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Characterization and genome analysis of novel bacteriophages infecting the opportunistic human pathogens *Klebsiella oxytoca* and *K. pneumoniae*

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Abstract *Klebsiella* is a genus of well-known opportunistic human pathogens that are associated with diabetes mellitus and chronic pulmonary obstruction; however, this pathogen is often resistant to multiple drugs. To control this pathogen, two *Klebsiella*-infecting phages, *K. oxytoca* phage PKO111 and *K. pneumoniae* phage PKP126, were isolated from a sewage sample. Analysis of their host range revealed that they infect *K. pneumoniae* and *K. oxytoca*, suggesting host specificity for members of the genus *Klebsiella*. Stability tests confirmed that the phages are stable under various temperature (4 to 60 °C) and pH (3 to 11) conditions. A challenge assay showed that PKO111 and PKP126 inhibit growth of their host strains by 2 log and 4 log, respectively. Complete genome sequencing of the phages revealed that their genome sizes are quite different (168,758 bp for PKO111 and 50,934 bp for PKP126). Their genome annotation results showed that they have no human virulence-related genes, an important safety consideration. In addition, no lysogen-formation gene cluster was detected in either phage genome, suggesting that they are both virulent phages in their bacterial hosts. Based on these results, PKO111 and PKP126 may be good candidates for development of biocontrol agents against members of the genus *Klebsiella* for therapeutic purposes. A comparative analysis of tail-associated gene clusters of PKO111 and PKP126 revealed relatively low homology, suggesting that they might differ in the way they recognize and infect their specific hosts.

Introduction

Klebsiella is a genus of well-known opportunistic human pathogens that primarily infect immunocompromised individuals who are hospitalized and suffering from severe underlying diseases, including diabetes mellitus or chronic pulmonary obstruction [33]. It has been estimated that this pathogen causes about 8% of all nosocomial bacterial infections in the United States and Europe, indicating that it is one of the eight most important global infectious pathogens in hospitals [25]. In Taiwan, *K. pneumoniae* was ranked second among the most prevalent pathogens causing nosocomial infections from 1991 to 2003 [39]. Interestingly, about 80% of global nosocomial infections are known to be caused by multidrug-resistant *K. pneumoniae* strains [9, 16, 26, 31, 40]. The prevalence of *Klebsiella* multidrug-resistant strains has been reported to be increasing, probably due to the misuse of various antibiotics for therapy [6]. Because the treatment of *K. pneumoniae* infections has made use of a combination of various beta-lactams [6], the number of strains producing extended-spectrum β-lactamase (ESBL) has increased in Eastern Europe and Latin America during the last few decades [25]. Due to the emergence of multidrug-resistant strains, a new alternative approach to controlling this

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pathogen is urgently needed. The bacteriophage has been proposed as a novel agent to control drug-resistant bacteria. In fact, it has been used to control various pathogens in Eastern European countries for several decades [11]. More recently, it has attracted interest in Western countries because of the emergence of various antibiotic-resistant bacteria.

A bacteriophage is a bacterial virus infecting only a specific host without causing human infection, meaning that it should be safe for human trials [13]. While lysogens are formed by bacteria infected with temperate phages, virulent phages can lyse and disrupt bacterial cells after infection, providing bactericidal activity [15]. Based on these characteristics, phages have been considered as novel biocontrol agents or a novel natural food preservative for various therapeutic and food applications.

To develop phages as novel biocontrol agents, the lytic bacteriophage KPO1K2 was first isolated with *K. pneumoniae* B5055 as its host and was shown to have a host range including *K. pneumoniae* and *E. coli*. Characterization of phage KPO1K2 revealed that it has a high burst size (>100) and high stability under various pH and temperature conditions, suggesting that it is a good candidate for *K. pneumoniae* therapy [37]. For clinical application of *Klebsiella* phages, a single dose of phage 1513 (2×10^9 PFU/mouse) was injected into mice that had previously been infected intranasally with clinically isolated multidrug-resistant *K. pneumoniae* KP 1513 from a patient with pneumonia. This treatment resulted in a significant improvement in the survival rate of the infected mice [7]. While all mice that were not treated with phage died within 24 h, only 20% of mice treated with phage at a multiplicity of infection (MOI) of 10 died by 72 h postinfection, suggesting that *Klebsiella* phage can be used for phage therapy. In addition, application of a mixture of *K. pneumoniae* and *K. oxytoca* phages to municipal sewage showed a 22-fold reduction in bacterial cells after 2 h of incubation, suggesting that a phage mixture is also a good alternative biocontrol agent [19].

The first phage therapy in humans involved direct injection into six patients with staphylococcal boils in 1921 [10]. Since then, various phages have been used for therapeutic purposes in Eastern Europe, and many phage therapy products have been developed. These include "*E. coli* bacteriophage" (Microgen, Moscow, Russia), "Complex pyobacteriophage" (Microgen), and "Phagestaph" (JSC Biochimpharm, Tbilisi, Georgia). *E. coli* bacteriophage is a pure lysate of specific phages infecting pathogenic *Escherichia coli*, intended for the treatment and prevention of diseases caused by *E. coli*. Complex pyobacteriophage is a mixture of various phages infecting *Staphylococcus, Enterococcus, Streptococcus, Pseudomonas aeruginosa, Klebsiella pneumoniae, K. oxytoca*, *E. coli*, *Proteus vulgaris,* and *P. mirabilis*, designed for the treatment of urogenital infections. Phagestaph is a mixture of sterile filtrates of phage lysates infecting *Staphylococcus aureus*, designed for the treatment of inflammatory infections.

In addition to their use in phage therapy, phages have been used to control food-borne pathogens in various foods, serving as novel natural food preservatives. Interestingly, "ListShield" (Intralytix, Baltimore, MD, USA), for use against *Listeria monocytogenes*, was approved by the United States Food and Drug Administration (FDA) as a novel natural food preservative in 2006 [18]. Since then, more than 10 phage products have become available on the market, including "Ecoshield" against *E. coli* O157:H7 (Intralytix), "SALMONELEX" against *Salmonella* (Micreos Food Safety, Wageningen, The Netherlands), and "LISTEX P100" against *Listeria* (Micreos Food Safety).

In this study, two *Klebsiella* phages (PKO111 and PKP126) were newly isolated and characterized. In addition, their genomes were completely sequenced and analyzed using various bioinformatics tools, and they were shown to be virulent phages and to have all the required components for reconstruction in host cells. These phages could be useful for the development of novel biocontrol agents against *Klebsiella* pathogens.

