Briefly, the isolated phage S1 (Accession number: LC779550) has a linear double stranded DNA genome with 46,366 bp and a G + C content of 34.07% (Fig. 5). Bioinformatics analysis revealed

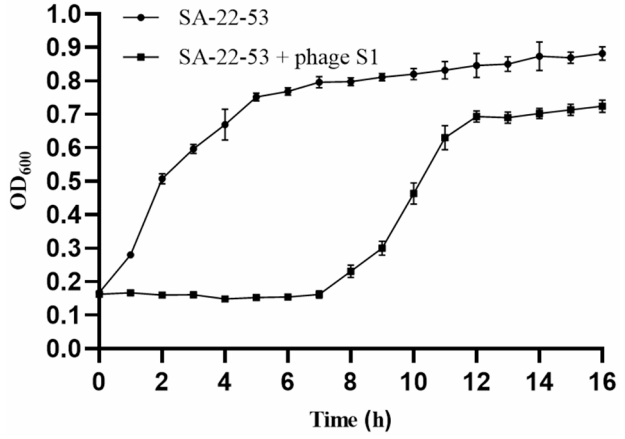

Fig. 3 The inhibitory effect of phage S1 against SA-22-53 at an MOI of 0.01

67 putative open reading frames (ORFs) and most of these (57%) encoded hypothetical proteins. The predicted ORFs with classified functional characteristics included those involved in replication/regulation, DNA packaging, structure/morphogenesis, lysis, and lysogeny. The integrase and transcriptional activator RinB are associated with integrative recombination of temperate phages. In addition, there were no tRNA nor antibiotic resistance genes predicted in the genome of phage S1. PVL leukocidin-associated protein (ORF24), Virulence associated (ORF27), and Virulence-associated E family protein (ORF38) were the major predicted virulence-associated proteins.

To explore the evolutionary relationships between phage S1 and other *S. aureus* temperate phages, evolutionary trees were constructed based on global mutation distances of whole genomes using Mash. The tree showed that phage S1 was closely related to the temperate phages tp310-2 (NC_009762) with genomic size of 47,785 bp, SA137ruMSSAST121PVL (NC_055043) with genomic size of 45,999 bp (Fig. 6). The integrase and transcriptional activator RinB existed in almost all temperate phages by BLASTx analysis (Fig. 6). Phage tp310-2 had the highest identity to S1 (97.64%) based on an 88% query coverage. This analysis suggests phage S1 lies within the *Triavirus* genus of the *Caudoviridae*. The similarity of phage S1 with four closely related phages is shown in Fig. 7.

Lysogenicity of phage S1

Spot tests showed that phage S1 could infect the wild-type strain 53 but could not infect the 53^{+} lysogen strain (Fig. 8A). To confirm the presence of the S1 phage genome in *S. aureus* lysogens, specific primers of phage lyase N-acetylmuramoyl-L-alanine amidase gene were designed for the detection of S1 phage genomic DNA. PCR results showed that the genome of the lysogen strain 53^{+} contained the phage S1 genome (Fig. 8B). ONT sequencing showed that phage S1 was integrated into the strain 53 genome between two lipase precursor open reading frames (Fig. 8C).

