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Characterization of Extended-Host-Range Pseudo-T-Even Bacteriophage Kpp95 Isolated on *Klebsiella pneumoniae*[▽]

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Kpp95, isolated on *Klebsiella pneumoniae*, is a bacteriophage with the morphology of T4-type phages and is capable of rapid lysis of host cells. Its double-stranded genomic DNA (ca. 175 kb, estimated by pulsed-field gel electrophoresis) can be cut only by restriction endonucleases with a cleavage site flanked either by A and T or by T, as tested, suggesting that it contains the modified derivative(s) of G and/or C. Over 26 protein bands were visualized upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the virion proteins. N-terminal sequencing indicated that the most abundant band (46 kDa) is the major coat protein (gp23) which has been cleaved from a signal peptide likely with a length similar to that of T4. Phylogenetic analyses based on the sequences of the central region (263 amino acid residues) of gp23 and the full length of gp18 and gp19 placed Kpp95 among the pseudo-T-even subgroup, most closely related to the coliphage JS98. In addition to being able to lyse many extended-spectrum β-lactamase strains of *K. pneumoniae*, Kpp95 can lyse *Klebsiella oxytoca*, *Enterobacter agglomerans*, and *Serratia marcescens* cells. Thus, Kpp95 deserves further studies for development as a component of a therapeutic cocktail, owing to its high efficiencies of host lysis plus extended host range.

The *Klebsiella* spp., ubiquitous in nature, are opportunistic human pathogens attacking primarily immunocompromised individuals who are hospitalized and suffering from severe underlying diseases such as diabetes mellitus or chronic pulmonary obstruction. It is estimated that *Klebsiella* spp. cause 8% of nosocomial bacterial infections in the United States and in Europe, placing these bacteria among the eight most important infectious pathogens in hospitals (35). Nosocomial *Klebsiella* infections are caused mainly by *K. pneumoniae*, the most clinically important species of the genus, present as a saprophyte in the human mouth, the nasopharynx, and the intestinal tract (35). In Taiwan, the high prevalence of *K. pneumoniae* has also been observed. For example, a survey taken during 1991 to 2003 at a university hospital in Taiwan indicates that *K. pneumoniae* ranked second among gram-negative bacteria causing nosocomial infections, only after *Escherichia coli* (22). Recently, *Klebsiella* infection-induced liver abscess was first reported in Taiwan, and *K. pneumoniae* has surpassed *E. coli*, the historically predominant causative agent of hepatic abscesses, as the number one isolate from patients with this disease (56, 57). Since the initial description of extended-spectrum β-lactamase production by *K. pneumoniae* strains in 1983 (24), this organism's resistance to expanded-spectrum β-lactam antibiotics has emerged quickly (7). A similar situation was encountered in Taiwan (61), and it has caused increasingly serious problems in treating *K. pneumoniae* infections. Thus, a different approach, such as treatments with specific lytic bacteriophages, could be a possible alternative therapy (for a review, see references 3, 9, 25, 46, 47, and 48).

In this study, the first effort toward phage therapy for treating *K. pneumoniae* infections, we isolated a lytic bacteriophage (Kpp95) from hospital samples, using a clinical isolate of *K. pneumoniae* as the indicator cell. Characterization of this phage is presented here.

MATERIALS AND METHODS

Bacterial strains and cultivation. Bacterial strains used in this study are listed in Table 1. All the strains were identified on the basis of microbiological methods and verified by using a VITEK system (BioMerieux Vitek Inc., Hazelwood, MO). Luria-Bertani (LB) agar and LB broth (36) were the media used for bacterial growth at appropriate temperatures with aeration. Antibiotics used were ampicillin (50 μg/ml) and kanamycin (50 μg/ml).

Spot test and plaque assay. To detect the presence of phage in a sample and the phage sensitivity of a bacterium, spot tests were carried out as described previously (11), except that LB broth and LB agar plates were used. To determine the phage titers, double-layered bioassays were performed on LB agar plates with the top and bottom layers containing 0.75% and 1.5% agar, respectively. One-tenth of a milliliter each of a phage suspension after serial dilutions and 100 μl of *K. pneumoniae* 10693 from an overnight culture were mixed with 3 ml of molten soft agar and poured onto the bottom solidified agar (12 ml). The numbers of plaques were counted after incubating the plates overnight. The same method was used to confirm phage susceptibility with the cells of different bacteria as the indicator hosts.

Propagation and purification of phage. To propagate the phage, overnight cultures of *K. pneumoniae* 10693 were diluted 20-fold into 125-ml flasks containing 20 ml of the LB broth. When the cultures reached mid-exponential phase, the phage particles were added at a multiplicity of infection (MOI) of 20 and further incubated until ca. 12 h postinfection. Crude phage suspensions were prepared by centrifugation (10,000 × *g*, 15 min, at 4°C) of the cultures to remove the cells and by passing the supernatants through a membrane filter (0.45-μm-pore size). Phage particles were purified by banding in ultracentrifugation as previously described (23).

