NOTES

Spontaneous Deletion Mutants of the Lactococcus lactis Temperate Bacteriophage BK5-T and Localization of the BK5-T attP Site

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Spontaneous deletion mutants of the temperate lactococcal bacteriophage BK5-T were obtained when the phage was grown vegetatively on the indicator strain Lactococcus lactis subsp. cremoris H2. One deletion mutant was unable to form stable lysogens, and analysis of this mutant led to the identification of the BK5-T attP site and the integrate gene (int). The core sequences of the BK5-T attP and host attB regions are conserved in a number of lactococcal phages and L. lactis strains.

BK5-T is a temperate lactococcal bacteriophage that can be induced from the lysogen Lactococcus lactis subsp. cremoris BK5 by mitomycin treatment (9). Under certain propagation conditions, BK5-T spontaneously loses the ability to lysogenize, possibly as a result of one or more deletion events (7). The availability of detailed information concerning the BK5-T genome (3, 11) prompted us to seek deletion mutants of BK5-T that had lost the ability to form stable lysogens as a strategy for identifying genes essential for the establishment and/or maintenance of lysogeny. Characterization of one of these mutants enabled us to identify and sequence the phage and host attachment sites, attP and attB, respectively. Deletion of a 536-codon open reading frame (ORF) and tandemly repeated segments within a 1,904-codon ORF in BK5-T did not affect the frequency of lysogeny, thereby eliminating the possibility that these gene products are required for lysogeny.

Deletions within the BK5-T genome during lytic propagation. The indicator strain L. lactis H2 (9) was infected with BK5-T.H2L (BK5-T isolated after induction of the lysogen L. lactis H2L [3]) and incubated until the culture lysed. The cell-free lysate was added to an appropriate volume of uninfected cells. This lysis/infection cycle was repeated 20 times, and the resulting phage was designated BK5-T.H2cyc20. Analysis of EcoRI-PstI digests of BK5-T.H2L DNA and BK5-T.H2cyc20 DNA, together with hybridization studies with EcoRI-a and EcoRI-b (11), showed that at least nine fragments (in addition to the fragments containing cos) in the BK5-T.H2cyc20 DNA digest were present in submolar amounts (Fig. 1). Five of these fragments were produced by specific deletions within EcoRI-b (11) (Fig. 1B, lane 2). EcoRI-b contains four perfect tandem repeats of 468 bp and a fifth incomplete tandem repeat within the large ORF(1904 (3). The 7.4-kbp fragment that hybridized to EcoRI-b was the full-length EcoRI-b(P1) fragment (11), while the sizes of four other submolar fragments (6.9, 6.4, 6.0, and 5.5 kbp) were consistent with their being produced by the loss of one, two, three, or four of the 468-bp tandem repeats, respectively. The other four submolar fragments (5.2, 4.3, 3.2, and 2.1 kbp in size) resulted from deletions within EcoRI-a (11) (Fig. 1C, lane 2).

Characterization of BK5-T deletion mutants. The presence of many submolar fragments in restriction digests of BK5-T.H2cyc20 DNA suggested that the BK5-T.H2cyc20 preparation comprised a mixed population. To characterize the deletions more precisely, three deletion mutants of BK5-T (BK5-T.H2Δ8, BK5-T.H2Δ10, and BK5-T.H2Δ11) were randomly selected from single plaques of BK5-T.H2cyc20. Deletions within BK5-T.H2Δ8, BK5-T.H2Δ10, and BK5-T.H2Δ11 DNA were located by restriction mapping, Southern hybridization (Fig. 1), and DNA sequencing. Each mutant contained a deletion within the tandem repeat region of ORF1904 (Fig. 2) (3), while BK5-T.H2Δ8 and BK5-T.H2Δ10 also contained deletions within EcoRI-a. The deletion within ORF1904 could be explained by a single crossover recombination event between two 5-bp “core sequences” of ACCGA situated at 4198 and 6070 bp (3) in BK5-T.H2Δ11 DNA or between two 8-bp homologous regions situated at 4222 and 6094 bp (3) in BK5-T.H2Δ8 DNA (Fig. 2). Each of these deletions removed 1,872 bp of DNA, equivalent to four of the 468-bp tandem repeats identified previously (3). These deletions shortened ORF1904 by 624 codons without changing the reading phase. BK5-T.H2Δ10 phage comprised a mixed population of phages carrying deletions within ORF1904 corresponding to the loss of two, three, or four 468-bp repeats. The precise endpoints of these deletions could not be determined. Each phage deletion mutant was able to propagate vegetatively on L. lactis H2, yielding slightly larger plaques (1 to 1.5 mm) than did BK5-T.H2L (0.5 to 1 mm).

