JOURNAL OF BACTERIOLOGY, Feb. 1995, p. 586–595 Vol. 177, No. 3
0021-9193/95/$04.00+0
Copyright © 1995, American Society for Microbiology

Characterization of Genetic Elements Required for Site-Specific Integration of *Lactobacillus delbrueckii* subsp. *bulgaricus* Bacteriophage mv4 and Construction of an Integration-Proficient Vector for *Lactobacillus plantarum*

LAURENCE DUPONT,* BRIGITTE BOIZET-BONHOURE,† MICHÈLE CODDEVILLE, FRÉDÉRIC AUVRAY, AND PAUL RITZENTHALER

Laboratoire de Microbiologie et Génétique Moléculaire du Centre National de la Recherche Scientifique, 31062 Toulouse, France

Received 12 August 1994/Accepted 28 November 1994

Temperate phage mv4 integrates its DNA into the chromosome of *Lactobacillus delbrueckii* subsp. *bulgaricus* strains via site-specific recombination. Nucleotide sequencing of a 2.2-kb *attP*-containing phage fragment revealed the presence of four open reading frames. The larger open reading frame, close to the *attP* site, encoded a 427-amino-acid polypeptide with similarity in its C-terminal domain to site-specific recombinases of the integrase family. Comparison of the sequences of *attP*, bacterial attachment site *attB*, and host-phage junctions *attL* and *attR* identified a 17-bp common core sequence, where strand exchange occurs during recombination. Analysis of the *attB* sequence indicated that the core region overlaps the 3′ end of a tRNA^{Ser} gene. Phage mv4 DNA integration into the tRNA^{Ser} gene preserved an intact tRNA^{Ser} gene at the *attL* site. An integration vector based on the mv4 *attP* site and *int* gene was constructed. This vector transforms a heterologous host, *L. plantarum*, through site-specific integration into the tRNA^{Ser} gene of the genome and will be useful for development of an efficient integration system for a number of additional bacterial species in which an identical tRNA gene is present.

Gram-positive *Lactobacillus* strains are extensively used as preservatives in food products and as lactic acid producers in dairy fermentations, but development of bacteriophages is the main cause of fermentation failures. Taxonomic studies on phages from *Lactobacillus delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis* led to the determination of two genetic groups (21, 32). The phage representative of the most widespread group, bacteriophage mv4, has been well characterized (13). Phage mv4 is a temperate phage which infects and lysogenizes *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis* strains. The 36-kb genome of the phage has been mapped physically, and its DNA is circularly permuted (20). Several genes have been characterized, such as those encoding structural proteins (52) or genes involved in cell lysis (6). The phage attachment site (*attP*) has previously been located on the mv4 genome, and several attachment sites on the chromosome of independently isolated lysogens have been identified (20).

Most temperate bacteriophages integrate their DNA into the host chromosome by a site-specific recombination process following the Campbell model (10). This mechanism involves two specific attachment sites, one on the bacterial chromosome (*attB*) and the other one on the phage genome (*attP*). The recombination process is catalyzed by a phage-encoded integrase. There are many well-characterized examples of site-specific recombination in gram-negative bacteriophages, and the best-studied system is that of bacteriophage λ (for a review, see reference 22). The integration system of phages of gram-positive bacteria is less well documented, but data on site-specific recombination are available for phages L54a, ϕ11, and ϕ13 of *Staphylococcus aureus* (14, 25, 55, 56), for mycobacteriophage L5 (26), and for phages of lactic acid bacteria (12, 30, 43, 51). The integration systems of integrative plasmids pSAM2, pSE211, pSE101, and SLP1 from actinomycetes appear to behave like those of temperate bacteriophages (5, 7–9).

To improve our understanding of host-phage relationships, we studied the site-specific integration of the mv4 genome into the *L. delbrueckii* subsp. *bulgaricus* chromosome. In this report, we characterize the phage *attP* and bacterial *attB* attachment sites, as well as the phage integrase gene required for site-specific recombination. A nonreplicative vector based on phage integration elements has been constructed and is able to integrate in a specific *attB* site in the *L. plantarum* chromosome.

MATERIALS AND METHODS

Bacteria, bacteriophages, and plasmids. The *L. delbrueckii* subsp. *bulgaricus*, *L. plantarum*, and *Escherichia coli* strains used in this study and the plasmids used for cloning experiments are listed in Table 1. Culture conditions for *E. coli* were as described by Sambrook et al. (45). *Lactobacillus* sp. strains were grown at 42°C in MRS medium (16). Bacteriophage mv4 was propagated on *L. delbrueckii* LT4c or LKT, and phage particles were purified as previously described (32).

DNA techniques. Phage DNA was extracted and purified as described by Mata et al. (32). *E. coli* transformation, plasmid DNA isolation, restriction endonuclease digestion, DNA ligation, and gel electrophoresis were performed as described by Sambrook et al. (45). *L. plantarum* LP80 was transformed by electroporation as described by Leer et al. (27). Lactobacillus chromosomal DNA was extracted as follows. Strains were grown in MRS broth (10 ml) to an optical density at 600 nm of 0.8, centrifuged, and resuspended in 1.2 ml of TES (100 mM Tris-HCl [pH 8], 20 mM EDTA, 20% sucrose) with lysozyme (25 μg/ml) and mutanolysin (5 U/ml) and further incubated for 30 min at 37°C. Then, 80 μl of 10% sodium dodecyl sulfate with proteinase K (400 μg/ml) was added and the mixture was incubated for 30 min at 60°C. After phenol-chloroform extraction, DNA was ethanol precipitated and resuspended in 50 μl of TE (10 mM Tris-HCl, 1 mM EDTA) and RNase (25 μg/ml). The nucleotide sequence was determined by the dideoxy-chain termination method (46) by using Sequenase version 2.0 (United States Biochemical). PCR amplification was accomplished with 2 to 10 ng of DNA in 100 μl of 1× Biolabs buffer containing, in addition, 400 μM

* Corresponding author. Mailing address: Laboratoire de Microbiologie et Génétique Moléculaire du CNRS, 118 route de Narbonne, 31062 Toulouse, France.

† Present address: CRBM-CNRS, 34033 Montpellier Cedex, France.

TABLE 1. Bacterial strains, plasmids, phages, and primers used in this study

Material	Explanation	Reference or source
Bacterial strains		
Lactobacillus delbrueckii subsp. *bulgaricus*		
LT4	Lysogenic for phage mv4	13
LT4c	Prophage-cured LT4 strain	20
Lactobacillus delbrueckii subsp. *lactis* LKT	Propagating strain for phage mv4	13
Lactobacillus plantarum LP80		27
Escherichia coli		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	
TG1	*supE hsd*Δ*5 thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	
Plasmids		
pBSII/KS$^+$	*lacZ bla*$^+$; 3.0 kb	Stratagene
pTZ18/19R	*bla*$^+$; 2.9 kb	34
pRC1	*ermAM*$^+$; 3.5 kb	23
pMC1	pRC1::1.6-kb *Eco*RV-*Pvu*II ϕmv4 *int*$^+$ *attP*	This study
pMC2	pMC1 *int* (4-bp deletion)	This study
pO7	pRC1::*ori* of LKT cryptic plasmid	Unpublished data
Phage mv4	Temperate phage isolated from strain LT4	13
Primers		
Ra	5′-ATCTCCATGGTAATATCGAT-3′	This study
Rb	5′-AAGCTTTCTAAATCAACTAGA-3′	This study
La	5′-CTGCAGCAAGTGGCTACCTTGGA-3′	This study
Lb	5′-GCGAAGTATCTCCGCATTTTA-3′	This study
Ba	5′-AAGCTTCACCATCTTAAAAAATA-3′	This study
Bb	5′-CATTTGATTTAGATGTCCTT-3′	This study
S3	5′-GTTCATCATCCTCAT-3′	This study
BX1	5′-GCATCTGGATCTTAT-3′	This study
BX4	5′-CCCGAGAATCAACAT-3′	This study
T1	5′-GAATTCGAGAGTTGGCAGAG-3′	This study
T2	5′-GTCGACACAGGATTTGAACC-3′	This study

deoxynucleoside triphosphates, 4 mM $MgSO_4$, 30 pmol of each primer, and 2 U of Vent DNA polymerase (Biolabs), overlaid with 100 μl of filtered paraffin to prevent evaporation loss. Routinely, 25 cycles were performed as follows on a PREM apparatus (LEP Scientific): 1 min at 95°C for denaturation (except 5 min at 95°C for the first cycle), 1 min for primer annealing at a temperature adapted to the set of oligonucleotides used in the amplification reaction, and 1.5 min at 72°C for strand synthesis. After amplification, the PCR products were purified by phenol-chloroform extraction and precipitated by addition of 1 volume of 4 M ammonium acetate and 2 volumes of 2-propanol. DNA-DNA hybridization experiments were done as previously described (20).