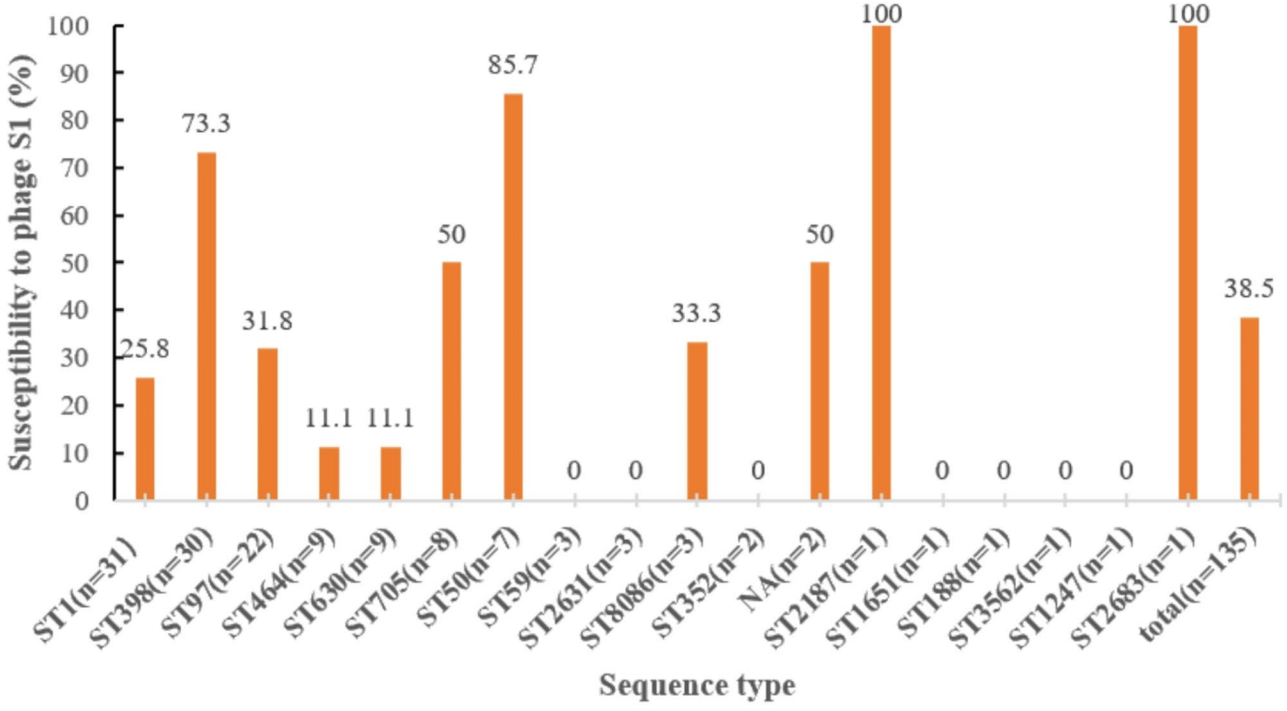

Fig. 4 Host range of phage S1 on 135 clinically isolated *Staphylococcus aureus* strains belonging to various ST types

