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Characterization of the Lactococcal Temperate Phage TP901-1 and Its Site-Specific Integration

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The temperate lactococcal phage TP901-1, induced by UV light from *Lactococcus lactis* subsp. *cremoris* 901-1, was characterized. The restriction map was found to be circular, and the packaging of TP901-1 DNA was concluded to occur by a headful mechanism. The *pac* region was localized on the 38.4-kb phage genome. TP901-1 belongs to the class of P335 phages (V. Braun, S. Hertwig, H. Neve, A. Geis, and M. Teuber, J. Gen. Microbiol. 135:2551–2560, 1989). Evidence is presented that the phages TP936-1 (V. Braun, S. Hertwig, H. Neve, A. Geis, and M. Teuber, J. Gen. Microbiol. 135:2551–2560, 1989) and C3-T1 (A. W. Jarvis, V. R. Parker, and M. B. Bianchin, Can. J. Microbiol. 38:398–404, 1992) are very closely related to or are identical to TP901-1. The lytically propagated TP901-1 phages were able to lysogenize both indicator strains *Lactococcus cremoris* 3107 and Wg2. Lysogenization resulted in site-specific integration of the phage genome into the bacterial chromosome. Only one chromosomal *attB* site was found in 20 independent lysogens. The *attP* region of TP901-1 and the *attL* and *attR* regions were cloned and sequenced. The results showed a core region of only 5 bp, in which the recombination occurs, followed after a 1-bp mismatch by a 7-bp identical region, TCAAT(T/C)AAGGTAA. This result was further verified by sequencing of the *attB* region obtained by PCR. An integration vector was constructed with the 6.5-kb *Eco*RI fragment from TP901-1 containing *attP*. This vector also functions in the plasmid-free strains MG1363 and LM0230 with only one specific *attB* site, strongly indicating a more general use of the TP901-1-based integration vector in lactococci.

Molecular studies of bacteriophages from *Escherichia coli* have revealed important gene regulatory mechanisms acting in the host strain. Also, many important genetic tools, such as phage cloning vectors and gene expression systems, have been developed from bacteriophages.

With the aim of developing genetic tools, such as a chromosomal integration system and regulated promoters, but also with the perspective later to be able to use the chosen phage as a tool for discovery of global gene regulatory systems in lactococci, we initiated a molecular study of the lactococcal temperate phage TP901-1.

Despite many cases of reported lysogeny among lactococcal bacterial strains, studies of the life cycles and molecular biology of the temperate phages from *Lactococcus* spp. have been scarce. One reason for this is probably the lack of suitable indicator strains for most of the induced phages.

The molecular biology of the temperate phages BK5-T and φLC-3—isolated from *Lactococcus lactis* subsp. *cremoris* (*L. cremoris*) BK5 and IMN-C3, respectively—however, has been studied in some detail (14, 22). Phage C3-T1 from *L. cremoris* C3 has also been characterized (13). Attachment and packaging sites were located in all three phages. In BK5-T and C3-T1, the phage genomes were shown to be linear, circular, permuted, and terminally redundant double-stranded DNA molecules (13, 14). Phage promoters affected by a phage gene were isolated and sequenced from BK5-T (15). Phage φLC-3 contained cohesive single-stranded DNA ends in a linear double-stranded phage genome (22). The integration system of φLC-3 has been analyzed and sequenced (21).

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In this report, we present the restriction map of phage TP901-1, including the location of the *attP* and *pac* regions. We have been able to monitor the lysogenic life cycle of the phage and demonstrate the presence of only one major attachment site. An integration vector based on TP901-1 DNA sequences was constructed, and the *attP*, *attL*, *attR*, and *attB* regions were identified, cloned, and sequenced. The integration system from TP901-1 was also shown to be functional and site specific in the laboratory strains often used for genetic studies of lactococci, namely *Lactococcus lactis* subsp. *lactis* MG1363 and LM0230. This strongly indicates that the integration system of TP901-1 may be of general use as a genetic tool with lactococci.

MATERIALS AND METHODS

Bacteria, plasmids, and phages. Bacterial strains and plasmids used in this work are listed in Table 1. Phage DNA from phages C3-T1 (13) and φLC3 (22) was obtained from A. Jarvis and D. Lillehaug, respectively.

Lactococcus strains were propagated at 30°C in M17 broth without shaking (28) (Oxoid Limited, Basingstoke, Hampshire, United Kingdom). Phage titers were determined as described by Terzaghi and Sandine (28). *E. coli* strains were grown with agitation at 37°C in Luria-Bertani broth (25) (Difco Laboratories, Detroit, Mich.). Bacto agar (Difco Laboratories) was used at 1.5% (wt/vol) in solid media and 0.7% (wt/vol) in top agar.

Temperate phages were induced from their hosts by UV irradiation. M17 broth was inoculated with 1% (vol/vol) of an overnight culture of the lysogenic strain. The culture was at an optical density at 600 nm of 0.15, harvested at 5,000 × *g* for 10 min, and resuspended in 0.5 volume of NC (0.5% NaCl [wt/vol], 5 mM $CaCl_2$). The suspension was pumped through a quartz flow cuvette placed at the bottom of a UV field (254

