Abortive infection mechanisms and prophage sequences significantly influence the genetic make-up of emerging lytic lactococcal phages

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ABSTRACT

In this study, we demonstrate the remarkable genome plasticity of lytic lactococcal phages allowing them to rapidly adapt to the dynamic dairy environment. The lytic double-stranded DNA phage ul36 was used to sequentially infect a wild-type strain of *Lactococcus lactis* and two isogenic derivatives encoding two phage resistance mechanisms, namely AbiK and AbiT. Four phage mutants resistant to one or both Abi mechanisms were isolated. Comparative analysis of their complete genomes as well as morphological observations revealed that phage ul36 extensively evolved by large-scale homologous and non-homologous recombinations with the inducible prophage present in the host strain. One phage mutant has exchanged as much as 79% of its genome as compared to the core genome of ul36. Thus, natural phage defence mechanisms and prophage elements found in bacterial chromosomes are significantly contributing to the evolution of the lytic phage population.

Bacteria and phages are linked by a long history of coevolution as prophage elements are found in the majority of the sequenced bacterial genomes (11). These prophages are not all functional as many appear to be defective or in a state of partial decay. However, genes on both intact and decaying prophage genomes can have important effects on the bacterial cell such as providing protection against phage infection or fitness factors that increase the selective advantage of the host in a particular niche (9). On the other hand, the diversification of a phage genome is driven by the accumulation of point mutations, gene disruption, and recombination (1). Owing to the latter, phages can significantly benefit from the acquisition of a genetic module from other phages or hosts (2). In fact, comparative analyses have concluded that phage genomes are composed of a mosaic of conserved modules (2) interspaced by non homologous sequences (12, 23, 25). It should be noted that our ideas on how bacteriophages evolved, particularly those with a double-stranded DNA (dsDNA) genome, are often inferred from bioinformatic analyses of the structure and sequence of phage genomes and not from direct observations of the evolution process.

The evolution of phages infecting the low G+C Gram-positive bacterium *Lactococcus lactis* is the subject of ongoing studies because of the economical value of the host strains in fermented dairy products as well as the frequent emergence of new virulent phages that are responsible for milk fermentations delays. Lactococcal phages have been recently reclassified into ten genetically-distinct groups of dsDNA and tail-containing phages (15). However, members of only three *L. lactis* phage groups (936, c2, and P335) are regularly isolated. While virulent members of the 936 and c2 species are rather homogeneous, their is a considerable genetic heterogeneity between members of the P335 group that contains both temperate and lytic phages (15).

One effective way to control lactococcal phages within the dairy processes is through the use, in rotation, of *L. lactis* strains harbouring phage defence mechanisms (8, 26). These mechanisms are divided based on their general mode of action: inhibition of phage adsorption, DNA ejection blocking, restriction/modification systems and the **ab**ortive **i**nfection mechanisms (Abi). The latter systems block phage multiplication and cause premature cell death upon phage infection. With the constant use of *L. lactis* strains carrying Abi systems, new phages resistant to Abi systems have emerged but they remain largely uncharacterized (4, 5, 16, 17, 19, 27).

In this study, the evolution of the lytic lactococcal phage ul36 (P335 species) was studied (25). Phage ul36 was sequentially propagated on a prophage-containing *L. lactis* strain (SMQ-86) harboring either AbiK (4-7, 20, 21) or AbiT (3). Phage mutants resistant to the Abi systems were isolated and characterized through genome sequencing and electron microscopy analyses.

Isolation and characterization of phage mutants. Lactococcal phage mutants resistant to Abi systems were isolated in laboratory from GM17 lysates (27) as illustrated in Figure 1A. The wild-type lytic phage ul36 (GenBank accession number AF349457) was first propagated on *L. lactis* SMQ-86 containing the AbiK system. A phage mutant resistant to AbiK was isolated at a frequency of 10^{-7} and named ul36.k1. This phage was then propagated on the same *L. lactis* host but carrying AbiT. Another phage mutant (ul36.k1t1) was obtained at a frequency of 10^{-8}, which was insensitive to both Abis. In parallel, a similar experiment was conducted by first propagating ul36 on an AbiT-containing host strain followed by propagation on an AbiK+ strain. Two phage mutants, ul36.t1 and ul36.t1k1, were isolated at a frequency of 10^{-8} and 10^{-7}, respectively.

