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ORIGINAL PAPER

Biological properties and genomics analysis of vB_KpnS_GH-K3, a *Klebsiella* phage with a putative depolymerase-like protein

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Abstract

Bacteriophages have been recently revisited as an alternative biocontrol tool due to the limitations of antibiotic treatment. In this study, we reported on the biological characteristics and genomic information of vB_KpnS_GH-K3 (abbreviated as GH-K3), a *Klebsiella* phage of the *Siphoviridae* family, which was previously isolated from a hospital sewage system. One-step growth curve analysis indicated that the burst size of GH-K3 was 291 PFU/cell. GH-K3 maintained a stable titer in a broad range of pH values (6–10) and temperature (up to 50 °C). Based on bioinformatics analysis, GH-K3 comprises of 49,427 bp containing a total of 77 open reading frames (ORFs), which share high degree of nucleotide similarity and close evolutionary relationships with at least 12 other *Klebsiella* phages. Of note, GH-K3 *gp32* was identified as a unique ORF. The major segment of gp32 sequence at the C-terminus (residues 351–907) was found highly variable as determined by its mismatch with the nucleotide and protein sequences available at NCBI database. Furthermore, HHpred analysis indicated that GH-K3 gp32 contains three domains (PDB ID: 5W6S_A, 3GQ8_A and 1BHE_A) similar to depolymerase (depoKP36) of *Klebsiella* phage KP36 suggestive of a potential depolymerase activity during host receptor-binding in the processes of phage infection. Altogether, the current data revealed a novel putative depolymerase-like protein which is most likely to play an important role in phage-host interaction.

Keywords Bacteriophage · *Klebsiella pneumoniae* · Genome sequencing · Depolymerase · Tail spike · Capsular polysaccharides

Introduction

Klebsiella pneumoniae, the world's second most common nosocomial infection-causing-pathogen, often causes pneumonia, liver abscess, urinary system symptoms, and even systemic blood infections [1]. The majority of *K. pneumoniae* strains can synthesize and secrete capsular

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Ruopeng Cai and Zijing Wang have contributed equally to this study.

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polysaccharides (CPSs), and these strains confer high virulence mainly by blocking antibiotic penetration, promoting pathogen colonization and evading host immunity [2]. The widespread prevalence of multidrug-resistant (MDR) *K. pneumoniae*, such as carbapenemase-producing and extended spectrum β-lactamase (ESBL)-producing strains, further brings threat to the public health [3, 4]. Infections caused by such MDR *K. pneumoniae* pathogens are difficult to treat by antibiotics raising the need for new alternative therapies.

The application of bacteriophages (phages) for the treatment of bacterial infections emerged in the early 20th Century, but gradually declined at the advent of antibiotics [5]. However, with the threat of a post-antibiotic era, bacteriophages have caught renewed attention for applications in diagnostics, therapeutics, and decontaminating antibiotic-resistant pathogenic bacteria [6–8]. Phage therapies have excellent curative effects against *K. pneumoniae*-triggered infections without any known side effects [9]. Whereas, for the treatments of multiple *Klebsiella* infections or even various bacterial mixed infections, therapeutic effectiveness of phages is usually limited due to restricted host range (the bacterial genera, species, and strains that a phage can recognize or lyse) of phages [10]. In addition, phage resistance limits the development of phage therapies [11]. To some extent, phage cocktail therapy can overcome these deficiencies [12], but, it is difficult to cure clinical infections fundamentally caused by *K. pneumoniae* for which at least 79 serotypes are known at the present [13, 14]. However, lysis cassette proteins (holins, endolysins, spanins) from phages have been shown to exhibit a broad spectrum bactericidal or attenuating effects, and rarely induce bacterial resistance mutations [15]. Additionally, phage-derived depolymerases also show potential as a new line of anti-virulence agents. Depolymerases specifically degrade CPSs of bacteria by recognizing the glycosidic bonds and releasing the repeating units of the sugar polymer [16]. So far, depolymerases have been shown to rapidly identify microbial capsule types [13], remove and inhibit biofilm [17], and reduce bacterial virulence [18]. With the popularity of next-generation gene sequencing and molecular cloning technologies, more and more studies on phage-derived depolymerases have brought a new dawn to the treatment of bacterial infections [5].

vB_KpnS_GH-K3 (abbreviated as GH-K3), a *Klebsiella* phage of *Siphoviridae* family, which forms large, clear, and round plaques with haloes on the lawn of *K. pneumoniae* K7, was isolated in our previous study [12, 19]. Outer membrane protein C (OmpC) has also been confirmed as adsorption receptor for this phage [19]. In this work, the biological characteristics of GH-K3, such as host range, one-step growth curve, stability studies, and bioinformatics analysis are reported. Furthermore, we report on the characterization of gp32, a putative depolymerase-like protein of GH-K3. Thus, our results paved the way for subsequent studies of GH-K3 derived depolymerase effective against *K. pneumoniae*.

Materials and methods

Bacterial strain and bacteriophage

K. pneumoniae K7 [12, 19] was isolated from a clinical specimen and was maintained in our laboratory. The strain was routinely cultured in Luria–Bertani (LB) broth at 37 °C on orbital shaker. Phage GH-K3 was isolated from sewage water samples using *K. pneumoniae* K7 as host strain in our previous study [12]. The counting and purification of phage was performed by double-layer agar plate method [20]. The purified phage was then amplified and stored at either 4 °C or at − 80 °C in glycerol (3:1 [*v*/*v*]) [20].