Materials and methods

Bacterial strains and growth conditions

All bacterial strains used in this study and their growth conditions are listed in Table 1. All bacteria were grown with shaking at 37 °C, except for anaerobic bacteria of the genera *Bacteroides* and *Clostridium*. These anaerobic bacteria were grown at the same temperature using a BD GasPak EZ container system (Franklin Lakes, NJ, USA). Of the strains used, *K. oxytica* ATCC 43863 and *K. pneumoniae* KCTC 2242 were chosen as indicator strains for isolation and characterization of phages PKO111 and PKP126, respectively.

Dotting assay and overlay assay

To determine the phage concentration, a dotting assay was used with 0.4% molten soft agar containing 100 µl of overnight-cultured host bacteria and a 1.8% agar base plate [12, 20]. After solidifying this overlay plate for 10 min at room temperature, 10 µl of each serially diluted sample (diluted with sodium chloride–magnesium sulfate (SM) buffer: 100 mM NaCl, 10 mM $MgSO_4 \cdot 7H_2O$, and 50 mM Tris·HCl, pH 7.5; Sigma, St. Louis, MO, USA) was spotted, and the plate was

Table 1 Host range and suitable growth media for (A) PKO111 and (B) PKP126

Bacteria	Plaque formation by PKO111[a]	Plaque formation by PKP126[a]	EOP of PKO111	EOP of PKP126	Reference or Source[b]	Medium[c]
***Klebsiella* and *Cronobacter* strains**						
K. oxytoca ATCC 43863	C	C	1.0000000	0.00000007	ATCC	LB
K. oxytoca KCTC 1686	C	C	0.8888889	0.00000052	KCTC	LB
K. pneumoniae KCTC 2242	C	C	0.0000003	1.00000000	KCTC	LB
C. sakazakii ATCC 29544	C	C	0.0000001	0.00000003	ATCC	TS
Gram-negative bacteria						
Escherichia coli MG1655	-	-	-	-	[14]	LB
E. coli O157:H7 ATCC 35150	-	-	-	-	ATCC	LB
ATCC 43888	-	-	-	-	ATCC	LB
ATCC 43890	-	-	-	-	ATCC	LB
ATCC 43894	-	-	-	-	ATCC	LB
ATCC 43895	-	-	-	-	ATCC	LB
E. coli DH5α	-	-	-	-	Invitrogen	LB
Pseudomonas aeruginosa KACC 10186	-	-	-	-	KACC	LB
Shigella boydii ATCC 8700	-	-	-	-	ATCC	TS
Shigella flexneri 2a strain 2457T	-	-	-	-	IVI	TS
Salmonella Typhimurium LT2	-	-	-	-	[21]	LB
DT 104	-	-	-	-	[28]	LB
Yersinia enterocolitica ATCC 55075	-	-	-	-	ATCC	TS
Gram-positive bacteria						
Bacillus cereus ATCC 13061	-	-	-	-	ATCC	BHI
ATCC 14579	-	-	-	-	ATCC	BHI
B. licheniformis JCM 2505	-	-	-	-	JCM	BHI
Bacteroides fragilis KACC 3688	-	-	-	-	KACC	RCM
Clostridium perfringens ATCC 3624	-	-	-	-	ATCC	RCM
ATCC 3629	-	-	-	-	ATCC	RCM
C. difficile ATCC 9689	-	-	-	-	ATCC	RCM
Staphylococcus aureus RN4220	-	-	-	-	[23]	TS
Staphylococcus epidermidis ATCC 35983	-	-	-	-	ATCC	TS
Enterococcus faecalis ATCC 29212	-	-	-	-	ATCC	MRS
Listeria monocytogenes ATCC 15313	-	-	-	-	ATCC	BHI

[a] C, clear plaques; -, no plaques; -, no susceptibility

[b] ATCC, American Type Culture Collection; KCTC, Korean Collection for Type Cultures; KACC, Korean Agricultural Culture Collection; JCM, Japan Collection of Microorganisms

[c] TS, tryptic soy broth; RCM, reinforced clostridial medium; BHI, brain heart infusion; MRS, Lactobacilli de Man-Rogosa-Sharpe medium

incubated at 37 °C for 12 h. To generate phage plaques, an overlay assay was used. Specifically, 100 µl of the diluted sample and 100 µl of overnight-cultured host bacteria were gently mixed with 6 ml of 0.4% molten soft agar and poured onto a 1.8% agar base plate, which was then incubated at 37 °C for 12 h.

Bacteriophage isolation and propagation

For phage isolation, 1 ml of each sewage sample was mixed with 5 ml of Luria-Bertani (LB) broth medium containing 50 µl of overnight culture of the indicator strain. This mixture was incubated with shaking at 37 °C for 9 h, followed by centrifugation at 8,000 rpm for 10 min. The supernatant was filtered using a 0.22-µm filter (Pall Corporation, Ann Arbor, MI, USA) in order to remove bacterial cells [24]. To verify the presence of phage, the supernatant was serially diluted, and 10 µl of the diluted supernatant was spotted onto an LB agar plate overlaid with 6 ml of 0.4% molten LB agar containing 100 µl of overnight culture of the indicator strain. After incubation, each single plaque was picked and resuspended in 300 µl of sterile SM buffer. After filtration of the solution using a 0.22-µm filter (Pall Corporation), an overlay assay was conducted to obtain a pure culture of the phage. This procedure was repeated five times to obtain the pure phage [25]. To achieve a high titer of the pure phage ($>10^{11}$ PFU), the standard phage propagation procedure and CsCl gradient ultracentrifugation were performed [24].

Host range analysis

A total of 28 test strains (*Klebsiella*, *C. sakazakii,* Gram-negative pathogens [*E. coli* O157:H7, *Pseudomonas, Shigella, Salmonella, Yersinia*], and Gram-positive pathogens [*Bacillus, Bacteroides, Clostridium, Staphylococcus, Enterococcus, Listeria*]) were incubated for 12 h at 37 °C (Table 1). Then, 100 µl of each strain of bacterial cells was mixed with 6 ml of 0.4% molten LB agar and poured onto a 1.8% LB agar plate. To measure the sensitivity of the test strains to each phage, the overlay assay and subsequent phage titer determination were performed using 10 µl of serially diluted phage suspension (from 10^1 to 10^{12} PFU/ml) [2]. The efficiency of plating (EOP) was determined by comparing the phage titer of the test strain with that of the indicator strain.