The efficiency of plaquing (EOP) of the wild type phage ul36 on strains carrying AbiK or AbiT was in the range of 10^{-6} to 10^{-8} while the two double phage mutants (ul36.k1t1 and ul36.t1k1) were totally resistant to both Abis (EOP of 1.0). As expected, phage ul36.k1 was only resistant to AbiK but phage ul36.t1 was insensitive to AbiT and surprisingly, also slightly resistant to AbiK (10^{-4}). The genomic DNA of these phages was isolated (Lambda Maxi Kit, Qiagen) and their EcoRI (Roche) restriction profiles indicated that they were related but distinct (Fig. 1B). Electron microscopy observations (Fig. 1A) confirmed that the wild-type phage ul36 has an isometric capsid, a non-contractile tail, and a two-disks baseplate, which mediates the initial interaction with the cell receptor (24, 29). Interestingly, only the phage mutant ul36.k1 possesses the double disk baseplate structure of the phage ul36. The three other phage mutants (ul36.t1, ul36.t1k1, and ul36.k1t1) have only a one-disk baseplate. The tail length of these three phages is also slightly longer (120 ± 6.2 nm) as compared to the tail of ul36 and ul36.k1 (99 ± 5 nm). In order to shed light on the origin of the phage mutants, their entire dsDNA genomes were sequenced (Integrated Genomics Inc. and Centre de Recherche du CHUL/CHUQ) (Fig. 2).

Phage ul36.k1. The linear genome of this AbiK-resistant phage has 37,131 bp with an overall G+C content of 35.7% (GenBank DB394806). This is 333-bp larger that the genome of the wild-type ul36 (36,798-bp, 35.8% G+C). However, both genomes are almost identical. The only difference was a 2,533-nucleotides region (coordinates 6,613 to 9,146) in phage ul36.k1, which represents 6.8% of the genome (Fig. 2). Interestingly, this acquired DNA segment by ul36.k1 is identical to one previously described for phage ul36.1, which is also resistant to AbiK (4).

Phage ul36.k1t1. This AbiK/AbiT phage resistant mutant has a genome of 35,594 bp with an overall G+C content of 35.7% (DB394807). Comparative analysis indicated that only 27% of the genome of phage ul36.k1t1 was found in the wild-type phage ul36 while 34% was present in the genome of ul36.k1. However, 23,430 bp of phage ul36.k1t1 were not found in the genomes of ul36.k1 or ul36. The divergent region contains all the genes involved in phage morphogenesis and cell lysis, which may explain the difference observed in the tail length and in the structure of the baseplate.

Phage ul36.t1. The genome of this AbiT-resistant phage has 35,992 bp with an overall G+C content of 36.0% (DB394808), which is 806-bp shorter that the genome of the wild-type ul36. Sequence analysis showed that 36% of ul36.t1 genome was found in the genome of ul36, confirming their relationship (Fig. 2). The remaining 23,179 bp (64%) of ul36.t1 were different from phage ul36, indicating a significant genomic modification. Again, the morphogenesis and lysis modules were divergent between both phages, which is in agreement with the morphological observations (Fig. 1A). Interestingly, phage ul36.t1 shares 78% of its genome with the genome of the phage ul36.k1t1 indicating that both phages have acquired the same DNA. The divergence between ul36.k1t1 and ul36.t1 was mainly in the area of the genes coding for proteins involved in DNA replication and transcription.

