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# Splicing of a Group II Intron Involved in the Conjugative Transfer of pRS01 in Lactococci

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**Analysis of a region involved in the conjugative transfer of the lactococcal conjugative element pRS01 has revealed a bacterial group II intron. Splicing of this lactococcal intron (designated Ll.ltrB) in vivo resulted in the ligation of two exon messages (*ltrBE1* and *ltrBE2*) which encoded a putative conjugative relaxase essential for the transfer of pRS01. Like many group II introns, the Ll.ltrB intron possessed an open reading frame (*ltrA*) with homology to reverse transcriptases. Remarkably, sequence analysis of *ltrA* suggested a greater similarity to open reading frames encoded by eukaryotic mitochondrial group II introns than to those identified to date from other bacteria. Several insertional mutations within *ltrA* resulted in plasmids exhibiting a conjugative transfer-deficient phenotype. These results provide the first direct evidence for splicing of a prokaryotic group II intron in vivo and suggest that conjugative transfer is a mechanism for group II intron dissemination in bacteria.**

Conjugation is an important mode of genetic exchange in bacteria. The specific mechanism of DNA transfer comprises two distinct functions, one being the enzymatic preparation of the plasmid DNA prior to replicative transfer and another involving formation of the mating channel through which DNA is transferred into a recipient cell. Initiation of plasmid transfer requires a single-stranded cleavage at a specific origin of transfer (*oriT*) produced by the action of a specialized nucleoprotein complex called a relaxosome (53). In many conjugative systems, transfer origins are flanked by genes involved in the formation of a functional relaxosome complex. One key feature of an *oriT* region is the ability of the region to confer mobilization in *cis*, provided the remaining transfer functions are present in *trans.*

The conjugative element pRS01 from *Lactococcus lactis* subsp. *lactis* ML3 and the sex factor from *L. lactis* subsp. *lactis* 712 are prototypical mobile elements in lactococci (15, 50). Both elements have been shown to mediate high-frequency transfer of genes encoding lactose utilization ($Lac^+$) by insertion sequence-directed conintegration with nonconjugative $Lac^+$ plasmids (19, 40). In addition, both elements confer a cell aggregation (Clu) phenotype (16, 52) associated with high-frequency conjugative transfer. Previous genetic analysis of these elements has identified the gene responsible for the aggregation phenotype (*clu*) associated within an inversion region (2, 20, 21). To date, however, the *oriT* and gene(s) encoding relaxosome components of pRS01 have not been localized within either element. In an effort to exploit conjugation as a means of lactococcal strain development, we have characterized the transfer regions of pRS01 by insertional mutagenesis via IS*946*-mediated cointegration with the 11-kb plasmid pTRK28 (43) (for a representation of pTRK28:: pRS01 cointegration, see Fig. 1 in reference 33). Analysis of the insertion site junctions of pRS01::pTRK28 cointegrate plasmids identified four distinct regions of pRS01 involved in conjugative transfer (33). Two of these regions, Tra1 and Tra2, were found to be unlinked to the previously known transfer regions (2). In this study, we have characterized the Tra1 region from pRS01. Complementation analysis of Tra1 region insertions with cloned Tra1 DNA resulted in mobilization of the complementing vector, which suggested that the conjugative origin of transfer of pRS01 is contained within the Tra1 region (32). Sequence analysis of the region encompassing Tra1 and Tra2 revealed six open reading frames (ORFs), *ltrC*, *ltrD*, *ltrE*, *ltrBE1*, *ltrA*, and *ltrBE2*. Further analysis indicated the presence of a bacterial group II intron (termed Ll.ltrB) within the gene encoding a conjugative relaxase (*ltrB*). Identification of insertion sites in Tra1 mutants revealed that all $Tra^-$ insertions fell within the relaxase upstream exon *ltrBE1* or the group II intron-encoded ORF *ltrA*. Splicing of the Ll.ltrB intron was demonstrated in vivo, and the sequence of the spliced product revealed an in-frame fusion of the *ltrBE1-ltrBE2* exons. This work provides the first description of splicing in vivo from a bacterial group II intron and indicates involvement of intron splicing in the pRS01 conjugative process.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** *Escherichia coli* DH5α was grown in LB medium (45). *E. coli* DH5α containing plasmid pLE12 or pE351 was grown in selective medium containing chloramphenicol (30 μg/ml). *L. lactis* strains were grown in GM17 (M17 medium [51] containing 0.5% glucose) at 30°C. Selective media for *L. lactis* strains contained the following antibiotics at the indicated concentrations: erythromycin, 10 μg/ml; chloramphenicol, 5 μg/ml; rifampin, 50 μg/ml; streptomycin, 600 μg/ml; and spectinomycin, 300 μg/ml. All plating media contained 1.5% Bacto Agar.

