

# Morphology, Genome Sequence, and Structural Proteome of Type Phage P335 from *Lactococcus lactis*<sup>∇†</sup>

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***Lactococcus lactis* phage P335 is a virulent type phage for the species that bears its name and belongs to the Siphoviridae family. Morphologically, P335 resembled the *L. lactis* phages TP901-1 and Tuc2009, except for a shorter tail and a different collar/whisker structure. Its 33,613-bp double-stranded DNA genome had 50 open reading frames. Putative functions were assigned to 29 of them. Unlike other sequenced genomes from lactococcal phages belonging to this species, P335 did not have a lysogeny module. However, it did carry a dUTPase gene, the most conserved gene among this phage species. Comparative genomic analyses revealed a high level of identity between the morphogenesis modules of the phages P335, ul36, TP901-1, and Tuc2009 and two putative prophages of *L. lactis* SK11. Differences were noted in genes coding for receptor-binding proteins, in agreement with their distinct host ranges. Sixteen structural proteins of phage P335 were identified by liquid chromatography-tandem mass spectrometry. A 2.8-kb insertion was recognized between the putative genes coding for the activator of late transcription (Alt) and the small terminase subunit (TerS). Four genes within this region were autonomously late transcribed and possibly under the control of Alt. Three of the four deduced proteins had similarities with proteins from *Streptococcus pyogenes* prophages, suggesting that P335 acquired this module from another phage genome. The genetic diversity of the P335 species indicates that they are exceptional models for studying the modular theory of phage evolution.**

*Lactococcus lactis* strains are extensively used by the dairy industry to manufacture several fermented milk products. However, virulent lactococcal phages are ubiquitous in dairy factories and, given the appropriate conditions, they can lyse these cultures and delay milk fermentation, which leads to low-quality products (43). Despite the wide range of strategies applied to control lactococcal phage populations in manufacturing facilities, new virulent phages are constantly emerging (24, 25). As such, new phage control tactics must be continuously developed (16, 40, 55). The development of antiviral measures starts with a thorough understanding of the phage population and its dynamic state. The morphology, host ranges, and genomes of hundreds of *L. lactis* phages have already been characterized (1). All lactococcal phages discovered to date belong to the *Caudovirales* order (double-stranded DNA tailed phages) (1, 38). Most lactococcal phages have long noncontractile tails and small isometric or short prolate capsids (*Siphoviridae* family). Lactococcal phages are currently classified into 10 species based on morphology and DNA ho-

mology (18). The phages of three species (936, c2, and P335) are mainly responsible for milk fermentation failures worldwide. Today, more than 20 complete lactococcal phage genome sequences are available in public databases, including 11 from phages belonging to the P335 species (6, 11, 14, 28, 30, 36, 51, 58, 60), 2 of the c2 species (33, 49), and 5 of the 936 species (13, 17, 37), as well as 3 sequences from rare lactococcal phage species such as the Q54 (21), KSY1 (15), and 1706 species (22). *L. lactis* genomic projects also revealed the presence of prophages and prophage remnants in these strains (64). Comparative analyses of these phage genomes are shedding light on their origins, evolution, and relationships with other phages. For example, rare phages KSY1 and 1706 share homology with T7-like marine phages and putative prophages found in other *Firmicutes*, respectively, while Q54 shares limited similarities with two of the main lactococcal phage species (c2 and 936), suggesting a phylogenetic link between these lactococcal phage groups (21).

Phages of the P335 species have been the subject of numerous studies mainly because they represent a remarkable group of diverse phages. In fact, it has been argued that this group of phages represents a polythetic species (18). Members of the P335 species are interconnected through shared properties or modules, but no single attribute is shared by all of them. The mosaic structure of their genomes is likely the consequence of recombination between related virulent phages and prophages (18). Their genome flexibility also provides a means to quickly adjust to a new host, particularly when facing phage resistance

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mechanisms (30). Members of the lactococcal P335 phage species have also become models in phage biology. For example, the biology of the temperate phage TP901-1 is among the best-studied gram-positive phages (10, 45, 46, 61–63).

Surprisingly, despite the body of knowledge on this group of lactococcal phages, the virulent type phage P335 that led to the designation of this species has not yet been thoroughly analyzed. This report outlines its morphology, genome sequence, and structural proteome.

## MATERIALS AND METHODS

**Strains, phages, and growth conditions.** The type phage P335 and its host strain *L. lactis* subsp. *lactis* biovar diacetylactis F7/2 (9) were deposited at the Felix d'Hérelle Reference Center for Bacterial Viruses ([www.phage.ulaval.ca](http://www.phage.ulaval.ca)). *L. lactis* strains were grown in M17 (Oxoid) (56) supplemented with 0.5% glucose (GM17) without shaking at 30°C. When phages were propagated, 5 to 10 mM CaCl<sub>2</sub> was added to the medium. Phages were purified by CsCl-gradient centrifugation as previously described (48). Plaque assays were conducted as outlined elsewhere (63). *Escherichia coli* XL1-Blue-MRF' (Stratagene) was grown in Luria-Bertani medium supplemented with 12.5 µg of tetracycline/ml (LB) (48) with shaking at 37°C.

**Electron microscopic observations.** Phage observations were performed as described elsewhere (61). Phages purified by CsCl gradient centrifugation were dialyzed against SM buffer (48). Staining was performed with 2% (wt/vol) uranyl acetate on freshly prepared carbon films. Grids were analyzed in a Tecnai 10 transmission electron microscope (FEI Company) at an acceleration voltage of 80 kV. Micrographs were taken with a MegaView II charge-coupled device camera (SIS). Phage dimensions are the means of at least 12 measurements.