Phage ul36.t1k1. The genome of this AbiT/AbiK resistant phage mutant contains 34,897 bp with an overall G+C content of 35.8% (DB394809). It has the shortest genome of the five lytic phages analyzed here. Comparative analysis demonstrated that ul36.t1k1 shares 84% of its genome with ul36.t1 (Fig. 2). The main divergence was again in the genes coding for proteins involved in DNA replication and transcription. Surprisingly, ul36.t1k1 kept only 21% of the original core DNA from ul36.

Taken altogether, these results showed that the four derivatives of phage ul36 have picked up new DNA. The acquisition of such large DNA segments is rather exceptional and it occurred without affecting the functional capacities of the resulting phage hybrid. It clearly points out to the existence of exchangeable and compatible modules. Thus, some of these gene products are highly fit to work together, which would be expected for proteins necessary to the phage structure. Therefore, this functional new DNA must have come from another phage. This prompted us to sequence the complete genome of the only known inducible prophage of *L. lactis* SMQ-86, namely ϕsmq86.

Prophage ϕsmq86. Following induction with mitomycin C (5 μg/ml), the prophage was purified for electron microscopy observations and its DNA was isolated for sequencing. The prophage ϕsmq86 had the same morphological features as the lytic phages ul36.t1, ul36.k1t1, and ul36.t1k1 (Fig. 1A). The genome of the prophage ϕsmq86 has 33,641 bp with an overall G+C content of 36.0% (DB394810). Thus, the genome of ϕsmq86 is smaller than the five lytic phages. Fifty-one ORFs of 40 codons or more were identified and a function was attributed to 17 ORFs based on their homology with proteins with putative function or conserved domains (Table 1). The genome of ϕsmq86 is organized into different functional modules as observed in other phages (10, 14, 25) (Fig. 2). All ORFs are encoded on the same strand, except for the four genes (*orf1* to *orf4*) of the lysogeny module. Many gene products possess homology with counterparts found in other phages of the P335 species, prompting the classification of ϕsmq86 within this group (15). However, the deduced proteins involved in packaging and capsid morphogenesis share no similarity with known proteins of *L. lactis* phages. Comparative nucleotide sequence analysis confirmed that most of the acquired DNA by the new lytic phages obtained in this study came from ϕsmq86.

Comparisons between ϕsmq86, ul36.t1, and ul36.t1k1. The entire new DNA picked up by ul36.t1k1 came from the prophage ϕsmq86, except for a region of 100 bp that is repeated three times from coordinates 8216 to 8315, 8316 to 8415 and 8416 to 8515 in ul36.t1k1. The same observation was made for ul36.t1 as a short insertion of 1,165 nucleotides within the *orf46,* possibly coding for the receptor-binding protein (RBP) (hatched region in Fig. 2) was not found in the inducible prophage. But, this 1.1-kb region of ul36.t1 possesses homology with the *rbp* of

other *L. lactis* phages, suggesting a second recombination event took place with another prophage (most likely defective) of *L. lactis* SMQ-86. It is known that *rbp* genes are the site of frequent DNA shuffling that favour the generation of phage variants with altered host range (18, 22, 28).

Comparisons between ϕsmq86, ul36.k1, and ul36.k1t1. As indicated previously, the exchanged DNA (2.5-kb) in phage ul36.k1 was already investigated (4). Phage ul36.k1t1 kept this 2.5-kb segment but also acquire a large DNA fragment from the prophage ϕsmq86 (Fig. 2). However, another region (2.4-kb) covering the *rbp* gene (*orf46*) and the lysis genes was unique to ul36.k1t1 (hatched region in Fig. 2). Again, this segment is likely the result of another recombination event with a resident prophage in *L. lactis* SMQ-86.

Comparison between ϕsmq86 and ul36. Pairwise comparison of the entire genomes of the lytic phage ul36 and the inducible prophage ϕsmq86 uncovered very limited nucleotide identity that could serve as substrate for homologous recombination. Most matching DNA regions were between the coordinates 11,000 and 14,000 in ul36. This finding supports the fact that one of the recombination events in phages ul36.k1t1 and ul36.t1 occurred by homologous recombination in this region. Otherwise, it appears that the other recombinations were illegitimate.

