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Isolation and characterisation of KP34—a novel φKMV-like bacteriophage for *Klebsiella pneumoniae*

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Abstract Bacteriophage KP34 is a novel virus belonging to the subfamily *Autographivirinae* lytic for extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* strains. Its biological features, morphology, susceptibility to chemical and physical agents, burst size, host specificity and activity spectrum were determined. As a potential antibacterial agent used in therapy, KP34 molecular features including genome sequence and protein composition were examined. Phylogenetic analyses and clustering of KP34 phage genome sequences revealed its clear relationships with "phiKMV-like viruses". Simultaneously, whole-genome analyses permitted clustering and classification of all phages, with completely sequenced genomes, belonging to the *Podoviridae*.

Keywords φKMV-like bacteriophage · Genome · *Klebsiella pneumoniae* · Phage therapy · *Podoviridae* · phiKMV-like viruses

Introduction

Most of the publications during the last decades regarding bacteriophages lytic for enteric rods described their application in phage typing (Ślopek et al. 1972; Dąbrowski et al. 1988) or phage therapy (Ślopek et al. 1981a, b, 1984; Sakandelidze and Meipariani 1974; Zhukov-Verezhnikov et al. 1978; Richardson et al. 1999; Weber-Dąbrowska et al. 2000, 2001,

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2003; Sulakvelidze et al. 2001). Data concerning newly isolated *Klebsiella* phages focus on biological characteristics or biodistribution and therapeutic efficacy in animal models (Bogovazova et al. 1991; Kumari et al. 2009; 2010a, b; Verma et al. 2009). None of the above described *Klebsiella* phages was tested regarding genome organisation or sequence similarity to already known viruses. Although fast and efficient techniques of DNA sequencing and proteomics are available today and the detailed analysis of genome structure and organisation is possible, there is not much molecular data about *Klebsiella* virulent bacteriophages. As of today, only four complete genomes of *Klebsiella*-specific lytic viruses have been deposited in GenBank, and three of them (KP15, KP32, KP34) were sequenced by our team. Recognition and analysis of genome structure and genes function is the required step before bacteriophages can be approved as therapeutic agents. The knowledge of protein composition allows one to definitively establish the absence of possible toxins potentially dangerous during phage application. The present study focused on novel "phiKMV-like virus" KP34 propagated on a multidrug-resistant *Klebsiella pneumoniae* strain producing an extended-spectrum β-lactamase (ESBL). Phage KP34 was fully characterised regarding its host specificity, activity spectrum and also morphological, biological, molecular and genomic features. This bacteriophage, which is a new member of the *Podoviridae*, is being considered for potential use in phage therapy.

Materials and methods

Isolation and purification of phages

K. pneumoniae-specific bacteriophage KP34 was isolated from a sewage sample by enrichment. A sewage sample was centrifuged (15,000×*g*/15 min) and the supernatant filtered through a 0.22-μm Millipore filter. Phage propagation followed the modified method of Ślopek et al. (1972). One millilitre of filtered sewage sample and 0.5 ml of *K. pneumoniae* 77 ESBL (+), grown overnight in Mueller Hinton Broth (MHB; bioMérieux Polska, Warsaw, Poland), were added to 10 ml of MHB and incubated at 37°C until complete lysis appeared (approximately 4–6 h). The suspension was then filtered through a 0.22-μm Millipore filter. The procedure was repeated three times to eliminated bactericidal activity of some chemical compounds probably presented in the sewage sample. The bacteriophage titer in the filtrate was assessed using the double-agar layer technique according to Adams (1959). The isolated phage was named KP34, with the first two letters indicating the host genus and the species name. This virus has been deposited in the Polish Collection of Microorganisms (Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland) under accession number F/00067.

Electron microscopy

A high-titer phage lysate previously filtered through a 0.22-μm Millipore filter was centrifuged at 25,000×*g* for 60 min, and the pellet was washed twice in ammonium acetate (0.1 M, pH 7.0). A portion of the resuspended sediment was deposited on carbon-coated Formvar films, stained with 2% uranyl acetate and examined in a JEM 100C (Joel LTD, Tokyo, Japan) transmission electron microscope at 80 kV with magnification of ×66,000. The phage size was determined from the average of five to seven independent measurements using T4 phage tail (114 nm) as the magnification control.

Phage adsorption procedure

The adsorption of phages to bacterial host cells was examined using previously described by (Adams 1959; Ackermann and DuBow 1987; Roncero et al. 1990; Gallet et al. 2009) with slight modifications introduced in our laboratory. Overnight cultures of *K. pneumoniae* 77 ESBL (+) grown on Mueller Hinton Agar (MHA; bioMérieux Polska, Warsaw, Poland) were used. Cells from the agar medium were suspended in enrichment broth (MHB) to an optical density at 600 nm of approximately 0.9–1.0. An equal volume of bacterial suspension and phage diluted to 10^5–10^6 pfu/ml were incubated at 37°C for 5 min. After incubation, the culture was filtered (0.22 μm) and the number of free phages was determined, in duplicate, in the filtrate using the double-agar-layer method. The reduction in phage titer was the number of phages adsorbed to the cells. No reduction in phage titer in control filtration (0.22 μm Millipore filters) was observed.

Burst size experiments

A one-step growth curve of KP34 was performed according to the method of Pajunen et al. (2000) with modifications. The density of a mid-exponential bacterial culture (MHB) was adjusted to 2×10^8 cfu/ml. To 0.9 ml of this cell suspension was added 0.1 ml of bacteriophage in order to achieve a multiplicity of infection of 0.005. Phages were allowed to adsorb for 5 min at 37°C, after which time the mixture was diluted to 10^{-5}, and samples, in triplicate, were taken at 5-min intervals for titration. Experiment was performed on three different occasions, and values depict the mean of three observations ± standard deviation.

Storage stability of phage culture

The stability of KP34 preparation, neat at −70°C, −20°C, 4°C and 20°C or in the presence of 25% (*v*/*v*) glycerol at −70°C and −20°C was determined after 3 months of storage using the double-agar-layer technique according to Adams (1959).

