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Complete Genomic Sequence of Bacteriophage ul36: Demonstration of Phage Heterogeneity within the P335 Quasi-Species of Lactococcal Phages

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The complete genomic sequence of the *Lactococcus lactis* virulent phage ul36 belonging to P335 lactococcal phage species was determined and analyzed. The genomic sequence of this lactococcal phage contained 36,798 bp with an overall G+C content of 35.8 mol %. Fifty-nine open reading frames (ORFs) of more than 40 codons were found. N-terminal sequencing of phage structural proteins as well as bioinformatic analysis led to the attribution of a function to 24 ORFs (41%). A lysogeny module was found within the genome of this virulent phage. The putative integrase gene seems to be the product of a horizontal transfer because it is more closely related to *Streptococcus pyogenes* phages than it is to *L. lactis* phages. Comparative genome analysis with six complete genomes of temperate P335-like phages confirmed the heterogeneity among phages of P335 species. A dUTPase gene is the only conserved gene among all P335 phages analyzed as well as the phage BK5-T. A genetic relationship between P335 phages and the phage-type of the BK5-T species was established. Thus, we proposed that phage BK5-T be included within the P335 species and thereby reducing the number of lactococcal phage species to 11.

Key Words: bacteriophage; classification; genome; *Lactococcus lactis*.

INTRODUCTION

Lactococcus lactis is a gram-positive bacterium used to manufacture a wide variety of fermented dairy products. Several *L. lactis* strains are susceptible to attack by virulent phages found in industrial milk environments. Phage infections can cause fermentation failures, which translate into significant financial losses for the dairy industry. Given their economic impact, lactococcal phages are the most intensively studied phages among the lactic acid bacteria (LAB) group. Many lactococcal phages have been isolated and are currently divided into 12 species (Jarvis *et al.,* 1991), all belonging the *Caudovirales* order (Ackermann, 1999). However, large-scale milk fermentation collapses in North American dairy factories are mainly caused by virulent members of three genetically distinct phage species, namely 936, c2, and P335 (Moineau *et al.,* 1992, 1996; Bissonnette *et al.,* 2000). Typically, phages of the 936 species are the most predominant, followed by c2 and P335. Common features among these three phage species include a double-stranded DNA, a long noncontractile tail, and their affiliation to the *Siphoviridae* family. The 936 and P335 phage species have an isometric capsid (B1 morphotype), while members of the c2 species have a prolate capsid (B2 morphotype). The 936 and c2 species are composed of only virulent phages, whereas the P335 species combine lytic and temperate members. The three species can now be identified and detected rapidly using a multiplex PCR method (Labrie and Moineau, 2000). To date, the complete genomic sequence is available for 11 distinct lactococcal phages, including lytic phages sk1 and bIL170 of the 936 species (Chandry *et al.,* 1997; Crutz-Le Coq *et al.,* 2002), lytic phages c2 and bIL67 of the c2 species (Schouler *et al.,* 1994; Lubbers *et al.,* 1995), the temperate phages r1t, TP901-1, Tuc2009, bIL285, bIL286, and bIL309 of the P335 species (van Sinderen *et al.,* 1996; Brøndsted *et al.,* 2001; Chopin *et al.,* 2001), and the temperate phage BK5-T, the reference phage of the lactococcal phage species of that name (Mahanivong *et al.,* 2001).

Studies on phage–host interactions in *Lactococcus* led to the discovery of natural plasmids that encode antiphage mechanisms (for a review, see Forde and Fitzgerald, 1999). These plasmids were transferred into industrial strains and the resulting phage-resistant cultures have been successfully used in industrial large-scale fermentations (Moineau, 1999). After considerable use of these strains, modified or new lytic phages capable of evading the host phage defense systems were isolated (Moineau, 1999). Comparison between wild-type and mutated phages of the c2 and 936 species showed that they differ only by point mutations or short insertions/deletions. Such modifications were also observed for the virulent phage mutants of the P335 species. However for the P335 species, large DNA fragments were also exchanged through homologous recombination with

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(cryptic) prophages present within the host chromosome (Moineau *et al.,* 1994; Bouchard and Moineau, 2000; Durmaz and Klaenhammer, 2000). Acquisition of a methylase gene from a plasmid has also been reported for P335 phages (Hill *et al.,* 1991). Thus, there is evidence of genome fluidity within this lactococcal P335 phage species.

A better understanding of the evolution mechanisms involved in the different lactococcal phage species is necessary for the development of long-term phage-resistant strains. Comparative genome analysis will provide precise information regarding the evolution of a species (Bouchard and Moineau, 2000). Pairwise comparisons of the two complete genomes of virulent phages of the 936 species indicated that they share a high degree of homology (>70%). Similar results were obtained with virulent phages of the c2 species (Chopin *et al.,* 2001). In contrast, recent studies have demonstrated that phages of the P335 species are much more genetically diverse than expected (Jarvis, 1995; Labrie and Moineau, 2000). The comparative genomic analysis of five temperate phages of the P335 species showed that they are formed by a mosaic of conserved regions interspersed by nonhomologous sequences (Chopin *et al.,* 2001). Although virulent phages are clearly responsible for milk fermentation defaults, there are no complete genome sequences available for lytic phages of the P335 species.

Phage ul36 is a lactococcal bacteriophage that was isolated in cheese whey from a failed fermentation as part of a study on phage ecology in Canada (Moineau *et al.,* 1992). DNA–DNA hybridization studies have previously shown that it belongs to the P335 species of the lactococcal phage classification (Moineau *et al.,* 1992). This phage has a small isometric head of 55 nm in diameter and a long noncontractile tail of 150 nm. Its burst size was estimated at 400 phages and its latent period at 45 min (Moineau *et al.,* 1993b). Phage ul36 has been used repeatedly as a reference lytic phage of the P335 species in a number of studies on phage–host interactions, phage ecology, and phage detection assays (recently reviewed by Boucher and Moineau, 2001). Another interesting feature of this lytic phage is the ability of its genome to recombine with the chromosome of its host (Moineau *et al.,* 1994; Bouchard and Moineau, 2000).

This study presents the analysis of the entire genomic sequence of phage ul36. This first complete genome of a virulent phage of the P335 species is compared with genomes of the temperate P335 phages.