**Sequencing strategy.** P335 DNA was isolated as previously reported (20). Partial cloning libraries of HindIII- and Tsp5091-digested P335 DNA were constructed into pBluescript II SK(+) (Stratagene). *E. coli* XL1-MRF' cells were made competent by using CaCl<sub>2</sub> and transformed by heat shock. Transformants were selected on LB agar containing 75 µg of ampicillin/ml, 12.5 µg of tetracycline/ml, 40 µg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside/ml, and 40 µg of isopropyl-β-D-thiogalactoside/ml. White colonies were propagated in LB medium containing 100 µg of ampicillin/ml. Plasmids were isolated by using a QIAprep spin miniprep kit (Qiagen). Clones were sequenced with a CEQ 2000 dye terminator cycle sequencing kit with a Beckman Coulter CEQ 2000XL DNA analysis system using vector or custom-made primers (MWG). The complete genomic sequence was obtained by primer walking using P335 genomic DNA as a template and a ABI Prism 3700 apparatus (Centre Hospitalier de l'Université Laval).

**Bioinformatics analysis.** Sequences were assembled by using the VectorNTI (version 7.1; Invitrogen) and Staden package programs (54). The assembled sequence was analyzed for open reading frames (ORFs) using AUG, UUG, or GUG as start codons. ORFs putatively coding for more than 30 amino acids and with a putative ribosomal binding site upstream (2 to 10 bp) from the start codon or with a putative translational coupling were identified by using VectorNTI and GeneMark (34). Translated ORFs were analyzed by the National Center for Biotechnology Information (NCBI) tBLASTn and PSI-BLAST programs (2). CD search at the NCBI (39) and InterProScan (67) were used to identify the conserved domains in the proteins.

**Structural protein analysis.** Phages purified on a continuous CsCl-gradient (~10<sup>11</sup> PFU/ml) were analyzed for structural proteins by standard Tris-glycine sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS-12% PAGE) electrophoresis (31). Nanosep 3k columns (Pall) were used to concentrate the phage suspensions before electrophoresis as described elsewhere (21). The highly concentrated phage sample was sonicated for 5 s (output control = 4; duty cycle = 40%) with a Ultrasonic Sonifier W-350 (Branson Sonic Power Co.). Samples were then mixed with 2× sample loading buffer and boiled for 5 min before loading. Protein bands were detected by Coomassie blue staining. Protein bands were extracted from the gels, digested with trypsin, and identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using the proteomics platform at the McGill University and Génome Québec Innovation Centre.

**RNA analysis.** Total RNA was extracted from uninfected *L. lactis* F7/2 cells and from P335-infected (multiplicity of infection of 10) *L. lactis* F7/2 cells as described elsewhere (21). Transcription was blocked by centrifuging the infected cells and snap-freezing the pellets in liquid nitrogen-ethanol mix. RNA was extracted by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions except that the cells were treated with lysozyme (60 µg/ml) for 20

min at 37°C. The mRNA transcripts were separated by formaldehyde agarose gel electrophoresis and then capillary transferred to a positively charged nylon membrane (Amersham). The membranes were blotted by using radiolabeled specific oligonucleotides as described elsewhere (21). Four oligonucleotide probes were used, namely, probe *orf23* (5'-GCGGCATGGATATCCCTCCTC TACCAATTTTC-3'), probe *orf24* (5'-GATATGGCTTTAACCAACTTGGAAAC GCTACC-3'), probe *orf25* (5'-GCAACGCCTAACCTATTCTTGATAATC AGGC-3'), and probe *orf26* (5'-GATATCAGGTCGTCATTGATGTTAGGC TGT-3').

**Nucleotide sequence accession number.** The sequence data reported in the present study has been deposited in GenBank database under accession no. AY365423.

## RESULTS

**Morphology.** The virulent phage P335 was isolated in 1979 from a problematic milk fermentation in Germany and has since become the type phage of the P335 species (9). It is a member of the *Siphoviridae* family and has an isometric capsid with a diameter of 53.4 ± 2.1 nm ( $n = 53$ ). Phage P335 also possesses a noncontractile tail (length, 130.1 ± 3.3 nm [ $n = 53$ ]; diameter, 11.1 ± 0.8 nm [ $n = 65$ ]) with a complex baseplate (Fig. 1). The baseplate is composed of an upper baseplate (BppU; diameter, 28.7 ± 2.0 nm [ $n = 49$ ]). Side views of the baseplate reveal a number of appendages (Fig. 1a to d and g to k) with a length of 9.8 ± 2.0 nm ( $n = 42$ ). These lower structures (BppL) reveal flexibility and various shapes. They appear as perpendicular fibrils below the BppU structure (Fig. 1a to d) but can also spread (fan-like) (Fig. 1d) or aggregate and form a conical structure (Fig. 1g to j). In the latter case, a distal protruding fiber is also visible (length, 10.6 ± 1.9 nm [ $n = 12$ ]). These structures are arranged in two subdisks of different length, which are visible in Fig. 1h to j. Apparently, these lower baseplate structures can also flap to the side (Fig. 1e and f). In these cases, they look like circular fluffy brushes. It is notable that more than 6 BppL fibrils are clearly visible on these micrographs with top-view baseplates (approximately 12 in Fig. 1f). This is also confirmed by micrograph Fig. 1l, where the BppL structures appear as a set (approximately 18) of globular structures with a diameter of 4.6 ± 0.7 nm ( $n = 24$ ). Another unique feature of the virulent lactococcal phage P335 is the presence of neck passage structures (NPS) composed of a collar with a diameter of 16.1 ± 1.5 nm ( $n = 32$ ) and of distinct short whiskers (length: 17.9 ± 2.3 nm [ $n = 24$ ]). A maximum of three of these whisker fibers are visible. At their distal ends, they are arranged in globular knob-like structures (diameter: 9.9 ± 1.1 nm [ $n = 24$ ]). Complex baseplates and collar/whisker structures have also been observed for other P335 phages, namely, Tuc2009 (41), TP901-1 (52, 61, 62), and ul36 (30, 42).

**Host range.** Phage P335 was found to infect 4 (F7/2, F4/2, 455, and IL1403) of 10 tested *L. lactis* strains that are sensitive to P335-like phages. The six other *L. lactis* strains (NCK203, SMQ-86, LLC509.9, IMN-C18, Wg2, and 205.RV) were not sensitive to P335 under the conditions tested.

