

Research Article

Identification of a phage-derived depolymerase specific for KL47 capsule of *Klebsiella pneumoniae* and its therapeutic potential in mice

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ABSTRACT

Klebsiella pneumoniae is one of the major pathogens causing global multidrug-resistant infections. Therefore, strategies for preventing and controlling the infections are urgently needed. Phage depolymerase, often found in the tail fiber protein or the tail spike protein, is reported to have antibiofilm activity. In this study, phage P560 isolated from sewage showed specific for capsule locus type KL47 *K. pneumoniae*, and the enlarged haloes around plaques indicated that P560 encoded a depolymerase. The capsule depolymerase, ORF43, named P560dep, derived from phage P560 was expressed, purified, characterized and evaluated for enzymatic activity as well as specificity. We reported that the capsule depolymerase P560dep, can digest the capsule polysaccharides on the surface of KL47 type *K. pneumoniae*, and the depolymerization spectrum of P560dep matched to the host range of phage P560, KL47 *K. pneumoniae*. Crystal violet staining assay showed that P560dep was able to significantly inhibit biofilm formation. Further, a single dose (50 µg/mouse) of depolymerase intraperitoneal injection protected 90%–100% of mice from lethal challenge before or after infection by KL47 carbapenem-resistant *K. pneumoniae*. And pathological changes were alleviated in lung and liver of mice infected by KL47 type *K. pneumoniae*. It is demonstrated that depolymerase P560dep as an attractive antivirulence agent represents a promising tool for antimicrobial therapy.

1. Introduction

Klebsiella pneumoniae is ubiquitous as it is found in animals and ecological environment. It causes septicemia, pneumonia, soft tissue infections, urinary tract infections, surgical site infections and catheter-related infections in human, particularly among immunocompromised peoples (Podschn and Ullmann, 1998). Besides, it also causes clinical mastitis in cow (Medrano-Galarza et al., 2012). The increasing carbapenem-resistant *K. pneumoniae* (CRKP) led to the loss of effectiveness of antibiotics against these infections, and made it become one of the most troublesome Gram-negative bacteria in the world (Gu et al., 2018; Pitout et al., 2015; Xu et al., 2017). Thus, the Centers for Disease Control

of the USA designated *K. pneumoniae* as an urgent threat to public health (CDC, 2019; Ventola, 2015).

K. pneumoniae is typically coated with a thick capsule polysaccharide (CPS) layer, which takes part in the evasion of the host defense system (Li et al., 2014). CPS also contributes to biofilm formation (Li et al., 2014; Wu et al., 2011). Capsule removal increases susceptibility to gentamicin *in vivo* (Bansal et al., 2014). From 1926 to 1977, a total of 77 capsular types were identified by serological reactivity tests (Ørskov and Fife-Asbury, 1977). From 2008, some new capsular types, KN1–KN5, have been defined by the CPS gene clusters in *K. pneumoniae* (Hsu et al., 2013; Pan et al., 2008, 2013, 2015, 2017). Capsule locus type (KL) KL47 and KL64 CRKPs are increasingly prevalent in China (Li et al., 2020a;

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Yang et al., 2020). Additionally, a part of ST11-K47 and K64 CRKP isolates are hypervirulent, which poses great challenges to antimicrobial therapy (Gu et al., 2018; Huang et al., 2018; Yang et al., 2020). Under this background, it is an urgent need to seek alternatives to conventional antibiotics to prevent and control severe CRKP infections.

Phage-derived proteins have been valued for controlling infections caused by Gram-positive bacteria, such as holin and endolysin, causing destruction of the inner membrane and cell wall, respectively (Drulis-Kawa et al., 2015; Roach and Donovan, 2015). However, Gram-negative bacteria prevent the access of natural endolysins to peptidoglycan layer because of their outer membranes, thus making the exogenously added endolysins limited (Lim et al., 2014). Hence, as one of phage-derived proteins, depolymerases gained increasing attention, because it can degrade extracellular polysaccharides (Drulis-Kawa et al., 2015). Phage-derived capsule depolymerases, have been considered as alternative antivirulence agents to prevent and control CRKP infections (Liu et al., 2020). Capsule depolymerases showed specificity for various capsular types, due to depolymerase contributed to the recognition and digestion of capsule. To date, various capsule depolymerases have been identified and reported for 24 *Klebsiella* capsular types, K1 (Lin et al., 2014; Pan et al., 2017; Solovieva et al., 2018), K2/K13 (Solovieva et al., 2018), K3, K21 (Majkowska-Skrobek et al., 2018), K5, K8, K30/K69 (Hsieh et al., 2017), K11, K25, K35, K64, KN4, KN5 (Pan et al., 2017), K23 (Gorodnichen et al., 2021), K47 (Liu et al., 2020; Wu et al., 2019), K51 (Blundell-Hunter et al., 2021), K56 and KN3, KN1 (Pan et al., 2019), K57 (N et al., 2020), K63 (Majkowska-Skrobek et al., 2016), KN2 (Hsu et al., 2013). Among them, two depolymerases Dpo42 and Dpo43 encoded by phage IME205 were active against different subsets of strains with a capsular serotype K47 (Liu et al., 2020).