Sensitivity of phage particles to temperature, chloroform and pH

A filter-sterilized bacteriophage preparation at 10^7 pfu/ml was incubated at 60°C for 10 min with intermittent shaking. An equal volume of bacteriophage (10^7 pfu/ml) was mixed with chloroform and incubated for 2 and 24 h at room temperature with intermittent shaking. Further preparations of KP34 (10^7 pfu/ml) was incubated at pH 2, 4, 5, 6, 8 and 10 for 1 and 5 h at room temperature and at 37°C also with intermittent shaking. After all these experiments, the bacteriophage titer was assessed using the double-agar-layer technique (Adams 1959).

Determination of KP34 phage bacterial host range

The strains used in this study are listed in Table S1 (in electronic supplementary material). Bacteria were stored at −70°C in Trypticase Soy Broth (Becton Dickinson and Company, Cockeysville, MD, USA) supplemented with 20% glycerol. Prior to phage sensitivity testing, bacteria were subcultured at least twice in Trypticase Soy Broth. Unless otherwise stated in all phage experiments, 4–6-h bacterial cultures were used. To determine bacterial susceptibility to KP34 phage-mediated lysis, bacteria grown in liquid MHB medium were transferred onto MHA agar plates (bioMérieux). After drying, a drop of the phage suspension (10^8 pfu/ml) was placed on the bacterial layer and incubated at 37°C. The plates were checked 4–6 h and again 18 h later for the presence of bacterial lysis. Spot testing is a rapid and efficient method for determining the host range in large collection of bacteria (Clokie and Kropinski 2009).

Bacteriophage structural protein analysis by SDS-PAGE

Phage particles were partially purified by PEG precipitation (Sambrook and Russell 2001). After centrifugation, the pellets were suspended in 100 mM NaCl, 100 mM Tris–HCl (pH 7.5) and 25 mM EDTA buffer. Further purification was carried out by extraction with chloroform (1:1 *v*/*v*) followed by centrifugation. The concentrated phage particles were collected from the aqueous phase, mixed with the sample buffer (62.5 mM Tris HCl, 2% (*w*/*v*) SDS, 10% (*w*/*v*) glycerol, 5% (*v*/*v*) 2-mercaptoethanol, 0.001% (*w*/*v*) bromophenol blue) and heated in a boiling water bath for 5 min. Polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). Discontinuous sodium dodecyl sulphate (SDS) gel electrophoresis was carried out on slabs of 10% acrylamide. After electrophoresis, the gels were stained with Bio-Safe Coomassie Stain (Bio-Rad, Hercules, CA, USA). Bio-Rad Quantity One software was used for the molecular analysis of the phage structural proteins based upon a Sigma-Aldrich wide-range molecular weight marker S8445 protein standard. Molecular mass of structural proteins (kilodalton) of identified open reading frames (ORFs) products were estimated on the basis of amino acid sequence composition (http://izoelektryczny.ovh.org/).

DNA isolation and restriction endonuclease analysis

Bacteriophage DNA was extracted and purified from phage lysate using a QIAGEN® Lambda Midi Kit (QIAGEN Inc., Valencia, CA, USA) and following the manufacturer's protocol. Phage DNA was digested with the restriction endonucleases (EcoRV, EcoRI, HindIII, NsiI, NcoI, PaeI, Fermentas Life Science, Vilnius, Lithuania) according to the supplier's recommendations. DNA fragments were separated by electrophoresis in 0.6% agarose gel containing ethidium bromide (0.5 μg/ml) in Tris–boric acid–EDTA buffer, at 90 V in a Bio-Rad agarose gel electrophoresis system. Restriction digestions were carried out in triplicate.

Nucleotide sequence

KP34 DNA was sequenced by a commercial company, Genomed Ltd. (Warsaw, Poland), and its annotated sequence has been deposited in GenBank under accession number GQ413938.

Clustering of phage genome sequences

Ninety-two complete *Podoviridae* genomic sequences were downloaded from GenBank (http://www.ncbi.nlm.nih.gov; see Table S2 in electronic supplementary material). Information about the classification of these phages was taken from the GenBank annotations and from Lavigne et al. (2008). To cluster phages based on their genome sequences, cluster analysis of sequences (CLANS) was applied, which uses a version of the Fruchterman–Reingold graph layout algorithm to visualize pairwise sequence similarities in either two-dimensional or three-dimensional space (Frickey and Lupas 2004). The program performs all-against-all BLAST searches and calculates pairwise attraction values based on *P* values of high scoring segment pairs (HSPs). Analysed sequences are represented in the graph by vertices

which are connected by edges reflecting attractive forces proportional to the negative logarithm of the HSP's *P* value. For the whole set of 92 *Podoviridae* genome sequences, we performed all-against-all TBLASTX searches assuming a word-size=2. The pairwise comparison of 36 *Autographivirinae* genome sequences was made by BLASTN assuming a word-size=7 to increase sensitivity.

The applied method differs from the approach developed by Lima-Mendez et al. (2008) for a reticulate classification of phages, which is based on gene content. Starting from gene families, the authors built a weighted graph, where nodes represented phages and edges represented phage–phage similarities in terms of shared genes.

Phylogenetic analyses

Homologous sequences of four selected *Klebsiella* phage KP34 proteins—capsid protein (accession number ACY66716.1), putative internal core protein (ACY66722.1) and tail tubular proteins A (ACY66718.1) and B (ACY66719.1)—were obtained through a thorough search of GenBank using BLAST. To verify the BLAST results and determine domain content, we searched Conserved Domain Database (Marchler-Bauer et al. 2005). Only sequences that showed significant hits to domains present in the phage KP34 proteins were included in final analyses. Amino acid alignments were obtained in MAFFT program using slow and accurate algorithm L-INS-i with 1,000 cycles of iterative refinement (Katoh et al. 2005). Sites suitable for further phylogenetic analyses were extracted from the alignments with Gblocks 0.91b assuming less stringent criteria (Castresana 2000). Phylogenetic trees were inferred by the maximum likelihood method in Phyml (Guindon and Gascuel 2003) and by a Bayesian approach in PhyloBayes (Lartillot and Philippe 2004) software. In Phyml, we used amino acid substitution models as proposed by ProtTest program 2.4 (Abascal et al. 2005): LG+Γ (for capsid protein), LG+Γ+F (for core protein) and LG+I+Γ+F (for tail tubular proteins A and B). We assumed five discrete categories for gamma distributed rates and the best heuristic search algorithms in Phyml, i.e. NNI and SPR. Edge support was assessed by the bootstrap analysis with 1,000 replicates and by the approximate likelihood ratio test based on χ^2 and Shimodaira–Hasegawa-like procedure. The minimum of these two support values was shown at nodes in presented trees. In PhyloBayes analysis, two independent Markov chains were run for 200,000 cycles assuming the CAT-Poisson+ Γ model with number of components, weights and profiles inferred from the data and five discrete categories for gamma distributed rates. After getting a convergence, the last 50,000 trees from each chain were collected to compute posterior consensus.