Bacterial challenge assay

After 1% inoculation of overnight-cultured indicator strain into 100 ml LB broth medium, the culture was incubated at 37 °C until the optical density (OD) reached 1.0 at a wavelength of 600 nm. After incubation, the culture was divided into equal volumes (50 ml each), and the phage (PKO111 or PKP126) was added to the divided culture at an MOI of 1 or 100. During incubation at 37 °C, a 1 ml sample was collected from the phage-treated culture every 1 or 2 h. After sample collection, the samples were serially diluted and spread onto agar plates for determination of the viable cell count (CFU/ml). This challenge assay was performed in triplicate.

Stability tests under various stress conditions

To measure the thermal stability of phages PKO111 and PKP126, the phage solution (10^8 PFU/ml) was incubated at various temperatures (4, 20, 30, 37, 50, 60, 70, and 75 °C) for 12 h. After incubation, each phage titer was determined using dotting and overlay assays. To measure the stability of phages under various pH conditions, the pH of the SM buffer was adjusted with HCl or NaOH to different pH values ranging from of 1 to 12. A 100-µl sample of phage suspension (10^8 PFU/ml) was added to each pH solution and incubated at 37 °C for 12 h, after which the phage titer was determined using dotting and overlay assays.

Genome sequencing and bioinformatics analysis

Genomic DNA of PKO111 and PKP126 was extracted and purified as described previously by Wilcox et al. [38]. Before isolation of the phage genomic DNA, the phage solution in SM buffer was treated with DNase I (final concentration, 20 units/ml; New England BioLabs, Ipswich, MA, USA) at room temperature for 30 min to remove DNA contaminants. After this treatment, 0.5 M EDTA (pH 8.0; final concentration, 20 mM), proteinase K (>600 mAU/ml), and 10% sodium dodecyl sulfate (SDS; final concentration, 0.5%) were added to the phage suspension, and the sample was incubated at 56 °C for 1 h. After treatment with proteinase K and SDS, the aqueous phase containing phage genomic DNA was separated by centrifugation at 13,000 rpm for 10 min. The phage genomic DNA in the aqueous phase was extracted twice with saturated phenol-chloroform/isoamyl alcohol and precipitated with ice-cold absolute ethanol. The DNA pellet was washed with 70% ethanol and resuspended in water. Sequencing of the genomic DNA was performed using a 454 pyrosequencer (GS-FLX Titanium; Roche, Mannheim, Germany) at Macrogen, Seoul, Korea. The qualified sequence reads were assembled using Newbler v2.3 (Roche). Open reading frames (ORFs) were predicted using Glimmers [3], FgenesV (Softberry, Inc., Mount Kisco, NY, USA) and GeneMarkS [5], and ribosomal binding sites (RBSs) were predicted using RBSfinder for confirmation of ORF predictions (J. Craig Venter Institute, San Diego, CA, USA). The predicted ORFs were annotated for specific functions using the BLASTP [4] and InterProScan programs with various protein domain databases [27]. Phage lifestyle was predicted using the PHACTS program [22]. Phage virulence factor analysis was conducted using Virulence Searcher [36]. The genome sequence was edited using Artemis16 [29], and sequence alignments with genome sequences of other phages were conducted using the MUMmer3 [30] and BLAST 2 Sequence programs [35]. Comparative genome analysis

was conducted using ACT12 [8]. The amino acid sequences of major capsid proteins (MCPs) from complete *Klebsiella* phage genome sequences available in the GenBank database were compared using the ClustalW sequence alignment program [32]. A phylogenetic tree of MCPs was generated using MEGA6 by the neighbor-joining method with p-distance values [34].

Nucleotide sequence accession number

The complete genome sequences of PKP126 and PKO111 (with their annotation information) were deposited in the NCBI database under the GenBank accession numbers KR269719 and KR269720, respectively.

Results

Isolation, morphology, and host range

The new phages PKO111 and PKP126, which specifically infected *K. pneumoniae* and *K. oxytoca*, respectively, were isolated from a sewage treatment facility in Suwon, South Korea, using the indicator strains *K. oxytoca* ATCC 43863 and *K. pneumoniae* KCTC 2242.

A host range test revealed that the phages PKO111 and PKP126 have different inhibition patterns against various host bacteria. Phage PKO111 was found to inhibit *K. oxytoca* but only partially inhibit *K. pneumoniae* and *Cronobacter sakazakii* (Table 1). In comparison, phage PKP126 inhibits *K. pneumoniae* but only partially inhibits *K. oxytoca* and *C. sakazakii*, suggesting that these phages have different host specificities for infection (Table 1). However, the host range test of these phages showed that neither of them inhibits other Gram-negative (*E. coli, Pseudomonas, Shigella, Salmonella,* and *Yersinia*) or Gram-positive (*Bacillus, Bacteroides, Clostridium, Staphylococcus, Enterococcus,* and *Listeria*) bacteria, suggesting that they probably have high host specificity (Table 1).

Phage stability under various stress conditions

For therapeutic purposes, isolated phages need to be stable under various stress conditions, including temperature and pH. To determine the stability of phages PKO111 and PKP126, they were tested under broad ranges of temperature (4 to 75 °C) (Fig. 1A) and pH (1 to 12) (Fig. 1B) for 12 h of storage time, showing that they are stable under these various stress conditions. While these phages are weak under strong acidic or alkaline conditions (pH <3 or >11) or at high temperatures (>70 °C), they remain active for infection in their specific bacterial hosts under general storage and transport conditions.

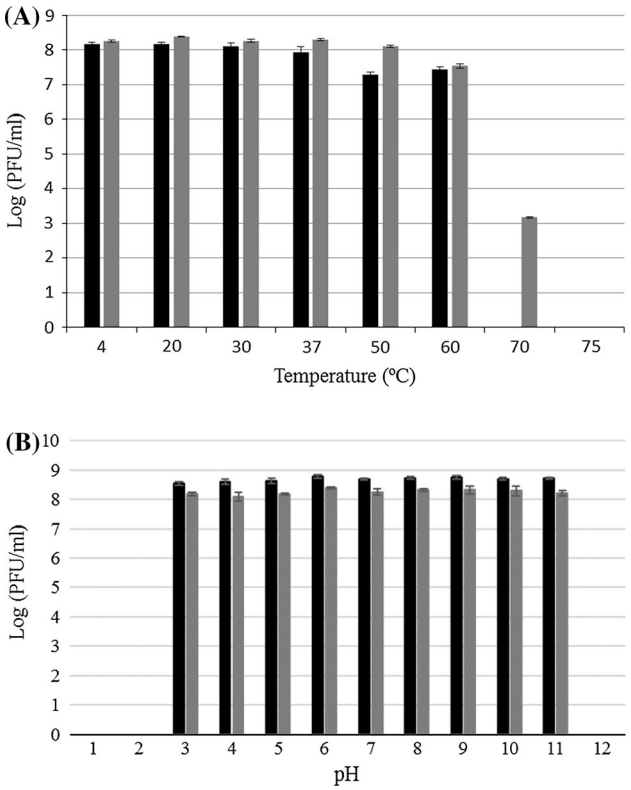

Fig. 1 Stability of phage PKO111 and phage PKP126 under various stress conditions. (A) Temperature stability of phages PKO111 and PKP126. (B) pH stability of phages PKO111 and PKP126. Black bar, phage PKO111; grey bar, phage PKP126