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TABLE 1. Bacterial strains used in this study

Strain(s)	Relevant characteristic(s)[a]	Source or reference
Acinetobacter baumannii		
Ab1–Ab10	Imi^r Mer^r Amp^r	This study[b]
Enterobacter agglomerans		
EaJ0–EaJ9	EaJ0 (ESBL strains), Amp^r	This study[b]
Enterobacter cloacae		
Ec1–Ec12	ESBL strains, Amp^r	This study[b]
Escherichia coli		
DH5α	*endA1 hsdR17* (rk$^-$ mk$^+$) *supE44 thi-1 recA1 gyrA relA1* ϕ80d *lacZ*Δ *M15*Δ (*lacZYA-argF*)*U169*	18
E1–E13	Amp^r	This study[c]
Klebsiella oxycota		
KoJ1–KoJ8	Amp^r	This study[b]
KoC1–KoC6	Amp^r	This study[c]
Klebsiella pneumoniae		
10693	Phage host, Amp^s	ATCC 23357
Kp1–Kp108[d]	ESBL strains, Amp^r	This study[c]
Proteus mirabilis		
Pm1, Pm3, Pm18, Pm40, Pm51, Pm59, Pm69, Pm70, Pm72, Pm73, Pm84, Pm97, Pm103, Pm111	ESBL strains, Amp^r	59
Pseudomonas aeruginosa		
27853	Amp^s	38
Pa1–Pa6	Imi^r Mer^r Amp^r	This study[b]
Serratia marcescens		
Sm1–Sm4, Sm8	ESBL strains, Amp^r	58

[a] Amp, ampicillin; Imi, imipenem; Mer, meropenem; r, resistant; s, susceptible; ESBL, extended-spectrum β-lactamase.
[b] Strains isolated in Jen-Ai Hospital, Taichung, Taiwan, Republic of China.
[c] Strains isolated in the Hospital of China Medical University, Taiwan, Republic of China.
[d] All of these strains were Amp^r, but only 25 of them were ESBL strains.

One-step growth curve. A culture (10 ml in a 125-ml flask) of *K. pneumoniae* 10693 grown to mid-exponential phase was harvested by centrifugation and resuspended in fresh LB broth (ca. 1×10^9 CFU/ml). Phage was added at an MOI of 0.003 and allowed to adsorb for 5 min at 4°C. The mixture was then centrifuged, and the pelleted cells were resuspended in 10 ml of LB, followed by incubation at 37°C. Samples were taken at 5-min intervals and immediately titered by the double-layered agar plate method (34). The first set of samples were immediately diluted and plated for phage titration. A second set of samples was treated with chloroform (1% final concentration) to release intracellular phage for determining the eclipse period before phage titration. Experiments were repeated three times with duplicate samples.

Isolation of phage DNA. Phage DNA was purified either by a Wizard Lambda DNA Preps DNA purification system (Promega, Madison, WI) or by phenol extraction of concentrated phage particles. In the latter case, the phage lysate was incubated for 30 min at 37°C in the presence of 20% polyethylene glycol 8000–2.5 M NaCl. The mixture was then incubated on ice for 60 min followed by centrifugation in 15-ml Corex tubes (Corning, Corning, NY) at 12,000 × *g* for 5 min. The pellet was suspended in 400 μl of 40 mM Tris-HCl (pH 7.4) containing 150 mM NaCl and 10 mM $MgSO_4$. The mixture was clarified by centrifugation in a microcentrifuge for 2 min. The supernatant was extracted twice with chloroform. Phage DNA was then released by gentle mixing in an equal volume of Tris-HCl (40 mM, pH 7.9)-buffered phenol for 5 min. After centrifugation for 5 min, the upper layer was extracted again with phenol and then with chloroform. DNA was precipitated by the addition of 1 ml of 95% ethanol and 50 μl of 3 M sodium acetate (pH 5.2), collected by centrifugation, rinsed with 300 μl of 70% ethanol, allowed to dry, and gently suspended in 50 μl of 10 mM Tris-HCl (pH 8.0) containing 0.1 mM EDTA.

Restriction endonucleases were purchased from Takara Shuzou (Shiga, Japan), Gibco BRL (Grand Island, NY), or Roche Molecular Biochemicals (Indianapolis, IN). Digestion of the phage DNA was performed according to the instructions provided by the suppliers. The samples were electrophoresed either in 0.8% agarose (0.5× Tris-borate-EDTA buffer [TBE]) at 50V for 1 h or in 1% agarose (0.5× TBE buffer) at 50 V for 3 to 4 h.

PCR amplification. To amplify the internal fragment of Kpp95 gene 23, we used primers P23F (5′-TGTTATAGGTATGGTACGACGTGCTAT-3′) and P23R (5′-TGAAGTTACCTTCACCACGACCGG-3′), which were previously used for PCR amplification of a 789-bp fragment internal to the gene 23 of various T4-type phages (49). PCR mixtures (10 μl) consisted of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 μM each of the primers, 0.16 mM deoxynucleoside triphosphates (Gibco BRL), 0.5 U of *Taq* polymerase (Gibco BRL), and 1 μl of phage lysate (10^4 to 10^7 PFU). Reactions were performed at 95°C for 2 min, followed by 30 cycles of 95°C for 60 s, 53°C for 60 s, and 72°C for 60 s. The sizes of the amplified fragments were checked with agarose gel (1.5% in 0.5× TBE buffer) electrophoresis run at 50 V, followed by ethidium bromide staining.