Influence of Abis and strain rotation on phage evolution. Viruses are known to mutate during amplification cycles and the existence of various selective pressures such as host diversity and barriers will influence the nature of their infectious population. Lactococcal phages typically face such shifting selective pressures in the dairy environment. It was previously reported that two general types of lactococcal phage mutants can be isolated through the selective pressure of Abi systems (13). The first class of Abi-resistant phage mutants carry only point mutations (5, 13), while the second class is the result of recombination with phage-related sequences present in the host chromosome (4, 19, 27). All the mutant phages isolated in this study belonged to the second class of mutants. To different extent, they have all acquired new DNA by homologous or illegitimate recombination mostly from an inducible prophage. The percentage of DNA exchanged in these genomes ranged from 6.8% (ul36.k1) to a remarkable 79% (ul36.t1k1). The frequency at which these ul36 derivatives were obtained (from 10^{-7} to 10^{-8}) suggests that they were already present in the high-titer lysate used to challenge the Abi-containing strains. In fact,

these lysates likely contain a population massively composed of phages with the ul36 genetic make-up but also with some functional and nun-functional derivatives carrying genetic variations. In the presence of a specific selective pressure, the fittest organisms will rapidly multiply while the others will be kept at low levels or will eventually be eliminated. This study provides biological evidences that Abi systems and prophage DNA can significantly influence the genetic make-up of lytic phages. It also demonstrates that the genome plasticity of lactococcal phages allow them to rapidly adapt to new environments. In fact, the practice of rotating isogenic strains carrying different antiphage systems appears to significantly contribute to the emergence of new lytic phage variants, at least for P335-like phages. Interestingly, the sequential encounter of a specific phage resistance mechanism will also determine the direction of the phage evolution.

Phage-bacterium co-evolution. Since prophage elements are found in many bacterial genomes (11), the probabilities that phages infecting a cell and recombine with prophage genome(s) by homologous or illegitimate recombination are rather high. This genomic reshuffling inexorably leads to emergence of new phages and quite possibly to new bacterial strains as well. Considering the astonishing genomic rearrangement observed in some of the phage ul36 derivatives and the presence of numerous phage defence systems in bacteria, the weight of this phage-bacterium co-evolution may be more significant than previously envisaged.

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Table 1. Features of prophage ϕsmq86 ORFs and the putative functions of their product.