**Genome sequence analysis.** The double-stranded DNA genome of P335 was 33,613 bp long. The overall G+C mol content was 35.5%, which is similar to that of other lactococcal phage genomes. The redundancy at both ends of the genome suggests that P335 uses a *pac*-type packaging mechanism (5). Computational analyses revealed that it had 50 ORFs (Table 1) covering 90.8% of the genome. Only ORF25 was not preceded by a suitable ribosome-binding site (RBS) (Table 1 and

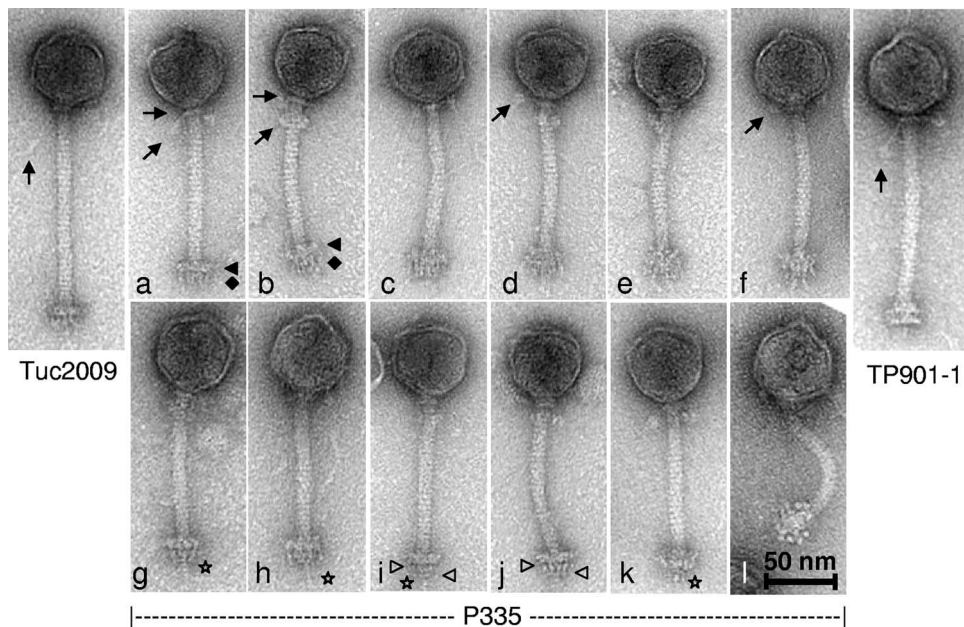


FIG. 1. Transmission electron micrographs of *L. lactis* phage P335 illustrating side views (a to d and g to k) or bottom views (e to f and l) of baseplate structures. Markings on selected micrographs indicate whiskers ( $\nearrow$ ), collar ( $\rightarrow$ ), upper baseplate ( $\blacktriangleleft$ ), lower baseplate ( $\blacklozenge$ ), upper plane of lower baseplate ( $\blacktriangleleft$ ), lower plane of lower baseplate ( $\blacktriangleright$ ), and protruding spike structure ( $\star$ ). *L. lactis* phages Tuc2009 and TP901-1 exhibiting longer tails, longer whiskers ( $\uparrow$ ), and differently shaped lower baseplate structures are also shown.

Fig. 2). The genome synteny was similar to the other lactococcal phages and all of the genes were on the same DNA coding strand. Interestingly, contrary to other phages of the P335 species, a lysogeny module is absent in the genome of phage P335. However, a putative *cro*-type function was assigned to ORF1.

**Early expressed genes and DNA regulation.** Based on previous gene expression studies of P335-like phages (35, 51), the putative early expressed region likely encompassed 22 ORFs (*orf1* through *orf22*). A putative function was assigned to nine of these ORFs (Table 1). The *erf* gene (*orf5*) is very common among lactococcal phages, and ORF5 likely belongs to the superfamily of single-strand annealing proteins, which plays a role in DNA annealing and homologous recombination (8). ORF5 was previously designated Sak2 since it is involved in the sensitivity of P335 to the abortive infection mechanism AbiK (8). A gene coding for a single-strand binding protein (SSB) is usually found downstream from an *erf*-like gene. The *orf6* possessed weak homology with the *ssb* gene of *Streptococcus suis* phage SMP, while *orf7* possessed strong homology with phage genes coding for replisome organizer proteins (44), which are usually located just downstream from the *ssb* gene. The SSB protein associates with the replisome organizer protein to initiate DNA replication. The origin of replication (*ori*) of phage P335 is probably located within *orf7*, since it contained direct repeats, had a very low G+C mol content (25%), and was highly homologous with the *ori* of phage *ul36.1* (7).

The *orf17* of phage P335, probably encoding a dUTPase, is the most conserved gene within the lactococcal P335 species. This gene has been targeted by PCR to rapidly detect P335 phage species (28). To date, only the virulent P335-phage 4268 does not possess a similar gene (58). *orf22* encodes the activa-

tor of late transcription (ALT) protein as it is highly similar (99% [211 of 213 bp]) to the characterized ALT protein from TP901-1 (12, 45, 46). A putative promoter region ( $P_{late}$ ; 100% identical to the characterized  $P_{late}$  of the phage TP901-1) was also found downstream of the *alt* gene (12). This region is composed of four conserved repeats (ALT boxes) just upstream the promoter (located at coordinates 9546 to 9589), which are identical to those found in phage TP901-1 (46). The ALT protein binds specifically to the ALT boxes to induce the expression of the late genes (46).