Pulsed-field gel electrophoresis. Pulsed-field gel electrophoresis (PFGE) was performed in a CHEF-DR II PFGE system (Bio-Rad Laboratories, Hercules, CA) as described previously (52). The 1% gels were made with Seakem Gold agarose (Rockland, ME) in 0.5× TBE buffer. The conditions for electrophoresis were 0.5× TBE running buffer, 1-s initial switch time, 40-s final switch time, 200 V, 20-h running time, and a buffer temperature of 14°C. Lambda DNA concatemers, HindIII-digested DNA, and *Saccharomyces cerevisiae* YPH80 chromosomes (New England Biolabs, Beverly, MA) were used as the molecular size markers. The gels were stained with ethidium bromide and visualized on a UV box.

SDS-PAGE and protein analysis. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation, sample containing the Kpp95 was mixed with the sample buffer and then heated in a boiling water bath for 3 min, as described previously (27), and electrophoresed in an SDS-polyacrylamide gel (12%) by the method of Laemmli (25). Protein bands were visualized by staining the gels with Coomassie brilliant blue. For determination of the N-terminal amino acid (aa) sequence, the Kpp95 was separated by SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane, and then the protein was eluted from the membrane and subjected to Edman degradation in an Applied Biosystems model 476A protein sequencer. Protein concentration was determined as previously described (26).

DNA cloning and sequencing. The Kpp95 DNA isolated from the phage particles was digested with SspI, and the resultant fragments were blunt ended and cloned into the *E. coli* vector pOK12 (54). Direct sequencing of both strands of the PCR products or the inserts was performed by dideoxy chain termination with an Applied Biosystems sequencer (ABI 377) (41) (Table 2).

Bioinformatics. Amino acid sequence identity was searched by using BLAST and Blastp (http://www.ncbi.nlm.nih.gov). Sequences were aligned by CLUSTALW (http://www.ebi.ac.uk/clustalw), and phylogenetic trees based on the neighbor-

TABLE 2. Proteins/protein segments deduced from sequenced Kpp95 DNA fragments and their identities to homologues of T4-type phages

Fragment (gene product)[a]	Corresponding aa positions at T4 homologue[b]	T4 protein	Identities shared (%) with corresponding proteins from T4-type phages	Accession no.
906 (gp17)	97–399 (610)	Terminase (central region)	RB69 (89); T4, RB32 and JS98 (87)	DQ845391
3788 (gp17)	414–610 (610)	Terminase (C terminus)	RB69 (83); RB32 and T4 (81); JS98 (79)	AY538772
3788 (gp18)	1–656 (659)	Tail sheath	JS98 (76); RB69, RB32 and T4 (71)	AY538772
3788 (gp19)	1–163 (163)	Tail tube	JS98 (82); RB43 (73); RB69 and 42 (69)	AY538772
3788 (gp20)	1–156 (524)	Head portal	JS98 (69); RB69 and RB32 (65); T4 (64)	AY538772
789 (gp23)[c]	98–360 (521)	Major capsid	JS98, T4 and JS9 (83); T6, RB69, RB32, AR1, MVSS and JSD1 (82)	DQ845392
858 (gp34)	1,062–1,288 (1,289)	Proximal tail fiber	RB43 (40); JS98 (31); RB32 (29); T4 (28)	DQ845394
333 (gp37)	96–165 (1,026)	Long tail fiber	K3 (49); T4 (47); T5 (46); JS98 and Ac3 (35)	DQ845393
1031 (gp41)	1–142 (475)	Primase-helicase	RB69, RB32 and T4 (69); JS98 (65)	DQ845390
678 (gp wac)	76–259 (487)	Whisker antigen	RB43 (43); T2 (38); T4 and K3 (37); JS98 and AR1 (36)	DQ845389

[a] gp18 and gp19 are full-length proteins, whereas the others are segments.
[b] Numbers in parentheses represent the full length of the T4 protein.
[c] A PCR fragment.

joining method were constructed by using PHYLIP (version 3.6). A bootstrap confidence value was assigned for each branch of the tree using the SEQBOOT program of PHYLIP (1,000 replicates).

Nucleotide sequence accession numbers. The Kpp95 sequences described here have been deposited in GenBank with the accession numbers DQ845389 to DQ845394 and AY538772 as listed in Table 2.

RESULTS AND DISCUSSION

Kpp95 is one of the phages isolated on *K. pneumoniae*. During this study, 254 hospital samples, including patient specimens, catheter washings, and wastewater from drainages, were collected. These samples were screened separately for the presence of phage by spot testing using *K. pneumoniae* 6 (a clinical strain) and *K. pneumoniae* 10693 as indicator host cells. Twelve phages were isolated after three consecutive single-plaque isolations and designated as Kpp2, -3, -4, -5, -6, -7, -10, -11, -30, -42, -50, and -95. They were indistinguishable based on preliminary results of study of phage properties, including morphology, EcoRV restriction patterns, and genome sizes estimated by PFGE (data not shown except for that of Kpp95 which is described below). Kpp95, capable of rapid lysis of host cells yielding high titers of progeny (3×10^{10} to 5×10^{10} PFU/ml), was studied further.

Electron microscopy showed that Kpp95 had a moderately elongated icosahedral head (ca. 101 nm long and 72 nm in diameter) connected to a sheathed tail (ca. 100 nm long and 13 nm wide) (Fig. 1). The distal end of the tail contained structural elements similar to those of the base plate, and a collar was localized faintly at the head-tail junction (Fig. 1). This virion morphology was similar to that of the T-even phages (2).

