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Sequence Analysis and Molecular Characterization of the *Lactococcus lactis* Temperate Bacteriophage BK5-T†

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The *Lactococcus lactis* temperate bacteriophage BK5-T is one of twelve type phages that define *L. lactis* phage species. This paper describes the nucleotide sequence and analysis of a 21-kbp region of the BK5-T genome and completes the nucleotide sequence of the genome of this phage. The 40,003-nucleotide linear genome encodes 63 open reading frames. Sequence runoff experiments showed that the cohesive ends of the BK5-T genome contained a 12-bp 3′ single-stranded overhang with the sequence 5′-CACACACATAGG-3′. Two major BK5-T structural proteins, of approximately 30 and 20 kDa, were identified, and N-terminal sequence analysis determined that they were encoded by *orf7* and *orf12*, respectively. A 169-bp fragment containing a 37-bp direct repeat and several smaller repeat sequences conferred resistance to BK5-T infection when introduced in *trans* to the host cell and is likely a part of the BK5-T origin of replication (*ori*).

Lactic acid bacteria are used extensively as starter cultures in the dairy industry. The bacteria ferment lactose to lactic acid, a crucial process in cheese manufacture. One of the major microbiological problems faced by the dairy fermentation industry is the susceptibility of the starter bacteria to bacteriophage infection. Such infections result in lysis of starter cultures and failure of the fermentation. Since reports of bacteriophage infection of starter strains as early as 1935 (64), an increasing number of bacteriophages have been identified and considerable research has been conducted to improve our understanding of these viruses and their interaction with their hosts.

BK5-T is a temperate bacteriophage with a small isometric head and a noncontractile tail of 232 nm in length (24) first isolated from *Lactococcus lactis* subsp. *cremoris* BK5 (23). Boyce et al. (8) determined the nucleotide sequence of approximately 19 kbp of the BK5-T genome, which defined the *Eco*RI restriction fragments *Eco*RI-a and *Eco*RI-b (32). Thirty-two open reading frames (ORFs) were identified, and the predicted amino acid sequences encoded by several of these ORFs demonstrated significant homology with sequences available in protein databases. Analysis of the partial genome sequence also identified a putative BK5-T immunity region containing regulatory elements involved in determination of the phage lysis-or-lysogeny decision (7). This report describes the nucleotide sequence of the remaining 21 kbp of the BK5-T genome and characterizes the phage termini, major structural proteins, and the putative origin of replication.

MATERIALS AND METHODS

Bacterial strains and media. The strains and plasmids used in this investigation are listed in Table 1. *Escherichia coli* strain JM107 was incubated at 37°C with shaking in 2TY medium (42). The BK5-T indicator strain *L. lactis* subsp. *cremoris* H2 was grown at 30°C for 16 h in M17 medium supplemented with 0.5% (wt/vol) glucose (M17G) (57). When necessary, the antibiotic erythromycin (2.5 μg/ml for *L. lactis* or 200 μg/ml for *E. coli*) or ampicillin (150 μg/ml for *E. coli*) was included in the media.

Phage preparation. Bacteriophage BK5-T was propagated by lytic infection of *L. lactis* H2 in M17G medium with 10 mM $CaCl_2$. The bacteriophage were precipitated with 1 M NaCl and 10% (wt/vol) polyethylene glycol (PEG), and when necessary, further purified and concentrated by CsCl density gradient centrifugation (49). Phage DNA was isolated from the PEG-precipitated phage by the procedure described for coliphage λ by Qiagen (Qiagen, GmbH, Hilden, Germany).

N-terminal amino acid sequencing of phage proteins. BK5-T phage purified by CsCl density gradient centrifugation (49) were heated at 95°C for 15 min in cracking buffer (50 mM Tris-HCl [pH 6.8], 1% [wt/vol] sodium dodocyl sulfate [SDS], 2 mM EDTA, 10% [vol/vol] glycerol, 1% [vol/vol] β-mercaptoethanol) and separated by SDS-polyacrylamide gel electrophoresis (PAGE) on a precast Novex (Amrad Biotech, Victoria, Australia) 4 to 20% acrylamide Tris-glycine gel. The denatured proteins were transferred by electroblotting to a Bio-Rad polyvinylidene difluoride membrane in a buffer containing 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) and 20% (vol/vol) methanol. The membrane was stained with 0.1% (wt/vol) Ponceau S containing 1% (vol/vol) glacial acetic acid, the phage protein bands were excised, and their N-terminal amino acid sequences were determined by the Australian Proteome Analysis Facility (Macquarie University, Sydney, Australia).

Nucleotide sequencing. The BK5-T *Eco*RI restriction fragments *Eco*RI-f, *Eco*RI-d, and *Eco*RI-g, which had previously been cloned in pACYC184 by Lakshmidevi et al. (33), were subcloned into the *E. coli* vector pGEM-3Zf (+) (Table 1). *Eco*RI-f and *Eco*RI-d were subcloned directly in both orientations and *Eco*RI-g was digested with *Hin*dIII and the five fragments generated were individually cloned in both orientations into pGEM-3Zf (+). All the clones were subjected to exonuclease III treatment and a series of subclones containing deleted fragments was used for sequencing templates. Plasmid DNA for sequencing was purified by the Qiagen miniprep procedure (Qiagen), followed by PEG precipitation as described by the Applied Biosystems (ABI) *Taq* DyeDeoxy terminator cycle sequencing kit protocol. Determination of the nucleotide sequence was conducted as outlined in the ABI sequencing manual. The sequencing reaction products were analyzed on an ABI model 373A automated sequencer.

The nucleotide sequence across junction points between clones was determined by chromosome walking, using synthesized oligonucleotides and purified BK5-T phage DNA as template. Assembly of the nucleotide sequence was conducted using Sequencher software (Sequencher 3.0, Gene Codes Corporation, Ann Arbor, Mich.). Computer analyses and database searches were conducted using programs available at the Australian National Genomic Information Service.

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† This report is dedicated to the memory of Barrie E. Davidson, who passed away in July 2000.

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TABLE 1. Strains and plasmids used in this investigation

Strain or plasmid	Relevant characteristics[a]	Reference
Strains		
JM107	*endA1 gyrA96 thi hsdR17 supE44 relA1* λ⁻ Δ*(lac-proAB)* (F′ *traD36 proAB lacI*^{q} *ZΔM15*)	65
H2	Indicator strain for BK5-T phage	CSIRO Dairy Research Laboratory
Plasmids		
pTRKH2	Em^r; *E. coli* lactococcal shuttle vector, high-copy-number vector	46
pJDC9	Em^r; *E. coli* vector, one functional transcription terminator, contains pUC19 polylinker and *lacZ′* region	12
pGEM-T	Ap^r; *E. coli* cloning vector	Promega, Madison, Wis.
pMU1252	Tc^r Cm^s; *Eco*RI-d from BK5-T cloned in pACYC184	32
pMU1253	Tc^r Cm^s; *Eco*RI-f from BK5-T cloned in pACYC184	32
pMU1254	Tc^r Cm^s; *Eco*RI-g from BK5-T cloned in pACYC184	32
pCM25	Em^r; BK5-T 2.7-kbp *Pst*I fragment cloned in pJDC9	This study
pCM29	Em^r; 921-bp PCR product containing BK5-T *orf49* cloned into the *Sma*I site of pTRKH2	This study
pCM30	Em^r; 306-bp PCR product containing the repeat (DR1 to DR3 and IR4) sequences in *orf49* cloned into the *Sma*I site of pTR KH2	This study
pCM30.2	Em^r; 250-bp exonuclease deletion version of pCM30	This study
pCM30.4	Em^r; 275-bp exonuclease deletion version of pCM30	This study
pCM31	Em^r; 306-bp PCR product containing the repeat (DR1 to DR3 and IR4) sequences in *orf49* cloned into the *Sma*I site of pTR KH2 in the opposite orientation to pCM30	This study
pCM31.8	Em^r; 111-bp exonuclease deletion version of pCM31	This study
pCM31.31	Em^r; 177-bp exonuclease deletion version of pCM31	This study
pCM31.35	Em^r; 205-bp exonuclease deletion version of pCM31	This study

[a] Em, Ap, Cm, and Tc refer to antibiotic resistance markers erythromycin, ampicillin, chloramphenicol, and tetracycline, respectively.

Sequence runoff experiments. Two oligonucleotides, COS1 (5′-GACCATCA TGGATAACTTGGC-3′) and COS2 (5′-CGCCAACAAGCACTTGCGAG-3′), were designed approximately 200 bp from either end of the linear BK5-T genome. These oligonucleotides were used for linear amplification of DNA using three different preparations of purified unligated BK5-T phage DNA as sequencing template, and the nucleotide sequences of the amplicons were determined as described above.

Nucleotide sequence accession number. The sequence reported here is available in GenBank under accession no. AF176025.

