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Characterization of Genetic Elements Required for Site-Specific Integration of the Temperate Lactococcal Bacteriophage φLC3 and Construction of Integration-Negative φLC3 Mutants

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The genetic elements required for the integration of the temperate lactococcal bacteriophage φLC3 into the chromosome of its bacterial host, *Lactococcus lactis* subsp. *cremoris*, were identified and characterized. The φLC3 phage attachment site, *attP*, was mapped and sequenced. DNA sequence analysis of *attP* and of the bacterial attachment site, *attB*, as well as the two phage-host junctions, *attR* and *attL*, in the chromosome of a φLC3 lysogen, identified a 9-bp common core region, 5′-TTCTTCATG′-3, within which the strand exchange reaction takes place during integration. The *attB* core sequence is located within the C-terminal part of an open reading frame of unknown function. The φLC3 integrase gene (*int*), encoding the φLC3 site-specific recombinase, was identified and is located adjacent to *attP*. The φLC3 Int protein, as deduced from the nucleotide sequence, is a basic protein of 374 amino acids that shares significant sequence similarity with other site-specific recombinases of the integrase family. Phage φLC3 *int*- and *int-attP*-defective mutants, conferring an abortive lysogenic phenotype, were constructed.

Most temperate bacteriophages characterized thus far insert their DNA into the chromosome of their bacterial host through site-specific recombination between specific phage (*attP*) and bacterial (*attB*) attachment sites, in accordance with the model of phage λ integration (10, 15). This integrative recombination is catalyzed by site-specific recombinases (integrases) identified in several temperate coliphages (for a review, see reference 37) but also in temperate phages from several other bacterial genera (14, 35, 70, 71). The integrase (Int) family of site-specific recombinases also includes the recombinases of several other genetic elements, such as certain transposons and plasmids (18, 20, 27, 41, 48, 51), and regulatory elements of fimbria synthesis in *Escherichia coli* (26). Although the members of the Int family constitute a highly divergent group of enzymes, they all share limited sequence similarity, especially in a C-termal region believed to make up part of the catalytic site of these recombinases (4, 56).

The integrative recombination system of bacteriophage λ has been extensively studied (46, 47, 65, 67) and has provided a model for the characterization of the components as well as for the elucidation of the regulatory mechanisms involved in this recombination process. Despite numerous reports on temperate bacteriophages from lactococci, there is so far no information on the molecular biology of lysogeny among this group of gram-positive bacteria. Bacteriophage φLC3, a temperate phage isolated from *Lactococcus lactis* subsp. *cremoris* after induction with UV light, recently was described (39). In the present study, the site-specific recombination system of phage φLC3 was analyzed. The φLC3 attachment sites were mapped and sequenced, and a common core sequence present in both *attP* and *attB* and in the prophage-host hybrid regions in lysogens was identified. The φLC3 integrase gene also was identified, and the amino acid sequence of the deduced Int protein was compared with those of other site-specific recombinases. Integrase-negative mutants of φLC3, with a phenotype similar to that of integrase-negative mutants of phage λ, were constructed. Phage φLC3 was shown to integrate into an open reading frame encoding a protein of unknown function, but DNA sequence analysis showed that the length of this reading frame was preserved in the lysogen.

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MATERIALS AND METHODS

Phages, bacteria, and plasmids. The bacterial strains, phages, and plasmid used are listed in Table 1. *L. lactis* subsp. *cremoris* IMN-C1819, carrying an integrase-defective φLC3 mutant prophage, was constructed by complementation of the defective *int* gene through coinfection of *L. lactis* subsp. *cremoris* IMN-C18 with φLC3 *int*ins1 and φLC3 *int*$^+$ *c*1 mutant phages. The ins1 mutation in the φLC3 *int*ins1 prophage and the location of the φLC3 *int*ins1 prophage in the IMN-C1819 chromosome were confirmed by a polymerase chain reaction (PCR) followed by DNA sequencing (see Fig. 5, lane 9) (38). Isolation of φLC3 wild-type (wt) lysogens and phage titration assays were done as described previously (39).

Media and enzymes. *L. lactis* subsp. *cremoris* and phage φLC3 were propagated in M17 broth or on M17 agar (64) at 30°C as previously described (39). *E. coli* was grown on LB agar or in LB broth (42). Mutanolysin and lysozyme were purchased from Sigma and Boehringer-Mannheim, respectively. DNA-modifying enzymes were purchased from Bethesda Research Laboratories. All enzymes were used as recommended by the manufacturers.

Preparation of φLC3 antiserum. φLC3 phage particles, purified twice on a CsCl gradient, were dialyzed against 0.9% NaCl and mixed with an equal volume of Freund's complete adjuvant, and the mixture was shaken to yield an emulsion. A 0.5-ml sample containing ca. 10^{12} PFU was injected subcutaneously into the neck of a rabbit. The rabbit was reinoculated after 4 and 6 weeks, with Freund's incom-

TABLE 1. Bacterial strains, phages, and plasmid

Bacterial strain, phage, or plasmid	Relevant characteristic(s)	Source or reference
Bacterial strains		
L. lactis subsp. *cremoris*		
IMN-C3	Original ϕLC3 lysogenic *L. lactis* subsp. *cremoris* strain	39
IMN-C18	Standard ϕLC3 host indicator strain	39
IMN-C17	Alternative ϕLC3 host indicator strain	This work
IMN-C1814	IMN-C18 (ϕLC3 wt)	This work
IMN-C1819	IMN-C18 (ϕLC3 *int*ins1)	This work
E. coli		
K-12	λ^+	29
C-600	λ^-	3
DH5α	Used for cloning into pGEM-7Zf(+)	19
JM109	Used for M13 cloning	68
Bacteriophages		
ϕLC3	Original isolate	39
ϕLC3 del1	Does lysogenize; *Xba*I-*Sal*I deletion	This work
ϕLC3 del2	*int* and *att* defective; *Bln*I-*Xba*I deletion	This work
ϕLC3 *int*ins1	Insertion and excision defective; 4-bp insertion in *int*	This work
ϕLC3 *c*1	*int*$^+$; does not lysogenize	39
M13mp18 and M13mp19	Used for cloning and sequencing of ϕLC3 DNA restriction fragments	50
Plasmid pGEM-7Zf(+)	Used for cloning of ϕLC3 DNA restriction fragments	Promega Biotec

plete adjuvant, and the serum was harvested 2 days after the last injection. For determination of the anti-ϕLC3 titer, 1-μl aliquots of antiserum were added to a dilution series of phage suspended in 1 ml of 75 mM $MgCl_2$. After 5 min of incubation at 30°C, PFU were determined by titration on *L. lactis* subsp. *cremoris* IMN-C18.