The one-step growth curve of Kpp95 was measured as described in Materials and Methods on *K. pneumoniae* strain 10693. As shown in Fig. 2, eclipse and latent periods of 20 to 25 and 30 min, respectively, were observed, indicating that Kpp95 requires a short growth period of about 5 to 10 min. The estimated burst size was 100 PFU per infected cell (Fig. 2). These values fit into the ranges observed for T-even group phages (1).

Kpp95 has a double-stranded DNA genome that appears to have base modifications. T-even genomes contain hydroxylmethylcytosine in place of cytosine, and these residues are generally glucosylated, rendering the DNA resistant to digestion by some restriction enzymes, which provides additional protection against host restriction systems (5, 28). In contrast, the genomes of the pseudo-T-even phages can be cut by restriction enzymes that are unable to digest T-even DNA, suggesting that only limited nucleotide modifications must be present in the pseudo-T-even genomes (32). We were interested in understanding whether the Kpp95 DNA could be cut by restriction endonucleases to give distinct fragments from which we might be able to sum up the total lengths for estimation of the genome size. To achieve this, DNA was prepared from Kpp95 phage particles and separately digested with 16 restriction endonucleases, including AseI, AvaI, BamHI, DraI, EcoRI, EcoRV, HaeIII, HincII, HindIII, KpnI, NaeI, PstI, SacII, Sau3A1, SmaI, and SspI. The DNA could be cut only by AseI, DraI, EcoRV, NdeI, and SspI into distinct bands, as visualized with an agarose gel after electrophoresis and staining with ethidium bromide (data not shown), but was refractory to digestion with the other 11 enzymes tested. Although the bands were not resolved well enough for estimation of the

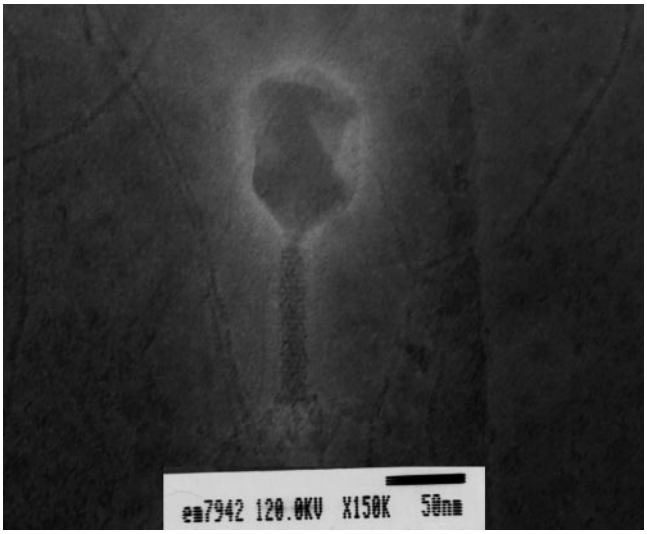

FIG. 1. Electron micrograph of bacteriophage Kpp95. The sample was stained with 2% uranyl acetate.

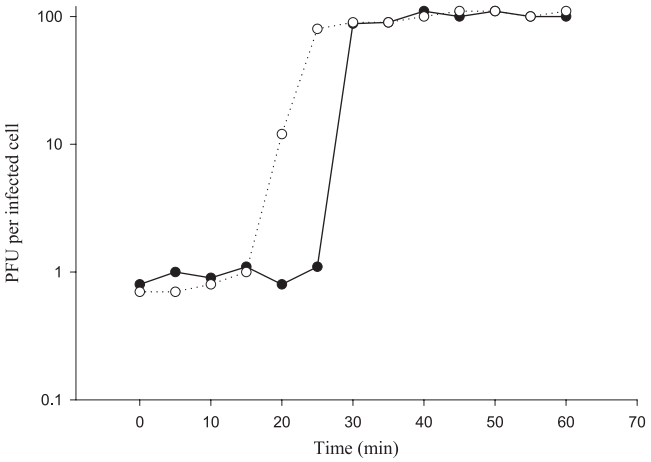

FIG. 2. One-step growth curve of bacteriophage Kpp95 on *K. pneumoniae* strain 10693 in chloroform-treated culture (○) and in untreated cultures (●).

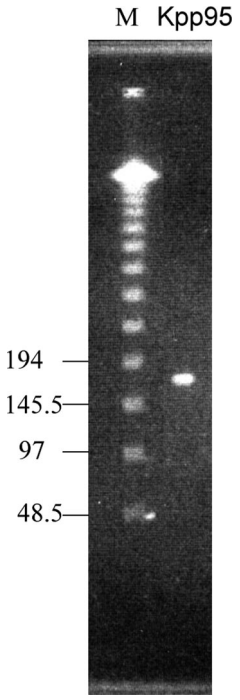

FIG. 3. PFGE of the Kpp95 genome. Electrophoresis was performed with 1% agarose gel at 14°C and 200 V, with an initial time of 1 s, a final time of 40 s, and a running time of 20 h. Lane M contains molecular size markers (Lambda DNA ladder).

total lengths, susceptibility to restriction digestion indicated that the Kpp95 genome is a double-stranded DNA molecule. Notably, each of the five competent enzymes has a cleavage site flanked by either A and T (DraI, EcoRV, NdeI, and SspI) or T (AseI), suggesting that the Kpp95 genomic DNA contains a modified derivative(s) of G and/or C, rendering the DNA resistant to enzymes with cleavage sites flanked by G or C. These findings suggest that base modifications similar to the cases in T-type phages occur in Kpp95.