Statistical analysis

A comparison of differences in the properties of phage susceptible strains basing on the ESBL carrying plasmid was performed by χ^2 test with Yates' correction. Statistical analysis of the data was done using StatSoft's (Tulsa, OK, USA) statistical package STATISTICA9, and the differences were considered significant at $P \leq 0.05$.

Results

Isolation and physicochemical properties of KP34

During this study, six different sewage sources were screened separately for phage presence using clinical *K. pneumoniae* strain 77 as the host, but in only a sample collected from a wastewater treatment plant located near Wrocław, Poland was lytic activity detected. Phage KP34 was purified by single plaque picking, dilution and titration. In the double-agar-layer technique, this virus produces plaques 5 mm in diameter with small clear centre surrounded by hazy ring (halo). The presence of the halo might suggest the production of the soluble phage enzymes, for example polysaccharide depolymerases, as suggested by Hughes et al. (1998). Phage KP34 was negatively stained with uranyl acetate and observed by electron microscopy (Fig. 1). It possesses an icosahedral head approximately 57×63 nm connected to short (15 nm) tail common to members of the *Podoviridae* family, morphotype C1 (Ackermann and DuBow 1987). These dimensions are consistent with T7 type phages.

Determination of KP34 phage bacterial host range and lytic potential

A high percentage (99.7%) of the KP34 particles adsorbed to *K. pneumoniae* 77 cells after 5 min of incubation. The one-step growth curve of KP34 indicated that the latent

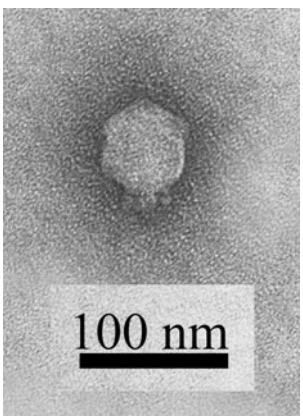

Fig. 1 Electron micrograph of phage KP34 negatively stained with uranyl acetate. The *bar* indicates 100 nm

Table 1 General characteristics of genes coded in the genome of *Klebsiella* phage KP34

Locus tag	ORF position	Length of product (aa)/molecular mass (kDa)	Percent identity with homologous proteins from other phages	Predicted molecular function	Characteristic domains
KP-KP34p09	3433–3777	114	30 (*Vibrio* phage VP93)	Conserved hypothetical protein	cl00204; phosphofructokinase superfamily
KP-KP34p11	5497–6543	348	42 (*Vibrio* phage VP93)	Putative peptidase	
KP-KP34p14	7207–7992	261	44 (*Vibrio* phage VP93)	Putative DNA primase	PHA02031; putative DnaG-like primase
KP-KP34p16	8184–9464	426	60 (*Vibrio* phage VP93)	Putative DNA helicase	GP4d_helicase, P-loop NTPase domain
KP-KP34p19	9812–10282	156	39 (Enterobacteria phage RTP)	Putative HNH endonuclease	PHA00280; putative NHN endonuclease
KP-KP34p20	10282–12642	786	62 (*Vibrio* phage VP93)	DNA polymerase	pfam00476; DNA_pol_A,DNA; polymerase family A
KP-KP34p24	14350–14775	141	39 (Enterobacteria phage TLS)	Putative HNH endonuclease	PHA00280; putative NHN endonuclease
KP-KP34p32	17394–18362	322	42 (*Vibrio* phage VP93)	Putative 5′–3′ exonuclease	cl00079; T5 type 5′–3′ exonuclease domain
KP-KP34p34	18513–18935	140	55 (*Vibrio* phage VP93)	Putative DNA endonuclease VII	pfam02945; endonuclease_7; recombination endonuclease VII
KP-KP34p37	19723–22191	822	52 (*Vibrio* phage VP93)	DNA-dependent RNA polymerase	PHA00452; T3/T7-like RNA polymerase; pfam00940; RNA_pol DNA-dependent RNA polymerase
KP-KP34p40	22926–24521	531/58.2	42 (*Vibrio* phage VP93)	Head–tail connector protein	pfam12236; head-tail_con; bacteriophage head to tail connecting protein
KP-KP34p41	24536–25378	280/30.1	41 (*Vibrio* phage VP93)	Putative scaffolding protein	PHA01929; putative scaffolding protein
KP-KP34p42	25404–26423	339/37.8	73 (*Vibrio* phage VP93)	Capsid protein	PHA02004; capsid protein
KP-KP34p44	26706–27266	18,621.3	42 (*Vibrio* phage VP93)	Tail tubular protein A	PHA00428; tail tubular protein A
KP-KP34p45	27276–29636	786/86.1	42 (*Vibrio* phage VP93)	Tail tubular protein B	
KP-KP34p46	29638–30225	195/20.4	38 (*Vibrio* phage VP93)	Putative internal virion protein B	
KP-KP34p47	30243–32927	894/97	35 (*Vibrio* phage VP93)	Conserved hypothetical protein	
KP-KP34p48	32978–36676	1,232/134	41 (*Vibrio* phage VP93)	Putative internal core protein	PHA02006; virion protein
KP-KP34p49	36678–37601	307/32.7	37 (*Vibrio* phage VP93)	Putative tail fiber protein	pfam03906; phage_T7_tail; phage T7 tail fiber protein
KP-KP34p50	37613–37915	100	66 (*Vibrio* phage VP93)	Putative DNA maturase A	PHA02046
KP-KP34p51	37915–39771	618	70 (*Vibrio* phage VP93)	Putative DNA maturase B	DEXDc; DEAD-like helicases
KP-KP34p55	40735–40986	83		Hypothetical transmembrane protein	Putative holin
KP-KP34p56	40970–41578	202	37 (Iodobacteriophage phiPLPE)	Endolysin	cd00737; endolysin_autolysin; COG3772; phage-related lysozyme; muraminidase