RESULTS

Complete nucleotide sequence of BK5-T. Boyce et al. (8) determined the nucleotide sequence of 18,935 bp of the BK5-T genome, and the remaining 21 kbp is reported here. The total circular length of the BK5-T genome was determined to be 40,003 bp. The codon usage of the BK5-T ORFs was determined and found to correlate with the codon usage of the host lactococcal genome.

Analysis of BK5-T ORFs. A schematic diagram of the BK5-T ORFs is shown in Fig. 1. The BK5-T ORFs were numbered sequentially along the genome, as described for other phages (10, 26, 27, 55), in contrast to the previous nomenclature of Boyce et al. (8), which was based on the number of codons in the ORF (Table 2). A total of 63 ORFs which met the following criteria were identified: (i) the ORF contained greater than 40 codons; (ii) the ORF was preceded by an identifiable ribosomal binding site (RBS) (4 to 12 nucleotides from the putative start codon) or was likely to be translationally coupled to the preceding ORF; and (iii) the ORF began with either an AUG, GUG, AUA, or UUG codon. In addition, several ORFs that had previously been identified by Boyce et al. (8) that did not meet all of the above criteria were included for consistency.

The deduced amino acid sequences of all the ORFs were compared with protein sequences in a nonredundant peptide sequence database encompassing GenPept, TREMBL, SWISS-PROT, and PIR and using the TFASTA, FASTA, or BLASTP comparison programs (2). Significant homologies are shown in Table 2 and are discussed below.

(i) ORF1 and ORF2. The predicted size of the gene products encoded by *orf1* and *orf2* and the location of these genes immediately downstream from the *cos* site are indicative of the terminase subunits of other phages, including coliphage λ (4), *Streptococcus thermophilus* phages φ7201, DT1, φSfi19, and φSfi21 (16), and *L. lactis* phage sk1 (10). Terminase subunits form a hetero-oligomeric complex which functions in concert to bind and nick DNA at the *cos* site prior to packaging of the DNA concatamers into phage heads. Terminase activity is an ATP-dependent process, and an A-type Walker nucleoside triphosphate-binding motif (62) was identified between residues 49 and 53 in the putative BK5-T large terminase subunit ORF2. No such motif was detected in ORF1. In addition to homology with streptococcal phages (Table 2), BK5-T ORF1 exhibited homology to putative packaging protein GP3 found in three *Salmonella enterica* serovar Typhimurium bacteriophages belonging to the Podoviridae family. Although the homology between the two proteins is low (23% over 163 amino acids), it suggests an evolutionary link between two quite distantly related phage species.

(ii) ORF5. The amino acid sequence of ORF5 showed 56 to 57% identity to four uncharacterized streptococcal phage proteins (16, 39, 58), 24% identity to ORF4 of *Staphylococcus aureus* φPVL (26), and 21% identity with GP34 of *Streptomyces actinophage* phage φC31 (54). The last two proteins showed sequence homology to the experimentally determined portal protein of coliphage HK97 (19). Portal proteins create a multisubunit ring structure that serves as an entry point for the translocation of DNA into the phage head (3).

(iii) ORF6. ORF6 shares homology with phage proteins of unknown function from *S. thermophilus, Lactobacillus gasseri*, and *Pseudomonas aeruginosa* (Table 2) and exhibits homology to a series of endopeptidase ClpP proteins encoded in the

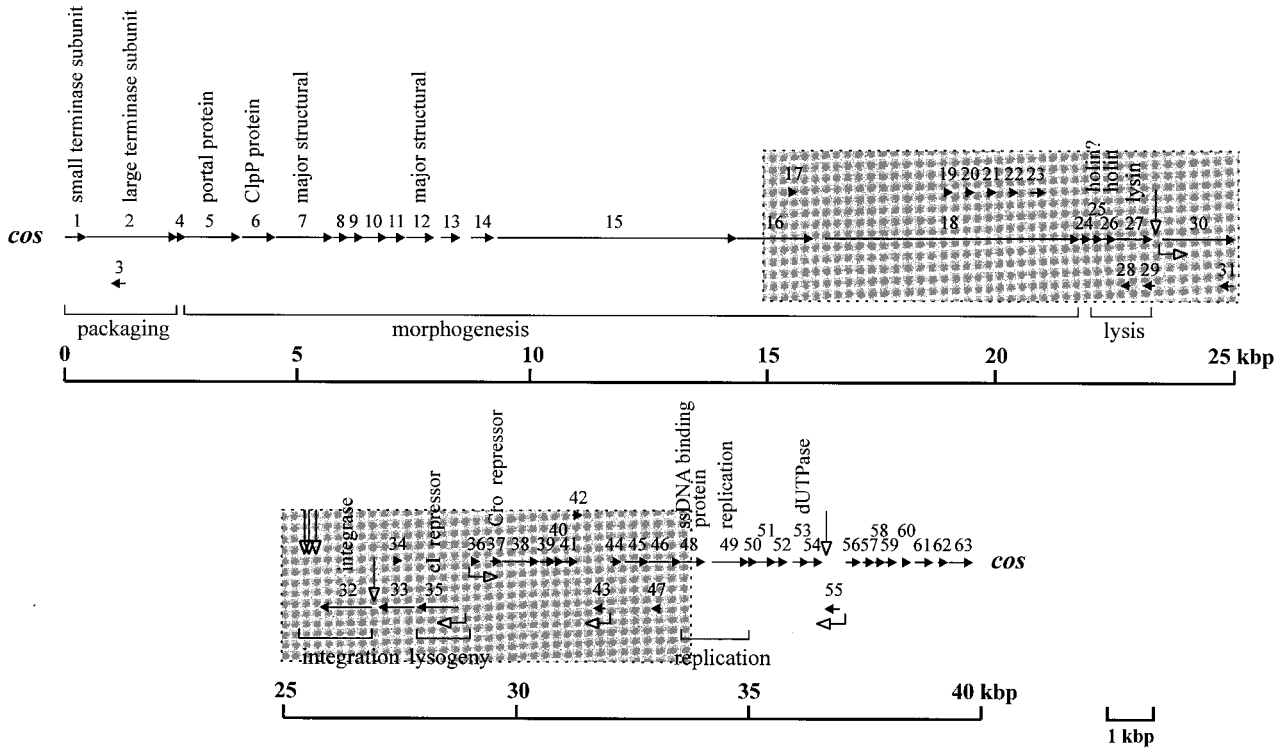

FIG. 1. Genetic map of the BK5-T genome. The map is linearized at the cohesive ends and arbitrarily split at the phage *attP* site located between *orf31* and *orf32*. The grey-boxed areas indicate ORFs previously determined by Boyce et al. (8). The horizontal arrows indicate ORFs and their orientation while the numbers above the arrows indicate ORF designation. Open-headed right-angled arrows indicate positions of putative promoter sequences and their direction of transcription initiation and open-headed vertical arrows indicate the positions of palindromic sequences that may be involved in termination of transcription. Bracketed regions below the map indicate the ORFs associated with the functional modules as described in the text. Putative functions assigned to the BK5-T ORFs are described above the relevant ORFs.

genome of *E. coli*, plants, and higher eukaryotes, but not to the ClpP protein from *L. lactis* (22). The highly conserved Ser^{85} and His^{108} and surrounding residues, which form the proteolytic cleavage site in ClpP protein sequences, were conserved in both BK5-T ORF6 and the *L. lactis* ClpP protein.

(iv) ORF7. The deduced amino acid sequence of ORF7 exhibited significant homology to major structural proteins from four *S. thermophilus* phages, *Bacillus subtilis* phage ϕ105 (ORF27), *Lactobacillus casei* A2, *P. aeruginosa* D3, and *S. aureus* phage ϕPVL (ORF7) (Table 2). The BK5-T *orf7* encodes a major structural protein (see below).

(v) ORF12. The predicted amino acid sequence of ORF12 showed approximately 45% identity with ORFs of unknown function from *S. thermophilus* phages ϕSfi19 (ORF203) and ϕSfi21 (ORF202) (15) and with an experimentally determined small major structural protein from *S. thermophilus* phage ϕ7201 (34). The gene product of BK5-T *orf12* is a major structural protein (see below).

(vi) ORF25, ORF26, and ORF27. The topological positions of *orf25, orf26*, and *orf27* are similar to the arrangement of the holin and lysin cassette observed in prophage-like elements found in the chromosome of many *Bacillus* strains (29) and in bacteriophages infecting *S. thermophilus* (15, 40, 52). The predicted amino acid sequence of ORF25 demonstrates 28% identity to the *Clostridium acetobutylicum* ORF2 of unknown function, which is found upstream from the *lyc* gene, whose gene product exhibits sequence homology to a number of autolytic lysozymes (13). The amino acid sequence of ORF26 shows homology to the putative holin proteins from *S. aureus* phage ϕPVL (26), *B. subtilis* phage SPP1, and experimentally determined holin proteins from *Bacillus licheniformis* (30, 44) (Table 2). The BK5-T ORF25 and ORF26 are therefore likely to encode a two-component holin system similar to that suggested for phages infecting *S. thermophilus* (15, 40, 52). Holin proteins function to disrupt the cell membrane to allow the lysin protein access to the cell wall (67).

