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Isolation of a Novel IS*3* Group Insertion Element and Construction of an Integration Vector for *Lactobacillus* spp.†

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An insertion sequence (IS) element from *Lactobacillus johnsonii* was isolated, characterized, and exploited to construct an IS-based integration vector. *L. johnsonii* NCK61, a high-frequency conjugal donor of bacteriocin production (Laf$^+$) and immunity (Laf$^-$), was transformed to erythromycin resistance (Em^r) with the shuttle vector pSA3. The NCK61 conjugative functions were used to mobilize pSA3 into a Laf$^-$ Laf^s Em^s recipient. DNA from the Em^r transconjugants transformed into *Escherichia coli* MC1061 yielded a resolution plasmid with the same size as that of pSA3 with a 1.5-kb insertion. The gram-positive replication region of the resolution plasmid was removed to generate a pSA3-based suicide vector (pTRK327) bearing the 1.5-kb insert of *Lactobacillus* origin. Plasmid pTRK327 inserted randomly into the chromosomes of both *Lactobacillus gasseri* ATCC 33323 and VPI 11759. No homology was detected between plasmid and total host DNAs, suggesting a Rec-independent insertion. The DNA sequence of the 1.5-kb region revealed the characteristics of an IS element (designated IS*1223*): a length of 1,492 bp; flanking, 25-bp, imperfect inverted repeats; and two overlapping open reading frames (ORFs). Sequence comparisons revealed 71.1% similarity, including 35.7% identity, between the deduced ORFB protein of the *E. coli* IS element IS*150* and the putative ORFB protein encoded by the *Lactobacillus* IS element. A putative frameshift site was detected between the overlapping ORFs of the *Lactobacillus* IS element. It is proposed that, similar to IS*150*, IS*1223* produces an active transposase via translational frameshifting between two tandem, overlapping ORFs.

The genus *Lactobacillus* is one of the largest groups of organisms used in fermentation processes worldwide and is thus of great economic importance. Lactobacilli also constitute a large portion of our intestinal microflora (52). Their purported association with the general state of our health is under active investigation. The application of genetic technologies to characterize and potentially manipulate lactobacilli remains highly significant on both economic and medical grounds.

Little information is available about the content and organization of the *Lactobacillus* genome. Genetic characterization and manipulation of the lactobacilli await development of effective gene transfer strategies and construction of suitable vehicles for insertional mutagenesis and stable integration of genes. Integration vectors based upon homologous recombination have been used for both the delivery of heterologous DNA (47) and insertional inactivation (3, 27) in a *Lactobacillus* background. Site-specific integration has been demonstrated in *Lactobacillus gasseri* with a vector bearing an *attP* site derived from a temperate bacteriophage (40). While both systems have proven effective, the integrative potentials of the homology-based vectors are limited to previously cloned regions, while that of the *attP*-based vector is limited to the availability of *attB* sites. An alternative integration vector, one based upon the transposition functions of an insertion sequence (IS) element, would provide an extended functional range. As the basis for constructing a random integration vector, we attempted to isolate an insertion element from *Lactobacillus johnsonii*, an intestinal bacterium.

Bacterial ISs are small (between 800 and 2,500 bp), compact genetic structures that are flanked by inverted repeats and generally encode their own transposition functions (14). ISs are widely distributed in both the chromosome and plasmid complement of lactic acid bacteria. Three families of IS elements in lactococci have been defined (37), and their copy numbers, host ranges, and positions have been shown to vary widely among strains (46). Until recently, only one insertion element had been isolated from a *Lactobacillus* background. ISL*1* was detected in a virulent phage derived from the temperate bacteriophage ϕFSW of *Lactobacillus casei* (54). Screening of 48 bacterial strains, including 31 lactobacilli, located ISL*1*-like sequences in only three *L. casei* subsp. *casei* strains (53). Underscoring a possible role in *Lactobacillus* genome plasticity, two IS elements were recently identified as factors in *lacZ* instability. ISL*2* was isolated as an insertion in spontaneous lactose-negative mutants of *Lactobacillus helveticus* (66), while ISL*3* was isolated from a deletion-prone region following the *lacZ* gene in *Lactobacillus bulgaricus* (16).

A variety of prokaryotic insertion elements have been isolated by different strategies (15, 30, 33, 49, 55). One method of retrieving IS elements which has proved fruitful in a gram-positive background is based upon the ability of IS elements to mediate cointegrate formation with plasmids that can be conjugally mobilized and then recovered from transconjugants as resolution products (18, 32, 38, 41, 56). A similar strategy was adopted in this study to trap an IS element from *L. johnsonii*. Conjugal transfer of genetic determinants encoding production and immunity to the bacteriocin lactacin F occurs in *L. johnsonii* VPI 11088 and has been associated with a 110-kb unstable plasmid (31). This plasmid exhibits the characteristics of an episomal element, since it reintegrates into the recipient's chromosome following conjugal transfer. This conjugation system was exploited to mobilize plasmid pSA3 (7). A new *Lactobacillus* insertion element, IS*1223*, was discovered in pSA3 resolution products recovered from

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant characteristics[a]	Source[b] or reference
L. johnsonii		
NCK88	VPI 11088, Laf$^+$ Laf^r, parental strain, pPM4, pPM27	VPI (31)
NCK89	VPI 11088 derivative, Laf$^-$ Laf^s *str-6 rif-7*, pPM4, pPM27, conjugal recipient	31
NCK61	VPI 11088 derivative (unstable transconjugant), Laf$^+$ Laf^r *spc-8 gen-9*, pPM4, pPM27, pPM68, conjugal donor	31
NCK676	Transconjugant resulting from mating between NCK61 containing pSA3 and NCK89, *str-6 rif-7* Em^r	This study
L. gasseri		
ATCC 33323	Neotype, DSM 20243 (Gasser, 63 AM)	ATCC (21, 24)
VPI 11759		VPI (21)
E. coli		
JM110	*rpsL* (Str^r) *thr leu thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ(lac-proAB) [F′ *traD36 proAB lacI^q*ZΔM15]	S
MC1061	*araD139* Δ(*ara-leu*)*7696* Δ(*lac*)*174 galU galK hsdR2* (r_k^- m_k^-) *mcrB1 rpsL* (Str^r)	S, 20
NCK662	JM110, pTRK327, Em^r, Cm^r, Tc^r	This study
DH5α	F′ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17*(r_K^- m_K^+) *deoR thi-1 supE44* λ*gyrA96 relA1*	BRL
NCK547	DH5α, pTRK327, Em^r Cm^r Tc^r in *E. coli*, Em^r in *Lactobacillus* species provided by pTRK327	This study
pBSKS+	pBluescript II KS$^+$; 2.9 kb; *lacZ* Ap^r	S
pSA3	10.2 kb; Em^r Cm^r Tc^r	7
pSA34	6.0 kb; Em^r Cm^r Tc^r, suicide vector for gram-positive organisms	44
pTRK371	11.7 kb; Em^r Cm^r Tc^r (pSA3::IS*1223*)	This study
pTRK327	8.2 kb; Em^r integration vector for lactobacilli; Em^r Cm^r Tc^r, and plasmid replication in *E. coli*	This study

[a] Laf$^+$, lactacin F producer; Laf^r, lactacin F resistant; Laf$^-$, lactacin F negative; Laf^s, lactacin F sensitive; *spc-8*, gene encoding spectinomycin resistance; *gen-9*, gene encoding gentamycin resistance; Em^r, erythromycin resistant; Cm^r, chloramphenicol resistant; Tc^r, tetracycline resistant.
[b] VPI, Virginia Polytechnic Institute; ATCC, American Type Culture Collection; S, Stratagene; BRL, Bethesda Research Laboratories.

transconjugants. The IS element was sequenced, characterized, and used in the construction of a suicide vector for lactobacilli.