In parallel experiments, the restriction enzymes EcoRI, HincII, HindIII, and PstI that could not cut the Kpp95 genome were found capable of cutting the chromosomal DNA prepared from *K. pneumoniae* 10693 (data not shown), indicating that the same base modification is not found in the host cell. In addition, randomly cloned fragments of Kpp95 genome prepared from *E. coli* (see below) were susceptible to digestion with these restriction enzymes. These observations together indicate that the modification system is encoded in Kpp95.

Kpp95 genome is ca. 175 kb in length. Since our efforts to estimate the Kpp95 genome size by summing the restriction fragment lengths were unsuccessful, we adopted pulsed-field gel electrophoresis to mobilize the intact genomic DNA prepared from the phage particles. As shown in Fig. 3, the Kpp95 genome migrated to a distance corresponding to a size of 170 to 180 kb. T4 packages its genome by a head-filling mechanism (45); and with similar head sizes, pseudo-T-even phages have genomes with sizes comparable to that of T4 (49). Thus, the Kpp95 has a genome size falling within the range of those of T4 (168,903 bp) and the pseudo-T-even phages (31, 49).

At least 26 virion proteins of Kpp95 can be visualized by SDS-PAGE. To analyze the virion structural proteins, the Kpp95 phage particles were purified by ultracentrifugation, treated by boiling with the cracking buffer, and separated by SDS-PAGE (Fig. 4). At least 26 distinct protein bands, with molecular masses ranging from 6 to 200 kDa, were visualized after the gels were stained with Coomassie brilliant blue. The most abundant one in the gel was a 46-kDa protein, most likely the major coat protein of Kpp95. The sequence of the N-terminal 15 aa chemically determined for this protein, AEIG GDHGYDAQNIA, was 14/15 identical to aa 67 to 81 of the SV14 and RB69 gp23 proteins and 13/15 identical to aa 66 to 80 of the T-even (T2 and T6) and AR1 gp23 proteins (32, 49, 53, 60). In T4, during phage maturation, the N-terminal 65 aa residues of gp23 (521 residues) are cleaved off to give rise to gp23* (residues 66 to 521) (44). Due to the high abundance of the protein and the high degree of identity to the mature N terminus of the T4 gp23, it is reasonable to conclude that this 46-kDa protein is the mature major coat protein of Kpp95 that has been N-terminally processed to cleave off a length similar to that of T4 gp23.

Random genome sequencing indicates Kpp95 is similar to T-even phages. In order to understand its similarity to other phages, the Kpp95 genome was digested with SspI, and the resultant fragments were blunt ended and cloned into pOK12. Six of the recombinant plasmids were subjected to nucleotide sequence determination of the inserts. Analysis revealed 906-, 3,788-, 858-, 333-, 1,031-, and 678-bp sequences. The G+C contents of these regions were around 40%, ranging from 38 to 42%, which deviated from that (55.5%) of the host genome (6). Notably, 2, 9, 3, 2, 2, and 2 recognition sequences for restriction endonucleases EcoRI, HincII, HindIII, KpnI, NaeI, and PstI, respectively, that could not cut the Kpp95 DNA were found in these sequenced fragments.

The sequences of the above-cited fragments showed low degrees of similarity at the nucleotide level to the entries in the database. However, alignment of the aa sequences deduced from these DNA sequences revealed that varied degrees of identity were shared with proteins from T4-type phages. Table 2 lists (i) the products of the genes residing in the fragments,

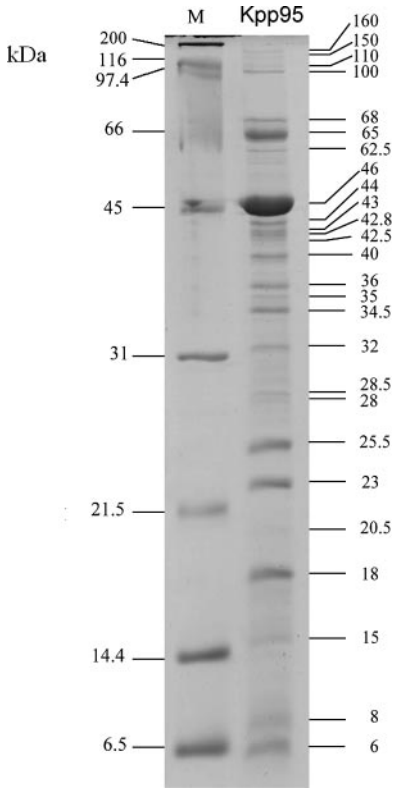

FIG. 4. SDS-PAGE of Kpp95 coat proteins. Lane M contains broad-range protein molecular mass markers.