The amino acid sequence of ORF27 showed 99% homology to the putative lysin of *L. lactis* ϕ31, 73% homology to the *N*-acetylmuramoyl-L-alanine amidase (Pal) of pneumococcal phage Dp-1 (51), and 30% identity to putative lysin proteins of *S. thermophilus* phages (Table 2), suggesting that ORF27 encodes the BK5-T lysin.

(vii) ORF32. Boyce et al. (9) showed that the gene product encoded by *orf32* exhibited significant homology to a number of lactococcal temperate phage integrase proteins and that it was essential for the establishment and/or maintenance of lysogeny in BK5-T.

(viii) ORF35. Boyce et al. (7) identified a helix-turn-helix DNA binding motif (amino acid positions 33 to 54) as predicted by the Dodd and Egan algorithm (18) and a putative RecA Ala-Gly cleavage site in ORF35. ORF35 showed homology to two putative cI repressor proteins from the lactococcal phages Tuc2009 and ϕLC3 and to an experimentally determined *c*I homologue from phage rlt (Table 2), suggesting that

TABLE 2. Position, predicted molecular sizes, RBSs, and database sequence homologies for the BK5-T ORFs

BK5-T ORF	Previous name[a]	Molecular size (kDa)	Predicted start position	Predicted stop position[b]	Putative RBS[c]	ORF homology[d]	Organism matched	% Identity (over no. of amino acids)	Accession no.
1		18.2	77	550	acuaaaaGAAAGGAGaaaaaaug	ORF21	*S. thermophilus* ϕ7201	46 (151)	AAF43514
						ORF2	*S. thermophilus* DT1	43 (151)	AAD21878
						ORF152	*S. thermophilus* ϕSfi21	44 (151)	AAD41028
						ORF161	*S. thermophilus* ϕSfi19	43 (151)	AAD44055
						ORF149	*L. gasseri* ϕadh	38 (144)	
						ORF4	*L. lactis* subsp. *cremories*	35 (139)	CAA11696
						ORF4	*L. casei* A2	24 (146)	CAA66177
2		75.6	537	2,510	ggcauucuuugauGGAGGugaug	ORF623	*S. thermophilus* ϕSfi19	64 (623)	AAD44056
						ORF623	*S. thermophilus* ϕSfi21	63 (623)	AAD41029
						ORF624	*L. gasseri* ϕadh	59 (625)	CAB52518
						ORF22	*S. thermophilus* ϕ7201	57 (624)	AAF43515
						ORF3	*S. thermophilus* DT1	64 (365)	AAD21880
						ORF4	*S. thermophilus* DT1	62 (231)	AAD21879
3		12.8	1,385	1,065	ucugGAuAGGAaGUugaaauuug				
4		7.7	2,479	2,688	ccuugaugacgaaauagaugaug	ORF5 (head-tail joining)	*S. thermophilus* DT1	45 (55)	AD21881
						ORF23	*S. thermophilus* ϕ7201	42 (55)	AAF43516
						ORF59c	*S. thermophilus* ϕSfi21	44 (55)	AAD41030
						ORF59	*S. thermophilus* ϕSfi19	43 (53)	AAD44057
5		43.5	2,685	3,863	auagaaaaGAAgGGAGGugauug	ORF387	*S. thermophilus* ϕSfi19	56 (389)	AAD44058
						ORF384	*S. thermophilus* ϕSfi21	56 (388)	AAD41031
						ORF6	*S. thermophilus* DT1	57 (381)	AAD21882
						ORF24	*S. thermophilus* ϕ7201	57 (383)	AAF43517
						ORF397	*L. gasseri* ϕadh	51 (356)	CAB52519
						ORFH	*L. casei* A2	24 (291)	CAB63683
						ORF4 (portal)	*S. aureus* ϕPVL	24 (306)	BAA31877
						GP34	*S. actinophage* ϕC31	21 (331)	CAA07104
						ORF1305	*P. aeruginosa* D3	22 (368)	AAD38955
6		2.6	3,906	4,619	uauucuuaGAAAGGAGGuaaaug	ORF7	*S. thermophilus* DT1	53 (238)	AAD21883
						ORF25	*S. thermophilus* ϕ7201	54 (237)	AAF43518
						ORF221	*S. thermophilus* ϕSfi21	52 (237)	AAD41032
						ORF242	*L. gasseri* ϕadh	41 (239)	CAB52520
						ORF891	*P. aeruginosa* D3	38 (167)	AAD38956
						ClpP	*Listeria monocytogenes*	29 (139)	AAF04744
						ClpP	*Streptomyces coelicolor*	25 (146)	AAC70947
7		44.7	4,631	5,845	gaaaaauAAAGGAGacucaaaug	ORF397—major structural	*S. thermophilus* ϕSfi19	60 (395)	AAD44059
						ORF397—major structural	*S. thermophilus* ϕSfi21	59 (399)	AAD41033
						MPL—major structural	*S. thermophilus* ϕ7201	59 (395)	AAD43519
						ORF8—major structural	*S. thermophilus* DT1	65 (289)	AAD21884
						ORF395—major structural	*L. gasseri* ϕadh	47 (409)	CAB52521
						ORF27	*B. subtilis* ϕ105	34 (396)	AB016282
						Major structural protein	*L. casei* A2	22 (357)	CAB63685
						ORF1188—major structural	*P. aeruginosa* D3	20 (400)	AAD38957
						ORF7—major structural	*S. aureus* ϕPVL	25 (359)	BAA31880
8		12.1	5,853	6,176	ugcagGAAAGuAGGuaauuuaug	ORF27	*S. thermophilus* ϕ7201	32 (103)	AAF43520
						ORF106	*S. thermophilus* ϕSfi21	29 (103)	AAC39275
						ORF104	*S. thermophilus* ϕSfi19	29 (103)	AAC39289
						ORF9	*S. thermophilus* DT1	27 (103)	AAD21885
						ORFK	*Leuconostoc oenos* L10	28 (86)	AAA66331
						ORF126b	*L. gasseri* ϕadh	29 (105)	CAB52522
9		1.3	6,151	6,504	uucguGgAAGGAGGcgcaagaug	ORF28	*S. thermophilus* ϕ7201	31 (113)	AAF43521
						ORF10	*S. thermophilus* DT1	30 (113)	AAD21886
						ORF116	*S. thermophilus* ϕSfi19	30 (113)	AAC39290
						ORF116	*S. thermophilus* ϕSfi21	27 (113)	AAC39276
						ORF126c	*L. gasseri* ϕadh	22 (120)	CAB52523
10		19.1	6,506	7,012	gaagaaGAAgGGAGcuuaauaug	ORFA	*L. oenos* L10	39 (147)	AAA66332
						ORF141b	*S. thermophilus* ϕSfi21	26 (163)	AAC39277
						ORF29	*S. thermophilus* ϕ7201	29 (165)	AAC43522
						ORF11	*S. thermophilus* DT1	30 (165)	AAC21887
						ORF140	*S. thermophilus* ϕSfi19	30 (162)	AAC44060
						ORF159b	*L. gasseri* ϕadh	28 (178)	CAB52524
11		14.7	7,009	7,404	gaaaAAAGaAGGaauaacuuaug	ORFI	*L. oenos* L10	37 (110)	AAA66333
						ORF123	*S. thermophilus* ϕSfi19	32 (107)	T09268
						ORF30	*S. thermophilus* ϕ7201	33 (107)	AAF43523
						ORF12	*S. thermophilus* DT1	33 (107)	AAD21888
						ORF123	*S. thermophilus* ϕSfi21	32 (107)	AAC39278
12		20.6	7,435	8,028	auuuaaGAAAGGAauuuaaaaug	ORF203—major structural	*S. thermophilus* ϕSfi19	46 (198)	AAC39293
						ORF13—major structural	*S. thermophilus* DT1	46 (195)	AAD21889
						ORF202—major structural	*S. thermophilus* ϕSfi21	45 (199)	AAC39279
						MPS—major structural	*S. thermophilus* ϕ7201	45 (198)	AAB71820
						ORF E	*L. oenos* L10	43 (193)	AAA66334
						ORF237—major tail	*L. gasseri* ϕadh	28 (134)	CAB52526
13		15.9	8,173	8,592	uaaaauagAGGAGauauacaaug	ORF14	*S. thermophilus* DT1	31 (121)	AAD21890
						ORF117	*S. thermophilus* ϕSfi19	28 (121)	AAC39294
						ORF117	*S. thermophilus* ϕSfi21	29 (121)	AAC39280
						ORF32	*S. thermophilus* ϕ7201	26 (121)	AAF43524

