Novel Phage Group Infecting *Lactobacillus delbrueckii* subsp. *lactis*, as Revealed by Genomic and Proteomic Analysis of Bacteriophage Ldl1

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Ldl1 is a virulent phage infecting the dairy starter *Lactobacillus delbrueckii* subsp. *lactis*. Electron microscopy analysis revealed that this phage exhibits a large head and a long tail and bears little resemblance to other characterized phages infecting *Lactobacillus delbrueckii*. In vitro propagation of this phage revealed a latent period of 30 to 40 min and a burst size of 59.9 ± 1.9 phage particles. Comparative genomic and proteomic analyses showed remarkable similarity between the genome of Ldl1 and that of *Lactobacillus plantarum* phage ATCC 8014-B2. The genomic and proteomic characteristics of Ldl1 demonstrate that this phage does not belong to any of the four previously recognized *L. delbrueckii* phage groups, necessitating the creation of a new group, called group e, thus adding to the knowledge on the diversity of phages targeting strains of this industrially important lactic acid bacterial species.

*Lactobacillus delbrueckii* subsp. *lactis* is a member of the lactic acid bacteria (LAB) and is commonly used in the production of commercial fermented milk products, such as Emmental-like cheeses, where it is employed as a starter culture contributing to the acidification and the organoleptic properties of the final product (1, 2). Bacteriophage (or phage) infection of these starter strains represents a major hurdle to their technological activity, as this may cause lysis of the starter, which in turn may lead to reduced acidification activity and a poor(er)-quality product, with a consequent negative economic impact (3).

The commercial importance of *L. delbrueckii* fermentations has catalyzed extensive research into the occurrence, diversity, and impact of its infecting phages. Currently, *L. delbrueckii* phages are organized into four distinct groups (designated groups a, b, c, and d), classified based on DNA homology by hybridization (4–6) and, more recently, by comparative genome analysis (7). *L. delbrueckii* phages belonging to groups a and c have enjoyed considerable scientific scrutiny, with phage LL-H representing a prototype *Lactobacillus* phage. LL-H was originally isolated in 1972 (8), and its full genome sequence and transcriptional map, which revealed two distinct phases of transcription, have been determined (9). Furthermore, research on phage-host interactions, culminating in the identification of gp71 as the receptor-binding protein of LL-H (10) and lipoteichoic acids as the recognized receptor, have significantly advanced our understanding of this phage and the way in which it recognizes its host (11, 12). Also, the prolate, head-tail, temperate phage JCL1032, being a member of group c *L. delbrueckii* phages, has been subjected to considerable scientific characterization, including genome sequencing (13), analysis of genomic integration (14), and identification of lipoteichoic acid as its receptor (12).

Due to their apparent increased frequency of isolation, research has recently focused on group b phages, facilitated by the sequencing of six phages, c5 and LL-Ku (13); Ld3, Ld17, and Ld25A (7); and phILdb (15). Ld17 is the most comprehensively studied group b phage, with a defined transcriptome consisting of two transcripts (E1 and L1), while structural proteins were identified not only for this phage but also for group b phages Ld3 and Ld25A (7). Finally, group d phages remain significantly understudied, being represented by just a single isolate, the prolate-headed phage 0252, which infects *L. delbrueckii* subsp. *lactis* (6).

Here we present the complete genome sequence of Ldl1, a novel phage infecting *L. delbrueckii* subsp. *lactis*, isolated in 2010 from a cheese manufacturing facility in Switzerland and representing a fifth, possibly newly emerging group of bacteriophages infecting *L. delbrueckii*.

**MATERIALS AND METHODS**

**Bacteriophages, bacterial strains, and growth conditions.** Phage Ldl1 was isolated from a Swiss whey sample by employing an Emmental-specific starter culture manufactured by Sacco s.r.l. in 2010. *L. delbrueckii* subsp. *lactis* strain Ld1S was used for the isolation, propagation, and enumeration of phage Ldl1. Both phage and host were provided by Sacco s.r.l. The strain was grown overnight at 42°C in MRS-LCT (MRS broth [Oxoid] supplemented with 1% lactose, 20 mM CaCl₂, and 0.5% tryptone). Phage propagation was performed by picking an individual plaque and infecting a host at an optical density at 600 nm (OD₆₀₀) of ~0.15 in MRS-LCT, followed by incubation at 42°C until lysis occurred. The resulting lysate was passed through a 0.45-μm filter to remove cell debris. Phage enumerations, expressed as PFU ml⁻¹, were carried out by employing the double-layer plaque assay method with single-plaque isolates (16), using MRS-LCT agar supplemented with 2% glucose (17).