Host range determination

The host range of the phages was tested against 72 clinical strains, preserved in our laboratory, as determined by spot testing and plaque assay [21]. Briefly, 5 µL of purified phage suspensions (10^{10} PFU/mL) were spotted onto freshly seeded lawns of the strains and left to dry before incubation for 6 h at 37 °C. Meanwhile, according to double-layer agar plate method, all the strains were analyzed by plaque assay at the titer of 10^5 PFU/mL. Additionally, an efficiency of plating (EOP) assay was also performed among phage-sensitive strains as previously described [22].

Growth characteristics of the phage

Multiplicity of infection (MOI) refers to the ratio of phage to host bacteria during the processes for infection [23]. *K. pneumoniae* K7 culture was grown to log phase and transferred to fresh LB broth at a final concentration of 2×10^7 CFU/mL ($OD_{600} \approx 0.4$). Phage GH-K3 was then added at different multiplicity of infections (MOIs) (phage/bacteria = 0.00000001, 0.0000001, 0.000001, 0.00001, 0.0001, 0.001, 0.01, 0.1, 1, 10, and 100) and the mixtures were incubated for 10 h. The phage titers of samples were then determined immediately by plaque assay after serial diluted [24].

For one-step growth curve determination, GH-K3 was added to an exponential phase K7 culture (1×10^7 CFU/mL) at an MOI of 0.1, and allowed to adsorb for 10 min at 37 °C. The mixture was then centrifuged at 12,000×*g* for 5 min at 4 °C, and the pellet was resuspended in 10 mL of LB broth. After that, the suspension was incubated at 37 °C with shaking at 180 rpm. Samples were collected at 5 min intervals until 120 min. Finally, the titers of the lysates were quantified by plaque assay [25].

Stability studies

To measure stability characteristics of GH-K3, survival rate was determined after treatment with diverse ranges of pH and temperature. Briefly, for pH stability tests, aliquots of phage suspensions were incubated at 37 °C (pH 1–14) for 1 h. For thermal stability tests, aliquots of phage suspensions were incubated at 4, 25, 37, 50, 60, 70, and 80 °C for 1 h, samples were taken at 20 min intervals until it was up to 80 min. Moreover, in order to test phage stability after long-term storage, aliquots of phage suspensions were stored at 4 °C for 1 year. All the samples after treatment were diluted and tested immediately by double-layer agar plate method.

Concentration and purification of the phage

For extraction of genomic DNA, the phage was concentrated and purified as previously described with few modifications [26]. Briefly, GH-K3 was propagated at large scale (1 L) and the lysates were centrifuged twice at 4000 rpm for 20 min at 4 °C. Next, DNase I and RNase A were added to the supernatants (1 µg/mL) followed by incubation for 30 min at room temperature. This was followed by addition of 1 M NaCl, and the supernatants were then put on ice bath for 1 h followed by addition of polyethylene glycol 8000 (PEG8000) (10% (*w/v*). The mixture was incubated at ice temperature overnight, and phage particles were collected by centrifugation (10,000 rpm, 10 min) and resuspended in Saline-Magnesium (SM) buffer. Finally, the phage suspensions were purified by chlorinated cesium (CsCl) density gradient ultracentrifugation (CsCl gradients: 1.32, 1.45, 1.50, and 1.70 g/mL) and then preserved at 4 °C.

Sequencing and analysis of the phage genome

The phage genomes were extracted from the concentrated phage as purified above using a Viral DNA Kit (Omega Bio-Tek Inc., Doraville, GA, USA) and was sequenced by an Illumina HiSeq 2500. Roche Newbler v.2.8 was used for sequence assembly [27]. Genes encoding tRNA and rRNA were identified by tRNAscan-SE [28] and RNAmmer [29], respectively. ORFs within phage genome were predicted by GeneMarkS (Georgia Institute of Technology, Atlanta, GA, USA) [30]. After searched and aligned through PSI-BLAST (Threshold = 0.005) at non-redundant protein sequence database (nr) of National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the functions of encoded regions in GH-K3 were annotated by the functions of the two most similar sequences [31]. The domain analyses were performed by HHpred at MPI bioinformatics Toolkit (https://toolkit.tuebingen.mpg.de/#/tools/hhpred) [32]. The schematic diagram of the gene functional module map was generated by CLC Main Workbench, version 8.0.1 (CLC Bio-Qiagen, Aarhus, Denmark). Once the gene sequences of terminase large subunit genes and alleles of GH-K3 *gp32* were aligned by ClustalW, phylogenetic trees were constructed by Neighbor-Joining Method (100 bootstrap replicates) through PHYLIP version 3.697 [33]. Global genome comparisons were launched using Mauve 2.3.1 [34]. Additionally, structural analysis was performed by I-TASSER [35].

Statistical analysis

GraphPad Prism 5 (GraphPad Software, Inc., CA, USA) was utilized for all statistical analyses. P values < 0.05 were considered statistically significant. Error bars represent standard deviation of the mean.

Results

The host range of GH-K3

Of the 72 *K. pneumoniae* strains screened, phage GH-K3 could form clear spots on the lawn of 9 strains including K7, indicating that these strains were sensitive to this phage. In our previous study, all of these nine strains were identified to possess K2 serotype CPS [19]. However, besides K7, phage GH-K3 could only form plaques on five of these strains, including KP1, KPP6, KPP7, KPP27, and KPP41 (Table S1). But the EOP of these strains was found much lower than that of K7 (Table 1). Additionally, plaques formed by GH-K3 on the lawns of *K. pneumoniae* K7 and KPP6 were surrounded by enlarged haloes after prolonged incubation (Fig. S1), suggesting that the phage most likely encodes a depolymerase with polysaccharide-degrading activity.

