

Identification of Prophage Genes Expressed in Lysogens of the *Lactococcus lactis* Bacteriophage BK5-T

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Bacteriophage BK5-T is a small isometric-headed temperate phage that infects *Lactococcus lactis* subsp. *cremoris*. Northern (RNA) analysis of mRNA produced by lysogenic strains containing BK5-T prophage revealed four major BK5-T transcripts that are 0.8, 1.3, 1.8, and 1.8 kb in size and enabled a transcription map of the prophage genome to be prepared. The position and size of each transcript corresponded closely to the position and size of open reading frames predicted from the nucleotide sequence of BK5-T. Analysis of the transcripts suggested that one of them was derived from the gene encoding the BK5-T integrase and another was from the gene encoding the BK5-T homolog of the λ *cI* repressor. Computer analysis of the nucleotide sequence upstream of the BK5-T *cI* homolog predicted the presence of a pair of divergent promoters and three inverted repeat sequences, features characteristic of temperate-phage immunity regions. By analogy with λ , the three inverted repeat sequences could be binding sites for *cI* or Cro homologs and the two divergent promoters could initiate transcription through the BK5-T equivalents of *cI* and *cro*.

BK5-T is a temperate lactococcal bacteriophage that can be isolated by mitomycin induction of lysogenic strains carrying the BK5-T prophage (14). The phage genome is 40 kbp in length and has cohesive ends. The nucleotide sequence of approximately half of the BK5-T genome has been determined, and the BK5-T *int* and *attP* regions of the phage have been identified (2, 3).

Because BK5-T can be induced by mitomycin, it is likely that maintenance of the lysogenic state is controlled by the expression of a phage repressor protein which represses expression of phage lytic genes and is inactivated by proteolytic cleavage during the mitomycin-induced SOS response (9, 12, 13, 19, 21, 31). To investigate the control of lysogeny in BK5-T, an analysis of BK5-T prophage transcripts was undertaken. The data presented here indicate that only a small number of BK5-T genes, clustered in two regions of the BK5-T genome, are expressed by the prophage. One of these regions contains an open reading frame (ORF) which shares significant sequence homology with a number of bacterial LexA repressor proteins and phage repressors. It appears likely that this ORF is the BK5-T homolog of the λ repressor.

MATERIALS AND METHODS

Phages, bacteria, and plasmids. *Lactococcus lactis* is used throughout the text to indicate *L. lactis* subsp. *cremoris*. The BK5-T lysogens *L. lactis* BK5 and H2L, the indicator strain *L. lactis* H2, and other phages and plasmids used in this study have been described previously (3, 14, 15). Growth conditions for bacterial cultures are described in one of the accompanying papers (3).

DNA techniques. Digestions, electrophoresis, and other DNA manipulations were performed as described elsewhere (25) or as specified by the manufacturers. Isolation of BK5-T DNA from lysogenic strains and computer analysis of nucleotide sequence data were performed as described previously (3).

RNA preparation. Total cellular RNA was prepared from *L. lactis* strains by a modification (5) of the method of van Rooijen and de Vos (35). All manipulations after cell lysis were carried out in the presence of 10 μ M vanadylribo-

nucleoside complexes (5 Prime \rightarrow 3 Prime Inc., Boulder, Colo.) to inhibit RNase activity. Preparation of Northern (RNA) blots and hybridization procedures were as described previously (5). Transcript sizes were determined by comparison of their electrophoretic mobilities with those of a set of single-stranded RNA markers (Bethesda Research Laboratories, Gaithersburg, Md.).

RESULTS

Transcripts produced by BK5-T prophage. A Northern blot of total RNA from two BK5-T lysogens, *L. lactis* BK5 and *L. lactis* H2L, and the indicator strain, *L. lactis* H2, was probed sequentially with the ³²P-labeled BK5-T *EcoRI* a, b, d, f, and g (*EcoRI*-c plus *EcoRI*-e) fragments, which cover the entire BK5-T genome (14).

Four different-sized transcripts (1.8, 1.3, 0.8, and 0.3 kb) produced by the lysogens *L. lactis* H2L and *L. lactis* BK5 hybridized strongly with *EcoRI*-a (Fig. 1A; Table 1). *EcoRI*-b and *EcoRI*-g did not hybridize significantly with any transcripts from either lysogen (Fig. 1B and E, respectively). *EcoRI*-d hybridized weakly with a 0.4-kb transcript (Fig. 1C), and *EcoRI*-f hybridized weakly with four transcripts in the size range of 0.5 to 5.5 kb (Fig. 1D). The transcripts that hybridized with *EcoRI*-d and *EcoRI*-f were not investigated further because of their low abundance.

