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A Resolvase-Like Protein Is Required for the Site-Specific Integration of the Temperate Lactococcal Bacteriophage TP901-1

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The integration system of the temperate lactococcal phage TP901-1 was characterized in *Lactococcus lactis* subsp. *cremoris* LM0230 and MG1363 with the use of deletion derivatives of the integration vector pBC143 (B. Christiansen, M. G. Johnsen, E. Stenby, F. K. Vogensen, and K. Hammer, J. Bacteriol. 176:1069–1076, 1994). The phage-encoded elements necessary for integration were localized on a 2.8-kb *Nsi*I-*Eco*RI fragment including the phage attachment site, *attP*. This fragment was DNA sequenced, and sequence analysis revealed three putatively expressed open reading frames, Orf1, Orf2, and Orf3. By the introduction of mutations within the *orf1*, *orf2*, and *orf3* genes, it was shown that only Orf1 was necessary for the integration process. Furthermore, it was found that Orf1, *attP*, and a 425-bp region upstream of the *orf1* gene are sufficient for integration. Orf1 contains 485 amino acids and is located just upstream of *attP*. The N-terminal 150 to 180 amino acids of Orf1 showed 38 to 44% similarity to the resolvase group of site-specific integrases, while no similarity to known proteins was found in the C-terminal end. Bacteriophage TP901-1 therefore contains a unique integration system that does not resemble the Int class of site-specific integrases usually found in temperate bacteriophages. The constructed integration vector, pBC170, integrates into the chromosomal attachment site very efficiently and forms stable transformants with a frequency corresponding to 20% of the transformation efficiency.

Most temperate bacteriophages from gram-negative and gram-positive bacteria, including the well-known λ bacteriophage, integrate their DNA into the host chromosome when they enter the lysogenic cycle. The integration often occurs site specifically and has, in these cases, been found to be mediated by an integrase (Int) belonging to the Int class of site-specific recombinases (1, 24).

TP901-1 is a *pac*-type, lactococcal temperate bacteriophage with a small isometric head and a long noncontractile tail. It belongs to a group of temperate bacteriophages which show a high degree of homology to a group of virulent bacteriophages, represented by the type bacteriophage P335 (3, 15). It is likely that TP901-1 is identical to TP936-1 and C3-T1 on the basis of DNA restriction patterns and hybridizations (5, 16). We have previously described the site-specific integration of the temperate lactococcal bacteriophage TP901-1 and the construction of a TP901-1-based integration vector (5). The phage attachment site (*attP*) and the chromosomal attachment site (*attB*) are unrelated to those found in other phages of lactic acid bacteria and their host strains, e.g., φadh, mv4, φLC3, Tuc2009, and BK5-T (2, 8, 10, 21, 36).

This communication contains a further characterization of the integration system from TP901-1 by deletion analysis, DNA sequencing, and mutational analysis. The data presented show that the integration system from TP901-1 is very different from the bacteriophage integration systems reported so far, since Orf1, which is identified to be necessary for integration, does not belong to the Int class of site-specific integrases but is related to the class of site-specific resolvases. The integration system from TP901-1 thus seems to constitute a unique recombination system among temperate bacteriophages.

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MATERIALS AND METHODS

Cell growth. *Lactococcus* strains were propagated at 30°C in GM17 medium, M17 broth (Oxoid Limited, Basingstoke, Hampshire, United Kingdom) containing 0.5% glucose (wt/vol), without shaking (35). *Escherichia coli* strains were grown with agitation at 37°C in Luria-Bertani broth (29) (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.

DNA preparation. Extraction of chromosomal DNA was performed as described for *E. coli* (29), with the modification that cells were treated with 20 mg of lysozyme per ml for 2 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 (29) or with QIAGEN columns as recommended by the supplier (QIAGEN Ltd., Hilden, Germany). 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 Biochemicals (Mannheim, Germany), as were Klenow enzyme, 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 8.0], 1 mM EDTA). DNA restriction fragments were isolated from excised agarose gel segments with the Gene-Clean kit (Bio 101, Inc., La Jolla, Calif.).

Construction of plasmids. The plasmids used in this study are listed in Table 1. The plasmids pG7f2, pG5f4, pG5f9, pG5f11, pG5f12, pBC104, pBC143, and pBC144 have been previously described (5). The plasmids pBC165, pBC166, pBC171, and pBC172 were constructed by inserting the fragments indicated in Table 1 into digested and calf intestine alkaline phosphatase-treated pGEM-7zf(+). pBC300 was constructed by treating the 1.8-kb *Pst*I-*Nsi*I fragment with Klenow enzyme, under conditions favoring exonuclease activity as recommended by the supplier. This fragment was subsequently cloned into the *Hin*cII site of pGEM-11zf(+). Plasmid pBC187 was obtained from pBC166 by filling in of the *Hin*dIII site with the Klenow enzyme, resulting in the introduction of an *Nhe*I site as expected. Plasmids pBC170 and pBC190 were constructed by insertion of the

