Bacteriophages LIMelight and LIMEzero of *Pantoea agglomerans*, Belonging to the “phiKMV-Like Viruses”[†]

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Pantoea agglomerans is a common soil bacterium used in the biocontrol of fungi and bacteria but is also an opportunistic human pathogen. It has been described extensively in this context, but knowledge of bacteriophages infecting this species is limited. Bacteriophages LIMEzero and LIMelight of *P. agglomerans* are lytic phages, isolated from soil samples, belonging to the *Podoviridae* and are the first *Pantoea* phages of this family to be described. The double-stranded DNA (dsDNA) genomes (43,032 bp and 44,546 bp, respectively) encode 57 and 55 open reading frames (ORFs). Based on the presence of an RNA polymerase in their genomes and their overall genome architecture, these phages should be classified in the subfamily of the *Autographivirinae*, within the genus of the “phiKMV-like viruses.” Phylogenetic analysis of all the sequenced members of the *Autographivirinae* supports the classification of phages LIMelight and LIMEzero as members of the “phiKMV-like viruses” and corroborates the subdivision into the different genera. These data expand the knowledge of *Pantoea* phages and illustrate the wide host diversity of phages within the “phiKMV-like viruses.”

Pantoea agglomerans bacteria are Gram-negative, nonsporulating, facultatively anaerobic rods that belong to the family of the *Enterobacteriaceae*. Synonyms of this species name are *Enterobacter agglomerans*, *Erwinia herbicola*, and *Erwinia milletiae* (6, 19). The genus name *Pantoea* is derived from the Greek word *pantoios*, which means “of all sorts and sources,” a descriptor well reflected in the habitat, since species of the genus have been isolated from plant surfaces, seeds, water, soil, and even humans and animals (3, 19, 41). *Pantoea agglomerans* is known in plant production as a biocontrol agent. It is used in postharvest control of a number of fungi (for instance, *Penicillium expansum*, *Rhizopus stolonifer*, *Monilinia laxa*, and *Botrytis cinerea*) on a wide variety of fruits (18, 44, 45, 54). Some strains of *P. agglomerans* or *E. herbicola* have been reported to produce an antibiotic compound against *Erwinia amylovora*, the cause of fire blight on apple and pear trees, and can be used in orchards (24, 26, 47). However, *P. agglomerans* has a malevolent side as well, as indicated by reports of clinical isolates that are opportunistic human pathogens (10). In most cases, the bacteria cause soft tissue or bone/joint infections (septic arthritis) after penetration of the skin by vegetation, e.g., thorns or wood slivers (11, 14, 17, 29, 38, 55, 57). The potential pathogenicity of the bacterium prevents its use in biocontrol in Europe, but recently a single marker characteristic that may be useful in biosafety determinations and regulation has been discovered in the biocontrol strains (48).

Bacteriophages (also referred to simply as phages) can be used as a means to control bacteria. For *P. agglomerans*, phage therapy can focus on different aspects, such as reduction of bacterial residues on apples and pears, sanitation of appliances used in agriculture, or use as an alternative for antibiotics in human disease control (40, 52, 53). Lehman (2007) has reported the use of *P. agglomerans* strains as carriers for phages in the biological control of fire blight (36). In this case, phages infecting both *P. agglomerans* and *Erwinia amylovora* are applied on orchards together with a nonpathogenic *P. agglomerans* strain in order to support a population of phages during bloom.

To date, only one genome sequence of a phage infecting *P. agglomerans* is publicly available: *Erwinia* phage phiEa21-4 (RefSeq accession no. NC_011811) has a genome of 84,576 bp and is a member of the family *Myoviridae* (37). In this paper, we report on the isolation and characterization of two bacteriophages of *Pantoea agglomerans*, called LIMelight and LIMEzero (Leuven ILVO Merelbeke). Genome analysis of these phages shows them to be members of the “phiKMV-like viruses.” This recently established phage genus is classified within the *Autographivirinae* subfamily of the *Podoviridae* along with the “T7-like viruses” and “SP6-like viruses.” It groups phages with a common transcriptional scheme, able to infect a wide range of environmentally important bacteria, such as *Vibrio* spp., *Pseudomonas* spp., and *Ralstonia solanacearum* (5, 8, 25, 30, 31, 32, 33, 46).