Bacterial challenge assay

To examine the host lysis activity of phages PKO111 and PKP126, the growth of host bacterial after phage infection was monitored using the viable-cell-count method. This bacterial challenge assay showed that phages PKO111 and PKP126 inhibit growth of their host strains by 2 and 3 log, respectively, in 2 h. However, host cell growth recovered completely by 12 h postinfection (Fig. 2). Interestingly, *K. pneumoniae* was highly sensitive to infection by phage PKP126, and a bacteriophage-insensitive mutant (BIM) was rapidly selected, whereas *K. oxytoca* was less sensitive to infection by phage PKO111, and its BIM emerged slowly.

To further investigate the infection of specific host strains by the phages, additional challenge assays at an MOI of 1 were performed with 12 h of incubation time (Fig. S1). A comparison of results determined using MOIs of 1 and 100 showed that these phages (PKP126 and PKO111) also inhibited the growth of the host bacterial strains at an MOI of 1, as expected. However,

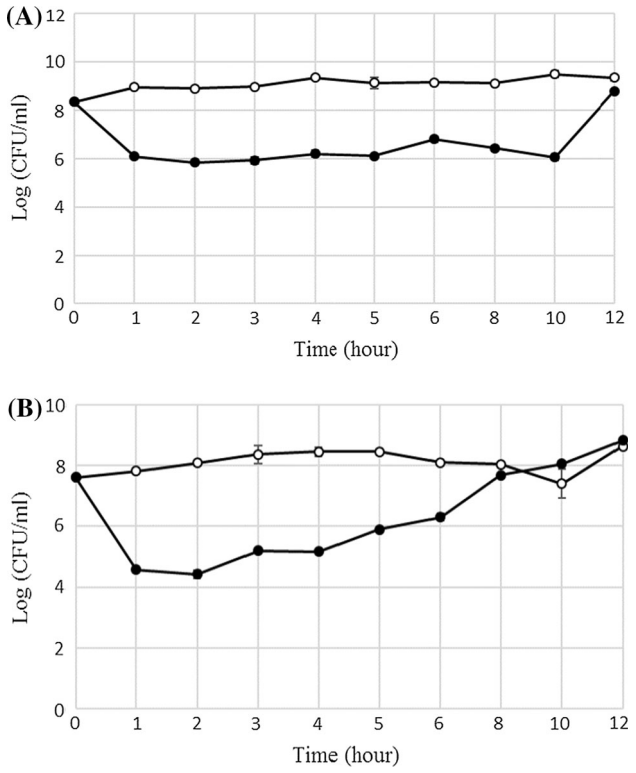

Fig. 2 Bacterial challenge assay of (A) phage PKO111 with *K. oxytoca* ATCC 43863 and (B) phage PKP126 with *K. pneumoniae* KCTC 2242. Closed circles, PKO111- and PKP126-infected samples; open circles, samples not infected with PKO111 or PKP126

while the challenge assay pattern of PKP126 at an MOI of 1 was similar to that of the same phage at an MOI of 100, the challenge assay pattern of PKO111 at MOIs of 1 and 100 were slightly different, suggesting that these two phages might differ in their effect on infection and growth inhibition in their respective host bacteria. Infection by phage PKP126 reduced the number of host cells by up to 10^4 CFU/ml at both MOIs (1 and 100). However, PKO111 infection reduced the number of host cells by up to 10^2 CFU/ml at an MOI of 100, and by up to 10^4 CFU/ml at an MOI of 1. These results suggest that PKP126 has a similar host infection pattern regardless of MOI; in contrast, increasing the MOI appears to affect the infection and lysis of the host cells by PKO111 (Fig. 2 and Fig. S1).

Genome characterization

In general, a phage genome analysis provides information about its genomic characteristics at the molecular level, as well as safety verification for applications of phage therapy. To obtain this information, the genomes of phages PKO111 and PKP126 were completely sequenced and analyzed using various bioinformatics tools.

The genome of PKO111 consists of 168,758 bp of DNA with a GC content of 39.39%; it contains 204 predicted open reading frames (ORFs) and 16 tRNA genes. The genome of PKP126 has 50,934 bp of DNA with a GC content of 50.37%; it contains 78 ORFs and no tRNA genes. Of all the predicted ORFs for phages PKO111 and PKP126, 113 ORFs of phage PKO111 (55.4%) and 26 ORFs of phage PKP126 (33.3%) were predicted to have specific functions. Others in both genomes encode hypothetical proteins (91/204 [44.6%] for PKO111 and 52/78 [66.7%] for PKP126), likely due to insufficient phage genome annotation information in the public databases. The annotation information for all of the ORFs in these two genomes is shown in Tables S1 and S2. Notably, these phage genomes may not be associated with their host strain genomes, because the GC content of the phage genomes was found to be quite different from that of the host genomes (39.39% for PKO111 vs. ~55% for *K. oxytoca*; 50.37% for PKP126 vs. 57.60% for *K. pneumoniae* KCTC 2242). These functional ORFs were categorized into six functional groups: DNA replication/modification (DNA terminase, DNA endonuclease, and DNA primase/helicase), structure and packaging (baseplate subunit, neck protein, and major capsid protein), transcription regulation (transcription regulator TetR, RNA polymerase binding protein, and RNA ligase), host lysis (tail lysozyme), tail structure (tail tube protein, long tail fiber, tail fiber connector, and tail pin protein), and additional function (membrane attachment protein, thymidine kinase, nucleotidyltranferase, ADP-ribose pyrophosphatase, thioredoxin, uracil reductase, glutaredoxin, sliding clamp protein, superinfection immunity protein, and thymidylate synthase). These results suggest that these phage genomes have all the required core phage genes, and they might share core functions (Fig. 3). However, an additional virulence factor analysis of both phage genomes using the Virulence Searcher program showed that human virulence genes are lacking in both genomes, suggesting that these phages would be safe for use in therapeutic applications.