**Moron genes.** Downstream of the early-expressed genes (*orf1* to *orf22*), the P335 genome contained four genes (*orf23* to *orf26*) that were homologous to genes found in *Streptococcus pyogenes* and *Enterococcus faecium* (Table 1). This module was not present in the other characterized P335 phages. In fact, in most other P335-like phages, *orf22*-like genes are usually adjacent to *orf27*-like genes with a 3- to 400-bp noncoding region in between (12, 46). Moreover, *orf23* to *orf25* homologous genes found in *S. pyogenes* M18 genome (SpyM18\_1784 to SpyM18\_1782) are adjacent to each other, suggesting that P335 has acquired these genes as a block. These observations also suggested that these four ORFs (*orf23* to *orf26*) might be an example of the genetic elements named morons (26). Morons are composed of a protein-coding region flanked by a promoter and a factor-independent transcription terminator (26). To determine whether *orf23* through *orf26* were transcribed autonomously, the total RNA of P335-infected cells was extracted at different times, and Northern assays were conducted. Three transcripts covering this region were detected (Fig. 2 and Fig. 3). The longest mRNA (2.8 kb) covered the entire region (*orf23* to *orf26*), while the 1.5-kb transcript covered *orf23* to *orf25*, and the smallest (1.0 kb) covered the 3'