(The preliminary results of this study were reported at the 93rd General Meeting of the American Society for Microbiology [62].)

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are presented in Table 1. Cultures were maintained as frozen stocks at −70°C in growth media containing glycerol at a final concentration of 10% (vol/vol). *Lactobacillus* cultures were propagated under static conditions in MRS broth (Difco Laboratories, Detroit, Mich.) or anaerobically on MRS agar (1.5%) at 37°C. For selection of lactobacilli, antibiotics were added at the following concentrations: erythromycin, 7.5 μg/ml; rifamycin SV, 100 μg/ml; and streptomycin, 2,000 μg/ml. *Escherichia coli* cultures were propagated at 37°C aerobically on Luria-Bertani (LB) agar or in LB broth shaken at 270 rpm. Antibiotics were added at the following concentrations: erythromycin, 200 μg/ml; chloramphenicol, 15 μg/ml; tetracycline, 10 μg/ml; and ampicillin, 50 μg/ml.

Plasmid DNA isolation and characterization. Alkaline lysis of *Lactobacillus* strains was performed essentially as described by Muriana and Klaenhammer (31) with the following modifications by C. Ahn (1): prior to lysozyme treatment, cultures grown overnight were diluted 1:5 in fresh MRS broth and incubated at 37°C for 2 h, the pH of the lysis solution was adjusted to 12.4 with 3 N NaOH just prior to addition to the cell pellet, and prior to the addition of phenol, a salt solution of 3 M potassium acetate and 1.8 M formic acid was added to lysed cells at a ratio of 1:3, respectively. Rapid isolation of *E. coli* plasmid DNA was accomplished by the protocol of Sambrook et al. (43) with the following modifications: after lysis, phenol-chloroform extraction was eliminated, the cell debris was removed by centrifugation, and the DNA was then promptly precipitated with 0.7% isopropyl alcohol. Large-scale isolation of *E. coli* plasmid DNA was obtained by using the QIAGEN plasmid kit (QIAGEN, Inc., Chatsworth, Calif.) following the manufacturer's instructions. QIAGEN-isolated DNA was of sufficient purity to use in most sequencing reactions. In some cases, plasmid DNA for sequencing was obtained by alkaline lysis and precipitated in 7.0% polyethylene glycol (PEG 8000)–0.5 M NaCl. All DNA-modifying enzymes and restriction endonucleases were used, following the individual manufacturer's instructions. All plasmid DNA for cloning was first purified in SeaKem GTG agarose (FMC BioProducts, Rockland, Maine) and extracted with glass milk, following the GeneClean II kit instructions (Bio 101 Inc., La Jolla, Calif.).

Electroporation and conjugation. Electrocompetent *E. coli* cells were prepared by the method of Dower et al. (12) and electroporated by using the Gene Pulser unit (Bio-Rad Laboratories, Richmond, Calif.). A 40-μl aliquot of cell-DNA mixture was electroporated in a 0.2-cm-wide cuvette (Bio-Rad) at 2.45 kV, 25-μF capacitance, and 200-Ω resistance. Following electroporation, the cells were resuspended in SOC medium (43) and incubated with shaking at 37°C for 1 h prior to spreading on selective media. Electrocompetent *Lactobacillus* cells were prepared by using 3.5× SMEB buffer according to the procedure of Luchansky et al. (28). An optimized transformation protocol of Holo and Nes (19) was adapted for use with *L. johnsonii* VPI 11088, which was poorly transformed by the basic protocol. The changes to the basic protocol included the following: cells were pregrown in MRS broth supple-

mented with 2.0% glycine and 0.5 M sucrose, 10% glycerol was added to the electroporation buffer, and the outgrowth medium consisted of MRS broth containing 0.5 M sucrose, 20 mM $MgCl_2$, and 2.0 mM $CaCl_2$. Conjugation experiments were conducted using the method of Muriana and Klaenhammer (31) with the following modifications: when the optical density at 590 nm reached 0.5 to 0.6, the cells were rinsed twice with MRS broth (pH 5.5) and resuspended in the same. Solid-surface matings were conducted by transferring these cells to MRS agar (pH 5.5) and incubating at 37°C for 18 h. Mating cell mixtures were resuspended in MRS broth (pH 6.8) with a glass hockey stick and plated for transconjugant recovery on MRS agar (pH 6.8) containing 7.5 μg of erythromycin per ml, 2,000 μg of streptomycin per ml, and 100 μg of rifamycin SV per ml.

Integration analysis by pulsed-field gel electrophoresis and Southern hybridization. *Lactobacillus* genomic DNA was isolated in situ in SeaPlaque agarose (FMC Bioproducts) after the method of Tanskanen et al. (58) with the following modifications: the starting cell suspension was concentrated 10-fold in cell wash buffer instead of fivefold, *N*-lauryl sarcosine was omitted from the lysis buffer, and *N*-acetylmuramidase SG (ICN Biochemicals, Lisle, Ill.) was added at 40 U/ml. Multiple agarose-DNA slices (0.5 to 1.0 mm thick) were incubated at ambient temperature for 16 h in 200-μl aliquots of restriction enzyme solution containing 30 U of *Sma*I. The digested total DNA was separated in a 1.1% agarose gel (Agarose-NA; Pharmacia-LKB, Piscataway, N.J.) in 0.5× TBE (Tris-borate-EDTA) by using a 2015 Pulsaphor electrophoresis unit (Pharmacia-LKB) with the hexagonal electrode array. The electrophoresis conditions were 200 V, constant with 1 to 20 s switching time over 22 h at 11°C. Ethidium bromide-stained agarose gels were photographed with a Polaroid MP-3 Land camera and Polaroid High Speed 57 film.

For Southern hybridization, the stained agarose gels were first treated with one UV-autocrosslinking cycle by using the UV Stratalinker 1800 (Stratagene). DNA was transferred to either MagnaGraph or MagnaCharge nylon membrane (MSI, Westboro, Mass.) for 18 h by the method of Southern (57). Following transfer, the DNA was UV-crosslinked to the nylon by one autocrosslinking cycle. Vector integration was analyzed by probing the filters with either pSA3 or pTRK327 digested with *Nru*I. An internal *Hin*dIII-*Eco*RV fragment from the *Lactobacillus* insertion was used to probe the filters for homology specific to the IS element. Hybridizations were carried out in Kapak/Scotchpak heat-sealable pouches (Kapak Corp., Minneapolis, Minn.) in a 68°C water bath. The probes were labeled with digoxigenin, and the membranes were developed by using the Genius kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) following the manufacturer's instructions.