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MATERIALS AND METHODS

Bacterial strains and growth. *Pantoea agglomerans* strain GBBC 2043 was isolated from potato fields in Merelbeke, Belgium (Institute for Agricultural and Fisheries Research [ILVO]) and was classified by sequencing of the 16S rRNA gene and the additional core genes *gyrB* and *rpoB* (EMBL accession no. FR832419, FR832420, and FR832421, respectively) according to the work of

TABLE 1. Bacterial strains used in this study

Species and strain	Yr of isolation	Location	Biological origin
<i>Pantoea agglomerans</i>			
GBBC 2043	2008	Belgium	Soil
LMG 2660	1970	Japan	<i>Wisteria floribunda</i>
LMG 2570	1980	United States	<i>Sorbus</i> sp.
<i>Pantoea stewartii</i>			
LMG 2712	1957	United States	<i>Zea mays</i>
LMG 2713	1963	United States	<i>Zea mays</i>
LMG 2714	1932	United States	<i>Zea mays</i>
LMG 2717	1954	United States	Beetle
LMG 2718	1963	United States	Unknown
LMG 2719	1975	United States	Corn flea beetle
<i>Erwinia amylovora</i>			
LMG 2024	1959	United Kingdom	<i>Pyrus communis</i>
GBBC 403	2000	Belgium	<i>Crataegus</i> sp.
<i>Erwinia mallotivora</i>			
LMG 1271	1975	Japan	<i>Mallotus japonicus</i>

Brady et al. (7). All strains used in this paper (Table 1) were grown at 30°C in standard LB medium. LB was supplemented with 1.5% agar for plating and with 0.7% agar for agar overlays.

Bacteriophage isolation, amplification, and purification. Phages LIMelight and LIMEzero were isolated from 20-g soil samples taken from the same potato fields from which the bacterial strain was isolated, shaken for 30 min in sterile, demineralized water, and filtered over a 0.45- μ m-pore-size membrane (Millipore). The filtrate was centrifuged for 90 min at 28,000 $\times$ g (Sigma 3K30 centrifuge; fixed-angle rotor, type 12156-H [B. Braun Biotech]), and the pellet was resuspended in phage buffer (10 mM Tris-HCl [pH 7.5], 10 mM MgSO₄, 150 mM NaCl). This suspension was spotted onto a plate with a soft agar overlay of a stationary *Pantoea agglomerans* culture. Lysis zones were picked up, and three successive single-plaque isolations were performed using the standard agar overlay method (2). Phage were amplified by plating out 10⁵ PFU per plate, scraping off the soft agar layer after overnight incubation, and suspending it in phage buffer. Polyethylene glycol 8000 (PEG 8000) containing 1 M NaCl was added to the suspension to a final concentration of 8%, followed by incubation for a minimum of 3 h at 4°C. Phage particles were then precipitated by centrifuging the solution for 30 min at 8,000 $\times$ g in a Sorvall Legend RT Plus centrifuge with a swing-out 4-place rotor, type 75006445 (Thermo Scientific, Waltham, MA), and were resuspended in 15 ml phage buffer. The phage suspension was subsequently layered on a CsCl step gradient (1.33, 1.45, 1.50, and 1.70 g/cm³) and was centrifuged at 140,000 $\times$ g for 3 h with a Beckman Coulter (Brea, CA) Optima LE-80K ultracentrifuge (rotor type, SW28). LIMelight and LIMEzero were both collected at the interface between the gradients of 1.45 and 1.50 g/cm³ and were dialyzed three times for 30 min against 300 volumes of phage buffer.

Electron microscopy. Phage particles were pelleted by centrifugation for 1 h at 25,000 $\times$ g and were washed twice in 0.1 M ammonium acetate (pH 7.0) using a Beckman (Palo Alto, CA) high-speed centrifuge and a JA-18.1 fixed-angle rotor. The pellets were then deposited on copper grids with carbon-coated Formvar films, stained with 2% (wt/vol) potassium phosphotungstate (pH 7.0), and examined with a Philips EM 300 electron microscope (1).