RESULTS

Genomic organization of bacteriophage ul36

The entire genomic sequence of the phage ul36 was resolved. Its linear DNA contains 36,798 bp with an overall content of 35.8 mol % G+C, which is very close to the 35.4 mol % G+C of the *L. lactis* strain IL1403 (Bolotin *et al.,* 1999). Bioinformatic analysis showed 59 open reading frames (ORFs) of at least 40 codons (Table 1). The ORFs were named according to the number of amino acids in the deduced protein. The gene organization was very compact, allowing several overlapping ORFs and resulting in only very few short intergenic regions. All ORFs, except five (associated with the cryptic lysogeny module, see below), were coded in the same orientation. A codon preference table was established and compared to the host *L. lactis* table. No significant differences were noted with the ul36 codon table and no biases in codon usage were found for a particular ORF. All ORFs were preceded by a region that shares variable homologies with a Shine–Dalgarno (SD) sequence complementary to the 3′ end of the 16S rRNA of *L. lactis*. These SD sequences were at an appropriate distance from one of the common initiation codons (AUG, UUG, GUG). Only ORF359 (integrase), ORF188 (cI-like repressor), ORF89b, ORF908 (putative anti-repressor), and ORF303 possessed weak SD sequences.

Using standard PSI-Blast protocol, 24 of the 59 (41%) ORFs showed homologies with a previously characterized gene or gene product; 30 displayed homologies to ORFs of unknown functions and 5 had no homologous proteins in the databases. The genome was divided into four regions (lysogeny, early, late, and lysis); only those ORFs for which a putative function can be attributed are discussed.

Cryptic lysogeny module

Although lysogens of ul36 have not been found, a lysogeny module comprising six ORFs from ORF359 to ORF74A (Fig. 1, Table 1) is present. A function was ascribed to only three of them: integrase (ORF359), CI repressor (ORF188), and Cro repressor (ORF74A) (Table 1).

Integrase-like gene. ORF359 exhibited a high level of identity with tyrosine recombinases of the LC3 subfamily (Esposito and Scocca, 1997; Nunes-Düby *et al.,* 1998). All specific patterns common to this type of enzyme were identified in ORF359 (e.g., R-H-R-Y motif, box I, box II, patch I, patch II, and patch III (Nunes-Düby *et al.,* 1998)). The presence of these elements suggests that this enzyme may be biologically active. ORF359 is virtually identical to the putative integrase of bIL286, a resident prophage of *L. lactis* IL1403 genome (Table 1). ORF359 showed 53% identity with the integrase of T12 and T270 phages of *Streptococcus pyogenes* (McShan *et al.,* 1997) and only 25–27% identity with the closest lactococcal phage integrase (prophage bIL310) (Table 1).

Putative CI and Cro repressors. The ORF188 protein was similar to several *cI*-like repressors, well characterized in *L. lactis* phages, such as BK5-T (Boyce *et al.,* 1995), r1t (Nauta *et al.,* 1996), TP901-1 (Madsen *et al.,*

TABLE 1

General Features of the Identified Open Reading Frames on the Genome of Bacteriophage ul36

ORF	Position Start	Position Stop	MW (kDa)	SD sequence AGAAAGGAGGT (6) ATG	Function/similarities	Size (aa)	Percentage of identity
359	1581	502	41.5	GTATAAAAAGCaact**TTG**	Integrase		
					int, probable integrase bIL286 *L. lactis* IL1403 (prophage)	360	358/360 (99%)
					int, Integrase *S. pyogenes* phages T12 and T270	362	192/358 (53%)
124	2075	1701	13.8	TTTAAGGAAAAataaaa**ATG**	yjqA, *B. subtilis*	125	44/104 (42%)
355	3185	2118	39.3	GGTTAGGAGAAagttt**ATG**	No significant homology		
195	3833	3246	22.9	AAATAGGAGGTttct**ATG**	ORF3, bIL286 *L. lactis* IL1403 (prophage)	195	195/195 (100%)
188	4409	3843	22.0	AAAAAGAAAACtctcaa**ATG**	cI-like repressor		
					Putative cI repressor, *L. lactis* phage phi31	180	164/172 (95%)
74A	4643	4867	8.6	GGCAGAAAGGAtgtatc**ATG**	cro-like repressor		
					Putative cro repressor, *L. lactis* phage phi-31	74	73/74 (98%)
238	4894	5610	27.7	AGAAAGGATTAgaaa**ATG**	ORF6, *L. lactis* phage Tuc2009	238	233/238 (97%)
					Putative antirepressor Orf238, *L. lactis* phage phi31.1	238	231/233 (99%)
111a	5623	5958	12.9	AATAGAGAGGAaaatcc**ATG**	ORF111b, *L. lactis* SMQ-86	111	111/111 (100%)
					ORF7, bIL285 *L. lactis* IL1403 (prophage)	112	109/111 (98%)
					ORF39, *L. lactis* phage BK5-T and ORF7, Tuc2009	111	109/111 (98%)
77	6021	6254	8.9	AGAAAAGAGAGatt**TTG**	ni-ORF, *L. lactis* SMQ-86	77	77/77 (100%)
					ORF9, bIL285 *L. lactis* IL1403, bIL286, and Tuc2009	64	63/64 (98%)
					ORF41, *L. lactis* phage BK5-T	113	61/64 (95%)
78a	6590	6826	9.1	AGAAAGGAAAAta**ATG**	ORF78, *L. lactis* phage phi31.1 and *L. lactis* SMQ-86	78	74/78 (94%)
61a	6845	7030	7.1	GCCTTCGAGGCaa**ATG**	ORF61b, *L. lactis* SMQ-86	61	56/61 (91%)
					ORF11, bIL309 *L. lactis* IL1403 (prophage)	57	54/57 (94%)
131	7129	7524	14.9	AGAAACGGAGAacatt**ATG**	ORF13, bIL286 *L. lactis* IL1403 (prophage)	131	128/131 (97%)
					ORF10, *L. lactis* phage TP901-1	129	106/131 (80%)
252	7533	8291	28.8	ATGCGTGAGGTaaatc**ATG**	ORF14, bIL286 *L. lactis* IL1403 (prophage)	253	251/252 (99%)
141	8284	8709	15.7	AAAAAGGAAGAacaaa**ATG**	Single-stranded binding protein		
					ORF15, ssb bIL286 *L. lactis* IL1403 (prophage)	141	133/141 (94%)
					ssbB, *L. lactis* IL1403	166	105/166 (63%)
255	8849	9616	29.3	AGAAAGGAGTAaac**GTG**	Replisome organizer		
					ORF16, bIL285 *L. lactis* IL1403; ORF255, *L. lactis* SMQ-86	256	255/255 (100%)
					Replisome organizer, *L. lactis* phage Q33	260	254/255 (99%)
241	9616	10341	27.2	CTCTTGGAGGTcttatcta**ATG**	ORF17, bIL285 *L. lactis* IL1403 (prophage)	242	234/241 (97%)
					ORF17, *L. lactis* phage Tuc2009	241	235/241 (97%)
129a	10,338	10,727	15.7	GTACAGGACAAcaaag**ATG**	RusA-like resolvase		
					RusA, bIL285 *L. lactis* IL1403 (prophage)	130	128/129 (99%)
					ORF14, resolvase *L. lactis* phage r1t	131	126/128 (98%)
					ORF19, *L. lactis* phage Tuc2009	129	126/129 (97%)
79	10,730	10,969	9.5	ATTACAGAGGTgtaaga**ATG**	Orf21, bIL286 *L. lactis* IL1403 (prophage)	80	73/79 (92%)
					ORF16, *L. lactis* phage r1t	79	76/79 (96%)
68a	11,080	11,286	8.2	AAATATGAGGTagtaat**ATG**	ORF8, *L. lactis* phage phi31	68	68/68 (100%)
					ORF53, putative dUTPase *L. lactis* phage BK5-T	115	45/45 (100%)
56	11,295	11,465	6.3	TGAAGTGAGGGatgag**ATG**	Orf24, bIL285 *L. lactis* IL1403; ORF20, bIL309	56	54/56 (96%)
68b	11,478	11,684	7.7	AAATATGAGGTagtaat**ATG**	ORF25, bIL285 *L. lactis* IL1403 (prophage)	69	68/68 (100%)
					ORF21, bIL309 *L. lactis* IL1403 (prophage)	55	55/55 (100%)
50	11,677	11,829	5.8	TTGGAGGACACgaaaa**ATG**	No significant homology		
237	11,765	12,478	28.1	GTTGATAAGGTttag**ATG**	ORF19, *L. lactis* phage r1t	213	158/224 (70%)
					ORF19, *L. lactis* phage TP901-1	210	119/221 (53%)