Isolation of *attL*, *attR*, and *attB* DNA fragments. An inverse PCR strategy was used to clone host-phage junction fragments. The *attR* site is carried on a 1.7-kb *Cla*I fragment, and the *attL* site is on a 2.8-kb *Sal*I fragment. Chromosomal DNA of lysogenic strain LT4 was digested with enzyme *Cla*I or *Sal*I. *Cla*I restriction fragments ranging from 1.5 to 2.0 kb and *Sal*I fragments ranging from 2.5 to 3.0 kb were extracted from agarose gel after electrophoresis and self-ligated. The positions and the sequences of the primers used (Ra, Rb, La, and Lb [Table 1]) were deduced from the nucleotide sequence of the mv4 integration region. Adjacent primers Ra and Rb, in the opposite orientation, were used to amplify a 1.7-kb fragment for *attR*, whereas for *attL*, a 2.7-kb fragment was amplified with primers La and Lb on LT4 chromosomal DNA. These fragments were cloned in plasmid pBS-KS at the *Sma*I site and sequenced by using appropriate primers. The sequences of oligonucleotides Ba and Bb were deduced from the bacterial nucleotide sequence of the host-phage junction fragments and used to amplify a 282-bp fragment (*attB*) on the chromosomal DNA of cured strain LT4c. This fragment was inserted at the *Sma*I site of plasmid pBS-KS and sequenced.

Construction of integrative plasmid pMC1 and plasmid pMC2. A 2.1-kb fragment obtained on mv4 DNA by amplification with primers S3 and BX1 (Table 1) was digested with *Eco*RV and *Pvu*II to generate a 1,620-bp fragment (Fig. 1B) which was cloned in vector pRC1 at the *Eco*RV site. The resulting 5.1-kb plasmid, pMC1 (see Fig. 6), carries the *int* gene, the *attP* site, and open reading frame I1 (ORFI1) with its 5′ part truncated. This plasmid was introduced by electroporation into *L. plantarum*, and transformants were selected on MRS agar containing 5 μg of erythromycin per ml.

To generate an *int* mutant, pMC1 was digested at its unique *Bgl*I site. Protruding ends were deleted by using the exonuclease activity of the Klenow fragment of *E. coli* DNA polymerase under the conditions described by the supplier (Biolabs), and the fragment was religated, yielding plasmid pMC2. A deletion of 4 bp was confirmed by sequencing reactions with appropriate primers on plasmid pMC2.

Construction of *attP*, *attL*, and tRNA^{Ser} gene probes. A recombinant plasmid carrying the *attP* region was digested with *Cla*I and *Pvu*II, and the 380-bp fragment generated was purified from a polyacrylamide gel and used as the *attP* probe (Fig. 1B). For the *attL* probe, a 480-bp fragment between the *Ava*I and *Bam*HI (B5) sites (Fig. 1B) was amplified by PCR with oligonucleotides BX1 and BX4 (Table 1). One set of primers (T1 and T2) was synthesized to amplify by PCR an 80-bp fragment on the chromosomal DNA of LT4c. This fragment was used in hybridization experiments as a tRNA^{Ser} gene probe.

Nucleotide sequence accession number. The 2,284-bp nucleotide sequence of the mv4 integration region has been deposited in GenBank under accession number U15564.

RESULTS

Earlier studies (20) showed that one integrated copy of phage mv4 DNA was present in the chromosome of the lysogenic strain *L. delbrueckii* subsp. *bulgaricus* LT4 and that the *attP* phage attachment site was located on a 5.9-kb *Sal*I fragment and more precisely on a 0.5-kb *Cla*I-*Pvu*II fragment. This was also the case in four other independent lysogenic strains analyzed, indicating the presence of one specific *attP* site on the phage genome.

Nucleotide sequence and genetic organization of the mv4 integration region. The precise restriction map of the mv4 5.9-kb *Sal*I fragment has been established (Fig. 1A). Representative restriction fragments of the *attP* region were cloned in *E. coli* vector pBlueScript or pTZ19R. By using these recombinant plasmids as templates, the 2,284-bp nucleotide sequence of the region between the *Sma*I (Sm2) and *Bam*HI (B5) sites (Fig. 1A) was determined (Fig. 2). The base composition of this sequence was 46% G+C, which is slightly lower

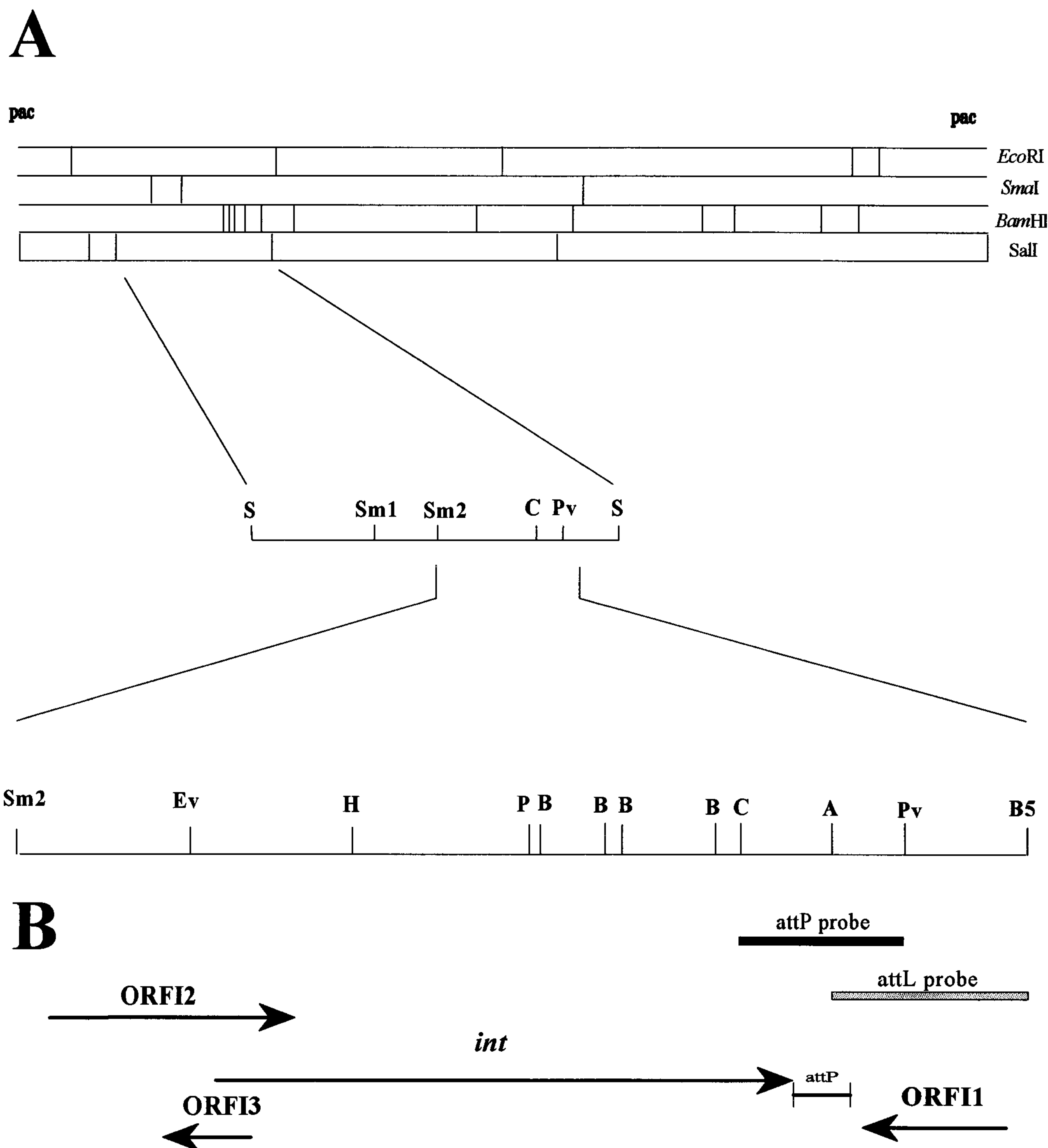

FIG. 1. Organization of the mv4 integration region. (A) Phage mv4 DNA restriction map. The 36-kb mv4 genome is shown linearized at its packaging site (pac). A detailed restriction map of the integration region is shown below. (B) Genetic organization of the integration region. The positions of the *attP* site and the ORFs deduced from the nucleotide sequence are indicated. The locations of the probes (*attP* and *attL*) used in the Southern experiments are shown. A, *Ava*I; B, *Bam*HI; C, *Cla*I; Ev, *Eco*RV; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; Sm, *Sma*I.

than that of the *L. delbrueckii* genome (50%). Within the interval, four closely spaced or overlapping ORFs were found: ORFI2 (bases 64 to 600) and the *int* gene (bases 437 to 1717), oriented from left to right (Fig. 1B), and ORFI1 (bases 2241 to 1915) and ORFI3 (bases 534 to 364), oriented from right to left.