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Origin	Resistance	Reference or source
Strains			
L. lactis subsp. *cremoris*			
3107	Indicator strain for TP901-1		3
901-1	Lysogenic for TP901-1		3
LM0230			9
MG1363			11
E. coli			
XL1-Blue	*recA endA1 gyrA96 thi-1 hsdR17 supE44 tet relA1 lac* [F′ *proAB lacI*^{q} *ZDM15* Tn*10* (Tet^r)]		Stratagene, La Jolla, Calif.
DH5α	ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*) *U169 recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1*		Lab strain
Plasmids			
pGEM-5Zf(−)	*lacZ*	Bla^r	Promega, Madison, Wis.
pGEM-7zf(+)	*lacZ*	Bla^r	Promega, Madison, Wis.
pGEM-11zf(+)	*lacZ*	Bla^r	Promega, Madison, Wis.
pIL253		Erm^r	34
pUC7,*erm*	pUC7::1.1-kb *Hin*PI pIL253 *erm*	Erm^r	de Vos, NIZO
pIC19-H	*lacZ*	Bla^r	22
pG7f2	pGEM-7Zf(+)::6.5-kb *Eco*RI TP901-1	Bla^r	5
pG5f4	pGEM-5Zf(−)::3.1-kb *Eco*RV TP901-1	Bla^r	5
pG5f9	pGEM-5Zf(−)::1.0-kb *Eco*RV TP901-1	Bla^r	5
pG5f12	pGEM-5Zf(−)::0.2-kb *Eco*RV TP901-1	Bla^r	5
pBC104	pGEM-7Zf(+)::2.3-kb *Cla*I TP901-1	Bla^r	5
pBC143	pG7f2::1.1-kb *Bam*HI *erm*	Erm^r Bla^r	5
pBC144	pGEM-7Zf(+)::1.1-kb *Bam*HI *erm*	Erm^r Bla^r	5
pBC154	pBC143 Δ1.8-kb *Eco*RI-*Cla*I	Erm^r Bla^r	This study
pBC156	pBC104::1.1-kb *erm*	Erm^r Bla^r	This study
pBC164	pBC143 Δ3.8-kb *Eco*RI-*Cla*I *erm*	Erm^r Bla^r	This study
pBC165	pGEM-7Zf(+)::2.4-kb *Eco*RI-*Cla*I TP901-1	Bla^r	This study
pBC166	pGEM-7Zf(+)::2.8-kb *Eco*RI-*Nsi*I TP901-1	Bla^r	This study
pBC170	pBC166::1.1-kb *Eco*RI *erm*	Bla^r Erm^r	This study
pBC171	pGEM-7Zf(+)::0.45-kb *Pst*I-*Hin*dIII TP901-1	Bla^r	This study
pBC172	pGEM-7Zf(+)::1.0-kb *Eco*RI-*Pst*I TP901-1	Bla^r	This study
pBC186	pBC300::1.1-kb *Bam*HI *erm*	Bla^r Erm^r	This study
pBC187	pBC166 Δ*Hin*dIII	Bla^r	This study
pBC190	pBC187::1.1-kb *Eco*RI *erm*	Bla^r Erm^r	This study
pBC191	pBC144::1.8-kb *Nsi*I-*Pst*I TP901-1	Bla^r Erm^r	This study
pBC300	pGEM-11Zf(+)::1.8-kb *Nsi*I-*Pst*I TP901-1	Bla^r	This study
pLB20	pIC19-H::580-bp *orf3*(Am) *Bam*HI[a]	Bla^r	This study
pLB21	pIC19-H::720-bp *orf2*(Am) *Bam*HI[a]	Bla^r	This study
pLB22	pIC19-H::675-bp *orf1*(Am) *Bam*HI[a]	Bla^r	This study
pLB23	pBC166::470-bp *Eco*RI-*Acc*I pLB20	Bla^r	This study
pLB24	pBC166::520-bp *Acc*I-*Pst*I pLB21	Bla^r	This study
pLB25	pBC166::450-bp *Pst*I-*Hin*dIII pLB22	Bla^r	This study
pLB26	pLB23::1.1-kb *Eco*RI *erm*[b]	Bla^r Erm^r	This study
pLB27	pLB24::1.1-kb *Eco*RI *erm*[b]	Bla^r Erm^r	This study
pLB28	pLB25::1.1-kb *Eco*RI *erm*[c]	Bla^r Erm^r	This study
pLB29	pBC166::1.1-kb *Eco*RI *erm*[c]; isogenic to pBC170	Bla^r Erm^r	This study
pLB44	pLB27 5.0-kb *Xba*I::pLB26 1.3-kb *Xba*I[b]	Bla^r Erm^r	This study
pLB45	pLB27 5.0-kb *Xba*I::pLB26 1.3-kb *Xba*I[c]	Bla^r Erm^r	This study
pLB67	pLB44 *Eco*RI[c]	Bla^r Erm^r	This study
pLB71	pLB23::1.1-kb *Eco*RI *erm*[c]	Bla^r Erm^r	This study
pLB72	pLB24::1.1-kb *Eco*RI *erm*[c]	Bla^r Erm^r	This study

[a] See Table 3 for formation of the PCR product.
[b] The *erm* gene is oriented in the same direction as *orf1*, *orf2*, and *orf3*.
[c] The *erm* gene is oriented divergently from *orf1*, *orf2*, and *orf3*.

1.1-kb *Eco*RI *erm* cassette from pUC7,*erm* into the *Eco*RI site of pBC166 and pBC187, respectively. Similarly, pBC156 and pBC186 were obtained by inserting the 1.1-kb *Bam*HI *erm* cassette into the *Bam*HI site of pBC104 and pBC300, respectively. Plasmid pBC191 was constructed by inserting the 1.8-kb *Nsi*I-*Pst*I fragment into the *Nsi*I site of pBC144. Finally, pBC154 and pBC164 were constructed as deletion clones from pBC143.

PCR fragments containing an amber stop codon (TAG) as well as an *Xba*I site (TC**TAG**A) in *orf1*, *orf2*, and *orf3* were constructed in a two-step PCR as described by Landt and coworkers (20). A third PCR was performed to increase the amount of the desired fragment (see Table 2 for information on the components of the PCRs and Table 3 for primer sequences). The amber stop codons were introduced into *orf1*, *orf2*, and *orf3* at the amino acid positions 8, 81, and 43, respectively. The introduction of the amber stop codon was confirmed by the presence of an *Xba*I site, which was introduced simultaneously. The resulting fragments from the third PCR were digested with *Bam*HI and ligated into pIC19-H *Bam*HI, giving rise to plasmids pLB20($orf3_{am}$), pLB21($orf2_{am}$), and pLB22($orf1_{am}$). Sequencing of the cloned fragments showed that one nucleotide substitution had occurred in *orf1* and *orf3* downstream of the amber stop codon.

TABLE 2. Components used in the three-step PCR for construction of the amber stop codons within *orf1*, *orf2*, and *orf3*

Gene	Component	PCR 1	PCR 2	PCR 3
orf1	Template	TP901-1 DNA	TP901-1 DNA	580-bp fragment from PCR 2
	Primer 1	P4	330-bp fragment from PCR 1	P4
	Primer 2	PZ (1-bp mismatch)	PC	PC
	PCR fragment	330 bp	580 bp	580 bp
orf2	Template	TP901-1 DNA	TP901-1 DNA	720-bp fragment from PCR 2
	Primer 1	P3	370-bp fragment from PCR 1	P3
	Primer 2	PY (3-bp mismatch)	PF	PF
	PCR fragment	370 bp	720 bp	720 bp
orf3	Template	TP901-1 DNA	TP901-1 DNA	675-bp fragment from PCR 2
	Primer 1	P2	220-bp fragment from PCR 1	P2
	Primer 2	PW (1-bp mismatch)	PB	PB
	PCR fragment	220 bp	675 bp	675 bp

This leads to a amino acid substitution at position 13 in Orf1 (from Thr to Pro) and at position 47 in Orf3 (from Asp to Glu).

Purified fragments from pLB20 (470-bp *Eco*RI-*Acc*I fragment), pLB21 (520-bp *Acc*I-*Pst*I fragment), and pLB22 (450-bp *Pst*I-*Hin*dIII fragment) were cloned in pBC166 *Eco*RI-*Acc*I, pBC166 *Acc*I-*Pst*I, and pBC166 *Pst*I-*Hin*dIII, respectively, and thereby plasmids pLB23, pLB24, and pLB25 were constructed. Subsequently, the *erm* gene (1.1 kb) from pUC7,*erm* was cloned with an orientation opposite to that of Orf1, Orf2, and Orf3, in the *Eco*RI site of pLB23, pLB24, pLB25, and pBC166, respectively, giving rise to the integration vectors pLB71, pLB72, pLB28, and pLB29 (isogenic to pBC170). In plasmids pLB26 and pLB27 the *erm* gene was cloned in the *Eco*RI site of pLB23 and pLB24, respectively, in the same orientation as that of Orf1, Orf2, and Orf3. Plasmids pLB44 and pLB45, containing deletions in *orf2* and *orf3*, were constructed by ligation of purified *Xba*I fragments from pLB26 (1.3 kb) and pLB27 (5.0 kb). In pLB44 the N-terminal part of *orf3* was just upstream of the C-terminal part of *orf2*, whereas in pLB45 the N-terminal part of *orf3* was separated from the C-terminal part of *orf2* by the *erm* cassette. The integration vector pLB67 was constructed from pLB44 by digestion with *Eco*RI and religation. The *erm* gene in pLB67 was then oriented divergently from Orf1, Orf2, and Orf3. Thus, in all of the integration vectors used (pLB28, pLB29, pLB45, pLB67, pLB71, and pLB72), the orientation of the *erm* gene is divergent from that of *orf1*, *orf2*, and *orf3*.