(ii) the aa positions corresponding to those of T4, (iii) the names of homologous proteins in T4, and (iv) at least four homologues from T4-type phages that shared the highest degrees of identity (36 to 89%) with the deduced Kpp95 proteins or protein segments. The 906-bp fragment encoded a region homologous to aa 97 to 399 of T4 gp17 (terminase, a packaging protein). The region homologous to aa 414 to 610 of the T4 gp17 was found in an upstream region of the 3,788-bp fragment. The remaining part of the 3,788-bp fragment encoded the complete gp18 (tail sheath protein) and gp19 (tail tube protein) and the N terminus of gp20 (head portal protein). The 858-, 333-, 1,031-, and 678-bp DNA fragments encoded protein segments homologous to the corresponding regions of T4 gp34 (proximal tail fiber protein), gp37 (long tail fiber protein), gp41 (primase-helicase), and gp wac (whisker antigen), respectively. Table 2 also shows the identities (82 to 83%) shared between the central region of the major capsid protein gp23 from T4-type phages (corresponding to aa 98 to 360 of T4 gp23) and that from Kpp95 revealed by sequencing a PCR fragment (263 aa) amplified on the Kpp95 genome by using a T4 gp23-specific degenerate primer pair (see below).

It is worth noting that (i) the sequences from JS98, RB69, RB43, RB32, and T4 were always the ones sharing the highest degrees of identity with the Kpp95 proteins and that (ii) while over 64 to 89% identities were shared among gp17, gp18, gp19, gp20, gp23, gp41, and the respective corresponding proteins, lower than 50% identities were shared among the gp34, gp37, and whisker protein homologues (Table 2).

Phylogenetic analysis of gp18, gp19, and gp23 indicated Kpp95 was most closely related to coliphage JS98, which classified it as a pseudo-T-even subgroup member. Based on relatedness of the sequences of three major virion structural proteins, the tail sheath protein (gp18), the tail tube protein (gp19), and the major capsid protein (gp23), the T4-type phages can be divided into four subgroups with increasing divergence from T4: the T-even, the pseudo-T-even, the schizo-T-even, and the exo-T-even phages (13). Although the phylogenetic trees obtained for all of the three virion proteins are similar, the ones based on gp23 are most commonly used for relatedness analysis (13, 15, 49). For the analyses, alignments were performed among the amino acid sequences encoded by the internal fragments, each of which was flanked by two regions of consensus among the gp23 of T4-type phages. In the T4 gp23 proteins, the internal region encompasses aa 95 to 375, and therefore, using the primer pair corresponding to aa 95 to 103 and 368 to 375 of the T4 gp23, the central portion of gene 23 (ca. 850 bp) of various T4-type phages can be amplified, and an internal region of 188 aa (aa 115 to 302, corresponding to the T4 gp23) was used for phylogenetic analysis (13, 49). In this study, we used the degenerated primers described in Materials and Methods for amplification, with the Kpp95 genome as the template, and an amplicon of 789 bp (encoding 263 aa) was obtained. To discern the relatedness, the aa sequence deduced from this Kpp95 fragment (instead of the 188-aa region, see below) was aligned with the corresponding region of gp23 from T4-type phages, which was available from the database, had a deposited length of at least 263 aa, and shared over 65% identity with that of the Kpp95 gp23. Eleven entries were found that met these criteria and were used for the construction of a phylogenetic tree as described in Materials and Methods. As shown in Fig. 5A, the analysis indicated that Kpp95 is most closely related to the coliphage JS98 (sharing the highest identity, 83%, with the Kpp95 gp23 region) belonging to the pseudo-T-even subgroup (Fig. 5A) (12, 13, 49).

Full-length sequences of gp18 and gp19 from some T4-type phages were also available in the database. Among them, 13 each of gp18 and gp19 sharing the highest identities with that of the Kpp95 sequence were aligned for phylogenetic analyses. As shown in Fig. 5B and C, they showed 50 to 76% and 57 to 82% identities to the Kpp95 gp18 and gp19 sequences, respectively, with the highest degree of identity shared with the proteins from JS98 (76% for gp18 and 82% for gp19). The phylogenetic analyses based on the gp18 and gp19 sequences indicated that JS98 was still the phage most closely related to Kpp95.

During these analyses, we also tried to use the internal 188-aa sequences of gp23 for tree construction as described previously (13, 49). However, the relatedness found was closest to that of *Burkholderia cepacia* phage 42 (data not shown), which did not even appear in the tree when the sequences of 263 aa were based for gp23 analysis (Fig. 5A). Furthermore, when the full-length sequences were used for analysis of gp18 and gp19, the phage 42 sequences were not as closely related as those of JS98. Since the three trees consistently favored relatedness to JS98, it appears that the 263-aa sequences of gp23 can provide a better basis than the 188-aa internal regions for the relatedness analysis of T4-type phages.