ORF	Size (aa)	Start	Stop	pI	MW (kDa)	SD Sequence (AGAAAGGAGGT(6)ATG)	Putative function; Best match	Size (aa)	Identity[a] (%)	ul36.k1t1[b]	ul36.t1[b]	ul36.t1k1[b]
1	510	1562	30	9.28	58.9	GTAGAGGAGGA(14)ATG	Integrase; INT, *L. lactis* phage TP901-1	485	368/485 (75%)			
2	179	2152	1613	7.87	19.9	AGGCAGGAGGTtttttATG	CTC00414, *C. tetanii* E88	137	39/109 (35%)			
3	194	2792	2208	4.52	22.8	AAATAAGAGGTgagctATG	ORF4, *L. lactis* phage bIL312	195	92/194 (47%)			
4	136	3213	2803	4.93	15.3	GTAAAGGATGTtttatttTTG	Repressor; Llacc01000251, *L. lactis* SK11	127	57/123 (45%)			
5	77	3390	3623	5.26	8.8	GGAAATAGGGAattgaATG	Repressor; tec, *L. lactis* phage r1t	80	25/66 (37%)			
6	229	3682	4371	6.93	26.4	AGAAAGGTAATgagATG	Antirepressor; ORF016, *S. aureus* phage EW	258	75/221 (52%)			
7	60	4387	4569	9.46	6.9	AGAAAGGAGCAtgagatATG	XIS, *L. lactis* phage TP901-1	64	23/37 (62%)			
8	40	4566	4688	9.52	4.8	AAAAAGGAGATtgcATG	ORF8, *L. lactis* phage BK5-T	42	19/37 (51%)			
9	82	4701	4949	4.83	9.6	AGATAGGAGAAaaaATG	ORF5, *L. lactis* phage 4268	82	69/82 (84%)			
10	58	4946	5122	7.97	6.8	AAGTTGGCGGTgattaaATG	ORF9, *L. lactis* phage TP901-1	58	55/58 (99%)			
11	245	5229	5966	4.82	27.8	AGAAACGGAGAatttaaaaATG	SSAP (Sak); ORF245, *L. lactis* phage ul36.1	245	242/245 (98%)			+
12	364	5941	7035	5.34	42.3	AGAAACTGGCGaagttattgATG	ORF364, *L. lactis* phage phi31.1	364	364/364 (100%)			+
13	299	7325	8224	5.61	34.3	–	Replicase; ORF201, *L. lactis* phage phiLC3	201	161/178 (90%)			+
14	67	8221	8424	5.24	7.8	TGAAATGGGGTtacgtaatggATG	EcolE2_01002138, *E. coli* E22	419	16/41 (39%)			
15	118	8303	8659	6.84	13.8	TGACAGATGGCttaaATG	rhaA, *E. feacalis* V583	428	23/72 (31%)			+
16	136	8672	9082	6.43	16,0	TTTAAAAAGGTcaatatATG	ORF17, *L. lactis* phage bIL309	136	135/136 (99%)			+
17	68	9190	9396	9.69	8.2	AAATATGAGGTagtaatATG	ORF68a, *L. lactis* phage ul36	68	68/68 (100%)	+		+
18	56	9405	9575	4.45	6.3	TGAAGTGAGGGatgagATG	ORF56, *L. lactis* phage ul36	56	56/56 (100%)	+		+
19	52	9588	9746	4.83	5.9	TTAAAGGAGATaagaaATG	ORF26, *L. lactis* phage bIL286	119	50/52 (96%)	+		+
20	60	9765	9947	4.31	6.9	AGAAATAGCAGaaaacctaATG	Excisionnase; ORF17, *L. lactis* phage 4268	119	60/60 (100%)	+		+
21	180	9940	10482	5.29	21.4	GGCTTGGAGGAcacgaaaaATG	ORF18, *L. lactis* phage 4268	196	102/180 (52%)	+		+
22	121	10479	10844	5.3	14,0	CAGATTGAGGTggaaaaATG	ORF184, *L. lactis* phage ul36.1	184	62/65 (95%)	+		+
23	139	10841	11260	5.15	15.1	CCTGTGGAGGAcggagaATG	dUTPase; ORF139b, *L. lactis* phage ul36.1	139	138/139 (99%)	+		+
24	119	11264	11623	5.57	13.5	ACCGGGGAGGTgtgaaaaATG	ORF118b, *L. lactis* SMQ86	118	112/116 (96%)	+	+	+
25	239	11650	12369	4.8	27.4	GTGGTGGAGGGgatagATG	ORF230, *L. lactis* phage phi31.1	230	225/230 (97%)	+	+	+