TABLE 1. Bacteria and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
L. lactis		
Wg2	Indicator strain for TP901-1 and TP936-1	2
3107	Indicator strain for TP901-1 and TP936-1	2
901-1	Lysogenic for TP901-1	2
936-1	Lysogenic for TP936-1	2
ES46	3107::TP901-1	This study
BK5	Lysogenic for BK5-T	14
LM0230		8
MG1363		9
BC1014	3107::pBC143	This study
BC1017	MG1363::pBC143	This study
BC1022	LM0230::pBC143	This study
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r)]	Stratagene, La Jolla, Calif.
Plasmids		
pBluescriptIISK(+)	*lacZ bla*	Stratagene, La Jolla, Calif.
pGEM-5zf(−)	*lacZ bla*	Promega, Madison, Wis.
pGEM-7zf(+)	*lacZ bla*	Promega, Madison, Wis.
pIL253	*erm*	27
pUC7,erm[a]	pUC7::1.1-kb *Hin*PI pIL253 *erm*	W. M. de Vos, NIZO, Ede, The Netherlands
pBC104	pGEM-7Zf(+)::2.3-kb *Cla*I TP901-1 *bla*	This study
pBC144	pGEM-7Zf(+)::1.1-kb *erm bla*	This study
pBC143	pGEM-7Zf(+)::6.5-kb *Eco*RI TP901-1::1.1-kb *erm bla*	This study
pBOP1	BC1014 *Pst*I-rescue *erm bla attL* (10 kb)	This study
pPOB1	BC1014 *Sac*I-rescue *bla attR* (14 kb)	This study
pPOB2	BC1014 *Bam*HI-rescue *bla attR* (12.5 kb)	This study
pBOP6	pGEM-7Zf(+)::3.6-kb *Cla*I 901-1 *bla attR*	This study
pG5f1, pG5f3-pG5f12	pGEM-5Zf(−)::*Eco*RV TP901-1 *bla*	This study
pBf2-1	pBluescriptIISK(+)::4.7-kb *Eco*RI-*Eco*RV TP901-1 *bla*	This study
pBf2-2	pBluescriptIISK(+)::2.5-kb *Eco*RI-*Eco*RV TP901-1 *bla*	This study
pG7f1-pG7f8a,b-pG7f13	pGEM-7Zf(+)::*Eco*RI TP901-1 *bla*	This study

[a] The pUC7,erm plasmid was obtained by cloning the 1.1-kb *Hin*P1 fragment from pIL253 into the *Hin*cII site of pUC7. The *erm* cassette can be moved on a 1.1-kb *Bam*HI or *Eco*RI fragment.

nm) (CN15; Vilber Lourimat, Marne La Vallée, France). The average exposure time was approximately 23 s. The suspension was transferred to an equal volume of twofold concentrated M17 and incubated in darkness at 30°C until lysis. Liberated phages were precipitated from the supernatant by incubation with NaCl (1 M) and polyethylene glycol 6000 (10% [wt/vol]) and further purified by CsCl step gradients as described for bacteriophage λ (25).

Lysogenization of the indicator strains. Indicator strains and temperate phage were mixed in a CFU/PFU ratio of 10^7:10^7 in a total volume of 400 μl. The phage were allowed to adhere to the bacterial cells for 20 min at 30°C. The mixture containing the bacterium-phage complexes was diluted and plated on M17 plates, each containing an additional 10^8 phage. The plates were incubated at 30°C for 2 days. Colonies were purified three times on M17 plates.

DNA preparation. DNA extraction from purified phage particles was performed as described for λ (25). Extraction of chromosomal DNA was performed as described for *E. coli* (25), with the modification that cells were treated with 20 mg of lysozyme per ml for 4 h before lysis. Recombinant plasmid DNA from *E. coli* was isolated by the alkaline lysis technique, and preparative portions were further purified by CsCl-ethidium bromide equilibrium gradient centrifugation (25). All DNA preparations were resuspended and stored in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA).

Recombinant DNA techniques. Restriction endonuclease enzymes and buffer systems were supplied by Boehringer Mannheim Biochemicals (Mannheim, Germany), as were T4 DNA ligase and calf intestine alkaline phosphatase. The enzymes were used as recommended by the supplier. Analytical and preparative agarose gel electrophoresis was conducted with TAE buffer (40 mM Tris-acetate [pH 8], 1 mM EDTA). DNA restriction fragments were isolated from excised agarose gel segments with the Prep-A-Gene kit (Bio-Rad Laboratories, Hercules, Calif.).

Construction of plasmids. Library plasmids were constructed by ligating purified TP901-1 fragments into corresponding endonuclease-digested and calf intestine alkaline phosphatase-treated vector pGEM-7zf(+) (*Eco*RI and *Cla*I fragments), pGEM-5zf(−) (*Eco*RV fragments) or pBluescriptIISK+ (*Eco*RI-digested EV2 fragment). The integration plasmid pBC143 was obtained by inserting a 1.1-kb *Bam*HI fragment, containing the *erm* cassette from pUC7,erm, into *Bam*HI-digested and calf intestine alkaline phosphatase-treated pG7f2. pGEM-7zf(+) containing the *erm* cassette (pBC144) served as a control.

The rescue plasmids, containing the *attL* and *attR* regions, were obtained by digestion of chromosomal DNA of *L. cremoris* BC1014 with *Pst*I, *Sac*I, and *Bam*HI. The digested chromosomal DNA was ligated at concentrations of 5 μg/ml.

The cloning of the *attB* region from *L. cremoris* 3107 was

performed by digesting the 1.5-kb PCR product with *Sau*3AI and ligating the 224-bp *attB*-containing fragment into *Bam*HI-digested and calf intestine alkaline phosphatase-treated pGEM-7zf(+).

Transformation and selection. *E. coli* XL1-Blue was made competent with $CaCl_2$ and was transformed as described by Sambrook et al. (25). Transformants were selected on Luria-Bertani plates containing 100 μg of ampicillin per ml, 10 μg of tetracycline per ml, 200 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml, and 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG). When introducing the *erm* cassette, selection was performed on 100 μg of ampicillin per ml and 30 μg of erythromycin per ml.

L. lactis MG1363 and LM0230 were transformed by electroporation according to the method described by Holo and Nes (12). *L. cremoris* 3107 was transformed with this procedure, slightly modified, by propagating cells in M17 broth containing 0.2 M sucrose instead of 0.5 M sucrose. Transformants were selected on 1 μg of erythromycin per ml.