Continued on following page

TABLE 2—*Continued*

BK5-T ORF	Previous name[a]	Molecular size (kDa)	Predicted start position	Predicted stop position[b]	Putative RBS[c]	ORF homology[d]	Organism matched	% Identity (over no. of amino acids)	Accession no.
14		18.2	8,821	9,312	uuaaaaaucAGGAGaaauucaug				
15		183.5	9,394	14,535	aaaauuAGAAAGGAGuaaaaaug	ORF48 (minor capsid)	*Lactobacillus plantarum* ϕgle	30 (1,369)	CAA66745
						ORF465	*L. casei* A2	30 (450)	CAB63691
						ORF16	*S. thermophilus* DT1	37 (320)	AAD21892
						ORF360	*Lactobacillus delbrueckii* LL-H	35 (306)	AAC00548
						ORF15	*S. thermophilus* DT1	22 (1,291)	AAD21891
						ORF33	*S. thermophilus* ϕ7201	24 (552)	AAF43525
						ORF1560 (minor tail)	*S. thermophilus* ϕSfi21	23 (1,753)	AAC39281
						ORF1626 (minor tail)	*S. thermophilus* ϕSfi19	23 (1,789)	AAC39295
16	′410	60	14,532	16,178	agauuAGGAGGuauuaugauuug	ORF34	*S. thermophilus* ϕ7201	25 (564)	AAF43526
						ORF17	*S. thermophilus* DT1	26 (564)	AAD21893
						ORF515	*S. thermophilus* ϕSfi19	27 (567)	AAC39296
						ORF515	*S. thermophilus* ϕSfi21	27 (567)	AAC39282
						ORF I—structural tail	*L. casei* A2	26 (236)	CAB63694
17	64#1	7.7	15,646	15,840	aggaggacaagaauuaaauaaug				
18	1904	205.8	16,178	21,892	uggaaAGAAAGGuaucuauaaug	ORF35	*S. thermophilus* ϕ7201	36 (515)	AAF43527
						ORF18	*S. thermophilus* DT1	32 (738)	AAF21894
						ORF45	*S. thermophilus* O1205	38 (509)	AAC79560
						ORF38	*S. thermophilus* ϕ7201	39 (560)	AAF43530
						ORF1276	*S. thermophilus* ϕSfi21	33 (1,058)	AAC39283
						ORF1291	*S. thermophilus* ϕSfi19	34 (1,058)	AAC39297
19	66	7.6	18,981	19,181	aaaagacGGAaauaacgguaaug				
20	71#1	8.3	19,434	19,649	uguagccuauaugGGUaccaaug				
21	71#2	8.3	19,902	20,117	uguagccuauaugGGUaccaaug				
22	71#3	8.3	20,370	20,585	uguagccuauaugGGUaccaaug				
23	69#1	8.1	20,838	21,044	uguagccuauaugGGUaccaaug				
24	78	8.7	21,907	22,143	aacuagaaacAAGGAGaaaaaug				
25	75	8.9	22,156	22,383	aaaaauAGAAAGcAGGguuaug				
26	95	10.5	22,396	22,683	gauucugAAGGAGaaagaacaug	ORF24	*S. aureus* ϕPVL	37 (70)	BAA31897
						BH0967 (holin)	*Bacillus halodurans*	36 (72)	BAB04686
						GP26	*B. subtilis* SPP1	39 (82)	CAA66519
						XPAF2—holin	*B. subtilis*	40 (81)	P36549
						XPAG2—holin	*B. licheniformis*	35 (81)	BAA08562
27	259	27.9	22,683	23,462	uucacagAAGGAGGcgaauaaug	Lysin	*L. lactis* ϕ31	9 (173)	AC04153
						N-acetylmuramoyl-L-alanine amidase	*Pneumococcus* sp. Dp-1	43 (148)	CAB07986
						ORF288	*S. thermophilus* ϕSfi21	30 (128)	AAC39288
						ORF288	*S. thermophilus* ϕSfi19	30 (128)	AAD44061
						ORF288	*S. thermophilus* ϕSfi11	30 (128)	AAC34421
28	74	8.1	22,996	22,772	ugugaguguaaGAGcagugaaug				
29	86	9.5	23,512	23,252	aguaaucuaaaaauaguuucaug				
30	536	61.9	23,679	25,289	cugauucGGAGugugugagaaug				
31	101	11.3	25,210	24,905	uuauuccuuuaccuucucccaug				
32	374	43.3	27,035	25,911	aaauGGAGuaaaaaucaaauaug	—Integrase	*L. lactis* Tuc2009	99 (374)	AAA32608
						—Integrase	*L. lactis* ϕLC3	99 (374)	AAA32254
						ORF1—Integrase	*L. lactis* rlt	98 (374)	AAB18676
						BH3551	*B. halodurans*	36 (376)	BAB07270
						ORF359	*S. thermophilus* TPJ34	33 (376)	AAC03454
						ORF1	*S. thermophilus* O1205	33 (373)	AAC79517
						ORF8 (Integrase)	*L. plantarum* ϕgle	31 (381)	CAA66758
						—Integrase	*L. lactis* TPW22	30 (379)	AAF12706
						ORFB—integrase	*L. casei* A2	26 (391)	CAA73344
						—Integrase	*S. aureus* ϕ13	30 (372)	CAA57755
						ORF2—integrase	*S. aureus* ϕ42	30 (372)	AAA91615
33	258	28.7	27,949	27,173	agcuagauaGGAGuauuuauaug	Exp3	*L. lactis* MG1363	43 (128)	AAC14595
34	64#2	7.6	27,496	27,690	uuuccauccauuauuuagcaaug				
35	297	33.3	28,898	28,005	aauauuaaggGGAGaugggaaug	cI repressor	*L. lactis* Tuc2009	73 (285)	AAA21825
						Rro—cI repressor	*L. lactis* rlt	74 (280)	AAB18678
						Repressor	*L. lactis* ϕLC3	99 (74)	AAB53017
36	63	7.5	29,155	29,346	gaaggcaaauGGAGGuucuaaug	ORF9	*L. lactis* TPW22	93 (55)	AAF12713
						ORF61b	*L. lactis* u136.1	56 (61)	AAF74094
						ORF61a	*L. lactis* ul36.2	56 (61)	AAF74109
						ORF57	*L. lactis* ϕ31.1	52 (54)	AAF43117
						ORF9	*L. lactis* TP901-1	52 (54)	CAA74618
						ORF8	*L. lactis* rlt	52 (54)	AAB18683
37	79	9	29,574	29,813	aagcaaAGAAAGGAGccaguaug	ORF29	*S. thermophilus* DT1	37 (46)	AAD21905
						ORF67 (Cro)	*S. thermophilus* TPJ34	32 (47)	AAC03458
38	266	30.6	29,826	30,626	cuaguuAGAAAGGcgaaaacaug	ORF5	*L. lactis* rlt	97 (266)	AAB18680
						ORFA	*L. casei* A2	50 (147)	CAA73339
						ORF291	*L. delbrueckii* LL-H	39 (280)	AAB06224
						ORF169a	*L. delbrueckii* mv4	44 (154)	AAG31333
39	111	12.9	30,639	30,974	cuagcuAGAAAGGgaagcacaug	ORF6	*L. lactis* rlt	97 (109)	AAB18681
						ORF13	*L. plantarum* ϕgle	26 (107)	CAA66763
						ORF96	*L. casei* A2	27 (96)	CAB63664
40	42	4.8	30,971	31,099	uuuucAGGAGGgaguggucuaug		*L. lactis* phage ul36.2	95 (42)	AF212847