Mapping of prophage transcripts within *EcoRI*-a. The prophage transcripts which hybridized to BK5-T *EcoRI*-a were mapped by probing Northern blots of total RNA isolated from *L. lactis* H2, *L. lactis* BK5, and *L. lactis* H2L with subfragments of *EcoRI*-a (Fig. 2A and 3; Table 1). With the exception of probes 5 and 7, which hybridized weakly with transcripts in all strains (Table 1), all probes hybridized with transcripts produced only in the BK5-T lysogens. These data were used as follows to construct a transcript map of *EcoRI*-a.

Probes 1 and 2 each hybridized strongly with a 1.3-kb transcript and less intensely with a 2.3-kb transcript (Fig. 3A and B, respectively). Neither probe 4 nor probe 13 hybridized with the 1.3-kb transcript (Fig. 3C and data not shown), suggesting that this transcript maps to a region between the *PfMI* site at 12198 bp and the attachment site at 10874 bp (Fig. 2A). The possibility that this transcript extends through *attR* (2) and into the *L. lactis* H2L chromosome cannot be discounted, because no

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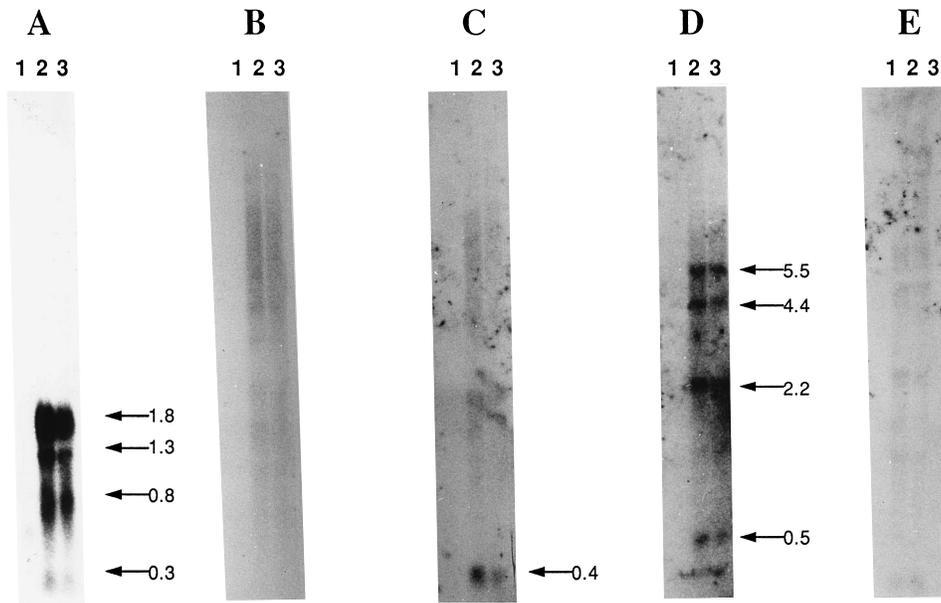


FIG. 1. Northern blots of total RNA from *L. lactis* strains hybridized with BK5-T *EcoRI* fragments. Equal quantities of total RNA from *L. lactis* H2 (lane 1), *L. lactis* H2L (lane 2), and *L. lactis* BK5 (lane 3) were separated by electrophoresis through an agarose-formaldehyde gel and transferred to a nylon membrane by capillary action. The same Northern blot was probed with the ^{32}P -labeled *EcoRI* a (A), *EcoRI* b (B), *EcoRI* d (C), *EcoRI* f (D), and *EcoRI* g (E) BK5-T fragments, derived from pMU1250 through pMU1254, respectively (14). Arrows and numbers at the right of panels A, C, and D indicate the positions and sizes (kilobases) of BK5-T prophage transcripts. The Northern blot was probed first with *EcoRI*-a and then sequentially with *EcoRI*-b, *EcoRI*-d, *EcoRI*-g, and *EcoRI*-f. Autoradiographs were exposed for 16 h (A), 4 days (B through D), or 5 days (E).

chromosomal fragments were used as probes. This transcript would encode ORF374, the BK5-T Int (3). The 2.3-kb transcript must also have contained *int*, but its location could not be precisely mapped because of its low abundance.