Sequencing and analysis. In order to sequence the *Lactobacillus* insertion element, a set of overlapping subclones was constructed in pBluescript II $KS^{+/-}$ (Stratagene) by utilizing both internal and external restriction sites. Double-stranded nucleic acid sequencing was performed on both strands with [^{35}S]dATP (New England Nuclear, Boston, Mass.) by the dideoxy-chain termination method of Sanger et al. (45), using Sequenase version 2.0 and the 7-deaza-dGTP kit (United States Biochemical, Cleveland, Ohio). The pBluescript II KS-specific primers T3, T7, KS, and SK (Stratagene) were used as well as seven insertion-specific, synthesized 17-mer oligonucleotide primers (Bio-Synthesis, Inc., Lewisville, Tex.). The sequence was assembled and analyzed with the PC/GENE software (IntelliGenetics, Inc., Mountain View, Calif.).

Nucleotide sequence accession number. The DNA sequence information presented in this report has been deposited in GenBank under the accession number U09558.

RESULTS

Conjugal mobilization and recovery of pSA3. *L. johnsonii* NCK61 (Laf^+ Laf^r) was transformed by electroporation with shuttle vector pSA3. Ten Em^r transformants were isolated and used individually as conjugal donors in subsequent mating experiments with *L. johnsonii* NCK89 (Laf^- Laf^s Em^s Str^r Rif^r). Em^r transconjugants were recovered at a low frequency (4.9×10^{-8} per input donor) from only 1 of the 10 donor-recipient mating pairs. Analysis of Em^r transconjugants did not reveal the acquisition of any new plasmids (data not shown). Miniprep DNA was isolated from 10 transconjugants and transformed separately into *E. coli* MC1061 (20). Em^r *E. coli* transformants were recovered at various frequencies, but most (28 of 36) were generated from one transconjugant (NCK676). All transformants were recovered and analyzed for plasmids. Of all 36 transformants recovered, 31 contained plasmids that were larger than pSA3, each bearing a 1.5-kb insertion.

Transposition functionality of the insert DNA. Restriction enzyme analysis was used to map the *Lactobacillus* DNA insert in one pSA3 resolution product, designated pTRK371. The insertion occurred between the *Ava*I and *Hin*dIII sites that closely follow the tetracycline resistance determinant (Fig. 1). The size of the insert (1.5 kb) and the manner of its isolation suggested the presence of an IS element. To examine this possibility, the pSA3 derivative was converted to a functional suicide vector by excision of the gram-positive replication region. This was accomplished through several rounds of partial *Hin*dIII digestion and religation, resulting in pTRK327 (Fig. 1).

The ability of the *Lactobacillus* insertion in pTRK327 to mediate integration was then evaluated in *L. johnsonii* VPI 11088, *L. gasseri* ATCC 33323, and *L. gasseri* VPI 11759. By using pTRK327, Em^r transformants were detected at frequencies of 21, 9.0, and 4.0/μg of plasmid DNA, respectively. In comparison, the transformation frequencies for the parent vector pSA3 were higher in all backgrounds (3.3×10^2, 2.4×10^4, and 5.1×10^3, respectively), while no Em^r transformants were detected in any strain following electroporation with pSA34, which is deficient in the gram-positive replication region (44) and lacks the *Lactobacillus* insert fragment. The relatively low pTRK327 transformation frequency suggested chromosomal integration, since the vector cannot replicate in a gram-positive background.

Total genomic DNAs from numerous Em^r transformants of *L. johnsonii* VPI 11088, *L. gasseri* ATCC 33323, and *L. gasseri* VPI 11759 were isolated, digested with *Sma*I, and separated by pulsed-field gel electrophoresis to generate a characteristic fingerprint. Integration events were detected visually by both shifts in the mobilities of restriction fragments and homology to vector (pSA3) or IS-specific (internal *Hin*dIII-*Eco*RV fragment of pTRK327) probes. In VPI 11088 transformed by pTRK327, all pTRK327 insertions occurred in four specific *Sma*I fragments with homology to the IS-specific probe (Fig. 2). These results demonstrated that sequences homologous to the insert DNA were distributed at four locations in the *L. johnsonii* genome. Integration of pTRK327 in strain VPI 11088 is likely occurring as a result of homologous recombination with these resident sequences. Restriction digests of pTRK327-transformed ATCC 33323 and VPI 11759 strains generated recognizable fingerprints composed of at least 18 fragments. Southern transfer and hybridization with pSA3 demonstrated that pTRK327 integrated randomly over the various *Sma*I fragments of both strains (Fig. 3). No homology was detected between the *Lactobacillus* insert DNA and either host chromosome, excluding the possibility of Rec-dependent homolo-