FASTA (15) comparison of the ORF1904 amino acid sequence with all protein sequences in GenBank showed significant homology with a number of proteins from the collagen family. This homology was centered around the repeated Gly-X-Y motif, found 64 times in ORF1904. FASTA analysis also indicated similarity between ORF1904 and an unidentified ORF (orfB35) from the lactococcal bacteriophage bIL67 (19) and less similarity to the lactococcal lytic bacteriophage US3.
lytic enzyme (16) and the *Bacillus subtilis* xylose isomerase (22). Analysis by COMPARE (18) indicated similarity between ORF1904 and numerous proteins involved in binding and/or degradation of cell wall glycoproteins. These sequence similarities and the observation that a number of cell wall-lytic enzymes contain repeated sequence motifs (5, 8, 10, 12) suggest that ORF1904 may be involved in cell lysis during the lytic cycle of BK5-T or in cell wall hydrolysis to enable phage DNA injection.

The genomes of BK5-T.H2Δ8 and BK5-T.H2Δ10 also con-
tained deletions within EcoRI-a (Fig. 2). These deletions resulted from a single-crossover recombination event between two 11-bp core sequences of TTTTTTTGTGT situated at 8677 and 10701 bp in BK5-T.H2Δ8 DNA (3) (Fig. 2) or between two 6-bp core sequences of GTGTTT situated at 8674 and 11737 bp in BK5-T.H2Δ10 DNA (3) (Fig. 2). Both of these deletions removed ORF536, while the BK5-T.H2Δ10 6-bp core sequences of GTGTTT situated at 8674 and 10701 bp in BK5-T.H2Δ10 resulted from a single-crossover recombination event between the ORF536-to-ORF374 intergenic DNA is essential for the BK5-T.H2Δ10 to form lysogens. The inability of ORF374 and/or prokaryotic proteins in the GenBank database. The inability of amino acid sequence exhibits no significant homology with any encode products that are not essential for normal propagation. (6). The function of the ORF536 protein is unknown, and its function of the ORF536 protein is unknown, and its name is likely to occur. Thus, BK5-T attP is located in a region of the BK5-T genome that was deleted in BK5-T.H2Δ10 but not in BK5-T.H2Δ11 or BK5-T.H2Δ8 (Fig. 2). The deletion of attP in BK5-T.H2Δ10 provides an explanation for the inability of this phage to form stable lysogens. This observation is of particular interest in an industrial context, since to our knowledge it is the first demonstration of spontaneous mutations.

Localmization of the BK5-T attachment site. Since BK5-T.H2Δ10 contained deletions within the EcoRI-a fragment, previously shown to contain attP (11), it was decided to locate attP more precisely to determine whether loss of this feature was related to the nonlysogenic nature of BK5-T.H2Δ10. Subfragments of BK5-T EcoRI-a were used to probe Southern blots of EcoRI digests of chromosomal DNA from the BK5-T lysogens L. lactis BK5 and H2L (data not shown). BK5-T subfragments spanning attP would hybridize to two chromosomal fragments, whereas subfragments which did not contain attP would hybridize to only one fragment. By using this approach, attP was localized between 10769 and 10999 bp (3) and the phage/host junctions were shown to be in chromosomal XbaI fragments of 90 and 18 kbp in L. lactis BK5 and H2L (data not shown).

The sequences of these phage/host junctions (attL and attR) in L. lactis H2L were then determined (Fig. 3B). For attL, this was done by sequencing a 900-bp PCR fragment, obtained by inverse PCR (14) with JB6 and JB7 as primers and a ligated Spel digest of the gel-purified 90-kbp XbaI chromosomal fragment as the template. The obtained sequence revealed 100% sequence identity between the 21 bp of host DNA adjacent to attL in BK5-T and in φLC3, another L. lactis temperate phage (13). The existence of this identity and of the 97% identity between the 1,627 bp of DNA surrounding attP of BK5-T and φLC3 (13) enabled us to use the nucleotide sequence surrounding attP in the φLC3 lysogen L. lactis IMN-C18 (13) to design PCR primers that amplified L. lactis H2 attP (JB48 and JB49 [Table 1]) and L. lactis H2L attR (JB8 and JB49 [Table 1]) and provided DNA for sequencing.

Comparison of the DNA sequences of the attP, attL, attR, and attB regions (Fig. 3B) identified a common 9-bp core sequence, 5′-TTCTTCATG-3′ (bases 10882 to 10874) (3), which within recombination between BK5-T and the host genome is likely to occur. Thus, BK5-T attP is located in a region of the BK5-T genome that was deleted in BK5-T.H2Δ10 but not in BK5-T.H2Δ11 or BK5-T.H2Δ8 (Fig. 2). The deletion of attP in BK5-T.H2Δ10 provides an explanation for the inability of this phage to form stable lysogens. This observation is of particular interest in an industrial context, since to our knowledge it is the first demonstration of spontaneous mutations.