TABLE 1. Size of prophage transcripts which hybridized to specific ^{32}P -labeled BK5-T fragments

Fragment used as probe	Transcript size(s) (kb)
<i>EcoRI</i> -a.....	1.8, 1.3, 0.8, 0.3
<i>EcoRI</i> -b.....	None
<i>EcoRI</i> -d.....	0.4 ^a
<i>EcoRI</i> -f.....	5.5, 4.4, 2.2, 0.5
<i>EcoRI</i> -g.....	None
Probe 1: 0.5-kbp (pHID2142) ^{b,c}	2.3, 1.3
Probe 2: 1.1-kbp <i>EcoRV</i> - <i>PflMI</i> ^b	2.3, 1.3
Probe 3: 1.4-kbp <i>HindIII</i> - <i>PstI</i> ^b	1.8, 1.3, 0.8
Probe 4: 0.7-kbp <i>PflMI</i> - <i>PstI</i> ^b	2.3, ^a 1.8, 0.8
Probe 5: 2.4-kbp <i>PstI</i> ^b	1.8 ^d
Probe 6: 0.9-kbp <i>EcoRI</i> - <i>HindII</i> (pHID498) ^{b,e}	2.3, ^a 1.8
Probe 7: 0.5-kbp <i>HindIII</i> - <i>EcoRV</i> ^b	None ^d
Probe 8: 2.8-kbp <i>PstI</i> ^b	0.3
Probe 9: 2.6-kbp <i>PvuII</i> - <i>EcoRI</i> ^b	0.3
Probe 10: 0.7-kbp <i>EcoRI</i> - <i>StyI</i> ^b	None
Probe 11: 2.1-kbp <i>StyI</i> - <i>NciI</i> ^b	1.8
Probe 12: 0.7-kbp <i>HindIII</i> ^b	1.8
Probe 13: 0.3-kbp (pHID1122) ^{b,f}	None

^a Transcript observed only after long exposures.

^b Fragments derived from BK5-T *EcoRI*-a (Fig. 2).

^c This probe was derived from a plasmid containing an exonuclease III-treated subfragment of *EcoRI*-a containing nucleotides 10999 to 11471 (3).

^d Two additional observed transcripts are not listed because they hybridized with equal intensity to *L. lactis* H2 indicator strain mRNA.

^e This probe was derived from a plasmid containing an exonuclease III-treated subfragment of *EcoRI*-a containing nucleotides 13173 to 15341 (3).

^f This probe was derived from a plasmid containing an exonuclease III-treated subfragment of *EcoRI*-a containing nucleotides 10452 to 10769 (3).

Probes 4, 6, 11, and 12 all hybridized with a 1.8-kb BK5-T transcript (Fig. 3). It was concluded that two different 1.8-kb transcripts were transcribed from BK5-T prophage, because two of these probes (probes 6 and 11) are separated by >1.8 kbp in both the prophage and circularized phage DNA. Since probes 2 and 7 did not hybridize with a 1.8-kb transcript, one of the 1.8-kb transcripts was mapped between the *PflMI* site at 12198 bp and the *HindII* site at 14048 bp (Fig. 2A). The nucleotide sequence predicts two genes, *orf258* and *orf297*, in this region of the BK5-T genome, and the size of the observed transcript corresponds closely to the combined length of these two genes.

Because probes 10 and 13 did not hybridize with a 1.8-kb transcript, it was concluded that the second 1.8-kb transcript mapped to the region between the *StyI* site at 8402 bp and the *HindIII* site at 10452 bp (Fig. 2A). The nucleotide sequence data indicated that a relatively large gene, *orf536*, was located in this region of the BK5-T genome. Transcription of this gene would require a transcript of at least 1.6 kb.

In addition to the 1.8-kb transcript, probe 4 hybridized with a 0.8-kb transcript (Fig. 3C). Because neither probe 2 nor probe 6 hybridized with this 0.8-kb transcript, it was mapped between the *PflMI* site at 12198 bp and the start of probe 6 at 13173 bp (Fig. 2). The only significant ORF in this region was ORF258, which would require a transcript of at least 674 bp for its expression. Probe 5 hybridized with a 0.8-kb transcript in the indicator strain *L. lactis* H2 (Table 1), making it impossible to determine unequivocally whether this probe hybridized with BK5-T transcripts of the same size.