Preparation of DNA. Large-scale purification of ϕLC3 particles and preparation of phage DNA were done as previously described (39). M13 clones and *E. coli* plasmid and chromosomal DNAs were prepared by standard procedures (59). The amount of free phage particles in a culture of a ϕLC3 lysogenic strain is about 10^6 PFU/ml, indicating a high frequency of spontaneous induction. Preliminary Southern analysis of a ϕLC3 lysogen to map the phage attachment site revealed the presence of a background of free intracellular phage DNA, possibly due to the high level of spontaneous prophage induction. DNA from lysogenic *L. lactis* subsp. *cremoris* was prepared from cells grown in the presence of ϕLC3 antiserum to prevent a massive and continuous superinfection by spontaneously released ϕLC3 phages. This antiserum treatment reduced the background of free ϕLC3 DNA, making it easier to identify the *attP*-carrying restriction fragment. The antiserum, enough to inactivate 10^8 phages per ml of culture, was added to the medium prior to inoculation and then to the bacterial cultures every hour during growth. The cultures were harvested in the mid-exponential phase, and the DNA was extracted from the cells essentially as described previously (36). DNA from nonlysogenic bacterial strains was similarly prepared from cells grown in the absence of ϕLC3 antiserum. *E. coli* strains were grown with constant shaking at 37°C. Total bacterial DNA, used as a template in quantitative PCR analysis, was extracted from log-phase cells essentially as described previously (36).

Recombinant DNA methodology. Agarose gel electrophoresis and molecular cloning were performed by standard procedures (59) unless otherwise stated. Southern analysis was done by blotting onto a GeneScreen Plus membrane (New England Nuclear, Boston, Mass.) with an LKB vacuum blotter as recommended by the manufacturer. The DNA on the membrane was hybridized with ϕLC3 DNA that had been labeled with ^{32}P by use of a nick translation system kit from Bethesda Research Laboratories and [α-^{32}P]dATP from Amersham. Hybridization was performed as described previously (12). The ϕLC3 DNA restriction fragments used as probes were purified by cloning in pGEM-7Zf(+) (Promega Biotec, Madison, Wis.) prior to labeling with ^{32}P. Electrophoresed DNA was extracted from agarose gels by use of a GeneClean or Mermaid kit (Bio 101, Inc., La Jolla, Calif.). Biotinylated oligonucleotide DNA primers were labeled with biotin during synthesis in an Applied Biosystems 380/381 DNA synthesizer by use of biotin phosphoramidite (Cambridge Research Biochemicals Ltd., Cheshire, United Kingdom) as described by the manufacturer.

Amplification and isolation of ϕLC3 attachment sites. The PCR (58) was done with *Taq* DNA polymerase by use of a GeneAmp kit (Perkin-Elmer Cetus) and a Perkin Elmer DNA thermal cycler under various sets of conditions. The concentrations of primers were as recommended by Perkin Elmer-Cetus. After amplification, the primers were removed from the PCR mixture by centrifugation through a Centricon-100 microconcentrator (Amicon Div., Grace & Co., Beverly, Mass.). The PCR primers used are listed in Table 2.

The *attP-attB* junction regions, *attR* and *attL*, were isolated by PCR amplification with the 320-kb *L. lactis* subsp. *cremoris* IMN-C1814 bacterial *Bam*HI restriction fragment carrying the complete ϕLC3 prophage (39) as a template. Prior to the PCR, this large DNA fragment was purified by extraction from an agarose gel after separation by pulsed-field gel electrophoresis (39). Amplification of the junction

TABLE 2. Nucleotide sequences of PCR primers[a]

Primer set	Sequence	Amplified region
1	5′-AATATATTCGGGATGATTGTGGGA-3′ (1505 to 1482) (t)/5′-CCTCCGTTAGCTTCTGAAAGTTC-3′ (w)	φLC3 *attL*
2	5′-AGTTAAGCCATTCTTCGGAGTGG-3′[b] (1464 to 1442) (i)/5′-CCTCCGTTAGCTTCTGAAAGTTC-3′ (w)	φLC3 *attL*
3	5′-CTAGGACACGCAAGTGTGGC-3′ (1067 to 1086) (t)/5′-GTATTAGCAGCAGCCCCAAAAGCG-3′ (w)	φLC3 *attR*
4	5′-AAAATAATTGAGCTGCTACTGGAG-3′[b] (1145 to 1168) (i)/5′-GTATTAGCAGCAGCCCCAAAAGCG-3′ (w)	φLC3 *attR*
5	5′-TGTTAAAGCAGGAATCAAAGG-3′/5′-AAATACCTAAGCACACGAAGGCC-3′[b]	φLC3 *attB*
6	5′-TGTTAAAGCAGGAATCAAAGG-3′/5′-AATATATTCGGGATGATTGTGGGA-3′ (1505 to 1482)	φLC3 *attL*
7	5′-CTAGGACACGCAAGTGTGGC-3′ (1067 to 1086)/5′-AAATACCTAAGCACACGAAGGCC-3′[b]	φLC3 *attR*
8	5′-AATGGAAATGATAAAGCCG-3′ (518 to 536)/5′-AATATATTCGGGATGATTGTGGGA-3′ (1505 to 1482)	φLC3 *int*ins1/del2
9	5′-CTAGGACACGCAAGTGTGGC-3′ (1067 to 1086)/5′-AATATATTCGGGATGATTGTGGGA-3′ (1505 to 1482)	φLC3 *attP*
10	5′-CAATCTATTGATTTAACTAAAGAATTGCAAACCTTG-3′/5′-AAATACCTAAGCACACGAAGGCC-3′[b]	φLC3 *attB*
11	5′-CAATCTATTGATTTAACTAAAGAATTGCAAACCTTG-3′/5′-AATATATTCGGGATGATTGTGGGA-3′ (1505 to 1482)	φLC3 *attL*
12	5′-CAAAAGCCAATGCCAGCGC-3′ (−81 to −99)/5′-GGCGCAATGCCATCTGGTATC-3′ (−83 to +63)	λ *attB*
13	5′-CAAAAGCCAATGCCAGCGC-3′ (−81 to −99)/5′-CTCGGGCATAAGTCGGACACC-3′ (+161 to +141)	λ *attL*

[a] Primer sets were as follows: primers used for amplification and purification of the φLC3-host DNA junction regions by the targeted gene walking PCR strategy (53) (primer sets 1 to 4); primers used for amplification and verification of φLC3 *attB* (primer set 5), φLC3 *attL* (primer set 6), and φLC3 *attR* (primer set 7) in strain IMN-C3 and in various IMN-C18 and IMN-C17 lysogens; primers used for amplification of the ins1 and del2 mutations (primer set 8); and primers used for quantitative PCR (primer sets 9 to 13). The coordinates are indicated in parentheses at the 3′ end of the primers. For φLC3-specific primers, the coordinates are relative to the sequence shown in Fig. 4. For λ *attL*- and λ *attB*-specific primers the coordinates are relative to the sequence presented by Landy and Ross (28). The φLC3 *attB*-specific primers are based on the φLC3 *attB* sequence (38). The walking primers (w) (53) consist of arbitrary sequences. Only the two most successful walking primer sets are shown. The targeted PCR primers (t) (53) and internal detection and PCR primers (i) (53) are based on the φLC3 DNA sequence shown in Fig. 4.

