ORIGINAL PAPER

Characterization and genome analysis of a novel bacteriophage vB_SpuP_Spp16 that infects *Salmonella enterica serovar pullorum*

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Abstract

A novel virulent bacteriophage vB_SpuP_Spp16 (hereafter designated Spp16) that infects *Salmonella enterica serovar pullorum* was isolated. Transmission electron microscopy showed that Spp16 possessed an isometric polyhedral head (60 nm in diameter) and a short tail (10 nm in length) belonging to the family *Podoviridae*. Its complete genome was determined to be 41,832 bp, with a 39.46% GC content by next-generation sequencing. The genome contains 53 proposed open reading frames that are involved in DNA replication and modification, transcriptional regulation, phage structural and packaging proteins and bacterial lysis. No transfer RNA genes were identified. The termini of genome were determined using our previously proposed termini identification method, which suggests that this phage has redundant termini with 421 bp direct terminal repeats. BLASTn analysis revealed the highest sequence similarity with *Yersinia* phage phi80-18, with a genome coverage of 33% and highest sequence identity of 69%. The phylogenetic analysis indicated that Spp16 forms a distinct branch of the subfamily *Autographivirinae*. Comparative genomics analysis showed that the phage Spp16 should be regarded as a new subcluster within the GAP227-like cluster in the *Autographivirinae* subfamily. The phage Spp16 has an obligate lytic life cycle demonstrated by experimental data and genomic analysis. These results suggest that Spp16 may be a proper candidate to control diseases caused by *Salmonella enterica serovar pullorum*.

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Introduction

Salmonella spp. is Gram-negative Enterobacteriaceae bacteria without producing any spores or capsule. *Salmonella* spp. comprise *S. enterica* and *S. bongori*. *S. enterica* is subdivided into six subspecies and contains > 2600 serovars [1]. While the serovars of *Typhimurium* and *Enteritidis* are globally distributed [2], other serovars are regionally distributed. *Salmonella* not only causes a variety of livestock and poultry diseases but can also be a foodborne pathogen endangering human health. Poultry and poultry products are considered one of the main sources of contaminated food in human salmonellosis outbreaks [3]. In recent years, due to the excessive use and abuse of antibiotics in the fields of medicine, livestock, and agriculture, the number of multidrug-resistant pathogenic bacteria, such as *Salmonella*, are increasing and the incidence of human salmonellosis has risen [4]. It is estimated that the economic burden of medical care and loss of productivity due to *Salmonella* is

as high as several billions of dollars per year in the United States [5]. At present, multidrug-resistant bacteria constitute a major threat to human health, making the discovery of new antibiotics or alternative antibiotic drugs a priority [6]. Bacteriophages are abundant bacterial viruses that only infect and reproduce in their particular host. It is estimated that there are 10^{31} phage particles worldwide [7]. Phages are considered alternatives to antimicrobial agents for the treatment of foodborne pathogens [8]. In this report, we isolated and characterized a novel lytic *Salmonella* phage Spp16. A genome analysis showed that Spp16 represented a new species in the subfamily *Autographivirinae*.

Materials and methods

Phage isolation

Bacteriophages were isolated from sewage using enrichment cultures [9]. Specifically, approximately 4 mL of filtered (Millipore membranes, pore diameter 0.22 µM) sewage was mixed with 10 mL of an exponential phase indicator bacterium. The mixture was incubated overnight at 37 °C and centrifuged at 12,000×*g* for 10 min. The supernatant was filtered (Millipore membranes, pore diameter 0.22 µM) to remove the residual bacterial cells. Then, 100 µL of supernatant was mixed with 300 µL of indicator bacterium in the exponential growth phase (OD600 = 0.2 to 0.5). The mixture was incubated at 37 °C for 5 min and added to about 5 mL of top agar (LB with 0.7% agar) at 50 °C, the mixture was poured onto an LB plate pre-warmed at 37 °C (double-layer method). The plaques were produced after an overnight incubation at 37 °C. Pure phage strain was obtained by three serial single-plaque isolations.

Electron microscopy

Bacteriophage morphology was examined by transmission electron microscopy (TEM) [10].The purified bacteriophage was applied to the surface of carbon-coated copper grids and the excess was drawn off with filter paper. The phage was negatively stained with 2% uranyl acetate, then uranyl acetate was removed after 1 min. Prepared samples were examined with a transmission electron microscope (JEM-1200EX, Japan) at 100 kv.