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Electron microscopic analysis. Bacteriophage lysates were purified on a continuous cesium chloride gradient and were dialyzed three times against TBT buffer (20 mM Tris [pH 7.2], 10 mM NaCl, 20 mM MgSO4). Staining was performed with 2% (wt/vol) uranyl acetate on freshly prepared carbon films. Grids were analyzed with 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 (Soft Imaging Solutions) at the Max Rubner Institute, Kiel, Germany (18).

One-step growth curve. A one-step growth experiment was performed to ascertain the burst size and latent, rise, and eclipse phases of Ldl1 by using a modification of the one-step growth curve protocol of Lu et al. (19). Briefly, L. delbrueckii subsp. lactis strain Ldl1 was grown in 50 ml of MRS-LCT to an OD600 of ~0.15. Cells were then harvested at 5,580 × g and resuspended in 500 µl of MRS-LCT. Five hundred micro liters of Ldl1 lysate was added to produce a multiplicity of infection (MOI) of ~0.001, and cells were incubated at 42°C for 5 min, followed by centrifugation at 18,000 × g for 30 s, removing unadsorbed phage and thus ensuring a synchronous infection. The resultant pellet was resuspended in 50 ml of MRS-LCT and incubated at 42°C. This step represents time zero (T0), where phage population monitoring was initiated by the removal of 100 µl every 10 min and centrifugation at 18,000 × g for 30 s. Phages in the supernatant were then enumerated by employing the double-layer titration technique mentioned above (16). The burst size was calculated by using the following formula, where “titer after burst” is the phage titer after the initial burst (50 min postinfection here) and “phage added” is the phage titer added before adsorption (1.78 × 106 PFU/ml): burst size = (titer after burst − titer at T0)/(phage added − titer at T0).

Genome sequencing. Five micrograms of DNA of Ldl1, as determined by Nanodrop quantification, was isolated from fresh CsCl-purified lysates according to a previously described method (20), prior to shipment to the contract sequencing facility (Macrogen Inc., South Korea). Sequencing of the genomes was conducted by using a GS-FLXTitanium sequencer, yielding a 109-fold coverage of the phage genome. The reads generated by the 454 FLX instrument were assembled with GSAssembler (454 Life Sciences, Branford, CT, USA) to generate a consensus sequence. Quality improvement of the genome sequence involved sequencing of PCR products across the entire genome to ensure correct assembly and double strandedness and the resolution of any remaining base conflicts occurring within homopolymer tracts.

In silico analysis. Protein-encoding open reading frames (ORFs) were predicted by using GeneMark (21). ORFs with an AUG, UUG, or GUG start codon, encoding at least 30 amino acids (aa), and preceded by a sequence resembling the consensus Shine-Dalgarno sequence (5′-AGG AGG-3′) (22) were accepted. Initial functional annotation of the ORFs and percent amino acid identity between the deduced proteome of Ldl1 and its nearest relative were determined by using BLASTP (23), and functions were further confirmed by querying the PFAM (24) and NCBI Conserved Domain Database (25) protein domain databases and by performing homology prediction searches using HHPred (26). Phage genome maps were built with CloneManager Suite 7 (Scientific & Educational Software, Morrisville, NC, USA), with identity scores being added between the ORFs in Adobe Illustrator v15.0.0 (Adobe, San Jose, CA, USA). The tRNAscan-SE Search server (27) was used to search for putative tRNAs.