### TABLE 1. Oligonucleotides used in this investigation

<table>
<thead>
<tr>
<th>Number</th>
<th>Oligonucleotide sequence (5′-3′)</th>
<th>Region of BK5-T&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>JB6</td>
<td>GATCATTTAGGAATACTCTCCC</td>
<td>10012–9994</td>
</tr>
<tr>
<td>JB7</td>
<td>GATCGACATGGGAGAGGTTAAAAAGG</td>
<td>10259–10281</td>
</tr>
<tr>
<td>JB8</td>
<td>CACACAGCACAACCTATATCC</td>
<td>11051–11032</td>
</tr>
<tr>
<td>JB48</td>
<td>TGGTAAAAGCAGGAATCAAAGG</td>
<td>Comp&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>JB49</td>
<td>AAATTCAAGGAACTGAGCTCCA</td>
<td>Comp&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Oligonucleotides were synthesized in an Applied Biosystems model 381 DNA synthesizer.

<sup>b</sup> Sequence numbers refer to the nucleotide sequence of BK5-T as determined previously (3).

<sup>c</sup> Comp, complementary to L. lactis IMN-C18 genomic DNA (13).
resulting in a lytic phenotype in a temperate lactococcal bacterial phage. These lytic phages are not virulent, because they cannot infect BK5-T lysogens and thus would grow only on strains which did not express the BK5-T repressor protein or a functional equivalent.

BK5-T attP is identical to attP of the lactococcal bacteriophages φLC3 and Tuc2009 (13, 20). Moreover, L. lactis H2 attB and the 21 bases on either side are identical to the corresponding bases in the L. lactis integrator strains IMN-C18 (φLC3), UC509 (Tuc2009), and UC506 (Tuc2009) and differ by 1 base from that of MG1363 (Tuc2009) (13, 20). Five (TTCTT) of the nine bases of the BK5-T attP core sequence are identical to bases present in the 16-bp core sequence of the Lactobacillus gasseri phage dadh attP (17). There was no apparent homology between BK5-T attP and attP of the temperate lactococcal phage TP901-1 (4). This indicates that there are at least two classes of integration system in temperate lactococcal phages.

The nucleotide sequence surrounding the BK5-T attP core sequence contained a number of repeated and/or palindromic sequences (Fig. 4). These regions of DNA may be important in binding integrase or an L. lactis IHF homolog. Identical sequences also surround the φLC3 and Tuc2009 attP sites (13, 20). Because the deletion mutant BK5-T.H2Δ was able to form stable lysogens, only the repeated sequences at positions >10701 bp (Fig. 4) can be essential for phage integration.

Deduced amino acid sequence of the BK5-T integrase. FASTA comparison of the deduced amino acid sequence of ORF374, which is adjacent to attP (Fig. 2), with all GenBank proteins revealed significant homology with a number of site-specific recombinases. In particular, ORF374 shared 99.4 and 98.7% identity with the deduced amino acid sequences of the integrase proteins from the Tuc2009 and φLC3 phages, respectively (13, 20). All of these lactococcal phage integrase proteins contain the highly conserved residues of site-specific recombinases of the Φ integrase family (1).

Overall homology between BK5-T, φLC3, and Tuc2009. Comparison of the nucleotide sequence surrounding int and attP in BK5-T (bases 12212 to 10341) and Tuc2009 (bases 1 to 1872 [20]) identified a region of 1,632 bp including int and attP that showed 97% identity at the nucleotide level. Proceeding this homologous region, the nucleotide sequences diverge and the deduced amino acid sequence of ORF536, the adjacent gene in BK5-T, shows no homology with the protein encoded by the adjacent gene in Tuc2009. It is not possible at this stage to compare sequences at the other end of the 1,632-bp homologous region. However, a similar pattern of homologous and divergent regions is seen within the putative ci genes of the two phages (2, 21). This is suggestive of a common cassette containing the integration region (attP and int) and possibly ci in BK5-T, Tuc2009, and φLC3. Despite these similarities in the integration region, DNA from φLC3 hybridizes with the type phage P335 whereas BK5-T DNA does not (4). Moreover, phage TP901-1, which contains an integration system different from those of BK5-T, φLC3, and Tuc2009, also belongs to the P335 group of phages (4). Two different types of DNA-packaging systems have also been identified in temperate lactococcal bacteriophages. The genomes of BK5-T and φLC3 contain cohesive ends, whereas Tuc2009 and TP901-1 contain a pac site and package DNA into the phage heads by a headful mechanism. Further comparison of nucleotide sequence data from these phages is necessary to clarify the evolutionary and functional relationships between them.

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REFERENCES