period was short (15 min) and the estimated burst size was ~40–50 phage particles per infected bacterial cell. The lytic activity of KP34 tested against the 332 bacterial strains (Table S1) was limited only to *K. pneumoniae* subsp. *pneumoniae* strains. All of 55 *Enterobacter* sp., 100 *Escherichia coli* and 72 *Klebsiella oxytoca* strains were found to be resistant to this phage. Among the 101 *K. pneumoniae* subsp. *pneumoniae* isolates 42 (41.6%) were susceptible to KP34, but none of the *K. pneumoniae* subsp. *ozaenae* or *K. pneumoniae* subsp. *rhinoscleromatis* strains was sensitive. To verify specificity of KP34 to ESBL(+) and ESBL(−) *K. pneumoniae* subsp. *pneumoniae* strains the NMI collection was analysed. In that group, all 50 ESBL(+) and 50 ESBL(−) isolates were recognised in typing by the randomly amplified polymorphic DNA technique as separated clones. It turned out that 47.1% of ESBL(+) versus 36% of ESBL(−) strains were susceptible to KP34 phage. However, KP34 phage exhibited lytic activity against ESBL(+) strains much faster (after 6 h of incubation) in comparison to ESBL(−) strains where visible plaques were better seen after 18 h of incubation.

Stability of phage particles

Storage stability is the one of the important parameters of phage application in therapy. A vital property of industrial phage preparations is low susceptibility to temperature fluctuations and possibility of storage under different temperature conditions. KP34 high-titer phage lysates were stored in different ways: at room temperature, 4°C, −20°C and −70°C and in 25% glycerol at −20°C and −70°C. After 3 month storage, there was no significant loss of active phage particles at all of the tested temperatures, except at room temperature, where the number of plaque-forming unit was reduced by three orders of magnitude. KP34 was found to be relatively sensitive to high temperature, with a 100-fold decrease in titer observed after 10 min at 60°C. KP34 was unaffected by chloroform treatment for 2 and 24 h. The susceptibility of KP34 to different pH conditions showed that the phage was totally destroyed at pH 2 after 1 h of incubation at both temperatures. Incubation at pH 4 and 37°C caused a three and seven log decrease in phage titer after 1 and 5 h of incubation, respectively. The same acidity reduced plaque-forming units per millilitre 10-fold at room temperature after 5 h. KP34 was stable within a pH range 5–10.

Genome analysis

KP34 DNA was sensitive to digestion by a variety of restriction endonucleases (EcoRI, EcoRV, HindIII, NsiI, NcoI, PaeI) indicated that it is unmodified double-stranded DNA. The sequence of phage KP34 consisted of 43,809 bp (G+C content 54%) which is close to the genome size of other members of the *Autographivirinae* (Sillankorva et al. 2008). A total of 57 ORFs were predicted in the KP34 phage genome (Table 1), talking up a total of 93% of the coding capacity of this virus. The predicted ORFs encoded hypothetical proteins from 36 to 1,232 amino acid residues. Twenty-two of the KP34 potential gene products showed significant sequence similarity to proteins from *Vibrio* phage VP93 classified to the "phiKMV-like viruses" (Bastías et al. 2010). Nine KP34 potential gene products showed sequence similarity to proteins from different viruses infecting *Escherichia*, *Burkholderia*, *Yersinia*, and *Iodobacteria* strains (Table 1). Fifty-three of the KP34 ORFs start with an AUG codon, while four begin with UUG. Among the stop codons, UAA was the most commonly used (32 ORFs). The remainder ended with UGA (17 occurrences) or UAG (eight).

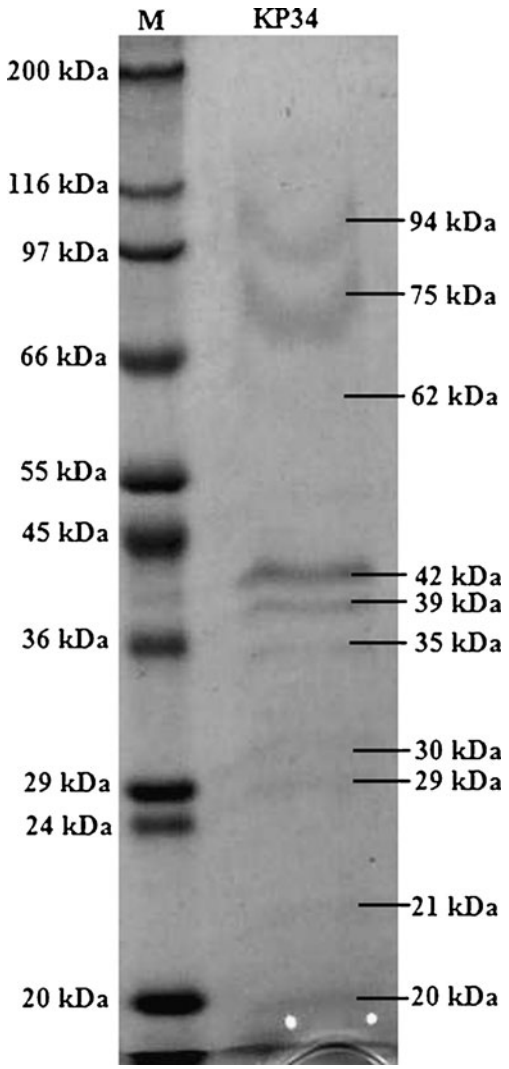

Fig. 2 SDS-PAGE analysis of purified KP34 virus particles with Sigma-Aldrich wide-range molecular weight markers in the *left lane*

Structural protein analysis

Analysis of genome sequence revealed presence of eight genes that coded for structural proteins (Table 1). All of these identified gene products showed identity with homologous proteins found in *Vibrio* phage VP93. The highest sequence similarity was detected for the capsid protein (73%) which possesses an estimated molecular mass of 37.8 kDa. Comparative proteomic analyses, using data on φKMV (Lavigne et al. 2006), allowed us to postulate that the product of gene 42 (locus lag KP-KP34p42) which is described as a conserved hypothetical protein could be additional structural protein (Fig. 3). In case of the *Vibrio* VP93, the predicted function of the protein homologous to KP34 gp42 is also designated as unknown. For further characteristics of KP34, its structural protein composition was analysed by SDS-PAGE (Fig. 2). At least ten different protein bands were detected in the gel ranging from approximately 20 to 97 kDa. According to genome analysis, nine structural proteins could be expected. The two most predominant peptide bands were approximately 42 and 39 kDa. The abundance and estimated mass of gp42, along with its homology to φKMV, led us to conclude that the 39-kDa band is the capsid protein.