Depolymerase Dep42 derived from phage SH-KP152226 specific for K47 capsule was able to significantly inhibit biofilm formation or degrade formed biofilms. Capsule depolymerases were also demonstrated to be potential anti-virulence agents that act by defeating resistance mechanism of *K. pneumoniae* against the innate immunity (Lin et al., 2014; Majkowska-Skrobek et al., 2018). Depolymerase also increases the lifespan of *Galleria mellonella* larvae (Majkowska-Skrobek et al., 2018) and survival rates of mice infected with *K. pneumoniae* (Hsieh et al., 2017; Lin et al., 2014; Pan et al., 2015; Wang et al., 2019). As more depolymerases were discovered, it will help to use synthetic biology to produce and design phage-derived proteins instead of whole phage particles showing a broader antibacterial spectrum, better tissue penetration, lower immunogenicity and low probability of bacterial resistance in the future (Drulis-Kawa et al., 2015).

In this study, we reported and characterized a *Klebsiella* phage P560 infecting KL47 *K. pneumoniae*, and identified a phage-derived capsule depolymerase specific for KL47 types CPS. Furthermore, we also investigated the potential application of depolymerase in the inhibition of biofilms as well as the prevention and control of CRKP infections.

2. Materials and methods

2.1. Bacteria and growth conditions

A total of 25 different KL types *K. pneumoniae*, including 9 CRKP (Li et al., 2020a) and 7 non-CRKP isolates from human and 9 *K. pneumoniae* from cows, used in this study were listed in Table 1. A total of 24 KL47 *K. pneumoniae* used in this study were listed in Supplementary Table S1. Multilocus sequence typing was used to investigate the sequence types (STs) (Diancourt et al., 2005). KLs were determined by wzi sequencing (Brisse et al., 2013). All isolates were routinely cultured in Luria-Bertani (LB) broth at 37 °C on orbital shaker with 180 rpm. Tryptic soy broth medium (TSB, Merck, Germany) were used to culture bacterial biofilms. Phosphate Buffer Saline (PBS, 0.1 mol/L Na₂HPO₄, 0.15 mol/L NaCl₂, pH 7.2) was used for dilution and wash of *K. pneumoniae* cultures.

Table 1

Host range of P560 and P560dep.

<i>K. pneumoniae</i>	Source	K type	P560 Plaques	P560dep
Kp1	Human	KL25	–	–
Kp3	Human	KL14	–	–
Kp29	Human	KL139	–	–
Kp30	Human	KL64	–	–
Kp32	Human	KL10	–	–
Kp36	Human	KL116	–	–
Kp42	Human	KL47	+	+
Kp55	Human	KL1	–	–
Kp56	Human	KL136	–	–
Kp68	Human	KL19	–	–
Kp71	Human	KL2	–	–
Kp73	Human	KL3	–	–
Kp75	Human	KL108	–	–
Kp76	Human	KL30	–	–
Kp84	Human	KL5	–	–
Kp86	Human	KL54	–	–
Kp225	Cow	KL61	–	–
Kp229	Cow	KL18	–	–
Kp241	Cow	KL6	–	–
Kp243	Cow	KL21	–	–
Kp248	Cow	KL149	–	–
Kp259	Cow	KL117	–	–
Kp269	Cow	KL24	–	–
Kp275	Cow	KL7	–	–
Kp299	Cow	KL34	–	–

+, clear lytic spots or plaques; –, no lytic spots or plaques.

2.2. Morphology of the phage

Phage P560 was isolated from sewage water in a hospital in Suzhou, China using CRKP Kp42 as host strain (Li et al., 2020a). The counting and propagation of the phage was performed by double-layer agar plate method (Adams, 1959). SM buffer [50 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 10 mmol/L MgSO₄ and 0.01% gelatin] was used for the dilution of phages. Phage suspensions were purified and stored as previous methods described (Li et al., 2021). Examination of bacteriophage morphology was performed by transmission electron microscopy (Li et al., 2020b).

2.3. Genome sequence analysis

Phage suspensions were concentrated and purified for genomic DNA extraction as previously described (Sambrook and Russell, 2006). Phage genome was sequenced using the Illumina HiSeq system (Illumina, USA). Sequencing reads were *de novo* assembled using Spades 3.11.1 (Bank-evich et al., 2012). Annotation of the phage genome was conducted by the RAST server (Aziz et al., 2008), followed by manual curation. The whole-genome sequence of phage P560 was deposited in the GenBank database. The closely related phages to P560 were searched using online BLASTn against the NCBI database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Genome alignment of these closely related phages was illustrated using EasyFig to analyze their similarities and differences. Further analysis of the product (putative tail fiber protein, TFP) of ORF43 gene from phage P560 was carried out using the Phyre2 server (Kelley et al., 2015).

2.4. Cloning, expression and purification of putative capsule depolymerase

To generate N-terminal (His)₆-tagged capsule depolymerase proteins, the depolymerase ORF43 gene (including stop codons) were inserted into the pET-28a expression vector (Novagen, Madison, WI, USA) via the *Nde* I and *Xho* I restriction sites. Primers used to amplify the capsule depolymerase gene are: forward, 5'-GGAATTCCA-TATGTTAAACAATCTGAATCAG-3'; and reverse, 5'-CCGCTCGAGT-TATGGACCAATGACCACACC-3'. PCR amplification program was 95 °C

for 3 min, followed by 30 cycles of 95 °C for 15 s, 50 °C for 15 s and 72 °C for 2 min. Then the PCR products were ligated into the pET-28a expression vector after restriction enzyme digestion. The recombinant plasmids were transformed into *Escherichia coli* BL21 (DE3) cells, and expression of recombinant proteins was induced by the addition of 0.3 mmol/L IPTG and the mixture was incubated at 16 °C overnight. The resulting His-tagged protein, P560dep, was purified using nickel beads (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions.

