Molecular Characterization and Genomic Analysis of Novel Phage vB_ ShiP-A7 Infecting Multidrug-Resistant Shiglla Fexneri and Escherichia Coli

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Research article

Keywords: bacteriophage vB_ShiP-A7, genome sequence, multi-drug resistance, Mass Spectrometry, comparative genome

Posted Date: October 26th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-96088/v1

Abstract

Background

Phage therapy has regained more attention due to the rise of multidrug-resistant (MDR) bacteria. Several case reports demonstrated clinical application of phage in resolving infections caused by MDR bacteria in recent years.

Results

We isolated a new phage, vB_ShiP-A7, and then investigated its characteristics. Phage vB_ShiP-A7 is a member of *Podoviridae* that has an icosahedral spherical head and a short tail. vB_ShiP-A7 has large burst size and short replication time. vB_ShiP-A7's genome is linear double stranded DNA composed of 40058 bp, encoding forty-three putative open reading frames. Comparative genome analysis demonstrated vB_ShiP-A7's genome sequence is closely related to fifteen different phages (coverage 74-88%, identity 86-93%). Mass Spectrometry analysis revealed that twelve known proteins and six hypothetical proteins exist in particles of vB_ShiP-A7. Genome and proteome analyses confirmed the absence of lysogen-related proteins and toxic proteins in this phage. In addition, phage vB_ShiP-A7 can significantly reduce the growth of clinical MDR stains of *Shigella flexneri* and *Escherichia coli* in liquid culture. Furthermore, vB_ShiP-A7 can disrupt biofilms formed by *Shigella flexneri* or *Escherichia coli in vitr*o.

Conclusion

Phage vB_ShiP-A7 is a stable novel phage, which has a strong application potential to inhibit MDR stains of *Shigella flexneri* and *Escherichia coli.* Comparing the genomes between vB_ShiP-A7 and other closely-related phages will help us better understand the evolutionary mechanism of phages.

Introduction

Growing level of multidrug resistance (MDR) bacteria have been reported, and the emergence of these MDR bacteria leads to serious systemic and biofilm-associated infections [1,2,3]. For example, infection caused by MDR *Enterobacteriaceae*, especially the β-lactam resistance *Enterobacteriaceae*, is too hard to be treated [4]. *Shigella* species and *Escherichia coli* (*E. coli*) are major members of *Enterobacteriaceae*, which are important enteric pathogens [5,6]. In the era of the antibiotic crisis, bacteriophages have been studied as alternatives biocontrol agents for these members of *Enterobacteriaceae* [7].

Phage was first used to treat dysentery caused by *Shigella* in 1920s [8]. After that, phages have long been used to treat dysentery in Eastern Europe [9]. In the 21st century, phages against MDR of *Shigella* have been investigated widely [10,11]. *Shigella* phage was successful applied to food safety [12]. Soffer *et al*. characterized five lytic bacteriophages and combined them as a cocktail ShigaShield™, which could inhibit the growth of *Shigellasonnei* in food, in the process of FDA and USDA assessment for the GRAS

status (GRN672) [13]. In mouse model, Mai *et al*. prepared a new reagent including phage cocktail and an antibiotic (ampicillin) named ShigActive™, which can inhibit *Shigella* effectively [14]. Bernasconi *et al.* proved three commercially available bacteriophage cocktails could suppress *Shigella* infections in human intestinal [15]. A cocktail including six lytic phages can inhibit *Escherichia coli* growth successfully [16]. A variety of mixed reagents including phages have been widely studied in the prevention and treatment of infections caused by *Shigella* species and *E. coli* in humans, suggesting that phages are promising for the treatment of infections caused by these members of *Enterobacteriaceae*.

Phage therapy needs appropriate lytic phages for different MDR bacteria. It has been shown that phage cocktail therapy can overcome the limitation of narrow host range of phage and phage resistance to bacteria [7, 17]. Cocktails of well-known lytic phages might open new perspectives for successful controling MDR bacteria. A comprehensive study of each phage is also required to avoid phage-encoded toxic proteins or lysogen-related proteins. Therefore, isolating and well-characterizing more phages will allow us to get enough stock phages for selecting against different MDR clinical bacterial strains.

Phages have been isolated from different environmental sources and fecal samples of humans and other animals [18,19,20,21,22,23,24]. In this study, we isolated a lytic phage named vB_ShiP-A7 using MDR *Shigella flexneri* as host from waste water in Nanjing, China. In addition, phage vB_ShiP-A7 can infect several clinically isolated MDR *E. coli* stains. Thus, this phage may be used to monitor, diagnose, and control infection caused by *Shigella flexneri* and *E. coli*.

Materials And Methods

Bacterial strains

All the bacterial strains used in this study were all grown in Luria–Bertani (LB) medium at 37°C (Table 1). *E. coli* wild-type strain MG1655 was a stock of our lab. *Shigella flexneri* A7, *Shigella sonnei* A5 and twenty-nine clinical stains of *E. coli* were isolated and cultured from different specimens of patients of the First Affiliated Hospital of Nanjing Medical University, Nanjing, China. *Shigella flexneri* A7 was deposited in China Center for Type Culture Collection (CCTCC Number is PB 2020012) in Wuhan, China.

Isolation and propagation of bacteriophages

vB_ShiP-A7 was screened from waste water in Nanjing (China) using multi-drug resistant *Shigella flexneri* A7 (*S. flexneri* A7) as host. Waste water samples were filtered through filters (0.45 μm Millipore, USA) first. Then the filtered liquid was added to early-log-phase culture of *S. flexneri* A7 and at cultured at 37°C for 4 hours to enrich phages. The cultures were spun down to removed bacterial cells. 10 μl supernatant, 100 μl *S. flexneri* A7 and 3ml melted top agar mixed up well, then the mixture was poured on the surface of LB plate. After cultured at 37°C about 12 h, plaques formed on the plates. Single clear plaque was selected to start new round of screening. After several round screening, plaques were homogeneous on double layer agar plate. The preliminary purified phage from single plaque was got and kept at 4°C.

Purification of Bacteriophage vB_ShiP-A7

Bacteriophage vB_ShiP-A7 was purified following the protocol of Yu's [25]. Briefly, phage vB_ShiP-A7 were added into the early-log-phase liquid culture of *S. flexneri* A7. Incubated at 37°C for another 2 h, the culture medium was spun down. The supernatant was collected and passed through filters (0.45-μm). The filtrate was concentrated by ultrahigh speed centrifugation. The supernatant was removed and the pellet was resuspended in SM buffer (10mM Tris–HCl, pH 7.5;100mM NaCl; 10mM $MgSO_4$). Further separation of the suspension by cesium chloride gradient ultrahigh speed centrifugation. We collected phage zone about 1 ml and diluted 10 times in SM buffer. Then the sample was participated at 200,000g for 3 hours to remove CsCl. The pellet was resolved in SM buffer, which is the purified phage particles of vB_ShiP-A7.

Electron microscopy

A drop of ultracentrifuge purified Phage vB_ShiP-A7 particles was dripped onto a copper grid. The phages on the copper grid were negatively stained using 2% (w/v) phosphotungstic acid. The morphology of phage vB_ShiP-A7 were observed using FEI Tecnai G2 Spirit Bio TWIN transmission electron microscope at 80 kV.

Analysis of the phage host range

Infection ability of phage vB_ShiP-A7 on different strains were using the standard spot tests [26]. 100 μl of log-phage bacterial culture of each strain was mixed up with 3 ml of melted soft agar (0.6% agar), which was poured on top of LB plate. After we prepared different concentration of vB_ShiP-A7 phage suspensions (10^{10}–10^2 pfu/ml), 5 μl of each concentration of the phage suspension were dropped onto the surface of the solidified plates containing different tested strains. After overnight cultured at 37 °C, the inhibition of bacterial growth by different concentration of vB_ShiP-A7 on each plate reflected the strain's sensitivity to vB_ShiP-A7. All experiments were conducted in accordance with the ethical rules of Nanjing Medical University (Nanjing, China) and the First Affiliated Hospital of Nanjing Medical University, and informed consents were obtained from all the patients.