Comparative genome analysis

A comparison of their genome sequences showed that the genome of PKO111 is ~120 kb longer than that of PKP126, suggesting that the PKO111 genome has more functional ORFs than the PKP126 genome. A comparative analysis of these additional ORFs in the PKO111 genome showed that it has many copies of similar functional genes in specific functional categories, including DNA replication and transcription. For example, the PKO111 and PKP126 phage genomes contain 26 and 7 ORFs for DNA replication and 15 and 2 genes for transcription, respectively. In addition, while these two genomes have similar

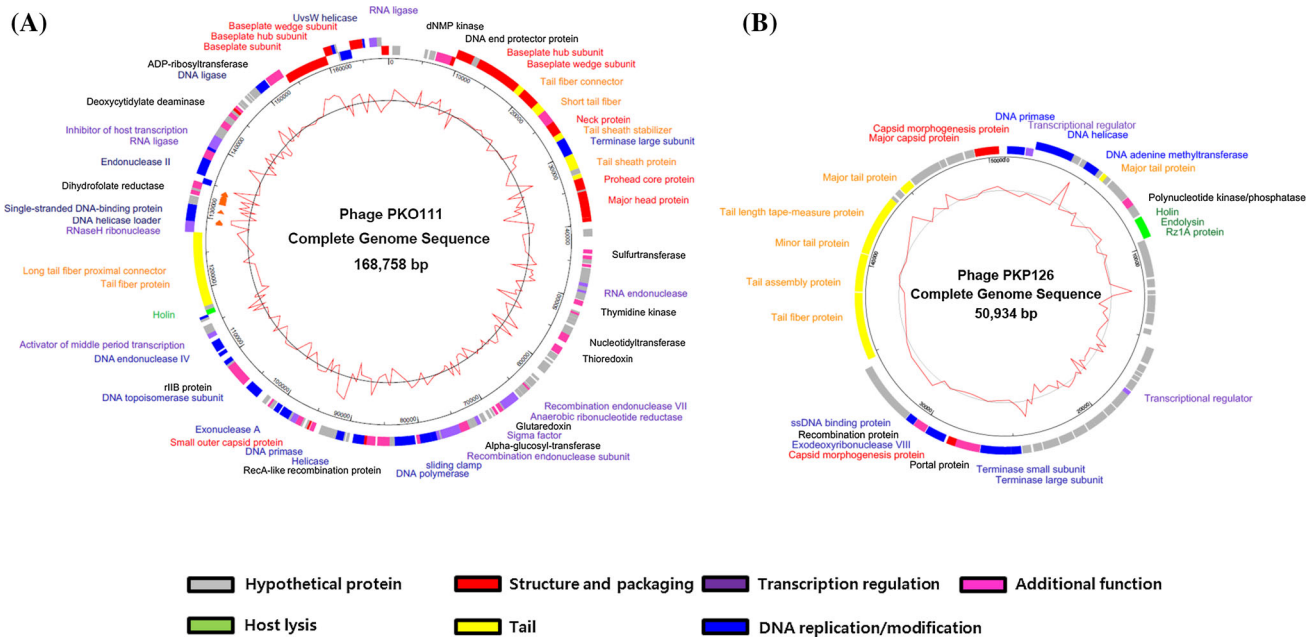

Fig. 3 Genome maps of (A) phage PKO111 and (B) phage PKP126

numbers of phage-associated genes, the PKO111 genome has more phage-tail-related ORFs than the PKP126 genome, which are probably related to the morphological characteristics of the tail in members of the family *Myoviridae*. However, the PKP126 genome has a complete host lysis gene cluster encoding endolysin, holin, and Rz/Rz1, while the PKO111 genome has only one ORF encoding holin. Nevertheless, the phages were found to have a similar host range (Table 1), suggesting that the reason that ORFs encoding endolysin and Rz/Rz1 were not detected in the PKO111 genome, was probably insufficient *Klebsiella* phage genome annotation information in the databases. To identify these genes, experimental characterization of hypothetical proteins in these genomes needs to be performed.

Although the phages PKO111 and PKP126 were found to have a similar host range, including *K. oxytoca, K. pneumoniae,* and even *C. sakazakii*, a comparison of their genome sequences showed very low DNA sequence similarity, suggesting that they evolved from different ancestors. Phylogenetic analysis of these two phage genomes revealed that they belong to different phylogenetic clusters, supporting our findings. To clarify these differences, tail-associated gene clusters in PKO111 and PKP126 were compared with closely related *Klebsiella* phage genomes and *C. sakazakii* phage genomes in the same phylogenetic trees. Phage-tail-associated genes in the gene cluster of PKO111 are very similar to those of *Klebsiella* phage JD18 but less similar to those of the *C. sakazakii* phage GAP161 (Fig. 4A). In addition, phage-tail-associated genes of PKP126 are very similar to those of the *Klebsiella* phage KLPN1 but less similar to those of the *C. sakazakii* phage ESP2949-1 (Fig. 4B). These observations suggest that the host ranges of *Klebsiella* phages in the same phylogenetic cluster are similar and that these phages weakly inhibit *C. sakazakii* phages in the same phylogenetic cluster (Table 2). However, the genomes of phages PKO111 and PKP126 are quite different from each other, despite their similar host range, suggesting that they have different infection mechanisms.

Discussion

Virulent bacteria phages generally have strong bactericidal activity, causing host cell lysis [3], and they therefore have potential as novel biocontrol agents to inhibit specific pathogenic bacteria. Members of the genus *Klebsiella* are major pathogens associated with chronic pulmonary obstruction and diabetes mellitus [3]. However, many *Klebsiella* infections are caused by multidrug-resistant strains that generally cannot be controlled using common antibiotics [6–10]. Therefore, the use of phage therapy to inhibit the growth of *Klebsiella* strains has been suggested. Single-phage or phage cocktail products are already available on the market for the control of various bacteria. However, previous *Klebsiella* phage studies have not been sufficient to support the development of new types of biocontrol agents with satisfactory results.