The purified protein was concentrated by centrifugation using a 30-kDa MW cut-off (MWCO) membrane (Thermo Scientific, USA) and stored at –80 °C. The concentration of the purified proteins was determined by the BCA Protein Assay Kit (Thermo scientific, USA). The purified proteins were analyzed in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels stained with Coomassie blue (Thermo Scientific, USA). A molecular weight (MW) standard (10–180 kDa) (Thermo Scientific, USA) was used for SDS-PAGE.

2.5. Activity spectrum of phage P560 and depolymerase

Spot testing often causes an overestimate of the host range (Mirzaei and Nilsson, 2015). Therefore, we used double-layer agar plate test to determine the host range of phage P560. A total of 25 clinical isolates of different KL types mentioned above were used to examine the host range of phage P560.

Log-phase bacterial cultures of *K. pneumoniae* were poured onto an LB soft agar overlay plate (LB with 0.5% agar) to form lawns. Purified P560dep protein was assessed for its polysaccharide-degrading activity with the concentrations from 2 mg/mL to 0.2 ng/mL on Kp42 CRKP strains (10 µL) using spot assay. PBS buffer was used as a negative control. The sensitivity of all 25 different KL types *K. pneumoniae* to P560dep (20 µg/mL) was determined by the single-spot assay.

2.6. Depolymerization activity against capsular polysaccharide

The bacterial capsular polysaccharide was extracted according to a previous method (Domenico et al., 1989). Depolymerization activity of P560dep against KL47 capsular polysaccharides was determined using our previous method (Li et al., 2021). All experiments were performed in triplicate. To investigate depolymerization activity against capsules, transmission electron microscopy was performed according to a previous study (Li et al., 2017, 2021).

2.7. Depolymerase potency against biofilm production

To assess the inhibition effectiveness of depolymerase P560dep against biofilm formation, 100 µL exponential-phase bacteria Kp57 (OD₆₀₀ ~0.4, ~2 × 10⁸ CFU/mL), 20 µL P560dep (4 µg, 0.4 µg, 0.04 µg, 4 ng, 0.4 ng, 0.04 ng and 0.004 ng, respectively), and 80 µL fresh TSB medium was added into each well of 96-well flat-bottomed polystyrene microtiter plates (Sigma-Aldrich, USA) as P560dep-treated groups. 100 µL bacteria mixed with 100 µL fresh TSB medium were used as control group. Then, the plates were incubated for 24 h at 37 °C without shaking. The supernatant in each well was removed carefully and the precipitates were washed twice with 200 µL PBS. The residues were stained with 1% crystal violet as described previously (Wu et al., 2019). Optical density at 595 nm (OD₅₉₅) values was measured using an ELISA microplate reader (Biotek, VT, USA). Each experiment was performed in triplicate.

2.8. Animal inoculation and capsule depolymerase treatment

Five-week-old specific-pathogen-free female BALB/c mice were procured from the Laboratory Animal Center of Yangzhou University (Yangzhou, China). All efforts were made to minimize the suffering of animals. Mice were divided into four groups (10 mice in each group), and

each group was kept in two clear polypropylene cages with free access to a standard antibiotic-free food and water.

In the prevention group, mice were infected with 2.5 × 10⁷ CFU of KL47 *K. pneumoniae* strain (Kp42) administered intraperitoneally after 1 h of inoculation intraperitoneally with 50 µg purified P560dep protein. In the treatment group, mice were infected with 2.5 × 10⁷ CFU Kp42. After 1 h of the bacterial challenge, mice were given 50 µg P560dep by intraperitoneal injection. Meanwhile, two control groups were administered with the same volume of *K. pneumoniae* strain Kp42 plus PBS buffer, and only PBS buffer, respectively. All groups were monitored for 7 days. To assess the effects of a single P560dep injection, heart, liver, spleen, lungs, kidney and brain were collected, and fixed in 4% paraformaldehyde buffer. Samples were dehydrated with a dehydrator, embedded in paraffin, and stained with hematoxylin and eosin (H&E). Further observation was carried out under a light microscope for histological analyses.

2.9. Statistical analysis

All statistical analyses in this study were carried out using the GraphPad Prism 5 software package (GraphPad, LaJolla, CA, USA). One-way ANOVA was used for biofilm assay and the bacterial survival assay in mice whole blood. For mice experiments, survival rates were analyzed with the log-rank test. Differences at *P* < 0.05 were considered significant.

3. Results

3.1. Morphological characterization of phage P560

Phage, named as P560, can lyse KL47 CRKP strains. Phage P560 formed big clear and round plaques with a diameter of approximately 0.3 cm surrounded by 0.1 cm enlarged haloes, indicating that they may encode depolymerases with polysaccharide-degrading activity (Fig. 1A). Transmission electron microscopy confirmed that it belonged to the order *Caudovirales*. The diameter of capsid of phage P560 was estimated at 50 ± 0.5 nm (Fig. 1B).

3.2. Genome analysis of phage P560

Next generation sequencing analysis showed that phage P560 was a linear dsDNA molecule of 40,562 bp and a GC content of 53.1%, and harbored 47 putative coding sequences. No lysogeny or virulence associated genes were identified in the genome of phage P560, showing its lytic nature and the potential for therapeutic application. Two TFPs, ORF43 and ORF44, were identified in phage P560 based on the RAST predication.