Temperature Stability

The thermal stability of phage vB_ShiP-A7 was determined under different temperature. Five test tubes containing 10^9 phages were immersed in different temperature water bath about 1 hour (4°C, 25°C ,37°C, 45°C, 50°C). Then the phage titers of all the samples were determined by the double-layer method. Three independent repeated experiments were carried out. The average value was used to generate the figure and the standard deviation was marked.

One-step growth curve of phage vB_ShiP-A7

One-step growth curve of phage vB_ShiP-A7 was drew following the protocol of Yang's with minor modification [27]. Phage vB_ShiP-A7 was added to the early-log-phase of *S. flexneri* A7 culture (1×10^8

CFU/ml) at a multiplicity of infection (MOI) of 10 and let them incubate with host strain for 10 min. After that, the phages were removed by centrifugation. The pellet was washed twice using fresh LB medium. Then, the precipitate was put into 50 ml LB medium and continue culture at 37°C. We got 1ml cell cultures at different time point and centrifuge at 14000 rpm for 1 minute remove the host bacteria. Free bacteriophage counts in these supernatants were counted using double-layer agar plate method. Three independent experiments were done to get the one-step growth curve of vB_ShiP-A7, in which the latency period, burst period and burst size of vB_ShiP-A7 were determined.

Bacterial challenge assay

Overnight culture of *S. flexneri* A7 was inoculated into LB medium at a ratio of 1:100, and continue cultured for 2.5 hours to logarithmic phase. Phage vB_ShiP-A7 were added (MOI=10,1,0.1) to log-phage cultures of *S. flexneri* A7. Bacterial culture was added with equal volume of SM buffer as negative control sample. Bacterial concentration was measured every 15 minutes for a total of 300 minutes.

Phage vB_ShiP-A7's genome isolation and sequencing

The ultra-purified ShiP-A7's particles were digested by DNase I (New England Biolabs) and RNase A (Tiangen Biotech) at 37°C for 2 h to remove the residual genome DNA and RNA of host bacteria. Then, the sample treated by proteinase K (Tiangen Biotech) at 55°C for 15 min. Then this sample was further purified using TIANamp Bacteria DNA Kit (Tiangen Biotech). The purified Phage DNA concentration was measured by a spectrophotometer (Nanodrop Technologies, USA). The whole genome was sequenced on Illumina platform (Illumina HiSeq 2500 sequencer). SOAPdenovov2.04 software and GapCloserv1.12 was used to analyze high throughput sequencing results and assemble reads into a whole genome.

Annotation and comparison

Artemis software (http://www.sanger.ac.uk/science/tools/artemis) and Glimmer 3 [28] were used to find putative open reading frames (ORFs) in vB_ShiP-A7's genome (The length of protein should not be less than 30 amino acids). Function annotation of vB_ShiP-A7's genome was conducted using the BLAST tools at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the non-redundant protein sequences database. tRNAscan-SE was used to find transfer RNAs (tRNAs) in vB_ShiP-A7's genome (v1.23, http://lowelab.ucsc.edu/tRNAscan-SE). RNAmmer was used to find ribosome RNAs (rRNAs) in vB_ShiP-A7's genome (v1.2, http://www.cbs.dtu.dk/services/RNAmmer/). Molecular masses and isoelectric points of all the predicted phage proteins was calculated using DNAman. NCBI Megablast analysis was used to compare the whole genome sequence similarities of vB_ShiP-A7 with all the other bacteriophages. EMBOSS Needle tool was used to compare the similarity of protein amino acid sequences (European Molecular Biology Laboratory-European Bioinformatics Institute). EasyFig was used to compare annotated proteins of vB_ShiP-A7 with those of relative phages (http://mjsull.github.io/Easyfig/files.html) [29]. Neighbor-Joining algorithm in MEGA was used to analysis phylogenetic relationships among phages.

Analysis particles proteins of phage vB_ShiP-A7

vB_ShiP-A7's particles were mixed up with loading dye and boiled at 100 °C water bath for 5 min. The boiled sample was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gel was stained with silver according to the protocol of Shevchenko's [30]. Liquid chromatography electrospray ionization with tandem mass spectrometry (LC-ESI MS/MS) was used to analysis proteins in vB_ShiP-A7's particles. vB_ShiP-A7 virions were digested with trypsin first, then the tryptic peptides were analyzed by Q Exactive mass spectrometer (Thermo Scientific, USA). MASCOT engine was used to find the corresponding peptides (Matrix Science, London, UK; version 2.2) against all putative ORFs predicted in vB_ShiP-A7's genome.

Biofilm biomass quantification using crystal violet staining

Overnight bacterial culture of *S. flexneri* A7 was subcultured into LB medium until early mid-logarithmic phase (colony-forming units about 10^8/ml). The bacterial culture was diluted to 10^7/ml using LB medium. 200 μL/well of diluted bacterial cells (10^7/ml) were added into 96-well plate (Corning Corp., United States). After incubated at 37 °C for 24 h, remove 100 μL of culture medium were removed from each well. At the same time, 100 μL fresh LB medium with phage vB_ShiP-A7 (10^9/ml) were added into sample wells, and 100 μL LB were added in control wells. Continue to culture for another 24 h, culture mediums of each well were gently removed. Then washed the wells twice using 1×PBS. Biofilm attached to the wells was stained by 0.5% (w/v) crystal violet (200 μL/well) for 20 min at 37 °C. In order to remove the extra crystal violet stain, the wells were washed three times with PBS. The pictures of different wells were taken underneath Microscope.

Nucleotide sequence accession number

The whole assembled genome sequence of vB_ShiP-A7 is deposited at the GenBank database under accession number MK685668.

Results

Morphology of Phage vB_ShiP-A7

Using *S. flexneri* A7 *E. coli* as host strain, a novel phage vB_ShiP-A7 was isolated from waste water in Nanjing, China. After overnight incubation with *S. flexneri* A7 at 37°C, phage vB_ShiP-A7 can form big clear round plague (diameter about 13 mm) on double-layer agar plate (Fig. 1A). We observed the morphology of this phage under Electron Microscope. vB_ShiP-A7 has isometric head with a mean diameter about 61.42±2.96 nm and noncontractile short tail about 13 nm in length (Fig. 1B). vB_ShiP-A7 is a member of viral family of *podoviridae*. This phage was named vB_ShiP-A7 followed the phage nomenclature defined by Kropinski *et al* [31].

Thermal Stability and population dynamics of phage vB_ShiP-A7

The thermal stability of vB_ShiP-A7 was measured at different temperatures (4°C, 25°C, 37°C, 45°C, 50°C) (Fig. 2A). The activity of phage vB_ShiP-A7 did not change much from 4°C to 37°C. When the temperature went up to 45°C, the phage started to lose their activity rapidly (Fig. 2A). These data suggested that vB_ShiP-A7 is stable over a relatively wide temperature range from 4°C to 37 °C, and therefore, it can be preserved well at 4°C in the laboratory and play the role in human body at 37 °C.

One-step growth experiment was conducted to assess the population kinetics of vB_ShiP-A7 using strain *S. flexneri* A7 as a host (Fig. 2B). vB_ShiP-A7 had been released 35 min after infection, with latent period about 30 min, and a burst size about 100 phage particles/cell (Fig. 2B).

Bacteriophage vB_ShiP-A7 inhibits planktonic bacterial growth

The efficacy of phage vB_ShiP-A7 on planktonic bacterial growth was assessed by inoculating a bacterial broth culture of *S. flexneri* A7 with different multiplicity of infectivity (MOI, 0.1, 1 and 10) of phage vB_ShiP-A7. Our results showed that growth of host strain *S. flexneri* A7 was completely inhibited within 90 min by phage vB_ShiP-A7 at different MOI, and this growth inhibition lasted until 300 min after infection (Fig. 2C). Compared to lower MOI (0.1, 1), higher MOI (10) of the phage can kill the host strain even faster (Fig. 2C).