TABLE 1—*Continued*

ORF	Position Start	Position Stop	MW (kDa)	SD sequence AGAAAGGAGGT (6) ATG	Function/similarities	Size (aa)	Percentage of identity
139a	12,475	12,894	15.3	CCTGTGGAGGAcggagaATG	dUTPase		
					ORF25, *L. lactis* phage Tuc2009	139	132/139 (94%)
					Orf27, *L. lactis* phage bIL286	139	130/139 (93%)
					ORF20, dUTPase *L. lactis* phage r1t; DUT, phage TP901-1	139	129/139 (92%)
					Orf139b, *L. lactis* SMQ86 and phage phi31.1	139	130/139 (93%)
					ORF24, dUTPase bIL309 *L. lactis* IL1403 (prophage)	140	128/139 (92%)
					Orf28, bIL285 *L. lactis* IL1403 (prophage)	139	128/139 (92%)
					ORF53, putative dUTPase *L. lactis* phage BK5-T	115	73/73 (100%)
118	12,897	13,253	13.2	ACTGGGGAGGTttgaaaATG	Orf118a, *L. lactis* phage ul36.1	118	118/118 (100%)
65	13,240	13,437	6.2	GTAAAAGTGAAtattatATG	ORF65, *L. lactis* phage u136.1	65	65/65 (100%)
					Orf64, *L. lactis* phage phi31.1	64	63/64 (98%)
120a	13,430	13,792	13.8	TGAAGGGAGCGgcgATG	Orf120, *L. lactis* SMQ-86 and phi31.1	120	120/120 (100%)
230	13,779	14,471	26.4	GTGGTGGAGGGgatagATG	Orf230, *L. lactis* phage phi31.1	230	220/230 (95%)
					ORF22, *L. lactis* phage r1t	228	216/227 (95%)
72	14,518	14,736	8.4	GGTGGGGAGGGattgaATG	No significant homology		
78b	14,733	14,969	9.5	TGGTATGAGGAtagttaaATG	Orf33, bIL286 *L. lactis* IL1403 (prophage)	64	40/78 (51%)
60	15,150	15,332	6.7	TGGATTGAAGTggatATG	Orf29, bIL309 *L. lactis* IL1403 (prophage)	61	34/61 (55%)
59	15,329	15,508	6.7	ATCGAGGAGGTtgcggaATG	No significant homology		
154	15,646	16,110	17.4	CAACTGGAGGAgaaataaaATG	ORF5, *L. lactis* S114 AbiN	154	153/154 (99%)
57	16,107	16,280	6.3	TTAAAGGAGAAaagaaATG	ni-ORF, *L. lactis* S114	58	52/57 (91%)
140	16,358	16,780	16.6	CAAAAGGAGAActtggttaATG	Activator of late transcription		
					ORF28 *L. lactis* phage Tuc2009	140	140/140 (100%)
					ALT, *L. lactis* phage TP901-1	140	102/140 (72%)
136	17,058	17,468	15.4	GGAAAGGAGATtaaATG	Terminase subunit 1		
					TerS, *L. lactis* phage TP901-1; ORF8, *L. lactis* S114 AbiN Operon	136	136/136 (100%)
462	17,461	18,849	53.0	AAGTTGGCGAGtcgtgATG	Terminase subunit 2		
					TerL, *L. lactis* phage TP901-1; ORF9 *L. lactis* S114 AbiN	462	450/462 (97%)
					ORF32, large subunit terminase *L. lactis* phage Tuc2009	402	382/400 (95%)
453	18,850	20,211	52.3	TCAAAGGAGGAttctaaTTG	Portal protein		
					N-terminal sequence identification		
					ORF32, *L. lactis* phage TP901-1	452	404/453 (89%)
					ORF33, minor structural protein 1 *L. lactis* phage Tuc2009	453	294/425 (69%)
346	20,215	21,255	39.6	ACAAATGAGGAgtaacctATG	Minor capsid protein		
					orf34, *L. lactis* phage Tuc2009	346	329/346 (95%)
					ORF33, *L. lactis* phage TP901-1	564	298/313 (95%)
69	21,306	21,515	7.4	GCAAATGAGCTaTTG	No significant homology		
89a	21,544	21,813	10.4	TGACAGGCGCTtttcttATG	ORF47, *L. lactis* phage Tuc2009	106	63/89 (70%)
					ORF44, *L. lactis* TP901-1	106	62/88 (70%)
198	21,938	22,534	22.1	GAGTAGGAGGAaccagaaATG	Scaffolding protein		
					orf36, minor structural protein 2 *L. lactis* phage Tuc2009	220	178/185 (96%)
					SFP, *L. lactis* phage TP901-1	118	181/185 (97%)
287	22,552	23,415	31.2	AAATAGGAGATctaaattATG	Major Capsid Protein		
					N-terminal sequence identification		
					ORF33, *S. pneumoniae* phage MM1	295	97/260 (37%)
110	23,686	24,018	27.2	AATAAGGAGTAatttATG	ORF38, *L. lactis* phage TP901-1	110	104/110 (94%)
					ORF41, *L. lactis* phage Tuc2009	110	102/110 (92%)
103	24,015	24,326	12.2	GATTGGGAGGTtcagattgtaTTG	ORF39, *L. lactis* phage TP901-1	103	92% (95/103)
108	24,323	24,649	12.1	TTGCAGGAGGTcaatcaATG	Structural protein		
					ORF42, *L. lactis* phage Tuc2009	102	94/102 (92%)
					ORF40, *L. lactis* phage TP901-1	103	94/103 (91%)