Three potential initiation codons were found for ORFI2 and *int*. The first ATG of ORFI2 and *int* is preceded by an adequate ribosome-binding site (underlined in Fig. 2) and might be the potential start codon, meaning that ORFI2 and *int* overlap by 164 bp. A unique probable ribosome-binding site was located upstream of the start of ORFI1, and none was found near ORFI3.

The *attP* site located on the 0.5-kb *Cla*I (C)-*Pvu*II (Pv) frag-

```
      SmaI
1     CCCGGGCAAGATCATCGGCAAGATGATCAAAATGTCAAGATTTCAATAGGAGGTAAAAGAAAAATGGAAAAGAAGAAATTAAAGAAATGGCAAATCGGAA
                                                                     M  E  K  K  K  L  K  K  W  Q  I  G  M
                                                               ORFI2 ▶
101   TGCTTATTTTCCTTGGCATGGCTGCCGTTGGTGCTGTAGAAGATGCCGTGTCACCAGAGCAGGACTCATCTCCAAAAACTGTTAAAAAGGCACATAAACA
        L  I  F  L  G  M  A  A  V  G  A  V  E  D  A  V  S  P  E  Q  D  S  S  P  K  T  V  K  K  A  H  K  Q

201   GAGCTCTAAAACGCCAAGTTCATCATCCTCATCTTCTTCCGTCTCGTCATCTTCATCTTCCTCATCGTCCTCATCTTCCTCCGAACCTGCCAAGGCTAAG
       S  S  K  T  P  S  S  S  S  S  S  S  S  V  S  S  S  S  S  S  S  S  S  S  S  S  S  E  P  A  K  A  K
                                                                                                    EcoRV
301   GAATCCGAAGACAACAAACGACTGGTTAAGGCTGTTTCTCAGTATCCTAAGGATTTCGAGTTAGCGGTGGTTAAAAGTGAAGTAGTAGGAAGAGATATCA
      E  S  E  D  N  K  R  L  V  K  A  V  S  Q  Y  P  K  D  F  E  L  A  V  V  K  S  E  V  V  G  R  D  I  N
                                                                       *  R  H  N  F  T  F  Y  Y  S  S  I  D

401   ACATTTATCTGTCTAATTCCTACTTGGAAGAAAAGGATGACAAGGAAAGGCTGTGCAGAAACTATTGGCAGTTGGCAGTCGATGACTTCAACAAATATAA
        I  Y  L  S  N  S  Y  L  E  E  K  D  D  K  E  R  L  C  R  N  Y  W  Q  L  A  V  D  D  F  N  K  Y  K
       V  N  I  Q  R  I  G  V  Q  F  F  L  I  V  L  F  P  Q  A  S  V  I  P  L  Q  C  D  I  V  E  V  F  I
                                                M  T  R  K  G  C  A  E  T  I  G  S  W  Q  S  M  T  S  T  N  I  K
                                          int ▶
501   AAAGTATACCGACCAATCCACGGCTTTTGTCCATATCTATGACCAATCTGGAAACGAACTGGCCAAAAGCAACTGGGGAAAATTTGAATATTTGGGTGAA
       K  Y  T  D  Q  S  T  A  F  V  H  I  Y  D  Q  S  G  N  E  L  A  K  S  N  W  G  K  F  E  Y  L  G  E
      F  L  I  G  V  L  G  R  S  K  D  M ◀ ORFI3
        S  I  P  T  N  P  R  L  L  S  I  S  M  T  N  L  E  T  N  W  P  K  A  T  G  E  N  L  N  I  W  V  N

601   TAAATATGCCAAAGCCGTAATCCTGCAATCAAAAAATACACCAGCCGGGGCCAAACAAAATACAAATTCCAGATTTACCTGGGCCAGGACGAAAGCGGAA
      *
       K  Y  A  K  A  V  I  L  Q  S  K  N  T  P  A  G  A  K  Q  N  T  N  S  R  F  T  W  A  R  T  K  A  E

701   AATCAATCAACACGACCCGGAGTGGTTTTAAATCTTACTCCCAATGCATCAGCAGCTTACAACAAGCTTAAGGCCCAAGGATTGGCCGCCAAAGCACCCA
      N  Q  S  T  R  P  G  V  V  L  N  L  T  P  N  A  S  A  A  Y  N  K  L  K  A  Q  G  L  A  A  K  A  P  K

801   AAAAAGCGACCACCGATGAGGGTTGGTCCGTTTGGTTTGATAGCTATAAAGGCGGAGTTAAAGAGTCGTCAGCAAACAAAACGCTGACTAGTTACAGAGT
        K  A  T  T  D  E  G  W  S  V  W  F  D  S  Y  K  G  G  V  K  E  S  S  A  N  K  T  L  T  S  Y  R  V

901   CCACATCAAGCCTGCTTTTGGTGATAAAATGATCAGCTCGATCAAGACGGCCACCGTACAACTCTGGGCAAACAATTTGGCCACCAAGCTGGTCAACTAC
       H  I  K  P  A  F  G  D  K  M  I  S  S  I  K  T  A  T  V  Q  L  W  A  N  N  L  A  T  K  L  V  N  Y

1001  AAGGTGGTTGTGCGCCTGCTAGGGACTCTTTTTGAATTTGCCAAGCGCCTGGACTATTGCAAGGACAACCCGGTCAAGCAGATCATCATGCCAAAAGCTA
      K  V  V  V  R  L  L  G  T  L  F  E  F  A  K  R  L  D  Y  C  K  D  N  P  V  K  Q  I  I  M  P  K  A  T

1101  CCTCCAGGCCTCGCAGAGACATCAGCACCAACTACTATAACCGTGATGAGCTTCAGCAGTTCCTGCAGGCCGCTAAAGAAGTAGGATCCCGGACTTATGT
        S  R  P  R  R  D  I  S  T  N  Y  Y  N  R  D  E  L  Q  Q  F  L  Q  A  A  K  E  V  G  S  R  T  Y  V

1201  CTTCTTTCTACTCCTTGCTACCACGGGCCTCCGAAAAAGGCGAAGCACTAGCCCTGGATTGGTCGGACATCGACTACGATCAAAGGAAAAATCTCCGTCA
       F  F  L  L  L  A  T  T  G  L  R  K  R  R  S  T  S  P  G  L  V  G  H  R  L  R  S  K  E  K  S  P  S
                            BglI
1301  CTAAAGACTCTTGCCTATGGCCTGGGTGGCAAGTACGGGATCCAGCCACCTAAGACTAAGGCAGGGATCCGCACGGTGCCACTGACTGATCAGATGGCAG
      L  K  T  L  A  Y  G  L  G  G  K  Y  G  I  Q  P  P  K  T  K  A  G  I  R  T  V  P  L  T  D  Q  M  A  A

1401  CCGTTTTAAAAGACTACCATAGTGATCTCTGCCCGCACCTTTTTCACACGCTTGATGGTGATTATCTCCGTCTTAGTAAGCCAGATCAGTGGCTTCAGGC
        V  L  K  D  Y  H  S  D  L  C  P  H  L  F  H  T  L  D  G  D  Y  L  R  L  S  K  P  D  Q  W  L  Q  A

1501  TGTTTATAAACACGACCCAGACCTCCGACAAATTAGAATCCATGGCTTCCGTCATACTTTTGCGTCCCTGCTCATCACTGCGGATCCGTCAATCAAGCCA
        V  Y  K  H  D  P  D  L  R  Q  I  R  I  H  G  F  R  H  T  F  A  S  L  L  I  T  A  D  P  S  I  K  P
                                                 ClaI
1601  ACAGACGTGCAAGCAATCCTGGGTCATGAATCAATCGATATTACCATGGAGATTTACATGCACGCCACTCAAGAAGGCAGGCGGAATGTTGAAAGAGTTC
      T  D  V  Q  A  I  L  G  H  E  S  I  D  I  T  M  E  I  Y  M  H  A  T  Q  E  G  R  R  N  V  E  R  V  L
                                                              r2
1701  TAAATCAACTAGATTTTTAACTAGAAAAAGTGAAACGCATGGAAACAACCAGAAACGCTTTTTAGCCCAAAAGCAAGATTCGAAACCTCCAGAACCCTCC
        N  Q  L  D  F  *       r1       r2         r2       r1                                  r2     r3        r3
                                 r4
      r1                                                           r1                                         r1
1801  AGAAACATGGTTGAAAGAA[CCTGTACTCTCCTTAAT]CAAAGCAATAATCCCCGAGAAATCAACATTCTCGGGGATTATTTTTGTTTTTAACTAGAAAATA
                                                  - - - - - - - ▶ tr-1 ◀ - - - - - - -                    r4
        r2
        r4
1901  ACTAGAAAGAGCTAGTTAATAGATGGCATTAGCGTGATAATCGCCTGCCCGTTTGACGGGTAGACCACTACACCAGTGTCGGTGGTTTGCCACAACTCGA
         r1            *  N  I  S  P  M  L  T  I  I  A  Q  G  N  S  P  Y  V  V  V  G  T  D  T  T  Q  W  L  E
                 PvuII
2001  CGGAGTACTGGCCAGCTGGCAGTCCAGCCAAATTGGCTGAGGATGCGATCAGCGTTCCATCGTCGTTAACTAGCGTGTCCAAGGTAGCCACTTGCTTCAA
      V  S  Y  Q  G  A  P  L  G  A  L  N  A  S  S  A  I  L  T  G  D  D  N  V  L  T  D  L  T  A  V  Q  K  L

2101  TTTGGCATCCCCGACTTTGACAACTACCTGGCTTTCGTCGGTAAAGTTTTGCGTGACGCCGTCTTGAGCTGCTGATAAGACAAAGCTGTGCGAAGTATCT
        K  A  D  G  V  K  V  V  V  Q  S  E  D  T  F  N  Q  T  V  G  D  Q  A  A  S  L  V  F  S  H  S  T  D
                                                                                            BamHI
2201  CCGCATTTTAAAGTGTCGTATTCGATTGACAGAGTGTTCATTTTTTATTTCTCCTATCATCAAAAAACGGACCCCGAAGGATCC
        G  C  K  L  T  D  Y  E  I  S  L  T  N  M
                                                ◀ ORFI1
```