Host range of phage vB_ShiP-A7

The ability of newly isolated phage vB_ShiP-A7 to infect different bacterial strains was estimated by the standard spot tests [26]. Phage vB_ShiP-A7 can infect MDR strain of *S. flexneri* A7, but not MDR strain of *S. sonnei* A5 (Table 1). vB_ShiP-A7 can also infect three MDR *E. coli* stains isolated clinically, which can form clear plaque on two of the MDR *E. coli* strains and turbid plaque on another MDR *E. coli* strain at low concentration of phage (Table 1). In addition, vB_ShiP-A7 can't infect wild-type *E. coli* stain MG1655 (Table 1), which means it may not affect the normal flora of human. Thus, vB_ShiP-A7 may be used as biocontrol agents to prevent or treat infection caused by MDR *S. flexneri* or *E. coli*.

Basic characteristics of vB_ShiP-A7 genome

To exclude the possibility that vB_ShiP-A7 contains any virulent proteins, it is necessary to understand complete genome sequence of vB_ShiP-A7. Next generation sequencing results suggested that the complete genome of phage vB_ShiP-A7 is a linear double-stranded DNA about 39881 bp. There is a single cut site of *Pst*I near one termini of phage vB_ShiP-A7's genome and a single cut site of *Eco*RI near the other termini of phage vB_ShiP-A7's genome. After digesting the phage genome with *Pst*I or *Eco*RI and re-sequencing the small fragments of these two enzymes' digestion products, we proved that the genome DNA of vB_ShiP-A7 contains 177 bp terminal repeats locating at the genome from nucleotides 1 to 177 and 39882 to 40058, respectively (Fig. 3). Therefore, the final genome length of phage vB_ShiP-A7 is 40058 bp with 49.4 % GC content (Table 2, Fig. 3). The general organization of vB_ShiP-A7's genome follows that of T7-like phages, in which forty-three putative open reading frames (ORFs) were predicted in complementary strand (Table 2). We did not find tRNA genes and rRNA genes in vB_ShiP-A7's genome.

The annotated 43 ORFs were summarized in Table 2. Twelve hypothetical proteins are predicted in vB_ShiP-A7's genome. Functions of these hypothetical proteins in vB_ShiP-A7 life cycle need to be determined in the future studies. Thirty-one ORFs (72.1%) were highly homologous to known functional genes, which were predicted to have similar functions with related genes (Table 2), and labeled in different colors in Fig. 3. The predicted functional proteins encoded by vB_ShiP-A7 can be divided into five categories: DNA/RNA replication/modification (DNA polymerase, DNA primase/helicase, ssDNA-binding protein, DNA ligase, RNA polymerase, bacterial RNA polymerase inhibitor, nucleotide kinase, exonuclease, endonuclease), host lysis (lysin protein, endopeptidase Rz), packaging (DNA packaging protein, DNA packaging protein A), structural proteins (tail fiber protein, internal virion protein D, internal core protein, DNA injection channel protein A , internal virion protein A, tail tubular protein B , tail tubular protein A, major capsid protein, capsid and scaffold protein, head-to-tail joining protein, tail assembly protein, host range protein), and additional functions (carbohydrate ABC transporter permease, N-acetylmuramoyl-L-alanine amidase, dGTP triphosphohydrolase inhibitor, putative protein kinase, putative S-adenosyl-L-methionine hydrolase, predicted antirestriction protein) (Table 2, Fig. 3). In addition, lysogen related proteins, such as integrase, recombinase, repressor and excisionase, were not presented in vB_ShiP-A7's genome. We believe that phage vB_ShiP-A7 is a lytic bacteriophage. We did not observe any poison proteins encoded by vB_ShiP-A7's genome. The characteristics of this lytic phage without harmful factors encoded make it an ideal antibacterial agent.

Comparative genome analysis of vB_ShiP-A7 with its related phages

Based on the result of BLAST analyses, vB_ShiP-A7's genome sequence is related to 15 different phages (coverage 74-88%, identity 86-93%), including *Escherichia* phage P483 [32], *Escherichia* phage P694 [32], *Enterobacteria* phage BA14 [33], *Escherichia* phage vB EcoP S523, *Enterobacteria* phage 285P, *Pectobacterium* phage PP74 [34], *Kluyvera* phage Kvp1[35], E*rwinia* phage FE44 [36], *Salmonella* phage BSP161, *Salmonella* phage BP12A, *Yersinia* phage PYPS50, *Yersinia* phage Yepe2, *Yersinia* phage YpP-G [37], *Yersinia* phage Berlin and *Yersinia* phage Yep-phi [38] (Fig. 4). Some of these phages contain terminal repeats (TRs) [32, 35, 38]. vB_ShiP-A7's TRs are very similar to those of Berlin, Yepe2, Yep-phi and Kvp1. Inverted terminal repeats at the genome ends are found in viruses which replicate by a protein-primed mechanism in phage life cycle [39]. vB_ShiP-A7 may have similar DNA replication strategy. Genome sequences of vB_ShiP-A7 and its related phages were aligned and concatenated. As observed in the phylogenetic tree, there are two major branches (Fig. 4). vB_ShiP-A7 is a novel bacteriophage that is closely related to phages *Escherichia* phage P483, *Escherichia* phage vB EcoP S523, *Yersinia* phage PYPS50, and *Salmonella* phage BSP161 (Fig. 4). Therefore, phage vB_ShiP-A7 is related to unclassified T7-like phages, which can infect members of *Enterobacteriaceae*. Currently, this group of phages are highly variable. In addition, vB_ShiP-A7 and its related phages are isolated from different places in the world and can infect bacteria of different species and genera, suggesting that the evolutionary relationships among these phages are complicated.

We compared vB_ShiP-A7 with its 15 related phages using EasyFig. Most of the proteins encoded by vB_ShiP-A7 and its 15 related phages are highly similar (Fig. 5). Several dissimilarity proteins among

these phages are showed in blank or light color in Fig. 5. DNA ligase encoded by ORF36 shows a divergence among these relative phages (100% coverage, 65-74% identity). Tail assembly protein encoded by ORF15 is different from the homologous proteins of other related phages (45-96% coverage and 57-89% identity). Tail fiber protein encoded by ORF5 of vB_ShiP-A7 has relatively lower homology with its related phages (37-46% coverage and 54-60% identity) (Fig. 5). The similarity of vB_ShiP-A7's tail fiber protein with other homologs is only found at the N-terminus, which is associated with the tail structure [35]. The C-terminus of this tail fiber protein, involved in ligand interactions, exhibits relatively large variability with tail fiber proteins of related phages (Fig. 5).

Structural Proteins of vB_ShiP-A7

Purified phage vB_ShiP-A7's particles were denatured and separated by SDS-PAGE. At least eight distinct protein bands were shown in the silver-stained SDS-PAGE gel, with molecular weights ranging from 17 to 180 kDa, (Fig. 6). Seven bands were speculated as structural proteins of vB_ShiP-A7 by estimated molecular weights (internal virion protein D, tail tubular protein B, tail fiber protein, head-to-tail joining protein, major capsid protein, capsid and scaffold protein, tail tubular protein A) (Fig. 6). To further confirm these structural proteins, phage vB_ShiP-A7' particle proteins were determined by mass spectrometry (Table 3). Total eighteen proteins were identified, including all the proteins showed on SDS-PAGE gel (Table 3, Fig. 6). Nine of them are known structural proteins. DNA primase/helicase was also determined in the phage particles. In addition, some hypothetical proteins (ORF17, ORF18, A7_225, A7_120, A7_146, A7_426, A7_68, A7_88) were also detected by Mass spectrometry, and their functions need to be determined further. Hypothetical proteins encoded by A7_225, A7_120, A7_146, A7_426, A7_68, A7_88 were only predicted when we used all the possible ORFs by artemis in vB_ShiP-A7' genome as reference (>30 amino acids). But they were omitted from annotation file of vB_ShiP-A7 (uploaded to NCBI under assigned number MK685668), since they don't have similar sequences with any predicted proteins at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or their genes may exist in the interior of known genes. Interestingly, A7_225, A7_120, A7_146, A7_68 and A7_88 were encoded by antisense RNAs on known genes of late operon of vB_ShiP-A7 (Table 3). A7_225 was translated on the opposite direction of ORF30 (endonuclease). A7_120 was existed on the opposite direction of ORF11 (tail tubular protein A). A7_146 was existed on the opposite direction of ORF14 (head-to-tail joining protein). A7_68 was existed on the opposite direction of ORF6 (internal virion protein D). A7_88 was translated on the opposite direction of ORF7 (internal core protein). In addition, no toxic protein was identified by Mass spectrometry in vB_ShiP-A7's particles.