**Cloning and DNA manipulation.** Restriction endonucleases, T4 DNA ligase, the Klenow fragment of DNA polymerase 1, and calf intestinal phosphatase were purchased from Gibco BRL Life Technologies (Gaithersburg, Md.), New England BioLabs, Inc. (Beverly, Mass.), or Promega (Madison, Wis.) and used as described by the manufacturers.

Large-scale isolation of plasmid DNA from *L. lactis* was performed as described by Anderson and McKay (3). Miniprep isolation of plasmid from *L. lactis* was performed as described by O'Sullivan and Klaenhammer (38). Rapid plas-

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mid isolation from *E. coli* was accomplished by the alkaline lysis method (45). Large-scale plasmid isolation from *E. coli* was performed by the polyethylene glycol precipitation procedure (4).

DNA fragments were analyzed by horizontal electrophoresis in agarose gels with Tris-borate-EDTA buffer (45). Lambda DNA cut with *Hin*dIII or a 1-kb ladder (Gibco-BRL Life Technologies) was used as a molecular weight standard as well as a concentration reference. DNA fragments were isolated from agarose gels by use of the GeneClean II kit (Bio 101, La Jolla, Calif.) as indicated by the manufacturer.

**Transformation.** Electroporation of *E. coli* or *L. lactis* was performed with a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.). Competent *L. lactis* cells were prepared as described previously (33). Competent *E. coli* cells were prepared as described elsewhere (4). All electroporations were conducted in a 0.1-cm cuvette at a voltage of 1.7 kV/cm and a capacitance setting of 100 Ω.

**DNA sequencing.** All plasmids containing insertions in the Tra1 or Tra2 region (for Tra1, pM5024, pM5015, pM1014, pM1005, pM3022, pM5086, pM3020, and pM5064; for Tra2, pM5008) and the plasmid pM2036 were cleaved with *Hin*dIII, and the products were self-ligated and transformed into *E. coli* DH5α (for a description of pRS01 subcloning strategy, see reference 33). The resultant subclones were subjected to dideoxy DNA sequencing with Sequenase 2.0 (U.S. Biochemicals, Cleveland, Ohio) and [α-$^{32}$S]dATP (Amersham Life Sciences, Arlington, Heights, Ill.) by using the p707out primer (43) to generate the initial Tra1 and Tra2 region DNA sequence flanking the pTRK28 insertion. Further sequencing of the Tra1 and Tra2 regions was accomplished by a primer-walking method on plasmid pE351, a subclone of pRS01 containing the complete Tra1 and Tra2 regions. Additional sequence was determined via fluorescent sequencing with the Applied Biosystems (Foster City, Calif.) Prism DyeDeoxy Terminator Cycle Sequencing Kit and analyzed on an Applied Biosystems model 373 DNA sequencer. Primers were synthesized with an Applied Biosystems model 391 automated synthesizer or purchased from Keystone Laboratories Inc. (Menlo Park, Calif.) or Gibco BRL Life Technologies. The *ltrB* reverse transcription-PCR product derived from *L. lactis* DM2036 RNA was purified with the Wizard PCR-purification system (Promega) and sequenced with the dsDNA Cycle Sequencing System Kit (Gibco BRL Life Technologies) and fluorescent sequencing.

**DNA and protein sequence analysis.** Sequence compilation and comparison were performed by use of Intelligenetics GeneWorks (Mountain View, Calif.) and Genetic Computer Group, Inc. (GCG) (Madison, Wis.) sequence analysis software. RNA folding analysis was performed with the MFOLD program provided within the GCG sequence analysis package. Amino acid alignments were performed by use of the FASTA (39) and BLAST (1) programs provided within the GCG sequence analysis package. Codon usage comparison was performed with the CODONPREFERENCE program from GCG. Phylogenetic analysis was obtained with the protein parsimony (PROTPARS) or protein distance (PROTDIST) programs provided within the PHYLIP 3.5c phylogeny inference package (12).