Thermal and pH stability

To measure the thermal stability of phages Spp16, the phage isolates (10^8 PFU/mL) were incubated at various temperatures (4, 25, 40, 50, 60, 70, and 80 °C) for 2 h. The aliquots were collected after 20, 40, 60, 80, 100, and 120 min and phage titer was assayed by the double-layer agar method

[11]. To evaluate the stability of phages under various pH conditions (2–12, adjusted using NaOH or HCl), a 100 µL sample of phage suspension (10^8 PFU/mL) was added to each pH solution and incubated for 2 h at 37 °C, after which the phage titer was determined using the double-layer agar plate method.

One-step growth curve

One-step growth experiment was carried out as described previously with little modification [12]. In brief, bacterial cells were harvested at an OD600 of 0.5 were harvested by centrifugation at 12,000×*g* for 15 min at 4 °C and resuspended in 10 mL fresh LB broth medium. The phage was mixed with its host at an MOI of 0.001 (the best MOI among all tested values) and allowed to adsorb for 5 min at 37 °C. Then, the mixture was centrifuged at 14,000×*g* for 1 min to remove unadsorbed phages. After washing twice with fresh LB medium and the pellet was resuspended in 20 mL of LB followed by incubation at 37 °C. Subsequently, the samples were taken at intervals of 10 min and titrated using the conventional double-layer agar method. The experiment was repeated three times.

Host range

Host range was determined using the double-layer agar plate method. To observe the host range, 200 µL of bacteria strains culture and 100 µL of phage culture were mixed with semi-solid LB medium and transferred directly onto plates already containing a layer of solid LB medium. After solidification, the plates were incubated for 8 h at 37 °C and then the degree of lysis was scored [13].

Bacteriophage genome extraction

Bacteriophage DNA was extracted based on a previous method [14]. In brief, 600 µL of the purified phage Spp16 (10^9 PFU/mL) were treated with DNase I and RNase A (Thermo Scientific, USA) to a final concentration of 1 µg/mL. The mixture was incubated for 30 min at 37 °C and then incubated at 80 °C for 15 min to deactivate DNase I. Final concentrations of 0.5% sodium dodecyl sulfate, 20 mmol/mL EDTA, 50 µg/mL proteinase K were added to the mixture followed by incubation for 1 h at 56 °C. An equal volume of phenol was added to extract the DNA. After centrifugation at 8000×*g* for 10 min, the aqueous layer was removed to a fresh tube containing an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) and centrifuged at 8000×*g* for 10 min. The aqueous layer was collected, mixed with an equal volume of isopropanol, and stored for 4 h at − 20 °C. The mixture was centrifuged for 15 min at 12,000×*g*, and the DNA pellet was washed with 75% ethanol, then air-dried

at room temperature, resuspended in 30 μL deionized water, and stored at − 20 °C.

Genome sequencing and termini identification

The DNA was used to construct a 600 bp insert length library using the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina. Briefly, 100 ng purified DNA was dissolved in deionized water to a total volume of 50 μL and fragmented by a Bioruptor Sonication System to a size of 500 bp. The sonicated fragments were end repaired and ligated with adapters. The adaptor-ligated fragments were PCR amplified, then, amplified products were purified with AMPure XP Beads. Prior to sequencing, the constructed library was subjected to quality control analysis using Bioanalyser 2100 (Agilent Technologies). Then subjected to high-throughput sequencing using Illumina Miseq (San Diego, CA, USA) according to manufacturer instructions. The qualified sequence reads were assembled using the de novo assembly algorithm Newbler Version 3.0 with default parameters. Assembled contigs were linked and extended to create a full-length sequence using Cytoscape v2.8.3 software [15]. Phage termini were identified using our proposed method [16].