Ability of vB_ShiP-A7 to destroy bacterial biofilms

Removal of biofilm is the key to treat chronic infectious diseases. We tested the effect of phage vB_ShiP-A7 on biofilm formed by *S. flexneri A7* and *E. coli* 395 B5, respectively. About 24 h post phage vB_ShiP-A7 addition, biofilm biomass of strain *S. flexneri* A7 (Fig. 7C) and *E. coli* 395 B5 (Fig. 7D) were significantly lower to that of the untreated controls (Fig. 7A, B). This analysis suggested that vB_ShiP-A7 can reduce

the biofilm formation on clinic strains of *Shiglla flexneri* A7 and *E. coli* 395 B5, raising a possibility of using phage vB_ShiP-A7 as a biofilm disruption agent.

Discussion

Shigella species and *E. coli* belong to *Enterobacteriaceae*, which can cause intestinal infection [6]. Of note, previous studies have demonstrated that bacteriophages can be used to treat this infection [15,16]. In this study, a new lytic phage vB_ShiP-A7 was isolated and characterized, with lytic activity against both MDR *S. flexneri* clinical strain and several MDR *E. coli* clinical strains (Table 1). Several phages were reported to have the ability to infect both *E. coli* and *Shigella* [40, 41, 42]. Since *E. coli* and *Shigella* are genetically similar, it is reasonable to expect some similar phages would target both strains. In addition, lytic phages are relative specific, and usually infect a subgroup of strains within one bacterial species or across closely-related species, which causes less disruption to gut flora than antibiotic treatment [14]. In addition, Phage vB_ShiP-A7 does not infect wild-type *E. coli* strain MG1655, suggesting that it may not affect the normal flora.

Phage vB_ShiP-A7 belongs to family *Podoviridae* according to its morphology under the Electron Microscope (Fig. 1). vB_ShiP-A7 has short latent time and a large burst size, suggesting that vB_ShiP-A7 can quickly increase the phage concentration [43]. Next-generation sequencing demonstrated that the genome of phage vB_ShiP-A7 does not encode integrases, recombinases, or harmful gene products (Table 2, Fig. 3). In addition, phage vB_ShiP-A7 has shown promising effects against bacterial growth in liquid and biofilm (Fig. 2, Fig. 6), suggesting that it may be used as an ant-infective agent.

Comparative genome analysis demonstrated that phage vB_ShiP-A7 is related to unclassified T7-like phages (Fig. 4, Fig. 5). Therefore, this phage can be assigned into virulent phage of T7-like family. Only several genes of vB_ShiP-A7 genome are dissimilar with their relative phages (Fig. 5), in which the tail fiber protein of vB_ShiP-A7 is obviously different with that of other phages. The tail protein of phage is the key protein to recognize host bacteria. Different tail proteins of phages can cause phages to infect different host bacteria [44]. Yosef *et al* reported that a small change in the tail protein sequence of a phage can lead to changes in host range [45]. The different tail fiber protein of vB_ShiP-A7 may allow this phage to infect some specific hosts which could be used as a component of phage cocktail. Most of the genes of vB_ShiP-A7 and 15 related phages are highly homologous. High homology of same functional phage genes was found in different phage species, illustrating that horizontal gene transfer between phages is a component of evolution [32,45]. Gene arrangement of phage vB_ShiP-A7 is different with some of related phages (Fig. 5). Gene rearrangement was also observed in other *Escherichia* phages [47]. vB_ShiP-A7 and its relatives may be evolved through horizontal exchange and rearrangement of their genes, which is a common phenomenon in the evolution of tailed phages [32,35,38].

Eighteen proteins were identified in vB_ShiP-A7 phage particles using mass spectrometry, including known structural proteins and hypothetical proteins (Table 3, Fig. 6). Interestingly, some of these hypothetical proteins were encoded by antisense RNA on late operon encoded structure proteins (A7_225,

A7_120, A7_146, A7_68, A7_88) (Table 3). Anne *et al* found PAK_P3 expressed antisense RNA elements targeting its structural region during the early stage of infection [48], which might be used to shutdown expression of late structural genes during the early stage of infection. An antisense RNA was also found in lambda phage genome, which was transcripted from paQ promoter and didn't encode protein or peptides [49]. We found several small peptides/proteins encoded by antisense RNAs in *E. coli* phage vB_EcoP-EG1 [50]. These small peptides/proteins encoded by antisense RNAs in late operon may exit in different phages and involve in the phage infection processes, however the detailed mechanisms remain to be determined.

The phage genome lays a foundation for studying the interaction between phage and its host. Comparative genome analysis of this phage with related phages shed light on the mechanisms of evolutionary changes of these T7-like family phage genomes. Fully characterized phage could be used as alternative treatment for the increasing number of MDR *Shigella* and *Escherichia*, and hence reducing the pressure to find new antibiotics.

Abbreviations

NCBI: National Center for Biotechnology; MDR: multidrug-resistant ; DNA: Deoxyribonucleic acid; RNA: Ribonucleic acid; ORF: open reading frame; MOI: multiplicity of infection; LC-ESI MS/MS: Liquid chromatography electrospray ionization with tandem mass spectrometry; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Declarations

Acknowledgments

The authors are appreciated to the bioinformatics platforms (http://mjsull.github.io/Easyfig/files.html and http://www.sanger.ac.uk/science/tools/artemis) and stations (http://lowelab.ucsc.edu/tRNAscan-SE and http://www.cbs.dtu.dk/services/RNAmmer) for providing computing and storage resources.

Author Contributions Statement

JX, YG, XY and XL conceived, designed and coordinated the study. JX, XY, RZ carried out the experimentation. JX, XY, YG and XL analyzed data. Contributed reagents/materials/analysis tools: GL, JX, XY, YG and XL. XZ helped edit the manuscript. All authors read and approved the manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (81501797 to XL) and (31800156 to XY) and the Natural Science Foundation of Jiangsu Province (BK20171044 to XY).

The role of the funders consisted on a financial support for all experiment realisations.

Availability of data and materials

The data for the genome of phage vB_ShiP-A7 is deposited at the GenBank database under accession number MK685668.

Ethics approval and consent to participate

Clinical strains were isolated from clinical samples of patients in the First Affiliated Hospital of Nanjing Medical University, Nanjing, China.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Host range spectrum of the bacteriophage vB_ShiP-A7

Strains	Source	Subtype	Resistance	Lysis or not
***E. coli* K-12 MG1655**	ATCC 700926			N
***Shigella flexneri* A7**			Ampicillin sulbactam Aminoglycoside streptomycin Spectinomycin	Clear plaque
***Shigella sonnei* A5**			Spectinomycin Gentamycin Streptomycin Aminoglycoside Ampicillin sulbactam	N
393 D3	Urine	ESBL	Levofloxacin Cefazolin Cefepime Cefotaxime Ceftazidime	Clear plaque
395B5	Urine	ESBL	Gentamycin Ampicillin sulbactam Aztreonam Cefepime Cefazolin Levofloxacin Cefotaxime Sulfamethoxazole and Trimethoprim	Clear plaque
397 D3	Urine	ESBL	Gentamycin Ampicillin sulbactam Levofloxacin Cefotaxime Cefepime Aztreonam Cefoxitin Cefazolin Sulfamethoxazole and Trimethoprim	Turbid plaque
389 A6	Urine	ESBL	Sulfamethoxazole and Trimethoprim Cefazolin Aztreonam Ceftazidime	N
389G7	Urine	ESBL	Ampicillin sulbactam Aztreonam Cefazolin Amikacin Levofloxacin Cefotaxime	N
393B7	Urine	ESBL	Levofloxacin Gentamycin Cefazolin Ampicillin sulbactam Ceftazidime Amoxicillin and Clavulanate Amikacin Aztreonam Sulfamethoxazole and Trimethoprim Minocycline Cefotaxime Cefepime Cefoxitin	N
393C8	Urine	ESBL	Sulfamethoxazole and Trimethoprim Cefepime Cefazolin Cefotaxime Levofloxacin	N
395G6	Urine	ESBL	Gentamycin Aztreonam Cefazolin Cefepime Ceftazidime Cefotaxime	N
396J1	Urine	ESBL	Levofloxacin Cefazolin Imipenem Cefepime Cefotaxime	N
394H7	Urine	ESBL	Cefotaxime Levofloxacin Ceftazidime Aztreonam Cefepime	N