Continued on following page

TABLE 2—*Continued*

BK5-T ORF	Previous name[a]	Molecular size (kDa)	Predicted start position	Predicted stop position[b]	Putative RBS[c]	ORF homology[d]	Organism matched	% Identity (over no. of amino acids)	Accession no.
							L. lactis strain SMQ-86	95 (42)	AF212844
41	113	13.7	31,112	31,453	uccgcuAGAAAGGAGagauuuug		*L. lactis* ul36.2	91 (68)	AF212847
							L. lactis phage ul36.1	91 (68)	AF212846
							L. lactis phage ul36	91 (68)	AF212845
							L. lactis strain SMQ-86	91 (68)	AF212844
42	72	8.5	31,333	31,551	acgcuagaaaaGAGaaauuuaug				
43	88	10.3	32,039	31,773	augGAAAGGAGGaaugaagcaug				
44	80	9.6	32,146	32,385	acauAGAAAGGAaauuaaaaaug				
45	169	20	32,457	32,966	auaacaaaucGGAGGAaauaaug	ORF169	*L. lactis* ϕ31	98 (169)	AAC48870
						ORF163	*L. casei* A2	31 (160)	CAB63666
						ORF157	*S. thermophilus* ϕSfi18	35 (165)	AAF63083
						ORF157	*S. thermophilus* ϕSfi11	35 (165)	AAF63054
						ORF157	*S. thermophilus* ϕSfi19	35 (165)	AAF63083
						ORF157	*S. thermophilus* ϕSfi21	35 (165)	AAC72433
						ORF8	*S. thermophilus* O1205	33 (165)	AAC79524
46	234	26.5	32,966	33,670	gaaaguuuGAGGuuuagauaaug	ORF9	*S. thermophilus* O1205	61 (230)	AAC79525
						ORF233	*S. thermophilus* ϕSfi21	61 (230)	AAC72434
						ORF233	*S. thermophilus* ϕSfi19	60 (230)	AAC97920
						ORF233	*S. thermophilus* ϕSfi11	60 (230)	AAC63055
						ORF32	*S. thermophilus* DT1	59 (230)	AAD21908
						ORF240	*L. casei* A2	56 (232)	CAB63668
47	69	7.5	33,231	33,021	ccaaguuaucguaaucaccuaug				
48	70′	19.4	33,670	34,191	aaauuugAAGGAGaaaaauaaug	SSB protein	*B. subtilis*	28 (103)	P37455
						ORFE13	*L. lactis* bIL170	31 (77)	AAC27225
						ORF34	*L. lactis* sk1	30 (76)	AAB70074
49		31	34,325	35,134	uauagaAGAAAGGAGaaaguuug	ORF269	*L. lactis* ϕ31.1	82 (269)	AAF43120
50		8.4	35,115	35,336	agaaguGGAGGcuuuaauaaaug	ORF73	*L. lactis* ϕ31.1	97 (73)	AAF43121
						ORF73	*L. lactis* ul36.1	97 (73)	AAF74098
51		16.2	35,314	35,715	agcaaccgaauuaaugauauaug	ORF14	*L. lactis* r1t	96 (130)	AAB18689
						ORF129	*L. lactis* ul36	97 (129)	AAF74082
						ORF19	*L. lactis* Tuc2009	96 (129)	AAD37104
52		9.4	35,719	35,958	aaauuacaGAGGuauaaaccaug	ORF20	*L. lactis* Tuc2009	99 (79)	AAD37105
						ORF16	*L. lactis* r1t	92 (79)	AAB18691
						ORF79a	*L. lactis* ul36	91 (79)	AAF74083
						ORF79b	*L. lactis* ul36.1	43 (46)	AAF74100
						ORF79	*L. lactis* ϕ31.1	43 (46)	AAF43123
53		13	36,069	36,416	cuaaaauauGAGGuaguaauaug	ORF139b (dUPTase)	*L. lactis* ϕ31.1	82 (92)	AAF43128
						ORF139a (dUPTase)	*L. lactis* ul36	82 (92)	AAF74088
						ORF139b (dUPTase)	*L. lactis* ul36.1	82 (92)	AAF74104
						ORF3 (dUPTase)	*L. lactis* S114	80 (92)	CAA72644
						ORF20 (dUPTase)	*L. lactis* r1t	80 (92)	AAB18695
54		11.1	36,413	36,712	ggcucaacuggGGAGGugugaug	ORF120	*L. lactis* ϕ31.1	39 (103)	AAF43131
						ORF120a	*L. lactis* ul36	39 (103)	AAF7409
55		12.9	37,084	36,746	aaaGAGGAAuggauauuuacaug				
56		12.7	37,207	37,530	ucaaugAAaGAGGUucaggaaug	ORF29	*L. lactis* sk1	94 (107)	AAB70069
						ORFE22	*L. lactis* bIL170	93 (107)	AAF009630
57		9.3	37,604	37,834	gacugguggGGAGGgauugaaug				
58		6.7	37,831	38,004	uuugauAAGGAGGacaaccaaug	ORF66	*L. lactis* ϕ31.1	55 (60)	AAF43134
59		6.9	38,001	38,180	aacguguucggGGAGGaaugaug				
60		5.5	38,444	38,587	uaaacucaacuGGAGGaaaaaug				
61		15.5	38,664	39,065	uacucGGAGGgacuuuuuaauug	16.9-kDa protein	*L. lactis* S114	35 (81)	CAA72647
62		4.8	39,288	39,416	caaguaguGAGugaaaguccuug				
63		20.6	39,434	39,949	ugauGAAAGGAGaugcuaucuug	ORF170	*L. gasseri* ϕadh	46 (162)	CAB52516
						ORF170	*S. thermophilus* ϕSfi19	44 (154)	AAD44054
						ORF20	*S. thermophilus* ϕ7201	44 (154)	AAF43513
						ORF175	*S. thermophilus* ϕSfi21	41 (155)	AAD41027
						ORF46	*S. thermophilus* DT1	42 (154)	AAD21922

[a] Boyce et al. (8) designation for previously identified ORFs.
[b] The stop position includes the stop codon.
[c] The sequence shown includes the putative start codon and the immediate upstream 20 nucleotides. The putative RBS is shown in uppercase letters.
[d] The protein sequences in the databases which show homology with the respective BK5-T ORFs are shown. Empty cells indicate that no significant homologies were observed; experimentally determined functions are indicated after a dash.

orf35 encoded the BK5-T *c*I repressor homologue. It was predicted that ORF35 facilitated lysogenic development by repressing a putative BK5-T promoter, P_2, found in the putative BK5-T immunity region (7).

(ix) ORF36. *orf36* was previously proposed to encode the BK5-T Cro protein homologue, based on its size and position relative to the putative BK5-T immunity region (7). However, no helix-turn-helix DNA binding motif was identified in ORF36, and preliminary data (Mahanivong, unpublished data) suggested that ORF36 did not bind to the putative BK5-T immunity region. ORF36 shares strong identity (93% over 55 amino acids) with lactococcal phage TPW22 ORF9 (47) and 52% identity over 54 amino acids with the uncharacterized ORF8 from lactococcal phage rlt (60), ORF57 from ϕ31.1 (20), and ORF9 from TP901-1. It is interesting that the relative position of BK5-T ORF36 is dissimilar to the homologues from

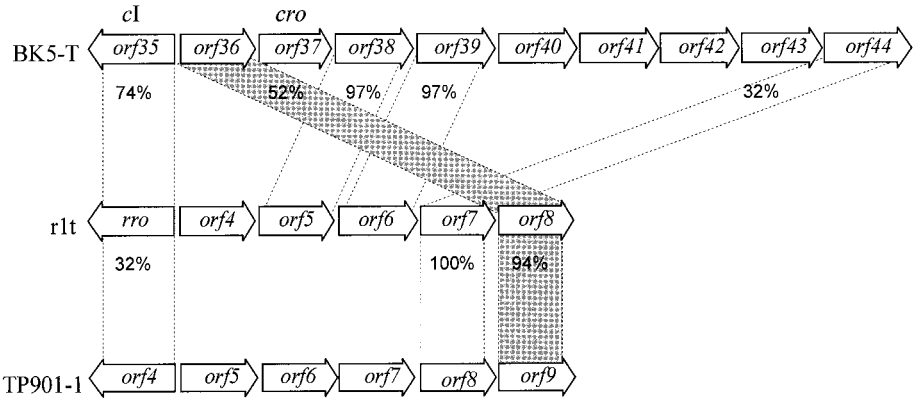

FIG. 2. Comparison of the gene location near the immunity region of *L. lactis* phages. The relative locations of the ORFs in BK5-T (7), rlt (43), and TP901-1 (25) are indicated. Horizontal arrows indicate individual ORFs, with the ORF name shown within the arrows and the direction of the arrowhead indicating its orientation. The global amino acid percentage of identity between ORFs is shown between the broken lines. The horizontal hatched area illustrates the discordant genetic arrangement of BK5-T *orf36* with the rlt and TP901-1 homologues *orf8* and *orf9*, respectively.

rlt and TP901-1 (Fig. 2), suggesting a horizontal introduction of the ORF into the genome or a genetic rearrangement event.

(x) ORF37. *orf37* is located 231 bp downstream of *orf36*. Analysis of the predicted amino acid sequence of ORF37 identified a putative helix-turn-helix DNA binding motif at amino acid positions 23 to 42. The predicted amino acid sequence of ORF37 demonstrates 30% identity to the Cro homologue from temperate streptococcal phage TPJ34 (ORF67) and ORF39 from lytic streptococcal phage DT1 (Table 2). Preliminary DNA binding studies (Mahanivong, unpublished data) suggest that ORF37 binds to the putative BK5-T immunity region and that it is likely to be the BK5-T Cro homologue.