Hybridization. DNA restriction fragments separated on agarose gels were transferred to GeneScreen+ membranes (Du Pont, NEN Research Products, Boston, Mass.) by vacuum blotting as recommended by Pharmacia (Uppsala, Sweden). Colonies were transferred to BA-S 85 nylon membranes (Schleicher & Schuell, Dassel, Germany) for colony hybridizations as recommended by Boehringer Mannheim (1). Recombinant plasmids used as probes were digested with restriction enzymes before denaturation. Probes were labeled with digoxigenin–11-dUTP, and hybridizations were performed as recommended by the supplier (Boehringer Mannheim). Digoxigenin–11-dUTP-labeled λ DNA digested with *Hin*dIII (Boehringer Mannheim) was used as a molecular marker for the hybridizations.

Amplification of the *attB*, *attL*, *attR*, and *attP* regions. The *attB* region from *L. cremoris* 3107 was isolated by PCR with oligonucleotides BI-POB1(inv) (biotin-CATCCCCTACTAAT CCGAAC) and P4-BOP6(inv) (GTATGCAGCGATGTCGT TACCC) obtained from *attL* and *attR* sequences, respectively, resulting in a product with a size of about 1.5 kb. The *attL*, *attR*, and *attP* regions were amplified with primers BI-EV11SP6(inv) (biotin-CACTCGCTCAAGCTCGTATG) and P4-BOP6(inv) for *attL*, BI-POB1(inv) and P4-Rb (GTGTGATTCAGGAAC TTATG) for *attR*, and BI-EV11SP6(inv) and P4-Rb for *attP*. The amplified products had sizes of approximately 1.5, 0.7, and 0.7 kb, respectively. The primers BI-EV11SP6(inv) and P4-Rb were obtained from known phage sequences. Annealing (1 min at 50°C) and extension (1 min at 72°C) were carried out on a Perkin-Elmer Cetus DNA Thermal Cycler 480 (Perkin-Elmer Cetus, Branchburg, N.J.). The GeneAmp PCR reagent kit with Ampli*Taq* DNA polymerase was used as recommended by the supplier (Perkin-Elmer Cetus).

DNA sequencing. The DNA sequences were determined by the method of Sanger et al. (26), performed as instructed by the Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio). Sequencing of the insert ends of library clones was performed by using 1 pmol of T7 (TAAT ACGACTCACTATAGGG) and SP6 (GATTTAGGTGAC ACTATAG) primers, respectively. The *attL* region sequence was obtained in clones pBOP1 and pBOP6, with 2 pmol of primer P1-EV11SP6(inv) (TAATGGTATTTCTAGGCAGA AACT) in the reaction mixture. The *attR* region sequence was obtained in clones pPOB1 and pPOB2, with 2 pmol of primer P1-EV9SP6(inv) (CGTCAACAACCTTGTATCAAAGG) in the reaction mixture. The flanking sequences of EI11 were obtained on pG5f1 by using two primers, P1-7f11.Trev (CATA AAGGGTTAATCCG) and P1-7f11.Srev (ACCCATTGAA CAGTAGC), reading out of the EI11 fragment. The primers P1-7f4.Srev (AAGCGCAAGGAAGTGTC), P1-7f9.SP6rev (GAGATATTCAATCCGTTC), and P1-7f10.Srev (TGATAC GAATGCTGAAG), reading out of the fragments EI4, EI9, and EI10, respectively, were also used on pG5f1.

Single-stranded templates of the amplified genomic *attL*, *attR*, and *attB* regions were prepared with Dynabeads M-280 streptavidin, as recommended by the supplier (Dynal, Oslo, Norway). The sequencing was performed with the internal primers P-ATTBL (CTACTGCTGCTTCACCAG) for the *attL* and *attB* regions and P-EV9SP6(inv) for the *attR* and *attP* regions. All primers used were delivered by P. Hobolth, Lyngby, Denmark. Computer analyses of the sequence data were carried out with the GCG Sequence Software Package (Genetics Computer Group, Inc., Madison, Wis.).

Nucleotide sequence accession number. The nucleotide sequence data shown in Fig. 6 have been deposited in GenBank under accession no. L19215.

RESULTS

Biological characterization of phage TP901-1. Braun et al. (2) demonstrated that the bacterial strains *L. cremoris* 901-1 and 936-1 carried the prophages TP901-1 and TP936-1, respectively. The prophages were furthermore shown to be inducible by UV light. We performed the initial studies with both phages and did not discover any differences between TP936-1 and TP901-1. However, since it was initially easier to make large-scale phage preparations by UV light induction from *L. cremoris* 901-1 than from 936-1, we have chosen TP901-1 for further studies. When similar data have been obtained for TP936-1, it will be mentioned.

Typically, phage titers of 10^9 PFU/ml were obtained for both phages after UV light induction. Both phages formed plaques on the two indicator strains, *L. cremoris* Wg2 and 3107. However, the plaques obtained with *L. cremoris* Wg2 were smaller and more turbid than those observed when *L. cremoris* 3107 was used. The phage titers were identical, independent of the indicator strain.

Extra additions of Mg^{2+} or Ca^{2+} to the M17 medium did not increase the phage titers. The plaque-forming ability of the phage lysates could, however, be reduced 4 orders of magnitude by addition of 20 mM citrate, indicating that divalent ions present in the M17 medium were necessary for phage infection.

Attempts to isolate a phage-cured derivative of *L. cremoris* 901-1 have so far been unsuccessful. However, we were able to demonstrate the complete lysogenic cycle in both indicator strains. TP901-1 lysogenic derivatives of *L. cremoris* Wg2 and 3107 were isolated. The resultant lysogens were immune to infections with TP901-1 and TP936-1. Similar results were obtained with isolates lysogenic for TP936-1.