396J5	Urine	ESBL	Levofloxacin□Aztreonam□Imipenem Cefepime□ Cefazolin□Cefotaxime	N
389 G7	Urine	ESBL	Cefazolin□Ampicillin sulbactam□ Levofloxacin□ Aztreonam□Amikacin□Cefotaxime	N
394F7	Urine	ESBL	Cefazolin□Aztreonam□Cefoxitin□ Ceftazidime□ Cefotaxime□ Amoxicillin and Clavulanate	N
389D9	Urine	Non-ESBL	Sulfamethoxazole and Trimethoprim	N
389G6	Urine	Non-ESBL	Levofloxacin	N
389G4	Urine	Non-ESBL	Gentamycin□Cefazolin□Ampicillin sulbactam□ Cefotaxime□Amikacin□ Levofloxacin□Ceftazidime□ cefoxitin	N
390B6	Urine	Non-ESBL	Sulfamethoxazole and Trimethoprim	N
390 J2	Urine	Non-ESBL	Minocycline□Levofloxacin□Sulfamethoxazole and Trimethoprim	N
389E6	Urine	Non-ESBL	Levofloxacin	N
390 G7	Urine	Non-ESBL	Sulfamethoxazole and Trimethoprim□ Gentamycin□Cefazolin□Ampicillin sulbactam□ Levofloxacin	N
390H2	Urine	Non-ESBL	Ampicillin sulbactam□Levofloxacin□ Sulfamethoxazole and Trimethoprim□ Gentamycin□Cefazolin	N
391D3	Urine	Non-ESBL	Gentamycin□Cefazolin□Sulfamethoxazole and Trimethoprim	N
389J4	Sputum	ESBL	Cefepime□Ceftazidime□Cefazolin□ Aztreonam	N
395 J2	Sputum	ESBL	Gentamycin□Ampicillin□Minocycline□Levofloxacin	N
397C8	Sputum	ESBL	Ceftazidime□Aztreonam□Cefepime□ Ceftazidime	N
396F3	Sputum	ESBL	Ampicillin□Gentamycin□Minocycline□Levofloxacin□ Amikacin	N
390A7	Sputum	Non-	Sulfamethoxazole and Trimethoprim	N

		ESBL		
391G4	Blood	ESBL	Cefepime□Cefazolin□Aztreonam□ Ceftazidime	N
393C1	Ascites	ESBL	Cefotaxime□Cefazolin□Cefepime□ Ampicillin sulbactam□Aztreonam□ Gentamycin□Ceftazidime□Sulfamethoxazole and Trimethoprim	N

Negative results are indicated by “N”.

Table 2. Predicted ORFs and genes encoded by the vB_ShiP-A7 genome.

ORFs	start	stop	Directions	No of residues	MW(da)	pI	Predicted molecular function
ORF1	848	2608	-	586	66236.1	5.35	DNA packaging protein
ORF2	2608	3054	-	148	16740.9	7.51	endopeptidase Rz
ORF3	3153	3419	-	88	10113.7	4.38	DNA packaging protein A
ORF4	3412	3618	-	68	7337.2	6.72	lysin protein
ORF5	3675	5540	-	621	68063.2	6.34	tail fiber protein
ORF6	5612	10546	-	1644	179456.2	7.02	internal virion protein D
ORF7	10570	12849	-	759	85637.0	5.78	internal core protein
ORF8	12856	13449	-	197	21320.4	10.26	DNA injection channel protein A
ORF9	13452	13865	-	137	15747.5	8.71	internal virion protein A
ORF10	13946	16342	-	798	89101.9	6.35	tail tubular protein B
ORF11	16365	16955	-	196	21966.7	4.22	tail tubular protein A
ORF12	17154	18188	-	344	36520.6	6.63	major capsid protein
ORF13	18321	19220	-	299	32722.7	3.91	capsid and scaffold protein
ORF14	19295	20902	-	535	58734.0	4.35	head-to-tail joining protein
ORF15	20916	21218	-	100	10201.8	10.64	tail assembly protein
ORF16	21221	21607	-	128	14558.0	6.78	host range protein
ORF17	21626	21904	-	92	9895.3	10.26	hypothetical protein
ORF18	21914	22159	-	81	9134.7	4.40	hypothetical protein
ORF19	22322	23236	-	304	34816.3	4.79	exonuclease
ORF20	23233	23442	-	69	7319.0	9.87	hypothetical protein
ORF21	23442	23744	-	100	11218.9	8.50	hypothetical protein
ORF22	23756	23983	-	75	9410.1	6.24	hypothetical protein
ORF23	23997	26111	-	704	79084.7	6.33	DNA polymerase
ORF24	26124	26540	-	138	15743.3	9.66	hypothetical protein

ORF25	26632	26934	-	100	10943.6	10.65	hypothetical protein
ORF26	26948	27181	-	77	8715.8	11.71	hypothetical protein
ORF27	27273	28985	-	570	63571.7	4.89	DNA primase/helicase
ORF28	29153	29245	-	30	3665.0	7.53	carbohydrate ABC transporter permease
ORF29	29250	29705	-	151	16950.3	8.10	N-acetylmuramoyl-L-alanine amidase
ORF30	29702	30148	-	148	17215.9	9.80	endonuclease
ORF31	30148	30852	-	234	26229.4	4.24	ssDNA-binding protein
ORF32	30922	31113	-	63	6972.6	4.93	bacterial RNA polymerase inhibitor
ORF33	31201	31374	-	57	6888.7	4.55	hypothetical protein
ORF34	31364	31657	-	97	11374.1	5.21	nucleotide kinase
ORF35	31657	31935	-	92	10188.1	11.30	hypothetical protein
ORF36	32108	33208	-	366	41577.2	4.68	DNA ligase
ORF37	33316	33588	-	90	10648.3	7.51	dGTP triphosphohydrolase inhibitor
ORF38	33588	33737	-	49	6078.8	11.37	hypothetical protein
ORF39	33829	36480	-	883	98990.8	7.32	RNA polymerase
ORF40	36554	37651	-	365	41750.0	7.61	putative protein kinase
ORF41	37673	37891	-	72	8446.1	11.76	hypothetical protein
ORF42	38113	38571	-	152	17040.3	6.74	putative S-adenosyl-L-methionine hydrolase
ORF43	38640	38984	-	114	13287.4	3.71	predicted antirestriction protein

Table 3. Phage vB_ShiP-A7 particle proteins identified in mass spectrometry.

First protein	Unique peptides	Unique sequence coverage [%]	Mol. weight [kDa]	Sequence length	Start	End
ORF6	111	72.9	179.52	1644	10546	10546
ORF7	56	69.7	85.783	760	12852	12852
ORF12	34	92.6	37.502	351	18188	18188
ORF14	25	56.6	58.744	535	20902	20902
ORF13	15	42.5	33.488	306	19220	19220
ORF8	11	65.2	22.545	207	13449	13449
ORF15	7	66	10.875	106	21218	21218
ORF17	4	51.1	9.8971	92	21904	21904
A7_225	3	25.4	7.7616	67	29770	29970
A7_120	2	28.1	10.122	89	16521	16787
A7_146	2	39	6.5657	59	19923	20099
ORF27	2	5.3	64.9	582	28985	27273
ORF18	2	43.9	9.2674	82	22159	21914
A7_426	2	46.7	3.5044	30	19069	18980
ORF11	2	18.4	21.97	196	16955	16365
ORF5	2	4.7	68.075	621	5540	3675
A7_68	2	93	4.7304	43	9639	9767
A7_88	2	34.8	7.6992	69	11963	12169

Figures

Figure 1

Isolation and morphology analysis of phage vB_ShiP-A7. (A) Plaques of phage vB_ShiP-A7 on top agar plate. (B). The morphology of phage vB_ShiP-A7 under transmission electron microscope Bar indicates 50 nm.