26	72	12388	12606	4.5	8.4	GGTGGGGAGGGattgaATG	No significant homology			+	+	+
27	71	12603	12818	7.71	8.5	GGTATGAGGGTaattaaATG	ORF72, *L. lactis* phage ul36	72	66/72 (91%)	+	+	+
28	102	12947	13255	8.83	12.2	CAACTGGAGGAgaaATG	Llacc01000146, *L. lactis* SK11	102	95/102 (93%)	+	+	+
29	129	13332	13721	8.69	15.2	CAAAAGGAGAAtttgattaATG	ORF6, *L. lactis* subsp. *cremoris* S114	141	44/107 (41%)	+	+	+
30	157	13892	14365	6.77	17.8	AGAAAGGAGGAgaaATG	ORF30, *L. lactis* phage Tuc2009	153	136/152 (89%)	+	+	+
31	483	14346	15797	6.12	55.4	ACTTCGGAGGTtagcaaATG	Large terminase; EF1455, *E. feacalis* V583	478	330/463 (71%)	+	+	+
32	509	15810	17339	5.24	56.1	TTTAGGGAGGTgataaaTTG	Tail protein; ORF52, *L. lactis* phage bIL309	511	236/512 (46%)	+	+	+
33	276	17332	18162	6.05	31.5	AATGCGGAGGGcgcgtgATG	lin1269, *L. innocua* Clip11262	582	86/266 (32%)	+	+	+
34	349	18178	19227	4.83	38.8	AGAAAGGATAAaaATG	ORF52, *B. subtilis* JH542	322	152/337 (45%)	+	+	+
35	305	19242	20159	6.33	33.2	CTAAAGGAGAAaaacaatATG	xkdG, *B. subtilis* 168 prophage PBSX	311	59/251 (23%)	+	+	+
36	78b	20188	20424	5.55	7.7	CCAACTGGGTaacgTTG	MS115, *Microscilla* Sp. PRE1	1330	41/74 (55%)	+	+	+
37	133	20498	20899	6.28	14.8	GGAAAGGAGGTcaaATG	gp8, *L. innocua* phage A118	131	44/126 (34%)	+	+	+
38	114	20889	21233	8.73	12.5	AAAAAGGAGGTaaaaataaGTG	gp9, *B. cereus* G9241	118	41/115 (35%)	+	+	+
39	109	21230	21559	9.3	12.4	CATTTGGAGGTacagttacaATG	No significant homology			+	+	+
40	144	21559	21993	4.73	16.5	ATTAAGGAGAAtctttgATG	No significant homology			+	+	+
41	158	22004	22480	5.35	17,0	AATAAGGAGAAaaaaaATG	Major capsid protein; gpP, *B. cereus* G9241	147	64/150 (42%)	+	+	+
42	135	22537	22944	8.63	15.8	TTTAAGGAGAAaaaaATG	No significant homology			+	+	+
43	235	22960	23667	4.77	28.1	AGACAGGAGGCtttacATG	ORFR198, *Lactobacillus* phage phi-g1e	198	55/165 (33%)	+	+	+
44	869	23657	26266	6.08	91,0	AGAAAGGAGGAagaattaaATG	TMP; ORF843, *L. lactis* phage phiLC3	843	302/838 (36%)	+	+	+
45	509	26276	27805	5.24	56.1	AAATAGGAGAGaaaaATG	Tail protein; ORF52, *L. lactis* phage bIL309	511	236/511 (46%)	+	+	+
46	1027	27802	30885	4.8	111.9	TCATGGGAGGAaaatatactTTG	RBP; ORF45, *L. lactis* phage 4268	1579	812/1410 (57%)	+[c]	+[a]	+
47	78c	30897	31133	4.37	8.4	TAAAAGGAGGAaaaATG	ORF24, *L. lactis* phage BK5-T	78	29/78 (37%)		+	+
48	116	31146	31496	5.62	13.4	AGAAAGTAGGGgttATG	ORF46, *L. lactis* phage r1t	115	101/114 (88%)		+	+
49	99	31509	31808	5.85	10.5	ATAAAGGAGAAagaacATG	Holin; ORF60, *L. lactis* phage bIL286	99	98/99 (98%)		+	+
50	259	31808	32587	9.23	27.9	CAGAAGGAGGCgaataATG	Endolysin; ORF61, *L. lactis* phage bIL286	256	254/259 (98%)		+	+
51	260	32739	33521	9.87	30.2	–	No significant homology					