**RNA purification.** Lactococcal RNA was purified from exponentially growing cells by the method of Shaw and Clewell (48) with the following modifications. All solutions, with the exception of growth media, phenol, and chloroform-isoamyl alcohol, were treated with the RNase inhibitor diethylpyrocarbonate prior to sterilization as described elsewhere (4). Eleven milliliters of GM17 was added to an 11-ml overnight culture of *L. lactis*, and the culture was incubated at 30°C for 2 h. The 22-ml culture was then mixed with an equal volume of an ice slurry solution of 0.02 M Tris (pH 7.3), 0.005 M $MgCl_2$, 0.02 M $NaN_3$, and 400 μg of chloramphenicol per ml in a sterile 50-ml conical tube. The culture was centrifuged at 4,350 × *g* for 5 min at 4°C. The resultant cell pellet was resuspended in 4 ml of 25% sucrose–50 mM Tris (pH 8.0). One hundred microliters of a solution containing 100 mg of lysozyme per ml and 1 μg of mutanolysin per ml was then added to the cell slurry, and the slurry was incubated for 5 min on ice. The slurry was then centrifuged at 3,030 × *g* for 5 min at 4°C. The resultant pellet was resuspended in 700 μl of lysis buffer (20 mM Tris-Cl [pH 8.0], 3 mM EDTA, 0.2 M NaCl), and the solution was divided between two sterile microcentrifuge tubes. Three hundred fifty microliters of prewarmed (95°C) lysis buffer containing 1.0% sodium dodecyl sulfate (SDS) was added to each tube, and the mixture was incubated at 95°C for 1 min. The solution was then thoroughly mixed with prewarmed (65°C) phenol saturated with lysis buffer and incubated for 3 min at 65°C with occasional mixing before chilling the solution on ice for 5 min. The cell-phenol mixture was centrifuged at 14,900 × *g* for 10 min at 4°C. The upper phase was removed, and the phenol extraction was repeated two more times. The resulting cell lysate was extracted three times with a mixture of chloroform-isoamyl alcohol (24:1). The cellular RNA was then precipitated by the addition of 1/10 volume of 1.5 M sodium acetate and 2.5 volumes of ethanol, followed by incubation at −20°C for 1 h and centrifugation at 14,900 × *g* for 20 min at 4°C. The resultant RNA pellet was washed twice with 70% ethanol, dried extensively prior to resuspension in 30 μl of $H_2O$, and stored at −70°C.

**Preparation of cDNA.** Ten micrograms of RNA was treated with 10 U of RNase-free DNase I (Gibco BRL Life Technologies) in 1× DNase buffer at 37°C for 15 min. The DNase I was inactivated by the addition of 10 μl of 20 mM EDTA, and the mixture was heated at 65°C for 10 min. The cellular RNA was then coprecipitated with 0.5 μmol of primer by the addition of 1/10 volume of 3 M sodium acetate and 2.5 volumes of ethanol, incubated at −20°C for 1 h, and centrifuged at 14,900 × *g* for 20 min at 4°C. The resultant RNA pellet was washed with 70% ethanol and dried prior to resuspension in 30 μl of diethylpyrocarbonate-treated $H_2O$ and divided into two microcentrifuge tubes. To each 15-μl aliquot was added 10 μl of extension mixture (0.675 mM [each] deoxynucleoside triphosphate, 5 U of RNasin [Promega] per ml, 2.5× Moloney murine leukemia virus reverse transcriptase buffer, 0.675 μg of actinomycin D) and 400 U of Moloney murine leukemia virus reverse transcriptase (RT) (Promega). The reaction mixture was incubated for 90 min at 42°C, and the reaction stopped by the addition of 0.5 M EDTA (pH 8.0). Cellular RNA was then removed by the addition of 1 μl of RNase A (2 mg/ml), followed by incubation at 37°C for 15 min. One hundred microliters of 7.5 M ammonium acetate was added, and the cDNA mixture was extracted with 125 μl of a phenol-chloroform (1:1) mixture. The 100-μl aqueous phase was removed, and the cDNA was precipitated by the addition of 300 μl of ethanol, followed by incubation at −20°C for 1 h and centrifugation at 14,900 × *g* for 20 min at 4°C. The cDNA pellet was washed with 500 μl of 70% ethanol and dried. The resulting pellet was resuspended in 50 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and stored at −70°C. For a typical reverse transcription-PCR, 1/20 of the cDNA preparation was used.