Probes 8 and 9 hybridized strongly with a 300-base transcript (data not shown), which must have been derived from the region between the *PvuII* site at 16304 bp and the *PstI* site at 18112 bp (Fig. 2A). There are a number of small ORFs in this region, and no attempt was made to determine which was present in this transcript. Transcripts smaller than 240 bases

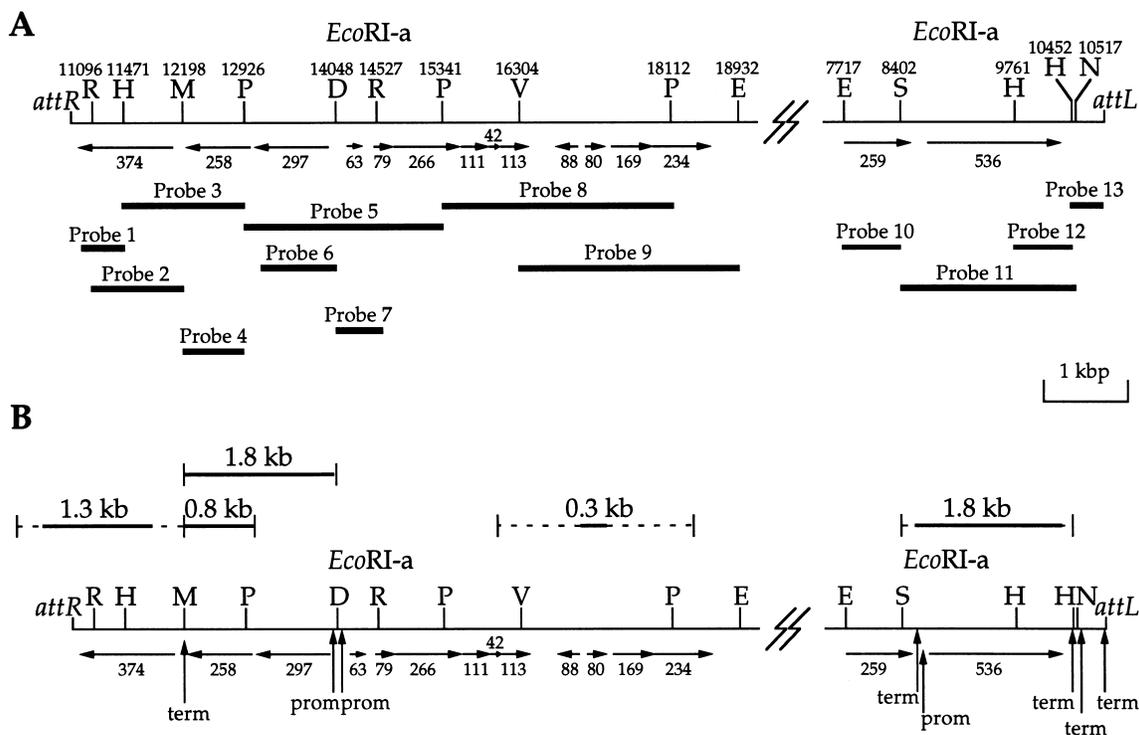


FIG. 2. Locations of BK5-T prophage transcripts. (A) Physical and genetic map of *EcoRI*-a of the BK5-T prophage showing subfragments used to probe the Northern blots shown in Fig. 3. All the sites for *EcoRI* (E), *PstI* (P), and *NciI* (N) are shown, but only relevant sites for *EcoRV* (R), *HindIII* (H), *HindII* (D), *StyI* (S), *PflMI* (M), and *PvuII* (V) are included. The location and numbering of restriction sites are from the accompanying paper (3). The map is linearized at *attL* and *attR*, as would occur in the prophage, and the intervening prophage DNA (*EcoRI*-g, -f, -d, and -b) is not shown. The orientation of relevant ORFs is shown below the restriction map, with corresponding numbers indicating the number of codons in each (3). (B) Map of *EcoRI*-a of the BK5-T prophage showing the positions of transcripts. The positions and lengths of prophage transcripts are shown above the map. Transcripts are represented by solid lines, and the numbers above the line represent the predicted length of the transcripts in kilobases. Uncertainties in the location of the ends of transcripts are indicated by broken lines. The positions of relevant putative promoters (prom) and terminators (term), deduced from the nucleotide sequence data (3), are indicated by vertical arrows below the map.

which hybridized with probes 2, 11, and 12 (data not shown) were not mapped precisely. It is not known whether these were full-length transcripts or degradation products of larger transcripts.