Online BLASTn analysis of P560 genomic sequence revealed that it is mostly close to *Klebsiella* phage KP32i196 (Accession no.: NC_047971) isolated from wastewater in Novosibirsk Oblast, Russian Federation, with 84% query coverage and 93.4% nucleotide identity and *Klebsiella* phage SH-KP152226 (MK903728) isolated from sewage in Shanghai, China, with 90% query coverage and 92.7% nucleotide identity. The results of genome structure and sequence similarity all suggested that phage P560 belonged to the genus *Przondovirus*, family *Autographiviridae*, the order *Caudovirales*.

The TFP ORF43 gene of P560 had low homology with that of phage KP32i196, high sequence identity to that of *Klebsiella* phage SH-KP152226 (Fig. 2A). Besides, it showed high sequence identity to that of *Klebsiella* phage vB_KpnP_IME205 and *Klebsiella* phage IME304. The enzyme activities of the TFPs mentioned above have not been examined. Phyre2 analysis revealed that ORF43 exhibited functional and structural similarity to galacturonase (96.4%; 11%), and lacked the N-terminal domain of Gp17 in T7 phage (Dunn and Studier, 1983) (Fig. 2B). The whole-genome sequence of phage P560 was deposited in the GenBank database under accession number MT966873.

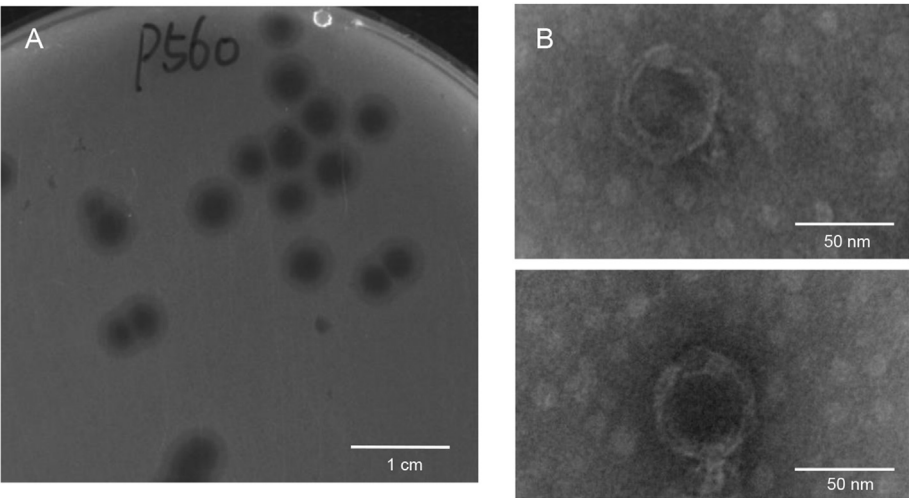


Fig. 1. Plaque and morphology of P560. **A.** Plaques of Phage P560 formed on the double-layer agar plate. Scale bar = 1 cm. **B.** Transmission electron micrograph of phage P560. The scale bar is 50 nm.

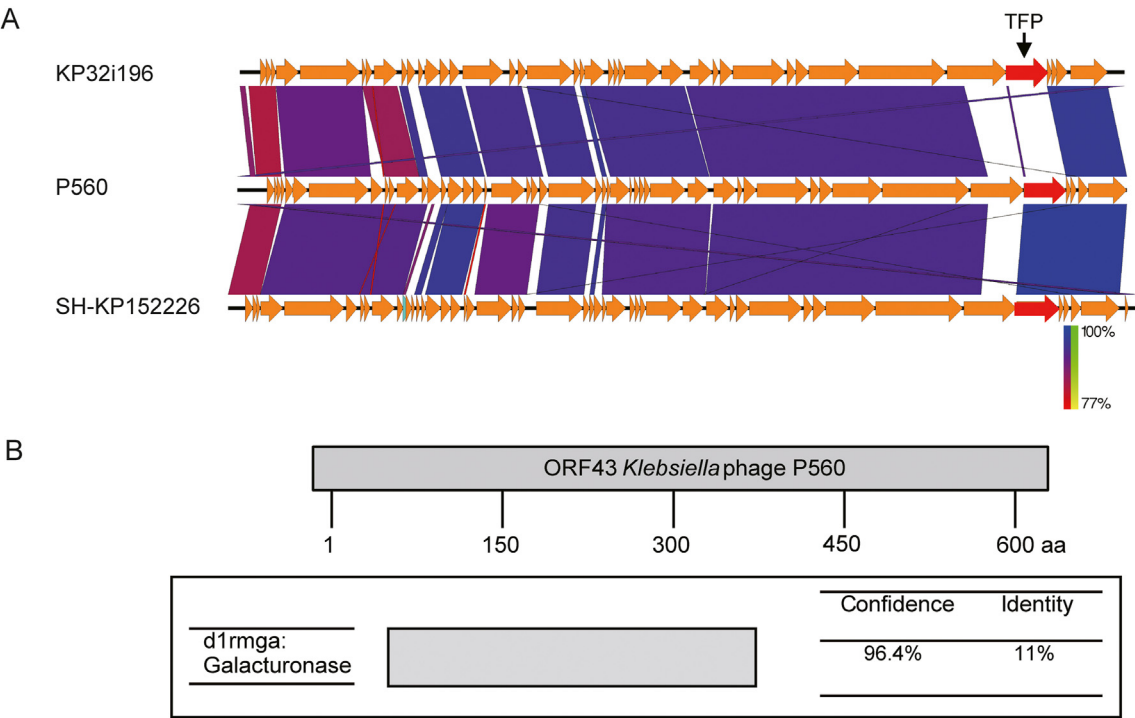


Fig. 2. Comparative genome analysis, and prediction of TFP structure and function. **A.** Schematic was produced using Easyfig_2.2.3_win software based on the whole genome nucleotide sequences of phage P560 compared to closely related phages KP32i196 (NC_047971) and SH-KP152226 (MK903728). TFP genes were showed as red arrows. Vertical blocks between sequences indicate regions of shared similarity shaded (blue and red for matches in the same direction or green and yellow for inverted matches; identity: 70%–100%). **B.** Prediction and analysis of TFP structure and function. The product of ORF43 gene (TFP) was analyzed by the Phyre2 server.