Another criterion of the lysogenic state, the spontaneous release of phages during growth of the lysogenic bacteria, was also fulfilled. Thus, overnight cultures of the lysogenized strains would typically contain 10^5 to 10^6 PFU/ml. Also, *L. cremoris* 901-1 and 936-1 released phages in the same order of magnitude. Both the UV light-induced phages and the lytical proliferated phages were able to lysogenize the indicator strains.

Molecular characterization of phage TP901-1. Restriction analyses of the DNA isolated from UV light-induced TP901-1 and TP936-1 were performed. The restriction fragment patterns were identical for all enzymes tested. The *Eco*RI digest was identical to the pattern for phage C3-T1 previously published (13). Also, the sizes of the restriction fragments from

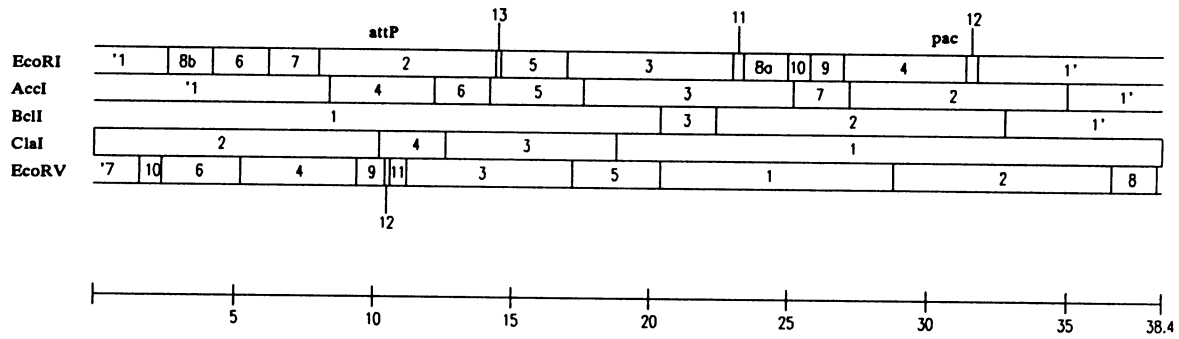

FIG. 1. Restriction map of TP901-1 phage genome. The circular phage TP901-1 DNA molecule has been opened at a *Cla*I site. Restriction enzyme digestions gave the following fragment sizes (in kilobases): *Eco*RI fragments, EI1, 9.5; EI2, 6.5; EI3, 6.0; EI4, 4.4; EI5, 2.4; EI6, 1.9; EI7, 1.8; EI8a, 1.5; EI8b, 1.5; EI9, 1.2; EI10, 0.8; EI11, 0.4; EI12, 0.3; EI13, 0.2; *Acc*I fragments, A1, 12.0; A2, 7.7; A3, 7.3; A4, 3.9; A5, 3.3; A6, 2.2; A7, 2.0; *Bcl*I fragments, B1, 26.0; B2, 10.5; B3, 1.9; *Cla*I fragments, C1, 19.6; C1a, 16.0; C2, 10.2; C3, 6.3; C4, 2.3; *Eco*RV fragments, EV1, 8.4; EV2, 7.6; EV3, 6.0; EV4, 4.3; EV5, 3.1; EV6, 2.7; EV7, 1.9; EV8, 1.6; EV9, 1.0; EV10, 0.9; EV11, 0.6; EV12, 0.2. Fragments A2, C1, B2, and EV2 are submolar but are present in the prophage at equimolar proportions and therefore are included in the calculation of the phage TP901-1 genome size, in contrast to C1a, which is submolar but is not present in the prophage at equimolar proportions. *attP*, phage attachment site; *pac*, phage packaging region. The linear scale is given in kilobases.

*Xho*I, *Sph*I, *Pvu*II, and *Bcl*I digestions of phage C3-T1 DNA (13) are the same as those found for TP901-1 (data not shown). Minor bands were observed in all digests, indicating that the phage DNA is terminally redundant, as a result of initial cleavage at the *pac* region followed by a headful packaging mechanism, with a concatemeric phage DNA substrate (5, 13, 14). Also, when phage DNA samples, ligated before digestion, were compared with unligated samples or when heated and unheated samples were compared, no differences in the patterns or intensities of the bands were observed (data not shown). This indicates that TP901-1 does not contain cohesive ends and thus belongs to the *pac*-type phages. A restriction map of the TP901-1 genome (Fig. 1) was constructed on the basis of data from single and double digestions in combination with Southern hybridizations with library clones (see below) or purified DNA fragments as probes (data not shown). The order EI8a-EI10-EI19-EI4 was verified by sequencing on pG5f1 (the EV1 clone) with primers reading out of the *Eco*RI fragments. The map was found to be circular. On the basis of the digests, an average genome size of 38.4 kb could be calculated.

Cloning of TP901-1 DNA. A complete library of the 14 *Eco*RI fragments from TP901-1 was constructed in pGEM-7zf(+). The library clones were designated pG7f1 to pG7f13 (including pG7f8a and pG7f8b). Similarly, 11 of 12 *Eco*RV fragments were cloned into pGEM5-zf(−) (designated pG5f1 to pG5f12, excluding pG5f2). It was not possible to obtain a clone containing the entire EV2 fragment. After cleaving the EV2 fragment with *Eco*RI, the two *Eco*RI-*Eco*RV fragments were cloned in pBluescriptIISK+ (pBf2-1 and pBf2-2). Also, the 2.3-kb *Cla*I (C4) fragment was cloned in pGEM-7zf(+) (pBC104).