TABLE 1—*Continued*

ORF	Position Start	Position Stop	MW (kDa)	SD sequence AGAAAGGAGGT (6) ATG	Function/similarities	Size (aa)	Percentage of identity
129b	24,646	25,035	14.9	AGAAAGGTTGAcgaa**ATG**	Structural protein		
					ORF44, *L. lactis* phage Tuc2009	129	122/129 (94%)
					ORF41, *L. lactis* phage TP901-1	129	122/129 (94%)
165a	25,046	25,543	18.2	CTAAGATAGGAgatttaat**ATG**	Major tail protein		
					N-terminal sequence identification		
					ORF45, *L. lactis* phage Tuc2009; MTP, phage TP901-1	165	165/165 (100%)
116	25,659	26,009	13.4	TTTAAGGAGAAatcaaa**ATG**	SPy0992, phage associated *S. pyogenes* M1 GAS	117	39/114 (34%)
89b	26,093	26,362	10.7	AGAGATATAGAgcgc**ATG**	ORF47, *L. lactis* phage Tuc2009	106	63/89 (70%)
					ORF44, *L. lactis* phage TP901-1	106	62/88 (70%)
737	26,378	28,591	78.4	AGAAAGGAGGAaaaaac**ATG**	Tape-measure protein		
					TMP, *L. lactis* phage TP901-1	937	219/518 (42%)
253	28,601	29,362	29.1	TTTTAGGAGGTagaa**ATG**	Structural protein		
					N-terminal sequence identification		
					ORF49, *L. lactis* phage Tuc2009	253	241/253 (95%)
					ORF46, *L. lactis* phage TP901-1	253	238/253 (94%)
908	29,389	32,115	100.9	TTATTTTTAGAtaag**ATG**	Putative anti-receptor		
					ORF47, *L. lactis* phage TP901-1	918	823/920 (89%)
					ORF50, *L. lactis* phage Tuc2009	906	818/908 (90%)
322	32,128	33,096	36.4	AGAAAGGGTCTatt**ATG**	Minor structural protein		
					N-terminal sequence identification		
					ORF51, *L. lactis* phage Tuc2009	322	309/322 (95%)
303	33,047	33,958	33.6	CTAATCTAATTgttt**ATG**	ORF52, *L. lactis* phage Tuc2009	286	254/286 (88%)
165b	33,977	34,474	18.0	AAATAGGAGAAataa**ATG**	Base plate protein		
					N-terminal sequence identification		
					ORF53, major struct. protein *L. lactis* phage Tuc2009	173	27/57 (47%)
					BPP, *L. lactis* phage TP901-1	163	22/65 (33%)
100	34,562	34,864	11.8	AGAAAGTAGGGggt**ATG**	ORF54, bIL309 *L. lactis* IL1403 (prophage)	109	59/106 (55%)
74b	34,854	35,078	8.1	ATAAGGTGGGAaaagaaa**ATG**	Holin		
					hol, *L. lactis* phage phiAM2	74	70/74 (94%)
429	35,075	36,364	46.4	GATACGGAGGAaaacaa**ATG**	Lysin		
					LYS, *L. lactis* phage TP901-1	429	412/429 (96%)
					lys, *L. lactis* phage phiAM2	429	409/429 (95%)
					LysB, *L. lactis* phage phi-LC3	429	404/429 (94%)

Note. Identity presented is significant using PSI-Blast algorithm.

1999), and Tuc2009 (van de Guchte *et al.,* 1994) (Table 1). In the N-terminal segment of the deduced protein, a helix-turn-helix (HTH) motif ordinarily involved in DNA binding was found. A similar ORF was found in other lactococcal phages of the P335 species (Nauta *et al.,* 1996; Madsen *et al.,* 1999, 2001; Blatny *et al.,* 2001). *cI*-type repressors usually show no or highly degenerative SD sequences, e.g., λ (Ptashne, 1986), *L. lactis* phages r1t (Nauta *et al.,* 1996), TP901-1 (Madsen *et al.,* 1999), ϕ31 (Madsen *et al.,* 2001), and LC3 (Blatny *et al.,* 2001), and is also observed for *orf188* of phage ul36. Thus, the mRNA may be poorly translated and lead to low production of CI repressor. ORF74A is likely the second component of a lysis–lysogeny switch, the Cro-like repressor. Proteins homologous to ORF74a were retrieved in several *L. lactis* and *S. thermophilus* phages (Table 1).

Early transcribed genes and DNA regulation

This region encompassed 29 ORFs from ORF238 to ORF140 (Fig. 1, Table 1). Putative functions were assigned to only five of them: single-stranded binding protein (ORF141), replisome organizer (ORF255), RusA-like resolvase (ORF129), dUTPase (ORF139A), and activator of the late transcription (ORF140). Eighteen ORFs (8320 bp, ORF111 to ORF120) in this region were previously identified (Bouchard and Moineau, 2000); their analysis is updated here.

The ORF141 showed several homologies with previously described SSB proteins as well as with the SSB motif Pfam 00436. ORF255 is nearly identical to many known *L. lactis* replisome organizers. During DNA replication, SSB proteins are associated with the replisome organizer protein. The origin of replication of phage ul36

has been previously identified within this ORF and may act as a weak terminator (Bouchard and Moineau, 2000). ORF129A matches a good number of *L. lactis* phages putative proteins, especially the resolvase of phage r1t (ORF14), which is similar to *Escherichia coli* RusA. As for *orf14* of phage r1t, complementation assays have shown that this gene suppresses UV light sensitivity and restores recombination events of *ruvAB* and *ruvC* in *E. coli* mutants. These data suggest that this protein is important in DNA repair and recombination (Nauta, 1997). ORF140 resembles several activators of late genes transcription (Table 1).