Table 1 Sensitivity of *Klebsiella pneumoniae* to phage GH-K3

ID	Spot	Plaque	EOP (%)
K7	+	2–3 mm with halo	100 ± 6.14
KP1	+	1 mm without halo	0.06 ± 0.02
KPP6	+	1 mm with halo	1.02 ± 0.13
KPP7	+	1–2 mm without halo	0.62 ± 0.24
KPP27	+	< 1 mm without halo	0.67 ± 0.07
KPP41	+	1–3 mm without halo	1.20 ± 0.11
KPP14	+	–	
KPP36	+	–	
KPP51	+	–	

+ GH-K3 can form spot or plaque, – GH-K3 cannot form spot or plaque

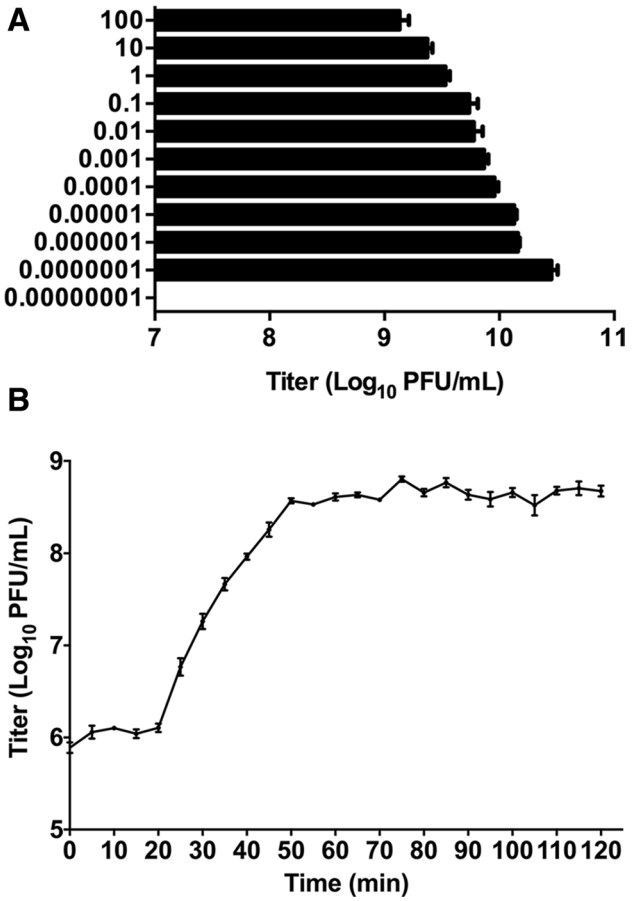

Fig. 1 The growth characteristics of GH-K3. **a** Titers of the phage under different MOI (phage/bacteria = 0.00000001, 0.0000001, 0.000001, 0.00001, 0.0001, 0.001, 0.01, 0.1, 1, 10, and 100), as indicated in the *Y*-axis. At the MOI of 0.0000001, GH-K3 reached maximum titers. **b** One-step growth curve of GH-K3 was carried out at MOI = 0.1. Each data is shown as mean ± SD from three biological experiments

The growth characteristics of GH-K3

When the MOI was 0.0000001, the phage titer of GH-K3 after propagation was found be the highest with approximate titer of 2.9×10^{10} PFU/mL (Fig. 1a). One-step growth curve experiments highlighted the latent period of GH-K3 was approximately 20 min, after which there was a rapid increase in the number of released viral particles. It took about 30 min for GH-K3 to reach the growth plateau phase with a burst size of approximately 291 PFU/cell (Fig. 1b).

Stability studies

The phage stability of GH-K3 at various pH values and temperatures were determined. The activity of GH-K3 was relatively stable at pH 6–10 (phage survival rate > 85%). The phage titers however decreased sharply at the acidic (pH 1–4) or alkaline (pH 12–14) conditions (Fig. 2a). High titers (10^9–10^{10} PFU/mL) were maintained between 4 and 37 °C, the activity of GH-K3 was decreased when the temperature was ≥ 50 °C (Fig. 2b). Additionally, the titer of GH-K3 almost did not change after 1 year of storage at 4 °C (Fig. 2c).

Bioinformatics analyses of GH-K3 genome

The genome of GH-K3 was sequenced and analyzed. The coverage of this genome was 100% and the average depth of sequencing was 2889.73. GH-K3 genome contains 49,427 bp, a coding density of 95.94%, and a G + C content of 50.2% including 77 coding proteins ranging from 37 to 1267 codons. Eighteen of these ORFs were positive stranded, while the others were negative stranded. The initiation codons of this phage include 93.5% ATG, 5.2% GTG,