Identification of *attP*, *attL*, and *attR*. In order to identify the region containing the attachment site (*attP*) on phage TP901-1, a series of hybridization experiments were conducted with labeled TP901-1 DNA as a probe. When *Cla*I-digested chromosomal *L. cremoris* 901-1 DNA was compared with *Cla*I-digested phage TP901-1 DNA, it was observed that a 2.3-kb *Cla*I fragment (C4) was not present in the chromosomal digest (data not shown). Instead, a 3.6-kb fragment and a 4.2-kb fragment, not present in the phage, hybridized to the TP901-1 probe. This was also seen when pBC104 (containing C4) was used as a probe (Fig. 2). The hybridization confirmed that the *attP* region was within this fragment and suggested that the two chromosomal junctions of *L. cremoris* 901-1, containing the *attL* and *attR* regions, were located on 3.6- and 4.2-kb *Cla*I fragments, respectively. Further hybridizations, with the three *Eco*RV library plasmid clones mapping within the C4 fragment used as probes, located *attP* on the 0.2-kb *Eco*RV (EV12) fragment (data not shown). One of the flanking *Eco*RV fragments (EV11) hybridized to the 3.6-kb *Cla*I junction (*attL* region), while the other (EV9) hybridized to the 4.2-kb junction (*attR* region).

When *Cla*I-digested chromosomal DNA from independently isolated TP901-1-lysogenized derivatives of *L. cremoris* 3107 and Wg2 (10 from each strain) were probed with pBC104, all 20 isolates gave a hybridization pattern similar to that of *L. cremoris* 901-1 (Fig. 2, lane 3). No signals from the parental

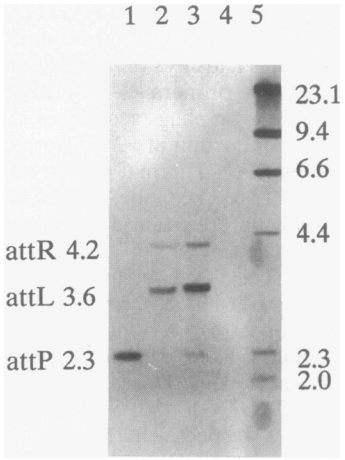

FIG. 2. Identification of the TP901-1 *attP* site in a Southern blot of *Cla*I-digested DNA. Lanes: 1, TP901-1 phage DNA; 2 to 4, chromosomal DNA from *L. cremoris* 901-1, ES46, and 3107, respectively; 5, *Hin*dIII-digested λ DNA. The DNA was hybridized to the digoxigenin-labeled probe of pBC104 containing the 2.3-kb *Cla*I fragment from TP901-1. The positions of the *attL* and *attR* junctions are indicated. The position of the TP901-1 *attP* fragment is also marked. The fragment sizes are given in kilobases.

strain *L. cremoris* 3107 were observed (Fig. 2, lane 4). Hybridization signals corresponding to the 2.3-kb *Cla*I fragment were also observed. This is probably due to concatemeric phage DNA in the chromosomal DNA preparations. These results show that the integration event is site specific and that only one major attachment site is found in *L. cremoris* 3107 and Wg2. This attachment site (*attB*) is located on a 5.5-kb *Cla*I fragment (data not shown).

Construction of an integration vector. Phage-encoded functions required for integration of temperate bacteriophages (i.e., *attP* and integrase) are often tightly clustered in the phage genome (20, 21, 23, 29, 30). The *attP* region of TP901-1 is located almost in the center of the 6.5-kb *Eco*RI fragment (EI2). This fragment might thus contain the putative phage-encoded integrase. Therefore, pBC143 and pBC144 were constructed by inserting a gram-positive selection marker into pGf72 and pGEM7-zf(+), respectively. The *erm* gene from pUC7,erm was used. Both plasmids lack an origin of replication which functions in lactococci. Putative transformants obtained from electroporation of pBC143 into lactoccocal strains should therefore be integrants.

Electrocompetent *L. cremoris* 3107 cells were transformed with pBC143, and 1 erythromycin-resistant (Em^r) transformant per μg of DNA was isolated (BC1014), while the control plasmid pBC144 did not give any Em^r clones. These experiments were difficult to evaluate because of the very low transformation efficiency of *L. cremoris* 3107. Even pIL253, which is able to replicate, gives only 10^3 transformants per μg of DNA. The strains *L. lactis* MG1363 and LM0230 are more easily made electrocompetent, and transformation frequencies of 10^4 and 10^6 transformants per μg of pIL253 DNA, respectively, were measured in our laboratory. We therefore tested whether the putative integration vector pBC143 could work in *L. lactis* MG1363 and LM0230. We obtained 10^2 and 10^4 Em^r colonies per μg of pBC143 DNA, respectively, while no colonies were found when the control plasmid pBC144 was used (e.g., $<1/\mu g$ of DNA). These numbers strongly indicate that integration occurs when pBC143 is used. This was confirmed by hybridization analysis of the chromosomal DNA of the corresponding Em^r transformants. One *L. cremoris* 3107 and three independently isolated Em^r transformants from both *L. lactis* MG1363 and LM0230 were analyzed. The chromosomal DNA was digested with *Cla*I and used for Southern blot experiments, with pBC104 as a probe. The hybridization results with one transformant from each strain are shown in Fig. 3. None of the transformants contained the 2.3-kb C4 fragment carrying *attP*, which is present in pBC143 (Fig. 3, lane 2). Instead, all transformants showed hybridization to two new *Cla*I fragments not present in pBC143. In *L. cremoris* BC1014, the two fragments were 3.6 and 4.2 kb, similar to the sizes found for the *attL* and *attR* fragments, respectively, in *L. cremoris* 901-1 (Fig. 3, lanes 3 and 5). In *L. lactis* LM0230::pBC143 (BC1022) and MG1363::pBC143 (BC1017), a 3.6-kb fragment was also present (*attL*), while the *attR*-containing fragment had a size of 11 kb (Fig. 3, lanes 7 and 9). All transformants showed hybridization signals corresponding to the vector *Cla*I fragments of 1.8 and 6.5 kb. No hybridization signals from the chromosomal DNA from the recipient strains *L. cremoris* 3107 and *L. lactis* LM0230 and MG1363 were observed (Fig. 3, lanes 4, 6, and 8).