Putative dUTPase. ORF139A is highly homologous to many dUTPases that play a role in DNA metabolism (Table 1). This ubiquitous enzyme (deoxyuridine triphosphate nucleotidohydrolase, EC 3.6.1.23) is widely distributed in viral, bacterial, and eukaryotic genomes. It catalyzes the cleavage of dUTP into dUMP and pyrophosphates, thereby preventing uracil incorporation into DNA by efficiently decreasing the dUTP/dTTP ratio during phage genome replication. The dUMP is also the precursor of dTTP biosynthesis. It was shown that dUTPase is not essential for phage T5 multiplication, but its alteration causes the incorporation of uracil residues instead of thymine during genome replication (Warner *et al.,* 1979). A dUTPase gene was found in phage BK5-T and in all the P335 phages analyzed in this study but one is absent in genomes of the 936 and c2 species sequenced to date. Despite the heterogeneity within P335 phage species, the dUTPase is highly conserved at both the nucleotide and the amino acid levels. Moreover, it is the only highly conserved DNA stretch (420 nt) of all P335-like phages (92–94% aa identity and 91–94% nt identity) and BK5-T (93% identity over a 258 nt stretch, 70% identity over full gene length). When compared to the dUTPase gene of P335 phages, the BK5-T gene is shorter due to a deletion of the first 69 bp (23 aa). These genes are significantly different to the *dut* gene of IL1403 *L. lactis* strain.

Late transcribed genes, DNA packaging, and morphogenesis

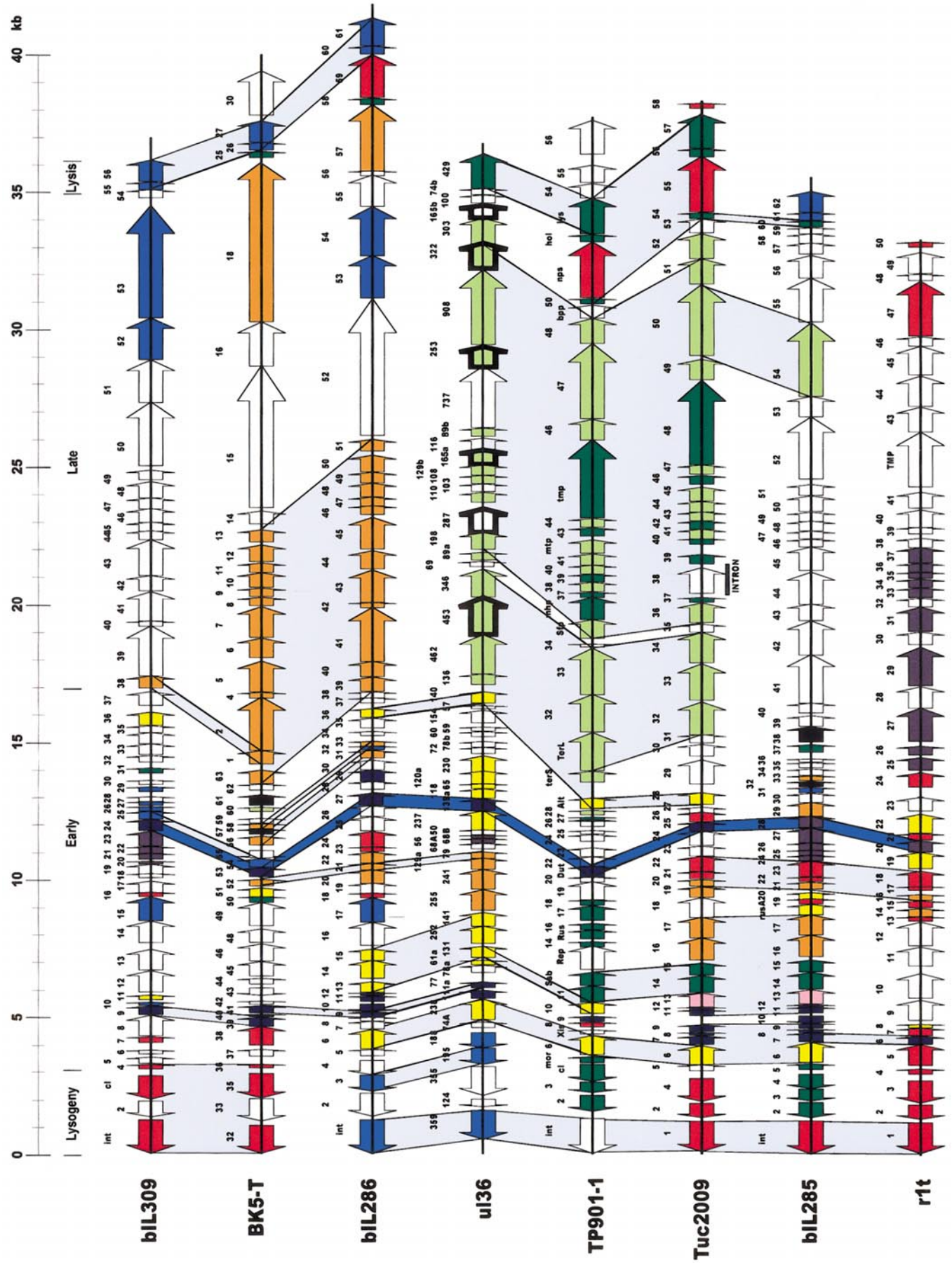
This region included 22 ORFs from ORF136 to ORF100 (Fig. 1, Table 1) and functions were attributed to 14 (65%).

Putative terminase subunits. ORF136 and ORF462 were identified as the small and large terminase subunits, respectively, according to sequence homologies with lactococcal phages and gene localization at the start of the late gene cluster.

Putative portal protein. ORF453 is similar to the portal protein of *Bacillus subtilis* phage SPP1 (Droge and Tavares, 2000; Droge *et al.,* 2000). Several structural proteins of phage ul36 were analyzed after SDS–PAGE separation and N-terminal sequencing. N-terminal amino acid residues were determined for 10 proteins recovered from the PVDF membrane (Fig. 2). The N-terminal sequence (MKYKP) of a 52-kDa protein was linked to ORF453 and corresponded well to the predicted MW of this ORF, indicating that the protein is indeed located within ul36 particle.