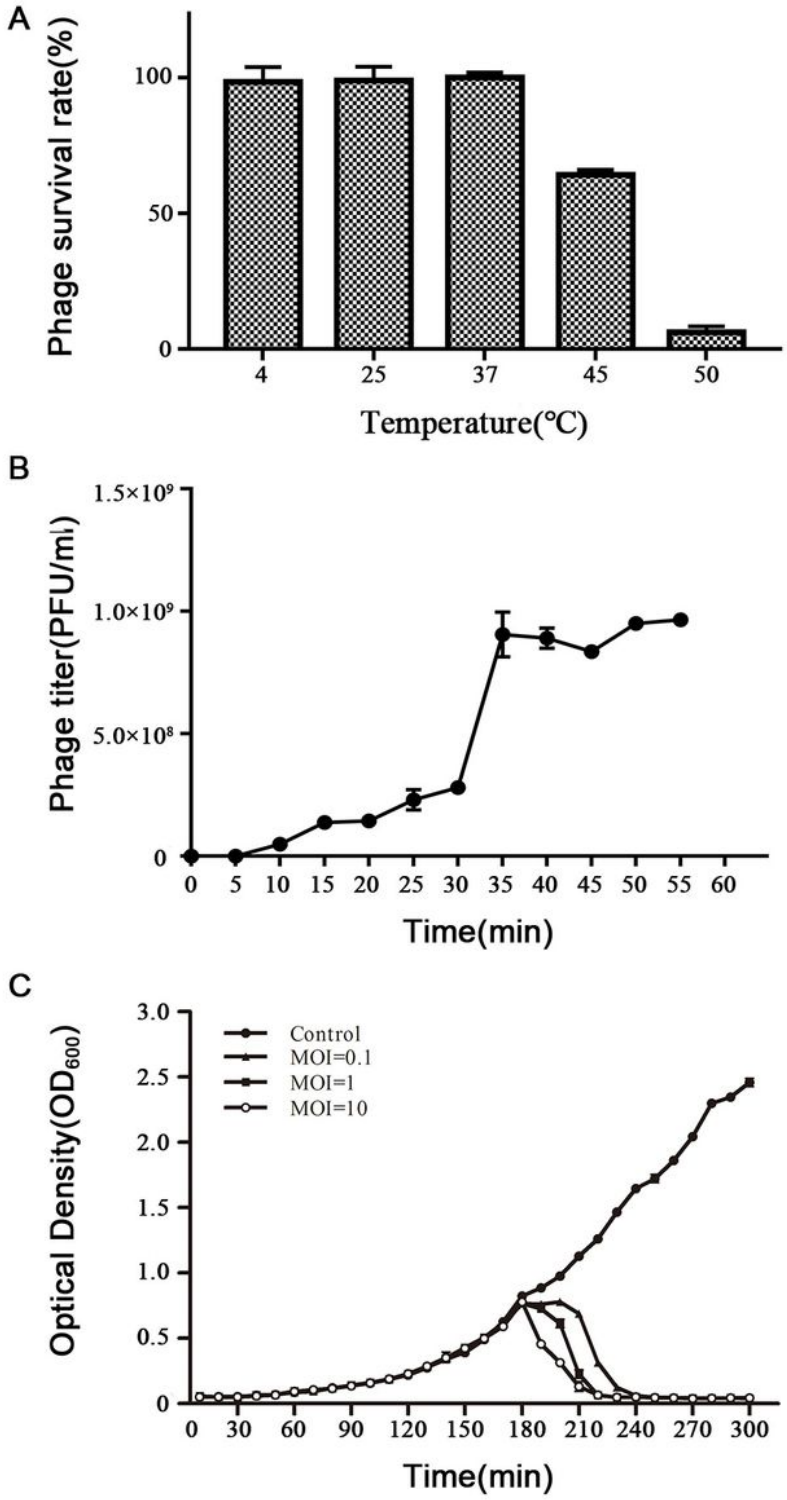

Figure 2

Biological characteristics of phage vB_ShiP-A7. (A) The surviving rate of vB_ShiP-A7 after treated under different temperatures for 1 h. (B) One-step growth curve of vB_ShiP-A7 on strain Shiglla flexneri A7 at

37°C. (C) Bacterial challenge assay with phage vB_ShiP-A7 to Shiglla flexneri A7. vB_ShiP-A7 was added at MOI of 0.1 (close triangles), 1 (close squares) and 10 (open circles) to different bacterial cultures after 2 h incubation (OD600 =0.25). SM buffer instead of vB_ShiP-A7 was inoculated into a bacterial culture and used as the negative control (close circles). OD600 of each sample was recorded at 15 min intervals, over a period of 300 min. In B and C, data from three independent experiments are combined and presented as the mean with standard deviation.

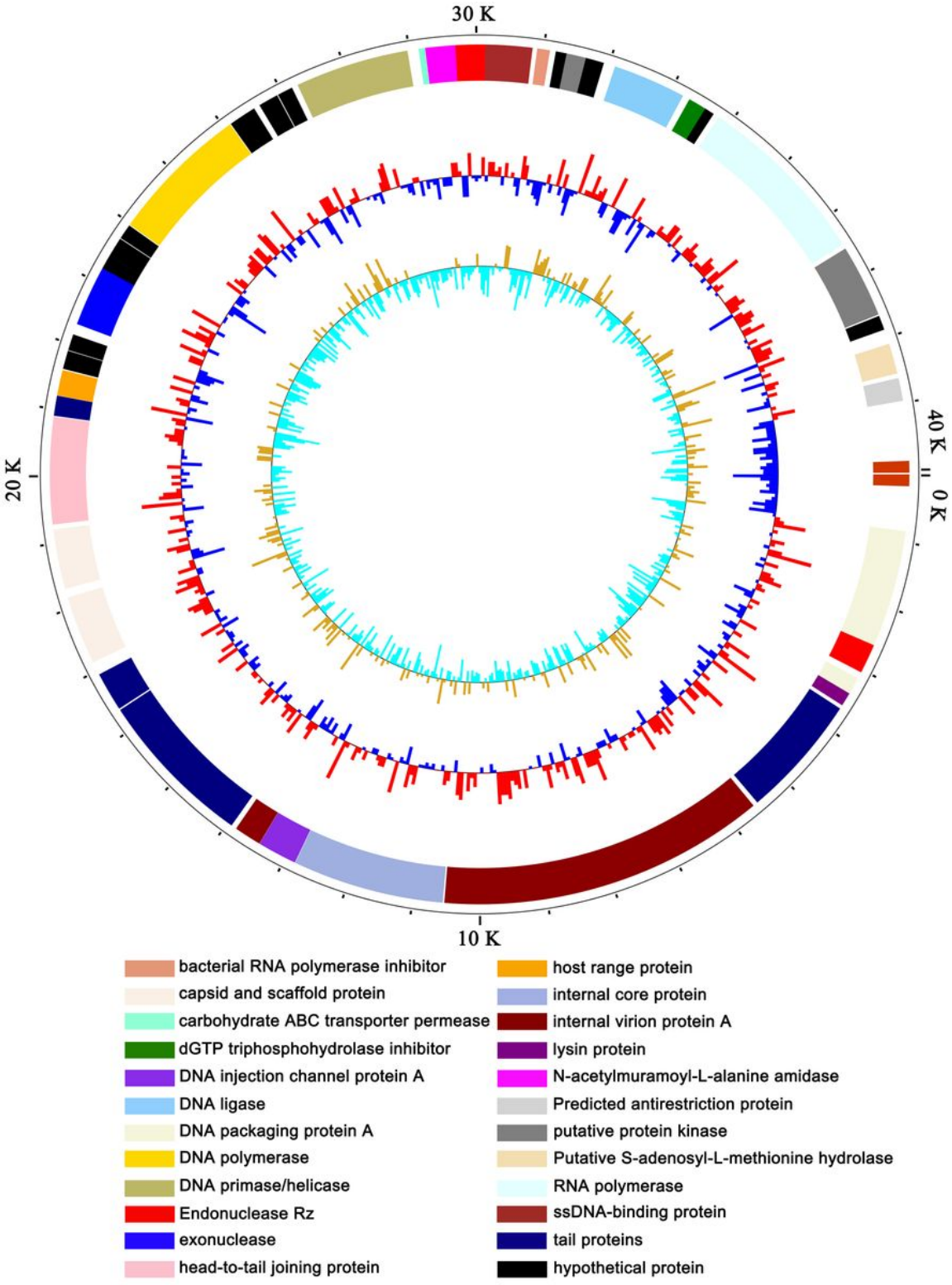

Figure 3

Map of the genome organization of bacteriophage vB_ShiP-A7. The predicted ORFs of vB_ShiP-A7 are indicated in different colors in outmost ring. All the ORFs predicted in vB_ShiP-A7 genome are in negative stain in inward. ORFs with predicted function marked in different colors. Hypothetical proteins are labeled in black. The second ring represents the GC content. Red outward indicates the GC content above average, and blue inward indicates that the GC content is below average. The innermost ring represents GC skew.