The results therefore demonstrate that pBC143 has integrated into the chromosome of all the recipient strains by site-specific integration with the *attP* region harbored on the plasmid.

Cloning of the *attL* and *attR* regions. Attempts to clone the *attL* and *attR* regions from the chromosome of *L. cremoris* 901-1 were successful only for *attL*. After isolation and cloning of chromosomal *Cla*I fragments in the 3- to 4-kb size range into pGEM-7zf(+), two clones hybridizing to the 2.3-kb C4 fragment of TP901-1 (containing *attP*) were identified. Both clones contained a plasmid having a *Cla*I insert of the expected size of 3.6 kb. Further restriction and hybridization analyses with *Cla*I and *Eco*RV confirmed that the plasmids did contain the *attL* region from the chromosome of the lysogenic *L. cremoris* 901-1. One of the plasmids, designated pBOP6, was used for DNA sequencing. By using another approach—the method of plasmid rescuing—both the *attL* and the *attR* regions were cloned from *L. cremoris* 3107. Advantage was taken of the integrant in *L. cremoris* BC1014. By digestion of the chromosomal DNA of *L. cremoris* BC1014 with a restriction enzyme unique for pBC143, religation of the chromosomal digest, and transformation in *E. coli*, the chromosomal fragment containing the *ori* of pGEM-7zf(+) and the *bla* gene could be selected for. A model of the rescue clonings is shown in Fig. 4. The enzymes *Pst*I, *Sac*I, and *Bam*HI were used and gave several identical clones with sizes of 10, 14, and 12.5 kb, respectively, as expected from Southern blot analysis (data not shown).

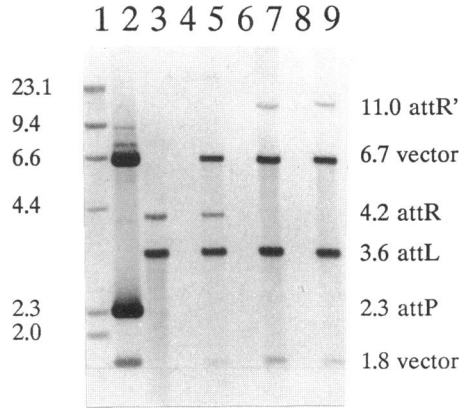

FIG. 3. Southern blot analysis of plasmid pBC143 integration in *L. cremoris* 3107 and *L. lactis* LM0230 and MG1363. pBC143 DNA (lane 2) and chromosomal DNAs of *L. cremoris* 901-1 (lane 3), 3107 (lane 4), and BC1014 (lane 5) and of *L. lactis* MG1363 (lane 6), BC1017 (lane 7), LM0230 (lane 8), and BC1022 (lane 9) were digested with *Cla*I and blotted. Digoxigenin-labeled pBC104 was used as the probe. The insert DNA in pBC104 is phage specific and does contain the *attP* region. pBC143 and pBC104 are derivatives of the same vector, pGEM-7zf(−). In lane 1, *Hin*dIII-digested λ DNA was used as a molecular marker. The fragment sizes are given in kilobases.

The *Pst*I clones were shown, by Southern blot analysis with pBC104 as probe, to harbor the *attL* region. They contained the 3.6-kb *Cla*I-*attL* fragment and the 0.65-kb EV11 fragment. Similarly, the *Sac*I and *Bam*HI clones were shown to contain the *attR* region. They contained the 4.2-kb *Cla*I-*attR* fragment and the 1.0-kb EV9 fragment. One representative of each type of rescue plasmid was used for DNA sequencing.

DNA sequences of *attP*, *attL*, *attR*, and *attB* and localization of the core region. The 189-bp EV12 fragment, on which the *attP* site had been localized, was sequenced. The *attP* sequence was further verified with a PCR product derived directly from TP901-1 DNA. The *attL* and *attR* sequences from *L. cremoris* BC1014 were obtained by sequencing the junction regions from the plasmid rescues. Additionally, the *attL* junction sequence from *L. cremoris* 901-1 was obtained from the pBOP6 plasmid. These sequences were verified with PCR products obtained from the chromosome of the original lyso-