**PCR.** All PCRs were carried out on an OmniGene thermocycler (Hybaid, Middlesex, United Kingdom). PCRs typically employed *Taq* polymerase with 0.1 μmol of primers specific to the *ltrBE1* and *ltrBE2* exons (A21230, 5′-TATTT CAAATCCAGACAAGA-3′; LtrBRT1, 5′-GAAGTCTTCAATATCTGATG ACATTTCTAA-3′, respectively) (see Fig. 1) and 60 ng of DNA template. For a typical reverse transcription-PCR, 1/20 of the cDNA preparation (see above) was used. All PCR products were purified with the Wizard PCR-purification system (Promega).

**Matings.** For determination of transfer frequencies, plate matings were performed on milk-agar plates (5% nonfat milk, 1% glucose, 1.5% agar) as described by Anderson and McKay (2). For a typical mating, donor and recipient cultures were grown in GM17 at 30°C, without shaking, to an optical density at 660 nm of 0.6. Donor and recipient cells were then centrifuged, and the pellet was resuspended in 1/50 volume of M17 medium. Typically, 50 μl of donor and 50 μl of recipient were mixed, spread onto a milk-agar plate, and incubated overnight at 30°C. The mating mixture was then washed off the milk-agar plate with 1 ml of sterile phosphate-buffered saline, diluted appropriately, and plated on selective media. For determination of mutant pRS01 plasmid transfer, transconjugants were selected on GM17 containing erythromycin (10 μg/ml). For determination of pLE12 plasmid transfer, transconjugants were selected on GM17 containing chloramphenicol (5 μg/ml). Transfer frequencies were calculated as the number of transconjugants recovered per input donor from at least two independent matings.

**Nucleotide sequence accession number.** The Tra1 DNA sequence reported here has been deposited in the GenBank database under accession number U50902.

## RESULTS

**The Tra1 region contains an intron.** Previous genetic analysis of pRS01 suggested that the Tra1 region might contain a *cis*-acting *oriT* (32). To complement Tra1 region insertions, a 7.5-kb *Pst*I fragment encompassing the Tra1 region was cloned from pRS01 into the nonmobilizable vector pLE1 (33), resulting in the plasmid pLE12 (Fig. 1). Plasmid pLE12 was transformed into several strains containing plasmids with insertions within Tra1. As indicated in Table 1, Tra1 insertion mutations were complemented for transfer with pLE12 in *trans*. Strains containing pLE12 exhibited plasmid transfer frequencies in the range of $10^{-4}$ to $10^{-5}$ transconjugants per donor, which was more than 1,000-fold higher than frequencies of strains containing the control plasmid pLE1. Mobilization of pLE12 was observed in all matings at approximately 1/10 the transfer frequency observed for the respective pRS01 Tra1 insertion mutant.

To localize the sites of insertion within Tra1 and Tra2, the complete region was sequenced, revealing six substantial ORFs (*ltrC*, *ltrD*, *ltrE*, *ltrBE1*, *ltrA*, and *ltrBE2*), all in the same orientation. All insertions which resulted in plasmids exhibiting transfer-negative phenotypes were localized to the *ltrBE1* and *ltrA* genes. *ltrA* encodes a putative 599-amino-acid product with extensive similarity to ORFs contained in eukaryotic group II introns (26, 44) and two recently identified bacterial group II introns (13, 14). Like group II intron-encoded ORFs, *ltrA* contained all seven polymerase-like domains conserved among retroelement reverse transcriptases (RT1 to RT7) (Fig. 2) (55). The *ltrA* protein also contained the following: domain