TABLE 1. ORFs examined in this study<sup>a</sup>

ORF	Size (aa)	Position		Size (kDa)	pI	RBS <sup>b</sup>	Putative function	Best match (extent, % aa identity)	Size (aa)
		Start	Stop			AGAAAGGAGGT-----ATG			
1	73	587	808	8.5	9.5	<u>AGAAAGGATA</u> AgataaATG	Cro/CI-like	<i>S. aureus</i> phage 29, ORF49 (26/66, 39)	75
2	102	852	1160	12.1	9.7	<u>TAAAAGGAGAA</u> aacaATG		No significant similarity found	
3	44	1190	1324	5.2	4.9	<u>CAAATGGAGGT</u> tctaATG	Transcription regulator	<i>L. lactis</i> phage ul36, ORF61a (41/44, 93)	61
4	131	1466	1861	14.9	8.0	<u>AGAAACCGGAG</u> AacattATG		<i>L. lactis</i> phage ul36, ORF131 (128/131, 97)	131
5	202	1872	2480	22.9	6.7	<u>ATGCGTGAGGT</u> tcgataaATG	Erf; recombinase protein	<i>L. lactis</i> SK11, Llac01001869 (196/202, 97)	202
6	116	2480	2830	13.0	5.1	<u>AACAAAGGAGA</u> AaaaataATG	SSB	<i>L. lactis</i> phage skl (936 species), ORF34 (56/119, 47)	119
7	269	2964	3773	31.4	9.1	<u>AGAAAGGAGA</u> AagTtTG	Replication protein	<i>L. lactis</i> phage BK5-T, ORF49 (253/268, 94)	269
8	73	3754	3975	8.5	5.0	<u>GAAGTGGAGG</u> CtttgataaATG		<i>L. lactis</i> phage BK5-T, ORF50 (70/73, 95)	73
9	129	3965	4354	15.7	9.8	<u>TATATCGAGA</u> AgcgaATG	RusA resolvase	<i>L. lactis</i> phage BK5-T, ORF51 (127/129, 98)	133
10	101	4359	4664	12.2	4.5	<u>ATTACAGAGG</u> TataataaATG		<i>L. lactis</i> phage bIL170 (936 species), E18 (61/80, 76)	83
11	79	4661	4897	9.4	9.3	<u>CTGAAGGAGAT</u> tttATG		<i>L. lactis</i> prophage bIL286, ORF21 (71/79, 89)	79
12	102	4916	5224	11.8	5.9	<u>AGAAATGGAT</u> AattaacATG		No significant similarity found	
13	59	5323	5502	6.8	9.1	<u>AAATATGAGG</u> TgctaaatATG		<i>L. lactis</i> phage P270, ORF17 (59/59, 100)	59
14	189	5492	6061	22.3	9.7	<u>AATTAGGAGG</u> AaaaaATG		<i>L. lactis</i> phage P270, ORF18 (184/185, 99)	186
15	131	6072	6467	15.6	4.7	<u>TTATAGGAGC</u> AgctagATG		<i>L. lactis</i> phage Tuc2009, ORF23 (122/131, 93)	131
16	190	6460	7032	22.3	5.2	<u>AGAGAGGAC</u> ACgaaaaATG		<i>L. lactis</i> phage LC3, ORF190 (172/188, 91)	190
17	139	7029	7448	15.4	7.0	<u>CCTGTGGAGG</u> AagaaaaATG	dUTPase	<i>L. lactis</i> phage TP901-1, ORF21 (172/188, 91)	139
18	176	7467	7997	19.9	9.4	<u>AGAAAGCTAG</u> TaaATG	HNH	<i>Acanthamoeba polyphaga</i> mimivirus (37/89, 41)	330
19	165	8000	8497	19.1	4.9	<u>ACTGGGGAGG</u> TtgaaaaATG		<i>L. lactis</i> prophage bIL285, ORF29 (162/165, 98)	165
20	81	8519	8764	8.9	5.6	<u>AAAGTGGT</u> GGAggATG		No significant similarity found	
21	31	8940	9035	3.7	4.5	<u>CAACTGGAGG</u> GaaaaatgaaATG		No significant similarity found	
22	140	9112	9534	16.7	6.4	<u>CAAAAAGGAGA</u> AtttgaaATG	ALT	<i>L. lactis</i> prophage bIL309, ORF36 (138/140, 98)	140
23	174	9959	10483	19.8	4.3	<u>ATTATGGAGG</u> AaaaATG		<i>S. pyogenes</i> MGAS8232, spyM18 1784 (101/174, 58)	172
24	225	10462	11139	25.5	5.8	<u>GTAGAGGAGG</u> GatatacATG	ABC transporter	<i>S. pyogenes</i> MGAS6180 (152/225, 67)	225
25	126	11136	11516	14.5	10.0	none		<i>S. pyogenes</i> MGAS8232, spyM18 1782 (46/88, 52)	89
26	236	11628	12338	28.1	9.5	<u>AGAAAGCGGA</u> AtttaaaATG		<i>E. faecium</i> EfaeDRAFT_2651 (125/229, 54)	230
27	162	12500	12988	18.9	9.1	<u>TGAAAGGAGA</u> AaataagATG	Small terminase	<i>L. lactis</i> phage Tuc2009, ORF30 (61/161, 37)	153
28	462	12978	14369	53.2	6.3	<u>AGAAAGTGG</u> CgagtcATG	Large terminase	<i>L. lactis</i> phage ul36, ORF462 (457/463, 98)	462
29	452	14370	15728	51.8	4.6	<u>TCAAAGGAGG</u> AttctaatTTG	Portal protein	<i>L. lactis</i> phage TP901-1, ORF32 (438/452, 96)	452
30	347	15732	16775	39.7	9.0	<u>AAGAATGGAG</u> AataacattATG	Minor capsid protein	<i>L. lactis</i> phage ul36, ORF346 (331/345, 95)	346
31	220	16890	17552	24.5	4.6	<u>GAGTAGGAGG</u> AaccagaaATG	Scaffolding protein, Sfp	<i>L. lactis</i> phage TP901-1, ORF35, Sfp (217/220, 98)	220
32	272	17554	18372	28.8	5.4	<u>TAACAGGAGG</u> CtattaaaATG	Major capsid protein	<i>L. lactis</i> phage TP901-1, ORF36, Mhp (266/272, 97)	272
33	66	18372	18572	7.7	8.9	<u>CATTTACGGG</u> TgtataATG		<i>L. lactis</i> phage Tuc2009, ORF40 (65/66, 98)	66
34	110	18556	18888	12.9	4.8	<u>AATTAGGAGT</u> TgcttATG	Structural protein	<i>L. lactis</i> phage Tuc2009, ORF41 (108/110, 98)	110
35	103	18885	19196	12.1	5.1	<u>GATAGGGAGG</u> T(10)TTG	Structural protein	<i>L. lactis</i> phage Tuc2009, ORF42 (97/102, 95)	102
36	119	19193	19552	13.4	10.0	<u>TTGCAGGAGG</u> TcaatcaATG	Structural protein	<i>L. lactis</i> phage Tuc2009, ORF43 (70/119, 58)	108
37	129	19549	19938	14.9	7.0	<u>CTAAAGGAGT</u> TgacgcaaATG	Structural protein	<i>L. lactis</i> phage Tuc2009, ORF44 (124/129, 96)	129
38	165	19949	20446	18.3	4.8	<u>AGATAGGAGT</u> TttaaatATG	Major tail protein	<i>L. lactis</i> phage ul36, ORF165a (155/165, 93)	165
39	116	20562	20912	13.4	5.0	<u>TTTAAAGGAG</u> AaatcaaaATG		<i>L. lactis</i> phage ul36, ORF116 (114/116, 98)	116
40	89	20996	21265	10.7	9.6	<u>AGAGATATGA</u> AataaaATG		<i>L. lactis</i> phage ul36, ORF89B (89/89, 100)	89
41	737	21281	23494	78.4	9.1	<u>AGAAAGGAGG</u> AaaaacATG	Tape measure protein	<i>L. lactis</i> phage ul36, ORF737 730/737, 99)	737
42	253	23504	24265	29.1	5.9	<u>TTTTAGCAGG</u> TtagaaATG	Distal tail protein, Dit	<i>L. lactis</i> phage Tuc2009, ORF49 (244/253, 96)	253
43	918	24265	27021	102.3	5.6	<u>AGAAAGCGG</u> TactttaATG	Tail-associated lysin	<i>L. lactis</i> phage ul36, ORF908 (870/909, 95)	908
44	322	27034	28002	36.4	5.1	<u>AGAAAGGTC</u> TattATG	Base plate protein, BppU	<i>L. lactis</i> phage ul36, ORF322 (319/322, 99)	322
45	277	27951	28786	31.0	5.3	<u>CTAATCTAAT</u> TgtttATG	Base plate protein, BppA	<i>L. lactis</i> phage ul36, ORF303 (177/312, 56)	303
46	160	28806	29288	17.2	5.9	<u>AAATAGGAGA</u> AattaaATG	RBP, BppL	<i>L. lactis</i> phage P475 (936 species) (103/179, 57)	269
47	74	29371	29595	8.7	5.4	<u>AGAAAGT</u> AGGggtATG		<i>L. lactis</i> prophage bIL285, ORF60 (71/74, 95)	74
48	658	29607	31583	75.0	4.8	<u>TTAAAGGAGT</u> AtacaATG	NPS	<i>L. lactis</i> phage Tuc2009, ORF55 (243/256, 94)	676
49	88	31811	32077	9.6	9.2	<u>AAATAGGAG</u> GtcaaaATG	Holin, LysA	<i>L. lactis</i> phage LC3, LysA (87/88, 98)	88
50	431	32074	33369	46.3	7.9	<u>AACTCAGAGG</u> AaaacaaATG	Endolysin, LysB	<i>L. lactis</i> phage ul36, ORF429 (367/431, 85)	429

<sup>a</sup> SSB, single-strand binding protein; ALT, activator of late transcription. ORFs in boldface are proteins found in the structure of the phage (Fig. 2). aa, amino acids.

<sup>b</sup> Underlining indicates nucleotides identical to the RBS consensus; lowercase indicates spacer nucleotides between the RBS and the start codon.

end of *orf25* and *orf26*. Putative promoters were identified at the beginning of each transcript and putative factor-independent terminators were identified at the end of the transcripts (data not shown), indicating that these four genes (*orf23* to *orf26*) were autonomously expressed.

**Late expressed genes and structural proteins.** This region most probably encompass 22 ORFs (*orf27* to *orf48*). Functions were assigned to 16 deduced ORFs, and most of them corresponded to structural phage proteins. This genomic region

contained several genes that were highly conserved in a group of three other studied P335-like phages, namely, ul36 (28), TP901-1 (11), and Tuc2009 (51). A BLAST analysis also revealed homology between the virulent P335 phage and two prophages (SK11-2 and SK11-3) in the *L. lactis* SK11 genome (66) (Fig. 2). The gene products of *orf27* to *orf29* are probably involved in packaging of the phage genome into the phage head (Table 1).