Major capsid protein. N-terminal sequencing indicated that ORF287 was the most abundant protein in the structure of phage ul36 (Fig. 2). Western blot assays and immunoelectron microscopy have previously identified this gene product as the major capsid protein (MCP) (Moineau *et al.,* 1993a). In comparison to other MCPs of lactococcal phages, the major capsid protein of phage ul36 is unique (Table 1). A significant level of identity was only detected with the ORF33 of *Streptococcus pneumoniae* phage MM1 (37% identity over full protein length). Four different peptides were linked to ORF287 during N-terminal sequence analysis of ul36's structural proteins. The 32-kDa protein detected on the SDS–PAGE corresponded to the calculated MW of the deduced ORF287 (31 kDa). The three other proteins observed on the SDS–PAGE (11, 28, and 44 kDa) may be the results of various modifications of ORF287 (processing or degradation) as reported for other structural proteins of lactococcal phages (Lubbers *et al.,* 1995; Chibani Azaïez *et al.,* 1998; Labrie and Moineau, 2000). The N-terminal sequence of the estimated 28-kDa protein corresponded to an internal portion of ORF287. The absence of a Shine–Dalgarno sequence within *orf287* suggests that this protein may be the product of the native protein degradation during sample preparation or that it results from a post-translational processing, as seen in the T4 major capsid protein gp23 (Black, 1989).

Other structural proteins. When compared to database assignments, ORF346, ORF108, and ORF129B are most likely phage structural components (Table 1) but they were not detected during N-terminal sequencing, which indicates that few, if any, copies of these proteins are in the phage particle. However, the presence of three other proteins (ORF253, ORF322, and ORF165B) was confirmed by N-terminal sequencing and their estimated size on the SDS–PAGE corresponded well to their predicted size based on the deduced proteins (Table 1, Fig. 2). However, ORF253 and ORF322 showed no or very low homologies with phage structural proteins of the databases, while ORF165B displayed a weak homology to baseplate proteins of lactococcal phages. The first methionine of the ORFs was absent in all these proteins, except ORF253, resulting in proteins starting at the second amino acid. This peptide maturation phenomenon was observed in many other phages and may be explained by the host methionine aminopeptidase activity (see Lowther and Matthews (2000) for a recent review). One of the protein detected by SDS–PAGE (41 kDa) could not be assigned to any ORF from phage ul36. It may be a host protein that was carried through the phage purification process.


kb
0
5
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15
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35
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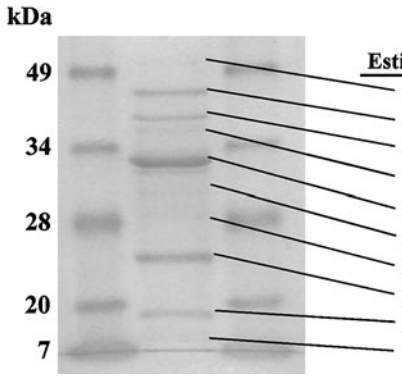
Lysogeny
Early
Late
Lysis
bIL309
BK5-T
bIL286
ul36
TP901-1
Tuc2009
bIL285
r1t
INTRON



MW (kDa) Estimated	MW (kDa) Calculated	N-terminal sequence	Description
52	52	MKYKP	ORF453, portal protein
44	31	AIKYFTK	ORF287, major capsid protein
41	—	DINGG	Not assigned
37	36	TEHFI	ORF322, minor structural protein
32	31	AIKYFTK	ORF287, major capsid protein, monomeric
30	29	MYKFR	ORF253, structural protein
28	31	ANVGF	Internal portion of ORF287
25	18	AELTAK	ORF165A, major tail protein
18	18	AELTKIY	ORF165B, base plate protein
11	31	AIKYF	Cleavage of ORF287, major capsid protein

FIG. 2. Protein profiles of phage ul36 as determined by SDS–15%PAGE stained with Coomassie blue. Lanes 1 and 3, prestained molecular mass markers (25, 32.5, 47.5, 62, 83, and 175 kDa) (New England Biolabs); lane 2, CsCl purified ul36 bacteriophage. N-terminal sequence was determined for protein bands identified by arrows and linked to corresponding data in the table.

Tail components. ORF165A and ORF737 revealed similarities with other phage tail structural components. ORF165A corresponded to the structural subunit for tail structure polymerization and was detected during N-terminal sequencing. The observed MW (25 kDa) was slightly higher than predicted (18 kDa). ORF737 is related to the tape-measure protein responsible for determining phage tail length (Katsura and Hendrix, 1984). Mutations in the corresponding gene of lactococcal phage TP901-1 have been shown to affect tail length (Pedersen *et al.,* 2000).

Host recognition. ORF908 appears to be the putative antireceptor of phage ul36 because a glycyl-glycine endopeptidase motif, frequently identified within host recognition proteins (Brøndsted *et al.,* 2001), is present. Interestingly, ORF303 shows homology with the variable region 1 (VR1) of the antireceptor of some *Streptococcus thermophilus* phages and may thus participate in phage–host interactions (Duplessis and Moineau, 2001).

Lysis module (putative holin and lysin). ORF74B gene product seems to be related to Class II holin. In fact, the only homology found was with *L. lactis* phiAM2 holin (Accession No. AAG24367). Also, two predicted membrane-spanning helical domains and a charged C-terminus, common to the holins (Blasi and Young, 1996), were found within the ORF74B peptide sequence. However, typical lambda dual start motifs were absent in phage ul36. Putative lysin function was associated to ORF429, as it was similar to several phages lysins of the muramidase group (Table 1).

Comparative lactococcal phage genome analysis

A comparative genome analysis of the 12 available and completely sequenced lactococcal phage genomes was performed at the nucleotide level. The nucleotide identity between each pair of *L. lactis* phages is presented in Table 2. This compilation confirms the high level of homology (73.3%) between the two members of the 936 species (Table 2). Similarly, the two members of the c2 species share a high level of identity (65.5%). An opposite outline is found with the so-called members of the P335 species (Table 2). Percentage of identity at the nucleotide level ranged from 7.0 to 51.2% between the seven P335 phages. Among all P335 phages, the highest homology was 51.2% between the temperate phages Tuc2009 and TP901-1. The virulent phage ul36 has the highest level of homology with Tuc2009 (46.6%), TP901-1 (40.5%), bIL285 (20.5%), and bIL286 (20.2%). Interestingly, the genome of phage BK5-T showed 44% of nucleotide identity with the prophage bIL286 of the P335 species. Phages BK5-T and bIL286 also have similar morphological proteins (Fig. 1) and perhaps should be included within the same group. In fact, comparisons of the nucleotide sequence of ul36 genome with other P335 phages as well as BK5-T demonstrate a genetic link between the P335 and the BK5-T species (Table 2).