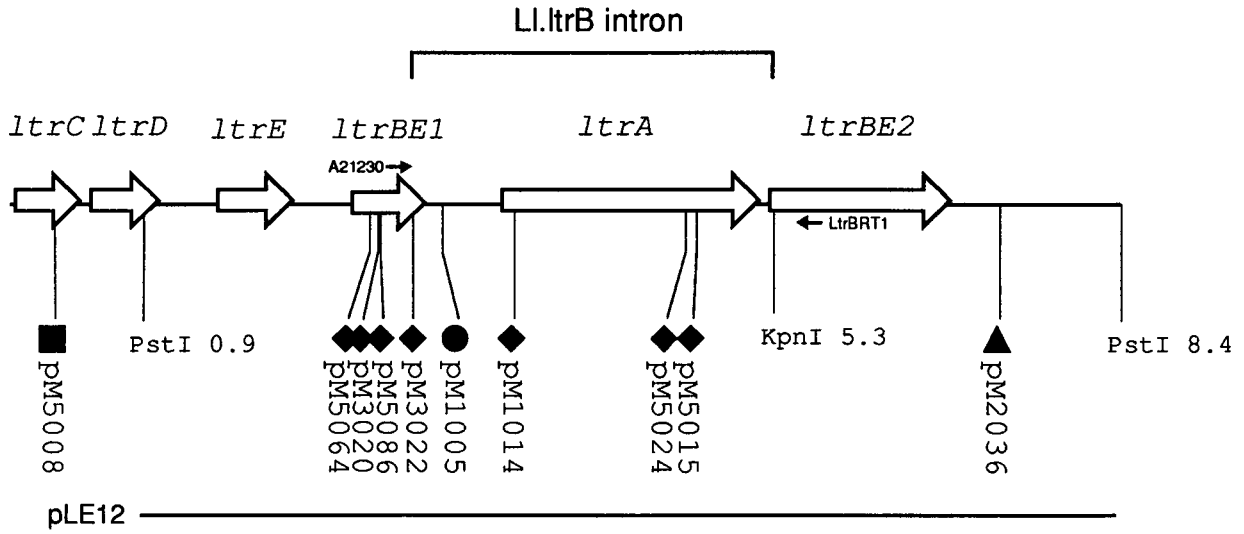


FIG. 1. Organization and insertion map of the pRS01 Tra1 and Tra2 regions. The figure is drawn from the sequence of the complete region (GenBank accession number U50902). The Ll.ltrB intron is bounded by the *ltrBE1* and *ltrBE2* exons. A21230 and LtrBRT1 are the primers used for PCR amplification. Insertions are indicated by the plasmid designation, and accompanying symbols indicate plasmid transfer frequency: ▲, $10^{-1}$ to $10^{-3}$ transconjugants per donor; ■, $10^{-4}$ to $10^{-6}$ transconjugants per donor; ●, $10^{-7}$ to $10^{-9}$ transconjugants per donor; ◆, $<10^{-9}$ transconjugants per donor category: as described (in reference 33). Plasmid pLE12 was used in complementation analysis of Tra1 insertions.

Z, a domain of undetermined function in non-long-terminal-repeat retroelements (29, 54); domain X, a domain suggested to be involved in a maturase-specific function for group II introns (35); and a C-terminal $Zn^{2+}$ finger-like region associated with DNA endonuclease activity (22, 36, 49). The predicted *ltrA* protein does not contain a P (protease) domain commonly found in retroviral polymerase genes and several fungal group II intron-encoded ORFs (10, 29, 56). Remarkably, the encoded *ltrA* protein is more similar to the group II intron-encoded ORFs predicted from *Saccharomyces cerevisiae* and *Podospora anserina* mitochondria than those which have been identified in a *Calothrix* sp. or *E. coli* (see Fig. 2 legend). Further support for the closer relationship between *ltrA* and mitochondrial group II intron-encoded ORFs comes from the fact that database searches with Ll.ltrB intron DNA identified several fungal and plant mitochondrial group II introns (51 to 56% identity primarily spanning the intron ORF coding region [34]) but failed to detect introns from *E. coli* and a *Calothrix* sp.

TABLE 1. Complementation of Tra1 region insertions

| Mutant pTRK28:: pRS01 plasmid[a] | Conjugation frequency[b] with pLE1[c] as coresident plasmid | Conjugation frequency with pLE12[d] as coresident plasmid | Mobilization frequency of pLE12 |
|---|---|---|---|
| *ltrBE1* insertions | | | |
| pM5064 | $<1.9 \times 10^{-8}$ | $1.3 \times 10^{-4}$ | $9.8 \times 10^{-6}$ |
| pM3022 | $<3.6 \times 10^{-9}$ | $2.9 \times 10^{-4}$ | $1.1 \times 10^{-5}$ |
| Intergenic region | | | |
| pM1005 | $2.0 \times 10^{-7}$ | $1.1 \times 10^{-4}$ | $4.6 \times 10^{-6}$ |
| *ltrA* insertions | | | |
| pM1014 | $<6.3 \times 10^{-8}$ | $7.6 \times 10^{-5}$ | $1.5 \times 10^{-5}$ |
| pM5024 | $<5.4 \times 10^{-9}$ | $8.1 \times 10^{-5}$ | $8.0 \times 10^{-6}$ |
| pM5015 | $<3.7 \times 10^{-8}$ | $1.1 \times 10^{-5}$ | $6.5 \times 10^{-7}$ |