Host range and infection assays. The host range of the bacteriophages was determined by spotting 10 μ l of phage suspension (10⁶ PFU/ml) on plates with a bacterial lawn and by the standard agar overlay where a phage suspension was added to the bacterial lawn. Phages were tested on 12 strains, summarized in Table 1. In adsorption experiments, the host strain GBBC 2043 was grown to an optical density at 600 nm of 0.4 and was infected with phages at a multiplicity of infection (MOI) of 0.001. After infection, 100- μ l samples were taken immediately and every minute. Samples were transferred to 850 μ l LB medium supplemented with 50 μ l CHCl₃. These mixtures were shaken gently for 15 min to lyse any remaining bacteria. The supernatant was titrated to determine the amount of nonadsorbed or reversibly adsorbed phages.

DNA isolation and sequencing. DNA was isolated according to the method of Sambrook and Russell (2001) (50). Approximately 1 μ g of DNA was sheared by sonication, and fragments of 1 to 2 kb were isolated. An end repair reaction was

performed with T4 and Klenow polymerase, and the fragments were ligated in the pJET1.2 blunt vector (Fermentas), which was transformed into *Escherichia coli* XL1-Blue cells. Individual colonies were selected, and the vectors were isolated and sequenced using vector primers on an ABI 3130 capillary sequencer. Contigs were aligned with Sequencher software (version 4.1; Gene Codes).

In silico analysis. The genomes of LIMelight and LIMEzero were scanned for potential open reading frames (ORFs) with ORF Finder (<http://www.ncbi.nlm.nih.gov/projects/gorf/>) and GeneMark.hmm software (39). Shine-Dalgarno sequences were verified manually upstream of each annotated ORF. Functional bioinformatic annotation was carried out by comparing translated ORFs in a BLASTP (20) analysis against the nonredundant GenBank protein database and by using the HHPred prediction software (51). The presence of transmembrane domains was verified with TMHMM software (43), and signal peptides were identified with SignalP (16). Host promoter regions were identified using the Nostradamus (21) prediction program. Potential phage promoter sites were scanned for using MEME (4) and PHIRE (34) software. Palindromic repeat regions were identified by TransTerm (28) and Mfold (59). Terminators were defined as palindromic sequences followed by a U-rich stretch and a stable secondary structure as calculated by Mfold. Nucleotide similarity between phages was compared using the Stretcher algorithm (42).

Comparative genomics. Phylogenetic trees were constructed using Phylogeny.fr (12). The nucleic acid sequences of the phages as found on NCBI were loaded in a FASTA file onto the server using the "One Click" option. Multiple alignment was carried out with the MUSCLE algorithm (15) without a curation step. Trees were constructed with PhyML (22) using the maximum-likelihood method and were drawn with TreeDyn.

RESULTS AND DISCUSSION

Phage isolation and morphological characteristics. Bacteriophages LIMEzero and LIMelight were isolated from soil samples of potato trial fields of the Institute for Agricultural and Fisheries Research (ILVO), Merelbeke, Belgium. Both formed clear plaques on *Pantoea agglomerans* strain GBBC 2043, an isolate from the same field soil. Two distinct plaque morphologies were observed: a clear zone surrounded by a turbid halo with a diameter of 2 mm for LIMEzero and a small clear plaque of 1 mm in diameter for LIMelight. The host range of phage LIMEzero was limited to the *P. agglomerans* strain on which it was originally isolated. LIMelight also infected *P. agglomerans* strain LMG 2660, as confirmed by a plaque assay and spot tests. In the spot tests, the phage showed an inhibitory effect, the formation of a turbid halo without lysis, on *Erwinia amylovora* strain GBBC 403, *Erwinia mallotivora* strain LMG 1271, and *Pantoea stewartii* strains LMG 2717 and LMG 2719, suggesting the presence of an exopolysaccharide (EPS) depolymerase (23). No plaques were formed on these strains.