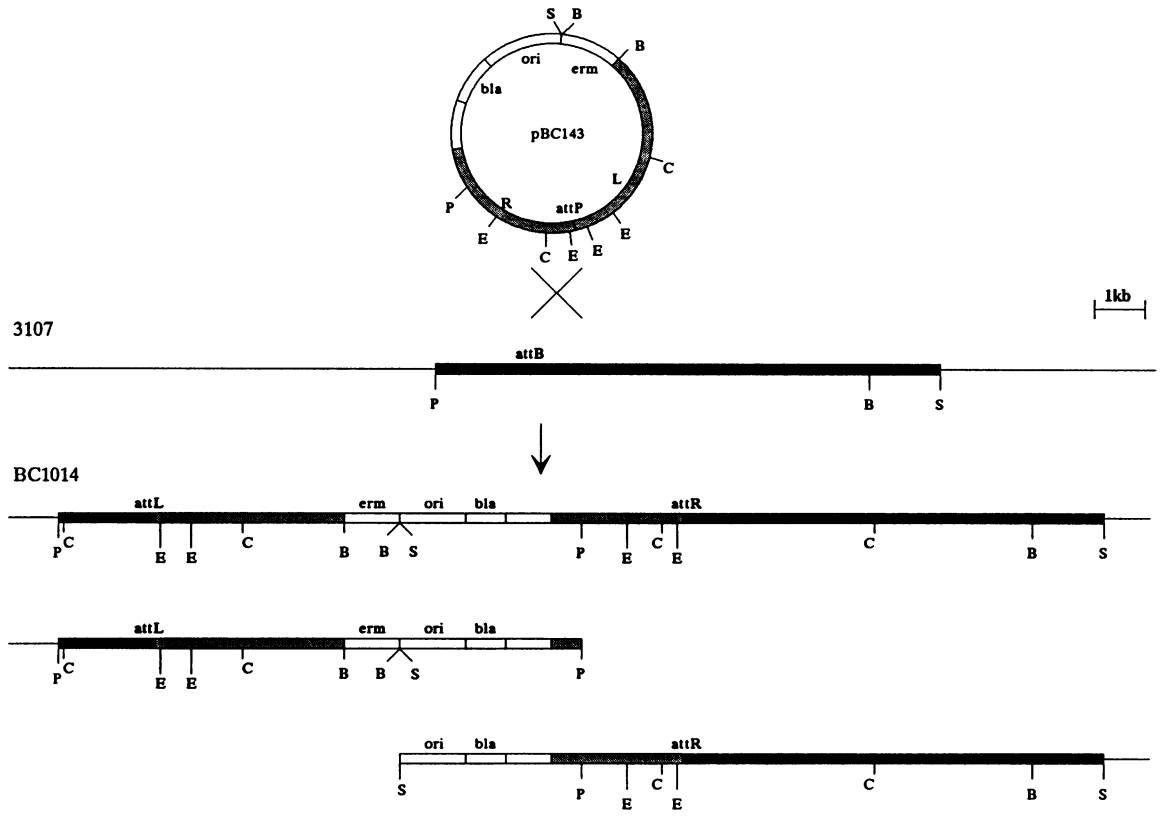

FIG. 4. Schematic presentation of pBC143 site-specific integration into the *L. cremoris* 3107 chromosome and the *Pst*I and *Sac*I plasmid rescues of *attL* and *attR*. Light grey boxes, EI2 *attP* fragment; solid boxes, the chromosomal *Pst*I-*Sac*I fragment containing *attB*; open boxes, vector sequences. The *Cla*I sites define the 3.7-kb *attL* and 4.2-kb *attR* junctions. The *Eco*RV sites indicate the EV11 and EV9 fragments flanking the *attP*-containing EV12 fragment. The *Eco*RV sites in the chromosome have not been mapped. B, *Bam*HI; C, *Cla*I; E, *Eco*RV; P, *Pst*I; S, *Sac*I.

genic strain *L. cremoris* 901-1, as well as with PCR products obtained from two independent lysogenic derivatives of *L. cremoris* 3107. The *attB* sequence from *L. cremoris* 3107 was obtained by cloning an internal 224-bp *Sau*3A fragment isolated from a PCR-amplified 1.5-kb fragment containing the *attB* region. Five clones were sequenced in order to avoid PCR-derived mutations. All five clones had identical sequences.

Comparison of these sequences identified a common 13-bp region, 5′-TCAAT(T/C)AAGGTAA-3′ with a 1-bp mismatch, as shown in Fig. 5. The integration event must have taken place within the left 5 bp (5′-TCAAT-3′), a region that is traditionally called the core region.

```
                                  core
attP  5'-GAGTTTTTATTTCGTTTATTTCAATTAAGGTAACTAAAAAACTCCTTTTAAGG-3'
        ooooooooooooooooooo       o       ooooooooooooooooooo

attL  5'-ATGCCAACACAATTAACATCTCAATTAAGGTAACTAAAAAACTCCTTTTAAGG-3'
        ....................      o       ooooooooooooooooooo

attR  5'-GAGTTTTTATTTCGTTTATTTCAATCAAGGTAAATGCTTTTTGCTTTTTTTGC-3'
        ooooooooooooooooooo       .       ....................

attB  5'-ATGCCAACACAATTAACATCTCAATCAAGGTAAATGCTTTTTGCTTTTTTTGC-3'
        ....................      .       ....................
```

FIG. 5. Nucleotide sequences of the regions containing the *attP*, *attL*, *attR*, and *attB* regions. The 5-bp core and the 7-bp identical sequences are in boldface. Phage TP901-1 DNA is underlined with open dots, and the *L. cremoris* 3107 chromosomal sequences are underlined with solid dots.

The *attB* core sequence was found to be located within an open reading frame 96 bp downstream of the start codon. Integration of TP901-1 disrupts the open reading frame, and a stop codon is introduced 24 bp downstream of the core sequence in the *attL* region. The open reading frame is more than 84 amino acids long, but the full size has not yet been determined. A computer analysis of the N terminal showed homology to precursors of fimbrial and secretion pathway proteins from different bacteria.

The 300-bp DNA surrounding the core sequence of TP901-1 was sequenced from the pBC104 plasmid (Fig. 6). Like the attachment site region of phage λ (16) and other temperate phages from lactic acid bacteria (21, 23), the region surrounding the TP901-1 core is relatively A+T rich. The 300-bp region flanking the TP901-1 core contains about 72% A+T. The TP901-1 *attP* region contains several direct and inverted repeats. The DNA analysis revealed four inverted repeats (r2, r3, r5, and r6) and two direct repeats (r1 and r4) (Fig. 6). These features are typical of other site-specific systems of recombination.