[a] Insertions were grouped by position within the Tra1 region gene map (see Fig. 1).
[b] Transfer of mutant pRS01 plasmids were scored for erythromycin-resistant CFU per input donor by using *L. lactis* subsp. *lactis* LM2345 as a recipient (33).
[c] Plasmid pLE1 is an *E. coli-L. lactis* shuttle vector generated previously (33).
[d] The pRS01 DNA cloned into pLE1 to generate pLE12 is shown in Fig. 1.

Several groups have developed phylogenetic trees based on the RT-like domains from various eukaryotic and eubacterial retroelements (10, 29, 55). We used a similar analysis to develop a tree from the RT-like domains from select mitochondrial, chloroplastic, and bacterial group II intron-encoded ORFs, including *ltrA* (Fig. 3). Consistent with the protein homology data, *ltrA* localized within the mitochondrial group II intron-encoded ORFs (13) and appeared distinct from other known bacterial group II intron-encoded ORFs.

The discovery that *ltrA* resembles a group II intron-encoded ORF suggested that intron splicing may be involved in conjugation of pRS01. Database analysis of the ORFs *ltrBE1* and *ltrBE2*, which flank *ltrA*, revealed homology to the conjugative relaxases from gram-positive bacteria (see below). This split gene organization of *ltrBE1* and *ltrBE2* combined with the homology between *ltrA* and other group II intron-encoded ORFs suggested that the conjugative relaxase gene (*ltrB*) from pRS01 is interrupted by an intron. We have designated this intron Ll.ltrB.

**Splicing of the lactococcal intron in vivo.** To demonstrate splicing of the lactococcal intron in vivo, RNA from the *L. lactis* strain DM2036, which exhibits high-frequency conjugative transfer (33), was reverse transcribed (4) and amplified by PCR with primers specific to the *ltrBE1* and *ltrBE2* exons (LtrBRT1 and A21230 [Fig. 1]). The resulting product (0.6 kb) (Fig. 4A, lane 2) was approximately 2.5 kb smaller than the product amplified by PCR from pM2036 DNA with the identical primers (Fig. 4A, lane 1). No product was observed from reverse transcription-PCRs on the plasmid-free host strain (*L. lactis* subsp. *lactis* MMS370) or the host strain containing plasmids with insertions within Ll.ltrB (pM1005, pM1014, pM5024, or pM5015) (34). In addition, unspliced *ltrB* pre-mRNA product was not observed from reverse transcription-PCRs with longer polymerase extension times. Direct sequence analysis of the 0.6-kb reverse transcription-PCR fragment revealed an in-phase ligation of the *ltrBE1* and *ltrBE2* reading frames (Fig. 4B). The 5′ and 3′ splice site junctions of the intronic sequence exhibited a strict conservation of the eukaryotic group II intron