To confirm which ORFs are coding for structural proteins, a

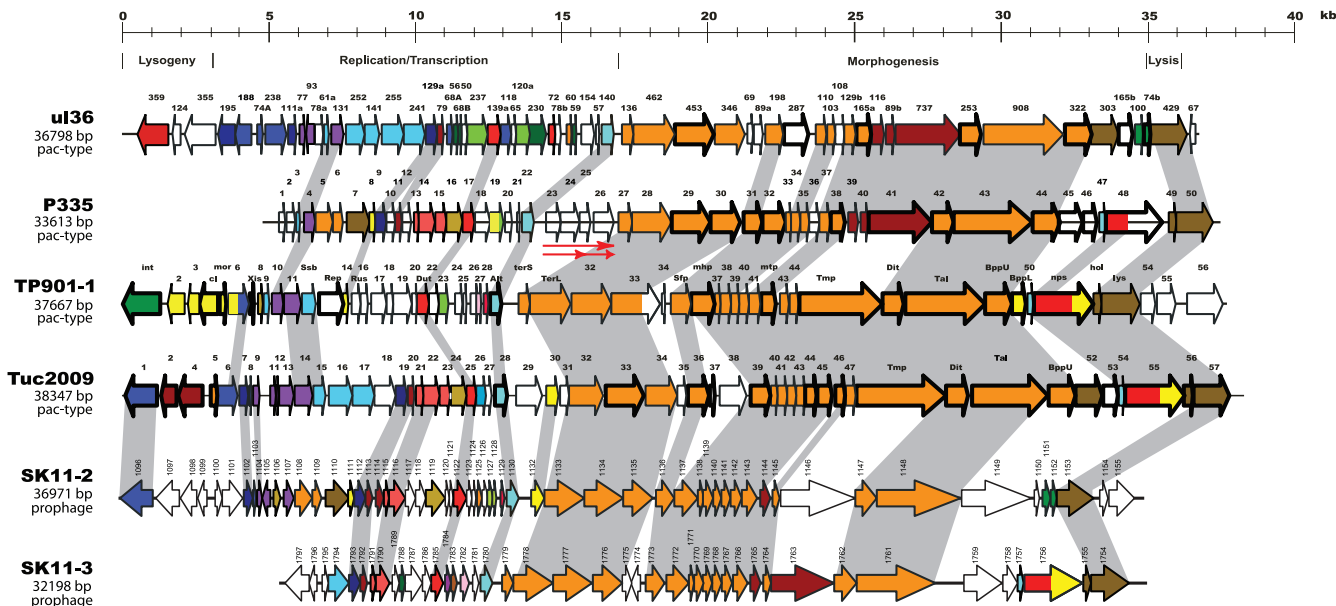


FIG. 2. Alignment of the genomes of six completely sequenced P335 phages (updated from Deveau et al. [18]). Regions of deduced amino acid identity exceeding 60% are represented by the same colors and are linked by gray shading when possible. ORFs with unique sequences are displayed in white. Red arrows under the genome of P335 represent the transcripts detected by Northern analysis. Proteins that were characterized in greater detail are represented by thick lines. A more complete figure with 12 genomes is available in the supplemental material.

purified phage sample was separated by SDS-PAGE and Coomassie blue-detected protein bands were analyzed by LC-MS/MS. Sixteen proteins were identified as structural proteins (Table 1 and Fig. 4). Most of the molecular masses estimated by SDS-PAGE were in line with the masses calculated from their sequences. The exceptions are discussed below.

**Capsid proteins.** The capsids are most probably encoded by *orf29* to *orf37*, with ORF33 apparently not being a structural protein. The ORF29 is probably the portal protein, since it is highly similar to the putative portal protein (ORF32) of TP901-1 (Table 1 and Fig. 4, band D) (11). The minor capsid protein encoded by *orf30* possessed a conserved domain (TIGR01641) found also in protein F of coliphage Mu (PF04133) and in GP7 of *Bacillus subtilis* phage SPP1. The GP7 protein was present in the capsid of SPP1 as a minor

constituent (4). The major capsid protein (ORF32) of phage P335 was the most abundant protein on the SDS-PAGE gel (Fig. 4) and also shared a high level of identity with the verified major capsid protein of TP901-1 (23). Interestingly, ORF32 was found in two bands (Fig. 4, bands G and K). Peptide analysis of the protein found in band K indicated that the N-terminal of the MCP was truncated. Processing of the MCP was previously observed for the P335-like phage ul36 (28).

**Putative tail structure proteins.** The tail/baseplate gene module most likely encompasses *orf38* through *orf48*. The *orf38* encoded the major tail protein (MTP), which shared 93% amino acid identity with the MTP of phage Tuc2009. Mc Grath et al. demonstrated that the tail shaft of Tuc2009 is mainly composed of MTP units (41). Based on the positions on their corresponding genes in the genome of P335, ORF39 and ORF40 were likely homologs of phage  $\lambda$  proteins gpG and gpT, respectively (32). Both are required for tail assembly, but neither was part of the mature tail structure. Accordingly, we could not find these two proteins in the structure of P335. In  $\lambda$ , a slippery sequence at the end of the gene G caused some ribosomes to slip back one nucleotide, leading to a gpG/gpT translational fusion product (32). A putative slippery sequence (5'-CGAAGAGTTAAAAAAGTCGGAATTT-3', coordinates 20834 to 20858; slippery sequence underlined) was also identified in the P335 phage genome, which could cause a frame-shift leading to an ORF39/ORF40 fusion protein. The ORF41 likely corresponded to the tape measure protein (TMP). The amino acid sequence of ORF41 shared 99% identity with ORF737 of phage ul36. The ORF41 is significantly shorter than the corresponding TMP in TP901-1 and Tuc2009 (47), which is in agreement with the shorter tail of P335 (Fig. 1). Peptide analysis suggested that ORF41 was processed because peptides were only identified in the N-terminal part. A pro-