(xi) ORF38. The identification of a putative helix-turn-helix DNA binding motif (18) at amino acid positions 174 to 193 suggests that *orf38* encodes a DNA binding protein. The predicted amino acid sequence of ORF38 shows 97% identity to ORF5 of *L. lactis* phage rlt (60) and both genes are located in the same relative position immediately downstream of their Cro protein homologues (Fig. 2). BK5-T ORF38 also shows 50% identity to *L. casei* phage A2 ORFA, which is located immediately downstream of its *c*I homologue (31).

(xii) ORF48. The predicted amino acid sequence of ORF48 exhibits limited homology with the putative single-stranded DNA binding (SSB) proteins encoded by *B. subtilis, S. aureus* phage ϕPVL, and *B. subtilis* phage SPP1 and with ORFs of unknown function from *L. lactis* phages sk1 and bIL170 (Table 2). SSB proteins are a ubiquitous class of proteins identified in prokaryotic and eukaryotic organisms. They function primarily to bind single-stranded DNA and play important roles in DNA replication, recombination, and repair. A number of SSB proteins have been characterized and some functionally relevant structural features have been identified. There are aromatic amino acid residues in the N terminus of *E. coli* and mitochondrial SSB proteins (36) that are important for binding single-stranded DNA, but these residues are not observed in BK5-T ORF48. The greatest region of homology between BK5-T ORF48 and the SSB protein sequences occurs at the acidic C-terminal end of the proteins. The C-terminal six amino acids (DEDLPF) of BK5-T ORF48 were compared with protein databases and showed matches to other putative SSB proteins of bacterial (*B. subtilis, E. coli, L. lactis*, and *Thermus aquaticus*) or phage (including Tuc2009, ϕadh, SPP1, and ϕ105) origin (data not shown). The functional relevance of this acidic domain is unknown; however, the loss of this domain from the *E. coli* SSB protein (14) results in a nonfunctional protein.

(xiii) ORF49. ORF49 contains a helix-turn-helix region (Fig. 3A), suggesting that it is a DNA binding protein, and its nucleotide sequence contains a series of direct and indirect repeats, which are often indicative of a phage origin of replication (10, 21). The amino acid sequence of BK5-T ORF49 shows 82% identity to ORF269 in the recombinant lytic lactococcal phage ϕ31.1 (20) and 80% identity to ORF235 of lactococcal phage ul36.1 (6) (Table 2). Mutant phage ϕ31.1 was isolated after ϕ31 infection of cells expressing a phage resistance phenotype (Per) due to a plasmid-encoded ϕ31 *ori*. Phage ϕ31.1 overcame the resistance by acquiring 7.8 kbp of DNA from the host chromosome, which contained an alternative phage *ori*. The *ori* was postulated to be contained within ORF269. Mutant phage ul36.1 arose as a variant resistant to the phage resistance mechanism AbiK and had incorporated noncontiguous sections of the host chromosome DNA, one of which contained ORF235, also postulated to contain a phage *ori*.

(xiv) ORF53. The deduced amino acid sequence of ORF53 demonstrates significant homology with ORF20 and ORF139 of *L. lactis* phages rlt (60) and ϕ31.1 (20), respectively. Furthermore, it shows homology to dUTPase enzymes of bacterial and eukaryotic origin. The function of this enzyme, which hydrolyzes dUTP to dUMP and pyrophosphate, is to regulate intracellular levels of dUTP and to prevent incorporation of dUTP into DNA (53). The absence of dUTPase in *E. coli* leads to an increased recombination and mutation rate during DNA replication (59).

(xv) ORF55. ORF55 did not show any significant homology with other protein sequences in the databases and its function is unknown. Interestingly, *orf55* is located in the opposite orientation to the surrounding ORFs and is preceded by a putative RBS (AGAGGAA) and promoter (−10 [TATAAT] and −35 [TTCAAT]) located 11 and 25 nucleotides upstream from the ATG start codon, respectively. The codon usage of ORF55 is similar to that of the rest of the phage. A region of dyad

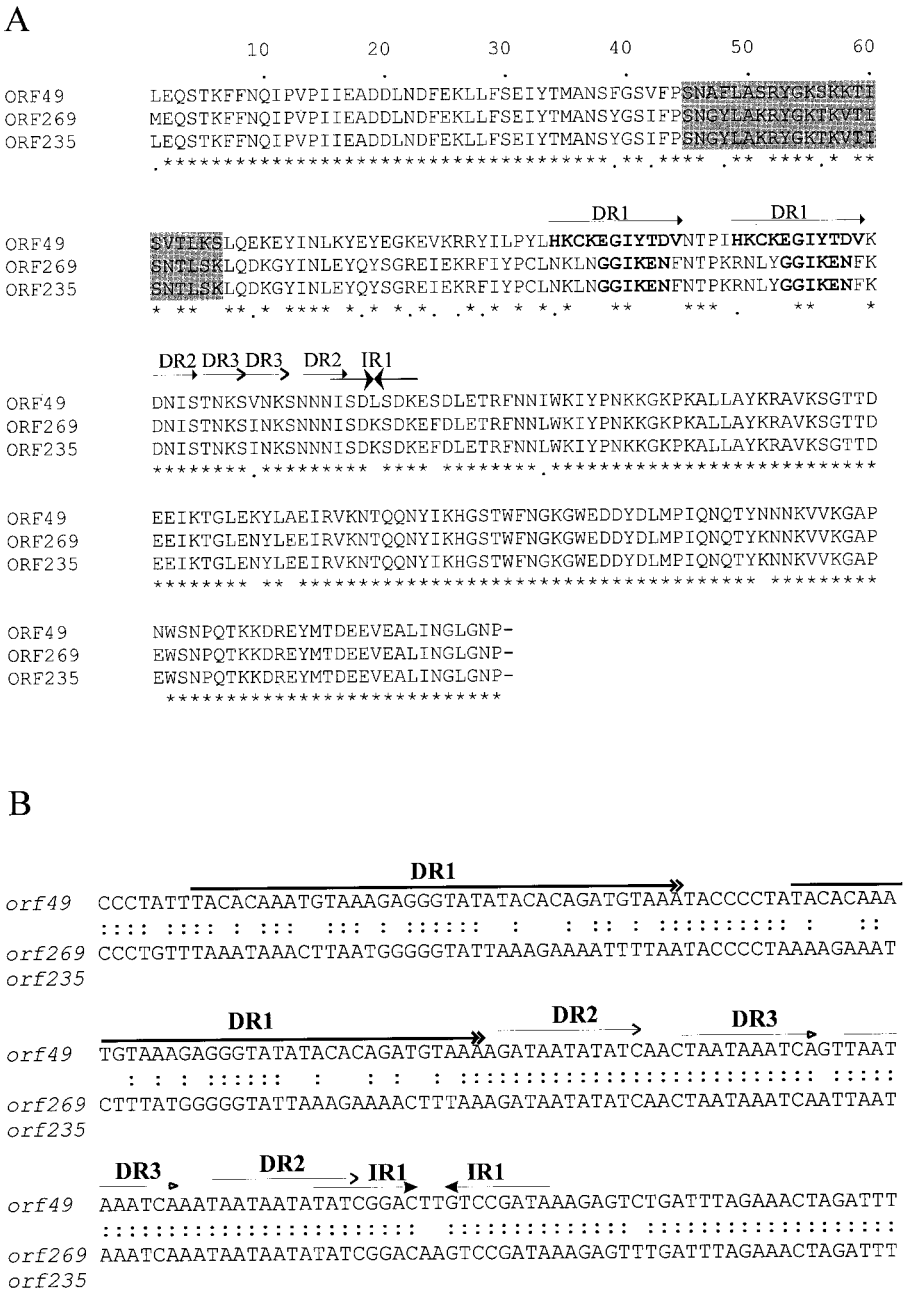

FIG. 3. (A) Comparison of the ORF49, ORF269, and ORF235 amino acid sequences from *L. lactis* phages BK5-T, φ31.1, and ul36.1, respectively. The asterisks indicate identical amino acid residues and the periods indicate residues conserved across all three sequences. Amino acid residues within the putative helix-turn-helix DNA binding motif and the longest direct repeat sequence are indicated by the shaded regions and in bold, respectively. The positions of the four repeat sequences (and their designations) found in all three ORFs are indicated by the horizontal lines with matching arrowheads. (B) The nucleotide sequences of the repeat sequences (DR1 to DR3 and IR1) in BK5-T *orf49* are shown in the top strand. The sequence below represents the homologous region from *orf235* and *orf269* from *L. lactis* phages ul36.1 and φ31.1, respectively, which are identical. The colons indicate identical nucleotides between BK5-T *orf49* and the other *L. lactis* phage sequences. The nucleotide positions of the four repeat sequences (DR1 to DR3 and IR1) in BK5-T are indicated by the corresponding arrows underneath the repeat label.