Electron microscopy revealed the phages to be members of the *Podoviridae*, with a morphotype identical to that of phiKMV (Fig. 1). The particles are indistinguishable from each other, with icosahedral heads of approximately 60 nm in diameter, a short, stubby tail 12 nm long, and a number of tail spikes (10 by 3 nm). The infection processes of LIMelight and LIMEzero were compared by infection and adsorption assays. New phage particles were released after 30 min for LIMEzero and after 270 min for LIMelight. The adsorption constants were 8.2 $\times$ 10⁻⁹ ml/min and 1.2 $\times$ 10⁻⁹ ml/min, respectively.

Genome analysis. The genomic sequences of phages LIMelight and LIMEzero were determined using DNA extracted from purified phage particles by shotgun sequencing and primer walking. For LIMelight, this resulted in a sequence of 44,546 bp delineated by direct terminal repeats (DTRs) of 277 bp. These DTRs were determined by direct sequencing with

FIG. 1. Electron microscopic images of phage LIMEzero (A) and phage LIMelight (B). Bar, 100 nm. Phages are colored with 2% phosphotungstate.

outward-directed primers located in and right outside the repeats. LIMEzero has a genome of 43,032 bp, but we were not able to perform primer walking for the determination of DTRs in this phage, although they are expected. The GC contents of LIMelight and LIMEzero were 54.0% and 55.4%, respectively.

The genome of LIMEzero consisted of 57 ORFs, 18 of which were similar to those of members of the “*phi*KMV-like viruses” at the amino acid level (see Table S1 in the supplemental material). For LIMelight, 55 ORFs were predicted, also with 18 ORFs similar to those of the “*phi*KMV-like viruses” (see Table S2 in the supplemental material). No marked DNA similarity exists between the two phages.

Phages of the *Autographivirinae* (35) share a genome organization and make use of both the host RNA polymerase and a phage-encoded RNA polymerase. In contrast to the “T7-like viruses,” these phages depend on the host RNA polymerase for the transcription of both early and middle genes. Therefore, the phages should encode both host σ^{70} promoters and phage-specific promoter sites (Fig. 2 and Table 2). In LIMelight, seven σ^{70} promoters were identified in intergenic regions; five of these are situated upstream of ORF1. One is located in front of the RNA polymerase gene and a final one in front of the sequence coding for tail protein A. A search for conserved intergenic motifs yielded four putative phage promoter sites: one in the early region, two in the DNA metabolism region, and one at the start of the predicted structural region (Fig. 2; Table 2). Two factor-independent terminators were located, one directly behind the capsid protein gene and one at the end of the genome, in front of the right DTR. In LIMEzero, only three potential host promoter sites were identified, all upstream of the first ORF. Putative phage-specific promoters were found in front of ORF17 in the beginning of the DNA metabolism region and in front of the RNA polymerase. Two terminators were identified; the first was located behind ORF16, in front of the first phage-specific promoter, and the second was located behind the capsid protein gene.

Interestingly, direct genomic sequencing of LIMelight led to a sudden drop in the signal strength of the chromatograms at

four specific locations on the noncoding strand, suggesting the presence of single-stranded breaks or nicks at these locations. A consensus sequence was found at the nick site (Table 3). In contrast, no nicks and no consensus sequence were identified in the genome of LIMEzero. The presence of such conserved nicks has also been observed in phages T5 (58) and phikF77 (31), but their consensus sequences bore no similarity to that of LIMelight (Table 3). The assumption has been made that these nick consensus sites function as phage-specific promoters (31), but for phage LIMelight, these promoters are clearly separate entities, with a different location and a different consensus sequence (Table 2). To date, no suitable explanation for the presence of these nicks has been offered. However, it should be noted that the presence of these nicks is conserved across different host species, but not within the phage genus or species.

Genome architecture. Two distinct genomic regions, marked by the location of the RNA polymerase gene, can be delineated in the genomes of the “*phi*KMV-like viruses.” A typical characteristic of these phages is that the early and middle region ends with the DNA-dependent RNA polymerase and encompasses the host conversion and DNA replication genes. The late region comprises the genes coding for structural and lysis proteins.