FIG. 2. Nucleotide sequence of the bacteriophage mv4 integration region. The amino acid sequences deduced from the different ORFs are given below the nucleotide sequence. Relevant restriction sites are indicated. Ribosome-binding sites are underlined. Arrows indicate the positions of the direct repeats (r1 to r4). The position of the putative terminator sequence is represented by opposed arrows with broken lines. The core region is boxed.

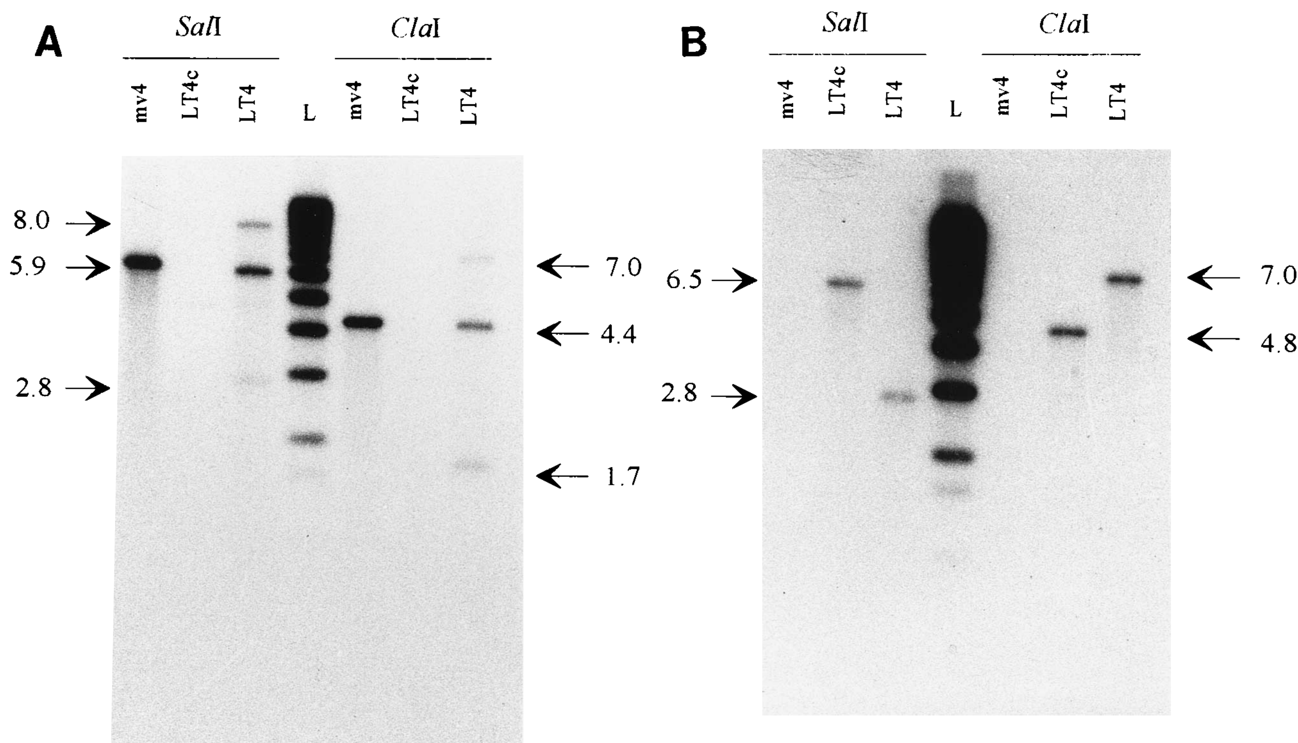

FIG. 3. Identification of *att*-bearing restriction fragments. Digested phage or chromosomal DNAs were separated on a 0.8% agarose gel and hybridized with the *attP* probe (A) or with the tRNA^{Ser} gene as a probe (B). The position of the hybridizing restriction fragments is indicated by an arrow, and molecular sizes are in kilobases. L, 1-kb ladder (Bethesda Research Laboratories).

ment appeared to be 380 bp long (bases 1635 to 2015). This interval can be reduced to 192 bp between the 3′ end of *int* (position 1720) and that of ORFI1 (position 1912). No ORF was detected in this segment, and numerous stop codons were found in all six possible frames. The *attP* site is AT rich (75%), and many direct and inverted repeats were found within the 192 bp (Fig. 2): the *attP* region is particularly rich in 5-bp direct repeats r1 (AGAAA) and r2 (GAAAC), which are present at six and five copies, respectively. Two large direct repeats of 9 bp (r3) and three of 10 bp (r4) were also detected. A perfect inverted repeat (tr-1) spaced by 7 bp could play a role as a terminator of transcription. It could form a hairpin secondary structure (−30 kcal/mol [1 cal = 4.184 J]) followed by a poly(T)-rich sequence characteristic of a rho-independent terminator.

Characterization of the *attL*, *attR*, and *attB* sites and localization of the core region. To identify the host-phage DNA junctions, chromosomal DNAs of strains LT4 and LT4c (Table 1) were digested with restriction enzymes *Cla*I and *Sal*I and hybridized with two phage probes represented in Fig. 1, an *attP* probe and an *attL* probe. On the *Cla*I-digested LT4 DNA, two bands of 1.7 and 7.0 kb corresponding to the junction fragments were detected with the *attP* probe (Fig. 3A), in addition to the 4.4-kb *Cla*I phage fragment containing *attP*. When *Sal*I-digested LT4 DNA was probed, the 5.9-kb *Sal*I mv4 fragment bearing *attP* was also present in the LT4 pattern and junction fragments of 8.0 and 2.8 kb appeared. The presence of the phage fragments containing an entire *attP* site in the LT4 pattern is not surprising, since it has been shown that in mv4 lysogenic strains, a linear extrachromosomal form of mv4 DNA coexists with the prophage integrated form (20). On *Sal*I and *Cla*I LT4 digests, the *attL* probe hybridized only with the 2.8-kb *Sal*I and 7.0-kb *Cla*I junction fragments, respectively (data not shown), suggesting that they carried the *attL* host-phage DNA junction and that the *attR* site was located on the 1.7-kb *Cla*I and 8.0-kb *Sal*I fragments.

The junction fragments were amplified by using the inverse PCR strategy on the 1.7-kb *Cla*I and 2.8-kb *Sal*I fragments for *attR* and *attL*, respectively. The PCR products were cloned into plasmid pBS-KS and sequenced at the phage-host junction. The results are presented in Fig. 4. Two primers derived from the host nucleotide sequence from *attL* and *attR* were used to PCR amplify a 282-bp fragment of nonlysogenic strain LT4c DNA. This fragment, containing the *attB* bacterial attachment site, was cloned and further sequenced. Sequence comparison with *attL*, *attR*, and *attP* (Fig. 4) identified a 17-bp region (5′-GGACATGAGAGGAATTA-3′) common to the four nucleotide sequences and located 100 bp downstream of the *int* gene (Fig. 2). This 17-bp sequence corresponds to the core region where the strand exchange reaction takes place during the phage genome integration event.