The genomes of the seven P335 phages (r1t, Tuc2009, bIL285, bIL286, bIL309, TP901-1, and ul36) and BK5-T were also compared at the protein level (Fig. 1). The most striking observation was the resemblance (amino acid sequence and gene order) between the deduced structural proteins of phage ul36, TP901-1, and Tuc2009. The major capsid protein (ORF287) of phage ul36 is different from all other corresponding genes of *L. lactis* phages. This observation explains the weak antigenic interactions of ul36 MCP-directed antibodies against other P335 phages (Moineau *et al.,* 1993a). Nonetheless, these alignments corroborate the observations on the conser-

FIG. 1. Alignment of the genetic maps of all completely sequenced lactococcal P335-like and BK5-T bacteriophages. The deduced proteins sharing more than 60% aa identity are represented using the same color and linked using gray shading when possible. ORFs with unique sequences are displayed in white. The dUTPase gene present in all the genomes is linked by blue shading. Phage r1t genes filled in purple (ORF25 to ORF37) are highly similar to *S. pyogenes* prophage M1 GAS. Proteins identified by N-terminal sequencing within the structure of phage ul36 are identified by thick lines.

TABLE 2

Nucleotide Identities between All Completely Sequenced *L. lactis* Bacteriophages

	Species	r1t	Tuc2009	TP901-1	bIL285	ul36	bIL286	bIL309	BK5-T	bIL170	sk1	c2	bIL67
r1t	P335	—											
Tuc2009	P335	22.1	—										
TP901-1	P335	11.7	51.2	—									
bIL285	P335	13.0	30.3	20.0	—								
ul36	P335	10.8	46.6	40.5	20.5	—							
bIL286	P335	10.7	14.3	10.7	16.7	20.2	—						
bIL309	P335	10.2	7.0	7.0	12.3	9.5	25.6	—					
BK5-T	BK5-T	14.8	12.1	4.8	16.6	7.3	44.0	16.7	—				
bIL170	936	3.6	4.1	3.9	0.2	0.3	3.4	0.2	1.1	—			
sk1	936	0.0	0.6	0.7	0.6	0.0	1.0	0.0	1.1	73.3	—		
c2	c2	0.2	0.8	0.2	0.0	0.1	0.3	0.6	0.7	0.4	0.0	—	
bIL67	c2	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.0	65.5	—

Note. Global identity between two phages was calculated using nucleotide comprised in the homologous regions as described under materials and methods.

vation of the gene order in the morphogenesis/packaging module as compared to the variable organization of the early module (Casjens and Hendrix, 1988; Chandry *et al.,* 1997; Desiere *et al.,* 2001). These alignments also confirm the extensively discussed high heterogeneity among phages of the P335 species genomes (Jarvis, 1995; Labrie and Moineau, 2000; Brøndsted *et al.,* 2001; Chopin *et al.,* 2001; Desiere *et al.,* 2001).

DISCUSSION

Historically, coliphage research followed the recommendation of Delbrück to study in detail a restricted group of phages (Sharp, 2001). Dairy phage research followed a different path because a large number of diverse phages were found to be responsible for milk fermentation failures, even within one dairy factory. Consequently, it was critical to study phage biodiversity to improve control strategies in dairy plants. Comparative genome analysis is now contributing information regarding the evolution of dairy phages.

This study provides the twelfth complete genomic sequence of a lactococcal phage, but the first from a virulent phage of the P335 species. The genome size of 36,798 bp, the overall G+C of 35.8 mol %, and the genomic organization as well as the gene order are in agreement with the other studied P335 genomes. Comparisons with databases indicate that homologous regions are separated by heterologous regions, which are indicative of previous recombination events. In fact, gene acquisition appears to be important in the evolution of this lactococcal phage species (Bouchard and Moineau, 2000; Durmaz and Klaenhammer, 2000). Conservation in the gene order and protein similarities have made it possible to assign functions to several ORFs (24 of 59).

The cryptic lysogeny module

One of the most surprising findings was the identification of a seemingly integral lysogeny module in the genome of the virulent lactococcal phage ul36. Other phages containing this module have been found in prophages induced from a *L. lactis* strain. Interestingly, all sequenced phage genomes belonging to the P335 species contain a lysogeny module or remnants of one. These observations confirm that virulent P335 phages are likely descendents of temperate phages (Chopin *et al.,* 2001). At this time, we do not know if this module is functional in phage ul36. Another unexpected feature of the lysogeny module was the weak homology of the integrase gene with other *L. lactis* phage integrases. In fact, ORF359, which is also found in the bIL286 prophage genome, is closer to integrases of *S. pyogenes* phages T12 and T270 (53% identity) than it is to LAB phages. It is tempting to speculate that a horizontal transfer of this gene occurred between *S. pyogenes* and *L. lactis*.

dUTPase, a target for phage detection assays

Recently, the first generation of the PCR-based phage detection system was optimized to classify lactococcal phages within the three main phage species 936, c2, and P335 (Labrie and Moineau, 2000). This method has the advantage of simultaneously detecting the presence of lactococcal phages and their species in one reaction using three pairs of primers. The primers used to detect the P335 species were based on a common genomic region found in 10 P335-like phages. This DNA segment corresponded to *orf17–18* of phage r1t and *orf20–orf21* of Tuc2009. The absence of these two genes in phage ul36 prompted us to sequence its genome to shed light on its evolution (Labrie and Moineau, 2000). Interestingly, we found a highly con-

served dUTPase gene among all P335 phages, including ul36 and BK5-T. Moreover, no dUTPase coding gene was identified in genomic sequence of 936 and c2-like phages. This gene could represent an interesting target for the design of new primers to improve the PCR detection of P335 phages.

Lactococcal phage classification and phage evolution

The bioinformatic analysis of the increasing number of lactococcal phage genomes has provided substantial knowledge on phage evolution, module diversity, horizontal/vertical genes transfers, and lytic/lysogenic cycles. Another area that considerably benefits from such studies is phage classification. According to the current ICTV classification, lactococcal phages are grouped into 12 species, using virion morphology and DNA–DNA hybridization as criteria (Jarvis *et al.,* 1991). Recently, this classification has been criticized because of newer sequencing data and the analysis of an enlarging set of P335-like phages (Jarvis, 1995; Labrie and Moineau, 2000; Chopin *et al.,* 2001).

The addition of the nucleotide sequence of phage ul36 to the phage genome databases prompts us to look into possible evolutionary links with other phages. Six complete lactococcal phage genomes (bIL285, bIL286, bIL309, TP901-1, BK5-T, and Tuc2009) were made available almost simultaneously last year thereby preventing their overall comparisons (Brøndsted *et al.,* 2001; Chopin *et al.,* 2001; Mahanivong *et al.,* 2001). The comparative genomic analysis of these genomes with ul36 was illustrated in this study. The full-length genome alignments unambiguously showed significant relationships at the DNA and protein levels of all sequenced P335-like phages (r1t, Tuc2009, TP901-1, bIL285, bIL286, and bIL309). The comparisons confirm that these P335 phages are genetic mosaics such as lambdoid phages (Juhala *et al.,* 2000) and support the idea that the P335-like phages be considered a quasi-species (Chopin *et al.,* 2001). The heterogeneity of this phage group also points out that novel phage resistance mechanisms should be tested against several P335 phages before attesting of their efficacy.