The genome architecture of all proposed “*phi*KMV-like viruses” (Table 4) is conserved despite the low DNA homology between a number of phages. *Pseudomonas* phages *phi*KMV, LKD16, LUZ19, phikF77, PT2, and PT5 show strong DNA similarity and can be considered the same species (with *phi*KMV as the type phage), whereas the other phages are each clearly distinct species. Phage KP34, not yet officially assigned to any genus, shows a similar genome architecture and is therefore included in this study.

Early and middle region. The early and middle region comprises the early genes and the DNA metabolism genes (Fig. 2). In LIMEzero and LIMelight there are, respectively, 16 and 19 small, early genes, which show no similarity to each other and have no counterpart in any public database. These genes are transcribed immediately after infection and are involved in

FIG. 2. Comparison of the genomes of *Pantoea* phages LIMEzero and LIMelight with those of *Pseudomonas* phage LKA1, *Vibrio* phage VP93, *Ralstonia* phage RSB1, and *Klebsiella* phage KP34. The predicted open reading frames are indicated by arrows. ORFs of unknown function in the *Pantoea* phages are in light gray, predicted DNA metabolism genes in green, structural genes in blue, and lysis genes in purple. (ORFs in lighter shades have no equivalents in the other phages.) Functionally equivalent genes are connected by thin lines, and putative functions are designated. Host promoters are indicated by bent arrows with triangular points and phage-specific promoters by smaller bent arrows; factor-independent terminators are shown with stem-loop structures.

host conversion. Recently, Roucourt and Lavigne (49) have shown that these proteins protect the bacteriophage from bacterial defense mechanisms or adapt the host cell mechanism (49). The strong divergence within the “phiKMV-like viruses” may thus be explained in part by the fact that they infect a different range of host bacteria or utilize different defense/adaptation mechanisms.

The first genes of the DNA replication region are the DNA primase (ORF17) in LIMEzero and the DNA ligase (ORF20) in LIMelight. In LIMEzero, no ligase could be identified by either BLAST or HHPred. Next is the DNA polymerase, followed by a 5′–3′ exonuclease, an endonuclease, and a polynucleotide 5′ kinase/3′ phosphatase, in the same order in both phages. This region ends with the DNA-dependent RNA polymerase. This gene order is preserved among most “phiKMV-like viruses.” Phage VP93 lacks a discernible DNA ligase, just like LIMEzero, whereas the DNA ligase in phage RSB1 is situated directly in front of the RNA polymerase instead of at the beginning of the region. In the latter, no predicted endonuclease has been found.

RNA polymerase and phage-specific promoters. The DNA-dependent RNA polymerases of phages LIMEzero and LIMelight are very similar (BlastP expectancy values, $10e-90$) and are also conserved throughout the genomes of all the “phiKMV-like viruses” (summarized in Table 4), with BlastP expectancy values between $10e-59$ and zero. Conservation of the catalytic residues Tyr639, Lys631, Asp537, and Asp812 of the T7 RNA polymerase (9) was observed for LIMEzero and LIMelight. No homology was detected with either the recognition loop (amino acids 93 to 101) or the specificity loop (amino acids 739 to 770) of the T7 RNA polymerase, suggesting divergence in phage-specific promoter sequences. Indeed, no T7-like phage-specific promoter sequences were identified in LIMEzero or LIMelight. As mentioned above, two and four phage-specific promoters were found in LIMEzero and LIMelight, respectively (Fig. 2). The location of these promoters does not seem to be conserved within the “phiKMV-like viruses,” although both LIMEzero and LIMelight have one at the start of the structural region.