Phage structural proteome and mass spectrometry. Purified phage proteins were extracted from CsCl-purified Ldl1 phage particles by performing a single methanol-chloroform extraction (1:10.75, vol/vol) and subsequently precipitated by the addition of an equal volume of methanol. Proteins were pelleted by centrifugation at 20,800 × g for 6 min and resuspended in 100 µl TBT buffer. The structural protein profile was generated by standard Tris-glycine sodium dodecyl sulfate (SDS)−12% polyacrylamide gel electrophoresis (PAGE). Gel slices were then excised, trypsinized, and analyzed by using electrospray ionization tandem mass spectrometry (ESI-MS/MS), as previously described (28).

Lactobacillus phage proteomic tree. To gain an understanding of the evolutionary relationship between Ldl1 and phages of other Lactobacillus species, a phylogenetic tree was constructed. The genomes of all available Lactobacillus phages were downloaded from the NCBI database and arranged as previously described (29). Briefly, all encoded proteins were extracted and concatenated beginning with the terminase, with all proteins following the same gene order as that of the phage genome. The resultant concatamers were then aligned by using ClustalW (30) and a BLOSUM matrix using MEGA 5.10 (31). A phylogenetic tree was constructed by using the neighbor-joining method, using the number-of-differences method. The phylogeny was then tested by using bootstrap assessment based on 2,000 replicates. Nucleotide sequence accession number. The complete sequence data for Ldl1 are available in the GenBank database under accession number KM514685.

RESULTS AND DISCUSSION

Morphological analysis of Ldl1. Ldl1 possesses a long noncontractile tail (399 ± 11 nm; n = 20) and a large head (73 ± 2 nm; n = 15) with a defined baseplate structure (Fig. 1). This phage morphology is unique among phages infecting L. delbrueckii, as the tail is significantly longer than those of other reported typical L. delbrueckii phages, which range from 116 to 290 nm (32). In fact, among phages that infect Lactobacillus species, the Ldl1 tail length is second only to that of L. plantarum phage B2, which was
isolated in 1971 and which was originally reported to display a tail length of 500 nm (33). However, a recent study found the tail length of this phage to be 240 \text{nm} (34). Also evident is a minihead variant of Ldl1, exhibiting a much smaller head with the long tail still present. Such a variant could represent a phage particle where the head has not been filled with DNA, thus preventing the expansion of the prohead following the DNA packaging process, as previously observed for bacteriophage T4 (35). Alternatively, these minihead variants may represent naturally occurring mutant phages similar to those observed for T4, where mutations in the major capsid protein resulted in variants with both smaller and larger heads, which were found to be capable of infection (36).

**Phage population dynamics.** The population kinetics of Ldl1 was investigated by performing a one-step growth curve (see Materials and Methods). The latent period was found to be 30 to 40 min (Fig. 2). This is one of the shorter latent periods reported for a phage infecting *L. delbrueckii* subsp. *lactis*, being almost 40 min shorter than that observed for the group a phage LL-H (37). The burst size was determined to be 59.9 ± 1.9 (n = 3) phage particles, which is comparable to those of other *L. delbrueckii* subsp. *lactis* phages, such as YAB and lb3, with burst sizes of 48 and 27, respectively (38), but lower than the value observed for the group a phage LL-H, with a burst size ranging from 100 to 200 (36).

**Genome analysis.** Ldl1 genomic DNA was isolated and sequenced, revealing a genome of 74,806 bp with 79 putative ORFs (see Table S1 in the supplemental material), overtaking the group c phage JCL1032 (49,433 nucleotides [nt]) in having the largest genome of an *L. delbrueckii*-infecting phage. The genomes of *L. delbrueckii* phages typically display a GC content of 49.6 to 49.7% (39–41), considerably higher than that of the Ldl1 genome (37.8%), suggesting that some elements of the phage genome may have been acquired from other phages infecting hosts with a lower GC content or could have evolved from an ancestor that infected a host with a lower percent GC content. Ldl1 bears little resemblance to any other phage infecting *L. delbrueckii*. The nearest relative of Ldl1 appears to be *L. plantarum* phage ATCC 8014-B2 (here called B2) (34), with limited levels of homology in the structural region (Fig. 3) and several other conserved regions, as discussed further below. The Ldl1 genome is organized into the following functional modules, which are discussed in further detail below: DNA packaging, morphogenesis, and DNA replication and lysis (Fig. 3).