In summary, the transcript-mapping data presented above indicated that at least four ORFs are transcribed in the BK5-T prophage. One of these, ORF536, is located near *attL*, while the other three are at the other end of the prophage, near *attR*. The orientation of all four ORFs is toward the attachment site and the host chromosome. ORF536 and ORF374 were observed in monocistronic transcripts, while ORF258 was found with ORF297 in a 1.8-kb bicistronic transcript and on its own in a 0.8-kb monocistronic transcript. The smaller transcript may have been a processing product of the 1.8-kb transcript.

Possible functions of ORFs expressed in the BK5-T prophage. Data presented in an accompanying paper indicated that ORF374 encoded the BK5-T integrase (2). Comparison of the deduced amino acid sequence of ORF297 with all GenBank proteins by using the FASTA (18) algorithm revealed an overall identity of 73% with the putative repressor protein of the temperate lactococcal phage Tuc2009 (32). The identity over the 190 C-terminal residues was greater than 99%. ORF297 also showed significant homology with a number of bacterial LexA repressor proteins and phage repressors (Fig. 4). The observed homology was centered around three highly conserved domains containing residues (underlined in Fig. 4) involved in the RecA-mediated cleavage of these proteins. Cleavage occurs between the conserved Ala-Gly residues, while the conserved Ser and Lys residues (Ser-119 and Lys-156

in LexA from *Escherichia coli*) are also essential for cleavage (30). At least nine other residues conserved across the LexA and phage repressor family are conserved in BK5-T ORF297. This conservation of sequence argues strongly that ORF297 is the BK5-T homolog of the λ repressor (the *cI* product) and that it is cleaved in the presence of activated RecA in the same manner as the λ repressor is cleaved.

The deduced amino acid sequence of ORF258 and ORF536 showed no significant homology with other prokaryotic proteins in the GenBank database. By analogy with λ prophage, the products of these ORFs may have a role in superinfection exclusion of BK5-T lysogens, although no evidence for this function was obtained in this study.

Analysis of the nucleotide sequence upstream of ORF297. The DNA upstream from *cI* in a number of phages, e.g., λ , P22 and 434, contains two divergent promoters (p_R and p_{RM}) and three repressor-binding sites, O_{R1} , O_{R2} , and O_{R3} (Fig. 5) (22). In BK5-T, a similar arrangement is found in the region upstream of ORF297 (Fig. 5D). Analysis revealed two divergent sequences ($p1$ and $p2$) with strong homology to the *L. lactis* promoter consensus sequence and three putative repressor-binding sites ($O1$, $O2$, and $O3$) characterized by strong dyad symmetry. $O1$ and $O3$ overlap $p2$ and $p1$, respectively, and $O2$ lies between the two promoters. Although the spacing between the two promoters in BK5-T is larger than the corresponding spacing in phages λ , P22, and 434, the overall arrangement of divergent promoters with overlapping twofold-symmetrical repressor-binding sites is very similar to that observed in these phages. The three regions of dyad symmetry observed up-