[b] Labeled with biotin at the 5′ terminus.

regions was performed in two steps, with two nested *attP*-specific primers and several nonspecific "walking" primers, based on a strategy referred to as the "targeted gene walking PCR strategy" (for details concerning this strategy, see reference 53). Our modification included the use of a biotinylated "internal detection primer" instead of a ^{32}P-labeled primer. Consequently, the target DNA was labeled with biotin at the "oligomer extension step" and could then easily be purified from the PCR mixture by magnetic separation with streptavidin-coated Dynabeads M-280 (Dynal AS, Oslo, Norway). In contrast to the strategy of Parker et al. (53), the internal detection primer was also used for reamplification of the target DNA. Magnetic separation was performed as described by the manufacturer after removal of the primers from the PCR mixture. For confirmation of the *attB* region in the nonlysogenic host indicator strains and the φLC3-host DNA junction regions, *attL* and *attR*, in φLC3 lysogenic strains, the various attachment site regions were amplified with specific primers located on each side of the common core and total bacterial DNA as a template.

Relative proportions of phage attachment sites, as measured by quantitative PCR. The relative proportions of the various attachment sites in lysogenic and nonlysogenic bacteria were determined by comparing the amounts of *attB*, *attP*, and *attL* PCR products produced from total bacterial DNA extracts. The primers used for quantitative amplification are shown in Table 2. The attachment site regions were amplified from a series of template DNA dilutions by a step-down procedure in which the denaturation and elongation steps were kept constant during the run (1 min at 94°C and 30 s at 72°C, respectively) but in which the annealing temperature was altered (2 cycles at 66 and 65°C, 4 cycles at 64°C, 6 cycles at 63°C, 8 cycles at 62°C, and 12 cycles at 61°C for amplification of the λ attachment site regions and 2 cycles at 59 and 58°C, 4 cycles at 57°C, 8 cycles at 56°C, and 12 cycles at 55°C for amplification of the φLC3 attachment site regions). For amplification of the φLC3 *attB* site in IMN-C1819 and for amplification of the φLC3 *attP* site, the number of cycles was extended by 10 and 5 additional cycles at 55°C, respectively. The λ attachment site PCR mixtures contained 2.5% formamide to avoid nonspecific priming (61). The amount of DNA obtained from the amplification reactions was proportional to the concentration of the template in all dilutions used in the PCR analysis (see Fig. 5 and reference 38), and the efficiency of the *attB*-specific primer sets was similar to the efficiency of the corresponding *attL*-specific primer sets (38), thus substantiating that the PCR analysis was quantitative.

Nucleotide sequencing. DNA sequencing was done by the chain termination method (60) with the Sequenase DNA sequencing kit (United States Biochemical) and with [α-^{35}S]dATP (Amersham). The primers used for sequencing were the universal forward and reverse pUC-M13 sequencing primers (Promega) in addition to oligonucleotide primers synthesized in an Applied Biosystems 380/381 DNA synthesizer. Cloned φLC3 restriction fragments were sequenced either as double-stranded plasmid DNA (55) or as single-stranded DNA after subcloning into M13. PCR-amplified *attB*, *attL*, and *attR* DNA was sequenced either directly as double-stranded DNA essentially as described previously (11, 73) or as single-stranded DNA prepared by magnetic separation as recommended by the manufacturer of Dynabeads M-280.

Directed deletion and insertion mutagenesis in the φLC3 *int-attP* region. φLC3 DNA was first circularized by ligation of the cohesive ends. Removal of the *Xba*I-*Sal*I fragment (del1) and insertion into the *Bln*I site (ins1) were done by digestion of the circular DNA with *Xba*I-*Sal*I and *Bln*I, respectively; this step was followed by Klenow filling in of the staggered ends and blunt-end ligation (59). The *Bln*I-*Xba*I deletion (del2) was constructed in the same way, but because the staggered DNA ends resulting from *Bln*I-*Xba*I cleavage were compatible, the Klenow filling in step was omitted. The ligation mixtures were used for transfection of the host strain, *L. lactis* subsp. *cremoris* IMN-C18, by electroporation (25). After incubation at 30°C for 1 h in 0.5 ml of M17 broth supplemented with 0.4% glucose–0.5 M sucrose–20 mM $MgCl_2$–2 mM $CaCl_2$, 100-μl aliquots were plated on 0.4% top agar together with a fresh overnight culture of indicator strain IMN-C18 for detection of infective

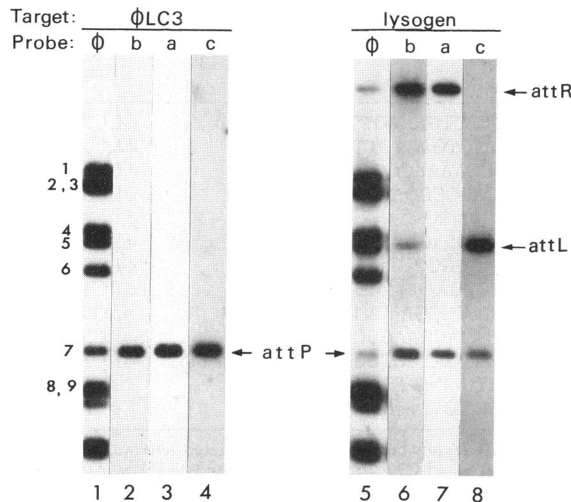

FIG. 1. Mapping of the bacteriophage attachment site. A Southern blot containing one single loading of DNA from a *Hinc*II φLC3 phage particle digest (lanes 1 to 4) and one single loading of a *Hinc*II DNA digest from the φLC3 lysogen *L. lactis* subsp. *cremoris* IMN-C1814 (lanes 5 to 8) was hybridized sequentially with the complete, ^{32}P-labelled φLC3 genome (φ) and with ^{32}P-labelled probes a, b, and c indicated in Fig. 2. The nine largest *Hinc*II restriction fragments are numbered the same way as in Fig. 2. Prior to rehybridization, the probe was removed from the membrane by 30 min of boiling in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (42)–1% sodium dodecyl sulfate.

centers. Between 20 and 700 PFU were obtained per μg of φLC3 DNA, depending on the batch of competent cells. The ins1 and del2 mutations were confirmed by PCR followed by DNA sequencing (38); the del1 mutation was confirmed by restriction analysis (38).

Computer-assisted sequence analysis. Sequence analyses were performed by use of PCGENE (IntelliGenetics, Mountain View, Calif.) microcomputer software. A search for nucleotide and amino acid sequence similarities in the GenBank and Swiss-Prot data bases was done by use of the FASTA (54) and the BLAST (2) programs. A comparison of protein sequences was performed by use of the ClustalV program (23).

Nucleotide sequence accession number. The nucleotide sequence of the φLC3 *int-attP* region (Fig. 4) will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under accession number L10286.