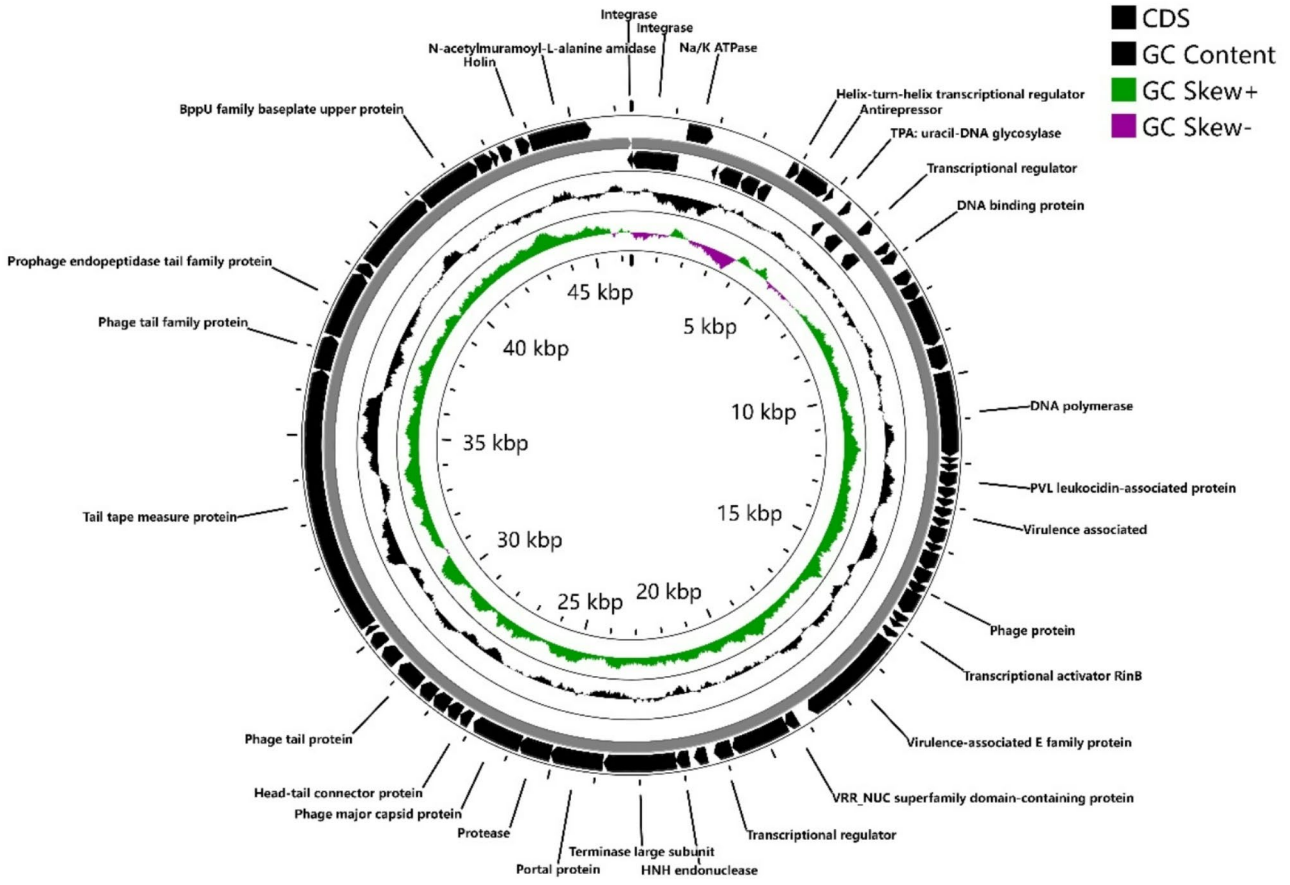

Fig. 5 Schematic representation of the phage S1 genome. Display (from the outside): (1) ORFs transcribed in the clockwise or the counterclockwise direction. (2) G + C % content. (3) GC skew

Effect of prophage S1 on host biofilm formation and invasion of MAC-T cells

Biofilm formation was enhanced in the lysogen strain compared with the wildtype strain (OD_{570} 0.61 to 0.78; $P < 0.01$**) (Fig. 9A). Comparison of the invasion rate of the lysogenic strain with the wildtype strain in bovine mammary epithelial cells (MAC-T) showed an increase of 10.39% ($P < 0.05$*) (Fig. 9B). The expression of six adhesion related genes (*icaA*, *icaR*, *clfA*, *clfB*, *fnbA*, and *fnbB*) was measured to study the influence of phage S1 integration. RT-qPCR data revealed that only the *clfA* expression level was significantly up-regulated in the lysogen strain compared with the wildtype strain ($P < 0.001$***) (Fig. 9C).

Effect of prophage S1 on host antibiotic sensitivity and pathogenicity

The MIC of the wildtype strain and lysogen strain were compared for 16 antibiotics. The data showed the MIC of wildtype and lysogen strains did not change (Table 2).

There were no obvious differences in morbidity and mortality observed when wildtype and lysogen strains were injected into model organisms of *G. mellonella* and mice. Mice injected with wildtype and lysogen strains were euthanized after 2 days and the bacterial load measured in the heart, liver, spleen, and lungs to be $\sim 10^5$ CFU/g, with no significant differences between wildtype and lysogen strains (Fig. 10).

Effect of prophage S1 on host sensitivity to virulent phages

The lysogen strain showed reduced sensitivity to virulent phage infection compared with the wildtype strain on solid media (Figs. 11A and B). The growth rate of the wildtype strain was also lower than that of the lysogen strain when the virulent phages JS25 and SD6 were mixed with the hosts for 10 h in liquid media (Figs. 11C and D). Results indicated that lysogen strain shows a reduced sensitivity to virulent phages.

Discussion

The *S. aureus* lineage ST398 has been shown to be highly transmissible between species, establishing itself as a successful pathogen in numerous environments and countries over the globe. There is increasing evidence about the reciprocal selection of bacteria on phage populations, and phages on the diversity of bacterial populations, influencing bacterial virulence, increasing bacterial