Clustering of phage genome sequences

We used the CLANS software package to reveal the relationship of *Klebsiella* phage KP34 genome to complete genome sequences of other bacteriophages. The graph presented in Fig. S1 (in electronic supplementary material) reflects global similarity of 92 *Podoviridae* genomic sequences at the amino acid level. In Table S2 (in electronic supplementary material), we list all analysed members of the *Podoviridae* with proposed taxonomic classification. Almost all phages clustered according to their taxonomic affiliation proposed by Lavigne et al. (2008). Interestingly, in the scale of the whole graph, a compact cluster is formed by BPP-1-, P22- and "ε15-like viruses" together with 13 currently unclassified phages (see Table S2).

In our analyses, *Lactococcus* phage ascc φ28 shows a close relationship with the *Picovirinae* which is in accord with the findings of Kotsonis et al. (2008). On the other hand, the connections of *Lactococcus* phage KSY1 to the "AHJD-like viruses" seen in the figure result from significant hits of protein KSY1p075 (tail protein) and KSY1p076 (hypothetical protein) to the corresponding homologues. Their genes were probably acquired by the *Lactococcus* phage KSY1 genome via horizontal gene transfer. *Lactococcus* phage KSY1 shows also quite strong attraction to *Lactococcus* phage ascc φ28 in the graph. This affiliation results from several significant hits from the lysin (amidase), which was also found in *Lactobacillus casei* and *Lactobacillus rhamosus* and in phages classified to *Siphoviridae*: *Lactobacillus* phages A2 and PL-1 and *Lactococcus* phage r1t. This suggests that the genomic region coding the lysin was probably

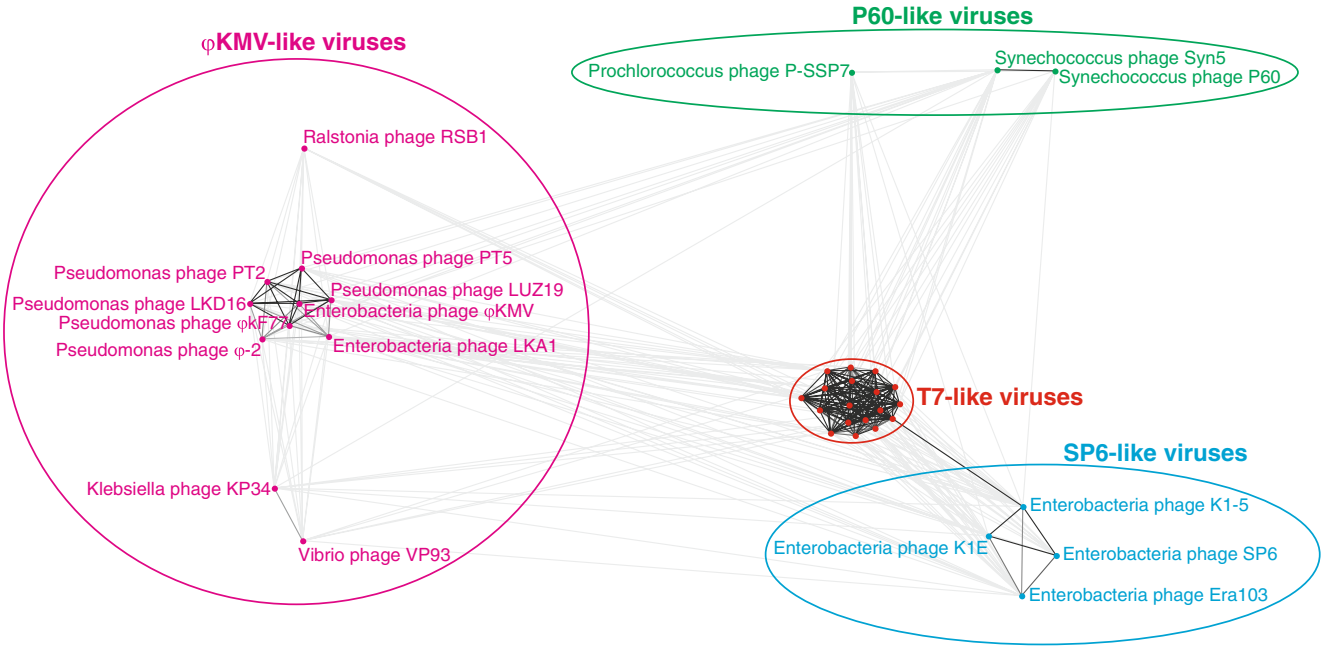

Fig. 3 Graphic layout made in CLANS software for 36 *Autographivirinae* genome sequences using BLASTN searches. Analysed sequences are represented by vertices connected by edges reflecting attractive forces proportional to the negative logarithm of the HSP's *P* value. The greyness intensity of the connections is proportional to these forces. The not shown phages names forming compact clusters are fully listed in Table S2 in electronic supplementary material

subjected to horizontal gene transfer similarly to other region encoding components of the distal tail structure (Chopin et al. 2007). The most distant position in the graph is occupied by two "119X-like viruses" which do not connect with any *Picovirinae* viruses which in turn show moderate affiliation to the rest of viruses. "LUZ24-viruses", Enterobacteria phage phiEco32 and *Roseobacter* phage SIO1 show the strongest attraction to *Autographivirinae*.

Since *Klebsiella* phage KP34 showed the closest affiliation to the *Autographivirinae* subfamily, we decided to carry out a separate whole-genome comparison of this subfamily using BLASTN to analyse this relationship in details (Fig. 3). This analysis revealed four distinct clusters corresponding to phiKMV-, P60-, SP6- and "T7-like viruses". *Klebsiella* phage KP34 belongs to the cluster of "phiKMV-like viruses" and is the most closely related to the recently sequenced *Vibrio* phage VP93 also classified to this genus (Bastías et al. 2010). Among "phiKMV-like viruses", six *Pseudomonas* and two Enterobacteria phages form the most compact grouping. The members of SP6-like viruses appear close relatives to "T7-like viruses" through the CLANS analysis. This agrees with genomic