[a] Identity presented is significant using PSI-Blast algorithm

[b] + mean the presence of the gene in the genome of phage mutants

[c] The N-terminal region is unique to the phage ul36.k1t1

[a] Possesses an insertion of 1,165 nucleotides compared to ϕsmq86

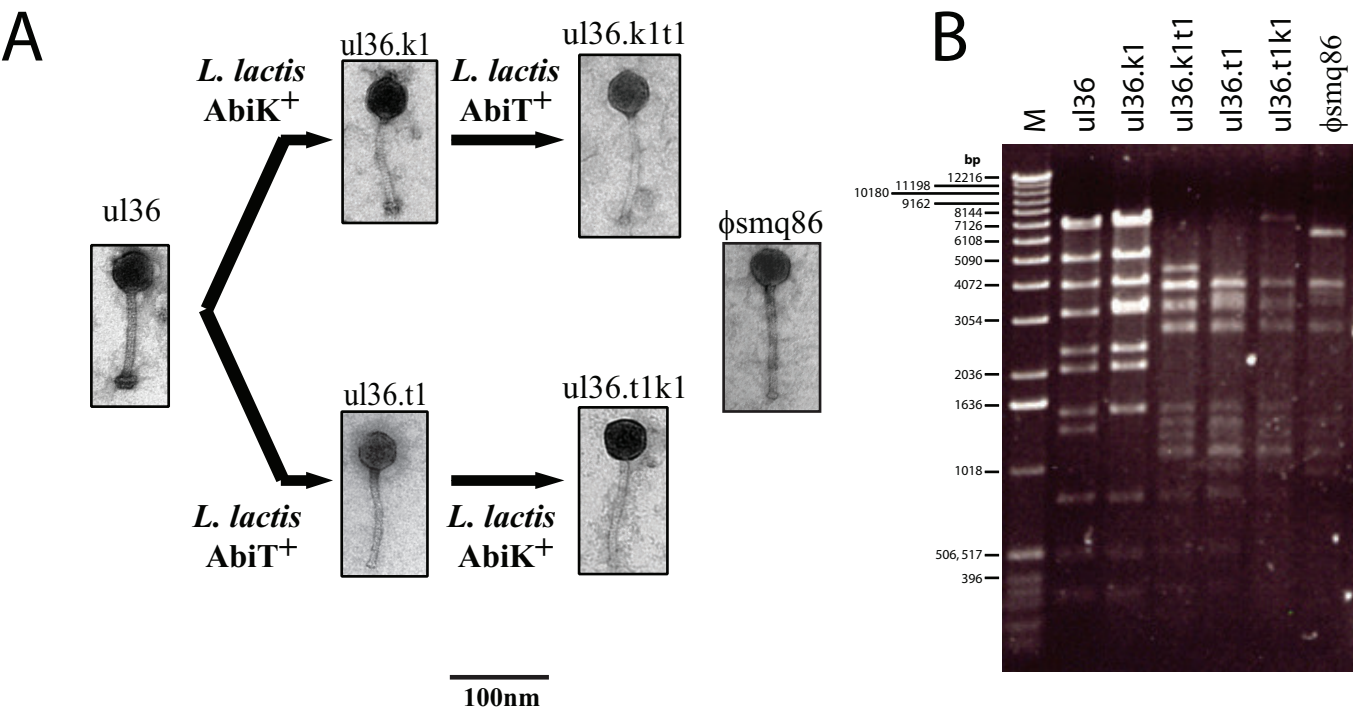

Figure 1. A) Electron micrographs and B) EcoRI restriction profiles of lactococcal phages analysed in this study. This figure also illustrates the methodology used to isolate the mutant phages.