TABLE 3. Oligonucleotides used in this study

Primer	Sequence
T7	TAATACGACTCACTATAGGG
SP6	GATTTAGGTGACACTATAG
P2Lb	CTTGAAATCCAATCAGGTTG
P3Lb	CCCTGACAGCATATATTTAGC
P4Lb	CCGATGTGTCCAGATTC
P1Ld2	GCTCATCAATTGAGAACC
P00Lc	CCCAGAAGTCGCAAAAGAG
P2Lc	CGTTCCAAATGTGTCAG
P3Lc	GAGTTCATACGCATCCATG
P4Lc	CTTGTTCTATTATCGTCAAATC
P1Rc	GTTATTGTGGACACACCC
P2Rc	CTTAAGTGGATATGCG
P3Rc	GCGCCATATATCGAAC
P4Rc	GTTTGTTGATGGAGATACTG
P5Rc	GTAGAGTAGAAGTTAAGAAC
P1Rd	CCGCTCAAGTTTGGCAACG
P1Rb	GGGCGAGCGAAATCTGG
P2Rb	CTGGACACATCGGTAAAG
P3Rb	CCCTAGACCTTTCCAAGC
P4Rb	GTGTGATTCAGGAACTTATG
P1-5f12T7	CATCAGTTATCTCATGC
PEV9	CGGAATTGAGCCCAACTGAG
PEV9T7inv	GAGAAACAAGCTACCCATAGCAG
P2int	CCCCTCGAGTCGACGCAATTAAGCGAGTTGGAATTT
PB	CGGGATCCATCGTTCCAAATGTGTCAGAAA
PC	CGGGATCCTACTTAACAAGATAAAGAGTAT
PF	CGGGATCCCGTTTACTATACCACATAGATT
PW	TCATCTCTGATCTAGATAATATCATATTCA
PY	CGGTAAACTTTTTCTAGAATATCATTCCAT
PZ	GGATACTCGTGTCTAGATTGCTACTTTCTT
P2	CGGGATCCGTGGACAGCTTAACGACAATGA
P3	CGGGATCCATGGATATGCGAATTAGTTATT
P4	CGGGATCCATAGATGCACCAATACTTGTCG

Transformation and selection in *E. coli* and *Lactococcus lactis* subsp. *cremoris*. *E. coli* XL1-Blue or DH5α was made competent with $CaCl_2$ and transformed as described by Sambrook and coworkers (29). Transformants in XL1-Blue based on pGEM vectors 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). For introduction of the *erm* cassette, selection was done with 100 μg of ampicillin per ml and 30 μg of erythromycin per ml.

L. lactis subsp. *cremoris* LM0230 and MG1363 were transformed by electroporation according to the method described by Holo and Nes (14), with 0.03 to 0.5 μg of DNA per electroporation. Transformants were selected on plates containing 2 μg of erythromycin per ml. Integration was analyzed by PCR of chromosomal DNA, as previously described, with representative transformants for the presence of *attL* and *attR* and the absence of *attB* (5). The presence of the *int* gene was determined by PCR, with the primers P4Rb and Pint2. The DNA concentration of the plasmid preparations was determined by comparing known amounts of λ DNA digested with *Hin*dIII (Boehringer) with digested plasmid DNA.

DNA sequencing and computer analysis. Plasmid DNA to be used for DNA sequencing was prepared from *E. coli* DH5α. Plasmids pG7f2, pG5f4, pG5f9, pGf12, pBC165, pBC171, and pBC172 were used as templates. The DNA sequences were determined by the method of Sanger and coworkers (30), as instructed by the supplier of the Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio). Sequencing of the 2.8-kb *Nsi*I-*Eco*RI fragment was performed with 2 pmol of the primers. All primers used are listed in Table 3. The primers used in this study were supplied by P. Hobolth, Lyngby, Denmark, or DNA Technology ApS, Århus, Denmark. Computer analyses of the sequence data were carried out with the Genetics Computer Group sequence software package, version 7 (6). The ΔG values were calculated as described by Salser (28), with the computer program RNAFOLD. The isoelectric points of Orf1, Orf2, and Orf3 were calculated with the computer program Isoelec (Ramcon A/S, Birkerød, Denmark).

Nucleotide sequence accession number. The nucleotide sequence data shown in Fig. 2 have been deposited in GenBank under accession no. X85213.

RESULTS

Deletion analysis of the integration vector pBC143. The temperate bacteriophage TP901-1 integrates specifically into only one chromosomal attachment site, *attB*. The *attB* region, the attachment site of the phage (*attP*), and the chromosomal junctions, *attL* and *attR*, between the phage genome and the host chromosome in the lysogens were cloned and sequenced (5). In all four cases this identified the core region, where the site-specific recombination takes place, as a sequence of 5 bp, 5′-TCAAT-3′. At all four sites the core region is followed by the same 7-bp region separated by a 1-bp mismatch. An integration vector, pBC143, containing 6.5 kb of DNA from bacteriophage TP901-1 was previously shown to mediate site-spe-