Genome annotation and bioinformatics analysis

The complete sequence was annotated by Rapid Annotation using Subsystem Technology (RAST, http://rast.nmpdr.org) and GeneMarkS [17, 18]. All predicted ORFs were manually verified by performing searches against the National Center for Biotechnology Information (NCBI) using PSI-BLAST. The Conserved Domain Database (CDD) was used for the putative functional annotation of protein sequences and the identification of proteins with similar domain architectures [19]. Putative transfer RNA (tRNA)-encoding genes were searched using tRNAscan-SE [20]. Prediction of transmembrane helices was performed using TMHMM 2.0 [21]. An online BLASTn search was used to calculate the percentage of DNA identity [22]. Comparisons of phage genomes were made using matrix dot plot-generating computer program Gepard [23]. To understand the phylogenetic relationship between Spp16 and other T7-like phages, three phylogenetic trees were constructed using MEGA version 6 [24]. Terminase etc. sequences were chosen and 30 sequences of different bacteriophages of the subfamily *Autographivirinae* in ICTV virus taxonomy current [25] release were downloaded from the NCBI database. Phylogenetic analysis was carried out using ClustalW and Maximum Likelihood method based on the JTT matrix-based model, with a bootstrap assessment based on 1000 replicates.
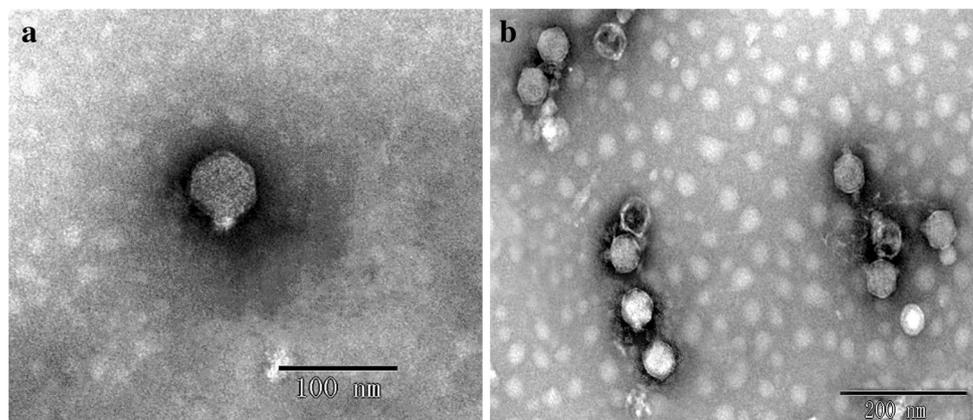
Nucleotide sequence accession number

The complete genome sequence of phage Spp16 with annotation was deposited in the NCBI nucleotide database under the accession number MG878892.

Results

Phage isolation and morphology

A novel bacteriophage Spp16 was isolated and purified from sewage taken from a poultry farm (Qingdao, Shandong Province, China). On 0.7% agar plates, Spp16 formed 1 mm clear plaques. Transmission electron microscopy showed that phage Spp16 possessed an isometric polyhedral head (60 nm in diameter) and a short tail (10 nm in length) (Fig. 1). According to the current International Committee on Taxonomy of Viruses classification system, Spp16 was classified as belonging to the family *Podoviridae* (order *Caudovirales*).

Fig. 1 Transmission electron microscopy of phage vB_SpuP_Spp16

Biological characteristics of Spp16

One-step growth curve experiment showed the phage Spp16 infection process took approximately 90 min, with a 40-min latent period and a 50-min burst period (Fig. 2a). The burst size was approximately 35 pfu/cell after 90 min. In thermostability test, phage Spp16 activity stayed at the similar high level at temperatures ranging from 4 to 60 °C. When heated to 70 °C, the titer of phage Spp16 rapidly decreased with no viral particles being detected after 60 min (Fig. 2b). The phage was relatively stable over a broad pH range (pH 4–10), which declined dramatically at pH 2 (Fig. 2c).

Host range

Host range was assessed by the ability of Spp16 to form plaques upon a library of Salmonella consisting of 30 different isolates. Spp16 can infect only *Salmonella enterica serovar pullorum*; other serovar were resistant to Spp16. For

Salmonella enterica serovar pullorum, Spp16 was able to form plaques on 7 of the 22 isolates tested (Table S1).