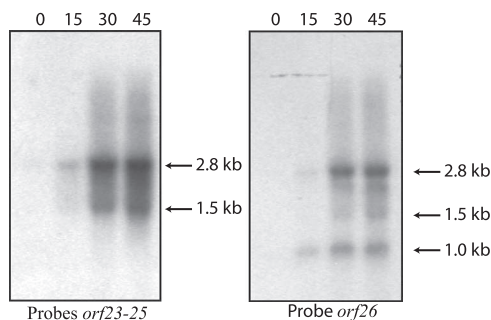


FIG. 3. Northern blot of the total RNA from P335-infected *L. lactis* cells at different times after the infection. Membranes were blotted by using four different radiolabeled oligonucleotide probes specific to each gene in the insertion module (*orf23* to *orf26*). The estimated size of the observed transcripts is indicated on the right side. Identical results were obtained with the three probes *orf23*, *orf24*, and *orf25*.

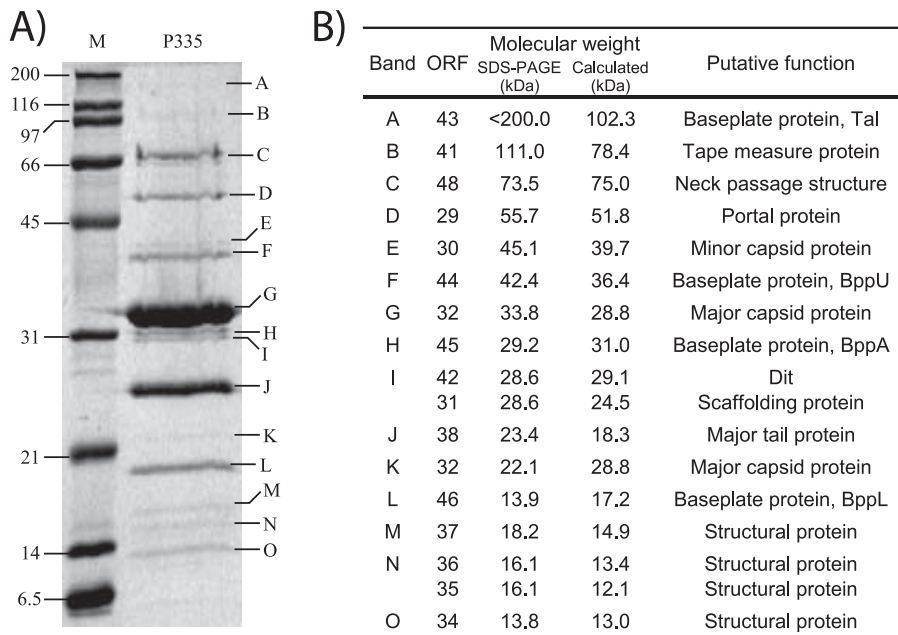


FIG. 4. LC-MS/MS analysis of structural proteins of phage P335. (A) Coomassie blue staining of a SDS–12% PAGE gel of the structural proteins of P335. Letters on the right side indicate the band analyzed and identified by LC-MS/MS. The numbers on the left indicate the size of the broad-range mass standard (M). (B) Identification and characteristic of the protein shown in panel A.

cessed TMP was also observed in the phage Tuc2009 and TP901-1 (41, 61). It has been also shown that the C-terminal portion of the TMP (gpH) of phage  $\lambda$  was cleaved prior to the joining of the capsid and tail structures (59).

ORF42 of phage P335 shared 96% identity with the distal tail protein (Dit) of TP901-1 (61), while ORF43 shared 90% identity with the Tal protein of Tuc2009, which had cell wall-degrading activity (27). In contrast to the Tal of phage Tuc2009, the complete ORF43 was found only in one band in the SDS-PAGE gel (band A). However, a Tal self-cleavage site (GGNSGGG) identical to the Tal<sub>TP901-1</sub> and closely related to the verified Tal<sub>Tuc2009</sub> cleavage site (GGSSG ↓ GG) is found in P335 (27, 61). In TP901-1 the cleavage product is a minor protein located just below the NPS protein that was only seen in silver-stained gels or by Western blotting (61).

*orf44*, which was highly conserved in TP901-1, Tuc2009, ul36, SK11-2, and SK11-3, encodes the putative upper baseplate protein (BppU). On the other hand, *orf45* and *orf46* were much less conserved. The *orf45* encodes the baseplate-associated protein (BppA), which was identified in phage Tuc2009 (41) but absent in phage TP901-1 (50, 63). The *orf46* encodes the lower baseplate protein (BppL), which was the least conserved protein in the tail of phage P335. This is in line with its distinct host range of phage P335 because this protein is likely responsible for the host specificity (63). The structure of the homologous BppL protein in phage TP901-1 was recently resolved (52) and found to contain three domains: shoulders, neck, and head. The C-terminal head domain directly interacts with the host cell receptor, while the shoulder domain interacts with the other baseplate proteins. Interestingly, the C terminus of ORF46 showed homology to the receptor-binding protein (RBP) of the virulent lactococcal phage P475 of the 936 species (Table 1). Finally, ORF48 was the NPS protein and

showed high homology to the N-terminal part of NPS from Tuc2009 and TP901-1 (41, 62).

**The lysis module.** The lysis module of P335 was composed of two genes that encode the holin (*orf49-lysA*) and the endolysin (*orf50-lysB*). ORF49 had a conserved domain (PF04531) that corresponded to class II phage holin (65) with two transmembrane spanning motifs and an N-terminal signal peptide. It was previously observed that P335-like phages possessed a class II holin (29). ORF50 possesses two domains that are well conserved among *Siphoviridae* phages. The N terminus contains the muramidase domain (glycoside hydrolase [PF01183]), while the C terminus contains the cell wall-binding LysM (PF01476, endolysin) domain.