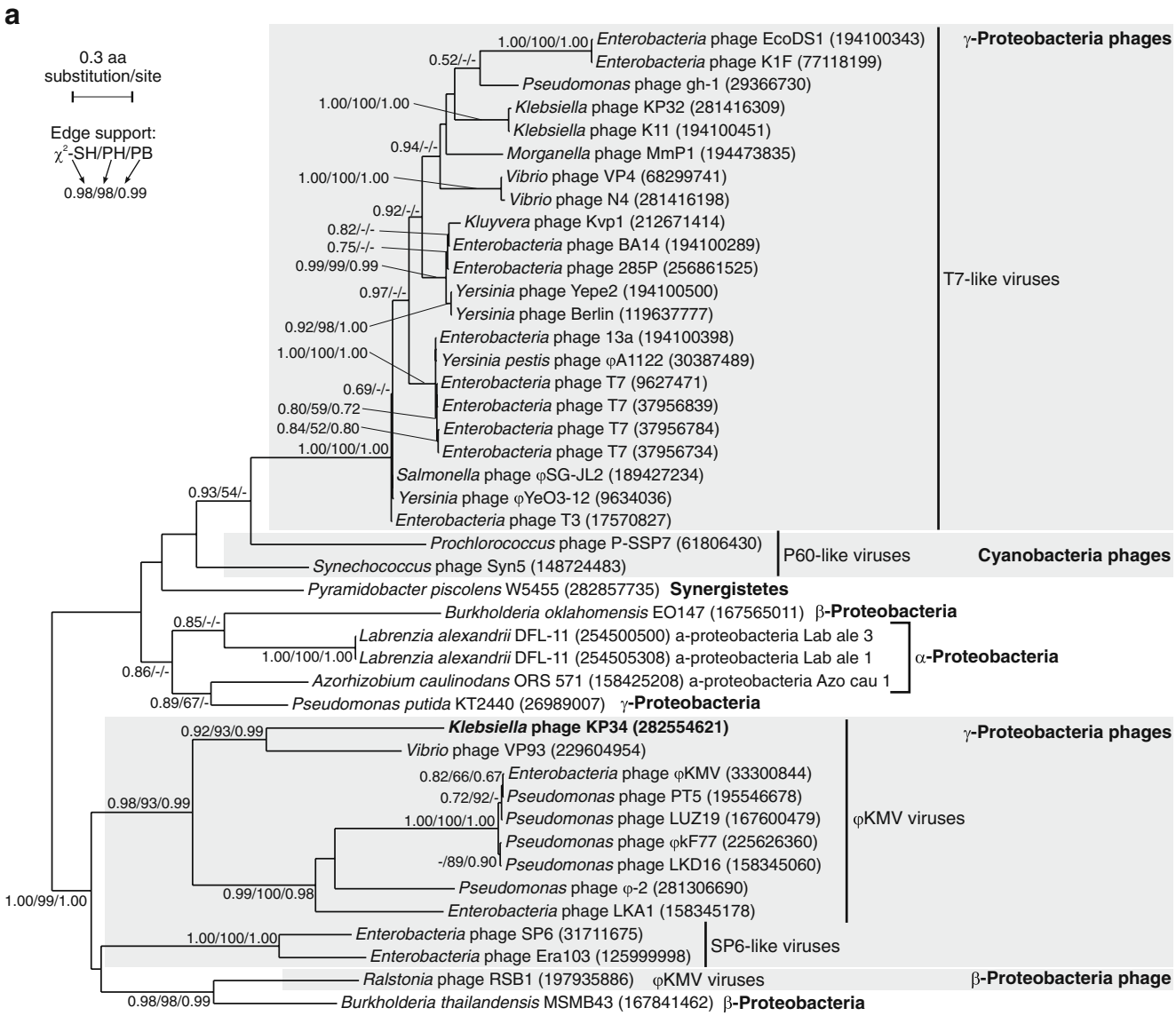

Fig. 4 Phyml tree of tail tubular proteins A (**a**) and tail tubular proteins B (**b**). Sequence from *Klebsiella* phage KP34 indicated in *bold font*. Sequences from phages are placed in the grey rectangle. *Numbers at nodes*, in the shown order, correspond to the minimum of support values calculated in Phyml by $\chi 2$ and Shimodaira–Hasegawa-like procedure (χ^2-SH); support values resulted from bootstrap analysis in Phyml (*PH*) and posterior probabilities estimated in Phylobayes (*PB*). Values of the posterior probabilities and bootstrap percentages lower or equal to 0.50 and 50%, respectively, were omitted or indicated by a *dash*. GenBank identifiers (gi) of sequences are shown in *parenthesis*

analysis of Enterobacteria phages SP6 and K1-5 which are considered an estranged subgroup of the T7-like viruses (Scholl et al. 2004).

Phylogenetic analyses

Phylogenetic analyses were performed on four selected *Klebsiella* phage KP34 proteins: capsid protein, putative internal core protein KP-KP34p48 (gene 48; Fig. S2A, B in electronic supplementary material), tail tubular protein A, and tail tubular protein B (Fig. 4a, b). Trees obtained both in Phyml and PhyloBayes gave identical or almost identical topologies for each of these proteins. In agreement with clustering analysis, the *Klebsiella* phage KP34 proteins clustered in all trees significantly with its homologues found in *Vibrio* phage VP93 and belonged to a major well-supported clade containing sequences from φKMV-like viruses infecting *γ-Proteobacteria*. All the analysed proteins exhibit homologues in members of different bacterial groups, which indicate that these phage genes were subjected to intensive transduction process. Of particular interest was the finding of tail tubular proteins A and B