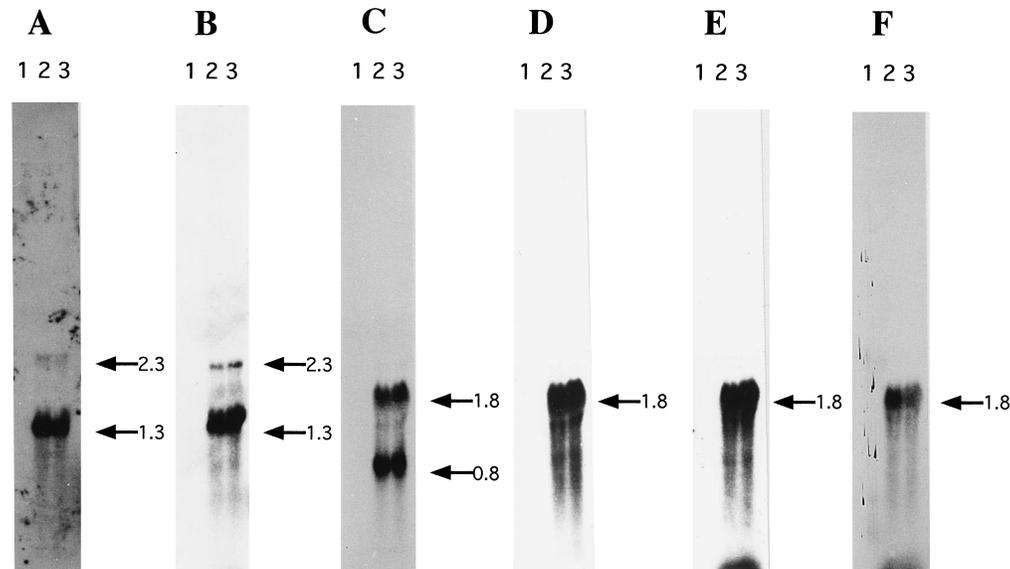


FIG. 3. Northern blots of total RNA from *L. lactis* strains hybridized with subfragments of BK5-T *EcoRI*-a. Total RNA from *L. lactis* H2 (lane 1), *L. lactis* H2L (lane 2), and *L. lactis* BK5 (lane 3) was separated by electrophoresis through agarose-formaldehyde gels and transferred to nylon membranes by capillary action. The Northern blots were then probed with the ³²P-labeled probes shown in Fig. 2: probe 1 (A), probe 2 (B), probe 4 (C), probe 6 (D), probe 11 (E), and probe 12 (F). Numbers and arrows at the right of each panel indicate the sizes (kilobases) and positions of major BK5-T prophage transcripts.

stream of BK5-T ORF297 are similar but not identical to each other. Five of the nine bases in each putative operator half-site are strongly conserved (Fig. 5D). This is similar to the λ, P22, and 434 operator half-sites (Fig. 5A, B, and C, respectively) in which small differences in repressor-binding-site sequences alter the affinities of cI and Cro for each site (12).

In lambdoid phages, the gene encoding the regulatory protein Cro, a competitor with cI for binding at O_R, is located downstream from p_R. A gene of similar size to *cro* (*orf63*) was identified downstream of p₂ in BK5-T (Fig. 5). ORF63, however, showed no significant homology to λ Cro or any other

characterized proteins, as determined by COMPARE (24) or FASTA (18) comparison with proteins in the GenBank database. The amino acid sequence of ORF63 was analyzed for the presence of possible helix-turn-helix structures, but no sequence fulfilling all the requirements of a helix-turn-helix motif was identified.

DISCUSSION

Analysis of transcripts produced by the lysogens *L. lactis* H2L and BK5 indicated that at least four major transcripts