**Morphogenesis module.** The structural module of Ldl1 bears resemblance in its gene organization and deduced amino acid sequences of its encoded proteins to the corresponding properties of *L. plantarum* phage B2. This applies in particular to the genes that are predicted to encode the portal protein through to that specifying the major tail protein (Fig. 3), with percent identities outlined in Table S1 in the supplemental material.

Downstream of the gene encoding the major tail protein, the Ldl1 genome encompasses additional genes that specify putative structural proteins. The putative tail tape measure protein (TMP), which is specified by ORF23Ldl1 and which is known to determine the tail length of phages, is 2,627 aa long, with a predicted molecular mass of 288.2 kDa, corresponding to its unusually long tail (Fig. 1; see also Table S1 in the supplemental material). In its C terminus, this predicted TMP contains a number of notable domains, including a LysM domain, which is known to bind peptidoglycan (42), and a transglycosylase (lytic transglycosylase [LT]) domain, which catalyzes peptidoglycan cleavage. The presence of peptidoglycan-binding and -degrading domains indicates that it plays an important, probably multifunctional role (in addition to determining tail length) in the phage infection process, similar to those described for coliphage T5 and *Staphylococcus aureus* phage vb_SauS-philPLA35 (43), where, for example, the T5 protein Pb2 performs tail length

![FIG 2 One-step growth curve of the *L. delbrueckii* subsp. *lactis* phage performed with MRS-LCT at 42°C. Three replicates were performed to generate the presented data.](image-url)
repeated regions in the antireceptors of the group B phages (loosely repeated 10-residue sequence that shows homology to the (S/Q)LQQ], and from residues 606 to 767, with 9 copies of a 11-residue repeat are observed [consensus, NAEGN(V/I)S]
bly those from residues 161 to 255 and 412 to 526, where 11 copies of an 11-residue repeat are observed [consensus, NAEGN(V/I)S]
determination functions as well as possessing confirmed in vitro muralytic activity (44).

HHPred analysis of the protein specified by ORF24, located downstream of the TMP-encoding gene, reveals significant similarity to the distal tail protein (Dit) of Bacillus phage SPP1 (E value, 5.3E−23) and lactococcal phage TP901-1 (E value, 1.8E−20), and therefore, it is proposed to encode the central hub upon which the baseplate apparatus is assembled, as has been established for the Dit proteins of both the Bacillus and lactococcal phage models mentioned above.

HHPred analysis of the deduced protein product of ORF25, located downstream of the TMP-encoding gene, reveals significant similarity (E value, 6.5E−38) to various predicted phage tail/baseplate proteins known as "Gp27-like" proteins, which are exposed at the distal region of the baseplate (45). The second half of ORF25 exhibits similarity to several beta propel-

It is probable that the four ORFs downstream of the TMP-encoding genes specify the baseplate structure at the tail tip of Ldl1 (Fig. 1). In well-characterized bacteriophages infecting Gram-positive organisms, the baseplate-containing tail tip consists of a TMP, a distal tail protein (Dit), and a protein with enzymatic activity (Tal), which together form the so-called initiation complex, which is used as a scaffold for one or more receptor-binding proteins/adsorption proteins, which then form the baseplate (44, 49–52).

**Ldl1 structural proteome.** Many of the proteins encoded by genes within the structural module of Ldl1 were confirmed to be part of the structural proteome by mass spectrometric analysis, including the predicted portal protein, the major capsid protein, the head-tail-joining protein, the major tail protein, the distal tail protein, and the two putative adsorption proteins (Table 1). The TMP was absent from the data, possibly due to its low abundance and/or size (predicted molecular mass of 288.2 kDa) preventing its migration through the SDS-PAGE gel. The deduced product of ORF25, i.e., the putative Tal-like protein, was present in the mass spectrometric data, but with only one unique peptide and 1.6% coverage, it fell below our limits for inclusion (two unique peptides and/or 5% coverage). Also present in the data was the}