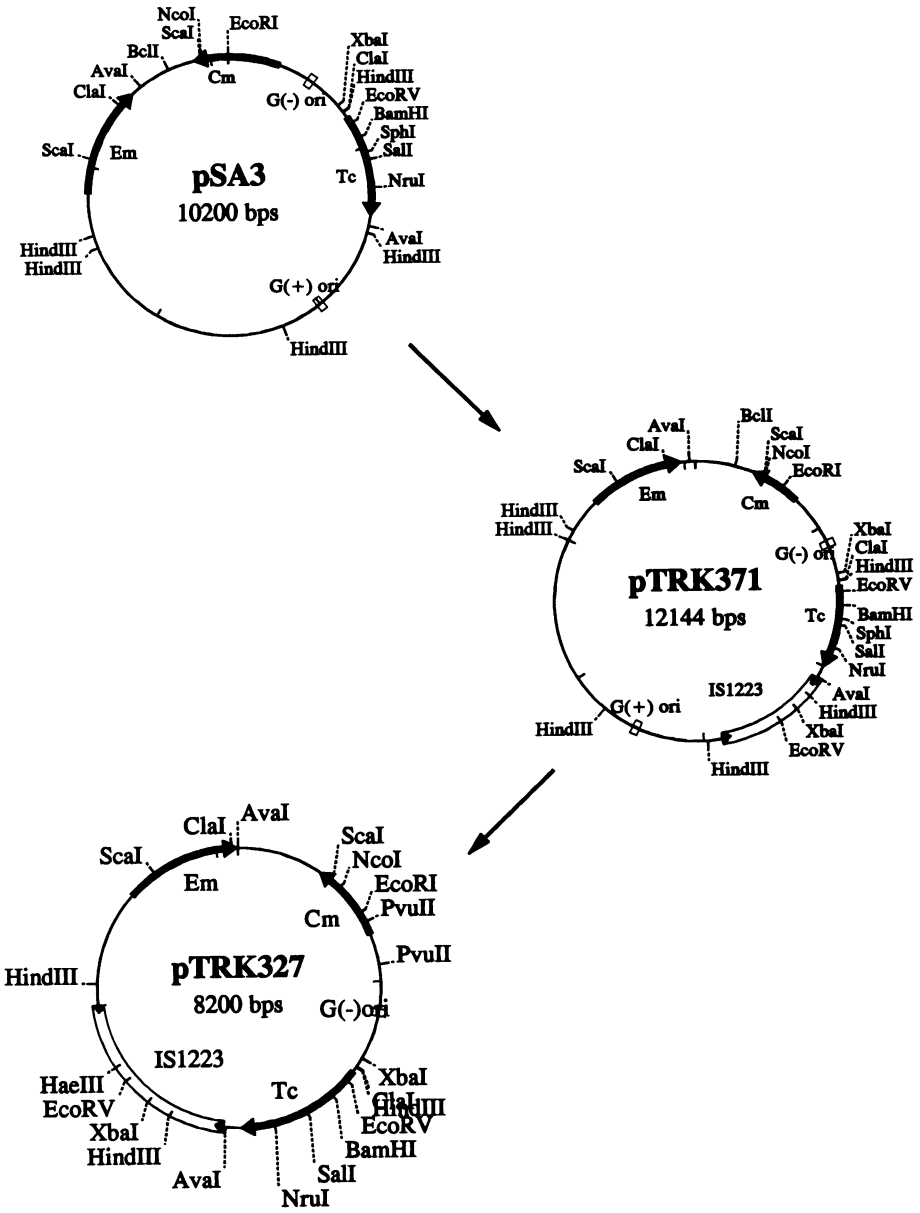

FIG. 1. Location of IS*1223* in pSA3 and construction of *Lactobacillus* integration vector pTRK327. pTRK371 was subjected to several rounds of partial *Hin*dIII restriction digestion and religation to remove the gram-positive replication region.

gous recombination as the basis of integration in *L. gasseri*. Random integration of pTRK327 in *L. gasseri* provided evidence that the insert DNA encoded transposition functions. From the genetic and physical analyses of the 1.5-kb insert fragment, this element was registered with the Plasmid Reference Center, Stanford, Calif., and designated IS*1223*.

Nucleotide sequence of IS*1223*. The *Ava*I-*Hin*dIII region of pTRK371 containing the *L. johnsonii* insert DNA fragment was subcloned via internal *Hin*dIII, *Eco*RV, and *Xba*I restriction sites into pBluescript II KS$^{+/-}$ (Stratagene) to create a set of overlapping subclones for sequencing. The complete sequence of the insertion is presented in Fig. 4. The borders of the insert DNA were determined by using the vector-specific 17-mer oligonucleotide primer pSA3-A (5′-CTTGGCAGAA CATATCC-3′) to sequence through the pSA3 region containing the insertion. The pSA3 insert junction region was detected as a site duplication (direct repeat [DR1]) of 5 bp with the sequence 5′-ATAAT-3′. IS*1223* was 1,492 bp long and flanked by two, 25-bp imperfect inverted repeats (IR1). A second set of 21-bp imperfect (16 of 21 conserved) inverted repeats (IR2) was found nested within the first set and flanked a 1,041-bp region beginning at nucleotide 440 and ending at nucleotide 1480 (Fig. 4 and 5B). This suggested that a previous transposition event had occurred to create a composite element and, in fact, a 3-bp target site duplication (5′-CTA-3′) was detected as direct repeats (DR2) bordering the nested set of inverted repeats. Two putative open reading frames (ORFs) were detected. The first, ORFA, was preceded by a putative ribosome binding site (GAGG) and could encode a predicted protein of 177 amino acids. The putative ribosome binding site

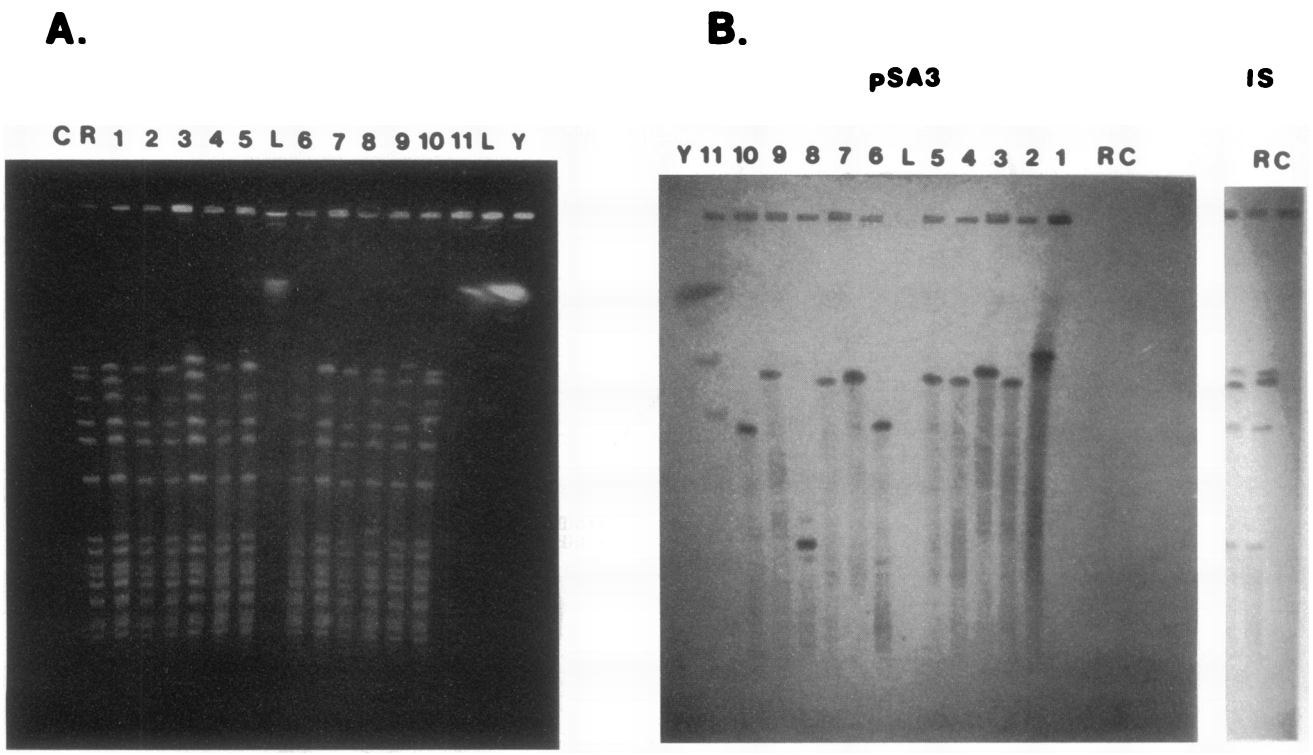

FIG. 2. Pulsed-field gel analysis of pTRK327 integration into the chromosome of *L. johnsonii* VPI 11088. (A) Total DNA was isolated in situ from 11 Em^r transformants (lanes 1 to 5 and 6 to 11), restricted with *Sma*I, and separated by pulsed-field gel electrophoresis (PFGE). λ DNA PFGE markers (lanes L) (size [kb] of 48.5 [lowest], 97.0, 145.5, 194.0, 242.5, and 291.0 [highest] visible), yeast DNA PFGE markers (lane Y) (size [kb] of 225, 295, and 375 visible) (Pharmacia LKB Biotechnology), VPI 11088 (lane C), and conjugal recipient (lane R). (B) Southern transfers of duplicate gels in which one was probed with pSA3 to detect integration sites and the other was probed with an *Eco*RV-*Hin*dIII fragment of IS*1223* to detect homology to the insertion element.