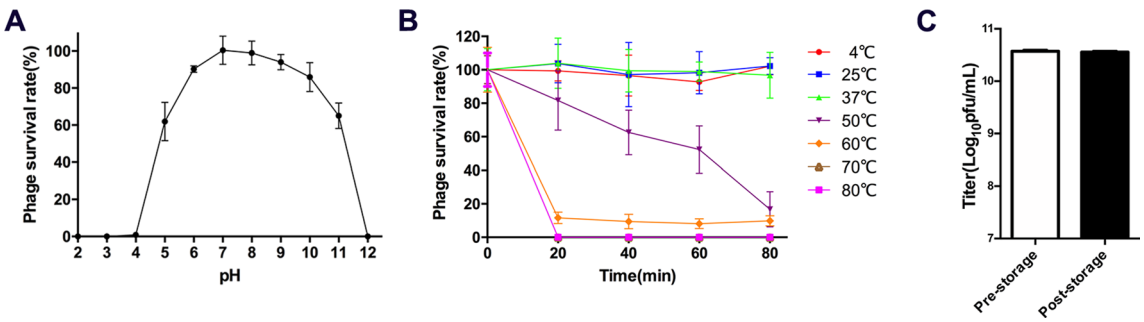

Fig. 2 Stability tests of GH-K3. **a** pH stability: phage particles were incubated under different pH conditions for 1 h. **b** Thermal stability: phage particles were incubated at various temperatures as indicated for 1 h. Then the samples were collected after 20, 40, 60, and 80 min. Phage survival rate = (titer after incubation)/(initial titer). **c** Long-term storage stability: initial titer (white column) and titer after storage for 1 year (black column) of GH-K3. Each data is shown as mean ± SD from three biological experiments

and 1.3% TTG. At the ends of GH-K3 genes, 54.5% TAA, 28.6% TGA, and 16.9% TAG serve as stop codons. Thirty-one of these ORFs (40.3%) show identity with proteins in the database with known functions, including nucleotide metabolism and replication (gp8, gp12, gp17–19, gp23, and gp25–31), DNA packaging (gp52 and gp53), morphogenesis (gp33–42 and gp49–51), and host lysis (gp14–16) (Fig. 3 and Table S2). However, no phage lysogeny modules, antibiotic-resistant genes, or putative virulence factors were detected in the genome of GH-K3.

At the nucleotide level, GH-K3 phage genome shows high identity to the genomes of *Enterobacter* phage F20 [36] and 12 *Klebsiella* phages, including phage 1513 [37], phage KP36 [38], phage KLPN1 [39], phage KOX1 [40], phage PKP126 [41], and phage Sushi [42], etc., which can be classified as a member of "*KP36likevirus*" genus [38]. However, phylogenetic analysis of the terminase large subunits revealed that GH-K3 has a close evolutionary relationship with a variety of *Klebsiella* phages, such as KLPN1, KP36, and 1513 (Fig. 5a). Interestingly, a fragment of more than 3000 bp in each homologous genome had extremely low sequence similarity (Fig. 4). The G + C content of these mismatched loci were found approximately 40%, lower than that found at the whole-genome level (approximately 50% G + C content, Table 2).

Our analysis indicates that *gp32* of GH-K3 is located in the nonmatching site described above (18,124 bp–20,847 bp) adjacent to the gene that encodes for putative tail fiber (Fig. 3). The N-terminus of gp32 has relatively high homology (47–88%) with the N-terminus of proteins derived from other homologous phages. However, a large part of the GH-K3 gp32 C-terminus (residues 351–907) shows very low similarity with the data available at NCBI. GH-K3 gp32 has a relatively distant evolutionary relationship with homologous alleles of other phages (Fig. 5b). Moreover, the C-terminus of gp32 and homologous proteins are highly variable and have extremely low identity with each other (Fig. 6). These may indicate that *gp32* and homologous alleles were derived from the late evolutionary stage during horizontal gene exchange processes [43].

With predominantly β-helical structures, GH-K3 gp32 shows some identity with domains such as tail spike