The case of BK5-T and the P335 quasi-species

Based on protein–protein comparisons, phage BK5-T was reported to be closer to *S. thermophilus* phage Sfi21 than to any other *L. lactis* phage (Desiere *et al.,* 2001). However, we found that phages BK5-T and Sfi21 shared only 1% identity at the nucleotide level, while the genome of BK5-T has between 5 and 44% nucleotide identity with the seven P335-like phages analyzed here (Table 2). The weak DNA homology between phages BK5-T and r1t was also previously established by DNA–DNA hybridization studies (Jarvis, 1984) and determined as 15% identity in the present study. Despite BK5-T genome possessing homology with *S. thermophilus* phages in the packaging/capsid morphogenesis gene cluster, these genes are more related to the newly sequenced *L. lactis* prophage bIL286 (44% nucleotide identity over the entire genome). Phage bIL286 has nucleotide identity with phage ul36 (20.2%), which definitely has an evolutionary relationship with phages Tuc2009 (46.5%), TP901-1 (40.5%), and bIL285 (20.5%) and therefore helps in linking BK5-T to P335-like phages.

The diversity within P335-like phages may be attributed to host–phage genomic recombination events allowing modular evolution (Bouchard and Moineau, 2000; Durmaz and Klaenhammer, 2000). As several DNA stretches from BK5-T showed high homologies with P335-like phages, it is reasonable to assume that genetic exchanges may have occurred between these phages. From this perspective, the P335 quasi-species may be considered as a heterogeneous group of related phages sharing a highly conserved genome organization with stretches of identical DNA and capable of genetic exchanges between themselves. Furthermore, it has previously been shown that P335-like phages have overlapping relationships with other *L. lactis* phage species (i.e., 1358 and 1483 species) (Jarvis, 1995; Chopin *et al.,* 2001). We confirmed here that the species BK5-T is also related to members of the P335 quasi-species. According to all these observations, we propose to assign BK5-T to the P335 quasi-species and thereby reduce the number of lactococcal phage species to 11. In light of these results, it appears that the genome of members of the eight other lactococcal phage species, particularly 1358 and 1483, should be analyzed prior to the in-depth revision of the lactococcal-phage classification.

MATERIALS AND METHODS

Bacterial strain, phage, and media

L. lactis SMQ-86, the host of phage ul36, was grown at 30°C in M17 (Terzaghi and Sandine, 1975) supplemented with 0.5% glucose. Phage ul36 (Moineau *et al.,* 1992) was propagated as described previously (Moineau *et al.,* 1994). Phages were amplified, purified, and concentrated according to Chibani Azaïez *et al.,* 1998.

DNA extraction and genome sequencing

Phage DNA was isolated using Maxi Lambda DNA purification kit (Qiagen, Chatsworth, CA) as outlined previously (Labrie and Moineau, 2000). Concentration of phage DNA was estimated with a Beckman DU Series 500 spectrophotometer as described by Sambrook *et al.* (1989). Phage DNA was digested with *Eco*RI and *Eco*RV endonucleases (Roche Diagnostic, Laval, Québec, Canada) and fragments were cloned into pBluescript KS vector (Stratagene, La Jolla, CA) using a shotgun strategy. Six *Eco*RI fragments (1.3, 1.5, 2.0, 2.3, 5.0, and 7.0 kb)

and two *Eco*RV fragments (2.7 and 3.6 kb) were successfully cloned into pBS. All cloned fragments were confirmed by Southern hybridization using ul36 genomic DNA as probe, as described previously (Labrie and Moineau, 2000). The *Tn1000* strategy (Stratagene) was used for sequence determination of the 2.7-, 3.6-, 5.0-, and 7.0-kb inserts. Transconjugants were obtained in sufficient numbers for efficient sequencing of cloned fragments using internal primers of *Tn1000* transposon and universal primers forward and reverse. Finally, gaps in the genome sequence were completed using synthetic primers (Applied Biosystems 394 DNA/RNA synthesizer) and the ul36 genome as template. DNA was sequenced on both strands with an Applied Biosystems 373A automated DNA sequencer.

N-terminal structural protein sequence determination

The phage ul36 structural proteins were separated on a sodium dodecyl sulfate–15% polyacrylamide gel using the Mini-Protean II apparatus (Bio-Rad Laboratories, Mississauga, Ontario, Canada) according to Chibani Azaïez *et al.* (1998). After separation, proteins were transferred with a Trans-Blot apparatus (Bio-Rad) to a PVDF Immobilion-P^{sq} membrane (Millipore, Bedford, MA) using CAPS buffer (10 mM 3-cyclohexylamino 1-propane sulfonic acid, pH 11). After membrane staining (0.1% Coomassie blue, 40% methanol, 1% acetic acid), the N-terminal sequencing of ul36 proteins was performed using Applied Biosystems 473A model pulsed liquid protein sequencer and five to seven amino acids were determined for assignation of the peptide to an open reading frame. The broad range protein marker and the prestained molecular mass markers (New England Biolabs, Mississauga, Ontario, Canada) were used to assign a molecular weight to phage ul36 structural proteins.

Bioinformatic analysis

The sequence assembly and analysis were performed using Genetic Computer Group sequence analysis software package, version 10.0, including GenBank release 120.0, GenPept release 120.0, EMBL (abridged) release 64.0, PIR-Protein release 66.0, NRL_3D release 27.0, SWISS-PROT release 36.0, SP-TREMBL release 15.0, and PROSITE release 15.0. All motif analyses were performed using tools available at http://www.motif.genome.ad.jp/, including Prosite Pattern, Prosite Profile, BLOCKS, ProDom, PRINTS, and Pfam. PSI-BLAST and Advanced Blast Search 2.1 were used for sequence comparisons with databases (Altschul *et al.,* 1997). To estimate the relationship between two phages, the nucleotide identity was calculated. The percentage of identity was obtained using only the identical nucleotides included in a significantly homologous stretch obtained using Blast search algorithm to eliminate random nucleotide identity in the calculation. The nucleic acid identity between two complete genomes was calculated as follows: standard nucleotide–nucleotide blast search was performed using complete phage genomes. The number of identical nucleotides in all segments showing homologies was then added and divided by the number of nucleotides of the smallest entire genome (nt identity/length of smaller genome × 100 and expressed as %). The complete genomic sequence of the lactococcal phage ul36 is available under the GenBank Accession No. AF349457.

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