Previously, the host range of the phage Kpp95, which infects *K. pneumoniae*, was evaluated [39]. This phage can infect *K. pneumoniae* as well as other related bacteria

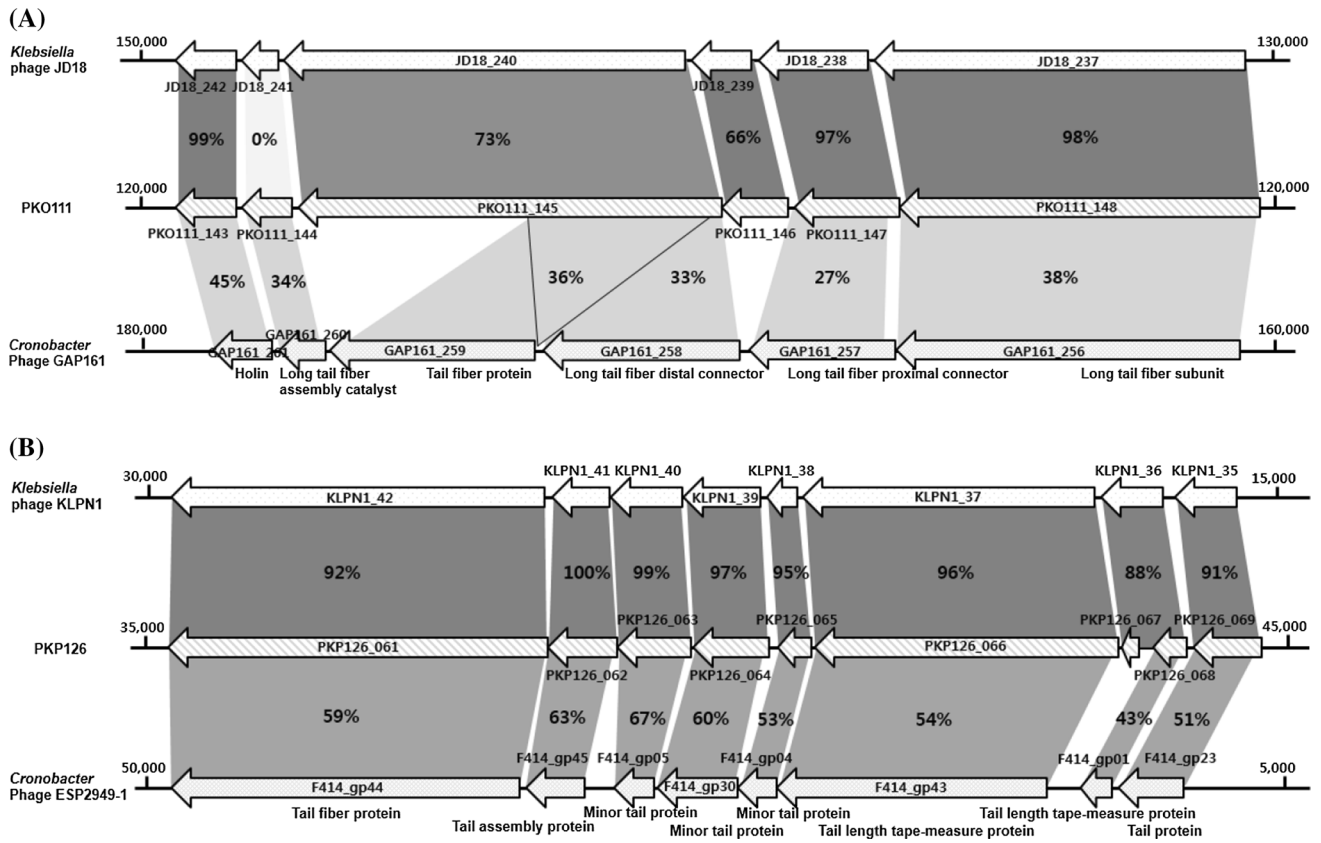

Fig. 4 Comparative genome analysis of phages PKO111, PKP126, closely related *Klebsiella* phages, and *C. sakazakii* phages. Organization of a tail-associated gene cluster (A) in PKO111 with two phage genomes (JD18 and GAP161) and (B) in PKP126 with two phage genomes (KLPN1 and ESP2949-1)

including *K. oxytoca, Serratia marcescens,* and *Enterobacter agglomerans*, suggesting that it has an extensive host range. Host range assays (Table 1) showed that PKP126 and PKO111 both infect *K. oxytoca*, *K. pneumoniae*, and *C. sakazakii*, but their host growth inhibition activities are slightly different. In particular, both of these phages weakly inhibit the growth of *C. sakazakii*. However, it is noteworthy that PKO111 and PKP126 can inhibit the growth of *Klebsiella* and *C. sakazakii* at the same time, which has never been reported previously. Although the *C. sakazakii* phage vB_CsaM_GAP161 (GAP161) was previously reported to share homology in its genomic DNA sequence with the *Klebsiella* phage KP15 [1], it was not shown that the *C. sakazakii* phage GAP161 can inhibit the growth of *Klebsiella* host strains. To investigate why these two *Klebsiella* phages weakly inhibited *C. sakazakii*, the genome sequences of these two phages, other related *Klebsiella* phages, and *C. sakazakii* phages were compared. Comparisons of the phage-tail-associated gene clusters in PKO111 and two phages with highly homologous genome sequences (*Klebsiella* phage JD18 and *C. sakazakii* phage GAP161) revealed that phage-tail-associated genes in the gene cluster of PKO111 are highly homologous to those of the *Klebsiella* phage JD18 but less similar to those of the *C. sakazakii* phage GAP161 (Fig. 4A). In addition, comparisons of the gene clusters in PKP126 and two phages with highly homologous genome sequences (*Klebsiella* phage KLPN1 and *C. sakazakii* phage ESP2949-1) gave the same result, suggesting that *Klebsiella* phages may specifically recognize *Klebsiella* but may only weakly recognize *C. sakazakii*. Furthermore, a subsequent comparison of the clusters containing PKO111 and PKP126 showed that phage-tail-associated genes in the clusters share relatively low sequence similarity, suggesting that they may have different host recognition and infection profiles in *Klebsiella*. This may explain why these two phages recognize and infect *K. oxytoca* and *K. pneumoniae* in different manners. To overcome the different infection patterns of PKP126 and PKO111 in *K. oxytoca* and *K. pneumoniae*, the phages were mixed in an equal ratio for preparation of a phage cocktail (data not shown). For evaluation of phage cocktails containing PKP126 and PKO111, four different combinations of phage samples were prepared: PKP126 only, PKO111 only, 1/2(PKP126) + 1/2(PKO111), and PKP126 + PKO111. *K. pneumoniae* KCTC 2242 and *K. oxytoca* ATCC 43863 served as indicator strains. While

Table 2 Comparative protein sequence analysis of host-specificity-related proteins in gene clusters of phages PKO111 and PKP126