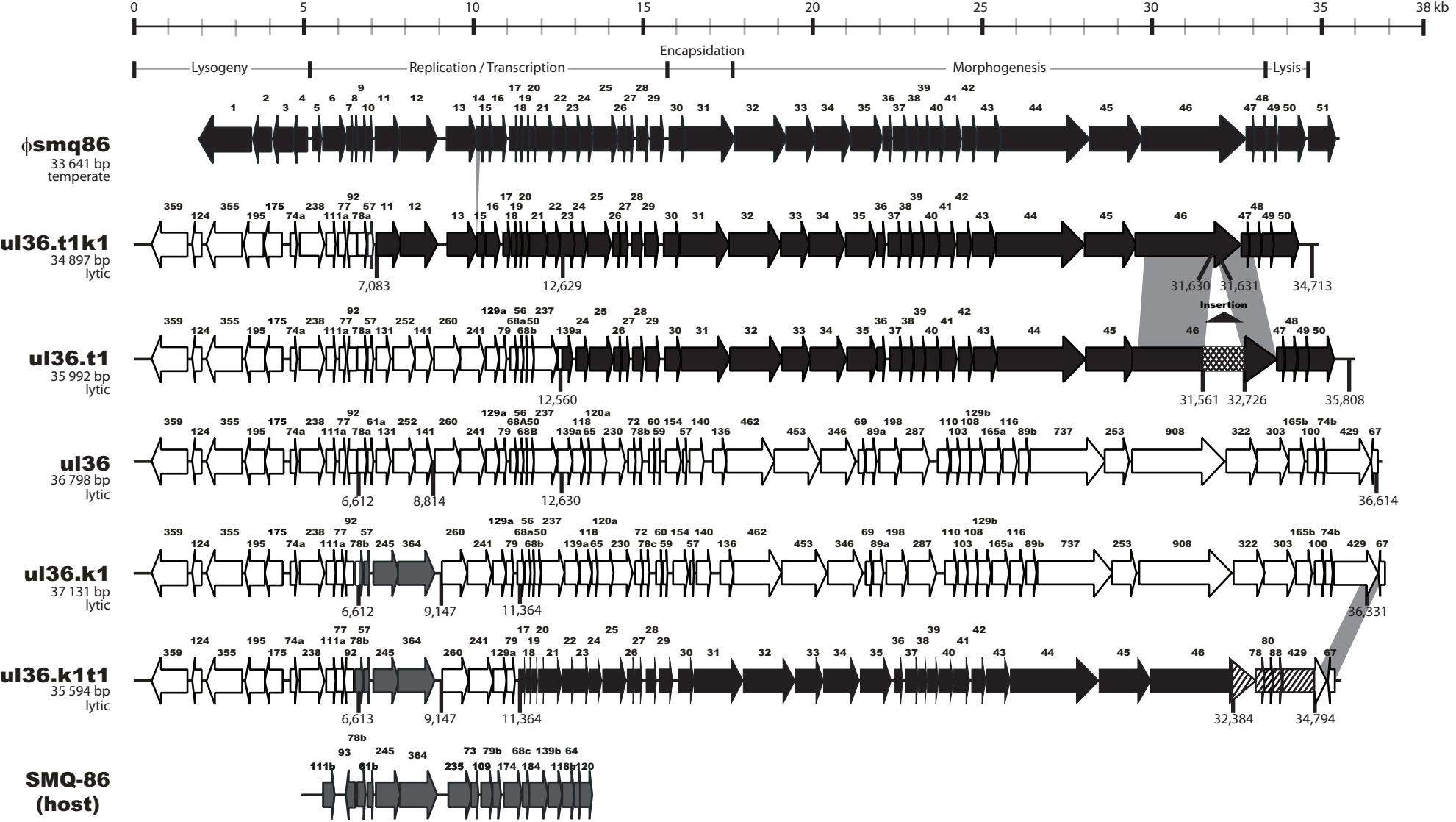

Figure 2. Alignment of the genetic maps of the wild-type phage ul36, the mutant phages ul36.k1, ul36.k1t1, ul36.t1, ul36.t1k1, the prophage ϕsmq86 as well as the host strain *L. lactis* SMQ-86 (4). Identical ORFs have the same color. Hatched regions are unique amongst the phage genomes illustrated in this figure.