TABLE 2. Regulatory elements in the genomes of LIMEzero and LIMelight

Position	ORF ^a	Regulatory element sequence ^b
Host promoters		
LIMEzero		
801_847	1	TAGTGCTTGTCAGATGGTTCGGATTGTTGTGTAATTCATCACCGA
928_974	1	CGCTCTTTATACAATCTGGTCTGGTTCGTGATAGTCCACCAATCAA
1399_1445	1	AAGTACTTGACGTTACAACGCAGTATGATGTAATTCGTCTCAAGCA
LIMelight		
500_546	1	GTATTTTGTATTTCGTTTCGTCGCCTCACTCTATAACGTCAATCATA
549_595	1	TATCTTTGAACGTAATCCGTTTATGATAGGTAACCTTGTGTACA
805_849	1	AGTGCTTGCAATCTCTAAATCATTCATC---TAGTATTCAATCCATC
967_1010	1	AGTGTTTGACAAGTGAATCCAGAAGGAT---TAGATTAGTTAGCAAC
1097_1140	1	AACGCTTGACAAGTTGAACGATAACGAT---TAACTTAGATAGCAAG
19347_19391	36	TCGTCTTGATAAAGCCGAGTGAGTGGTGT---TATAATCAATGGACAT
26470_26513	43	ATATTTTCAGTGTTCCTACGGGAGCACT---TATAAATACCATTAC
Consensus promoter, <i>E. coli</i>		-----TTGACA (−35) -----TATAAT (−10) -----
Phage promoters		
LIMEzero		
7294_7313	17	TTTAAAGGCAATCACTAAGG
20330_20349	34	TATAAAGGCAAGCACTATGA
LIMelight		
4898_4922	12	AATGGTGATGCACGTTATAGACCGA
14061_14085	27	ATTAAAGAGTGACGTTATAGAGAGA
15535_15559	29	TTTGAAGATACACGTTATAGAGAGC
22024_22048	37	AATGATGATGTACGTTATAGAGAGA
Factor-independent terminators		
LIMEzero		
7255_7284	17	AAGCCCATTTAAATAAGTGGGCTTTATTAT
25202_25236	41	AGCCCTGGACTCGTCCAGGGTTTGATTATGTTTT
LIMelight		
26239_26279	42	TCTAAGGGAGCCTTTTCGAGGTTCCCTTATTTGCC
44216_44250	DTR	GCAGCTGGTGTAATCCAGCACCTGAAATTTAT

^a Number of the ORF downstream of the element.^b Underlined sequences represent the −35 and −10 boxes for the host promoters and the palindromic sequence for the terminators.

Structural region. The structural region starts downstream of the RNA polymerase gene with several small genes, 4 in LIMEzero and 2 in LIMelight, followed by the first functionally identified gene, the head-tail connector (Fig. 2). The two small genes of LIMelight encode a conserved hypothetical protein (Gp37) and a conserved structural protein (Gp38) and are related to Gp36 and Gp37 of LIMEzero. These show similarity to the proteins of unknown function of phiKMV, Gp28 and Gp29, respectively, located in front of the phiKMV head-tail connector gene. In both LIMEzero and LIMelight, the scaffolding protein gene was found next, followed by the genes for the major capsid protein and tubular tail proteins A and B. This gene order was observed in all “phiKMV-like viruses.” Three internal virion proteins were identified in the

Pantoea phages, one of which has a lysozyme domain as predicted by HHPred. Similar internal virion proteins have been confirmed by mass spectrometry for LKD16 and LKA1 (8), but they have not been identified in other phages. However, three genes of the same size are present in the same location in the other phages (Fig. 2). On this basis, it can be assumed that these are also internal virion protein genes, despite the lack of sequence or amino acid homology. The tail fiber proteins were the most strongly divergent proteins between LIMEzero and LIMelight. The former has two tail fiber proteins and a tail spike protein, like phiKMV; the latter encodes a single tail fiber protein with a predicted EPS depolymerase. This EPS depolymerase showed strong homology to counterparts in *Erwinia amylovora* phages phi-Ea1h (27) and Era103 (56), ex-

TABLE 3. Nicks in the genome of LIMelight

Nick	Position	Sequence (5′-3′) ^a
n1	7422	CAGGGCCGTGCGCTATGCT▼CAAGCAGAACAAATT
n2	9551	GCCGCGTGTGCAGCCTGCA▼CCAGCAGACGTAATC
n3	15580	GGAGCTGGTGCCTGCTTACA▼CCAGCACAACAAAAC
n4	20424	CGGATGGGTGCAGGCTGCA▼CGAGCACAAGGTATC
Consensus		GTGC-N ₄ -TRCW▼CVAGCA
T5 consensus		CCCT▼CGCG
phikF77 consensus		CGAC-N ₅ -CCTA▼CTCCG

^a Underlined sequences mark the consensus site.