Characterization of the phage Spp16 genome

The phage Spp16 possesses a linear dsDNA genome of 41,832 bp with a G+C content of 39.46%. In total, 53 putative open reading frames (ORFs) were predicted. Based on the results of BLASTp and Conserved Domain Database (CDD) analysis, 20 ORFs were annotated to be functional proteins, and other ORFs shared homology with genes encoding hypothetical proteins in several bacteria or phages (Table S2). No tRNA genes were identified. All the ORFs were detected on the reverse strand, accounting for 93.6% (39,144 bp) of the total genome. The average ORF length was ∼ 740 bp. Of the 53 ORFs, 49 initiated with the start codon ATG, three with GTG, and one with TTG. The annotated proteins were then categorized into five functional groups (Fig. 3). These included

Fig. 2 One-step growth curve (**a**), thermostability (**b**) and pH stability (**c**) of phage vB_SpuP_Spp16

Fig. 3 Genome map of the phage Spp16. Arrows indicate predicted open reading frames (see legend for description)

DNA replication and modification (DNA primase, DNA helicase, DNA ligase, DNA polymerase I, HNH homing endonuclease, DNA exonuclease, putative HNH endonuclease, DNA endonuclease); transcriptional regulation (phage RNA polymerase); phage structural and packaging proteins (major capsid protein, phage capsid and scaffold, head–tail connector protein, tail tube protein A, tail tube protein B, tail fiber protein, small terminase subunit, large terminase subunit), and proteins involved with host lysis (phage lysin, lytic transglycosylase, holin). These compact modules, similar to T7-like phage, suggested that phage Spp16 may be a new member of the T7 family of phages.

In the DNA replication and modification module, the DNA primase gene was ORF 15, and the helicase gene was ORF 16. ORF 20 was predicted to be a phage DNA polymerase containing a 5′-3′ polymerase domain whose primary function was to fill DNA gaps generated during DNA repair, recombination and replication. The phage Spp16, like all other phages from the subfamily of the *Autographivirinae* contained a single T7-like RNA polymerase (ORF31) gene necessary for self-replication. All known phage-encoded transcriptases belong to the ssR-NAP family. Most double-stranded DNA bacteriophages use host RNAP throughout the infection and regulate their function to allow for the ordered expression of phage genes [26]. Some bacteriophages such as T7-like phage use the host RNAP to transcribe the phage's own RNAP gene at the initial stage of infection, and then rely on bacteriophage-encoded RNAP to transcribe late phage genes [27]. The packaging module of Spp16 phage contained ORF46 and ORF47, which encode the large terminase subunits and small terminase subunits, respectively. The large terminase subunit shares high amino acid sequence similarity with *Yersinia* phage vB_YenP_ISAO8. Normally, these two proteins work together as a complex, with the small terminase subunits determining the specificity of DNA packaging, while the large terminase subunit cuts phage DNA and packages it at the prohead [28]. The host lysis functional proteins are located behind the structural proteins. Lysin is a phage protein that degrades the bacterial host cell wall before phage release and has been reported as the best example of a bacteriophage antimicrobial agent [29]. ORF49 encoded one of the phage lysis enzymes and had 61% identity to a lysozyme from *Yersinia* phage phi80-18. A holin is a small phage-encoded protein that forms pores in the cytoplasmic membrane during the infection, where it helps lysin to enter the periplasm [30]. Holins can be categorized into three subtypes: class I, class II, and class III [31]. The holin protein of phage Spp16 had two potential membrane-spanning domains and belongs to class II and shares 41% identity with *Pectobacterium* phage PP2.

Genomic termini identification

Previous studies have demonstrated that the genomes of T7-like phages have direct terminal repeat sequences generated by terminases [32]. We could find which reads are located at the genome termini, given the large volume of sequence data. Thus, We have previously proposed that the reads with high frequency represent the termini of the sequenced genome based on NGS data [16]. To identify the termini of the Spp16 genome, we calculated the occurrence of each unique sequence and mapped the raw sequence reads onto the assembled Spp16 genome. The results revealed that two types of reads with extremely high frequency were mapped at the end of the assembled genome (Fig. S1). The read average frequency occurrence was calculated to be 4.2 [(total reads)/(genome length × 2 (forward and reverse direction) 350,554/(41,832 × 2)]. Thus, the ratios of the highest forward and reverse frequencies versus the average frequency were 131.9(554/4.2) and 113.1(475/4.2), respectively, which further suggested that these HFSs were the phage termini. The two high frequency reads were 421 bp apart (length = reverse termini position–forward termini position), indicating the phage Spp16 has redundant termini with 421 bp direct terminal repeats.