was separated from the flanking inverted repeat by a set of nearly perfect, 16-bp inverted repeats that could direct the formation of a 21.4-kcal stem-loop structure (SL1) (Fig. 4 and 5A). The second ORF, ORFB, displayed no discernible ribosome binding site, overlapped ORFA by 70 bp, and could encode a protein of 313 amino acids.

The EMBL data bank (version 33) was screened for similarity to IS*1223*. Three of the four highest scoring sequences matched insertion elements. Nucleotide sequence overlaps were 55.3% for *Mycoplasma incognitus* insertion sequence MIIS, 53.7% for *Streptococcus agalactiae* insertion sequence IS*861* and 52.9% for *E. coli* insertion element IS*150*. The two putative proteins encoded by IS*1223* were screened against the proteins in the protein data bank. Significant homology was detected between the putative ORFB protein and both the proposed 31-kDa protein of *Shigella sonnei* IS*600* (61.5% similarity and 24.3% identity) and the proposed ORFB protein of *E. coli* IS*150* (71.1% similarity and 35.7% identity). A multiple sequence alignment of all three putative proteins displayed 28.9% similarity and 17% identity (Fig. 6B). No significant homology was detected between ORFA and the proteins in the data base. However, ORFA did contain two putative helix-turn-helix (HTH) DNA-binding motifs with standard deviation (SD) scores of 3.28 (amino acid positions 21 to 42) and 4.97 (amino acid positions 78 to 99) as calculated by the method of Dodd and Egan (11). Putative HTH motifs have been reported for the ORFA proteins of both IS*150* and IS*600* (39), and their scores were independently calculated at 2.92 and 3.06, respectively. When the putative ORFA proteins of IS*1223*, IS*150*, and IS*600* were aligned, the lower scoring HTH motif of IS*1223* overlapped that of IS*150*, while the higher scoring IS*1223* HTH motif overlapped that of IS*600* (Fig. 6A). Both IS*150* and IS*600* have been assigned to the ubiquitous IS*3* superfamily of insertion elements, a group characterized, in part, by a similar organization (13). Most members of this family exhibit a short ORF followed by an overlapping, longer ORF in −1 phase. IS*1223* displays a molecular organization that is consistent with this family of IS elements.

DISCUSSION

We have isolated and characterized a genetic element from *L. johnsonii* that mediates genetic recombination in *L. gasseri* and *L. johnsonii*. The molecular organization of the element and its ability to mediate random integration events in *L. gasseri* indicate that it is an insertion element, designated IS*1223*. This is the first IS element discovered in lactobacilli used for the construction of an IS-based, integration vector in this important genus.

A native conjugation system in *L. johnsonii* was exploited to entrap a functional *Lactobacillus* insertion element. Conduction, a mobilization process requiring plasmid-plasmid association (6), has been cited by Guyer (17) as the most likely explanation for the F-mediated conjugal transmission of pBR322, which was recovered in transconjugants bearing the insertion element γδ. Similarly, a resolution product of pSA3 was recovered from the transconjugants that contained the 1.5-kb insert of *L. johnsonii*. Therefore, it is likely that pSA3