RESULTS

Localization of the φLC3 *attP* site. A comparison of Southern blots of DNA extracted from phage particles and from lysogenic strains, probed with labeled phage DNA, was used for mapping of the phage attachment site (*attP*) on the chromosome of several temperate bacteriophages (9, 17, 34, 62, 66). A restriction fragment harboring the *attP* site in a digest of phage DNA will be absent in the digest of prophage DNA, but instead, two new bands representing the hybrid phage-host junction fragments (*attR* and *attL*) will appear. Hybridization of φLC3 total DNA to a Southern blot containing φLC3 phage and prophage *Hinc*II digests demonstrated that the φLC3 *attP* site is located on 1.5-kb *Hinc*II fragment 7 (Fig. 1). Southern analyses of several other restriction digests confirmed the presence of *attP* in this region (38). The 1.5-kb *Hinc*II fragment was cloned from φLC3 DNA, and the location of *attP* within this 1.5-kb *Hinc*II fragment was further mapped by use of probes from three different regions of this cloned fragment (Fig. 2, probes a, b, and c). Two fragments, present only in the digest of DNA from the lysogen, hybridized to probe b (Fig. 1), indicating that this probe hybridizes to both junction fragments (*attR* and *attL*) and that *attP* is located on this 800-bp *Hind*III-*Xba*I probe b restriction fragment. Probes a and c, which flank probe b (Fig. 2), hybridized to different junction fragments (Fig. 1), confirming that φLC3 has integrated into the bacterial chromosome at a site located between the *Hind*III and *Xba*I restriction sites. Furthermore, stronger hybridization of probe b to the *attR* than to the *attL* junction fragment (Fig. 1) suggested that *attP* is located quite close to the *Xba*I site.

A φLC3 deletion mutant (φLC3 del1), constructed in vitro by deletion of the 700-bp *Xba*I-*Sal*I fragment (Fig. 2), gave rise to stable lysogens, indicating that the region essential for integration is located to the left of the *Xba*I site. The φLC3 del1 mutation did not seem to affect either lytic or lysogenic functions. The results of a Southern analysis of several independent φLC3 lysogenic isolates of *L. lactis* subsp. *cremoris* IMN-C18 and IMN-C17 and φLC3 parental strain IMN-C3 were all similar (34), indicating the existence of only one *attP* site on the φLC3 genome.

Sequence analysis of *attP*, *attR*, *attL*, and *attB* and identification of the core sequence. The probe b restriction fragment (Fig. 2), on which the φLC3 *attP* site had been localized, and the φLC3 prophage-host junction regions, *attR* and *attL*, were sequenced. Sequence comparisons identified a 9-bp core region, 5′-TTCTTCATG-3′, that is common to all three sequences (Fig. 3). Within *attP*, this core sequence is located 200 bp upstream from the *Xba*I site (Fig. 4). Sequence analysis of the *attB* region of nonlysogenic *L. lactis* subsp. *cremoris* IMN-C18 confirmed the presence of the core sequence in the bacterial chromosome (Fig. 3). The DNA sequences following and preceding the core in *attR* and *attL* are fully conserved in *attP* and *attB*, indicating that the strand exchange reaction between phage DNA and host DNA takes place by a crossover event within this 9-bp common core sequence, in accordance with the model for phage λ integration (10). Like the attachment site region of phage λ (28), the region surrounding the φLC3 *attP* core sequence is relatively rich in A+T. The 200-bp region flanking the φLC3 core contains about 78% A+T, compared with 63.5% A+T on average in the φLC3 genome (39). The φLC3 *attP* region contains several direct and inverted repeats, asymmetrically located relative to the core, a characteristic that is also typical of phage attachment site regions (33, 67, 72). PCR amplification and DNA sequencing of the *attR* and *attL* junction regions in two lysogenic isolates of IMN-C17, five lysogenic isolates of IMN-C18, and φLC3 parental strain IMN-C3 demonstrated that the same *attP* and *attB* sites had been used for integration in all the lysogenic strains examined. This result supports the Southern analysis results (Fig. 1) and previous data (39) indicating that the *attB* site described here could be the only attachment site or at least a strongly preferred site used for φLC3 integration.

Analyses of φLC3 lysogens. As mentioned in Materials and Methods, a background of unintegrated phage DNA was present in the φLC3 lysogens and detected as an *attP* DNA restriction fragment on Southern blots (Fig. 1, lanes 5 to 8). Cultures of φLC3 lysogens may contain 10^6 free phage particles. This amount is about 100 times more than that in cultures of, for instance, λ lysogens (45), indicating a relatively high level of spontaneous induction. Apparently, some

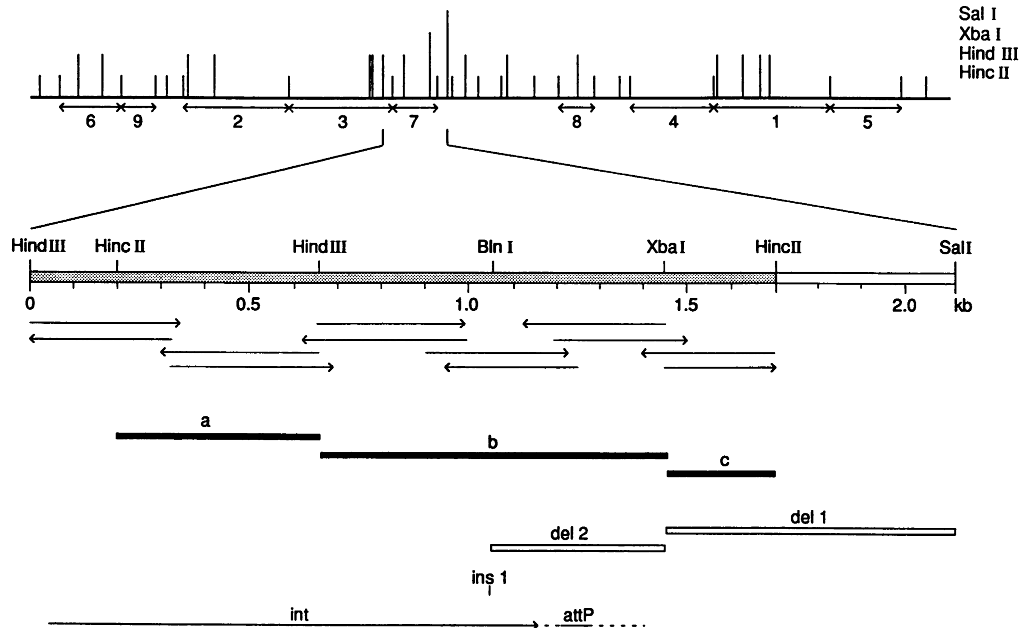

FIG. 2. Restriction map of φLC3 (39) and physical and genetic maps of the 2.1-kb region carrying the *int* gene and *attP*. The nine largest φLC3 *Hin*cII restriction fragments are numbered. The stippled region represents the 1.7-kb region that has been characterized by DNA sequencing (see Fig. 5). The restriction sites shown were used for subcloning and mutagenesis. Also shown are the sequencing strategy (pairwise arrows below the stippled region), the locations of the probes (a, b, and c) used in the Southern analysis, deletion mutations del1 and del2, *int* insertion mutation ins1, the coding region for the *int* gene, and the location of *attP*.