3.3. Expression and activity spectrum test of P560dep

The recombinant depolymerase P560dep were successfully expressed in *E. coli* BL21 cells. The purified recombinant depolymerase P560dep migrated as a single band with an approximate MW of 70 kDa on a 10% SDS-PAGE gel (Fig. 3A). The purified depolymerases were diluted to 2 mg/mL using PBS buffer. Phage P560 only can form plaques on KL47-type strains Kp42 by double-layer agar plate method. The recombinant depolymerase P560dep exhibited capsule-digesting activity against KL47-type *K. pneumoniae*, indicating that activity spectrum of P560dep

matched to the host range of phage P560 (Table 1). However, depolymerase P560dep only can degrade 19 out of 24 KL47 capsule of *K. pneumoniae* (Supplementary Table S1).

3.4. Depolymerization activity of P560dep

The agar plate showed that depolymerase P560dep was active against Kp42 strain of KL47 capsule. The translucent halo zones of P560dep were detected at the concentrations as low as 0.2 µg/mL (Fig. 3B). The results of punch tests revealed that the holes treated with P560dep presented

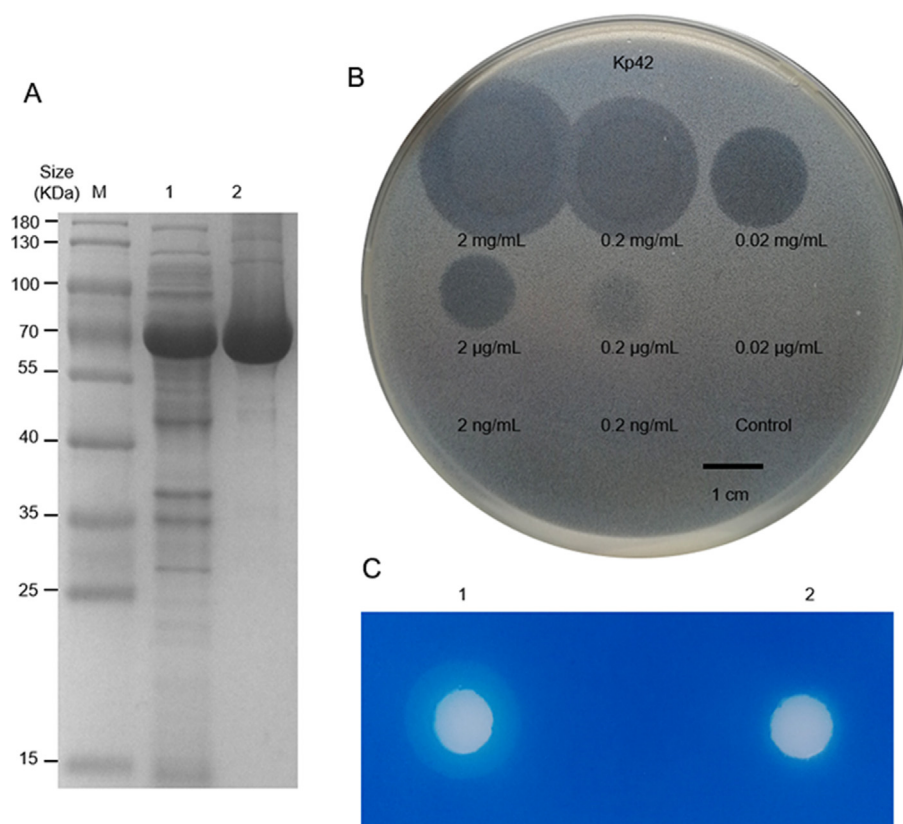


Fig. 3. Expression, purification and identification of recombinant depolymerase P560dep. **A.** The recombinant proteins before and after purification by nickel beads and concentration by a 30-kDa MW cut-off (MWCO) membrane were separated by 10% SDS-PAGE, along with the prestained protein ladder. Lane M, protein marker; lane 1, P560dep before purification and concentration; lane 2, P560dep after purification and concentration. **B.** Depolymerase activity against KL47 capsules of CRKP Kp42 strain. P560dep with different concentrations (ranging from 2 mg/mL to 0.2 ng/mL) were applied to an agar plate containing CRKP Kp42. Phosphate buffer saline was used as a control. **C.** CPS degradation by P560dep was detected using diffusion method. Hole 1, P560dep (20 µL, 2 mg/mL) against Kp42 CPS; hole 2, PBS (20 µL) against Kp42 CPS (as control).

degradation-rings, due to CPS degradation. In contrast, when the holes were treated with PBS buffer (as a control), no degradation-rings appeared (Fig. 3C).

Transmission electron microscopy showed significant differences on the morphology characteristics of P560dep treated group and PBS control group. The surface of Kp42 treated with P560dep was smooth (Fig. 4A), suggesting that “capsule-stripped” bacteria were generated. The surface of Kp42 treated with PBS was rough (Fig. 4B).