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<table>
<thead>
<tr>
<th>ORF</th>
<th>Function</th>
<th>No. of unique peptides</th>
<th>Coverage (%)</th>
</tr>
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<tbody>
<tr>
<td>11</td>
<td>Terminase small subunit</td>
<td>1</td>
<td>8.1</td>
</tr>
<tr>
<td>13</td>
<td>Portal</td>
<td>21</td>
<td>46.6</td>
</tr>
<tr>
<td>14</td>
<td>Prohead protease</td>
<td>9</td>
<td>14.8</td>
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<tr>
<td>15</td>
<td>Capsid</td>
<td>31</td>
<td>75.4</td>
</tr>
<tr>
<td>21</td>
<td>Major tail</td>
<td>7</td>
<td>38.1</td>
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<tr>
<td>24</td>
<td>Distal tail protein</td>
<td>4</td>
<td>5.4</td>
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<td>26</td>
<td>Adsorption protein</td>
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<td>5.3</td>
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<tr>
<td>27</td>
<td>Adsorption protein</td>
<td>4</td>
<td>4.6</td>
</tr>
<tr>
<td>56</td>
<td>ATP-binding cassette transporter</td>
<td>2</td>
<td>7.7</td>
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*Structural proteins extracted from purified phage particles were identified by ESI-MS/MS after separation on a 12% SDS-PAGE gel. A minimum of two independent unique peptides or 5% coverage was used as the threshold value.*
FIG 4 Proteomic tree of all Lactobacillus phages constructed by using the neighbor-joining method according to the number-of-differences model. The species that each phage on the tree is known to infect are indicated at the bottom.
ATP-binding cassette transporter, the deduced product of ORF50_{Ldl1}, although this protein is unlikely to be part of the structural proteome. It is possible that the gene encoding this function has been integrated into the Ldl1 genome from the host. Therefore, if this protein is expressed at a high level by the host, it is plausible that it could have been present in the lysate as contamination. The presence of the prehead protease in the structural proteome in group b phages Ld17 and Ld25A was observed previously (7) but may be explained by the protease remaining bound to the head after assembly.

**Lysogeny elements.** Ldl1 contains genetic elements associated with the lysogenic life-style, such as those encoding six putative nonidentical antirepressors (specified by ORFs 67, 69, 76, 77, 78, and 79) and a predicted recombinase/integrase (ORF35) showing 53% amino acid identity to the recombinase/integrase of B2. Despite the presence of these ORFs associated with the lysogenic life-style, Ldl1 does not appear to exhibit lysogenic characteristics, suggesting that it may have evolved from a lysogenic ancestor.

**Replication module.** The replication module of Ldl1 shares limited homology with a similarly annotated region of the *L. plantarum* phage B2 genome. Like B2, Ldl1 appears to encode its own DNA polymerase, which is specified by ORF37_{Ldl1} and which is similar to the deduced products of ORF45_{ATCC 8014-B2} and ORF47_{ATCC 8014-B2}. The latter two proteins in turn exhibit similarity to a DNA polymerase III protein (α subunit) from *Bacillus* phage SPB2 (34). ORF37_{Ldl1} also shows 41% identity to a putative DNA polymerase of *Bacillus subtilis* subsp. *natto* across the entire protein. ORF45_{ATCC 8014-B2} and ORF47_{ATCC 8014-B2} of B2 appear to be interrupted by a group I intron, which harbors ORF46_{ATCC 8014-B2}, thus indicating that the corresponding mRNA transcript will be processed to be translated as a single protein with very a high level of similarity to the product of ORF37_{Ldl1} across its full length. It is plausible to suggest that the phage-encoded polymerase is utilized for replication of the phage genome. The protein product of ORF66_{Ldl1} exhibits substantial similarity (37%) to that of ORF70_{ATCC 8014-B2}, which is probably a double-stranded DNA repair enzyme due to the presence of the Mre11 nuclease and an N-terminal metallophosphatase domain (42).