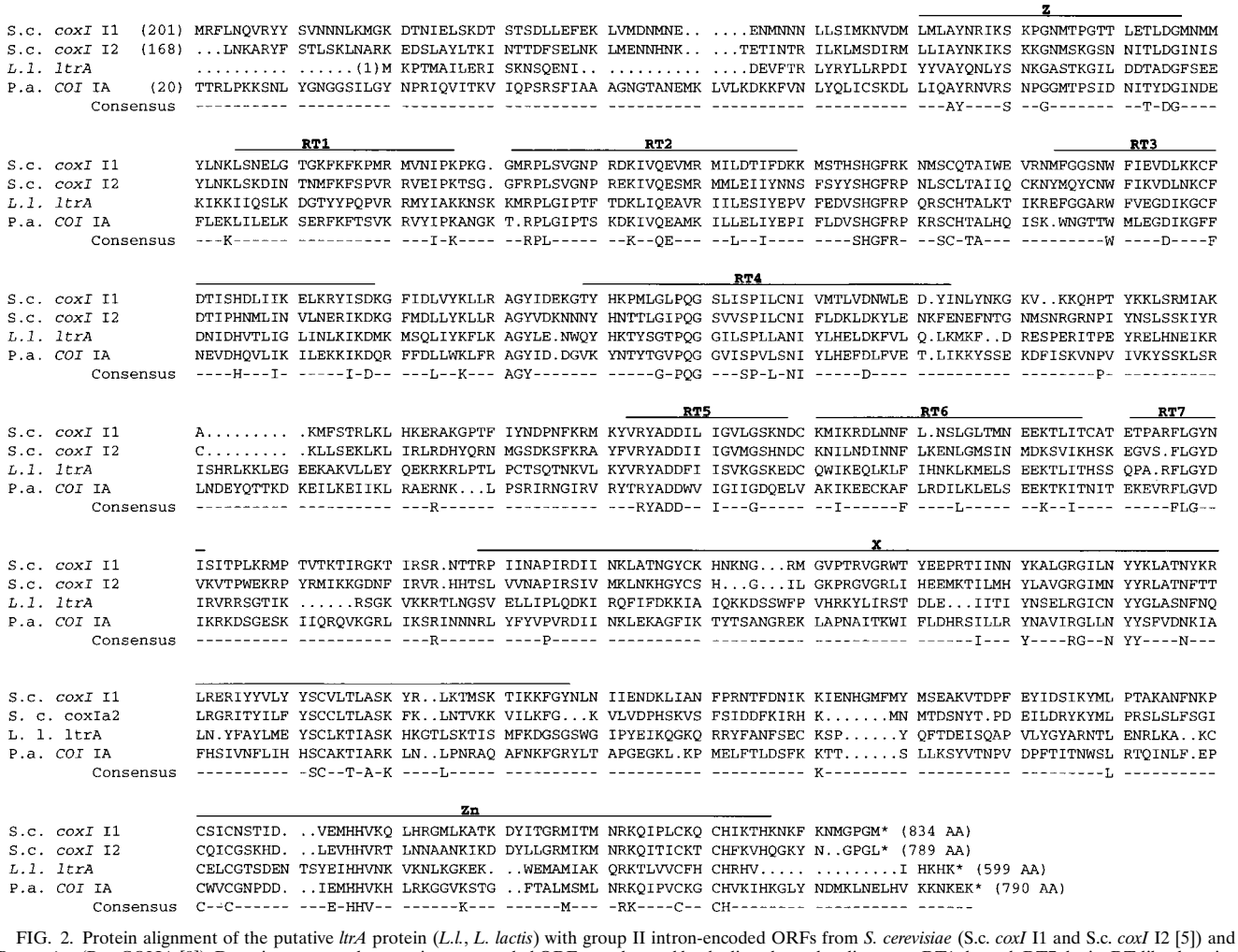


FIG. 2. Protein alignment of the putative *ltrA* protein (*L.l.*, *L. lactis*) with group II intron-encoded ORFs from *S. cerevisiae* (S.c. *coxI* I1 and S.c. *coxI* I2 [5]) and *P. anserina* (P.a. *COI* IA [9]). Domains conserved among intron-encoded ORFs are denoted by the line above the alignment. RT1 through RT7 depict RT-like domains (55). The maturase-specific domain is designated X, (35), and the zinc finger-like domain is designated Zn (49). Individual similarities to *ltrA*: for P.a. *COI* IA, BLAST analysis score of 253, Poisson probability [*P*] of $3.3 \times 10^{-106}$, and FASTA analysis results of 34.1% identity over 589 amino acids; for S.c. *coxI* I1, BLAST analysis score of 247, Poisson probability [*P*] of $2.4 \times 10^{-68}$, and FASTA analysis results of 30.1% identity over 612 amino acids; for S.c. *coxI* I2, BLAST analysis score of 235, Poisson probability [*P*] of $2.5 \times 10^{-71}$, and FASTA analysis results of 30.2% identity over 597 amino acids). For comparison, similarities to the *Calothrix* sp. intron-encoded ORF (BLAST analysis score, 137; Poisson probability [*P*] of $2.2 \times 10^{-31}$; FASTA analysis results, 32.5% identity over 237 amino acids) or *E. coli* intron-encoded ORF (BLAST analysis score, 143; Poisson probability [*P*] of $4.6 \times 10^{-26}$; FASTA analysis results, 32.3% identity over 186 amino acids) were lower than those of the fungal and plant mitochondrial group II intron-encoded ORFs.