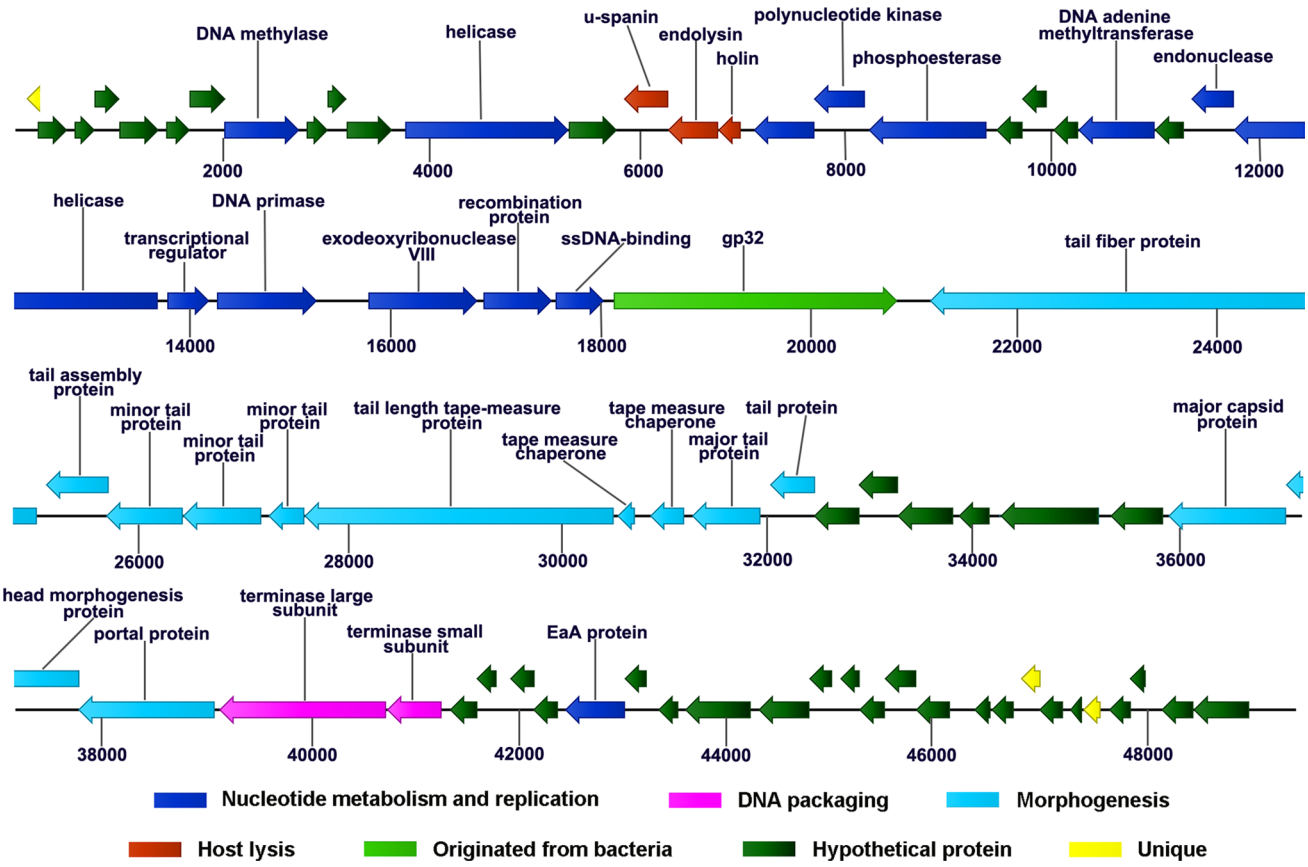

Fig. 3 Graphical representation of the phage GH-K3 genome. Schematic of the phage genome with predicted open reading frames (ORFs) indicated by arrows with different colors. The arrow represents the direction of transcription. The genome map was generated using CLC Main Workbench (version 8.0.1)

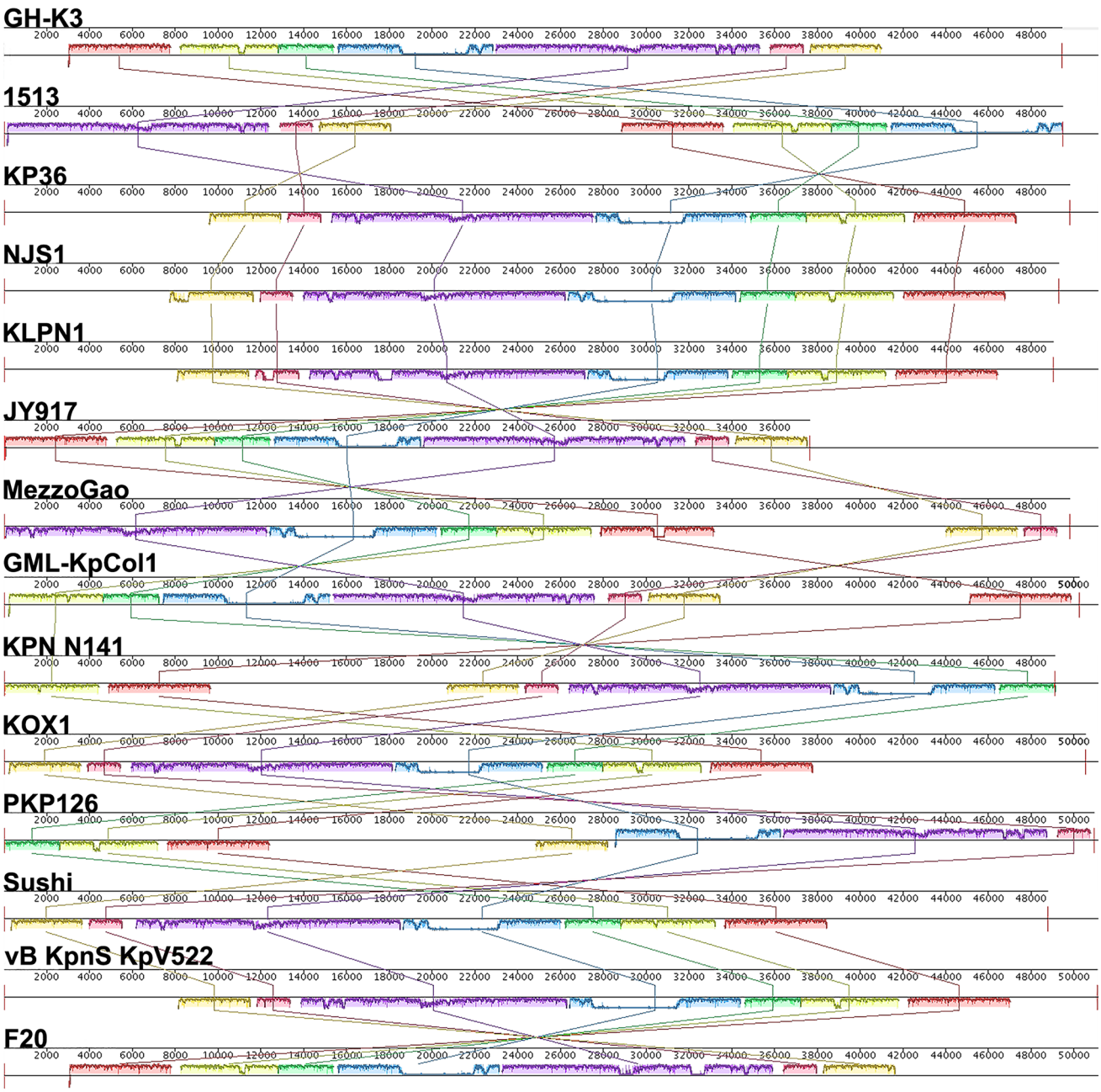

Fig. 4 Multiple genome alignments among GH-K3 and homologous phages. This figure was generated using Mauve software. Similarity is represented by the height of the bars, which correspond to the average level of conservation in that region of the genome sequence. Completely white regions represent fragments that were not aligned or contained sequence elements specific to a particular genome. Boxes with identical colors represent local collinear blocks (LCBs), demonstrating homologous DNA regions shared by two or more genomes without sequence readjustments

(residues 183–565, PDB ID: 5W6S_A), pre-neck appendage protein (residues 273–595, PDB ID: 3GQ8_A) and polygalacturonase (residues 265–583, PDB ID: 1BHE_A). Interestingly, depoKP36, the depolymerase of *Klebsiella* phage KP36 also exhibits low homology to the above mentioned domains [38] (Fig. 7), suggesting that GH-K3 gp32 may also be a depolymerase.