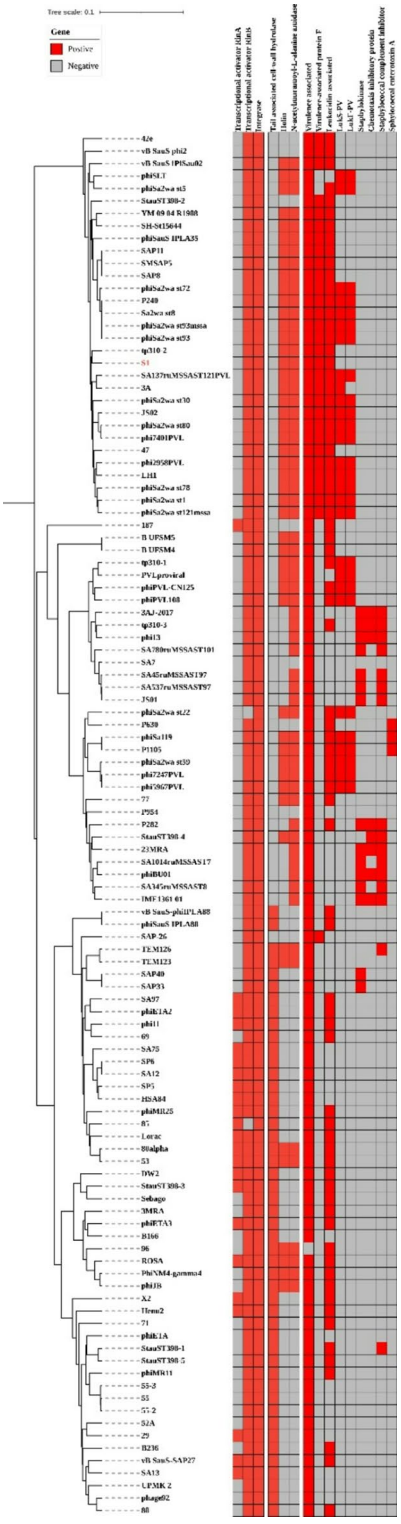

Fig. 6 Heat map showing the distribution of lysis, lysogeny, and virulence genes in 111 *Staphylococcus aureus* temperate phages. The phylogenetic trees were constructed based on the global mutation distance of the whole genome using Mash (v2.1), and overlayed with information of heat map using ITOL

evolvability, and even shaping the stability of ecosystems [17]. It is reported that *S. aureus* ST398 may be an increasing threat to public health due to the acquisition of prophages encoding virulence and antibiotic resistance genes [34].

In this study, we successfully isolated a temperate phage from dairy farm sewage using clinical *S. aureus* ST398 strains as an indicator. We demonstrate that phage S1 is a novel temperate phage based on genotype analysis and phenotypic verification. Morphologically, S1 is a Siphovirus with a rarely reported prolate capsid. Phylogenetically, S1 is a new member of the *Triavirus* genus of the *Caudoviridae*. The S1 genome size is 46,366 bp, which is consistent with other *S. aureus* temperate phages which typically have genomes of 40 ~ 45 kb [35], and include an integrase and transcriptional activator RinB. The integrase is normally present in other temperate phages [20], and transcriptional activators RinA and RinB are usually both required for expression of the *int* gene which is responsible for integrative recombination (e.g. in *phi*11 [36]). A few studies have characterized *Triaviruses* of *S. aureus*, and the majority of them are temperate phages. For example, phages B_UFSM4 and B_UFSM5 were experimentally characterized and proven to be a temperate phage by Bibiana et al. [37].

The temperate phage typically exhibits superinfection immunity after lysogen, which makes them insensitive to prophages [38]. The host range of phage S1 on 135 *S. aureus* isolates from raw milk revealed phage S1 could infect 38.5% (52/134), with ST398 (22/30) and ST50 (6/7) notably more susceptible compared to other STs, though some STs had fewer representatives tested. This may be due to the fact that ST398 and ST50 genomes do not contain S1 homologous prophage, which increases the interaction between phage S1 and these STs of *S. aureus*. Further host range testing of additional reference isolates of each of the STs may be able to definitively determine if there is a link between ST and S1 phage susceptibility in *S. aureus* but this was beyond the resources of the project. Evaluation of phage antibacterial activity is crucial for assessing potential bio-control effectiveness. Phage S1 completely inhibited the growth of *S. aureus* 53 for 7 h. Although phage S1 showed efficient antibacterial activity, it has virulence related ORF [PVL leukocidin-associated protein (ORF24), Virulence associated (ORF27), and Virulence-associated E family protein (ORF38)] and can lysogenize into host cell genome as a temperate phage, which may limit its utility as a biocontrol agent.

Bacteria can obtain new genetic traits and environmental adaptability through acquisition of temperate phage genetic material, indicating that phage genetic diversity affects the evolutionary direction of bacteria [39]. Therefore, we evaluated the effect of prophage S1 on the adaptability and pathogenicity of the host bacteria. Previous