Phylogenetic and comparative genomic analysis

Using the BLASTn program at NCBI to analyze the whole-genome sequence, the genome of phage Spp16 had the highest sequence identity of 69%, with *Yersinia* phage phi80-18 and *Yersinia* phage fHe-Yen3-01, with 33% and 32% genome coverage, respectively. RNA polymerase is the most significant protein with high conservation [33]. The large subunit, which is a key component of the terminase holoenzyme, is involved in translocation of the cleaved DNA into the empty prohead to ensure the encapsidation of one single genome [34]. Because the packaging reaction catalyzed by terminase is highly specific, terminase was used to construct phylogenetic relationships and to decipher the evolutionary relationships among phages belonging to different families [35]. Thus, phylogenetic analysis of Spp16 indicated that the phage was a novel bacteriophage and formed a distinct branch of the subfamily *Autographivirinae* (Fig. 4). A comparative genomic analysis of the phage spp16 with the genomes of closely related phage phi80-18, fHe-Yen3-01, phiAS7, GAP227 and phiR8-01 revealed similar length (41.8 kb for Spp16 with 421 bp terminal repeats, 42.4 kb for phi80-18 with 385 bp terminal repeats, 41.5 kb for phiAS7 with 147 bp terminal repeats, 42.7 kb for fHe-Yen3-01, 41.7 kb for GAP227 and 42 kb for phiR8-01 with 385 bp terminal repeats) and the phage Spp16 G+C content is much lower than other phages(39.5% for spp16, 47.6% for phi80-18,
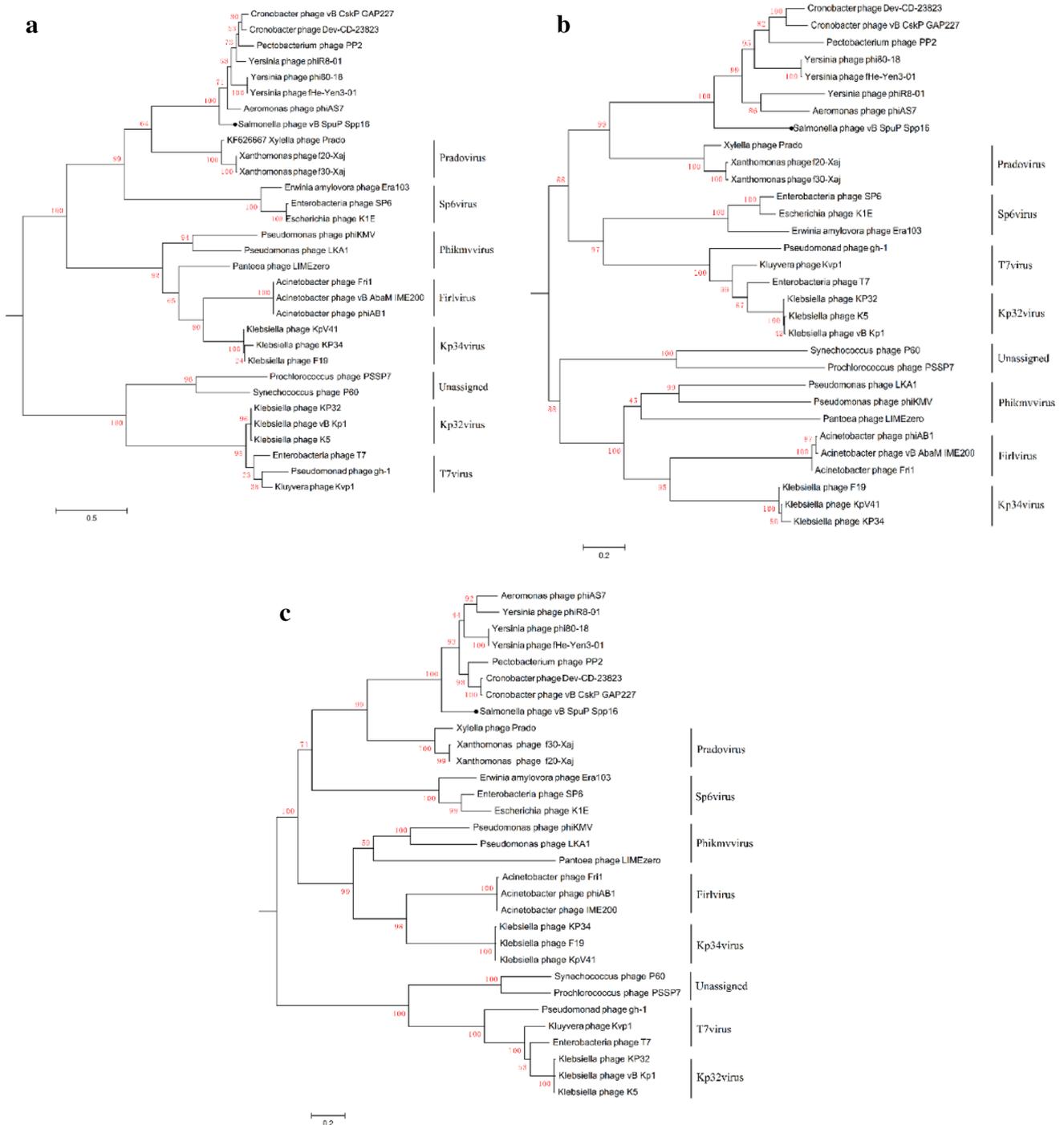
Fig. 4 Phylogenetic analysis of vB_SpuP_Spp16. Phylogenetic trees were used the Maximum Likelihood method with 1000 bootstrap replicates. Major capsid **(a)**, RNA polymerase **(b)** and terminase large subunit **(c)** amino acid sequences of the investigated phage and other phages that are classified within the subfamily *Autographivirinae* were aligned in the MEGA6