Discussion

In the present work, the biological properties and genomics of the phage GH-K3 were investigated. GH-K3 was identified as a virulent phage with high lysis efficiency against K2 capsular type *K. pneumoniae*. Moreover, the phage cocktail containing GH-K3 reduced the number of *K.*

Table 2 Global genome comparison of GH-K3 with homologous phages

	GH-K3	1513	KP36	NJS1	KLPN1	JY917	MezzoGao
Host strain type	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*
GenBank number	MH844531.1	KP658157.1	JF501022.1	MH445453.1	KR262148.1	MG894052.1	MF612072.1
G + C content (%)	50.2	50.6	50.7	50.7	50.5	50.4	51.0
Genome size (bp)	49,427	49,462	49,797	49,292	49,037	37,655	49,807
Identity of GH-K3 BLAST*N* (%)	100	97	96	96	95	96	96
Query coverage of GH-K3 (%)	100	91	87	87	83	69	87
	GML-KpCol1	KPN N141	KOX1	PKP126	Sushi	vB_KpnS_KpV522	F20
Host strain type	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	*Klebsiella oxytoca*	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	*Enterobacter aerogenes*
GenBank number	MG552615.1	MF415412.1	KY780482.1	KR269719.1	KT001920.1	KX237515.1	JN672684.1
G + C content (%)	51.0	51.0	51.2	50.7	50.8	50.8	47.9
Genome size (bp)	50,249	49,090	50,526	50,934	48,754	51,099	51,543
Identity of GH-K3 BLAST*N* (%)	95	95	84	89	97	85	84
Query coverage of GH-K3 (%)	89	89	78	86	87	79	75

pneumoniae in the blood from 3.6×10^6 to 3.6×10^1 CFU/mL within 2 h [12]. These indicate that phage GH-K3 has potential application value in rapid diagnosis and treatment of clinical infections. However, physical stability and chemical stability are foundation for phages to use them as clinical antimicrobial preparations [44]. In this work, the titers of GH-K3 were stable in the floating range of conventional pH (6–10) and temperature (< 50 °C), and the titer of this phage can be maintained for a long time at 4 °C. In addition, antibiotic resistance genes or putative virulence factors were not found in this phage by genomics analysis [45–47]. All these factors strongly suggest GH-K3 could be a potential therapeutic phage against multiple *K. pneumoniae* infections.

In addition to biological characteristics, this study also analyzed the genomic information of GH-K3 and a putative depolymerase-like protein, GH-K3 gp32. In spite of C-terminus of GH-K3 gp32 share an extremely low similarity with data published by NCBI, it contains three domains (PDB ID: 5W6S_A, 3GQ8_A, and 1BHE_A) similar to depoKP36. Thus, GH-K3 gp32 may be a novel depolymerase, but exhibits similar function as depoKP36. With the ability of bacterial capsule degradation during the first step of phage invasion, depolymerases promote recognition, endocytosis, and complement-mediated killing of CPS-producing bacteria by immune cells [48]. So far, depolymerases have been identified to improve the survival rate to both wax moth larvae (*Galleria mellonella*) and mice infected with *K. pneumoniae* [18, 38, 49]. Therefore, as a potential depolymerase, gp32 has important research value in combating *K. pneumoniae* infections. Further, HHpred BLAST analysis shows that the homologous proteins of gp32 also contain potential depolymerase domains which are dominated by hydrolases, except for 1513 gp71. Besides, the hydrolase