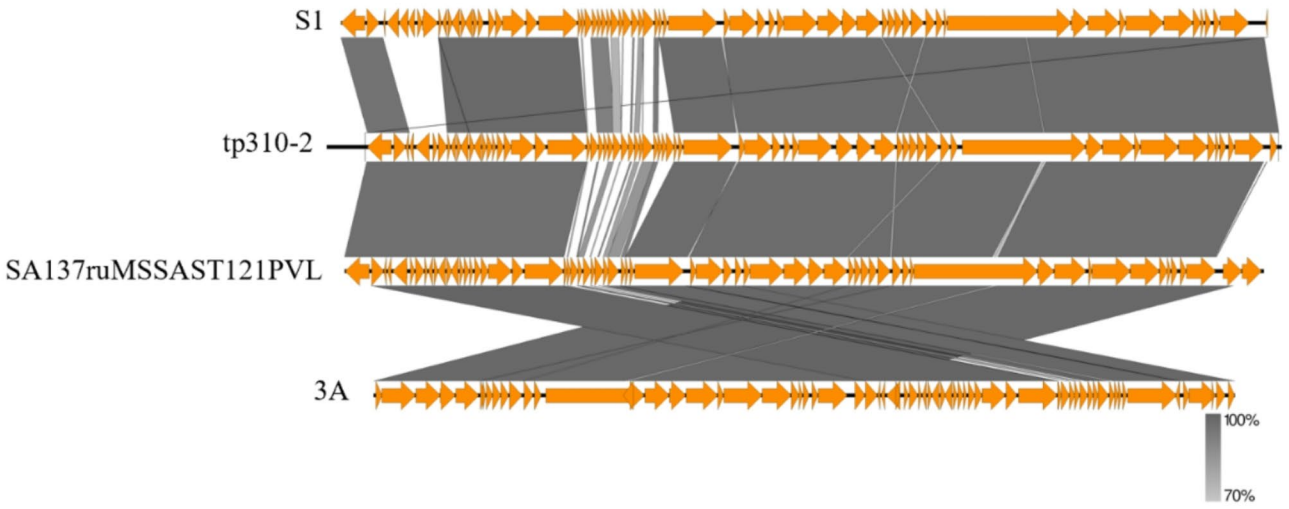

Fig. 7 Comparison of the genome of phage S1 with the closest related *Staphylococcus aureus* temperate phages (phage tp310-2, SA137ruMSSAST-121PVL, 3 A) at the nucleotide level

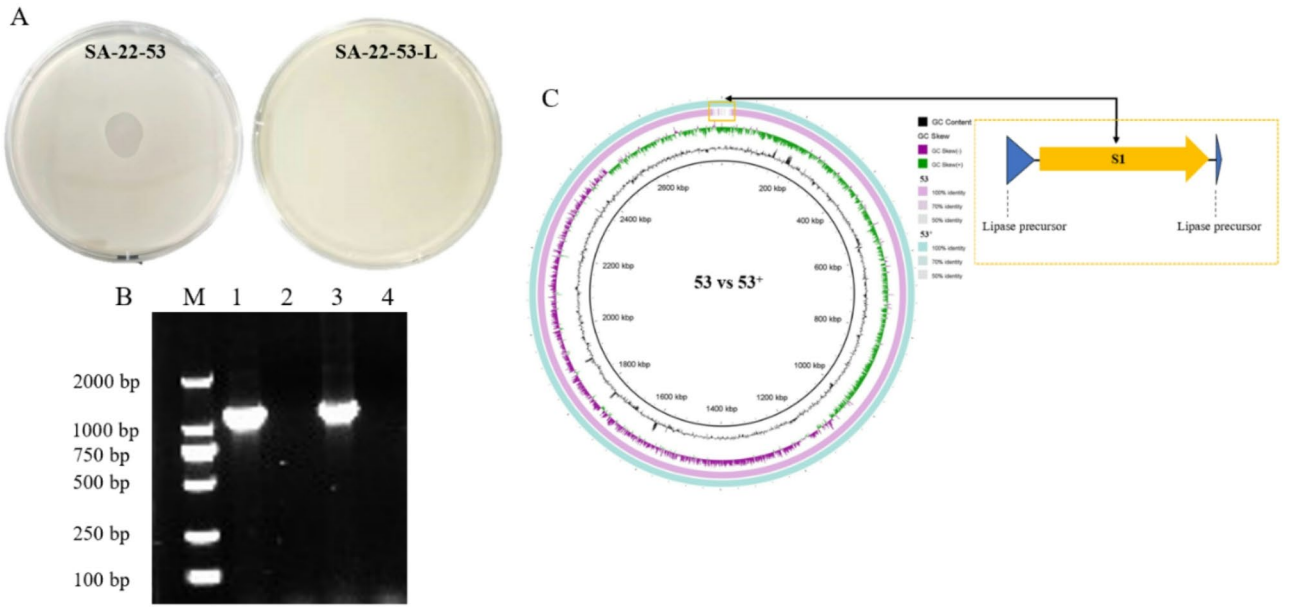

Fig. 8 Characterization of phage S1 lysogen *Staphylococcus aureus* strain and determination of integration site. (**A**). Infectivity of the temperate phage S1 against wildtype strain 53 and lysogen strain 53^+; (**B**). PCR analysis of 53^+ lysogen strain genome containing prophage S1, Note: M. Marker; (1) Phage S1; (2) wildtype 53; (3) lysogen 53^+; (4) Negative; (**C**). Comparative genome analysis of wildtype 53 and lysogen 53^+ strains, showing S1 integration site