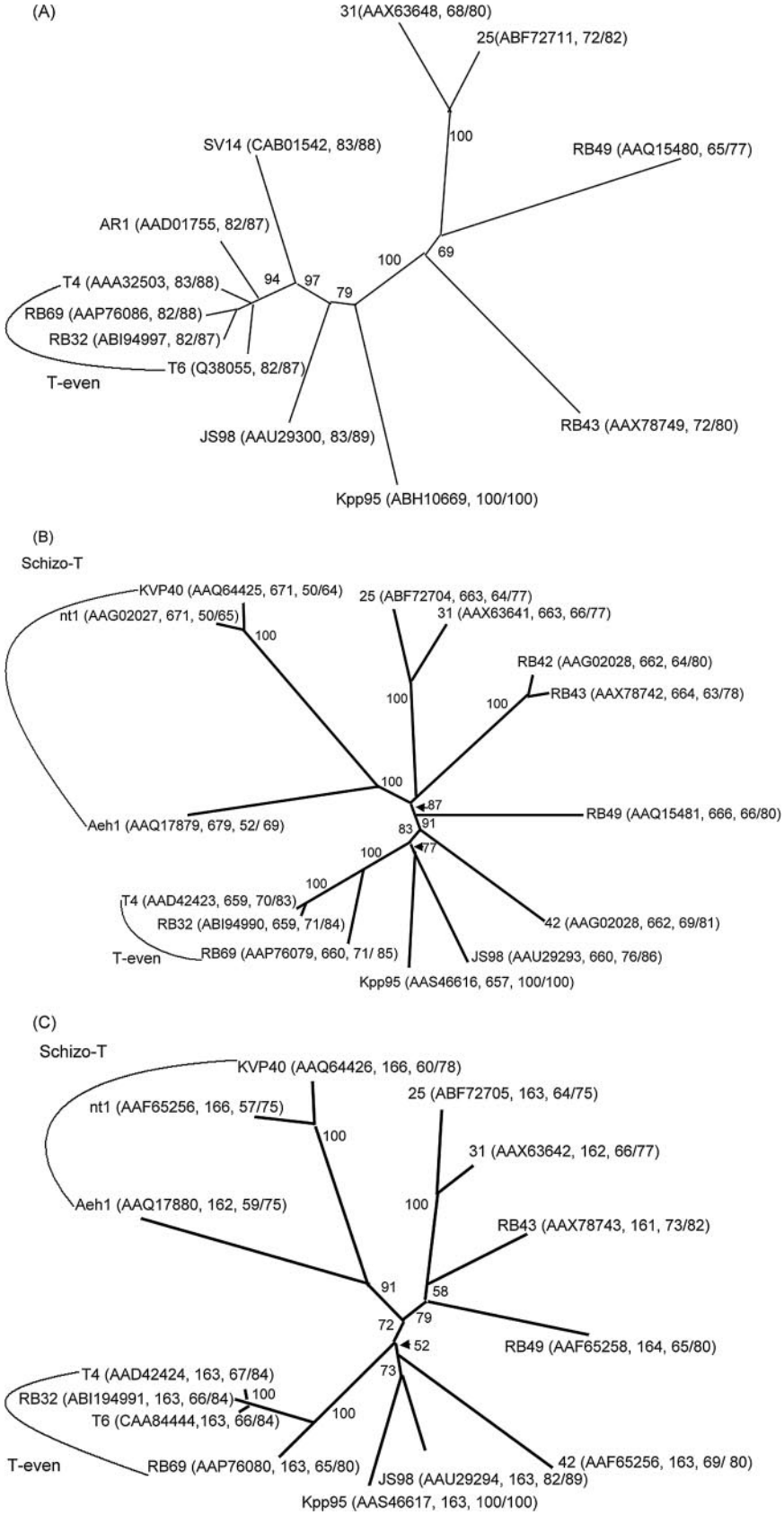

FIG. 5. Phylogenetic trees based on the aa sequence of gp23 (A), gp18 (B), and gp19 (C) from T4-type phages. The numbers at branching points are bootstrap values based on 1,000 replicates. Parentheses contain the GenBank accession numbers, followed by the lengths (aa) of the protein/protein segment (except that each of the gp23 segments was 263 aa in length) and then identity/similarity. The sequences include (i) T-even phages T4, T6, RB32, and RB69 infecting *E. coli*; (ii) pseudo-T-even phages 25 and 31 infecting *Aeromonas salmonicida*, phage 42 of *Burkholderia cepacia*, and coliphages AR1, JS98, RB42, RB43, RB49, and SV14; and (iii) schizo-T-even phage Aeh1 of *Aeromonas hydrophila*, KVP40 of *Vibrio cholerae*, and nt1 of *Vibrio natriegens*.

Kpp95 has an extended host range. To evaluate host susceptibility to Kpp95, the bacterial lawns of 107 *K. pneumoniae* strains, except for the indicator host strain 10693, were subjected separately to spot testing (Table 1). Completely clean clearing zones were formed on 40 (47%) of the strains, 25 (23.2%) strains gave turbid clearing zones, and 43 (39.8%) strains exhibited resistance to the phage. In plaque assays, the degrees of plaque clarity were consistent with those of the clearing zones.

Susceptibility to Kpp95 was also tested with 86 clinical strains representing eight bacterial species other than *K. pneumoniae* (Table 1). Results (the number of sensitive strains over the total strains tested) indicated that *Enterobacter agglomerans* (7/10), *Klebsiella oxytoca* (14/14), and *Serratia marcescens* (5/5) were susceptible to Kpp95. All strains shown to be positive by spot test gave clear plaques in plaque assays, except for *S. marcescens*, on which turbid plaques were formed. In these positive cases, plating efficiencies were similar when the same batches of the phage lysate were used for different host strains. *Acinetobacter baumannii* (10), *Enterobacter cloacae* (12), *E. coli* (14), *Proteus mirabilis* (14), and *Pseudomonas aeruginosa* (7), with the total numbers of strains tested in parentheses, were found to be resistant to Kpp95.