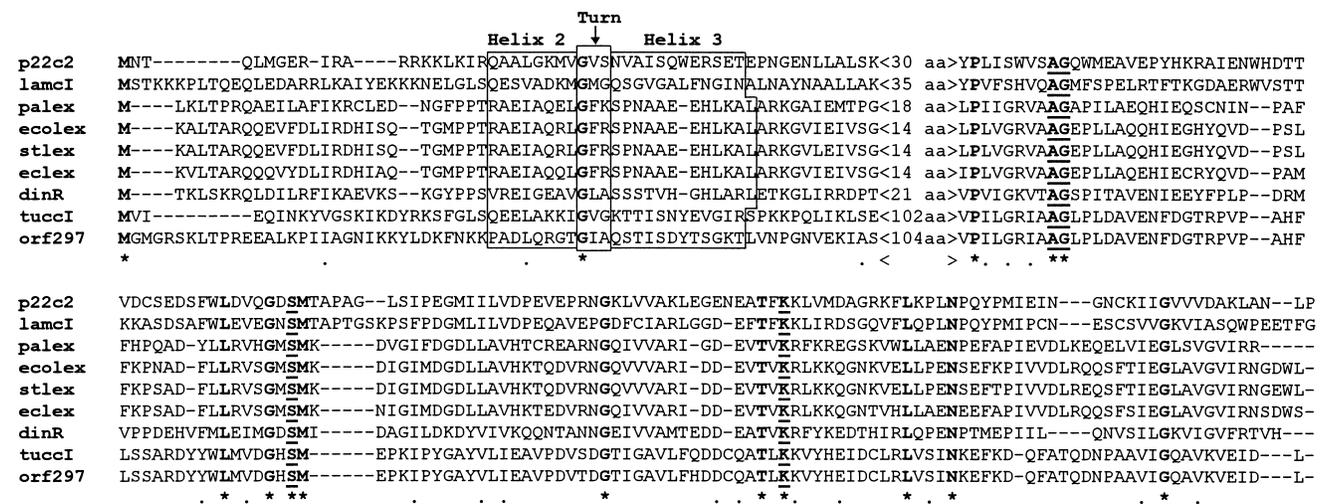


FIG. 4. Alignment of the amino acid sequences around the RecA-mediated cleavage site of various repressor proteins. Alignments were prepared with the CLUSTAL V (10) algorithm. Identical amino acids in all sequences are shown in boldface type and marked with asterisks, while conserved amino acids are marked with a period. The conserved residues essential for the RecA-mediated cleavage reaction (30) are underlined. Amino acids known or presumed to form a helix-turn-helix motif are boxed (27). Sequences are listed in order of increasing homology with ORF297 and are as follows: p22c2, phage P22 repressor protein (28); lamcI, phage λ repressor protein (26); palex, *Pseudomonas aeruginosa* LexA protein (7); ecolex, *E. coli* LexA protein (11); stlex, *Salmonella typhimurium* LexA protein (7); eclex, *Erwinia carotovora* LexA protein (7); dinR, *B. subtilis* DinR protein (23); tucci, *L. lactis* phage Tuc2009 putative repressor protein (32); orf297, phage BK5-T putative repressor protein.

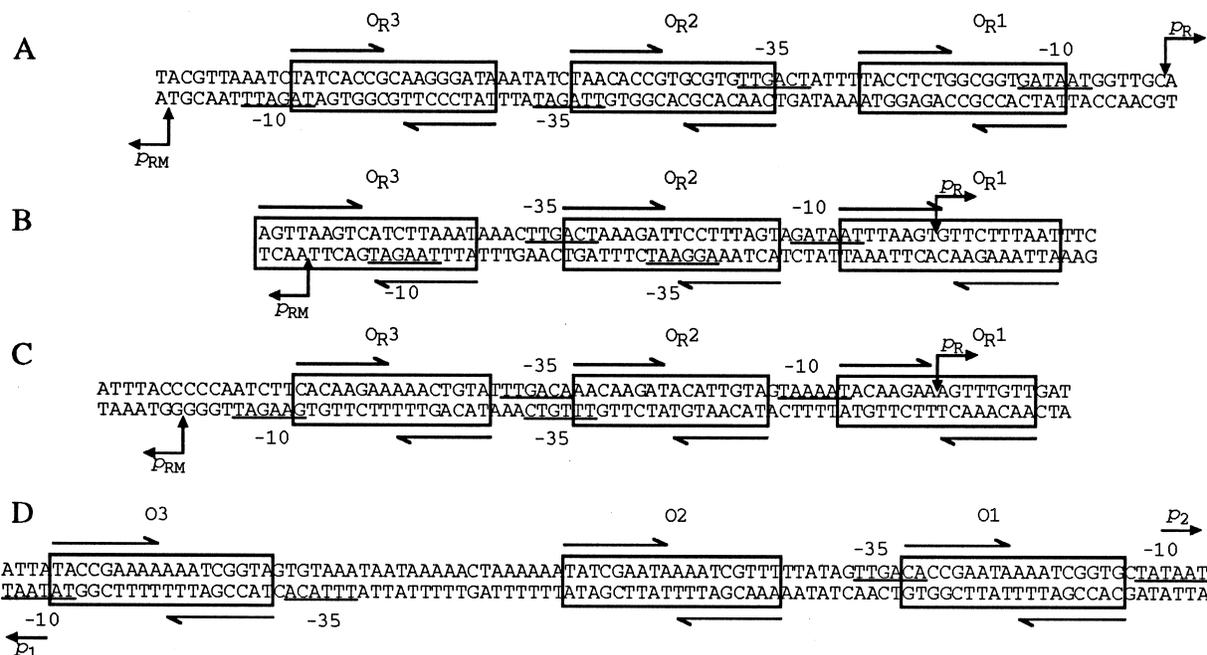


FIG. 5. Comparison of regulatory elements upstream from *cI* homologs from various phages. (A) λ (8); (B) P22 (20); (C) 434 (13); (D) BK5-T. Repressor-binding sites are enclosed in boxes and marked O_{R1} , O_{R2} , and O_{R3} (for λ , P22, and 434) and $O1$, $O2$, and $O3$ (for BK5-T). The -10 and -35 regions of p_R and p_{RM} are underlined on the upper and lower strands, respectively, and the start points of transcription, where known, are marked by vertical arrows. The -10 and -35 hexamers of two regions in the BK5-T DNA which exhibit homology to *L. lactis* promoter consensus sequences (34) are underlined and marked p_1 and p_2 . The dyad symmetry in each repressor-binding site is indicated by half-headed arrows. Sequences are aligned at the central repressor-binding site.

were expressed from the BK5-T prophage in these lysogens. Mapping of these transcripts indicated that four ORFs, ORF536, ORF374, ORF258, and ORF297, were expressed.