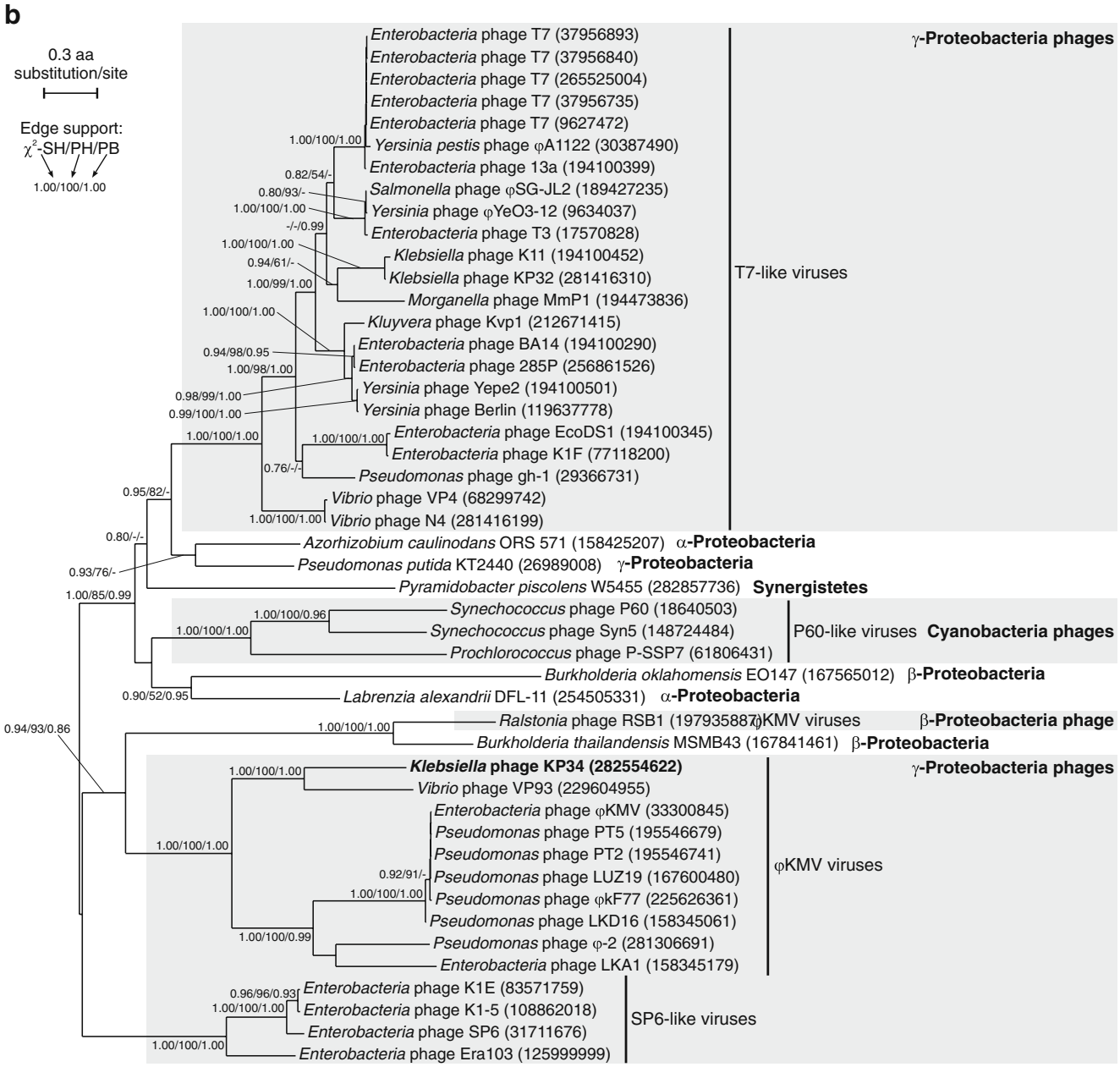

Fig. 4 (continued)

homologues in a number of bacterial genomes including α-, β- and γ-*Proteobacteria* and S*ynergistetes*. The latter are a phylum of obligately anaerobic Gram-negative bacteria (Vartoukian et al. 2007). While a T7-like prophage had been identified in the genome of *Pseudomonas putida* strain KT2440 (Canchaya et al. 2003, 2004), this is the first time that similar prophages have been reported for the strains indicated here.

Discussion

K. pneumoniae is one of the most common etiological factors of nosocomial infections particularly of Gram-negative bacteraemias and urinary tract infections (Matsen 1973; Center for Disease Control 1977; Jones et al. 2000; Coque et al. 2008). Widespread use of antibiotic treatment has been held responsible for the occurrence of *Klebsiella* strains producing ESBLs resulting in multidrug resistance (Gniadkowski 2001; Nijssen et al. 2004; Coque et al. 2008; Miriagou et al. 2010). At present, the prevalence of ESBL-producing *Klebsiella* strains in Europe has reached 10–30% among invasive isolates (European Antibiotic Resistance Surveillance System, http://www.ecdc.europa.eu/en/activities/surveillance/EARS-Net). Since multidrug-resistant bacteria appeared, therapeutic options have become limited. One possible method of bacteria eradication could be the application of bacteriophages. The idea of using phages as natural bacterial parasites is well-known, but most of available virus collections are ineffective against currently isolated clinical strains. There is a need to look for novel phages with lytic potential to multidrug resistance *K. pneumoniae* isolates. Recently isolated *Klebsiella* phages belonged to *Podoviridae* family (Kumari et al. 2009; 2010a; b; Verma et al. 2009) or pseudo-T-even family (Wu et al. 2007). During our team's experiments, five different viruses from the *Podoviridae*, *Siphoviridae* and *Myoviridae* were characterised regarding their biology and genomics (data not shown) and genomes of three phages have already been deposited in GenBank. In the present study, one of these phages (KP34) was described. The KP34 virus was propagated on *K. pneumoniae* subsp. *pneumoniae* isolate carrying ESBL plasmid. The host specificity of KP34 among most common *Enterobacteriaceae* rods was limited to one species, but the lytic activity was found to be in the high range (41.6%) regardless of whether they were ESBL(+) or ESBL(−) strains. *Klebsiella* KP34 phage, with its narrow host specificity, could be applied in phage cocktail as alternative or as supportive treatment simultaneously with antibiotics, for *K. pneumoniae* subsp. *pneumoniae* strains eradication with no harmful action on normal bacterial flora.