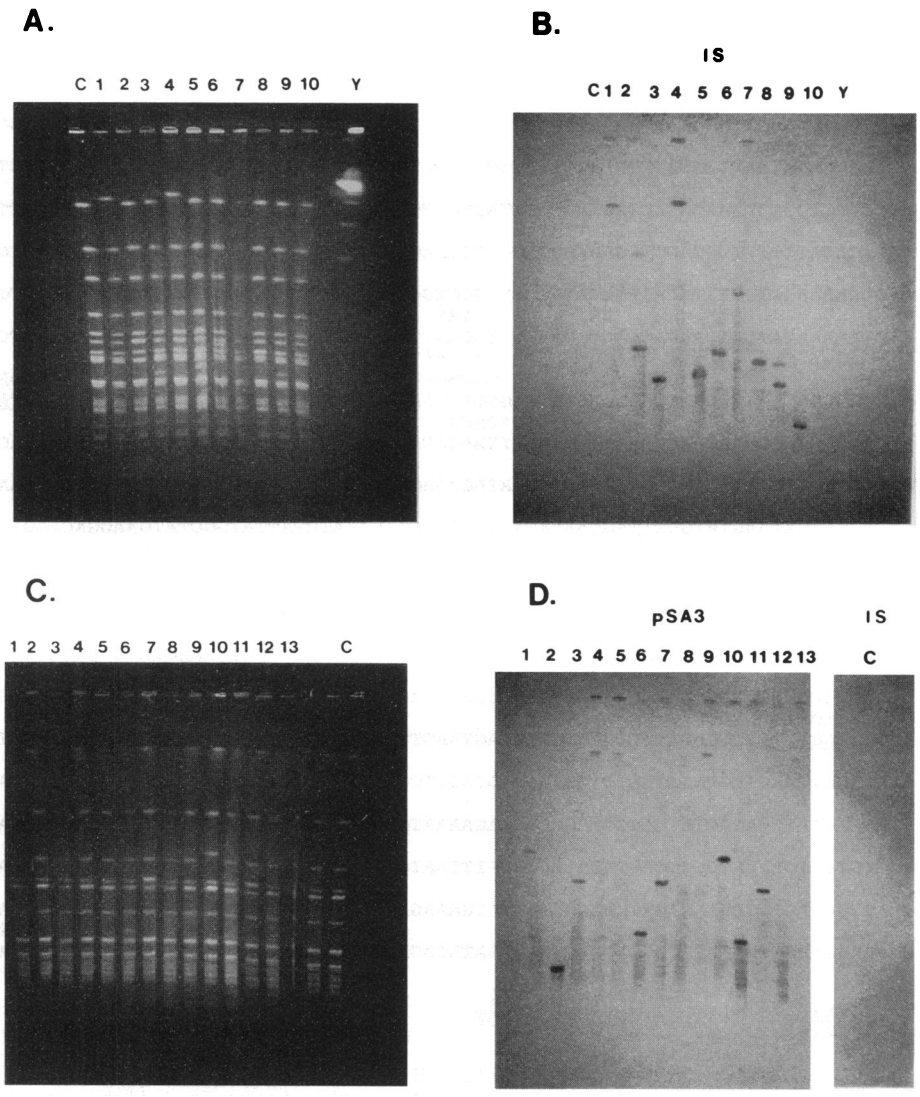

FIG. 3. (A) Pulsed-field gel analysis of pTRK327 integration into the chromosome of *L. gasseri* ATCC 33323. *Sma*I-digested total DNA was separated by pulsed-field gel electrophoresis (PFGE). The host chromosome (lane C), pTRK327 integrants (lanes 1 to 10), and yeast DNA PFGE markers (lane Y) are shown. (B) Southern transfer of *L. gasseri* ATCC 33323 DNA probed with an *Eco*RV-*Hin*dIII internal fragment of IS*1223* to both localize insertions and detect homology to the IS element. (C) Pulsed-field gel analysis of pTRK327 integration into the chromosome of *L. gasseri* VPI 11759. *Sma*I-restricted total DNA was separated on duplicate gels by PFGE. There are two lanes of VPI 11759 host chromosome indicated by C, and lanes 1 to 13 contain pTRK327 integrants. (D) Southern transfers of *L. gasseri* VPI 11759 DNA in which one is probed with pSA3 to localize insertion events and the other is probed with an *Eco*RV-*Hin*dIII internal fragment of IS*1223*.

was mobilized by conduction and cointegrate formation was mediated via IS*1223* with a *Lactobacillus* conjugal element.

The general structure of IS*1223* is consistent with the IS*3* group of insertion elements, but several novel characteristics are noted. The 25-bp terminal inverted repeats (IR1) are composed of outer and inner perfectly conserved domains of 8 and 9 bp, respectively (Fig. 4). These domains are separated by a less-conserved, 8-bp region. Dual functional domains have been detected in the inverted repeats of several bacterial insertion elements, including IS*903* (9) and IS*1* (65). In the case of IS*903*, the evidence indicates that the inner domain is important for transposase binding and that the outer domain is important for a later transposition step. An unusual structural feature of IS*1223*, and one that suggests it is a composite element, is the occurrence of a nested set of direct (DR2) and inverted repeats (IR2). The secondary transposition of one insertion element into another is not an uncommon occurrence and has been reported in IS*1* (48) and IS*15*-R (60).

Of the two putative ORFs identified in IS*1223*, only ORFA contains a possible ribosome binding site. A nearly perfect set of 16-bp inverted repeats between bases 7 and 45 (Fig. 4) could direct the formation of a −21.4 kcal stem-loop (SL1) structure in response to a read-through transcript. On formation, SL1 would sequester both the ribosome binding site and most of the terminal inverted repeat (IR1) (Fig. 5A). A similar stem-loop structure has been identified in IS*3* (59) just downstream of the left inverted repeat. It was identified as a possible mechanism for the control of extraneous activation of the insertion element, since the stem-loop could form when a transcript begins outside the element and incorporate the −10

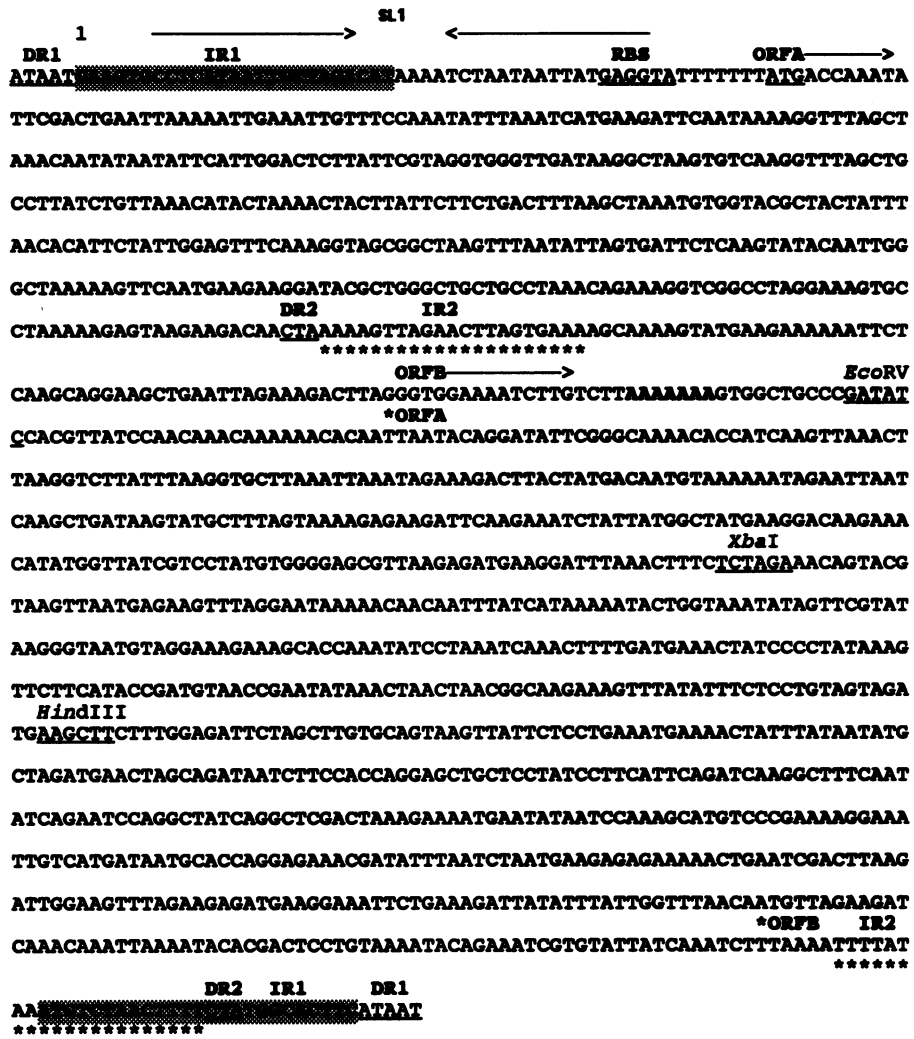

FIG. 4. Nucleotide sequence of *L. johnsonii* insertion element IS*1223*. DR1 (underlined) indicates the direct repeats generated by IS*1223* in pSA3. IR1, which is highlighted, indicates the terminal inverted repeats of the insertion element. The bold characters within IR1 indicate two strictly conserved regions within the inverted repeats. DR2 and IR2 represent a set of nested direct repeats (underlined) and inverted repeats (underlined by asterisks), respectively. SL1 indicates an additional set of inverted repeats. The two putative ORFs are indicated at their start sites and an asterisk indicates the base at which they terminate. Sequence numbering begins with the leftmost IR1.

region and ribosome binding site for the two overlapping ORFs. The occlusion of translation initiation regions by the formation of similar secondary structures may also occur in both IS*50* (22) and IS*10* (8).