3.5. Antibiofilm activity of the recombinant depolymerase P560dep

To further test the impact of depolymerase P560dep on biofilm formation, we investigated the inhibition effect of P560dep with different concentrations on exponential-phase bacteria Kp57. The results of crystal violet staining assay showed that the OD₅₉₅ values of five different P560dep-treated groups (4 µg, 0.4 µg, 0.04 µg, 4 ng and 0.4 ng) decreased significantly in comparison to that of untreated group (Fig. 5, $P < 0.001$).

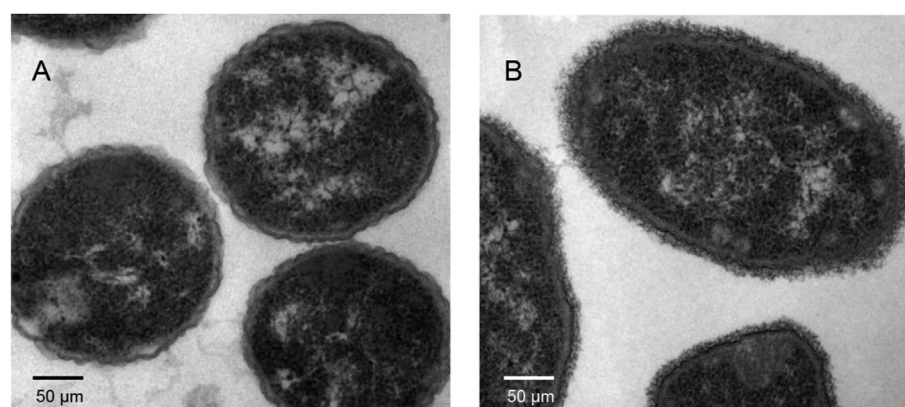


Fig. 4. Cell morphology analyses by transmission electron microscopy. **A.** Kp42 was treated with 20 µL 2 mg/mL P560dep. **B.** Kp42 was treated with 20 µL PBS (as control).

These results suggested that when P560dep ≥ 0.4 ng, it can significantly inhibit biofilm formation.

3.6. Depolymerase prevention and treatment in mouse infections

To verify the protection effect of depolymerase P560dep, three groups of BALB/c mice ($n = 10$) infected with bacteria were then treated with P560dep before and after infection. The results showed that 9 of 10 mice infected with 2.5×10^7 CFU of KL47 CRKP Kp42 strain died within one days without depolymerase P560dep treatment. In contrast, mice treated with 50 μ g P560dep before infected with Kp42 all survived ($P < 0.001$); nine of the 10 infected mice treated with 50 μ g enzyme at 2 h post-infection survived ($P < 0.001$, Fig. 6A). Intraperitoneal administration of 2.5×10^7 CFU of CRKP Kp42 in mice led to bacteremia within 8 h (Fig. 6B). In contrast, intraperitoneal administration of a single dose of (50 μ g/mouse) depolymerase P560dep 8 h post infection resulted in complete elimination of bacteria in the blood (Fig. 6B). H&E staining showed infiltration of neutrophils in lung and vacuolation in liver of Kp42 infected mice treated with PBS, however, no obvious pathological changes in mice treated with P560dep (Fig. 6C). Besides, no obvious pathological changes were observed in heart, spleen, kidney and brain tissues in either group (Supplementary Fig. S1). These results demonstrated that depolymerase P560dep can prevent and treat CRKP infections in mice.

4. Discussion

The world-wide prevalence of carbapenem-resistant *K. pneumoniae* poses a big threat to the public health. In China, most of CRKPs belonged to the clonal complex CC11, especially K64/KL64 and K47/KL47 types of ST11 (Chen et al., 2018a; Li et al., 2020a; Yu et al., 2019; Zhang et al., 2017). In this study, we isolated phage P560, and identified depolymerase P560dep targeting KL47 *K. pneumoniae*. Previous studies also reported phage SH-KP152226 (Wu et al., 2019), IME205 (Liu et al., 2020) and SRD 2021 (Hao et al., 2021) specific for CRKP with capsular K47 serotype. Most *Klebsiella* phages belonged to *Autographiviridae*, *Drexelvriidae* and *Myoviridae* family. *Przondovirus* genus in *Autographiviridae* family and *Webevirus* in *Drexelvriidae* family were the main parts of all reported *Klebsiella* phages (Walker et al., 2021). Phage P560 belonging to the genus *Przondovirus* showed high genome sequence

similarity to phage KP32i196 and SH-KP152226, and ORF43 gene of phage P560 also showed high sequence similarity to that of phage SH-KP152226. However, the enzyme activity of ORF43 from SH-KP152226 has not been analyzed yet.