With respect to its physicochemical stability, KP34 is similar in its sensitivity to chemical agents such pH and chloroform to the previously described T7-like *Klebsiella* phage, KPO1K2 (Verma et al. 2009), but is much more susceptible to high temperature. The average burst size determined from the one-step growth experiment is three times lower for KP34 (~50 virions per cell) comparing to KPO1K2 (~140 virions per cell). We identified ten KP34 structural proteins by SDS-PAGE, but only nine were

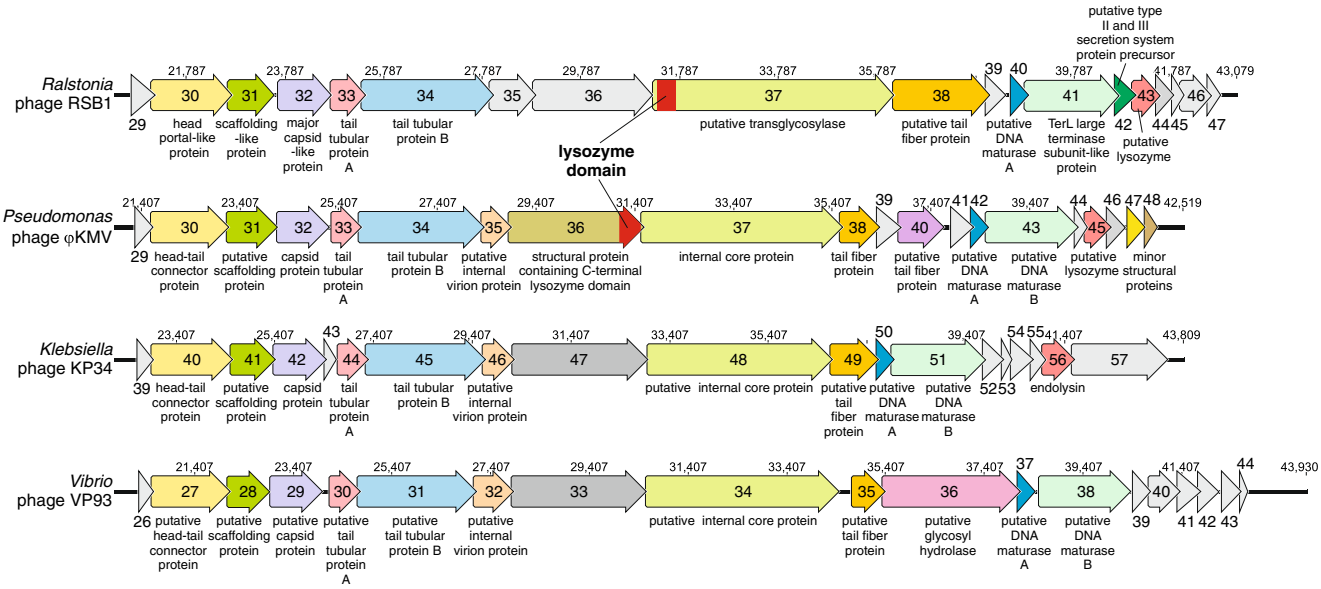

Fig. 5 Comparison of 3′-end genomic sequence in four phages classified to "φKMV-like" viruses. ORFs whose products show significant sequence similarity are presented in *the same colour*. ORFs in *grey* have their products annotated as hypothetical protein

predicted on the basis of our bioinformatic analysis of the sequence data. The presence of an additional 42-kDa protein in the gel could be the result of a frameshifting event in the major capsid gene as was observed previously with coliphage T7 phage (Condron et al. 1991). Similar results were also obtained with the T7-like *Pseudomonas* phage φIBB-PF7A (Sillankorva et al. 2008). Other investigators analysing structural proteins of *K. pneumoniae* T7-like phages by SDS-PAGE technique obtained wide range of band numbers, from 12 (29 to 120 kDa) with *K. pneumoniae* phage B5055 (Verma et al. 2009) to two to five bands representing proteins from 20 to 55 kDa in five phages (Kumari et al. 2010b). This would suggest that considerable variation occurs in this group of bacteriophages with respect to protein number and size. It is also significant that KP34 phage genome encoded only one type of tail fiber protein (307 amino acid; 32.7 kDa) as opposed to the closely related φKMV described by Lavigne et al. (2006) which produces two fiber proteins (gp38 and gp40).

This bacteriophage was also characterised genomically. Genetic and proteomic analysis helped us to discover new types of viruses and determine the phylogenetic relationships within phage groups. The whole-genome comparisons and phylogenetic studies showed a clear relationship between *K. pneumoniae* phage KP34 and the φKMV-like bacterial viruses. Among phages with completely sequenced genomes, *Vibrio* phage VP93 seems to be the closest relative of KP34. However, detailed comparison of genomes from KP34 and other φKMV-like viruses revealed some differences in gene organisation at the 3′-end genomic region in these phages and lysozyme domain distribution (Fig. 5). Lavigne et al. (2006) described that one of the features distinguishing *Pseudomonas* phage φKMV from "T7-like viruses" is that the gp36 protein containing a functional C-terminal lysozyme domain. They reported that the corresponding gp35 proteins in two "SP6-like viruses" also have a proper lysozyme domain at their C-terminal end. However, the "T7-like viruses" have a functional transglycosylase domain at the N-terminal end of gp16 proteins. We found that proteins with the C-terminal lysozyme domain are also coded by genomes of other "phiKMV-like viruses" (listed in Table S2 in electronic supplementary material) with exception of *Ralstonia* phage RSB1, *Klebsiella* phage KP34 and *Vibrio* phage VP93. Interestingly, *Ralstonia* phage RSB1, similar to "T7-like viruses", have a lysozyme domain at the N-terminal end of gp37 protein which is annotated as putative transglycosylase (Fig. 5). Although gene 46 (locus tag: KP-KP34p47) from *Klebsiella* phage KP34 and ORF33 (locus tag: VPP93_gp33) from *Vibrio* phage VP93 are in the same gene context as gp36 (locus tag: phiKMVp36) from *Pseudomonas* phage φKMV, their products show neither significant similarity to the gp36 nor to the lysozyme domain. We found only marginal similarity in pairwise BLAST comparison the C-terminal lysozyme domain proteins from "phiKMV-like" viruses with KP-KP34p47 and VPP93_gp33 proteins (identity ~20%; similarity ~40%; *E*-value=0.001 and 0.014, respectively). Probably, the genes in *Klebsiella* phage KP34 and *Vibrio* phage VP93 have undergone a high rate of divergence rate which minimized the sequence similarity. The most striking differences in the analysed genome region are visible just at its 3′-end (Fig. 5). Among the compared genomes only *Vibrio* phage VP93 does not code for a lysozyme but instead has a putative glycosyl hydrolase. *Pseudomonas* phage φKMV genome encodes additional putative tail fiber proteins and two minor structural proteins while *Ralstonia* phage RSB1 has a gene coding a putative type II and III secretion system protein precursor. The different gene contents may be responsible for differences in host recognition and infection mechanisms between "typical" φKMV viruses and *Klebsiella* phage KP34, *Ralstonia* phage RSB1 and *Vibrio* phage VP93. This is in agreement with whole-genome clustering analysis which showed outstanding position of three latter phages from the "typical" φKMV viruses (Fig. 3).

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