ORF536, which is separated by 531 bp at its 3' end from *attP*, showed no significant homology with other proteins in the GenBank database. We have shown previously that deletion of DNA encoding this ORF has no apparent effect on the ability of BK5-T to grow vegetatively or to form lysogens (2). In coliphage λ , the genes located to the left of *attP* are not essential and can be deleted with no apparent effect on λ growth.

ORF374 has previously been shown to encode the BK5-T integrase homolog (2). Low, constitutive levels of transcription of the integrase gene (*int*) from the nearby (p_1) promoter have been observed in λ prophage (29), and it has been suggested that continued production of integrase stabilizes the lysogen against nonlytic excision (6). The expression of BK5-T integrase from the prophage suggests a similar role for the BK5-T integrase.

Comparison of the deduced amino acid sequence of ORF297 with all GenBank proteins revealed significant homology with the sequence of a number of cellular LexA repressors and phage repressors (Fig. 4), centered around three highly conserved domains involved in the RecA-mediated cleavage of these proteins. A number of amino acid residues conserved among all the LexA proteins studied, as well as the repressors of a number of phages, were identified in ORF297 of BK5-T.

Some repressor proteins interact with DNA through a helix-turn-helix DNA-binding motif located in the N-terminal region of the protein (17, 27). Within proteins containing this motif, the turn is usually centered on a Gly residue surrounded by two hydrophobic amino acids (27). CLUSTAL V (10) alignment of phage and cellular repressors identified this conserved Gly residue to be at position 42 in ORF297 (Fig. 4). Interestingly,

this region of the BK5-T protein does not meet all the requirements suggested by Sauer et al. (27) for helix-turn-helix motifs. In particular, the predicted start of the first helix contains a proline residue, which is a helix-breaking amino acid. Similarly, while satisfying the general criteria proposed by Brennan and Matthews (4) for a helix-turn-helix motif, it did not meet the criteria used to identify "strong candidate(s) for a helix-turn-helix motif."

Analysis of the nucleotide sequence upstream of the gene encoding ORF297 identified a region of divergent putative promoters and overlapping regions of dyad symmetry strikingly similar to the *imm* region of λ , P22, and 434 (Fig. 5). By analogy with these phages, BK5-T ORF297 would positively regulate its own synthesis while simultaneously repressing the synthesis of the BK5-T homolog of λ Cro. The location and size of ORF63 suggest that it may be the BK5-T equivalent of the λ Cro. Experiments are in progress to study the function of the putative BK5-T analogs of the λ *cI* and Cro proteins and to investigate the regulation of expression of the genes encoding these proteins.

ORF297 was highly homologous to the putative repressor protein from Tuc2009, with greater than 99% identity between the 190 C-terminal amino acids of the two proteins (32). The DNA sequences of the regions encoding this domain are also more than 99% identical. By analogy with other repressor proteins, the C-terminal domain is likely to be involved with oligomerization of the protein monomers. By contrast, the N-terminal 102 amino acids of the BK5-T and Tuc2009 repressors showed only 17% identity, and there was little similarity at the predicted DNA-binding motifs of the two proteins (Fig. 4). It is unlikely, therefore, that the BK5-T and Tuc2009 proteins recognize the same operators, and BK5-T and Tuc2009 lysogens would not be expected to be homoimmune. The nucleotide sequence of putative operator regions in BK5-T has been

determined (Fig. 5), but the corresponding regions for Tuc2009 have not yet been reported.

Comparison of the known nucleotide sequences of BK5-T and Tuc2009 suggests that there is a high degree of identity from the 3' end of the putative *cI* gene through *int* and *attP* (1–3, 32, 33). The nucleotide sequences of *int* and *attP* of the temperate lactococcal phage ϕ LC3 are also almost identical to those of BK5-T (2, 16). Immediately outside of these regions, there is little identity between the BK5-T and Tuc2009 genomes. These data are suggestive of the presence of an “integration cassette” in temperate lactococcal phages, which can exchange between phages with different immunity specificities.

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