ORFA could code for a protein 177 amino acids in length and was found to contain two putative HTH DNA-binding motifs with SD scores of 3.28 and 4.97, as calculated by the method of Dodd and Egan (11). These scores correspond to a probability of HTH occurrence of greater than 50% for the first and 100% for the latter. SD scores are calculated for a target sequence of 22 amino acids by scoring the amino acid at each position against a weight matrix derived from a master set of HTH sequences. On the basis of the calculated scores, it is highly probable that ORFA contains one and possibly two HTH motifs. Potential DNA-binding HTH motifs have been detected in the ORFA proteins of at least ten members of the IS*3* family (39), which are probably involved with end recognition and regulation of transposition activity. This has proven to be the case with IS*1* in which the InsA protein contains a strong HTH DNA-binding motif near its carboxy terminus (63). InsA binds specifically to the inverted repeats of the IS element (65) and negatively regulates transposition activity (29, 64). The DNA-binding motif is conserved in the InsA-InsB fusion protein (51), thought to be the active transposase, and probably competes with InsA for the same binding site.

The second open reading frame of IS*1223*, ORFB, contained no discernible ribosome binding site, could code for a protein of 313 amino acids and overlapped ORFA by 70 bp. The overlapping region between ORFA and ORFB was found to contain the nucleotide sequence 5′-TTAAAAAAAGTG-3′, a site where a translational frameshift could occur within the run of seven adenines. A −1 frameshift at this site would generate an ORFA-ORFB fusion protein (Fig. 5B). The production of a functional transposase by programmed translational frameshifting has been confirmed in IS*911* (34), IS*150* (61), and IS*1* (50) and has been proposed as an alternative form of translational regulation of insertion element transposition (5). In the translational frameshift site of IS*150*, the

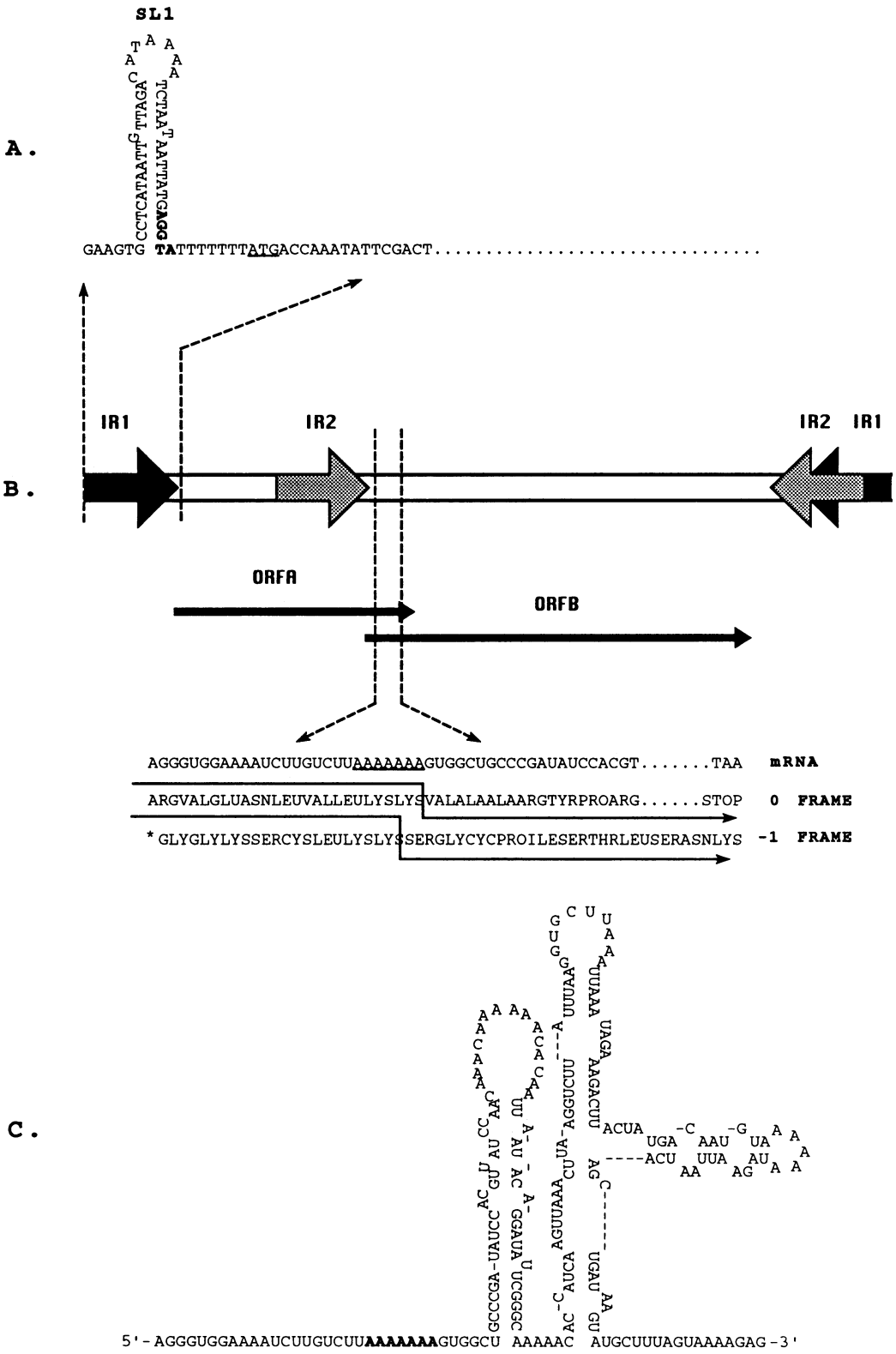

FIG. 5. (A) A potential stem-loop structure (SL1) that can incorporate and sequester part of the left terminal inverted repeat (IR1) and the putative ribosome binding site (characters in bold type). (B) Organization of *Lactobacillus* insertion element IS*1223* and proposed frameshift mechanism for translation of a functional transposase. The outer inverted repeats that form the boundaries of the IS element (IR1) and an inner set of inverted repeats that overlap at the right (3′) end of the IS element (IR2) are shown. The two putative overlapping ORFs, mRNA, and site of potential translational frameshifting are represented. The proposed transframe protein is delineated by the arrows. (C) A potential stem-loop structure with a calculated free energy of −19.8 kcal is shown 6 bp downstream of the putative frameshift site.