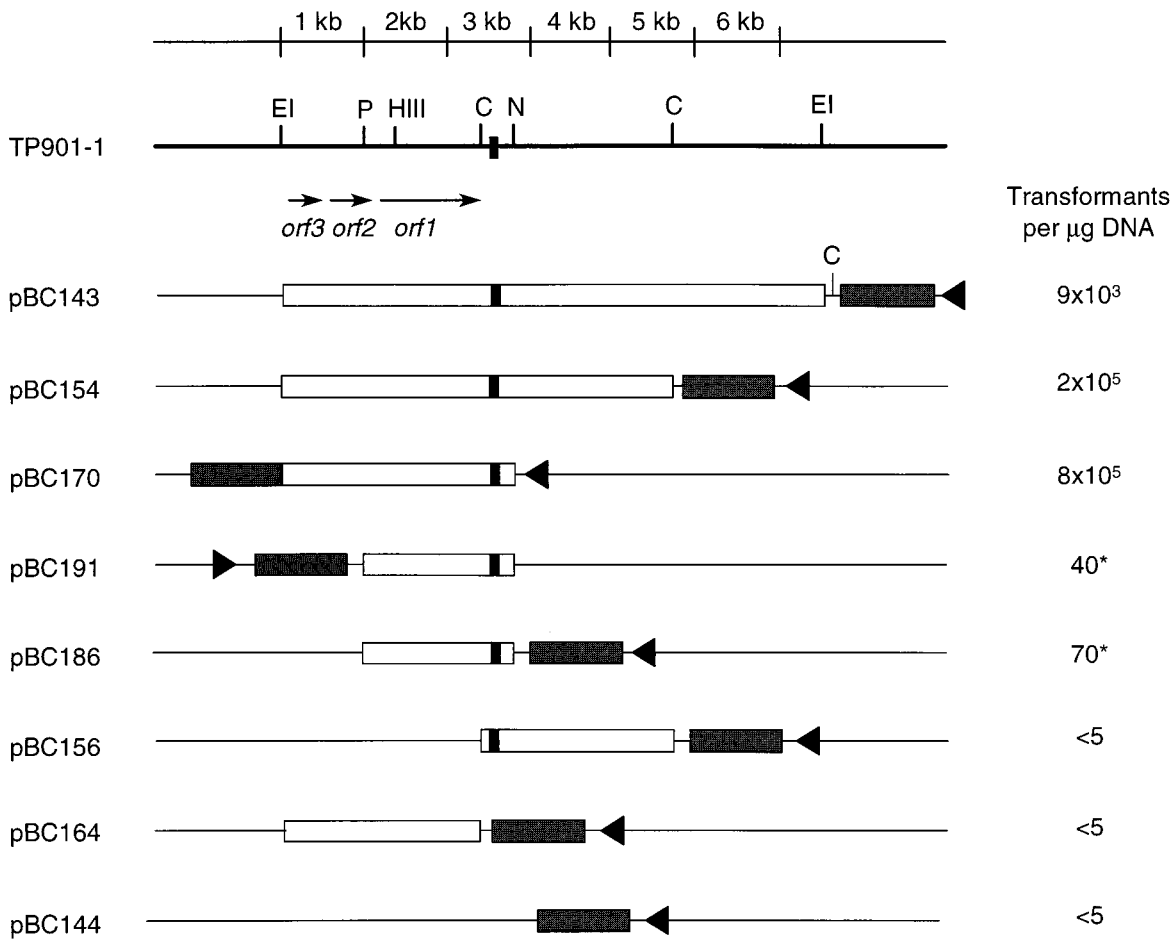

FIG. 1. Deletion analysis of 6.5-kb *Eco*RI fragment from the TP901-1 phage genome. *E. coli* plasmid clones contain the indicated inserts of the TP901-1 phage genome. The transformation frequencies in *L. lactis* subsp. *cremoris* LM0230 are indicated on the right; numbers with asterisks indicate nonviable colonies. Thin arrows indicate ORFs *orf1*, *orf2*, and *orf3* deduced from the nucleotide sequence. Open boxes, TP901-1 insert; large closed boxes, *erm* gene; small closed boxes, *attP* site of TP901-1. Thin lines indicate pGEM-7zf(+) DNA with no lactococcal origin of replication, and closed arrowheads indicate the orientation of the *lacZ* promoter in the vector. EI, *Eco*RI; C, *Cla*I; HIII, *Hin*dIII; N, *Nsi*I; P, *Pst*I.

cific integration into the indicator strain for the bacteriophage *L. lactis* subsp. *cremoris* 3107, as well as in the plasmid-free laboratory strains *L. lactis* subsp. *cremoris* MG1363 and LM0230 (5).

In order to localize the phage genetic elements, in addition to *attP*, involved in this process, deletion derivatives containing only parts of the 6.5-kb *Eco*RI phage DNA fragment were constructed. The putative integration vectors were all derivatives of the *E. coli* plasmid pGEM-7zf(+) or pGEM-11zf(+), containing a selectable marker (*erm*) functional in *Lactococcus* spp. but no origin of replication functional in gram-positive bacteria. By selection for erythromycin-resistant transformants after electroporation of *L. lactis* subsp. *cremoris* LM0230 or MG1363, the ability of the plasmids to function as integration vectors was tested. As shown in Fig. 1, the sequences to the right of the *attP* region, from the *Eco*RI site to the *Nsi*I site located 95 bp downstream of the core sequence, could be deleted without loss of the ability to integrate (plasmids pBC154 and pBC170). As expected, the *attP* region was found to be necessary since pBC164 could not integrate. On the other hand, *attP* alone cannot promote integration since pBC156 was not able to integrate. Therefore, sequences to the left of *attP* are needed for the integration. Vectors containing the 1.8-kb *Pst*I-*Nsi*I fragment (pBC186 and pBC191) gave only a few primary transformants (less than 100 transformants), and these transformants could not be propagated further; consequently, the 1.8-kb *Pst*I-*Nsi*I fragment was not sufficient to give viable transformants in *L. lactis* subsp. *cremoris*. Thus, from these studies, the smallest fragment which was found to promote integration was the 2.8-kb *Eco*RI-*Nsi*I fragment contained in pBC170. The presence of *attL* and *attR* was verified by PCR analysis of the integrants arising from pBC170, proving that pBC170 integrates by site-specific recombination into the chromosomal *attB* site of *L. lactis* subsp. *cremoris* LM0230. The presence of site-specific integrants in *L. lactis* subsp. *cremoris* 3107 was also tested.

The number of integrants obtained in LM0230 ranged from 9×10^3 to 8×10^5 integrants per µg of DNA and increased with the reduction in the size of the vector. With the use of the 4.8-kb plasmid pIL253, which is able to replicate in lactococci, as the control, 4×10^6 transformants per µg of DNA were obtained. The constructed vector pBC170 therefore functions as a highly efficient integration vector, resulting in formation of integrants with a frequency corresponding to 20% of the transformation efficiency.

Stability of integrants. The integrants obtained with the integration vector pBC170 were tested for the stability of the site-specific integration. Two independent derivatives were