TABLE 4. Summary of the proposed “phiKMV-like viruses”

Phage	Host	Genome (bp)	% DNA homology with:		GenBank accession no.	Source or reference(s)
			phiKMV	LKA1		
phiKMV	<i>Pseudomonas aeruginosa</i>	42,519	100	53.0	NC_005045	33
LKD16	<i>P. aeruginosa</i>	43,200	84.1	53.1	NC_009935	8
LUZ19	<i>P. aeruginosa</i>	43,548	89.1	52.8	NC_010326	32
phikF77	<i>P. aeruginosa</i>	43,152	86.5	52.9	NC_012418	30, 31
PT2	<i>P. aeruginosa</i>	42,961	96.7	52.9	NC_011107	Unpublished
PT5	<i>P. aeruginosa</i>	42,954	95.2	53.0	NC_011105	Unpublished
LKA1	<i>P. aeruginosa</i>	41,593	53.0	100	NC_009936	8
Phi-2	<i>P. fluorescens</i>	43,144	52.5	52.7	NC_013638	46
VP93	<i>Vibrio parahaemolyticus</i>	43,931	47.6	47.5	NC_012662	5
RSB1	<i>R. solanacearum</i>	43,079	50.2	49.7	NC_011201	25
KP34	<i>Klebsiella pneumoniae</i>	43,593	48.1	48.2	NC_013649	13
LIMELight	<i>Pantoea agglomerans</i>	44,546	49.7	49.2	FR687252	This study
LIMEzero	<i>Pantoea agglomerans</i>	43,032	50.1	49.5	FR751545	This study

plaining the results of the host screen assay and suggesting lateral acquisition of this gene. Phages LKA1, RSB1, and KP34 also have only one tail fiber gene each, showing little homology with each other (8, 13, 25, 34). VP93 is the only phage with two tail fiber genes (5). The last two functionally annotated structural protein genes code for DNA maturase A and B and are well conserved among all “phiKMV-like viruses.”

Lysis genes. Phages LIMEzero and LIMELight both encoded endolysin proteins (Gp55 in both phages), but only LIMELight had a clearly definable holin (Gp54). In LIMEzero, a presumptive holin gene was identified 5' of the endolysin gene, based on the presence of a transmembrane domain and the overlap of the ORF with the endolysin. The holin-lysin mechanism for lysis of bacteria has been proposed for phages phiKMV and LKD16 (8, 33), and based on the amino acid homology of their holins and endolysins, it can be considered a common mechanism for the entire phiKMV species. Between the other “phiKMV-like viruses” there is limited protein similarity in the lysis genes. LKA1 does encode a presumed holin (based on the presence of two hydrophobic domains) and an endolysin (8). For the other phages in this group, no holins have been identified, only endolysins, except for VP93, for which neither has been found. The internally overlapping Rz/Rz1 proteins, generally encoded downstream of the endolysin gene, have been located in phiKMV, LKA1, and LKD16 (8) but not in any of the non-*Pseudomonas* phages. LIMEzero and LIMELight do not seem to encode such proteins downstream of their endolysin but have an interesting feature in front of the presumed holin. In both phages, ORF53 has a signal peptide and a transmembrane domain, suggesting a function in the lysis mechanism.

Comparative genome analysis. A hallmark of the genus of the “phiKMV-like viruses” is the presence of a DNA-dependent RNA polymerase located directly in front of the genes encoding the structural proteins. The other members of the *Autographivirinae* subfamily also encode an RNA polymerase, but in a different location in the genome, leading to two other genera, the “T7-like viruses” and the “SP6-like viruses.” Phylogenetic analysis of the RNA polymerase, DNA polymerase, and major capsid protein genes of all *Autographivirinae* with available sequences was carried out to investigate the clustering of the phages in these genera. The novel *Pantoea* phages

LIMEzero and LIMELight were also included, as was *Klebsiella* phage KP34. The *Siphovirus* phage Xp10, infecting *Xanthomonas*, was also added because of the presence of a T7-like RNA polymerase.