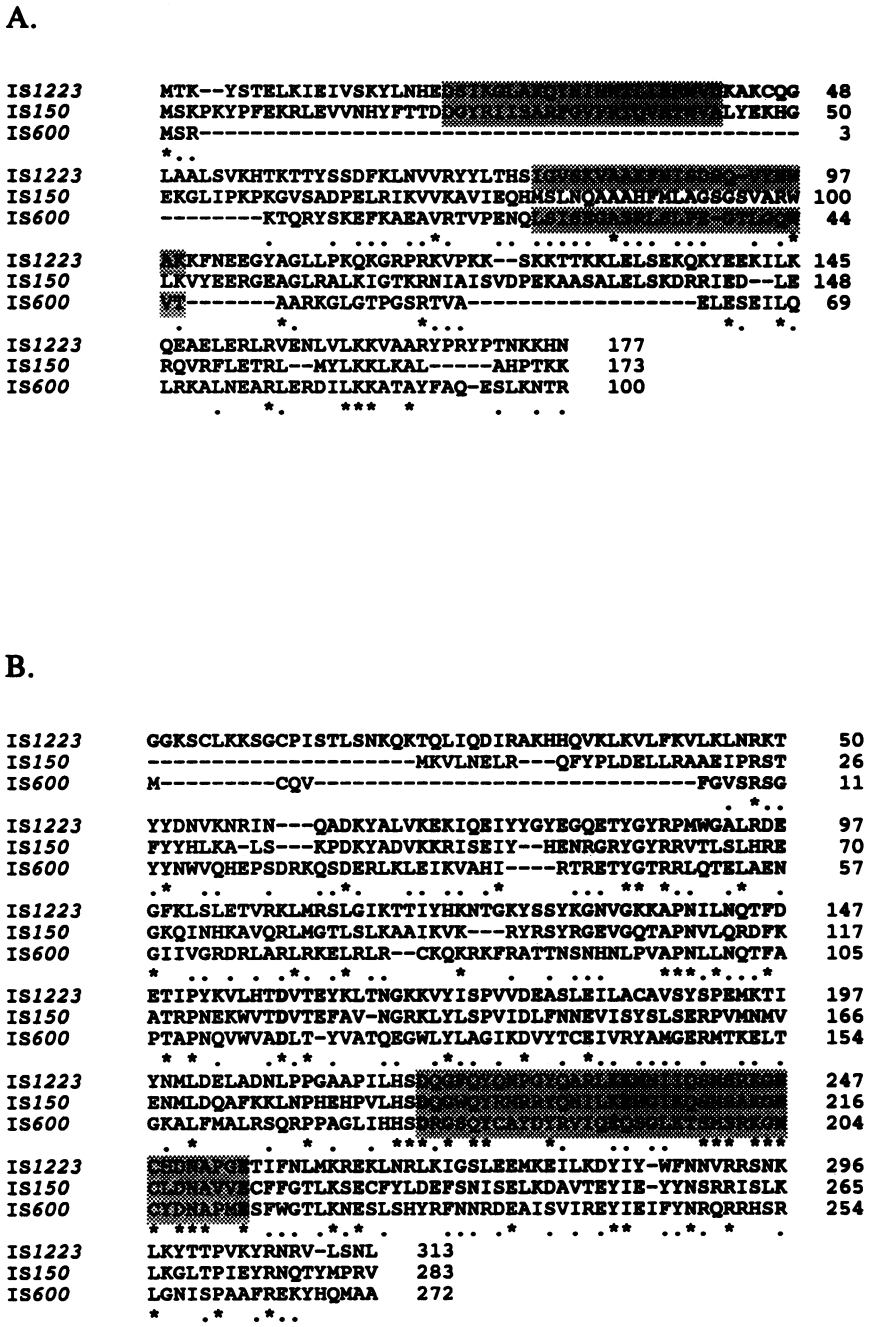

FIG. 6. Alignment of the proposed proteins of *L. johnsonii* IS*1223*, *E. coli* IS*150*, and *S. sonnei* IS*600*. (A) Proposed ORFA proteins of the three insertion elements with their putative HTH DNA-binding motifs highlighted. The HTH motif of IS*150* was calculated to have an SD score of 2.92 by the method of Dodd and Egan (11) and aligned with one of the IS*1223* HTH motifs (SD score of 3.28). The HTH motif of IS*600* was calculated to have an SD score of 3.06 and aligned with the other IS*1223* HTH motif (SD score of 4.97). The alignment of the three sequences yielded a similarity of 20.3% and an identity of 7.1%. (B) Proposed ORFB proteins with the D,D (35-amino-acid) E region highlighted. The alignment of the three sequences yielded a similarity of 28.9% and an identity of 17%. Perfectly conserved (*) and well-conserved (.) amino acids are shown.

heptanucleotide AAAAAAG is followed 6 bp downstream by a stem-loop structure (61). Stem-loop structures of −11.5, −15.6, and −19.8 kcal can be predicted to occur starting exactly 6 bp downstream of the potential IS*1223* frameshift sequence and ending at sequence positions 604, 654, and 708 (Fig. 5C), respectively. Stem-loop structures have been cited as one stimulator of high-level frameshifting (1a).

IS*1223* appears to be a new member of the IS*3* group of insertion elements, previously cited to contain at least 23 members (5). Members of this divergent group display limited homology at the nucleotide level, while maintaining structural similarities that include terminal inverted repeats, the occurrence of two overlapping ORFs (11 of 23 containing a potential frameshift region), and significant amino acid similarity in the

larger, downstream ORF. In recent comparisons of the amino acid sequences of the IS*3* family with those of retroviruses (13) and of both retroviruses and retrotransposons (23), invariant residues were identified that were necessary for transposition activity. Those conserved acidic residues, identified alternately as either D...35 amino acids...E or D,D...35 amino acids...E, span a highly conserved region which is present in the ORFB protein of the *Lactobacillus* insertion element IS*1223* (Fig. 6B). It has been suggested that the invariant aspartate (D) and glutamate (E) residues may be positioned at the catalytic center and be involved in binding a metal cofactor (23). Two insertion elements previously isolated from lactic acid bacteria, ISL*1* and IS*981*, have also been assigned to the IS*3* group (35). The D(35)E conserved acidic residues are also conserved in the longer second ORFs of both elements.

IS*1223* is the first insertion element isolated in lactobacilli for which functionality has been demonstrated. The utility of IS-based integration vectors has been adequately demonstrated in lactic acid bacteria. Random integration of heterologous DNA by vectors pRL1 and pTRK145 has been demonstrated in the genome of *Lactococcus lactis* subsp. *lactis* based upon the transposition functions of the insertion elements ISS*1*RS (25) and IS*946* (42), respectively. In addition, the utility of the IS*946*-based integration vector pTRK145 as an effective insertional mutagen has been established (10). One consequence of IS element-based transposition activity is the production of mobile regions of homology. This property has been exploited in the development of a temperature-sensitive vector (pKM10) that carries an internal fragment of IS*981* which mediates Campbell-like integration into the *L. lactis* subsp. *lactis* genome within resident copies of the same insertion element (36). With the construction of IS*1223*-based integration vector pTRK327, similar approaches can now be exploited in lactobacilli.

IS*1223*-based integration vectors could be exploited to integrate a range of genes in the interest of *Lactobacillus* strain improvement or to map the *Lactobacillus* genome when coupled with two-dimensional pulsed-field gel electrophoresis. Integration vector pRL1 was designed in this way and proved an effective tool for mapping the *L. lactis* subsp. *lactis* IL1403 chromosome (26). Multiple strategies also exist for utilizing IS*1223*-based vectors as insertional mutagens (2, 4, 10). The availability of IS*1223*-based vectors will provide a number of new opportunities to genetically characterize and manipulate the genome of this industrially important bacterium.

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