symmetry is located 9 bp downstream of the stop codon of *orf55* but is surrounded by a string of A nucleotides rather than the T nucleotides indicative of a rho-independent transcription terminator. The location and orientation of this ORF have not been reported in the genomes of other characterized lactococcal temperate phages (26, 27, 60). Located within ORF55, but oriented in the opposite direction, is another putative promoter (−10 [TATAAT] and −35 [ATGTTC]), which could direct transcription through *orf56* and downstream genes. The arrangement of oppositely oriented putative promoters within and upstream of *orf55* would generate transcripts with overlapping and complementary 5′ ends. Similar overlapping tran-

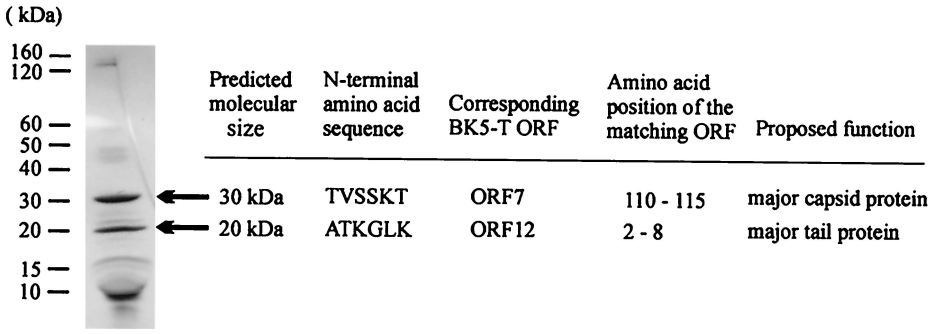

FIG. 4. SDS-PAGE analysis of the BK5-T structural proteins. The N-terminal amino acid sequence of the marked proteins is indicated, together with the position of the N-terminal sequence within the corresponding BK5-T ORFs and their proposed functions. The size and location of the molecular mass markers used (Benchmark ladder; Life Technologies Inc., Rockville, Md.) are given in the left margin.

scripts are observed in the λ *oop* region (28), and this region may represent a control point in the BK5-T gene expression.

Determination of the sequence of the BK5-T *cos* termini. BK5-T had previously been demonstrated to contain cohesive termini (8). Sequence runoff experiments were conducted on purified phage DNA to determine the DNA sequence of *cosN*, which defines the single-stranded overhang. COS1 and COS2 were used as primers to determine the sequence of ligated and unligated *cos* ends. The nucleotide sequences obtained from unligated phage DNA and ligated DNA were compared, and the absence of a 12-bp sequence in the unligated preparations indicated that BK5-T contained a 3′ overhang of 12 bp with the sequence 5′-CACACACATAGG-3′. Thus, the BK5-T *cos* site, like those of all other phages infecting gram-positive organisms studied to date, possesses a single-stranded 3′ overhang (11, 26, 35, 60).

N-terminal sequence of phage structural proteins. BK5-T phage was purified by CsCl density gradient centrifugation, and the proteins were analyzed by SDS-PAGE. Two major bands, with molecular masses of 20 and 30 kDa, and several bands of lower molecular mass were observed (Fig. 4). The proteins were transferred to a polyvinylidene difluoride membrane and the N-terminal sequence of the 20- and 30-kDa proteins was determined. The first six amino acids of the 30-kDa protein were TVSSKT. This sequence was identical to residues 110 to 115 of the BK5-T ORF7. The absence of the first 109 amino acids suggests that ORF7 is cleaved prior to the formation of the mature protein. The estimated molecular mass of the full length ORF7 is 45 kDa, whereas a 32-kDa protein is predicted for the mature ORF7 protein, which is consistent with observed experimental results (Fig. 4).

N-terminal sequence analysis of the 20-kDa BK5-T structural protein revealed the sequence ATKGLKM, which is identical to amino acids 2 to 8 of BK5-T ORF12. It appears that the N-terminal methionine residue was removed in the mature protein. The amino acid sequence of ORF12 showed homology to major structural proteins from a number of S. thermophilus phages, a leuconostoc phage protein, and the experimentally determined major tail protein from *L. gasseri* ϕadh (Table 2).

Identification of the putative BK5-T origin of replication. Comparison of the genome organization of BK5-T with that of other lactococcal bacteriophages (41) suggested that the BK5-T *ori* was likely to be located in *orf49*. ORF49 contains a helix-turn-helix DNA binding motif (amino acid positions 47 to 66) (Fig. 3A) and may bind to the various repeat sequences within *orf49* as has been observed in coliphage λ (48) and phage Tuc2009 (41). The genetic arrangement of the BK5-T *orf48* and *orf49*, encoding a putative SSB protein and a replication protein, respectively, is similar to that of phage Tuc2009 (41), but there is no sequence similarity between the analogous proteins.

It has been shown previously that cloned lactococcal phage *ori* can maintain plasmid replication (63), and similar experiments were conducted to analyze the BK5-T putative *ori*. A BK5-T-derived 2.7-kbp *Pst*I restriction fragment containing *orf46* to *orf51* was ligated into pJDC9 and the plasmid (pCM25) was cloned into *E. coli*. pCM25 does not contain a gram-positive replicon and therefore is unable to replicate in *L. lactis* unless the cloned fragment contains a functional *ori*. All attempts to introduce pCM25 into *L. lactis* were unsuccessful. This suggested that either the fragment did not contain sufficient elements to sustain autonomous replication or expression of phage-encoded proteins was necessary for DNA replication.

Attempts to introduce the same *Pst*I fragment downstream from the constitutive *L. lactis* promoter P32 in a modified pJDC9 plasmid were unsuccessful in both *E. coli* and *L. lactis*.

Consequently, an alternative approach was used to investigate the BK5-T putative origin of replication. It has been previously shown that when a phage *ori* is introduced to host cells in *trans*, it confers a resistance phenotype to the host strain (21, 45). It is hypothesized that the cloned *ori* acts as a false target for phage replication and causes a decrease in phage DNA replication. This can be monitored by a reduction in both plaque numbers and quantity of phage genomic DNA (21, 41). A 921-bp PCR product containing BK5-T *orf49* was cloned into the high-copy-number vector pTRKH2 to form pCM29, and cells containing this plasmid showed increased phage resistance as evidenced by reduced plaque size and number (efficiency of plating [EOP] = 1×10^{-5}). Control strains containing pTRKH2 with no insert or an insert of BK5-T DNA from the structural region were sensitive to phage infection (EOP = 1).

A number of direct and inverted repeat sequences were identified within *orf49* (Fig. 3B) and these sequences are likely to define the BK5-T *ori*. A 306-bp PCR product containing this region was cloned into pTRKH2 in both orientations (plasmids

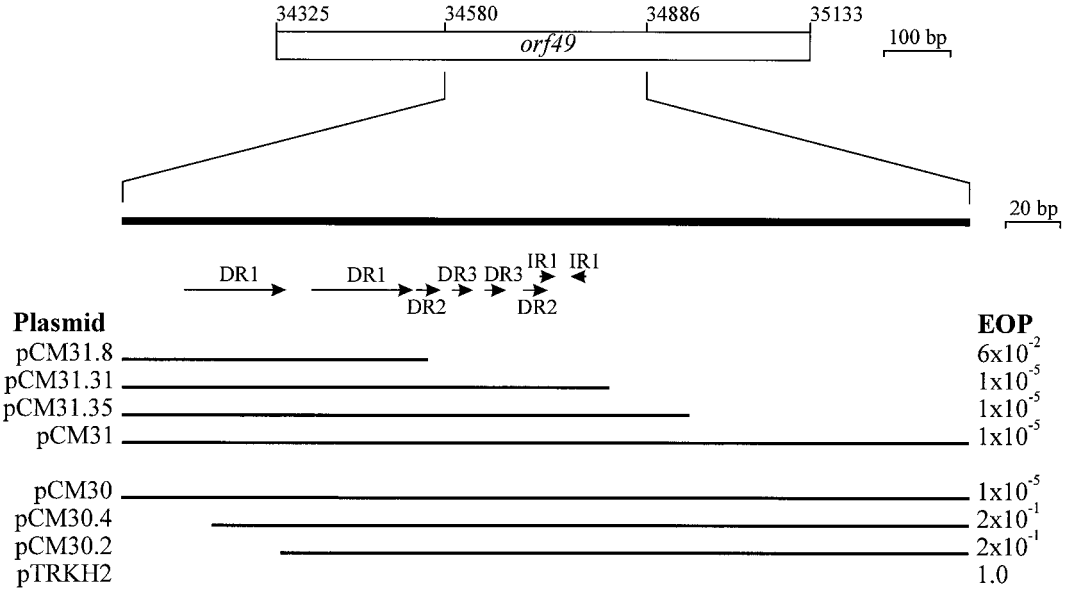

FIG. 5. Delineation of the putative BK5-T origin of replication. The thick solid bar represents the internal *orf49* sequence generated by PCR that contained the putative *ori*. Horizontal arrows shown underneath the bar indicate the positions of the four repeat sequences (DR1 to DR3 and IR1). The plasmid designations of the clones containing deletion fragments are listed on the left and the EOP values (averaged from at least four different experiments) are given on the right. The horizontal thin lines indicate the DNA fragments cloned in the plasmid constructs. The plasmids were introduced into lactococcal host strain H2 (Table 1).

pCM30 and pCM31) and shown to confer a similar level of phage sensitivity to the clone containing the complete *orf49* sequence (Fig. 5). Exonuclease III deletion constructs were generated to further define the region required to confer resistance to BK5-T infection. Removal of the first 10 bp of the 5′ end of the DR1 sequence in clone pCM30.4 caused a 4-log decrease in phage resistance, indicating that the DR1 sequence was necessary to effect phage resistance.