The chromosomal *attB* site is located in a tRNA^{Ser} gene. A nucleic acid database search showed a strong similarity between a 288-bp segment present on the *attB* sequence of strain LT4c and an *L. delbrueckii* subsp. *bulgaricus* sequence containing the tRNA^{Ser} gene (UCG), positions 49 to 333 in reference 19. The 89-bp tRNA^{Ser} gene was perfectly conserved between both strains (100% identical nucleotides), and the 200 bp downstream of this gene was highly homologous (92% identical nucleotides). The core region previously determined overlapped the 3′ end of this gene (Fig. 4). These observations indicate that the insertion of phage mv4 DNA into the bacterial chromosome takes place in the tRNA^{Ser} gene. To confirm this result, digested LT4 and LT4c chromosomal DNAs were

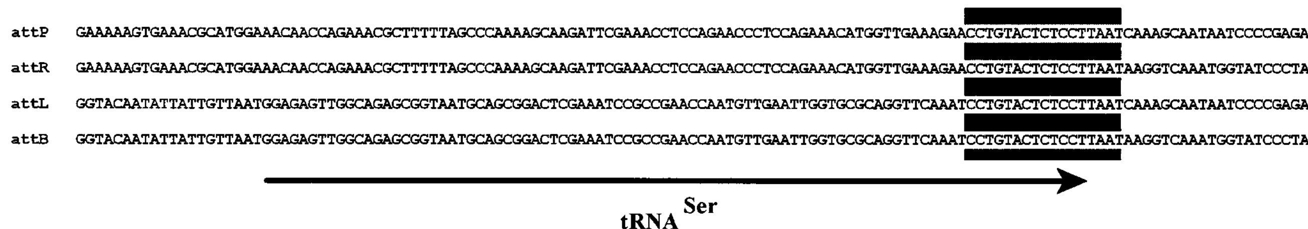

FIG. 4. Comparison of the DNA sequences of attachment sites. The shaded boxes indicate the 17-bp segment of identity among the four sequences, corresponding to the core region. The position of the tRNA^{Ser} gene is represented by the arrow below the *attB* sequence.

hybridized with the tRNA^{Ser} gene as a probe (Fig. 3B). A 6.5-kb *Sal*I fragment (*attB*) present in LT4c was replaced with a 2.8-kb fragment (*attL*) in LT4 chromosomal DNA after mv4 DNA integration, and a 4.8-kb *Cla*I fragment was replaced with the 7.0-kb fragment containing *attL*. As only 5 of 13 bp of the tRNA gene present in *attR* remained in the tRNA gene probe (PCR amplification with primers T1 and T2), the 8.0-kb *Sal*I and 1.7-kb *Cla*I fragments containing *attR* were not detected. These data demonstrate that mv4 DNA integration occurred specifically in the tRNA^{Ser} gene of strain LT4. The phage insertion generates on the left (*attL*) a functional hybrid tRNA gene (a 5′ bacterial sequence and a 3′ phage sequence) and on the right (*attR*) a 3′ part of the tRNA gene (core sequence) (see Fig. 6).

Another DNA sequence in the databases, encoding a minor tRNA^{Ser} (UCG) of *L. delbrueckii* (58), was also highly homologous to the *attB* sequence (95% identity in a 163-bp overlap). The homology extends over the tRNA gene and stops exactly at the 3′ end of the core region. Downstream of this point, the published sequence is almost identical to the right part of the mv4 *attP* region, starting at the 3′ end of the core region and covering ORFI1 (85.2% identity in a 285-bp overlap). This sequence seems to be an *attL* site, originating from an *L. delbrueckii* strain lysogenic for a temperate phage closely related to mv4.

Homology of the *int* gene product with site-specific recombinases. The location of the structural genes encoding the recombination proteins, immediately adjacent to the *attP* site, is a common feature of all site-specific recombination systems examined to date (29). Upstream of the *attP* site, a large ORF of 1,277 bp encoding a 427-amino-acid basic protein (molecular weight, 47,973; pI, 10.6) was found. This protein showed significant homology with the integrase family of proteins which promote site-specific integration of temperate bacteriophages (2). Although integrase proteins are poorly conserved at the amino acid level, their carboxy-terminal parts could be aligned in two domains (Fig. 5) which are involved in the recognition of specific DNA sequences. In the first domain, an arginine residue is conserved among all of the integrases (1). In the second domain, three perfect conserved amino acids residues, histidine, arginine, and tyrosine, were found (2). It has been suggested that the His residue could be available for hydrogen bonding and the Arg residue could be available for ionic interaction with the phosphate backbone of the target DNA (2, 39). The Tyr residue (position 342) of the λ Int protein was shown to be part of the active site of the integrase and to form a phosphodiester bond with DNA at the sites of strand exchange (38). These four perfectly conserved amino acids are present in the mv4 Int protein at the expected positions (Fig. 5), as are other residues highly conserved in the two domains. The greatest similarity, extending outside of the two C-terminal conserved domains, was found with the integrase of

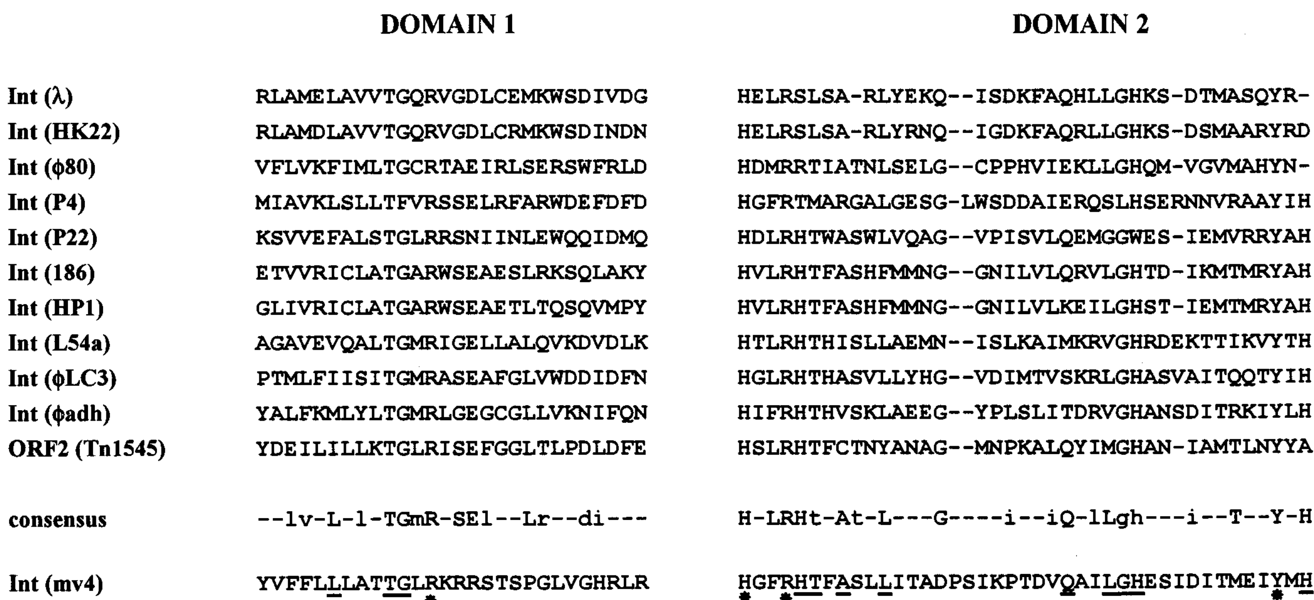

FIG. 5. Amino acid sequence comparison of the C-terminal parts of integrases of the Int family and the mv4 Int protein. The four invariant amino acids are indicated by an asterisk, and highly conserved amino acids are underlined in the mv4 Int sequence.

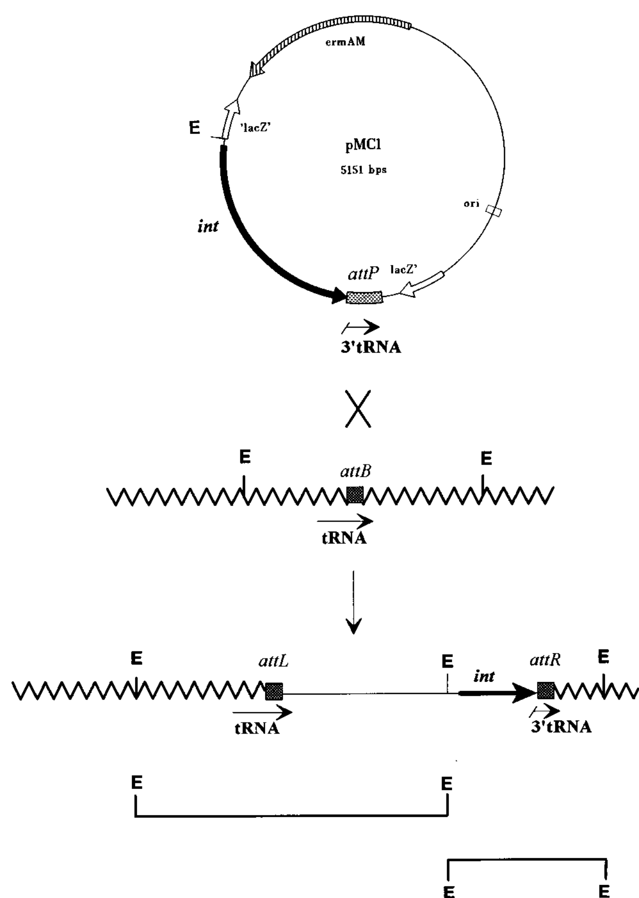

FIG. 6. Schematic representation of pMC1 site-specific integration into the tRNA^{Ser} gene of the *L. plantarum* LP80 chromosome. E, *Eco*RI.

lactococcal temperate phage φLC3 (30) and with the integrases of conjugative transposons Tn*1545* of *Streptococcus pneumoniae* (42) and Tn*916* of *Enterococcus faecalis*. With φLC3 Int (374 amino acids), 22.4% identical amino acids and 65.3% similar amino acids were found in a comparison with mv4 Int (427 amino acids) in a 326-amino-acid segment. No similarity was detected between the 50 N-terminal amino acids of φLC3 Int and the 120 N-terminal amino acids of mv4 Int. With the transposon integrases, 22.9% identical amino acids and 62.3% similar amino acids in a 284-amino-acid overlap were found. On the basis of these homologies, *int* might be the gene coding for the mv4 integrase.