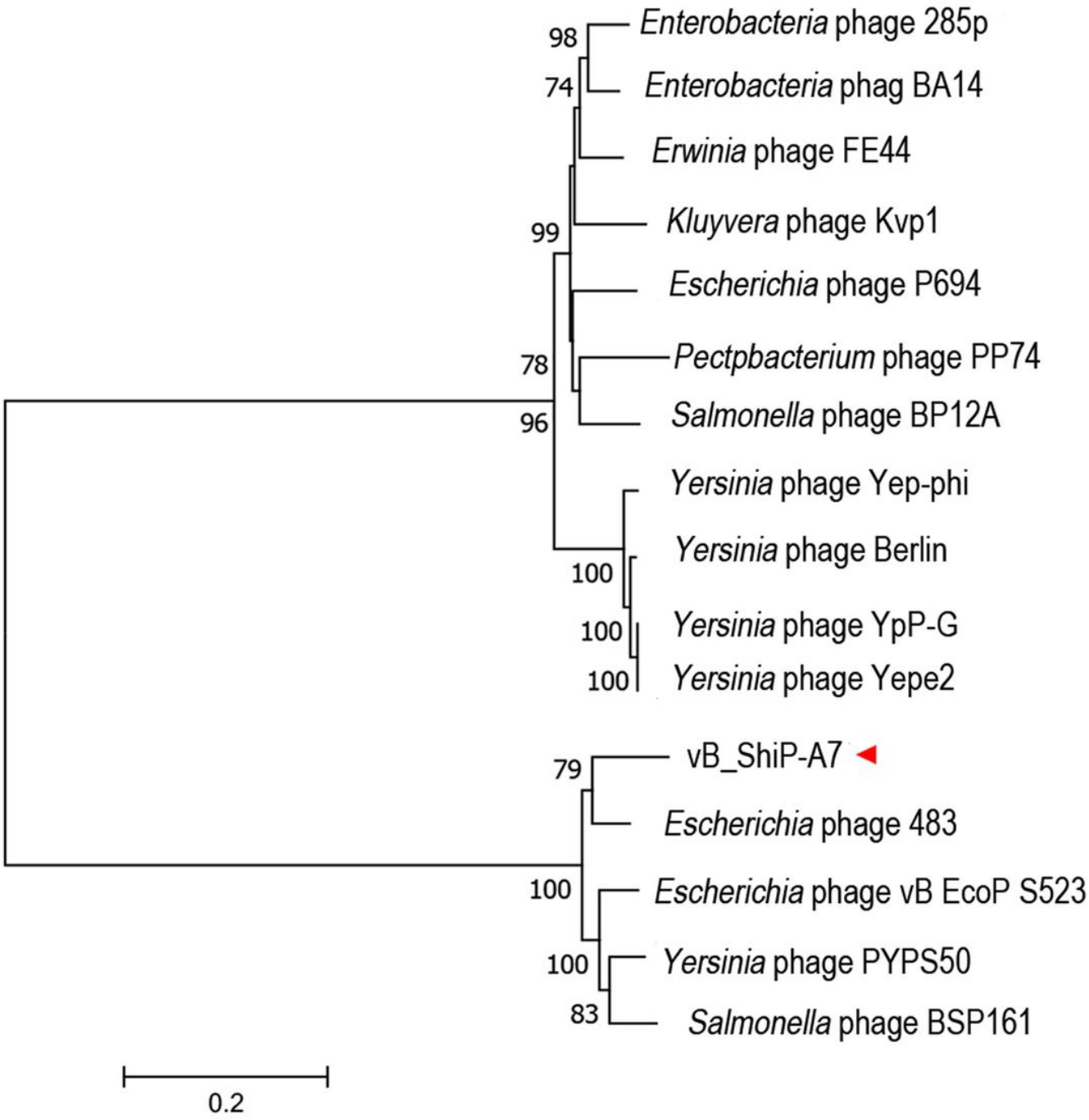

Figure 4

Phylogenetic tree based on whole genome sequence of vB_ShiP-A7 and its related phages. After compared genome sequences of these phages using the ClustalW program, phylogenetic tree was

generated using the neighbor-joining method with 1000 bootstrap replicates. vB_ShiP-A7 was marked by red arrow.

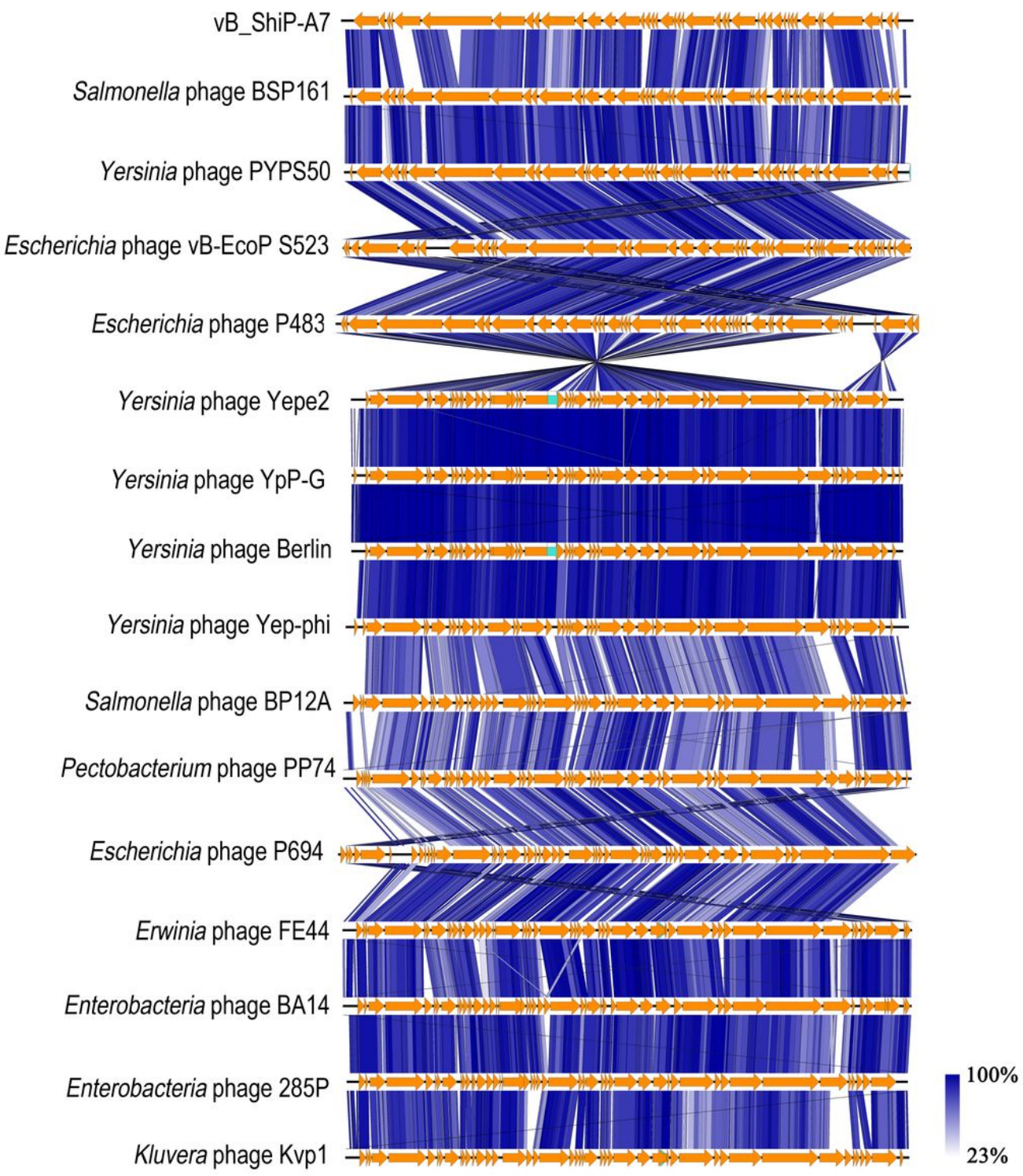

Figure 5

Schematic genomic alignment of the phage vB_ShiP-A7 with its related phages. Homologous ORFs or genes are present in brown, and amino acid identities of different proteins are indicated by different shades of blue.