## DISCUSSION

In this study, we report an international effort to characterize a lactococcal phage of historical and practical importance in order to shed light on its genome, proteome, and morphology. Comparative analyses of *L. lactis* phages will inevitably provide critical information on their diversity and their means of evolution, which should help develop improved phage control strategies in the dairy industry. In the past decade, considerable progress has been made in the genomics of *L. lactis* phages. These analyses have indicated significant intraspecies homogeneity in the widespread 936 and c2 species, while the P335 species exemplified the polythetic species concept (18). Studies on rare lactococcal phages have also suggested that they are most likely the result of genetic exchanges between phages and bacteria from diverse environments (15, 21, 22). On the other hand, the diversification of any given phage genome is driven by the accumulation of point mutations, gene disruption, and recombination events (3).



Comparative analyses of the genome of the type phage P335 with those of phages Tuc2009, TP901-1, and ul36 (Fig. 2) certainly illustrate the accumulation of point mutations, as well as the successful recombination events between various phage genomes. These comparisons may be pointing toward the emergence of subgroups within this diverse group of lactococcal phages. For example, the morphogenesis module of phages P335, Tuc2009, TP901-1, ul36, SK11-2, and SK11-3 are highly related, and they are only distantly connected to the other characterized P335 phages (Fig. 2 and see also the supplemental material). A similar subgroup could be made with phages BK5-T, 4268, and bIL286, as well as for phages r1t and  $\phi$ LC3 (see reference 18 and the supplemental material). In contrast, such grouping cannot be made based on the early expressed gene modules since they share homology with genes from an array of phages from other species. However, P335-like phages are prone to genomic reshuffling and can extensively evolve by large-scale recombination events with virulent phages or prophages (30). Thus, from a practical point of view, it would probably be better to keep P335-like phages in a loosely defined group such as the polythetic P335 species.

The analysis of the structural proteome of phage P335 confirmed the relatedness of the morphogenesis module with other lactococcal phages of the P335 species. It is notable that the tail length of phage P335 (130 nm) is significantly shorter than the well-studied P335-like phages TP901-1 and Tuc2009 (160 nm) and the P335-like phages r1t and  $\phi$ LC3 (140 nm). The use of sonication (to disrupt phage particles and DNA) prior to loading the concentrated phage sample on the SDS-PAGE gel led to the identification of 16 structural proteins of P335. To our knowledge, this represents the highest number of structural proteins identified for a lactococcal phage. One protein of particular interest in the structure of P335 was the lower baseplate protein (BppL), which is also the RBP (53, 57). Although the shoulder and neck domains of this protein were conserved among other P335-like phages, the C-terminal portion (head domain) is unique to the virulent phage P335. It is interesting that the head domain of BppL shows homology to the putative RBP of the virulent phage P475 of the 936 species (19). P335 was shown to infect a unique set of *L. lactis* strains, and it included two strains that were also sensitive to P475, namely, *L. lactis* strains IL1403 and 455. This suggests that exchange of head domains of RBP between lactococcal phage species may be a way to quickly adapt to frequent host changes in the dairy environment. The unique BppL/RBP of phage P335 was also in line with our transmission electron microscopic analysis. A sixfold symmetry has been suggested for the BppL structures of phages TP901-1 and Tuc2009 (50). Apparently, the symmetry of the P335 BppL structure is more complex, since approximately 12 fibrils and up to 18 globular structures could be counted in P335 micrographs. The different shapes of these structures do also indicate that the BppL structures are prone to conformational changes. These micrographs also illustrated the presence of two lower baseplate proteins (likely BppL and BppA), as supported by the sequence analysis. This suggested that not only BppL but also BppA were exposed at the bottom side of the P335 baseplate and may also interact with receptor components of the lactococcal host cells.

The nature of the singular spike-like fiber structure under the baseplate of the P335-like phages TP901-1 and Tuc2009

has recently been identified as the tail associated lysin (Tal) (61). In fact, the length (approximately 10 nm) of the protruding fiber was similar for these two phages and for phage P335.

Phage P335 also possesses a unique neck passage structural protein. Although its collar diameter is similar to the collar size of phages TP901-1 and Tuc2009 (15 to 16 nm), the P335 whiskers are significantly shorter (half the length of the TP901-1 and Tuc2009 whiskers). Although the TP901-1 and Tuc2009 whiskers exhibit tiny distal knobs, the distal globular substructures of phage P335 are significantly larger. This result is in accordance with the sequence analysis because the C-terminal part of the NPS<sub>P335</sub> is not homologous to the Tuc2009 and TP901-1 counterparts.

One of the most fascinating findings of this study was the discovery of moron genes in the genome of phage P335 that appears to have been acquired from other gram-positive bacteria. Obviously, it is also possible that these bacterial strains have acquired this piece of DNA from invading phages. These autonomously transcribed four genes were inserted between the genes coding for the activator of late transcription (*alt*) and the small terminase subunit (*terS*) of phage P335. They are probably transcribed under control of ALT<sub>P335</sub>, since four ALT-boxes identical to those found in TP901-1 (46) are located just upstream of *orf23*. Interestingly, phages bIL309, BK5-T, bIL286, and r1t possess a gene coding for a putative endonuclease between their respective *alt* and *terS* genes. These genes are also homologous to sequences found in the genomes of *Enterococcus faecalis*, *Streptococcus pyogenes*, and *Listeria innocua* prophages. It is tempting to speculate that the intergenic region between the *alt* and *terS* genes may be a hotspot for the acquisition of new DNA to perhaps provide fitness factors to virulent phage or to the bacterial strain (in the case of a prophage).

In summary, the present study revealed many similarities between the type phage P335 and other phages of the P335 species that have been studied in greater detail. Therefore, the lactococcal phages Tuc2009, TP901-1, and ul36 represent excellent models to study this group of dairy-adapted phages. Understanding how *L. lactis* phages evolve and acquire new host recognition domains certainly holds the key to reveal novel phage control strategies.

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