ORF	Predicted function	Length[a]	% Identity[b]	BLASTP best match	Accession no.
Klebsiella phage PKO111					
PKO111_143	Phage holin	218	138/218 (63%)	Holin [*Citrobacter* phage Moon]	NC_027331.1
PKO111_144	Long tail fiber assembly catalyst	175	43/69 (62%)	Histal long tail fiber assembly catalyst [*Klebsiella* phage KP27]	YP_007348901.1
PKO111_145	L-shaped tail fiber protein	1349	724/848 (85%)	L-shaped tail fiber protein [*Klebsiella* phage Matisse]	AKU44573.1
PKO111_146	Hinge connector of long tail fiber distal connector	222	134/229 (59%)	Hinge connector of long tail fiber distal connector [*Enterobacter* phage phiEap-3]	ALA45370.1
PKO111_147	Hinge connector of long tail fiber proximal connector	374	203/371 (55%)	Hinge connector of long tail fiber proximal connector [*Enterobacter* phage phiEap-3]	ALA45369.1
PKO111_148	Long tail fiber distal subunit	1264	584/1303 (45%)	Gp34 long tail fiber, proximal subunit [*Enterobacteria* phage CC31]	YP_004010112.1
Klebsiella phage PKP126					
PKP126_061	Putative tail fiber protein	1233	1137/ 1233(92%)	Tail fiber protein [*Klebsiella* phage KLPN1]	AKS10681.1
PKP126_062	Putative tail assembly protein	200	200/200 (100%)	Tail assembly protein [*Klebsiella* phage KLPN1]	AKS10680.1
PKP126_063	Putative minor tail protein	245	242/245 (99%)	Minor tail protein [*Klebsiella* phage KLPN1]	AKS10679.1
PKP126_064	Putative minor tail protein	261	242/250 (97%)	Minor tail protein [*Klebsiella* phage KLPN1]	AKS10678.1
PKP126_065	Putative minor tail protein	114	108/114 (95%)	Minor tail protein [*Klebsiella* phage KLPN1]	AKS10677.1
PKP126_066	Putative tail length tape-measure protein	981	944/981 (96%)	Tail length tape-measure protein [*Klebsiella* phage KLPN1]	AKS10676.1
PKP126_067	Tape measure chaperone	213	50/55 (91%)	Tape measure chaperone [*Klebsiella* phage Sushi]	AKQ07486.1
PKP126_068	Tape measure chaperone	108	95/108 (88%)	Tape measure chaperone [*Klebsiella* phage Sushi]	AKQ07485.1
PKP126_069	Putative major tail protein	218	199/218 (91%)	Major tail protein [*Klebsiella* phage KP36]	AEX26798.1

[a] Number of amino acids

[b] Amino acid sequence identity

single-phage tests were consistent with the previous results, phage cocktail tests revealed no synergistic activity. Nevertheless, the cocktails successfully inhibited the growth of both indicator strains, comparable to results for the single-phage treatment and its specific indicator strain. Therefore, to control both *K. pneumoniae* and *K. oxytoca*, treatment using a phage cocktail containing PKP126 and PKO111 should be more efficient for growth inhibition than a single-phage treatment.

To form lysogens, the lysogen decision gene cluster encoding Cro, CI, CII, CIII, N, or Q must be present in the genome. However, we could not find this gene cluster, suggesting that PKO111 and PKP126 are virulent phages. To verify this result, we used the PHACTS program to predict a virulent life cycle for both phages [22] (data not shown). Furthermore, a virulence factor analysis of these phage genomes with the Virulence Searcher program did not reveal the presence of human virulence genes, suggesting that these two phages may be suitable for further therapeutic applications. Previously, the *K. pneumoniae* phage Kpn5 was evaluated for therapeutic application [17]. This phage was used to treat a clinical burn wound infection with *K. pneumoniae* B5055, showing complete elimination of *K. pneumoniae* seven days after treatment. This demonstrated that phage therapy using *Klebsiella* phages could be an alternative treatment for *Klebsiella* infections.

To investigate the evolutionary relationship of the *Klebsiella* phages PKO111 and PKP126, amino acid sequences of the major capsid proteins (MCPs) deduced from these two phage genome sequences, as well as from another 54 complete genome sequences of *Klebsiella* and *C. sakazakii* phages, were compared using the neighbor-

joining method with p-distance values (Fig. S2). A phylogenetic tree constructed using phage MCP protein sequences contained two major phylogenetic groups. All phages in group I were *Klebsiella* or *C. sakazakii* phages belonging to the family *Myoviridae*, except for the *Klebsiella* phage Kp3 (*Podoviridae*) and the *C. sakazakii* phage phiES15 (*Siphoviridae*). This suggests that phages in group I may be closely related in terms of their evolution and have *Myoviridae* morphology. Most phages in group II are *Klebsiella* phages belonging to the family *Podoviridae*, except for four *C. sakazakii* phages, Dev2, Gap52, ESSI-2, and CR5, which belongs to the family *Myoviridae*. This suggests that these *Podoviridae*-associated phages may be evolutionarily related. However, other ungrouped phages (mostly members of the family *Siphoviridae*) showed little to no evolutionary relationship. Based on this analysis, *Klebsiella* and *C. sakazakii* phages belonging to the family *Myoviridae*, or *Klebsiella* phages belonging to the family *Podoviridae*, may have evolved individually with close relationships.

A characterization and genome analysis of the *Klebsiella* phages PKO111 and PKP126 showed that they have high host specificity and fitness, strong host lysis activities via virulent lifecycles, and no human virulence factors. Therefore, they may be good candidates for the development of novel biocontrol agents to treat *Klebsiella* infections in therapeutic settings. Further studies, including those focused on host receptor identification, phage cocktail optimization, and clinical *in vivo* effectiveness need to be performed.

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Compliance with ethical standards