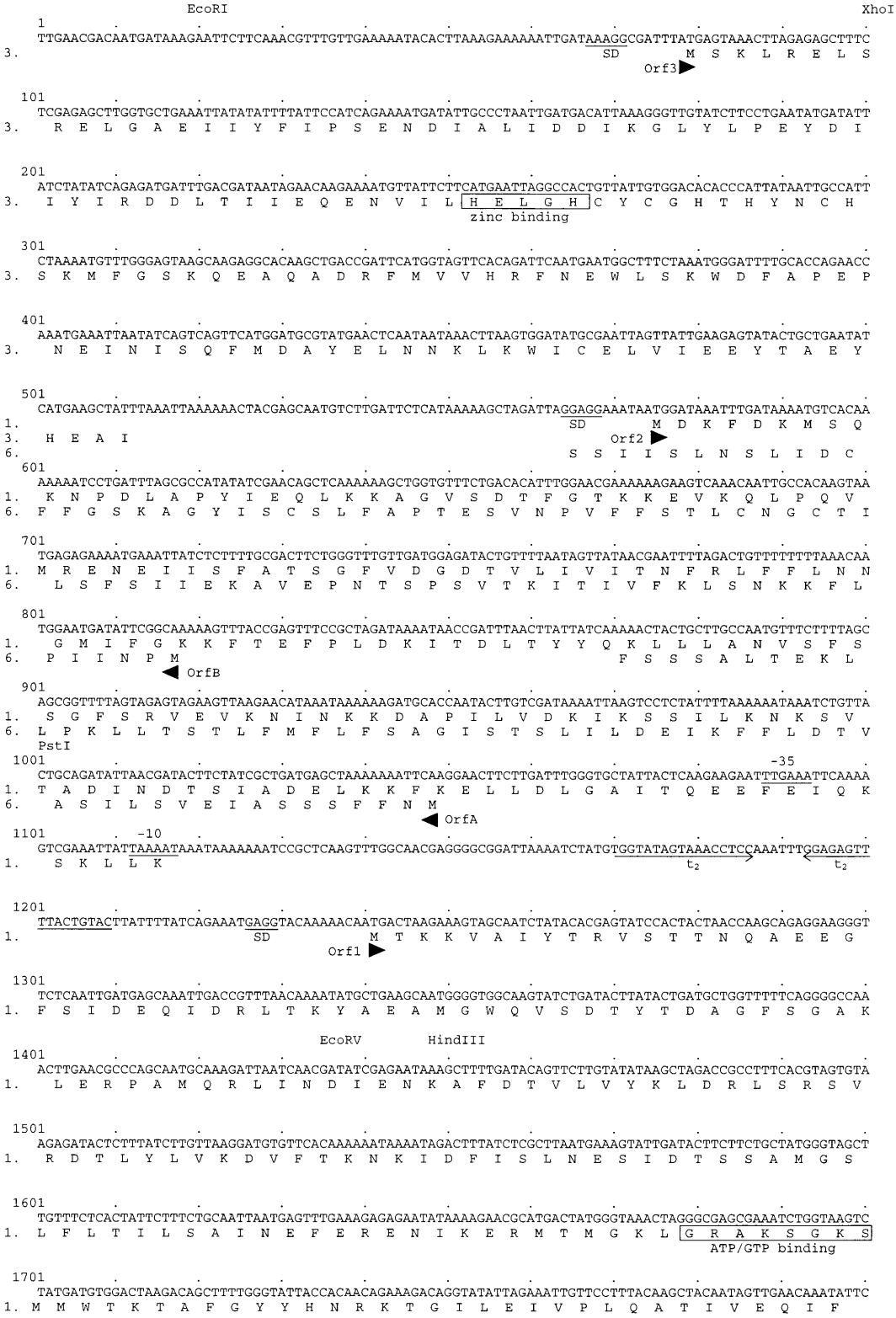

FIG. 2–*Continued.*

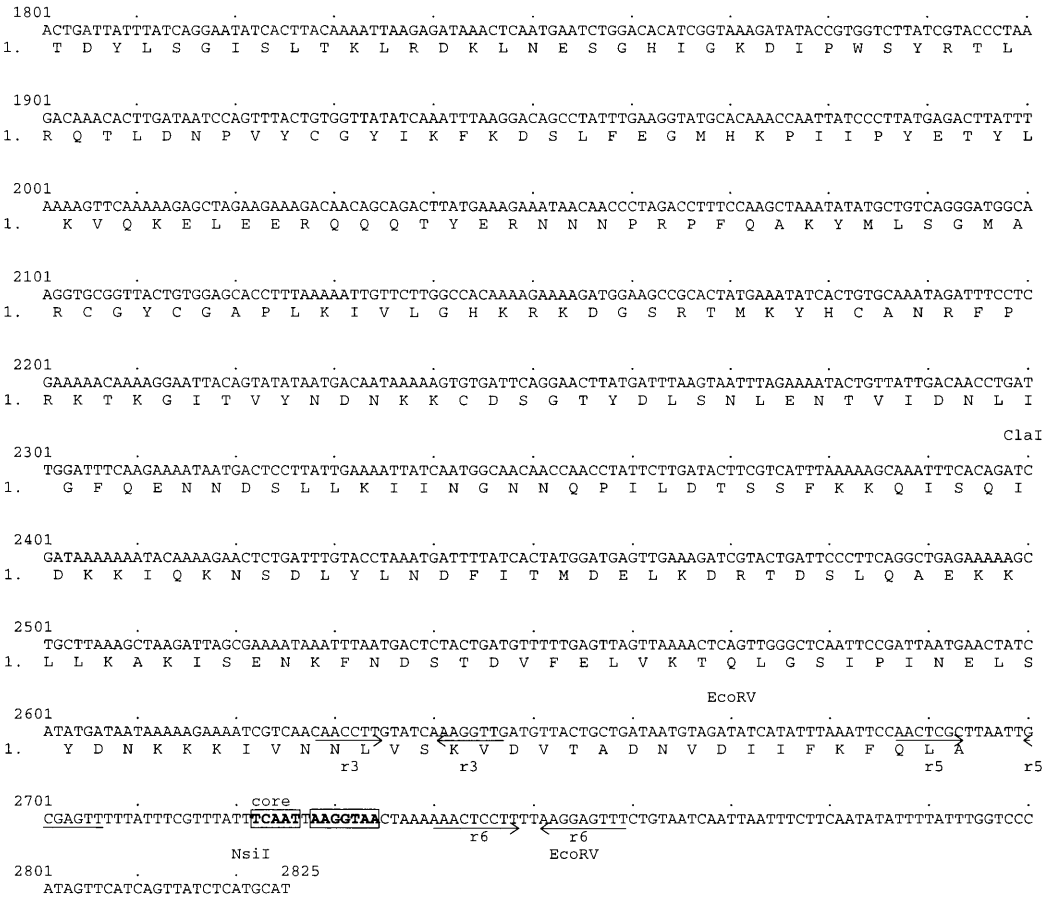

FIG. 2. Nucleotide sequence of 2.8-kb *Eco*RI-*Nsi*I fragment, including a 20-bp DNA sequence upstream of *Eco*RI. The fragment contains the putative integrase gene from TP901-1, *orf1*, and the ORFs Orf2, Orf3, OrfA, and OrfB. Deduced amino acid sequences and relevant restriction sites are given. Preceding the ORFs, potential SD sequences are underlined. A potential transcription terminator, t2, downstream of the *orf2* gene and the inverted repeats r3, r5, and r6 are shown in dyad symmetry. The core sequence is boxed and in boldface. A potential ATP/GTP binding site in Orf1 and a potential zinc-binding motif in Orf2 are depicted.

grown for 70 generations in GM17 medium at 30°C without selection (no erythromycin was added). Subsequently, 200 colonies were tested, and erythromycin resistance was preserved in all colonies. Therefore, the vector pBC170 is a useful tool for the construction of stable chromosomal integrants.

Nucleotide sequence and coding capacity of the region from TP901-1 needed for integration. The precise restriction map of the *Eco*RI-*Nsi*I fragment was established, and representative restriction fragments were cloned in *E. coli* vector pGEM-7zf(+) or pGEM-5zf(−). By using these recombinant plasmids as templates, the nucleotide sequence of the 2,801-bp *Eco*RI-*Nsi*I region was determined. The deduced amino acid sequences are shown in Fig. 2. The nucleotide sequence was searched by using three possible start codons (ATG, GTG, and TTG). The sequence revealed five open reading frames (ORFs), Orf1 (485 amino acids, bases 1240 to 2694), Orf2 (181 amino acids, bases 574 to 1116), and Orf3 (145 amino acids, bases 78 to 512), oriented from left to right upstream of the *attP* core sequence, and OrfA (60 amino acids, bases 1049 to 870) and OrfB (85 amino acids, bases 818 to 564), oriented from right to left. Orf1, Orf2, and Orf3 were preceded by possible Shine-Dalgarno (SD) sequences as indicated in Fig. 2, whereas OrfA and OrfB were not. The predicted Orf1, Orf2, and Orf3 sequences all start with ATG and end with TAA; OrfA and OrfB both start with TTG and end with TTT and TAA, respectively.

Only one alternative start codon was found, the ATG at position 592 as a start for Orf2; however, this ATG is not preceded by an SD sequence. The proposed start and stop positions are given in Fig. 2, and Orf1, Orf2, and Orf3 do not overlap but have intercistronic regions of 62 bp (Orf3 to Orf2) and 124 bp (Orf2 to Orf1). OrfA and OrfB are interspaced with 51 bp; Orf2 overlaps both OrfA and OrfB but reads in the opposite direction. Taken together, OrfA and OrfB are not likely to be expressed. The gene order is *orf3*, *orf2*, *orf1*, and core, with the end of *orf1* located 27 bp upstream of the core sequence.