of this background was caused by superinfection of the spontaneously released phages, because the background on Southern blots was clearly reduced by growth of the lysogens in the presence of φLC3 antiserum (38). However, since some intracellular unintegrated phage DNA still was present in DNA preparations from the lysogens, it could be argued that this extrachromosomal phage DNA might represent a P1-like φLC3 plasmid form (69). However, the fact that we were not able to isolate stable lysogens with the φLC3 *int*ins1 mutant (see below) strongly suggests that integration is a crucial step in the establishment of stable lysogeny. Also, previous pulsed-field gel electrophoresis analysis and Southern analysis showed that a large percentage of lysogenic cells carries an integrated φLC3 prophage (39). This result was examined further in the present study by the use of quantitative PCR for analysis of the proportion of the phage-host hybrid site, *attL*, in cultures of φLC3 lysogens (Fig. 5). The results of this analysis indicated that the φLC3 prophage is integrated into the same chromosomal *attB* site in more than 99% of the cells (Fig. 5, lane 8 versus lanes 3 and 4). The proportion of intact *attB* sites in a culture of a lysogen, estimated to be ca. 1:200 by quantitative PCR analysis (Fig. 5, lanes 3 and 4 versus lanes 1, 2, and 8), may be representative of cells in which the *attB* site has been restored following excision of the prophage because of spontaneous induction. Induction of a φLC3 lysogen with mitomycin C led to a dramatic increase in the proportion of uninterrupted *attB* sites (Fig. 5, lanes 5 and 6 versus lanes 3 and 4), demonstrating that *attB* is restored during the recombination process. Moreover, no intact *attB* site was detected in cells of IMN-C1819, carrying the excision-defective φLC3 *int*ins1 mutant (see below and Fig. 5, lane 7), an observation that also supports the notion that the intact *attB* sites found in φLC3 wt lysogens are a result of spontaneous induction. For comparison, we also estimated the proportions of uninterrupted versus interrupted *attB* sites in cultures of the *E.*

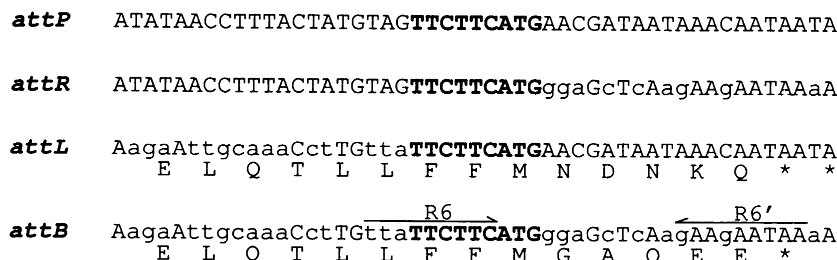

FIG. 3. Comparison of DNA sequences flanking the 9-bp common core (shown in boldface type) in the four attachment sites, *attP* (POP′), *attR* (POB), *attL* (BOP′), and *attB* (BOB′), and the C-terminal amino acid sequence of a putative gene product of an open reading frame overlapping the *attB* and *attL* core sequences. The termination codons are indicated by asterisks. Bases in *B* and *B′* that are divergent from those in *P* and *P′* are shown in lowercase letters. Inverted repeat R6, which overlaps the *attB* core, is also indicated.

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     HindIII
   1 AAGCTTTTTACTATACCATTTTATCAGAAATGAGGTATAAAAAGCAAATATGGCTACATATCAAAAGCGTGG
                                  RBS                   M  A  T  Y  Q  K  R  G

  73 TAAAACTTGGCAGTATTCAATATCAAGAACAAAACAAGGACTTCCTCGTCTAACAAAGGGTGGTTTTTCTAC
       K  T  W  Q  Y  S  I  S  R  T  K  Q  G  L  P  R  L  T  K  G  G  F  S  T
                                                                    HincII
 145 AAAGTCCGATGCACAAGCTGAAGCAATGGATATTGAAAGCAAACTAAAAAAAGGATTTATTGTTGACCCCAT
       K  S  D  A  Q  A  E  A  M  D  I  E  S  K  L  K  K  G  F  I  V  D  P  I

 217 TAAGCAAGAAATTTCCGAATATTTTAAAGACTGGATGGAACTTTATACGAAAAATGCAATTGATGAAATGAC
       K  Q  E  I  S  E  Y  F  K  D  W  M  E  L  Y  T  K  N  A  I  D  E  M  T

 289 TTATAAAGGTTATGAGCAAACGTTAAAATATTTAAAAACCTATATGCCAAATGTTTTAATTTCCGAAATAAC
       Y  K  G  Y  E  Q  T  L  K  Y  L  K  T  Y  M  P  N  V  L  I  S  E  I  T

 361 AGCATCTTCTTATCAAAGAGCGCTAAATAAATTTGCTGAAACACACGCCAAAGCATCTACAAAAGGGTTTCA
       A  S  S  Y  Q  R  A  L  N  K  F  A  E  T  H  A  K  A  S  T  K  G  F  H

 433 TACTAGAGTTAGAGCATCTATTCAACCACTCATTGAAGAGGGACGACTGCAAAAAGATTTTACCACTCGTGC
       T  R  V  R  A  S  I  Q  P  L  I  E  E  G  R  L  Q  K  D  F  T  T  R  A

 505 AGTAGTTAAAGGTAATGGAAATGATAAAGCCGAGCAAGACAAGTTTGTAAATTTTGATGAATACAAGCAATT
       V  V  K  G  N  G  N  D  K  A  E  Q  D  K  F  V  N  F  D  E  Y  K  Q  L

 577 AGTTGATTATTTCAGAAATAGACTTAATCCAAACTATTCATCTCCCACTATGCTGTTTATAATTTCAATTAC
       V  D  Y  F  R  N  R  L  N  P  N  Y  S  S  P  T  M  L  F  I  I  S  I  T
                         HindIII
 649 TGGCATGAGAGCCAGTGAAGCTTTTGGCTTAGTCTGGGATGATATTGATTTTAATAATAACACTATCAAGTG
       G  M  R  A  S  E  A  F  G  L  V  W  D  D  I  D  F  N  N  N  T  I  K  C

 721 TCGCAGAACTTGGAATTACAGAAATAAAGTAGGTGGTTTCAAAAAGCCCAAAACAGATGCTGGAATAAGAGA
       R  R  T  W  N  Y  R  N  K  V  G  G  F  K  K  P  K  T  D  A  G  I  R  D

 793 TATTGTTATAGATGATGAAAGTATGCAATTGCTAAAAGATTTTAGAGAACAGCAAAAAACATTATTTGAAAG
       I  V  I  D  D  E  S  M  Q  L  L  K  D  F  R  E  Q  Q  K  T  L  F  E  S

 865 TTTGGGTATAAAACCGATACATGACTTTGTTTGTTATCATCCTTATAGAAAAATAATAACTCTCTCAGCTTT
       L  G  I  K  P  I  H  D  F  V  C  Y  H  P  Y  R  K  I  I  T  L  S  A  L