Phage elements required for site-specific integration: construction of an integration vector. To confirm in vivo that the *int* gene product and the *attP* site are sufficient to mediate site-specific recombination, an integration vector was constructed. A 1.6-kb *Pvu*II-*Eco*RV fragment containing *int* and the *attP* site was cloned into pRC1 (Table 1), yielding plasmid pMC1 (Fig. 6). This plasmid is nonreplicative in gram-positive hosts and harbors an erythromycin marker (*ermAM*) expressed both in *E. coli* and in lactic acid bacteria. Plasmid pMC2 is derived from pMC1 and carries a deficient *int* gene because of the introduction of a 4-bp deletion at the *Bgl*I site (Fig. 2), leading to the synthesis of a truncated Int protein (deletion of the 131 C-terminal amino acids). As *L. delbrueckii* subsp. *bulgaricus* remains refractory to all of the techniques used for DNA transformation (35, 37), plasmids pMC1 and pMC2 were introduced in a transformable heterologous host, *L. plantarum* LP80. A transformation frequency of 10^4 transformants per μg of DNA was usually reached with replicative Em^r plasmid pO7 (unpublished data). With integration vector pMC1, 10^2 Em^r transformants per μg of DNA were obtained while no colonies were found with either control plasmid pRC1 or plasmid pMC2. Chromosomal DNA from Em^r transformants was digested with *Eco*RI and used for Southern blot experiments with pMC1 as the probe (Fig. 7A). No hybridization signal was detected with genomic DNA of strain LP80 (lane 1), while two fragments of 2.0 and 5.1 kb were detected in four independently isolated transformants (lanes 2 to 5). Plasmid pMC1 appeared to integrate into the host chromosome at a specific site. To demonstrate that integration occurred in a tRNA^{Ser} gene of *L. plantarum* LP80, hybridization experiments were realized with the *L. delbrueckii* subsp. *bulgaricus* tRNA^{Ser} gene as the probe (Fig. 7B). A homologous tRNA gene is located on a 2.0-kb *Eco*RI fragment of chromosomal DNA of strain LP80 (lane 1). This fragment disappeared in the transformants for a 5.1-kb junction chromosome-vector *Eco*RI fragment (lanes 2 to 5). These results show that the integration event in this heterologous *Lactobacillus* sp. occurred specifically in the tRNA^{Ser} gene, as depicted in Fig. 6, and that the *int* gene product is necessary for the integrative recombination process.

DISCUSSION

In this study, the nucleotide sequence and genetic organization of the mv4 region involved in the site-specific recombination process between the phage and host chromosome were determined. The *attP* region, 192 bp long, is located between two ORFs convergently transcribed towards this site. The *attP* site had different characteristics in common with other temperate phage *attP* sites. The significance of its high AT content (75%) is related to the fact that negatively supercoiled DNA, required as a substrate for integrative recombination, tends to partially denature in regions of high AT content and the local instability would facilitate the integration of phage DNA (54). As is usually described for other *attP* sites, the mv4 *attP* site is rich in direct and inverted repeats which could be the target sites for phage-encoded proteins such as integrase or excisionase or for host factors analogous to the *E. coli* integration host factor (15) or FIS protein (50). It is known that *attP* arms of λ and related phages contain several binding sites for the phage and host factors and exhibit highly ordered structures in the process of recombination (3, 4, 18, 28, 36, 47).

The nucleotide sequence of the mv4 integration region revealed an ORF encoding a polypeptide of 427 amino acids located adjacent to *attP* and convergently transcribed towards this site. Its location, size, and homology to site-specific recombinases of the integrase family strongly suggest that the ORF encodes the mv4 integrase. This was further confirmed by the fact that the presence of a functional *int* gene in addition to the *attP* region is necessary to promote site-specific integration.

The characterized site-specific integration systems comprise the integrase (*int*) and excisionase (*xis*) genes, located next to each other and transcribed convergently (φ80) or divergently (L54a) (29). By comparison to these systems, we proposed that ORFI3 might code for the mv4 excisionase. ORFI3 and the *int* gene are transcribed from the opposite strands and overlap by 75 bp (Fig. 2). The alignment of the L54a excisionase (56) and the small, basic ORFI3 protein (57 amino acids; 6,609 Da) indicated that 42% of their respective amino acid residues were similar (data not shown). Excision of phage mv4 seems to involve site-specific recombination between *attL* and *attR*, since after induction of the prophage by mitomycin C treatment, cured strain LT4c contained an intact tRNA gene. More

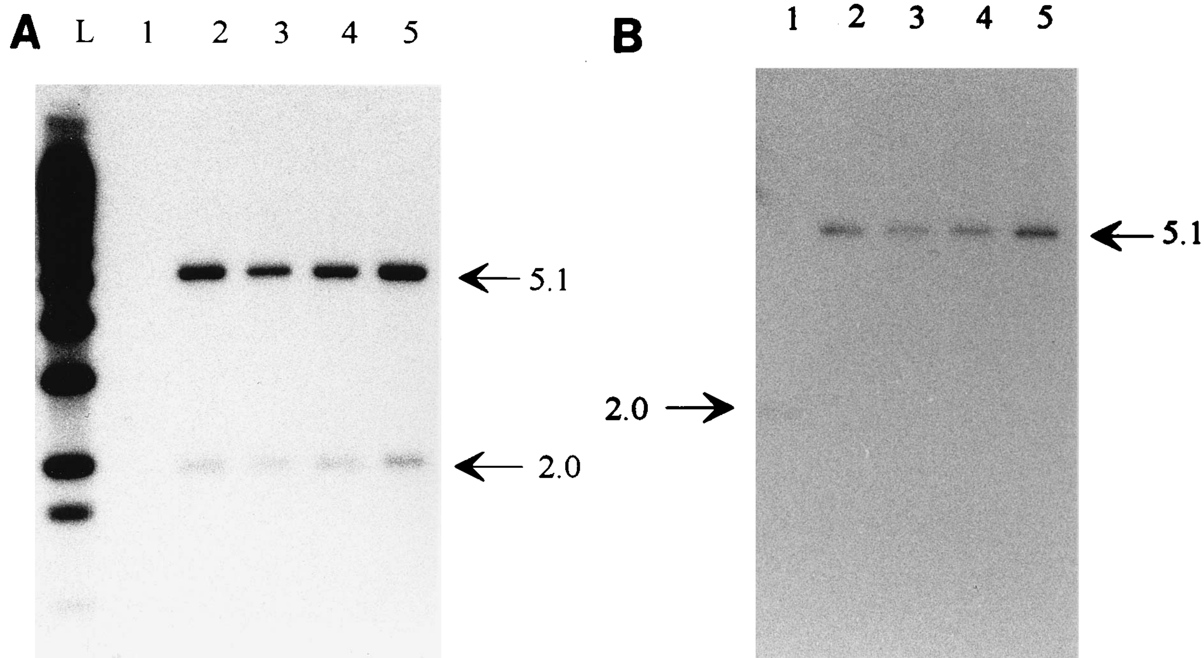

FIG. 7. Southern hybridization analysis of *L. plantarum* LP80 pMC1 integrants. Chromosomal DNAs from LP80 (lane 1) and four independently isolated pMC1 transformants (lanes 2 to 5) were digested with *Eco*RI and hybridized with pMC1 DNA (A) or with the tRNA^{Ser} gene (B). Restriction fragment sizes are in kilobases. L, 1-kb ladder (Bethesda Research Laboratories).

experiments are required to clearly demonstrate whether a phage-encoded excisionase is involved in mv4 excision or *int* alone is sufficient to stimulate excision.

By comparison of the nucleotide sequences of *attP*, *attL*, *attR*, and *attB*, a 17-bp common core sequence was identified. This core sequence does not show any homology to the core sequences of phages from lactic acid bacteria characterized to date (ϕLC3 9 bp, [30]; TP901-1 5 bp [12]; ϕadh 16 bp, [43]) or with that of other gram-positive or gram-negative bacteriophages.