Conservative phage therapy brings with drawbacks, including rapid emergence of phage-resistant bacteria and the potential for horizontal gene transfer of virulence and drug resistance genes, etc (Labrie et al., 2010). Hence, some phage-derived enzymes are highly valued. In Gram-positive bacteria, phage-derived endolysins can be applied exogenously, presenting a clear advantage over the whole-phage application (Kaspar et al., 2018). It also proved to have protective efficacy in different animal models (Entenza et al., 2005; Gilmer et al., 2013; Nelson et al., 2001) and human clinical trials (Totte et al., 2017). However, in Gram-negative bacteria, the outer membrane prevents endolysins from accessing and degrading the underneath peptidoglycan layer, thereby protecting them from endolysin attack (Lai et al., 2020). Therefore, the application of phage-derived depolymerases as one of the promising alternative approaches for Gram-negative bacteria infection, gained increasing attention to explore their potential as antimicrobial agents. In this study, it was demonstrated that phage P560 can infect KL47-type *K. pneumoniae*, while capsule depolymerase P560dep had capsule depolymerization activity against KL47-types capsule of *K. pneumoniae*. Except the depolymerases identified for 24 capsular types of *K. pneumoniae* mentioned above, future studies will be carried out to identify novel depolymerases for other 58 capsular types of *K. pneumoniae*. It is confirmed that the capsule depolymerases are host specificity determinants of these phages (Pan et al., 2019). P560dep treatment also led to the generation of “capsule-stripped” bacteria, which also demonstrated the polysaccharide-degrading activity of depolymerase P560dep targeting KL47 capsule. However, depolymerase P560dep cannot degrade all KL47 capsule of *K. pneumoniae*, that is, 5 out of 24 KL47-type strains (Kp5, Kp6, Kp9, Kp18 and Kp28) were insensitive to phage P560 and its depolymerase (Supplementary Table S1), which may be due to the subtle differences in capsule composition that existed within KL47 serotype. This phenomenon was similar to a recent report that two depolymerases (Dpo42 and Dpo43) from *Klebsiella* phage IME205 targeted different subsets of K47 capsule (Liu et al., 2020).

Bacterial biofilms are communities of bacteria and surrounded self-produced matrix composed of polysaccharides, proteins, lipids and nucleic acids (Flemming and Wingender, 2010). Biofilm plays an important role in the evasion of the host immune system and antimicrobial agents, and is a leading pathogenic factor in the medical devices and clinical settings (Clegg and Murphy, 2016). Phage-derived polysaccharide depolymerases were valuable for inhibiting and/or eradicating *Klebsiella* biofilms (Wu et al., 2019), *E. coli* O157 biofilms (Park and Park, 2021) and Shiga toxin-producing *Escherichia coli* biofilms (Chen et al., 2020). In this study, P560dep can inhibit biofilms formation of KL47 CRKP Kp42 stains. Additionally, it have been demonstrated that various depolymerases reduced colonization of the surfaces by interfering with the formation of *Proteus mirabilis* biofilms (Rice et al., 2021), *Acinetobacter baumannii* biofilms (Shahed-Al-Mahmud et al., 2021) and *Klebsiella* biofilms (Chai et al., 2014; Chen et al., 2020). In fact, depolymerase-resistant mutants emerged during the treatment of *K. pneumoniae* biofilm, which resulted in beneficial consequences, i.e., the sensitization to the innate immune response (Kaszowska et al., 2021).

Phage-derived depolymerases showed a therapeutic effect in the treatment of *K. pneumoniae*-induced infections. Previous studies reported that depolymerases treatment significantly increased survival in *Galleria mellonella* infected with *K. pneumoniae* of capsular type K3, K21 (Majkowska-Skrobek et al., 2018) and K63 (Majkowska-Skrobek et al., 2016), in mice infected with *K. pneumoniae* of capsular type K1 (Lin et al., 2014), K57 (N et al., 2020), K64 (Pan et al., 2015) and KN1 (Majkowska-Skrobek et al., 2016; Wang et al., 2019). Additionally, phage-derived depolymerases rescued mice infected with drug-resistant *A. baumannii* (Liu et al., 2019; Wang et al., 2020), a lethal bolus of *Pasteurella multocida* (Chen et al., 2018b), a lethal dose of *E. coli* (Lin et al., 2017). In our study,

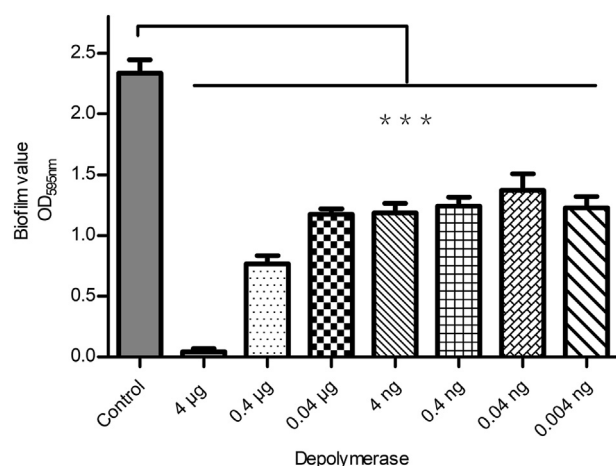


Fig. 5. Antibiofilm activity of depolymerase P560dep. PBS (20 μ L, as control group) and equivoluminal P560dep (4 μ g, 0.4 μ g, 0.04 μ g, 4 ng, 0.4 ng, 0.04 ng and 0.004 ng, respectively) was incubated with CRKP Kp42 (2×10^8 CFU/well) in 96-well plates for 24 h. The biofilms were assessed by crystal violet staining, and the absorbances were measured at 595 nm. Each assay was performed in duplicate and repeated three times. Significant differences are indicated (*** $P < 0.001$). The data are shown as the mean \pm SEM.