 937 GCAAAATACATTAGAACATGCATTAAAAAAACTAAAGATTTCTACTCCACTTACTGTACACGGTTTAAGGCA
       Q  N  T  L  E  H  A  L  K  K  L  K  I  S  T  P  L  T  V  H  G  L  R  H
                                                                  BlnI
1009 TACTCATGCTTCTGTTCTCCTCTATCATGGAGTTGATATCATGACTGTTTCAAAACGCCTAGGACACGCAAG
       T  H  A  S  V  L  L  Y  H  G  V  D  I  M  T  V  S  K  R  L  G  H  A  S

1081 TGTGGCTATCACACAGCAAACCTATATTCATATTATAAAAGAGCTAGAAAATAAAGATAAGGATAAAATAAT
       V  A  I  T  Q  Q  T  Y  I  H  I  I  K  E  L  E  N  K  D  K  D  K  I  I

1153 TGAGCTGCTACTGGAGTTATAATTTTCTTACAACAAAAATACAACAAATCATTAAAAACTAAGAAGAAACCC
       E  L  L  L  E  L  *                    R2
                                     R1           R1

1225 ATTATCTAAAGCATATATAACCTTTACTATGTAGTTCTTCATGAACGATAATAAACAATAATAGAATAAATA
                                         core

1297 TAAAATAAAAACGGCTTAATATAGCCGTTTTTCCTGTTTTTAAAACAACTAAAAAATATAGAAATAAAAAAC
             R3          R3'
                R4

1369 TCGTACAACAAAATTACAACATATCATTATGTTTCTGATAGTATTGTTGTAAATTTACAACAAACAAAAAAA
                 R2                                        P1
         R1          R1                          R1'            R1
                            XbaI
1441 GCCACTCCGAAGAATGGCTTAACTCTAGAAATAGGATGAAATCCCACAATCATCCCGAATATATTATAGCAC
      R5             R5'
                     R4