T4 infects only *E. coli* and the closely related *Shigella* sp., with the adsorption specificity being determined by the tip-of-tail fibers (gp37), which bind to receptors on the surface of the bacteria, and its host range is expanded by duplications of a small region of the tail fiber adhesin (50). Several other bacteriophages exhibiting an intrinsic broad host range have been reported, such as LG1 (a *Myoviridae* coliphage with 49.5-kb genomic DNA), the T4-like coliphage AR1, and the T4-like vibriophage KVP40. LG1 and AR1 can infect many serotypes of *E. coli* and enterobacteria, including *Proteus mirabilis*, *Shigella dysenteriae*, and two *Salmonellas* trains (16). KVP40 can infect eight vibrio species, including *V. parahaemolyticus*, *V. cholerae*, the nonpathogenic *V. natriegens*, and *Photobacterium leiognathi* (30). However, the mechanisms involved in these phages' abilities to infect different hosts have not been elucidated. To determine the sequence which might be involved in Kpp95 adsorption, we tried to obtain Kpp95 gene 37 by PCR amplification with primers specific to T4 gene 37 (37RV-1F [5′-GTTCTGGTAATTTTGCTAAC-3′] and 37RV-1R [5′-AACAGCTAACTTTGGATATG-3′], FR86 [5′-GCTTCAAGTACTGACTTAGG-3′], and FR89 [5′-ACAGTGATAGTATGACCATGTGATCC-3′]) (37, 49). However, the reactions failed to give a PCR product. This result was consistent with the low identity (47%) revealed by sequencing the fragment of g37 (Table 2) and indicated that the two phages have very divergent tail fibers.

Haggard-Ljungquist et al. (1992) have summarized the conditions that may cause a phage to alter or extend its host range, as follows (17). First, point mutations in the tail fiber genes can alter the host range. This might have evolved to counteract host range mutants of the bacterial hosts. Second, a new host range may be acquired by obtaining a portion of a tail fiber gene from another phage or from a gene left in the bacterial chromosome by another phage. This has been evidenced by (i) similarities in the tail fiber genes of such unrelated phages as P1, P2, Mu, λ, and T4, and (ii) by segments similar to genes 36 and 37 (tail fiber gene) of T2, and a cryptic gene (CRF86) similar to the tail fiber assembly gene have been detected in the *E. coli* chromosome (39). Third, phages Mu and P1 are capable of phase variations that alternate between two mutually exclusive sets of tail fiber genes, rendering the phages able to infect different hosts. Fourth, some phages such as λ and T5 may simultaneously use two separate systems of tail fibers, each recognizing a different receptor (8). In λ, the J gene product forming the single terminal tail fiber is used to recognize its normal receptor, LamB (38). In addition, the laboratory strain of λ carries defective genes *stf* and *tfa*, which encode the side-tail fibers. It is believed that reversion of the 1-bp deletion in *stf* by compensatory DNA replication can enable the phage to use the outer membrane protein C (OmpC) as a receptor (33). Phage T5 has two sets of tail fibers, the L-shaped tail fibers (which bind to lipopolysaccharide) and a single straight terminal tail fiber (which binds to FhuA receptor protein) (8, 19, 20, 40). Recently, a similar finding has been extended to the T7-like coliphage ϕK1-5, which has two different tail fiber proteins capable of infecting strains with a K1 or K5 polysaccharide capsule (42). Fifth, the same tail fiber of a phage may have the ability to recognize two separate receptors, e.g., T4 can use its tail fibers to recognize OmpC or the lipopolysaccharide as a receptor with equivalent efficiency (33). This last possibility may be the case for Kpp95, because (i) although the presence of two sets of tail fiber has been observed in some of the phages mentioned above, a similar situation has not been reported for T4-type phages, and (ii) the tail fiber adhesins of T-even phages are hotspots of lateral gene transfer not only between T4-type phages but also between different groups of morphologically distinct coliphages (17), giving hypervariable properties to the gp37 of T-even phages (reviewed in reference 21) which may also be exploited by Kpp95 for receptor recognition and adaptation to extended host range.

The emergence of pathogenic bacteria resistant to multiple drugs has posed a growing threat, and exploring alternative approaches such as phage therapy is a worthwhile task. While efforts are being increasingly directed along this line in North America and western Europe, numerous cases of successfully treated bacterial infections have long been known in Poland, Georgia, and the former Soviet Union, about which voluminous reports have been published, mostly in languages other than English, and some of these cases involved treatment of *K. pneumoniae* infections with phages (for a review, see references 3, 9, 29, 46, 47, and 48). In phage therapy, one of the problems that remains to be solved is the development of phage resistance by the bacterial hosts. Therefore, it is generally accepted that the isolation of host range mutant phages and the use of cocktails containing several phages in one preparation should be necessary to reduce the probability of resistance development and to cover a breadth of host ranges (31, 40). The lytic phage Kpp95, able to cause rapid and clear lysis of many *K. pneumoniae* strains, is worthy of development into a component of a therapeutic cocktail, maybe in conjunction with *K. pneumoniae* phages, which have previously been characterized as virulent (4, 10, 14, 43, 51, 55), or with antibiotics. Furthermore, with a broad host range, a therapeutic agent based on Kpp95 has the potential to treat coinfections by *E. agglomerans*, *K. oxytoca*, and *S. marcescens*.

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