The characterization of the *attB* site showed that mv4 DNA integrates into a tRNA^{Ser} gene. The 17-bp core sequence overlaps the 3′ end of this gene. Integration into a tRNA gene is a common occurrence in gram-negative phages (P22, P4, 186, HP1, 16-3, and ϕCTX) and in actinomycete integrative plasmids (SLP1, pSAM2, pMEA100, and pSE211). In contrast, except for mycobacteriophage L5, which integrates its DNA into a tRNA gene (26), phages of gram-positive bacteria integrate either into a gene coding for a protein (L54a [24], ϕ13 [14], ϕLC3 [30], or TP901-1 [12]) or into an intergenic region (ϕ11 [25]). The use of the 3′ ends of tRNA genes for the phage integration sites is a general phenomenon among various bacterial species (11, 44). One explanation for this target site preference is that integrase uses the symmetric sequence in the tRNA gene as a recognition site (11). For the elements inserting their DNAs into a tRNA gene, the length of the homology between *attB* and *attP* is usually large (average of 40 to 60 bp), from 20 bp for P4 (40) to 182 bp for HP1 (53). The mv4 17-bp core sequence is the shortest described to date, since the *attB*-*attP* homology begins in the T arm of the tDNA, as in P4 (20 bp) (40) and retronphage ϕR73 (29 bp) (49), while most core segments overlap the anticodon loop.

The (UCG) tRNA^{Ser} gene corresponds to a codon rarely used in *L. delbrueckii* (41). However, phage insertion into this gene is not lethal since it does not disrupt the gene because of the presence of a 5′ truncated tRNA gene (13 of 89 bp) in *attP*. The presence of a 3′ gene end in *attP* sites is common in genetic elements which insert into genes coding for a protein or more often into a tRNA gene.

After integration, the hybrid tRNA^{Ser} gene at the *attL* site is followed by a putative strong terminator structure tr-1 (Fig. 2) brought by the phage and present downstream of the *int* gene in the nonintegrated phage genome. The tr-1 structure might prevent bacterial transcription from entering the prophage sequence. At the *attR* site, integration spaced the *int* gene away from its putative terminator tr-1, as is the case for the λ *sib* site (17) or the P2 *int* terminator (57), and since no equivalent terminator exists downstream of the tRNA^{Ser} gene, the *int* transcript could proceed through the *attR* junction in the bacterial chromosome. As suggested for phage P2, mRNA with a long 3′ untranslated sequence might have a half-life shorter than that of the *int* transcript of the unintegrated phage DNA. Moreover, translation of ORFI2 might interfere with *int* initiation of translation since ORFI2 overlaps *int* by 164 nucleotides.

The highly conserved nucleotide sequence of the tRNA^{Ser} gene would provide efficient integration sites for mv4 DNA in nonisogenic strains of *L. delbrueckii* species and subspecies. Their sequence similarity among heterologous species might give phage mv4 the possibility to lysogenize a broad host range of bacteria. The conservation of the tRNA^{Ser} gene between *L. delbrueckii* subsp. *bulgaricus* and *L. plantarum* explains the finding that integration vector pMC1 is able to promote site-specific integration into the latter host. If this tRNA gene is conserved in other *Lactobacillus* spp. or more generally in lactic acid bacteria, it could be used as a target site to introduce genetic markers with biotechnological or medical potential. In other gram-positive species, such wide-spectrum integrative vectors have been constructed on the basis of the mycobacteriophage L5 (26, 48) or pSAM2 (31, 33) integrative function.

ACKNOWLEDGMENTS

We thank Antti Vasala for helpful suggestions.

This work was supported by grants from the Centre National de la Recherche Scientifique (UPR 9007), from the CEC BRIDGE program (BIOT-CT91-0263), and from the Région Midi-Pyrénées.