1513 AAAAAAAGCGCCCCAGTTAGGAGAGGGACGCTTAGGATAAACTTATGAAAAAAGTTTTTCGGAATAAGAACA

1585 TTATATAACTTTCCGTTCTTTTTGTAAAGAAAAAGCCCCGGAGGGCGAGTGATATATTAACCATATATCAGC

1657 AAAAGATATTACAAAAGTGCTTTTAAAACACATGTTTTTTTGGTTGAACGGTC
```

FIG. 4. Nucleotide sequence of the 1.7-kb φLC3 DNA region carrying *attP* and *int*. The deduced amino acid sequence of the Int protein is given below the nucleotide sequence. The *int* termination codon is indicated by an asterisk. Relevant restriction sites are indicated. The putative ribosome-binding site (RBS), the core sequence, the repeated sequences (R1 to R5) and inverted repeats (R′, R1′, R5′, and R6′), and the 20-bp palindrome (P1) are also indicated.

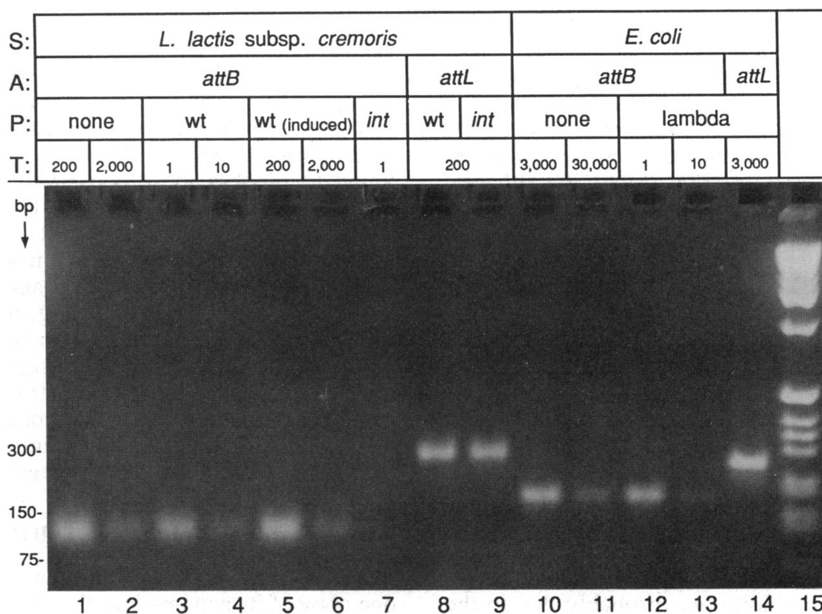

FIG. 5. Proportions of *attB* and *attL* DNAs in cultures containing φLC3 lysogenic *L. lactis* subsp. *cremoris* and λ lysogenic *E. coli*, as measured by quantitative PCR. The attachment site regions were amplified by use of bacterial chromosomal DNA from the lysogenic and nonlysogenic bacterial strains as a template. The figure shows the various *attB* and *attL* PCR products, amplified from different dilutions of 200 ng of chromosomal DNA, after separation by electrophoresis through a 2.5% agarose gel (lanes 7 to 14). Ten-microliter aliquots of the PCR mixtures were applied to the gel. Only the DNA from one representative amplification is shown. S, bacterial strain; A, amplified region; P, prophage carried by the lysogen; T, template dilution factor; wt, φLC3 wt (prophage in IMN-C1814); *int*, φLC3 *int*insl (prophage in IMN-C1819); wt $_{(induced)}$, template DNA extracted from IMN-C1814 1 h after φLC3 prophage induction. Lane 15 contains 1-kb DNA ladder (Bethesda Research Laboratories). The *L. lactis* subsp. *cremoris* and *E. coli* nonlysogenic strains and the *E. coli* λ lysogenic derivative were IMN-C18, C600, and K-12, respectively. Induction of the φLC3 prophage was performed by the addition of 2 mg of mitomycin per ml to a bacterial culture in its early exponential phase as described previously (44). The proportion of intact *attB* sites in a culture of a lysogen was measured both as the amount of *attB* PCR DNA relative to the amount of *attL* PCR DNA produced from the lysogen and as the amount of *attB* PCR DNA produced from the lysogen relative to the amount of *attB* PCR DNA produced from the nonlysogen. Results obtained from the amplification of *attL*, *attR*, and *attB* in four independent IMN-C18 lysogenic isolates, two φLC3 lysogenic derivatives of IMN-C17, and φLC3 parental strain IMN-C3 were all similar (38). The identities of all PCR products were confirmed by DNA sequencing.

coli λ lysogen K-12 and found them to be 1:3,000 (Fig. 5, lanes 12 and 13 versus lanes 10, 11, and 14). The data in Fig. 5 agree with the frequency of spontaneous λ induction (22, 45). Similarly, the proportion of intact *attB* sites in cultures of φLC3 lysogens, which was found to be about 10 times higher than that in cultures of λ lysogens (Fig. 5), probably corresponds to the higher level of φLC3 spontaneous induction (see above). If, on the average, 50 copies of the φLC3 genome are produced in each induced cell, as indicated by burst size experiments (39), the ratio of integrated versus unintegrated DNAs in a lysogen culture may be postulated to be ca. 1:4. The background of extrachromosomal versus integrated φLC3 DNAs may be estimated to be between 1:5 and 1:10, as judged by the relative intensities of the *attR*-, *attL*-, and *attP*-containing bands on the Southern blot (Fig. 1, lanes 7 and 8). Hence, our results strongly indicate that the unintegrated phage DNA observed in φLC3 lysogens is representative of a relatively high proportion of cells in which phage DNA is actively replicating because of spontaneous induction and that integration into the bacterial chromosome is a prerequisite for the establishment of φLC3 lysogeny.

The φLC3 *attB* site is located within a protein coding region. The *attB* core sequence was found to be located within an open reading frame 15 bp upstream from the stop codon of this reading frame (Fig. 3). Thus, φLC3 integrates into this open reading frame but, interestingly, the integration leads to the substitution of the five C-terminal amino acids with five other residues encoded by phage DNA at the *attL* junction region (Fig. 3). The size of this open reading frame is thus preserved in the lysogen, and it is possible that the slight change in the C terminus caused by the integration does not disrupt the function of the putative gene product. The actual size of this open reading frame has not yet been determined, but preliminary sequence analysis indicates a length of more than 600 bp (38). A search for amino acid sequence similarities did not reveal any significant similarity between this putative protein and other polypeptides in current data bases.

Identification and characterization of the φLC3 *int* gene. Sequence analysis of a 1.7-kb region surrounding the φLC3 *attP* site, between the *Hin*dIII and the *Hin*cII sites, as shown in Fig. 2, revealed a 1.1-kb open reading frame ending 87 bp to the left of the core sequence (Fig. 4). This open reading frame, encoding a basic protein of 374 amino acids, begins with an ATG initiation codon that is preceded by an 11-bp putative ribosome-binding site with almost complete homology to the 3′ end of the *L. lactis* 16S rRNA (40). A computer search of protein data bases revealed significant homology between the putative *orf374* gene product and several phage and plasmid site-specific recombinases belonging to the integrase family (1, 4, 56), strongly suggesting that *orf374* encodes the φLC3 site-specific recombinase. The location of *attP* close to the C-terminal end of the integrase gene is also

typical of the organization of these integrative recombination systems.

The *Bln*I site in *orf374* (Fig. 2 and 4) is unique in the φLC3 genome and was used for the in vitro construction of a mutation in *orf374*. A 4-bp insertion mutation was made by Klenow filling in of *Bln*I-digested φLC3 phage DNA, blunt-end ligation, and transfection of strain IMN-C18. The 4-bp insertion leads to a frameshift mutation, replacing the 34 C-terminal amino acids with a 22-amino-acid terminus. Thus, the mutated gene encodes a 362-amino-acid fusion protein retaining the first 340 amino acids of the *orf374* gene product. A φLC3 mutant (φLC3 *int*ins1) carrying this 4-bp insertion was unable to give rise to stable lysogens, although the plaques formed were turbid and indistinguishable from φLC3 wt plaques. Plaques made by the φLC3 wt are turbid, because lysogens that have acquired immunity to φLC3 arise and grow during the formation of the plaques. When cells picked from plaques made by the φLC3 mutant were streaked onto agar plates, the colonies formed had an irregular appearance and were more transparent than the colonies isolated from φLC3 wt plaques. Repeated attempts to isolate stable lysogenic clones from the φLC3 mutant plaques failed, and their turbidity was probably due to the growth of abortive lysogens in which φLC3 was not integrated and which were therefore unstable. However, when cells of strain IMN-C18 were picked from a phage spot containing a mixture of φLC3 *c*1 clear-plaque mutant phage (39) and *orf374* mutant phage, stable lysogens carrying the *orf374* mutant phage were obtained (38). This result demonstrates that the 4-bp insertion in *orf374* affects a diffusible factor that can be supplied in *trans* by a coinfecting phage and not a structural element of the φLC3 attachment site. Thus, in addition to the similarity between *orf374* and other phage integrases, the φLC3 mutant carrying the 4-bp insertion in *orf374* is phenotypically similar to phage λ *int* mutants (16). This similarity identifies *orf374* as the φLC3 *int* gene. In the φLC3 del2 mutant, the C-terminal region of *int* and the complete *attP* site are deleted by removal of the 398-bp *Bln*I-*Xba*I fragment (Fig. 2 and 4). This mutant confirmed the abortive lysogen phenotype.

A culture of strain IMN-C1819, an IMN-C18 derivative lysogenized with the *int*-defective φLC3 *int*ins1 mutant phage by complementation with the φLC3 int^+ *c*1 clear-plaque mutant phage, contains ca. 10^4 times fewer free phage particles than does a culture of a strain lysogenized with φLC3 wt phage. Thus, the φLC3 *int*ins1 lysogen is phenotypically similar to λ lysogens carrying an excision-defective λ *int* mutant prophage (15). The low level of φLC3 *int*ins1 spontaneous induction was confirmed by quantitative PCR analysis, in which the relative amounts of unintegrated intracellular φLC3 DNA in IMN-C1814 and IMN-C1819 were measured as the relative amounts of *attP*-containing PCR products produced by use of *attP*-specific primers. This analysis showed that cultures of IMN-C1819 contain at least 10^4 times fewer intracellular unintegrated φLC3 DNA than cultures of IMN-C1814. The fact that no uninterrupted φLC3 *attB* sites were detected in cultures of IMN-C1819 (see above and Fig. 5, lane 7) also confirms the excision-defective phenotype. Hence, the φLC3 Int protein, like that of phage λ (15, 16), is required for efficient excision as well as for integration of the φLC3 prophage.

The φLC3 Int protein is a member of the integrase family of recombinases. As mentioned above, amino acid sequence similarity between the φLC3 Int protein and several phage and plasmid integrases was found. The strongest overall homology was found to the integrase of temperate phage L54a, from *Staphylococcus aureus*, which shares 20.9% amino acid identity with the φLC3 integrase (Fig. 6A). Although the integrase family of recombinases is a diverse group of enzymes, all members share three moderately conserved regions (1, 4, 56) that are believed to make up part of the catalytic site (1, 52). The φLC3 integrase was also found to possess these conserved regions, including four invariant amino acid residues (1). Within the C-terminal conserved region, the strongest sequence similarity was found to transposase A (TnpA), from *S. aureus* transposon Tn*554* (48), which shares 53.8% amino acid sequence identity with the φLC3 integrase (Fig. 6B). Furthermore, many of the sequence differences represent conservative amino acid substitutions. The φLC3 *int*ins1 mutation disrupts the *int* reading frame 12 codons upstream of the codon for the invariant tyrosine residue, thus demonstrating that the region carrying the invariant tyrosine residue is also essential for the function of the φLC3 integrase.

DISCUSSION

Previously, we showed that bacteriophage φLC3 inserts one copy of its genome into the chromosome of its bacterial host, *L. lactis* subsp. *cremoris* IMN-C18 (39). Here, we demonstrated that the establishment of lysogeny by the temperate lactococcal bacteriophage φLC3 involves insertion of its genome into the bacterial chromosome through site-specific recombination between phage and bacterial attachment sites, *attP* and *attB*, respectively, after circularization of the linear φLC3 genome within the host cell. However, Southern analyses of DNA from φLC3 lysogens revealed the presence of some phage DNA in an unintegrated state, and it could be argued that this unintegrated state might represent a P1-like φLC3 plasmid form. However, as outlined in detail in Results, our results clearly demonstrate that this extrachromosomal φLC3 DNA represents replicating phage DNA that has been excised from the chromosome because of spontaneous induction and does not represent a P1-like φLC3 plasmid form.

Sequence and mutational analyses showed that the integration of φLC3 into the bacterial chromosome requires a φLC3-encoded site-specific recombinase, clearly related to the integrase family of recombinases found in various plasmids, transposons, and temperate bacteriophages. Moreover, the phenotype of the lysogen carrying the *int*-defective φLC3 *int*ins1 mutant prophage was similar to those of lysogens carrying *int*-defective λ mutant phage (15, 16), demonstrating that the φLC3 Int protein, like the λ Int protein, is needed for efficient excision as well as for integration of the prophage.

The phage and bacterial attachment sites have been identified and sequenced, but the exact boundaries of the functional φLC3 *attP* and *attB* sites are not yet known. However, the *attP* sequence needed for integration is probably located within the 290-bp region between the end of the *int* gene and the *Xba*I site (Fig. 2 and 4), because the φLC3 del1 mutant, which deletes the 700-bp *Xba*I-*Sal*I restriction fragment localized to the right end of this 290-bp region (Fig. 2), gives rise to stable lysogens. The various direct and inverted sequence repeats located in this region could represent recognition sites for the φLC3 integrase and other proteins possibly binding within *attP*, such as a φLC3 analog of the λ excisionase (24) and lactococcal homolog of IHF or FIS (5, 6, 65). The R3 inverted repeat located 130 bp downstream from the C-terminal end of the *int* gene represents a potential hairpin loop that could function as a termination signal for *int*

A