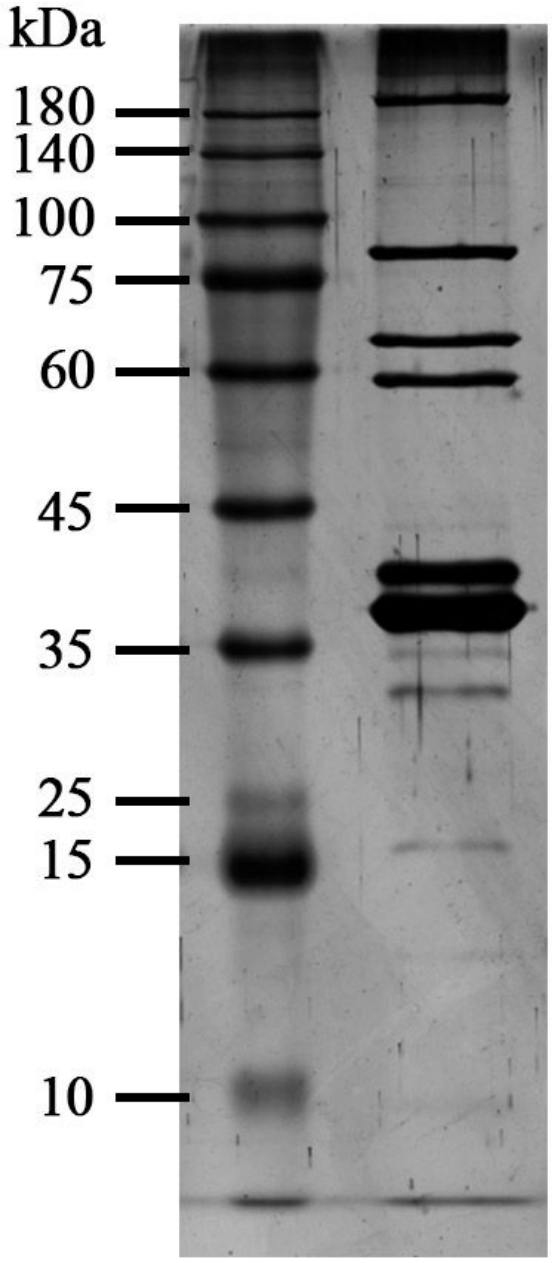

Figure 6

Particle proteins of vB_ShiP-A7 separated by SDS-PAGE gel. The purified vB_ShiP-A7 particles were separated by SDS-PAGE and stained with silver. The eight bands from top to bottom corresponding to different particle proteins of vB_ShiP-A7 are internal virion protein D, tail tubular protein B, tail fiber protein, head-to-tail joining protein, major capsid protein, DNA ligase, capsid and scaffold protein, tail tubular protein A. Protein molecular mass markers are indicated on the far left.

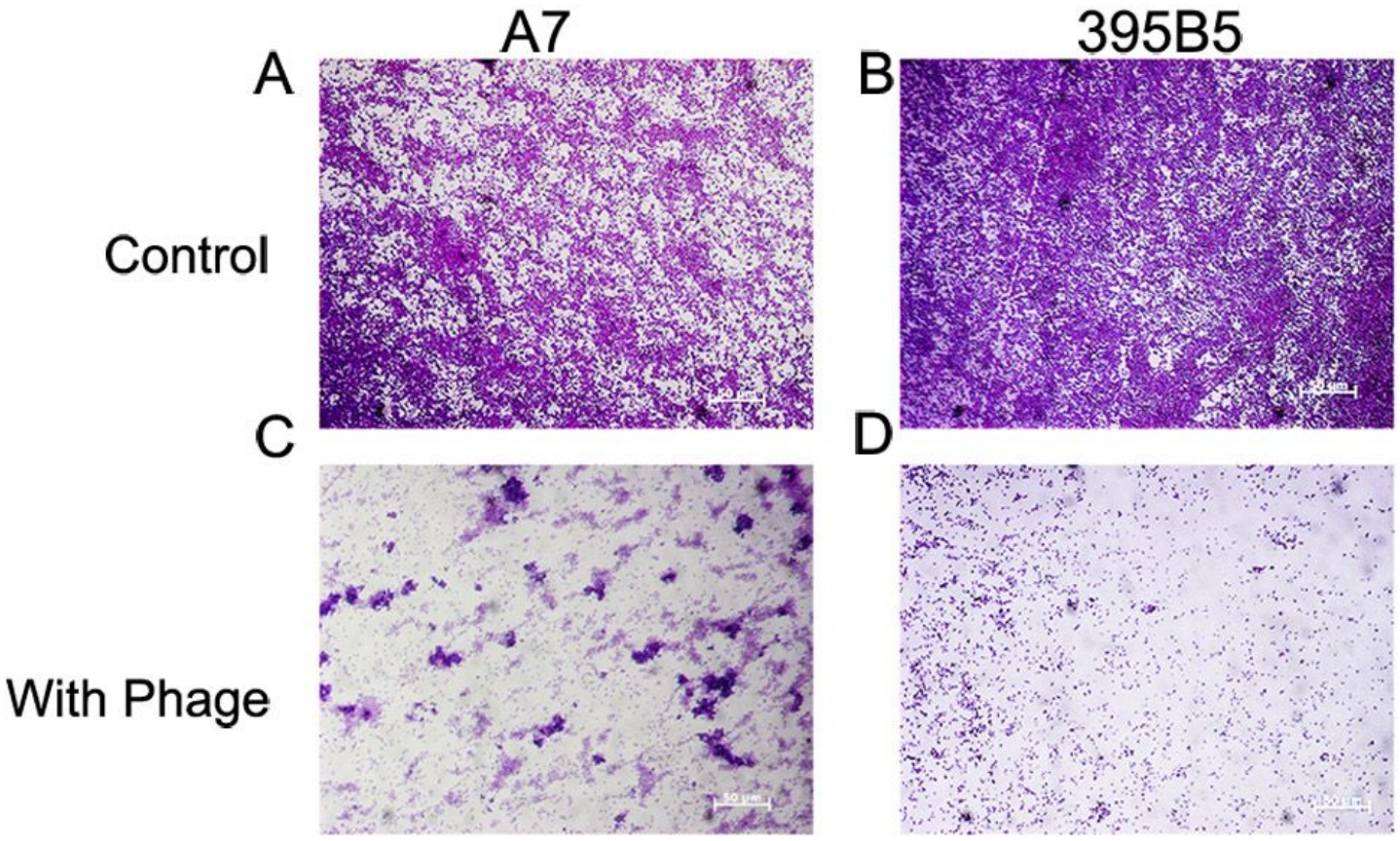

Figure 7

Crystal violet assay showing biofilm biomass of Shiglla flexneri A7 and E. coli 395B5 grown in presence or absence of phage vB_ShiP-A7. (A) Shiglla flexneri A7 strain without phage. (B) E. coli 395B5 strain without phage. (C) Shiglla flexneri A7 exposure to phage vB_ShiP-A7. (D) E. coli 395B5 exposure to phage vB_ShiP-A7.

Supplementary Files

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