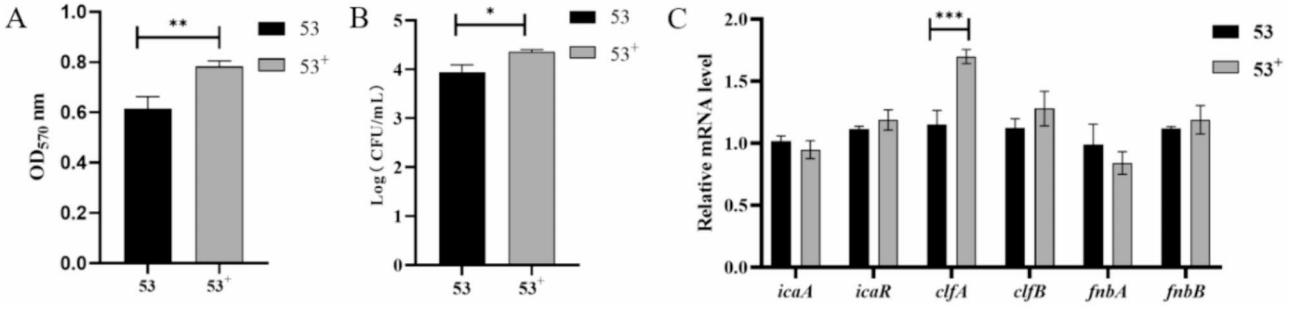

Fig. 9 Biofilm, adhesion, and invasion characteristics of 53 wildtype and 53$^+$ lysogen *Staphylococcus aureus* strains. (**A**). Biofilm formation of wildtype 53 and lysogen 53$^+$ strains; (**B**). Invasion capacity of wildtype 53 and lysogen 53$^+$ strains into MAC-T cells; (**C**). mRNA expression levels of adhesion related genes in wildtype 53 and lysogen 53$^+$ strains. Note: *($P<0.05$), ** ($P<0.01$) and *** ($P<0.001$) indicates extremely significant difference

Table 2 MIC distribution of wildtype 53 and lysogen 53$^+$ strains

Antibiotic	53 MIC(μg/mL)	53 + MIC(μg/mL)
TET	< 0.06	< 0.06
PEN	32	32
OXA	0.25	0.25
VAN	0.5	0.5
GEN	16	16
CIP	16	16
AMI	1	1
CLI	0.25	0.25
ERY	< 0.06	< 0.06
CHI	2	2
STR	32	32
KAN	16	8
AMP	16	16
SUL	64	64
MOX	4	4
RIF	0.25	0.25

studies have shown that temperate phages enhanced the biofilm formation and cell adhesion of host bacteria, even without the presence of virulence genes [30, 40, 41, 42], which is consistent with our results. We also demonstrated an increase in the invasion of MAC-T cells with lysogens carrying S1 compared to WT cells. However, this is not always the case, Chen et al. found that temperate phage PHB09 lysogen reduced the adhesion and invasion of lysogen strain to HEp-2 cells, and inferred that the phage lysogen may interfere with the expression of fimbriae protein at the insertion site, thereby reducing the adhesion and invasion to cells [43]. Previous studies have shown that prophage *phiv*142-3 encodes adhesion related genes which are inserted into bacterial adhesion related regions, and regulate their transcription level, thereby affecting the bacterial adhesion phenotype [44]. However, RT-qPCR data did not show significant differences in the expression of bacterial adhesion genes between wildtype and lysogen strains in this study, which may be related to the insertion site of temperate phage S1 in the two lipase precursors. It also suggested that bacterial biofilm formation and cell invasion may not be solely increased by regulating the expression of related genes [45].

Previous studies have reported that temperate phage can alter host bacteria virulence [40]. However, in this study, the S1 prophage did not significantly change the virulence of host bacteria in the virulence models of *G. mellonella* and mice, and also indicated that the pathogenicity of *S. aureus* ST398 isolate SA-22-53 was relatively low. Compared with the wildtype strain, the lysogen was not only insensitive to S1 reinfection but also less sensitive to infection by other virulent phages, which makes the phage S1 lysogen enhance the immunity of the lysogen strain to the related phage infection, to regulate the steady state of the flora. Lucie Kuntová et al. showed that a *S. aureus* temperate phage encoded membrane-anchored protein *PdpSau* which mediated tolerance to virulent phages [46]. The pathway of temperate phage S1 which mediates phage tolerance remains to be further studied. This study is consistent with previous studies that found no antibiotic resistance genes were encoded in the *S. aureus* phage genome. The prophage S1 did not change the sensitivity of the lysogen strain to antibiotics. On the whole, the data contributes to track of the co-evolutionary relationships between temperate phages and the multi-host zoonotic pathogen *S. aureus* ST398.