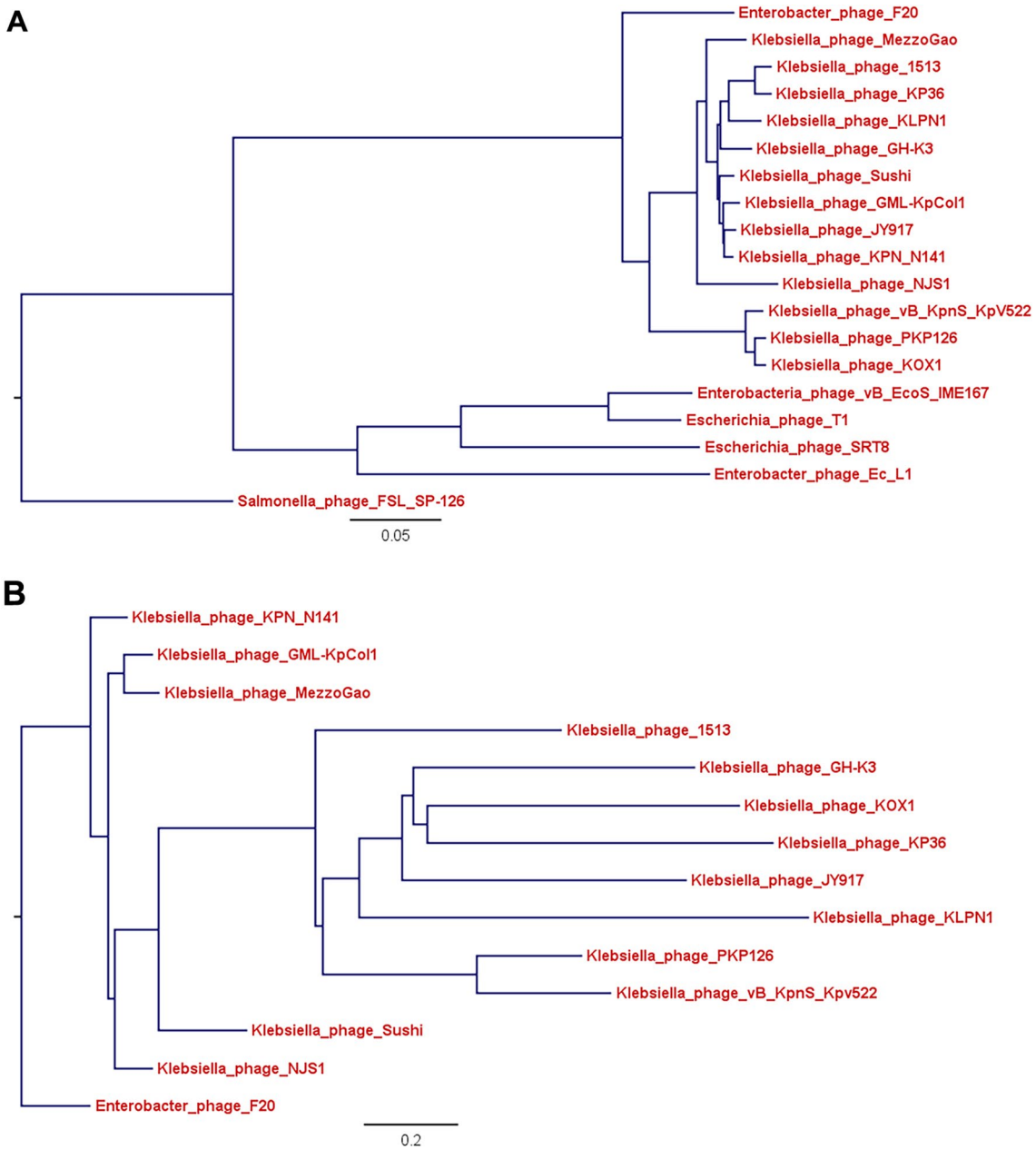

Fig. 5 Bioinformatics analyses of GH-K3 gp32. **a** Phylogenetic analyses based on terminase large subunit genes. **b** Phylogenetic analyses based on alleles of GH-K3 *gp32*. These sequences were compared using ClustalW, and the phylogenetic trees were generated using the Neighbor-Joining method and 100 bootstrap replicates through PHYLIP version 3.697

domains of Sushi gp24, MezzoGao gp18, NJS1 gp42, GML-KpCol1 gp13, F20 gp32, and KPN N141 gp71 are similar to an endo-N-acetyl neuraminidase of *Enterobacteria* phage K1F (PDB ID: 3GW6_B) (Table S3). Thus, these homologous proteins of gp32 may also exhibit depolymerase activity, and the diversity of these proteins may endow its specificity (target on different capsular types).

Majority of depolymerases have been reported as integral components of phage, encoded by the same or the adjacent ORF of phage structural genes (Mostly the tail fibers, base plates, even the neck or the capsid) [16]. GH-K3 gp32 is the adjacent protein of tail fiber and have a tail spike-like domain, suggesting that the potential depolymerase may also belong to the structural protein of GH-K3. Generally, phage