ORF46_{Ldl1} and ORF48_{Ldl1} appear to specify a primase and a helicase, respectively, and thus are presumed to have crucial functions in replication, while ORF41_{Ldl1} and ORF60_{Ldl1}, whose deduced products contain a thymidine kinase domain and a nucleoside 2-deoxyribosyltransferase domain, respectively, may play a role in nucleotide modification. The presence of a single tRNA gene in the Ldl1 genome at positions 66964 to 66893 is also noted, and this tRNA corresponds to the amino acid isoleucine (anticodon, UAU). Comparison of the usage of codon ATA in the genomes of Ldl1 and two *L. delbrueckii* strains (*L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365 and *L. delbrueckii* subsp. *bulgaricus* ATCC 11842) shows a frequency of 45.3% in the Ldl1 genome, compared to frequencies of 19.1 and 19.7% in the genomes of the other *L. delbrueckii* strains, respectively, suggesting that this tRNA may compensate for the relatively low abundance of the TAT anticodon in the host.

**Ldl1 necessitates the creation of a new group of *L. delbrueckii* infecting phages.** In order to classify Ldl1, a prototrophic tree was constructed for comparison with all previously sequenced *Lactobacillus* phages. The tree also provides valuable insights into the diversity of *L. delbrueckii* phages and also provides some interesting clusters of phages that infect different species of *Lactobacillus*. Particularly evident in the tree is the relationship between Ldl1 and B2, where Ldl1 is shown to be more closely related to *L. plantarum* phages B2 and Sha1 than to any of the other previously sequenced *L. delbrueckii* phages. The previously established groups of phages infecting *L. delbrueckii* are evident in the tree (Fig. 4), with group a (indicated in red), group b (green), and group c (purple) occupying distinct branches on the tree, confirming their division into specific groups based on genetic distinction. This leads to the proposal of a new group (group e) of phages (Fig. 4, blue) infecting *L. delbrueckii*, of which Ldl1 is, as yet, the only member. Also evident are the close phylogenetic relationships between phages infecting different species of *Lactobacillus*, particularly the relationship between phages infecting *Lactobacillus rhamnosus* and those infecting *Lactobacillus casei*. This is highlighted by *L. rhamnosus* phages Lrm1 and Ln-Nu sharing a clade with *L. casei* phages PL-1, J1, A2, and phiAT3 (Fig. 4). This observed relationship between these phages may be explained by a common temperate evolutionary origin due to their ability to infect these closely related host species (53). Furthermore, this finding suggests that the related phages Ldl1 and B2 may also have shared a common evolutionary host albeit more distant than the *L. casei* and *L. rhamnosus* phages.

**Conclusion.** In this study, we describe the isolation and characterization of a novel phage, Ldl1, infecting *L. delbrueckii* subsp. *lactis*.

The discovery of Ldl1 highlights the heterogeneity of phages infecting *L. delbrueckii* but also accentuates the crucial common elements that appear to be necessary for *L. delbrueckii* infection. It is clear that Ldl1 represents a new group of phages infecting *L. delbrueckii*, as the Ldl1 structural proteins and genome architecture exhibit remarkable similarity to those of a phage infecting another *Lactobacillus* species, *L. plantarum* phage B2. Interestingly, the baseplate region of Ldl1 is distinct from that of B2 and, as expected given its host preference, is more akin to the receptor-binding proteins of phages infecting *L. delbrueckii*. Two potential receptor-binding proteins show amino acid identity to those of *L. delbrueckii* phages LL-H and JCL1032, members of groups a and c, respectively, linking the three groups. The second putative receptor-binding protein shares homology with the previously noted (7, 13) repeat sequence in the antireceptors of group b phages, showing that there are common elements among all sequenced groups infecting *L. delbrueckii* in the receptor-binding regions, allowing them to infect strains of the same species.

The Ldl1 phage is one of the largest phages described for the genus *Lactobacillus* and is unlike any previously reported *L. delbrueckii* phage from both a morphological and a genomic perspective. This led us to propose the creation of a new group, group e, of which Ldl1 is currently the sole member. Thus, our discovery appears to be due to the recent emergence of a new phage group whose ancestor possibly originated from another (*Lactobacillus*) host yet has acquired characteristics to attack commercial *L. delbrueckii* starter strains.

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