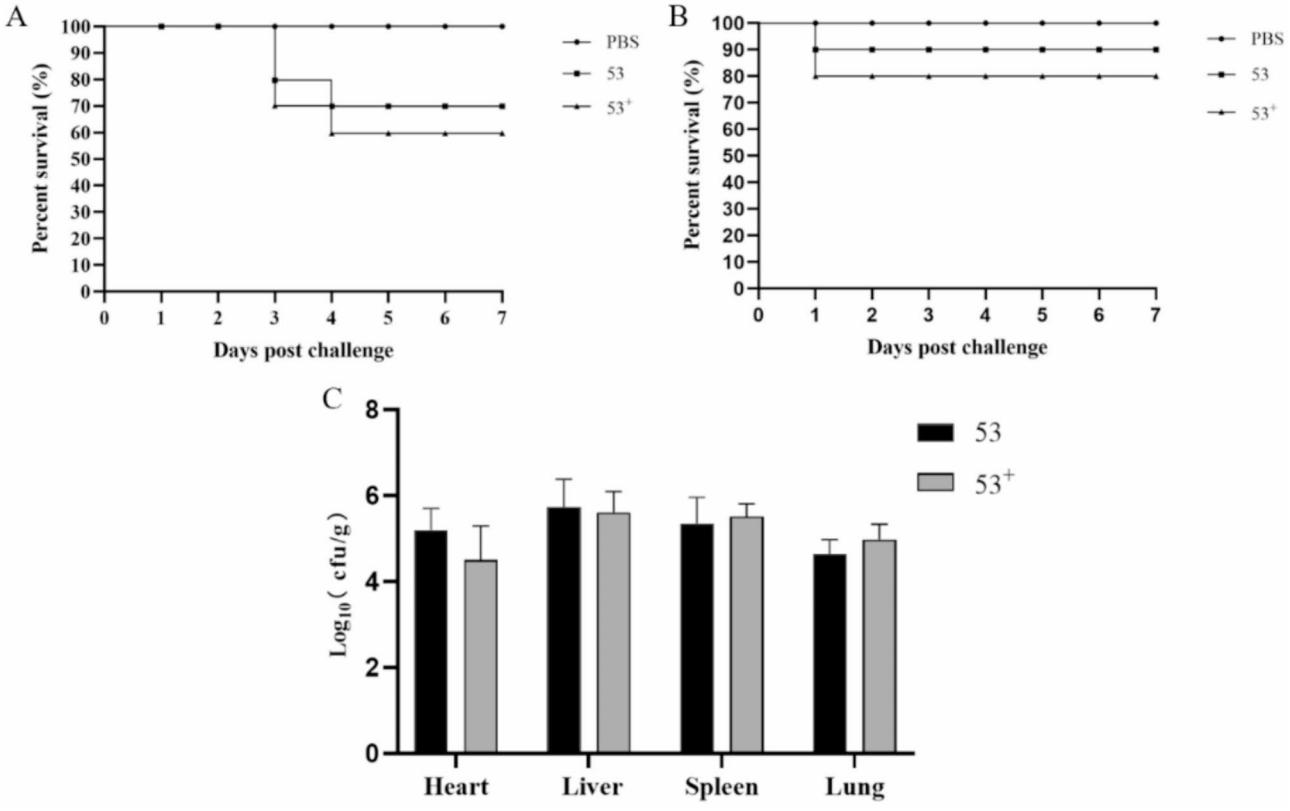

Fig. 10 Effect of the phage S1 lysogen on virulence of *Staphylococcus aureus* ST398 strains. (**A**). Survival curve of *Galleria mellonella* after intraperitoneal injection of wildtype 53 and lysogen 53^{+}strains; (**B**). Survival curve of mice after intraperitoneal injection of wildtype 53 and lysogen 53^{+} strains; (**C**). Bacterial colonization of mice organs