The three phylogenetic trees (Fig. 3) supported the current genus subdivision within the *Autographivirinae*. As expected, the “T7-like viruses” all clustered within the same group, supplemented by the unclassified T7-like phages. The “SP6-like viruses” also formed a distinct clade, showing more relatedness to the “T7-like viruses” according to the RNA and DNA polymerase trees (Fig. 3A and B) but clustering closer to the “phiKMV-like viruses” according to the major capsid protein gene tree (Fig. 3C). The last genus is the most diverse, with all but one of its phages clustering together. *Ralstonia* phage RSB1 does not fit in with any genus. Instead, it clusters together with Xp10 for the polymerases and with the “SP6-like viruses” for the major capsid protein genes. The new *Pantoea* phages were assigned to the “phiKMV-like viruses” cluster, and their presence in this genus is thereby confirmed. The unassigned members of the *Autographivirinae*, *Prochlorococcus* phage P-SSP7 and *Synechococcus* phages P60 and Syn5, formed a separate clade, suggesting that they all belong to a new genus.

Conclusion. In this paper we studied two newly isolated *Pantoea agglomerans* phages, LIMEzero and LIMELight. They are the first members of the *Podoviridae* infecting this host species whose genomes have been fully sequenced. Based on their overall genome architecture, they are classified within the subfamily *Autographivirinae*, in the genus of the “phiKMV-like viruses.”

The “phiKMV-like viruses” grouping was first individualized by Ceyssens et al. in 2006 with the discovery of *Pseudomonas* phages LKD16 and LKA1 (8), which showed marked similarity to another *Pseudomonas* phage, phiKMV (33). The genus was extended with other *Pseudomonas* phages (PT2, PT5, phikF77, and LUZ19) (30, 31, 32), *Vibrio* phage VP93 (5), and *Ralstonia* phage RSB1 (25). Recently, *Pseudomonas fluorescens* phage phi-2 has been proposed as a member of this genus (46). In addition, *Klebsiella* phage KP34 (13), currently assigned to the unclassified *Podoviridae*, also shares the typical genome architecture. In this paper we were able to extend the genus

FIG. 3. PhyML phylogenetic trees of the RNA polymerases (A), DNA polymerases (B), and major capsid genes (C) of the phages of the *Autographivirinae*. Red lines indicate the clade of “phiKMV-like viruses”; blue lines, the “SP6-like viruses”; and green lines, the “T7-like viruses.” The unassigned *Autographivirinae* are indicated by black lines, as are *Siphovirus* phage Xp10 and *Ralstonia* phage RSB1, which do not cluster in any of the previous groups.

“phiKMV-like viruses” with the novel *Pantoea* phages LIMEzero and LIMELight.

The known “phiKMV-like viruses” encapsulate a genome of approximately 42 kb; LIMELight had the largest genome, at 44.5 kb. Within this genus, all the phages have relatively long direct terminal repeats of 277 to 488 bp.

In the previously described “phiKMV-like viruses” LKD16, LUZ19, phikF77, PT2, PT5, and phiKMV, the overall gene sequences are highly conserved, with the exception of the early region and the tail fiber genes. They show more than 84% DNA homology with phiKMV and can be thus considered members of the same species, with phiKMV as the type phage. Bacteriophages LKA1, phi-2, VP93, RSB1, and KP34, and the new phages LIMELight and LIMEzero, show little DNA homology with phiKMV or with each other but display a conserved genome organization and gene order (Fig. 2).

We conclude that the genus “phiKMV-like viruses” is still expanding and is ubiquitously distributed, since isolates have been collected from all over the world (e.g., in Russia, Belgium, Chile, and Japan) and infect a range of different hosts.

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