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

References

1. Abbasifar R, Kropinski AM, Sabour PM, Ackermann HW, Lingohr EJ, Griffiths MW (2012) Complete genome sequence of *Cronobacter sakazakii* bacteriophage vB_CsaM_GAP161. J Virol 86:13806–13807
2. Adams MH (1959) Enumeration of bacteriophage particles. Bacteriophages. Interscience Publishers, New York, pp 27–30
3. Altermann E, Klaenhammer TR (2003) GAMOLA: a new local solution for sequence annotation and analyzing draft and finished prokaryotic genomes. OMICS 7:161–169
4. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402
5. Besemer J, Lomsadze A, Borodovsky M (2001) GeneMarkS: a self-training method for prediction of gene starts in microbial genomes. Implications for finding sequence motifs in regulatory regions. Nucleic Acids Res 29:2607–2618
6. Bouza E, Cercenado E (2002) *Klebsiella* and *Enterobacter*: antibiotic resistance and treatment implications. Semin Resp Infect 17:215–230
7. Cao F, Wang X, Wang L, Li Z, Che J, Wang L, Li X, Cao Z, Zhang J, Jin L (2015) Evaluation of the efficacy of a bacteriophage in the treatment of pneumonia induced by multidrug resistance *Klebsiella pneumoniae* in mice. Biomed Res Int 2015:752930
8. Carver TJ, Rutherford KM, Berriman M, Rajandream M-A, Barrell BG, Parkhill J (2005) ACT: the Artemis comparison tool. Bioinformatics 21:3422–3423
9. Demirdag K, Hosoglu S (2010) Epidemiology and risk factors for ESBL-producing *Klebsiella pneumoniae*: a case control study. J Infect Dev Ctries 4:717–722
10. Deresinski S (2009) Bacteriophage therapy: exploiting smaller fleas. Clin Infect Dis 48:1096–1101
11. Duckworth DH, Gulig PA (2002) Bacteriophages: potential treatment for bacterial infections. BioDrugs 16:57–62
12. Failla ML, Benedict C, Weinberg E (1975) Bacterial and fungal growth in total parenteral nutrition solutions. Antonie Van Leeuwenhoek 41:319–328
13. Gyles C (2007) Shiga toxin-producing *Escherichia coli*: an overview. J Anim Sci 85:E45–E62
14. Hayashi K, Morooka N, Yamamoto Y, Fujita K, Isono K, Choi S, Ohtsubo E, Baba T, Wanner BL, Mori H (2006) Highly accurate genome sequences of Escherichia coli K-12 strains MG1655 and W3110. Mol Syst Biol 2(2006):0007
15. Hendrix RW (2003) Bacteriophage genomics. Curr Opin Microbiol 6:506–511
16. Hirsch EB, Tam VH (2010) Detection and treatment options for *Klebsiella pneumoniae* carbapenemases (KPCs): an emerging cause of multidrug-resistant infection. J Antimicrobe Chemother 65:1119–1125
17. Kumari S, Harjai K, Chhirbber S (2010) Topical treatment of *Klebsiella pneumoniae* B5055 induced burn wound infection in mice using natural products. J Infect Dev Ctries 4:367–377
18. Lang LH (2006) FDA approves use of bacteriophages to be added to meat and poultry product. Gastroenterology 131:1370
19. Maal KB, Delfan AS, Salmanizadeh S (2014) Isolation and identification of *Klebsiella pneumonia* and *Klebsiella oxytoca* bacteriophages and their application in wastewater treatment and coliforrn's phage therapy. Res J Environ Sci 8:123–138
20. Maki DG (1976) Growth properties of microorganisms in infusion fluid and methods of detection. In: Phillips I, Meers PD, D'Arcy PF (eds) Microbiological hazards of infusion therapy. Microbiological hazards of infusion therapy. MTP Press, Lancaster, pp 13–47
21. McClelland M, Sanderson KE, Spieth J, Clifton SW, Latreille P, Courtney L, Porwollik S, Ali J, Dante M, Du F (2001) Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature 413:852–856
22. McNair K, Bailey BA, Edwards RA (2012) PHACTS, a computational approach to classifying the lifestyle of phages. Bioinformatics 28:614–618
23. Park K-H, Kurokawa K, Zheng L, Jung D-J, Tateishi K, Jin J-O, Ha N-C, Kang HJ, Matsushita M, Kwak J-Y (2010) Human serum mannose-binding lectin senses wall teichoic acid Glycopolymer of *Staphylococcus aureus*, which is restricted in infancy. J Biol Chem 285:27167–27175
24. Park M, Lee J-H, Shin H, Kim M, Choi J, Kang D-H, Heu S, Ryu S (2012) Characterization and comparative genomic analysis of a novel bacteriophage, SFP10, simultaneously inhibiting both

Salmonella enterica and *Escherichia coli* O157:H7. Appl Environ Microbiol 78:58–69
25. Podschun R, Ullmann U (1998) *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin Microbiol Rev 11:589–603
26. Pomakova D, Hsiao C, Beanan J, Olson R, MacDonald U, Keynan Y, Russo T (2012) Clinical and phenotypic differences between classic and hypervirulent *Klebsiella pneumonia*: an emerging and under-recognized pathogenic variant. Eur J Clin Microbiol Infect Dis 31:981–989
27. Quevillon E, Silventoinen V, Pillai S, Harte N, Mulder N, Apweiler R, Lopez R (2005) InterProScan: protein domains identifier. Nucleic Acids Res 33:W116–W120
28. Ribot EM, Wierzba RK, Angulo FJ, Barrett TJ (2002) *Salmonella enterica* serotype Typhimurium DT104 isolated from humans, United States, 1985, 1990, and 1995. Emerg Infect Dis 8:387–391
29. Rutherford K, Parkhill J, Crook J, Horsnell T, Rice P, Rajandream M-A, Barrell B (2000) Artemis: sequence visualization and annotation. Bioinformatics 16:944–945
30. Shen Y-J, Jiang H, Jin J-P, Zhang Z-B, Xi B, He Y-Y, Wang G, Wang C, Qian L, Li X (2004) Development of genome-wide DNA polymorphism database for map-based cloning of rice genes. Plant Physiol 135:1198–1205
31. Shoma S, Kamruzzaman M, Ginn AN, Iredell JR, Partridge SR (2014) Characterization of multidrug-resistant *Klebsiella pneumoniae* from Australia carrying blaNDM-1. Diagn Microbiol Infect Dis 78:93–97
32. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, Thompson JD, Higgins DG (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol 7:539
33. Struve C, Krogfelt KA (2004) Pathogenic potential of environmental *Klebsiella pneumoniae* isolates. Environ Microbiol 6:584–590
34. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729
35. Tatusova TA, Madden TL (1999) BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences. FEMS Microbiol Lett 174:247–250
36. Unerwood AP, Mulder A, Gharbia S, Green J (2005) Virulence Searcher: a tool for searching raw genome sequences from bacterial genomes for putative virulence factors. Clin Microbiol Infect 11:770–772
37. Verma V, Harjai K, Chhibber S (2009) Characterization of a T7-like lytic bacteriophage of *Klebsiella pneumoniae* B5055: a potential therapeutic agent. Curr Microbiol 59:274–281
38. Wilcox S, Toder R, Foster J (1996) Rapid isolation of recombinant lambda phage DNA for use in fluorescence in situ hybridization. Chromosome Res 4:397–404
39. Wu L-T, Chang S-Y, Yen M-R, Yang T-C, Tseng Y-H (2007) Characterization of extended-host-range pseudo-T-even bacteriophage Kpp95 isolated on *Klebsiella pneumoniae*. Appl Environ Microbiol 73:2532–2540
40. Zhu J, Jiang R, Kong H, Zhang R, Lü H, Sun C, Huang Z (2013) Emergence of novel variants of *gyrA*, *parC*, *qnrS* genes in multidrug resistant *Klebsiella* caused pneumonia. Zhonghua Liu Xing Bing Xue Za Zhi 34:61–66