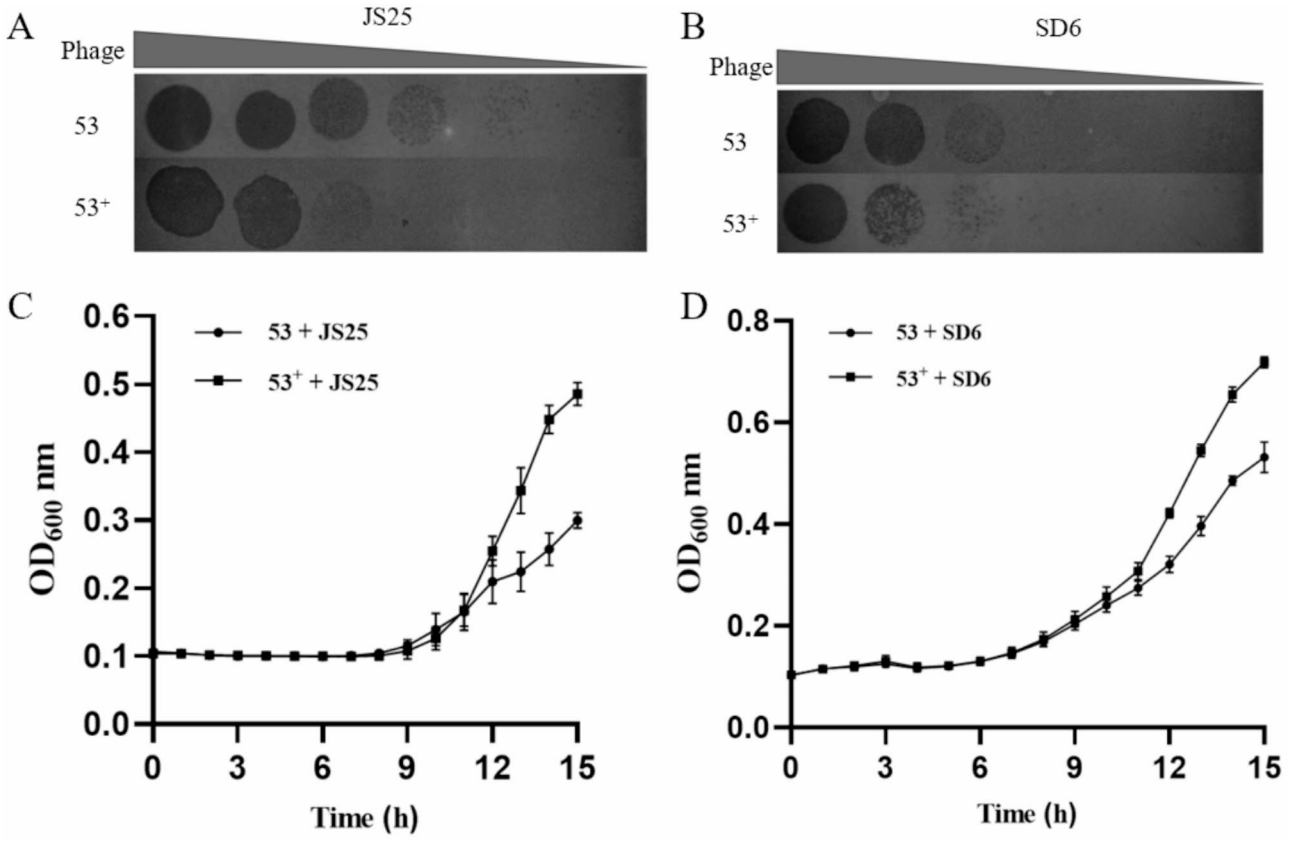

Fig. 11 Effect of virulent phages on wildtype 53 and lysogen 53^+ strains. (**A**). JS25 10-fold dilution spot-on lawn; (**B**). SD6 10-fold dilution spot-on lawn; (**C**). Bacterial growth curves of wildtype 53 and lysogen 53^+ strains with virulent phage JS25; (**D**). Bacterial growth curves of wildtype 53 and lysogen 53^+ strains with virulent phage SD6

Conclusion

In this study, the biological and genomic characteristics of *S. aureus* ST398 temperate phage S1 were evaluated. Morphologically, S1 had an uncommon prolate head and was genomically assigned to the *Triavirus* genus of the *Caudoviridae*. Compared to the wildtype host, S1 lysogens showed stronger biofilm formation and increased MAC-T invasion but did not significantly change the sensitivity to antibiotics or pathogenicity in a *G. mellonella* and a systemic murine infection model. However, the lysogen did show superinfection immunity and reduced the sensitivity to virulent phages. Thus, this study contributes to a better comprehension of the co-evolutionary relationships between temperate phages and the multi-host zoonotic pathogen *S. aureus* ST398.

Author contributions

LLZ and HYW conceived and designed the study. HL performed the experiments. JX, HCS and ASD analysed the data. HL wrote the paper and CB, XH and RW edited and commented on the paper. All authors reviewed and approved the final manuscript.

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Data availability

Data is provided within the manuscript. The genomic sequence of phage vB_SauS_S1 has been submitted to the NCBI Gen Bank database under the accession number LC779550.

Declarations

Ethics approval and consent to participate

This study was performed following the Chinese guidelines for animal welfare, and the animal protocol was approved by the Animal Welfare Committee of Jiangsu Academy of Agricultural Sciences (permit number: IACUC-RE-2024-06-007). The study was conducted in accordance with the local legislation and institutional requirements.

Consent for publication

Not Applicable.

Competing interests

The authors declare no competing interests.

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