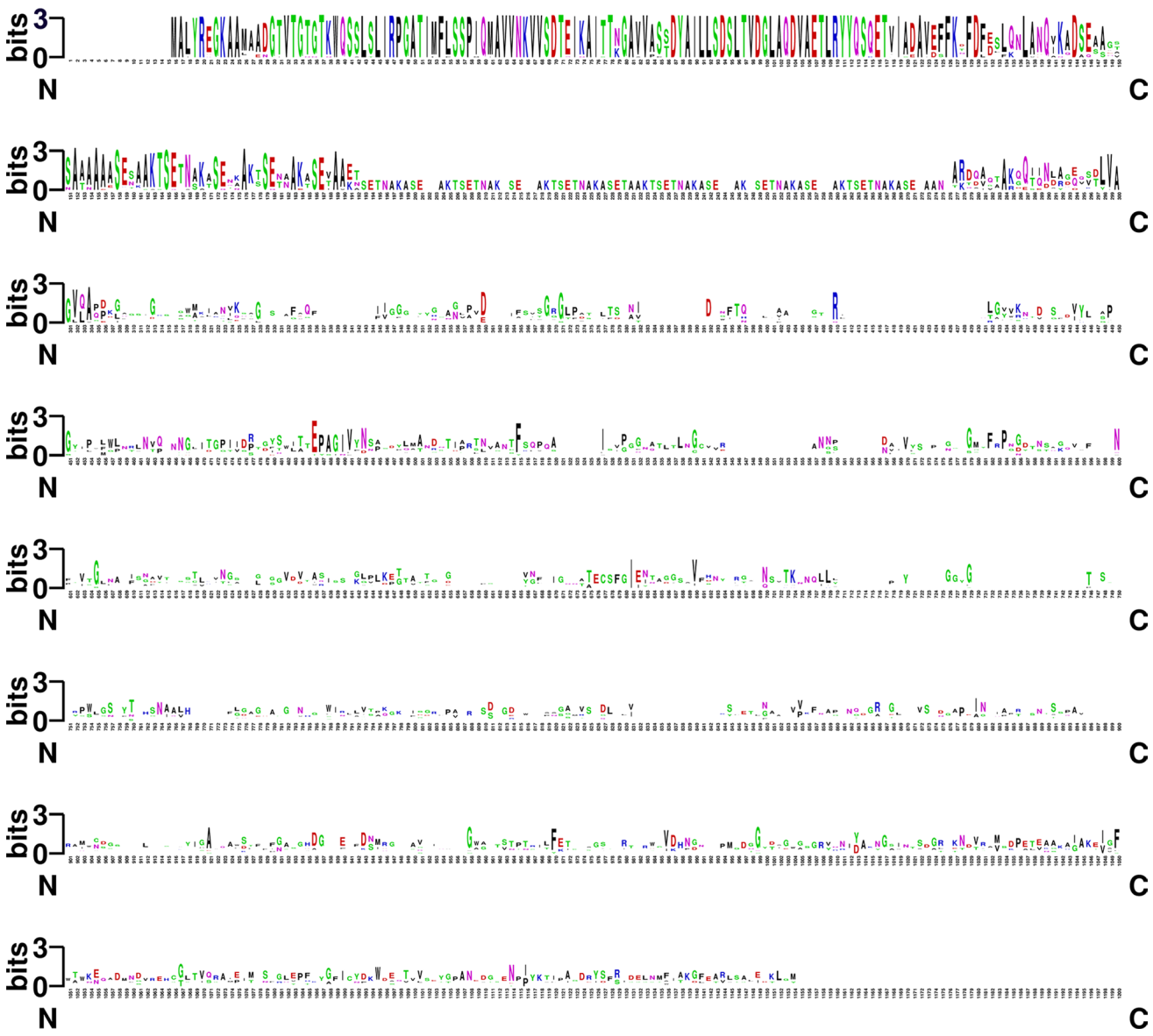

Fig. 6 Sequence conservation of GH-K3 gp32 in comparison with other homologous sequences. GH-K3 gp32, KP36 gp50 (depoP36), JY917 gp23, KXO1 gp23, 1513 gp71, KLPN1 gp43, Sushi gp24, PKP126 gp59, MezzoGao gp18, NJS1 gp42, GML-KpCol1 gp13, vB_KpnS_KpV522, F20 gp32, and KPN N141 gp71 were aligned and shown as sequence logo, which was created online at http://weblogo.berkeley.edu/logo.cgi

tail spike proteins not only digest the cell surface polysaccharides, but also mediate specific binding of phage to the outer membrane proteins [50, 51]. In our previous study, GH-K3 has been confirmed to infect its host by adsorbing onto the cell surface polysaccharides, and then specifically binding to the outer membrane protein C(OmpC) [19], suggesting that gp32 with a tail spike-like domain (PDB ID: 5W6S_A) and may play a key role in host specificity of GH-K3. Furthermore, the phage-encoded protein may also be a key factor of inducing bacterial anti-phage mutation in host bacteria. Thus, the interaction relationships of GH-K3 gp32 and host bacterial proteins are also worthy of further investigation.

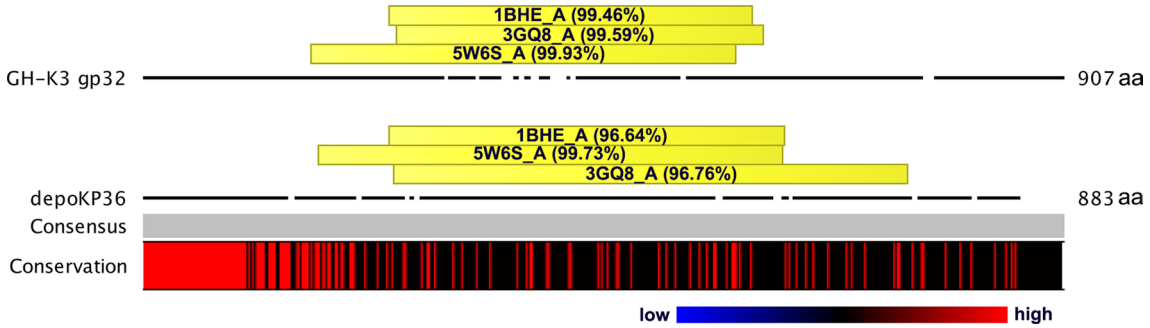

Fig. 7 Amino acid sequence alignments and domain analyses of GH-K3 gp32 and depoKP36. By HHperd analyses (https://toolkit.tuebingen.mpg.de/#/tools/hhpred), both of GH-K3 gp32 and depoKP36 show structural similarity with tail spike (residues 183–565, PDB ID: 5W6S_A), pre-neck appendage protein (residues 273–595, PDB ID: 3GQ8_A) and polygalacturonase (residues 265–583, PDB ID: 1BHE_A). The schematic shows the position and probability of the three domains in the two sequences

Nucleotide sequence

Whole-genome sequence of *Klebsiella* phage GH-K3 has been deposited in NCBI GenBank (accession number: MH844531).

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Author contributions JMG and WYH conceived and designed the study; RPC, ZJW, GW, and SUR contributed to the writing and revision of the manuscript; RPC, ZJW, HZ, MJC, ZMG, YLJ, HYX, XWW, YBX, SUR, CJS, XF, and LCL performed laboratory testing; RPC, ZJW, and YGT contributed to the genome sequencing and analysis. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts 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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