The predicted amino acid sequences of Orf1, Orf2, and Orf3 were compared with protein sequences in the SwissProt database. Orf2 and Orf3 showed no similarity to any of the known protein sequences, while the N terminus of Orf1 was found to have 38% to 44% similarity to 150 to 180 amino acids of several proteins (Fig. 3), all belonging to the resolvase family of site-specific recombinases (33), including the Tn*3* resolvase and the invertases Hin, Pin, Gin, and Cin. The homologous domain is involved in the recombination process and includes

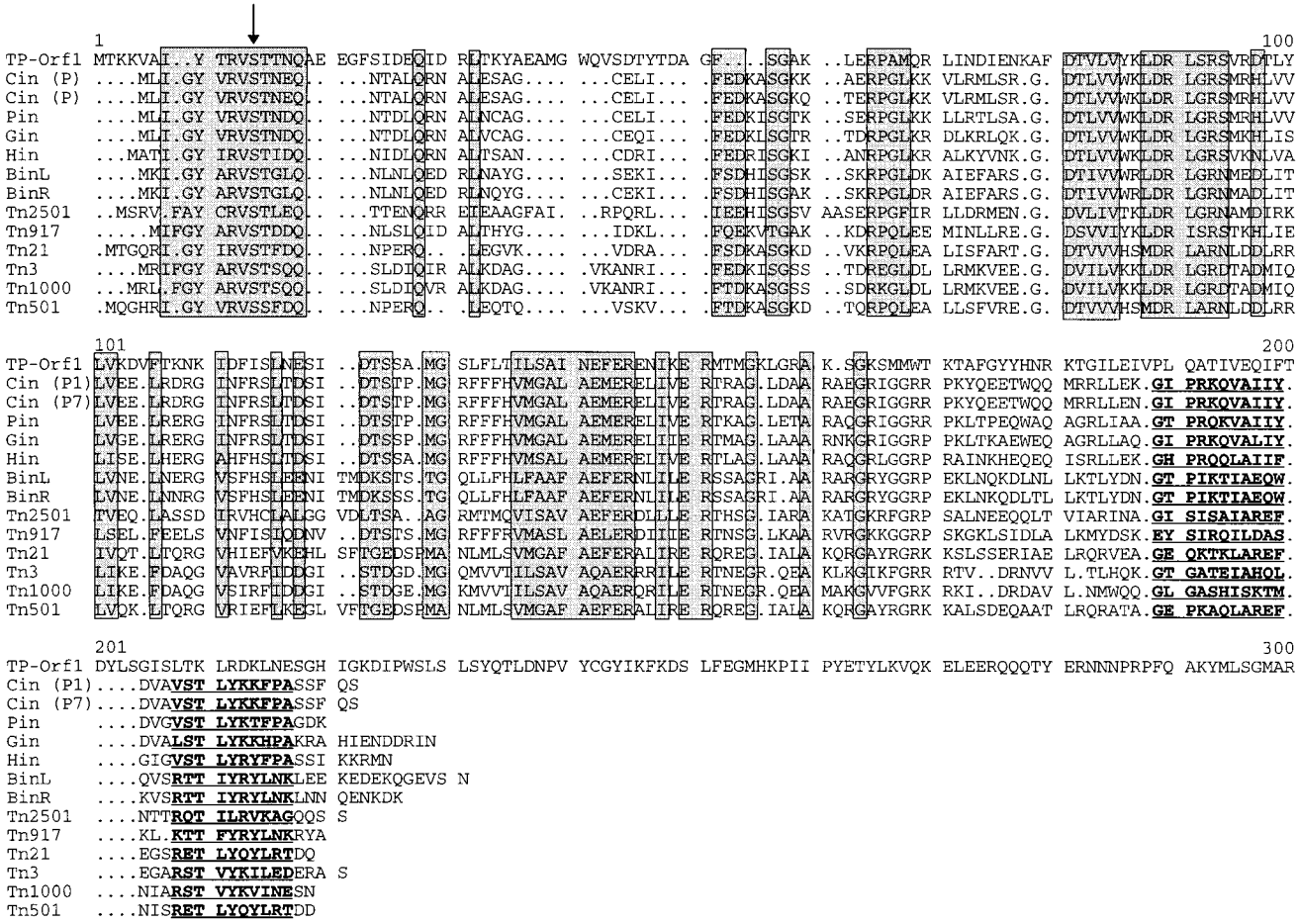

FIG. 3. Alignment of amino acid sequences deduced from the *orf1* gene of TP901-1, encoding the protein needed for integration, and representative resolvases and invertases. Sequences are aligned to show conserved regions. Highly conserved regions are boxed and shaded. The presumptive serine involved in the 5′-phosphoseryl linkage to DNA is indicated by an arrow. The helix-turn-helix motifs in the Tn*3* family of resolvases and of the invertases are boldface and underlined. The full-length amino acid sequences of the resolvases and invertases are shown, whereas only the homologous N-terminal sequence of the TP901-1 Orf1 protein is shown. The amino acid sequences, except the TP901-1 Orf1 sequence, were adapted from data reported by Sherratt (33).

serine at position 12, which directly participates in this process by the formation of covalent 5′-phosphoseryl intermediates with DNA (13, 26). The sizes of the resolvase family of proteins are less than half the size of Orf1, and they have a helix-turn-helix motif in the C-terminal end, which is supposed to be involved in the specific DNA binding (around position 200 [Fig. 3]). No such helix-turn-helix motif could be found in Orf1, neither at this position nor in the rest of the 485 amino acids. This finding made us repeat the sequencing of the central part of *orf1* (from nucleotide 1702 to nucleotide 2483) using the integration vector pBC170 as a template. The DNA sequence shown in Fig. 2 was verified. Furthermore, the *orf1*-encoded protein was visualized by in vivo induction of T7 polymerase in a strain carrying pBC186, which has *orf1* located on the *Pst*I-*Nsi*I fragment inserted after the T7 promoter. A protein band with a size of 55 kDa, which was not present in the control strain carrying pBC144, was observed, thus verifying the size of *orf1* (data not shown).

Two examples of site-specific recombinases have previously been reported to have C-terminal extensions of about 300 amino acids, as well as an N terminus showing significant similarity to that of the resolvase family, just like Orf1 from TP901-1. The total size of these peptide chains is about 500 amino acids, and they also do not contain the DNA binding motif found in the true resolvase proteins. The two examples are the site-specific recombinase encoded by *spoIVCA* in *Bacillus subtilis* (32) and the site-specific recombinase encoded by *xisF* in several *Anabaena* species (4). Both proteins are involved in chromosomal rearrangements during differentiation (sporulation and heterocyst formation, respectively). No significant similarities to these site-specific recombinases or to other proteins were, however, found in the C-terminal half of Orf1. Further analysis of the predicted amino acid sequence of Orf1 resulted in the discovery of a possible phosphate binding loop (P-loop) with the consensus sequence (G)XXXXGK(S) at positions 1678 to 1701 in the nucleotide sequence (Fig. 2) and position 146 to 154 in the aligned amino acid sequence (Fig. 3). The P-loop is a common motif in ATP/GTP-binding proteins (31). The P-loop was specific for Orf1 and was not found in the rest of the resolvase family or in *spoIVCA* or *xisF*. The significance of this finding may therefore be limited, also, since false positives were found. In Orf3, a possible zinc binding motif, HELGH, encoded by bp 252 to 266 was observed (Fig. 2), but the relevance of this observation is not known. The isoelectric points of the predicted proteins were calculated as 6.6, 9.2, and 4.5 for Orf1, Orf2, and Orf3, respectively.