```
φLC3   MATYQKRGKTWQYSISRTKQGLPRLTKGGFSTKSDAQAEAMDIESKLKKGFIVDPIKQEI    60
L54a   M--FRLEEKIKEKLNNKSSSELKTLT---FHALLD---EWLEYHIK-TSGFKVTTLDN--    49
       *  ..   *  .   ...   *  **   * .  *   * .. . *   ** *  . .

φLC3   SEYFKDWMELYTKNAIDEMTYKGYEQTLKYLKTYMPNVLISEITASSYQRALNKFAETHA   120
L54a   ---LKTRIKNIKKNSSQNLLLNKIDT--KYMQTFI-NELSNVYSANQVKRQLGHMKEA-I   102
          .* ..    **. ...  .  .   **..*.. * * .  .*.  .* *    *.

φLC3   KASTKGFHTRVRASIQPLIEEGRLQKDFTTRAVVKGNGNDKAEQDKFVNFDEYKQLVDYF   180
L54a   KYAVKFYNY----PNEHILNSVTLPKKSKT---IEDIEKEEAKMYNYLEMEQVIQIRDFI   155
       * . * ..     . . ...   * *   *   . .  .. *   .... ..  *. *..

φLC3   RNRLNPNYSSPTMLF----IISITGMRASEAFGLVWDDIDFNNNTIKCRRTWNYRNKVGG   236
L54a   LNDNNMQYRARILVAGAVEVQALTGMRIGELLALQVKDVDLKNKTIAINGTIHRIKCNAG   215
        *  * .* .  ..     ... ****.* ..*   *.*..*.**    * .  .  .*

φLC3   F---KKPKTDAGIRDIVIDDESMQLLKDFREQQKTLFESLGIKPIHDFVCYHPYR-KIIT   292
L54a   FGHKDTTKTAGSKRKIAINSRIANVLKKIMLENKKMQQWEPSYVDRGFI-FTTCQGNPMQ   274
       *      ** .. * * *.    ..** .  ..* . .       ..*. .   . . .

φLC3   LSALQNTLEHALKKLKISTPLTVHGLRHTHASVLLYHGVDIMTVSKRLGHASVAITQQTY   352
L54a   GSRINKRLSSAAESLNINKKVTTHTLRHTHISLLAEMNISLKAIMKRVGHRDEKTTIKVY   334
        * ... *  *   *.*.  .* * ***** *.*    . . .. **.**     * . *

φLC3   IHIIKELENKDKDKIIELLLEL                                         374
L54a   THVTEKMDRELEQKLEKLVY                                           354
        *.   ..    .*.  *.
```

B

```
Tn554 TnpA   302   HMLRHTHATQLIREGWDVAFVQKRLGHAHVQTTLNTYVH   339
                   | ||||||• |• •| |•  | ||||||••  | •||•|
φLC3 Int     263   HGLRHTHASVLLYHGVDIMTVSKRLGHARLAITQQTYIH   302
                   | |||| || |   |         |||         | |
Int family         H-LRHT-AS-L---G-----IQ--LGH---------Y-H
```

FIG. 6. (A) Comparison of the Int proteins of φLC3 and L54a. Asterisks and dots indicate identical and similar amino acids, respectively. The four invariant amino acids are boxed. Dashes represent gaps in the sequence. (B) Alignment of the conserved C-terminal region of φLC3 Int with the conserved C-terminal region of transposon Tn*554* TnpA protein (48) and with the conserved C-terminal region of the integrase family of site-specific recombinases (56). Identical and similar amino acids are indicated by vertical lines and dots, respectively. The three invariant amino acids are underlined. Dashes represent nonconserved and moderately conserved amino acids.

transcription. The φLC3 *attB* site was found to be located within a region encoding the C-terminal part of an open reading frame of unknown function. Thus, the integration of φLC3 disrupts this putative gene, but in the lysogen, the five C-terminal amino acids are replaced by five other residues, thus preserving the length of the open reading frame. As long as we do not know the role of this open reading frame, we can only speculate as to whether its function is preserved in the lysogen. To our knowledge, only the two *S. aureus* temperate phages, L54a and φ13, have been shown to integrate into a protein coding region, disrupting it in the process (13, 30, 31). Most bacteriophages and integrating plasmids so far characterized insert their DNA either into nonessential regions of the bacterial chromosome (7, 32) or into tRNA genes (8, 21, 35, 43, 57, 63).

A 9-bp core region, within which the strand exchange reaction takes place, has been identified. The size of the common core sequence of the integrase family of site-specific recombinases varies from a hexanucleotide sequence in transposon Tn*554* (49) to a 182-bp region in temperate bacteriophage HP1 (14). To our knowledge, the 9-bp φLC3 core sequence is the smallest core sequence found in a temperate bacteriophage.

Thus, analyses of φLC3 lysogens, sequence analyses of the φLC3 attachment site regions, and the amino acid sequence and functional similarities found between the φLC3 Int protein and the λ Int protein as well as other site-specific recombinases strongly suggest that the mechanism for φLC3 Int-mediated site-specific recombination corresponds to the model for bacteriophage λ (10, 15, 65, 67). Further characterization of the region upstream from the φLC3 *int* gene is currently in progress to elucidate the mode of *int* regulation.

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