REFERENCES

1. **Abremski, K. E., and R. H. Hoess.** 1992. Evidence for a second conserved arginine residue in the integrase family of recombination proteins. Protein Eng. **5:**87–91.
2. **Argos, P., A. Landy, K. Abremski, J. B. Egan, E. Haggard-Ljungquist, R. H. Hoess, M. L. Kahn, B. Kalionis, S. V. L. Narayana, L. S. Pierson III, N. Sternberg, and J. M. Leong.** 1986. The integrase family of site-specific recombinases: regional similarities and global diversity. EMBO J. **5:**433–440.
3. **Ball, C. A., and R. C. Johnson.** 1991. Efficient excision of phage λ from the *Escherichia coli* chromosome requires the Fis protein. J. Bacteriol. **173:**4027–4031.
4. **Ball, C. A., and R. C. Johnson.** 1991. Multiple effects of Fis on integration and the control of lysogeny in phage λ. J. Bacteriol. **173:**4032–4038.
5. **Boccard, F., T. Smokvina, J.-L. Pernodet, A. Friedmann, and M. Guérineau.** 1989. The integrated conjugative plasmid pSAM2 of *Streptomyces ambofaciens* is related to temperate bacteriophages. EMBO J. **8:**973–980.
6. **Boizet, B., Y. Lahbib-Mansais, L. Dupont, P. Ritzenthaler, and M. Mata.** 1990. Cloning, expression and sequence analysis of an endolysin-encoding gene of *Lactobacillus bulgaricus* bacteriophage mv1. Gene **94:**61–67.
7. **Brasch, M. A., G. S. Pettis, S. C. Lee, and S. N. Cohen.** 1993. Localization and nucleotide sequences of genes mediating site-specific recombination of the SLP1 element in *Streptomyces lividans*. J. Bacteriol. **175:**3067–3074.
8. **Brown, D. P., K. B. Idler, D. M. Backer, S. Donadio, and L. Katz.** 1994. Characterization of the genes and attachment sites for site-specific integration of plasmid pSE101 in *Saccharopolyspora erythraea* and *Streptomyces lividans*. Mol. Gen. Genet. **242:**185–193.
9. **Brown, D. P., K. B. Idler, and L. Katz.** 1990. Characterization of the genetic elements required for site-specific integration of plasmid pSE211 in *Saccharopolyspora erythraea*. J. Bacteriol. **172:**1877–1888.
10. **Campbell, A.** 1962. Episomes. Adv. Genet. **11:**101–145.
11. **Campbell, A. M.** 1992. Chromosomal insertion sites for phages and plasmids. J. Bacteriol. **174:**7495–7499.
12. **Christiansen, B., M. G. Johnsen, E. Stenby, F. K. Vogensen, and K. Hammer.** 1994. Characterization of the lactococcal temperate phage TP901-1 and its site-specific integration. J. Bacteriol. **176:**1069–1076.
13. **Cluzel, P.-J., M. Vaux, M. Rousseau, and J.-P. Accolas.** 1987. Evidence for temperate bacteriophages in two strains of *Lactobacillus bulgaricus*. J. Dairy Res. **54:**397–405.
14. **Coleman, C., J. Knights, R. Russel, D. Shanley, T. H. Birkbeck, G. Dougan, and I. Charles.** 1991. Insertional inactivation of the *Staphylococcus aureus* β-toxin by bacteriophage phi-13 occurs by site- and orientation-specific integration of the phi-13 genome. Mol. Microbiol. **5:**933–939.
15. **Craig, N. L., and H. A. Nash.** 1984. *E. coli* integration host factor binds to specific sites in DNA. Cell **39:**707–716.
16. **De Man, J. C., M. Rogosa, and M. E. Sharpe.** 1960. A medium for cultivation of lactobacilli. J. Appl. Bacteriol. **23:**130–135.
17. **Echols, H., and G. Guarneros.** 1983. Control of integration and excision, p. 75–92. *In* R. W. Hendrix, J. W. Roberts, F. W. Stahl, and R. A. Weisberg (ed.), Lambda II. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Han, Y. W., R. I. Gumport, and J. F. Gardner.** 1993. Complementation of bacteriophage lambda integrase mutants: evidence for an intersubunit active site. EMBO J. **12:**4577–4584.
19. **Hottinger, H., T. Ohgi, M. C. Zwahlen, S. Dhamija, and D. Söll.** 1987. Allele-specific complementation of an *Escherichia coli leuB* mutation by a *Lactobacillus bulgaricus* tRNA gene. Gene **60:**75–83.
20. **Lahbib-Mansais, Y., B. Boizet, L. Dupont, M. Mata, and P. Ritzenthaler.** 1992. Characterization of a temperate bacteriophage of *Lactobacillus delbrueckii* subsp. *bulgaricus* and its interactions with the host cell chromosome. J. Gen. Microbiol. **138:**1139–1146.
21. **Lahbib-Mansais, Y., M. Mata, and P. Ritzenthaler.** 1988. Molecular taxonomy of *Lactobacillus* phages. Biochimie **70:**429–435.
22. **Landy, A.** 1989. Dynamic, structural, and regulatory aspects of lambda site-specific recombination. Annu. Rev. Biochem. **58:**913–949.
23. **Le Bourgeois, P., M. Lautier, M. Mata, and P. Ritzenthaler.** 1992. New tools for the physical and genetic mapping of *Lactococcus* strains. Gene **111:**109–114.
24. **Lee, C. Y., and J. J. Iandolo.** 1986. Integration of staphylococcal phage L54a occurs by site-specific recombination: structural analysis of the attachment sites. Proc. Natl. Acad. Sci. USA **83:**5474–5478.
25. **Lee, C. Y., and J. J. Iandolo.** 1988. Structural analysis of staphylococcal bacteriophage φ11 attachment sites. J. Bacteriol. **170:**2409–2411.
26. **Lee, M. H., L. Pascopella, W. R. Jacobs, and G. H. Hatfull.** 1991. Site-specific integration of mycobacteriophage L5: integration-proficient vectors for *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, and bacille Calmette-Guérin. Proc. Natl. Acad. Sci. USA **88:**3111–3115.
27. **Leer, R. J., N. Van Luijk, M. Posno, and P. H. Pouwels.** 1992. Structural and functional analysis of two cryptic plasmids from *Lactobacillus pentosus* MD1363 and *Lactobacillus plantarum* ATCC 8014. Mol. Gen. Genet. **234:**265–274.
28. **Leong, J. M., S. Nunes-Düby, C. F. Lesser, P. Youderian, M. M. Susskind, and A. Landy.** 1985. The phi-80 and P22 attachment sites. J. Biol. Chem. **260:**4468–4477.
29. **Leong, J. M., S. E. Nunes-Düby, A. B. Oser, C. F. Lesser, P. Youderian, M. M. Susskind, and A. Landy.** 1986. Structural and regulatory divergence among site-specific recombination genes of lambdoid phages. J. Mol. Biol. **189:**603–616.
30. **Lillehaug, D., and N.-K. Birkeland.** 1993. Characterization of genetic elements required for site-specific integration of the temperate lactococcal bacteriophage φLC3 and construction of integration-negative φLC3 mutants. J. Bacteriol. **175:**1745–1755.
31. **Martin, C., P. Mazodier, M. V. Mediola, B. Gicquel, T. Smokvina, C. J. Thompson, and J. Davies.** 1991. Site-specific integration of the *Streptomyces* plasmid pSAM2 in *Mycobacterium smegmatis*. Mol. Microbiol. **249:**2499–2502.
32. **Mata, M., A. Trautwetter, G. Luthaud, and P. Ritzenthaler.** 1986. Thirteen virulent and temperate bacteriophages of *Lactobacillus bulgaricus* and *Lactobacillus lactis* belong to a single DNA homology group. Appl. Environ. Microbiol. **52:**812–818.
33. **Mazodier, P., C. J. Thompson, and F. Boccard.** 1990. The chromosomal integration site of the *Streptomyces* element pSAM2 overlaps a putative tRNA gene conserved among actinomycetes. Mol. Microbiol. **22:**431–434.
34. **Mead, D. A., E. Szczesna-Skorupa, and B. Kemper.** 1986. Single-stranded DNA "blue" T7 promoter plasmids: a versatile tandem promoter system for cloning and protein engineering. Protein Eng. **1:**67–74.
35. **Mercenier, A., P. H. Pouwels, and B. M. Chassy.** 1994. Genetic engineering of lactobacilli, leuconostocs and *Streptococcus thermophilus*, p. 252–293. *In* M. J. Gasson and W. M. de Vos (ed.), Genetics and biotechnology of lactic acid bacteria. Blackie Academic & Professional, Glasgow, Scotland.
36. **Moitoso de Vargas, L., C. A. Pargellis, N. M. Hasan, E. W. Bushman, and A. Landy.** 1988. Autonomous DNA binding domains of lambda integrase recognize two different sequence families. Cell **54:**923–929.
37. **Murphy, C. M., B. Boizet, J. L. Flickinger, and B. M. Chassy.** 1993. Studies on the transformation of *Lactobacillus delbrueckii* subsp. *bulgaricus*. FEMS Microbiol. Rev. **12:**P9.
38. **Pargellis, C. A., S. E. Nunes-Duby, L. Moitoso de Vargas, and A. Landy.** 1988. Suicide recombination substrates yield covalent lambda Int-DNA complexes and lead to the identification of the active site tyrosine. J. Biol. Chem. **263:**7678–7685.
39. **Parsons, R. L., B. R. Evans, L. Zheng, and M. Jayaram.** 1990. Functional analysis of Arg-308 mutants of Flp recombinase. Possible role of Arg-308 in coupling substrate binding to catalysis. J. Biol. Chem. **265:**4527–4553.
40. **Pierson, L. S., III, and M. L. Kahn.** 1987. Integration of satellite bacteriophage P4 in *Escherichia coli*. J. Mol. Biol. **196:**487–496.
41. **Pouwels, P. H., and J. A. M. Leunissen.** 1994. Divergence in codon usage of *Lactobacillus* species. Nucleic Acids Res. **22:**929–936.
42. **Poyard-Salmeron, C., P. Trieu-Cuot, C. Carlier, and P. Courvalin.** 1989. Molecular characterization of two proteins involved in the excision of the conjugative transposon Tn*1545*: homologies with other site-specific recombinases. EMBO J. **8:**2425–2433.
43. **Raya, R. R., C. Fremaux, G. L. De Antoni, and T. R. Klaenhammer.** 1992. Site-specific integration of the temperate bacteriophage φadh into the *Lactobacillus gasseri* chromosome and molecular characterization of the phage (*attP*) and bacterial (*attB*) attachment sites. J. Bacteriol. **174:**5584–5592.
44. **Reiter, W.-D., P. Palm, and S. Yeats.** 1989. Transfer RNA genes frequently serve as integration sites for prokaryotic genetic elements. Nucleic Acids Res. **17:**1907–1914.
45. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
46. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
47. **Segall, A. M., and H. A. Nash.** 1993. Synaptic intermediates in bacteriophage lambda site-specific recombination: integrase can align pairs of attachment sites. EMBO J. **12:**4567–4576.
48. **Stover, C. K., V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennet, G. P. Bansal, J. F. Young, M. H. Lee, G. F. Hatfull, S. Snapper, R. G. Barletta, W. R. Jacobs, Jr., and B. R. Bloom.** 1991. New use of BCG for recombinant vaccines. Nature (London) **351:**456–460.
49. **Sun, J., M. Inouye, and S. Inouye.** 1991. Association of a retroelement with a P4-like cryptic prophage (retronphage φR73) integrated into the selenocystyl tRNA gene of *Escherichia coli*. J. Bacteriol. **173:**4171–4181.
50. **Thompson, J. F., L. Moitoso de Vargas, C. Koch, R. Kahmann, and A. Landy.** 1987. Cellular factors couple recombination with growth phase: characterization of a new component in the lambda site-specific recombination pathway. Cell **50:**901–908.
51. **Van de Guchte, M., C. Daly, G. F. Fitzgerald, and E. K. Arendt.** 1994. Identification of *int* and *attP* on the genome of lactococcal bacteriophage

Tuc2009 and their use for site-specific plasmid integration in the chromosome of Tuc2009-resistant *Lactococcus lactis* MG1363. Appl. Environ. Microbiol. **60:**2324–2329.

52. **Vasala, A., L. Dupont, M. Baumann, P. Ritzenthaler, and T. Alatossava.** 1993. Molecular comparison of the structural proteins encoding gene clusters of two related *Lactobacillus delbrueckii* bacteriophages. J. Virol. **67:** 3061–3068.
53. **Waldman, A. S., S. D. Goodman, and J. J. Scocca.** 1987. Nucleotide sequences and properties of the sites involved in lysogenic insertion of the bacteriophage HP1*c*1 genome into the *Haemophilus influenzae* chromosome. J. Bacteriol. **169:**238–246.
54. **Weisberg, R. A., and A. Landy.** 1983. Site-specific recombination in phage lambda, p. 211–249. *In* R. W. Hendrix, J. W. Roberts, F. W. Stahl, and R. A. Weisberg (ed.), Lambda II. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
55. **Ye, Z.-H., S. L. Buranen, and C. Y. Lee.** 1990. Sequence analysis and comparison of *int* and *xis* genes from staphylococcal bacteriophages L54a and φ11. J. Bacteriol. **172:**2568–2575.
56. **Ye, Z.-H., and C. Y. Lee.** 1989. Nucleotide sequence and genetic characterization of staphylococcal bacteriophage L54a *int* and *xis* genes. J. Bacteriol. **171:**4146–4153.
57. **Yu, A., L. E. Bertani, and E. Haggard-Ljungquist.** 1989. Control of prophage integration and excision in bacteriophage P2: nucleotide sequences of the *int* gene and *att* sites. Gene **80:**1–12.
58. **Zwahlen, M. C., and H. Hottinger.** 1989. Nucleotide sequence of a *Lactobacillus delbrueckii* gene encoding a minor (UCG) $tRNA^{Ser}$. Nucleic Acids Res. **17:**1772.