Similarly, deletions from the right-hand end (Fig. 5) indicated that the smaller direct and/or inverted repeat sequences were also important, as removal of these sequences (pCM31.8) caused a 3-log decrease in phage resistance.

DISCUSSION

The nucleotide sequence of the temperate lactococcal bacteriophage BK5-T was completely determined and enabled comparative genomic sequence analysis. Molecular characterization of the phage structural proteins, *cos* termini, and replication functions were conducted.

The BK5-T 12-nucleotide single-stranded *cosN* sequence was determined. In numerous other phages, the region surrounding *cosN* contains recognition sequences for the binding of terminase (*cosB*), integration host factor, and other host- or phage-encoded proteins. In phage λ, there are three direct repeat sequences in *cosB*, referred to as R sites, which have been shown to be important for terminase binding (4). Similar repeat sequences were identified in phage sk1 (11) and *P. aeruginosa* phage D3 (50). Although no such repeat sequences were found in the BK5-T genome, it does contain the sequence C_3TC_5 located 20 nucleotides 5′ of *cosN* and a string of 7 consecutive G nucleotides occurs 43 nucleotides 3′ of *cosN* (Fig. 6). *S. thermophilus* phage ϕ7201 contains the sequence C_5GC_5 16 bases 3′ of *cosN*, and ϕSfi19 and ϕSfi21 both contain the sequence C_5GC_4 21 nucleotides 5′ of *cosN* (39). *L. lactis* phage sk1 also contains a string of 8 consecutive C nucleotides found 30 bases 5′ of *cosN* (11). The occurrence of such sequences in these low-GC organisms is unusual and these sequences may constitute recognition sites for terminase binding and activity, although this remains to be determined. A 130-bp region surrounding BK5-T *cosN* contains a number of runs of four to seven identical bases, primarily A or T (Fig. 6). Similarly, the *cos* site of coliphage λ contains runs of adenines and thymines that are important to DNA bending which occurs upon integration host factor binding (66).

BK5-T *orf7* and *orf12*, which encode the putative major head and tail proteins, were identified. N-terminal amino acid sequence analysis indicated that the BK5-T putative major capsid protein was processed to generate the mature protein.

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                                                                +1
TAATTTTTAATAA***CCCTCCCCCC***TATCTTTTCACTGGGAAAG**CACACACATAGG**TATCGT   60

CTTGCGTGAAAACCCATTTTTCAAAATTTTTATATA***GGGGGGG***TCAAAACACTAAAAGAA 120

AGGAGAAAAA 130
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FIG. 6. The 130-bp intergenic *cosB* sequence. The *cosN* sequence is shown in bold. Underlined residues indicate runs of at least four identical bases. The C_3TC_5 and G_7 sequences are indicated in bold italics.

Similar proteolytic cleavage was observed in the mature capsid proteins of a number of phages, including ϕPVL (26) and *L. gasseri* ϕadh (1). BK5-T *orf5, orf6*, and *orf7* encode gene products that are involved with phage head structure and assembly and are likely to have been acquired as a functional unit from a common ancestral phage (54). They encode the putative portal, ClpP protease, and major capsid proteins, respectively, and show identical arrangement to three functionally equivalent genes found in four phages infecting *P. aeruginosa* (50), *S. aureus* (26), *S. actinophage* (54), and *E. coli* (19). It is tempting to speculate that the putative BK5-T ClpP protease (ORF6) may be involved in the cleavage of ORF7, as observed in coliphage HK97, where the phage-encoded GP4 is a protease that cleaves the N-terminal 102 amino acids from the major capsid protein encoded by the adjacent downstream gene (19).

Investigations of the BK5-T replication functions identified a repeat-rich region within *orf49* which conferred phage resistance in a manner suggestive of a phage replication origin. However, the specific function of the repeat sequences in the replication process remains to be determined. ORF49 showed significant homology with ORF269 and ORF235 from lactococcal phages ϕ31.1 (20) and ul36.1 (6). Cloned DNA fragments containing these latter ORFs also conferred resistance to their respective phages (6). These ORF homologues are likely to encode replication proteins which bind to the repeat regions in their coding sequences to initiate phage DNA replication in a similar manner to the coliphage λ O replication protein (56). The greatest region of sequence diversity between BK5-T and its homologues occurs at amino acid positions 40 to 120 (Fig. 3A). This region contains the putative helix-turn-helix DNA binding motif (amino acid positions 45 to 66) and DR1, a possible binding site on the DNA. Interestingly, the nucleotide sequences of the other repeat structures downstream from DR1 were identical (Fig. 3B). The variation in amino acid sequence in the helix-turn-helix motif could indicate different DNA targets for the homologues, as represented by the differences in nucleotide sequence in the region of DR1.

The organization and orientation of the ORFs in BK5-T were similar to those observed in other temperate phages infecting lactic acid bacteria (1, 27, 39, 55, 60). The majority of the ORFs are oriented in one direction, while ORFs involved in lysogeny (ORFs 32, 33, and 35) and possibly regulation (ORF43 and ORF55) are oriented in the opposite direction. Moreover, organization of functional modules within BK5-T revealed a striking correlation with the pattern of functional modules observed in the genomes of many Siphoviridae phages (26, 38, 39, 58, 61), viz. packaging, structure and morphogenesis, lysis, integration, lysogeny (in temperate phages), and replication. A detailed comparison of the genetic organization of BK5-T compared with other phages is presented elsewhere (17).

Examination of the BK5-T genome allowed comparative analyses of genomic exchange at three levels: functional modules, individual genes, and gene segments. The strong conservation of the genetic organization, ORF sequence, and gene sequence of the packaging and morphogenesis modules (ORF63 to ORF18) observed between BK5-T and the streptococcal phages (15, 17) suggests recent divergence of these modules from a common ancestor. In contrast, ORF30 to ORF61 showed greater identity with phages infecting lactococci. This latter region encompasses the integration, lysogeny, and replication modules. This structure suggests that BK5-T is a recently evolved chimeric phage containing modules that are derived from two distinct ancestral phages. Within modules, differences were also observed in the relative location of homologous ORFs. The BK5-T *orf36* was located upstream of the putative BK5-T *orf37 cro* homologue (Fig. 2). In contrast, the ORF36 homologues from rlt and TP901-1 were both located four ORFs downstream from their corresponding *cro* gene homologues. Also the BK5-T *orf63* was located 5′ of the *cos* site, whereas the homologous ORFs from the skI and bIL170 were located 3′ of their respective large terminase subunits.

The acquisition or deletion of entire ORFs was also evident. The BK5-T ORF14 homologue was absent in the streptococcal phages, indicating that it was either introduced into the BK5-T genome via a nonhomologous event or lost from the streptococcal phage genomes since their divergence. The BK5-T *orf56* is surrounded by a 30-bp direct repeat sequence, which could facilitate its acquisition or deletion by a specific recombination event. ORF56 showed significant homology to ORFs from lactococcal phages sk1 and bIL170 (Table 2); however, no repeat sequences were found surrounding the ORFs in these phages. A single copy of the 30-bp direct repeat in rlt may be the remnant of an ORF deletion by recombination occurring between the repeat sequences. Examples of ORF deletions within flanking direct repeat sequences were also observed between phage bIL67 and *c*2 (37).

Evolution within ORF sequences was evident, as indicated by the conservation of functional domains linked to regions that show no apparent homology. Comparison of the amino acid sequence of the BK5-T *c*I homologue ORF35 indicates that the C-terminal domain had greater than 94% identity with the homologous proteins from lactococcal phages Tuc2009 and rlt. In contrast, there was negligible homology in the N-terminal domain of these proteins. The N-terminal domain contains the putative DNA binding motif and this divergence presumably accommodates phage-specific DNA recognition sequences.

As more sequence data on a variety of bacteriophage genomes becomes available, there is much focus on the genomic evolution and relationships between phages. These data suggest that phages have evolved by exchange of functional modules, individual genes, or gene segments by various genetic recombination events (5, 38).

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