56.9% for phiAS7, 47.7% for fHe-Yen3-01, 55.7% for GAP227 and 51.8% for phiR8-01). The 53 ORFs found in Spp16 are organized in modules similar to these phages but the primary sequences differ, showing a maximum amino acid identity range from 36 to 78% (Fig. 5). The

phage tail fiber protein encoded by ORF14 shows a greater divergence between these phages (20–42% coverage and 42–56% identity), which is associated with host specificity. Comparative genomic analysis of the *Enterobacteriaceae* T7-related phages has been reported previously,
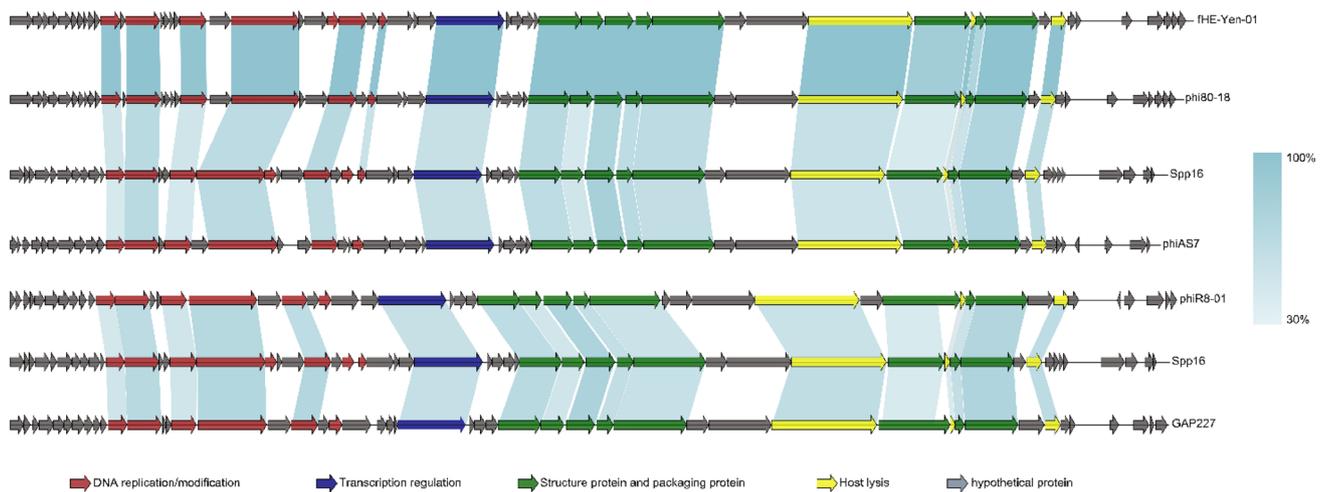
Fig. 5 Comparative genome map of phage Spp16, closely related phage phi80-18, fHe-Yen3-01, phiAS7, GAP227 and phiR8-01. Genes are displayed as colored arrows. Blocks connecting the compared genomes correspond to homologous regions in both genomes as determined using BLASTp
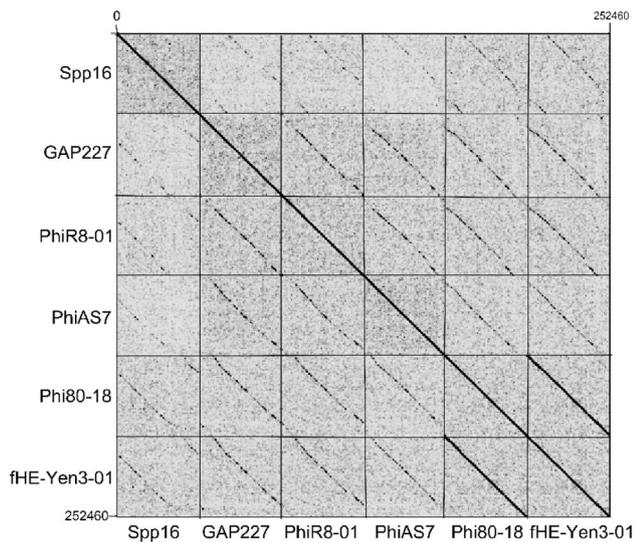