The nucleotide sequence was also analyzed for the occurrence of dyad symmetries. Hairpin structures followed by runs

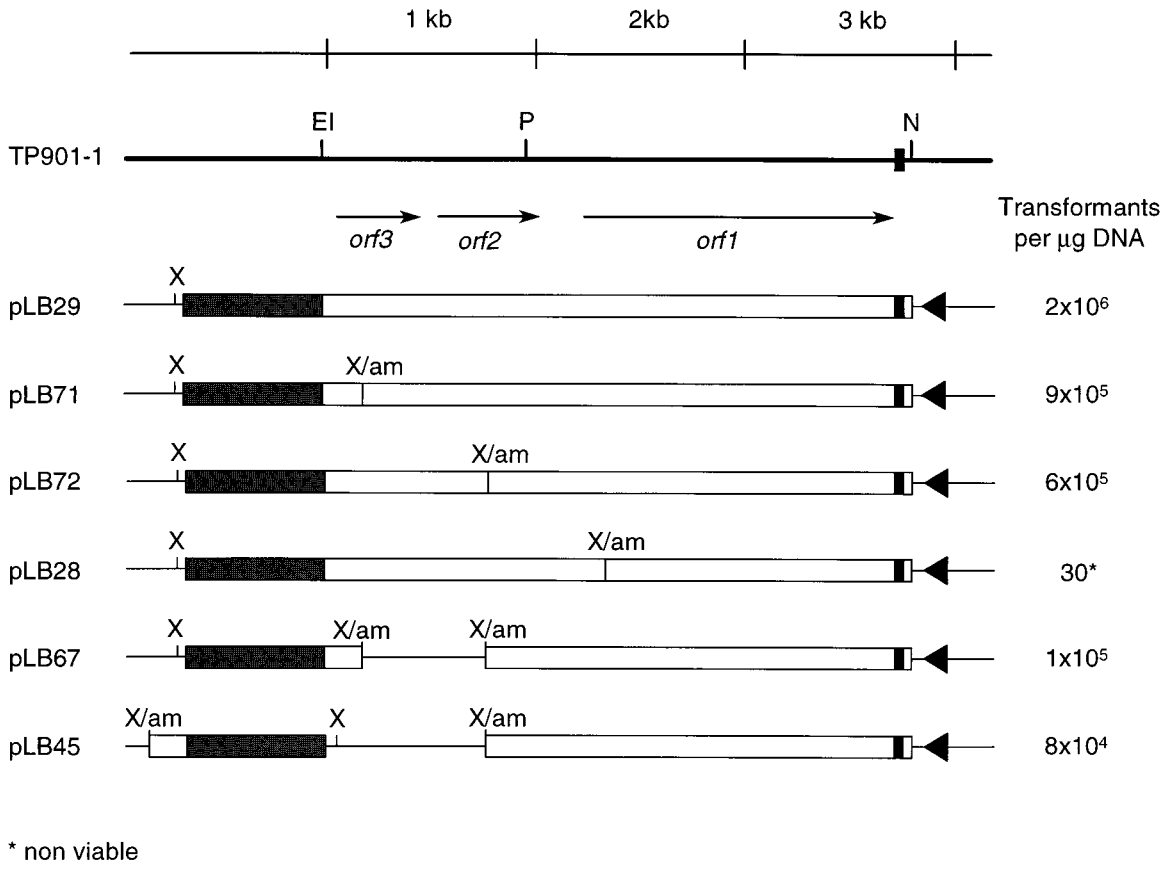

FIG. 4. Mutational and deletional analyses of the 2.8-kb *Eco*RI-*Nsi*I fragment from the TP901-1 phage genome. *E. coli* plasmid clones containing the 2.8-kb *Eco*RI-*Nsi*I fragment (or parts thereof) are shown. The transformation frequencies in *L. lactis* subsp. *cremoris* MG1363 are expressed as the number of transformants per microgram of plasmid DNA; an asterisk indicates nonviable colonies. Thin arrows indicate ORFs *orf1*, *orf2*, and *orf3* deduced from the nucleotide sequence. With a plasmid which is able to replicate in lactococci as a control, 3×10^7 transformants per µg of DNA were obtained (data not shown). Open boxes, TP901-1 insert; large closed boxes, *erm* gene; small closed boxes, *attP* site of TP901-1. The *erm* gene is not drawn to scale. The thin line indicates pGEM-7zf(+) DNA, with no lactococcal origin of replication, and closed arrows indicate the orientation of the *lacZ* promoter in the vector. EI, *Eco*RI; N, *Nsi*I; P, *Pst*I; X, *Xba*I; X/am, *Xba*I site containing an amber stop codon.

of U's could be factor-independent terminators. Two such sequences were located; t2, located downstream of *orf2* at positions 1172 to 1209, has a stem with a ΔG value of −18.9 kcal/mol (−79.1 kJ/mol), and r5 at positions 2687 to 2706 has a stem with a ΔG of −10.5 kcal/mol (−43.9 kJ/mol). In order to form the stem-and-loop structure and thus to function as a terminator, the dyad symmetry must be located in untranslated RNA, as is the case for t2; therefore, t2 could be a functional transcriptional terminator. In r5 the last three codons of *orf1* overlap. The leading ribosome must therefore be partially delayed in relationship to the RNA polymerase near the end of *orf1* if r5 functions as the transcriptional terminator for *orf1*.

The palindromic structures r3 and r6 (Fig. 2) found in positions 2628 to 2647 and 2740 to 2759, respectively, have ΔG values of −8.2 and −9.2 kcal/mol (−34.3 and −38.5 kJ/mol), respectively. However, they are not followed by runs of U's and are therefore not likely to function as factor-independent terminators. They may be involved in the formation of the protein-DNA complex involved in the site-specific recombination process. A putative consensus promoter was found upstream of *orf1* with the −35 region at nucleotides 1088 to 1093 and the −10 region at nucleotides 1112 to 1117, as indicated in Fig. 2.

Identification of TP901-1 integrase. The homology to the resolvase family, along with the location just upstream of the core sequence, strongly indicates that *orf1* encodes the site-specific recombinase of the TP901-1 integration system. In order to prove this, and to investigate the influence of *orf1*, *orf2*, and *orf3* on the integration process, integration vectors carrying an amber stop codon in each of the genes, as well as an additional missense mutation in *orf1* and *orf3*, were constructed (Fig. 4). The frequency of integrant formation in *L. lactis* subsp. *cremoris* MG1363 was unaffected by the mutations in the *orf3* and *orf2* genes (pLB71 and pLB72, respectively), whereas the mutations in the *orf1* gene (pLB28) abolished the formation of viable integrants in MG1363. The few colonies arising from transformation with pLB28 could not be further propagated (Fig. 4). The amber mutations do function as stop codons, since it was found that no amber suppressor was present in *L. lactis* subsp. *cremoris* MG1363: no erythromycin-resistant transformants were obtained when MG1363 was transformed with pFDi10, which contains an amber stop codon within the *erm* gene (7) (data not shown). Deletions of the *orf3* and *orf2* genes (pLB67 and pLB45) confirmed that *orf2* and *orf3* are not needed for integration. The data in Fig. 4 show that the *orf1* gene, the upstream 425-bp region, and the *attP* region are sufficient for integration in MG1363 (pLB45). Furthermore, it was found that Orf1 is required for integration, since pLB28 is not able to promote integration. This observation was verified by transformation of LM0230 with pBC190

(contains a frameshift mutation in *orf1*), since no erythromycin-resistant transformants were found (data not shown).