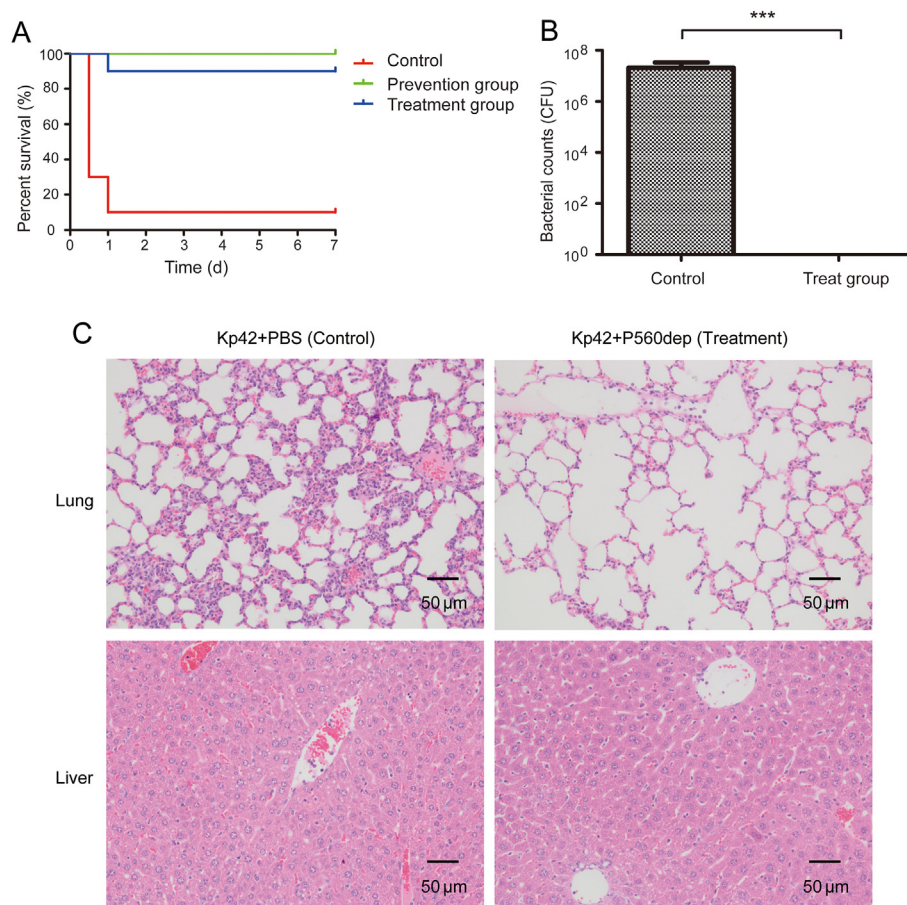


Fig. 6. Capsule depolymerase treatment in carbapenem-resistant *K. pneumoniae*-infected mice. **A.** Preventative and therapeutic efficacy of depolymerase P560dep in mice. Female BALB/c mice were infected with 2.5×10^7 CFU bacteria and treated with 50 μ g P560dep 1 h before (prevention group, $P < 0.0001$) and 2 h after infection (treatment group, $P = 0.0004$) (10 mice in each group). Control group was administered with the same volume of *K. pneumoniae* strain Kp42 plus PBS buffer. Statistical analysis was performed using the Kaplan-Meier method. The x axis represents days after inoculation, and the y axis represents the survival rates of mice. **B.** Bacterial load (CFU) in the blood of mice 8 h post infection. Mice were infected via intraperitoneal route with 2.5×10^7 CFU CRKP Kp42 and treated with 50 μ g P560dep. Error bars represent SEM from independent values. **C.** Depolymerase P560dep alleviated pathological changes of lung and liver in mice infected with CRKP Kp42. Representative H&E-stained lung and liver were shown. Scale bar = 50 μ m.

the treatment of the recombinant capsule depolymerase P560dep also effectively reduced bacteremia and deaths in mice infected with KL47 *K. pneumoniae*. A single dose (i.p., 50 μ g/mouse) of P560dep exhibited both preventative (100%) and therapeutic efficacy (90%) against the bacterial infection. Although capsule depolymerase alone is not sufficient to destroy the bacterial outer membrane and kill the bacteria, its unique ability to degrade bacterial capsular material can be applied to decrease pathogen virulence and expose the underlying bacterium to the immune attack such as complement-mediated killing (Majkowska-Skrobek et al., 2018). The complete eradication of bacteria in blood and the therapeutic efficacy on reducing deaths in animals infected by *K. pneumoniae* may be because depolymerases stripped capsules and exposed the underlying bacteria to innate immune attack. Considering the potential of phage-derived depolymerase P560dep as an anti-virulence compound for antimicrobial therapy, its three dimensional structure and catalytic mechanism need to be determined in future study.

5. Conclusions

Phage P560 isolated from sewage showed specific for capsule locus type KL47 *K. pneumoniae* with the enlarged haloes around plaques. We demonstrated that a capsule depolymerase from *Klebsiella* bacteriophage P560, had depolymerization activity against KL47-types capsule of

K. pneumoniae, and its lysis spectrum matched to the host range of phage P560. Recombinant depolymerase can significantly inhibit biofilm formation, prevent and treat CRKP infection in mouse models. Depolymerase as an attractive antivirulence agent represents a promising tool for antimicrobial therapy.

Data availability

The annotated whole-genome sequences of phage P560 were deposited in the GenBank database under accession number: MT966873.

Ethics statement

All animal studies were approved by the Experimental Animal Welfare and Ethics Committee of Nanjing Agricultural University (Approval ID: PZB2020002), and were performed according to Animal Welfare Agency Guidelines.

Author contributions

Min Li: conceptualization, data curation, formal analysis, validation, investigation, writing original draft, writing-review and editing. Hui Wang: data curation. Long Chen: resources. Genglin Guo: formal analysis.

Pei Li: validation. Jiale Ma: resources. Rong Chen: methodology. Hong Du: resources, funding acquisition. Yuqing Liu: supervision, funding acquisition. Wei Zhang: conceptualization, funding acquisition, resources, supervision, project administration, writing-review and editing.

Conflict of interest

All authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.virs.2022.04.005>.

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