DISCUSSION

Phage TP901-1 has been characterized, and a circular restriction map of the phage genome, including the *attP* and *pac* regions, has been constructed. Phage TP901-1 and the type phage TP936-1 both belong to the P335 group of phages (2). According to our results, both phages seem to be identical. The restriction patterns are identical, with five enzymes. Also, the

FIG. 6. Nucleotide sequence of the 300-bp region surrounding *attP* of TP901-1. Repeats (→) are indicated. The core sequence is marked.

phages TP901-1 and TP936-1 are homoimmune (i.e., *L. cremoris* 3107 and Wg2 derivatives lysogenic for TP936-1 are resistant to infections by TP901-1 and vice versa, and TP901-1 hybridizes to all *Eco*RI bands of TP936-1 [6]). In addition, the two major phage proteins of TP901-1 were estimated to be 31 and 23 kDa, respectively, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (6); these are the same sizes reported for the two major proteins of TP936-1 (2). Phage C3-T1 also seems to be identical to TP901-1, or at least seems to be very closely related. The sizes of the restriction fragments from *Eco*RI, *Xho*I, *Sph*I, *Pvu*II, and *Bcl*I digestions of phage C3-T1 DNA (13) are the same as those found for TP901-1 (6). Furthermore, the *Bcl*I maps of the two phages seem identical, as do the locations of the *att* and *pac* regions (Fig. 1). Also, TP901-1 DNA hybridizes to all *Eco*RI fragments of C3-T1 (6). The sizes of the major phage proteins could not be compared, since the protein profile for phage C3-T1 has not been reported. The genome sizes of the three phages have been estimated to be 38.4 kb for TP901-1 (this study), 37.8 kb for C3-T1 (13), and 37.8 kb for TP936-1 (2). Finally, DNA sequences of 200 bp covering the *attP* region were found to be identical in all three phages (6). Phage C3-T1 thus belongs to the P335 group of phages and does not represent a new phage species, as suggested by Jarvis et al. (13). Of the two other well-characterized lactococcal phages, φLC3 and BK5-T, only φLC3 hybridizes to TP901-1 and thus belongs to the P335 group of phages. The restriction pattern of phage φLC3 is clearly different from that of TP901-1, and φLC3 has cohesive ends, in contrast to the *pac*-type phage TP901-1. Furthermore, the *attP* and *attB* sequences determined for TP901-1 (Fig. 5) are clearly different from those reported for φLC3 (21), indicating that the two phages contain different integration systems.

Our results demonstrate that phage TP901-1 is able to lysogenize the indicator strains *L. cremoris* 3107 and Wg2. This was not shown for phage C3-T1, and, to our knowledge, among lactococcal phages, this has been shown only for the phages φT712 and φLC3 (10, 22). Lysogenization by phage TP901-1 results in integration of the phage genome into the bacterial chromosome by a site-specific recombination process following Campbell's classic model of integration for phage λ (3). Integrative recombination takes place between the *attP* region residing within the 2.3-kb *Cla*I fragment (C4) of TP901-1 and the *attB* region found within a 5.5-kb *Cla*I fragment of the host chromosome of the indicator strains. As a result of the integration event, the *attP*- and *attB*-containing fragments split into two *Cla*I junction fragments with sizes of approximately 3.6 and 4.2 kb (*attL* and *attR*, respectively).

Analysis of 20 independently isolated lysogens showed that only one major *attB* site exists in the indicator strains. The same *attB* site was used when the vector pBC143—containing the 6.5-kb EI2 *attP* fragment from TP901-1—was integrated into the chromosome. Isolation and sequencing of *attP*, *attL*, *attR*, and *attB* regions resulted in the finding of a 5-bp core followed by a 7-bp identical sequence. The identities of the *attR* and *attL* sequences were confirmed by sequencing of PCR products from several independent lysogens and plasmid insertions of *L. cremoris* 3107, as well as those from the lysogenic *L. cremoris* 901-1. The T-to-C mismatch was found in all *attR* regions (a total of nine were sequenced) and is thus not due to a mutation. Also, the *attP* sequence was verified by PCR with both the phage TP901-1 DNA and the integration vector pBC143 as the template. The presence of 1 bp separating the 5-bp core and the 7-bp identical sequence was found in *attP* and *attL* and *attR* and *attB*, respectively. Hence, the recombination between the phage genome and the host chromosome must occur in the identical 5-bp segments and not in the 7-bp region. This is, to our knowledge, the shortest core region described for a temperate phage.

The core regions have been determined from the temperate phages φLC3, φadh, L54a, φ13, and φ11 isolated from lactococci, lactobacilli, and staphylococci. The sizes were 9 (21), 16 (23), 18 (17), 14 (7), and 10 (18) bp, respectively. When the core regions and the surrounding *attP* sequences from these phages were compared with those determined for TP901-1, the DNA sequences from TP901-1 were found to be clearly different, indicating that the integration system of TP901-1 is a unique system that has not been previously reported. The lactococcal phages φLC3 and TP901-1 probably use common host proteins for the site-specific recombination process. It may be of significance for the binding of such proteins that the r1 repeat sequences in TP901-1 are almost identical to the R4 repeats in φLC3 (21). Also, the palindromic r6 sequences in TP901-1 show considerable sequence homology to the R3 palindrome reported in φLC3 (21).

The *attB* region from *L. cremoris* 3107 does not show any homology to genes encoding tRNA, a preferred insertion site for several bacteriophages (4, 11, 19, 24). In contrast, the TP901-1 *attB* was found to be located in a region encoding the N terminal of a putative open reading frame with a size of more than 84 amino acids. Insertion of the phage disrupts the reading frame (24 bp) downstream of the core sequence.

The integrative properties of plasmid pBC143 clearly show that the phage-encoded functions necessary for the site-specific integration are present on the EI2 fragment. Apart from the *attP* region, we expect to find the *int* gene of TP901-1, and experiments with the purpose of identifying *int* are in progress. The finding that the integration system of TP901-1 is functioning in the plasmid-free laboratory strains *L. lactis* MG1363 and LM0230 is very important. Derivatives of the integration vector should therefore be able to function as important tools for single-copy cloning of genes and for the construction of cloning vectors for identification of promoters, terminators, and signal sequences, etc. Furthermore, the integration vectors, on the basis of TP901-1, may be working in many different *Lactococcus* strains and thus may be useful for the construction of recombinant derivatives of industrial starter cultures.

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