The presence of *attL* and *attR* was verified by PCR analysis with chromosomal DNA extracted from erythromycin-resistant integrants from *L. lactis* subsp. *cremoris* MG1363.

DISCUSSION

We have shown that the bacteriophage-encoded functions necessary for the site-specific recombination of the temperate bacteriophage TP901-1 are present on the 2.8-kb *Eco*RI-*Nsi*I fragment (Fig. 1). The nucleotide sequence of the TP901-1 integration region revealed three putative ORFs, Orf1 (485 aa), Orf2 (181 aa), and Orf3 (145 aa), all preceded by possible SD sequences and transcribed in the same direction upstream of the *attP* core region. Two putative ORFs, OrfA and OrfB, transcribed from the opposite strand but not preceded by SD sequences are probably not expressed (Fig. 2).

The location upstream of the core and the high degree of homology (38 to 44% in 150 to 180 amino acids) to site-specific recombinases of the *res* family suggest that the Orf1 encodes the TP901-1 integrase. This possibility was demonstrated by deletion and mutational analyses. The results proved that Orf1 and *attP* are necessary for integration, whereas Orf2 and Orf3 are not. Furthermore, it was shown that *orf1*, *attP*, and the 425-bp upstream region of *orf1* were sufficient for integration.

The N-terminal part of the *res* family of proteins, homologous to the N-terminal part of Orf1, constitutes the recombinational domain. The serine at residue 12, which is important for recombination, is conserved in Orf1, indicating that this serine could be involved in recombination catalyzed by Orf1. The C-terminal end of the *res* family of proteins contains a helix-turn-helix motif involved in the site-specific DNA binding. No such helix-turn-helix motifs were found in Orf1. In contrast, Orf1 seems to be a member of a new family consisting of site-specific recombinases that are more than twice the size of the resolvases but showing significant similarity to the resolvases in the N terminus of about 150 amino acids. The two other identified members of this class are the site-specific recombinases from *B. subtilis* and several *Anabaena* species involved in chromosomal inversion and deletion events, respectively, during differentiation into spores or heterocysts (4, 32).

Orf1 does not contain the helix-turn-helix DNA-binding domain found in the resolvase family. Instead the additional 300-amino-acid C-terminal domain found in Orf1 may determine molecular arrangements during the specific recombination process quite different from those found in the genuine resolvase family. This is supported by analysis of the possible recombination sites on the DNA substrate. Recombinases of the resolvase family cut and religate the DNA within a region of perfect dyad symmetry at the center of *res* subsite 1 (25). The *res* site typically contains three subsites, each of imperfect dyad symmetry and each believed to bind a dimer of resolvase (12, 19). A specific recombination site with three subsites was searched for within the *attP* region of TP901-1, but no obvious sites were found. Also, the consensus sequences of the Tn*3* and γδ (Tn*1000*) *res* sites [5′-TGTCYR(A/T)TA-3′] (12) and the consensus sequence of the Tn*501*, Tn*1721*, and Tn*21 res* sites (5′-YGTCARRNTA-3′) (27) were searched for without success (data not shown). However, three regions of dyad symmetry around the 5-bp core sequence in the *attP* region were found and could represent binding sites for the site-specific TP901-1 DNA recombinase. Further study is necessary to confirm this observation.

The function of Orf2 and Orf3 is not known. Orf2 is a basic protein that has no homology to any known protein sequences. Orf3 is an acidic protein that is not homologous to any known protein sequences. A zinc-binding motif often found in metallopeptidases/proteases (18, 23) was found in the predicted Orf3 protein sequence; therefore, the Orf3 protein could be a zinc-dependent protein.

The observation that Orf1, *attP*, and the 425-bp region upstream of *orf1* were sufficient for integration suggests the presence of a promoter within the 425-bp fragment upstream of *orf1*. This promoter is not functional on pBC186 and pBC191, containing 240 bp of the upstream region, in addition to *orf1* and *attP*, since these plasmids were not able to integrate. This indicates either that the promoter is located upstream of the *Pst*I site or that a region necessary for the initiation of transcription from this promoter is present upstream of the *Pst*I site. By sequence analysis, a putative consensus promoter was found upstream of *orf1* but downstream of the *Pst*I site. Preliminary promoter screening data indicate that a functional promoter is present on the *Eco*RI-*Pst*I fragment but not on the *Pst*I-*Nsi*I fragment.

We have presented data showing that the site-specific integration of the temperate bacteriophage TP901-1 into the chromosome of *L. lactis* subsp. *cremoris* is mediated by a resolvase-like protein. This is a unique finding for site-specific integration systems originating from temperate bacteriophages and hence also among phages from lactic acid bacteria. The first integration vector based upon a phage system in lactic acid bacteria was derived from the temperate bacteriophage ϕadh from *Lactobacillus gasseri* (10). A second lactobacillus integration system from the temperate bacteriophage mv4 from *Lactobacillus delbrueckii* subsp. *bulgaricus* has been sequenced (8). The attachment sites of these two bacteriophages were not identical, and only distant similarity was observed between the integrases, both belonging to the Int family. The integrase of mv4, however, showed 65% similarity to the lactococcal integrase first identified in ϕLC3 but with different attachment sites. In lactococci, the integration systems of TP901-1 and three other temperate bacteriophages, ϕLC3, Tuc2009, and BK5-T, have now been characterized (2, 21, 36). In contrast to the unique integrase in TP901-1, the integration systems of the three other lactococcal bacteriophages were found to be almost identical, having identical *attP* and *attB* sequences and containing the same integrase, except for a few amino acid substitutions. The identity of this part of the phage genomes was surprising, as BK5-T is classified in a lactococcal bacteriophage group unrelated to the P335 group, while ϕLC3, TP901-1, and probably also Tuc2009 belong to the P335-type bacteriophages (17).

The resolvase-like integrase encoded by *orf1* in TP901-1 showed no similarity to the Int family and hence to the integrases identified from phages from lactic acid bacteria; neither was any similarity identified in the attachment site regions of TP901-1, indicating that the integration system of TP901-1 is a unique system for phage integration, one which has not previously been described. The integration is functional in all three strains of *L. lactis* subsp. *cremoris* tested (3107, LM0230, and MG1363). The constructed integration vector pBC170 is highly efficient, giving integration with a frequency of about 20% of the transformation frequency by using a replicating plasmid. The integration vector may therefore also be useful for integration in strains which can be transformed only at low frequency. The integrants were shown to be very stable; thus, the vectors derived from TP901-1 could be powerful tools for stable, single-copy constructions in industrial and laboratory strains.

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ADDENDUM

After submission of this paper, the *sre* gene (ORF469) of the *Streptomyces* bacteriophage R4 has been shown to encode a site-specific integrase with a size similar to that of Orf1 from TP901-1. This phage integrase also contains an N-terminal resolvase domain (22a).

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