Fig. 6 Dot plot analysis of the genome sequence of phage Spp16 and those of selected phages belonging to the GAP227-like cluster in the *Autographivirinae* subfamily. A continuous diagonal line indicates the alignment of the genome sequence with itself. The center slash indicates the similarity and synteny between the two phage genomes

these phages naturally form six clusters with relatively few nucleic acid sequence similarity, including T7-like, SP6-like, KP34-like, GAP227-like, LIMEzero and phiKT-like cluster [36]. Based on dot plot analysis, the definition of such a cluster is that > 50% of the nucleotide sequence of the genome is identifiably similar and is in line with other members of the cluster [36]. A genomic dot plot matrix analysis of Spp16 and GAP227-like phages was constructed and the result showed the Spp16 was defined

a new subcluster within the GAP227-like cluster in the *Autographivirinae* (Fig. 6).

Conclusions

Due to its natural antibacterial properties and the effectiveness of controlling bacterial pathogens, phage therapy has been proposed to replace antibiotics in humans and veterinary medicine [37]. In this study, we have isolated and characterized a novel lytic phage vB_SpuP-Spp16 which belongs to the family *Podoviridae*. The phage Spp16 is very stable maintaining high titers for a long period of time at 4 °C and remaining active when heated to 50 or 60 °C. In addition, phage activity was relatively stable over a wide pH range of 4–10. This stability would be very advantageous for the scale production and long-term storage of phage preparations.

To investigate the evolutionary relationship of the phage Spp16, the constructed phylogenetic tree using Spp16 and other representative phages of the *Autographivirinae* suggested that the phage Spp16 is novel and does not belong to any known genus in the *Autographivirinae*. Along with other *GAP227-like* cluster phages, Spp16 remains unclassified according to the 10th report of the International Committee on Taxonomy of Viruses (ICTV), suggesting the need to establish a new genus.

In conclusion, our data demonstrates that the phage Spp16 is classifiable as a novel member of the *GAP227-like* phage in the *Autographivirinae*. The Spp16 genome has all the necessary core genes for its own replication, phage reconstitution, and host lysis. No genes associated with pathogenicity, virulence and lysogeny (e.g. integrase) were identified. In addition, its lytic activity suggest that this

bacteriophage may be a candidate novel biocontrol agent for the prevention and control of *Salmonella* in food. Furthermore, complete genomic analysis of this bacteriophage provided new insights into the relationships between related *Salmonella* bacteriophages. Our findings therefore provide basic data for further research on the interaction between the *Salmonella* phage and their hosts. Further research will determine whether Spp16 will be useful in the prevention and treatment of *Salmonella enterica serovar pullorum.*

Author contributions Yigang Tong and Huiying Ren conceived and designed the experiments and critically evaluated the manuscript. Feiyang Zhao carried out the data analysis and wrote the manuscript. Guangqin Liu isolated and identified the phage. Huzhi Sun and Xiangying Zhou carried out the experiments. Manli Li analyzed the phage sequences. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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