5′ and 3′ consensus splice site sequences (GUGYG and AY, respectively) (31).

**Characterization of the Ll.ltrB intron structure.** Identification of the splice junction indicates that the Ll.ltrB intron spans 2,492 nucleotides and is similar in size to other ORF-containing group II introns (26, 31, 44). Ll.ltrB does not appear to be a recent genetic acquisition in lactococci, since the G/C ratio of the complete intron (36%) and codon usage for *ltrA* are consistent with lactococcal genomes (codon usage for *ltrA* is most similar to a codon usage table complied from *L. lactis* genes as determined by CODONPREFERENCE provided by GCG) (6, 46). A combination of comparative analysis and computer modeling of the lactococcal intron RNA secondary structure revealed that Ll.ltrB can be folded into the six RNA secondary-structure domains common to group II introns (31) (Fig. 5). The Ll.ltrB intron belongs to the group II intron subgroup IIA and shares many of the common nucleotides and potential tertiary pairings identified in a consensus model (31). These include a bulging adenine residue on the 3′ side of helix domain VI (seven nucleotides from the 3′ splice site) which has been shown to constitute a 2′ to 5′ lariat branch point in several group II introns (30, 31). Other common structures within the Ll.ltrB RNA folding domains are intron-exon pairings EBS1-IBS1 and EBS2-IBS2 and potential internal interactions, namely, α-α′, ε-ε′, δ-δ′, γ-γ′, β-β′, and ζ-ζ′ (7, 24, 30, 31). Two potential EBS2 sites were identified within the domain I (Fig. 5, EBS2$^a$ and EBS2$^b$), both of which can pair with the IBS2 sequence over 6 nucleotides.

***ltrB* resembles several bacterial relaxases.** Database searches with the spliced *ltrB* ORF revealed strong similarity to predicted relaxase proteins from *Streptococcus pneumoniae* Tn*5252* (GenBank accession number L29324) and the *Staphylococcus aureus* plasmids pC221 (41), pS194 (42), and pC223 (11). As shown in Fig. 6, a protein alignment of *ltrB* and several relaxase ORFs revealed sequence motifs indicative of initiator proteins of rolling circle DNA replication (23). Analysis of the *ltrB* amino acid sequence suggests it falls into family 1 of the mobilization (Mob) protein class of rolling circle DNA replication

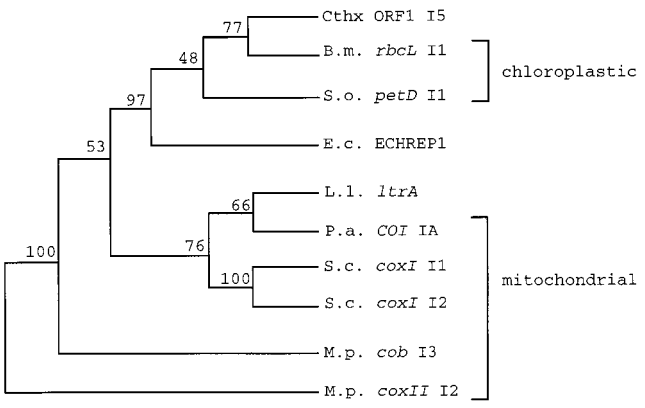


FIG. 3. Phylogenetic analysis of *ltrA*. An unrooted phylogenetic tree was constructed from the predicted *ltrA* protein and select group II intron-encoded ORFs representing the major phylogenetic groupings (13); bootstrap values (100 bootstrap resamplings) are presented at the nodes. Species abbreviations: L.l., *L. Lactis*; B.m., *Bryopsis maxima*; M.p., *Marchanta polymorpha*; S.c., *S. cerevisiae*; P.a., *P. anserina*; S.o., *Scenedesmus obliquus*; E.c., *E. coli*; Cthx, *Calothrix* sp. Acronyms and accession numbers: eukaryotic group II intron-encoded ORFs, B.m. *rbcL* I1 (X55877), M.p. *coxII* I2 (S25949), M.p. *cob* I3 (S25952), S.c. *coxI* I1 (P03875), S.c. *coxI* I2 (P03876), P.a. *COI* IA (E48327), S.o. *petD* I1 (P19593); bacterial group II intron-encoded ORFs, *E. coli* ECHREP1 (X77508), Cthx ORF1 I5 (X71404), and L.l. *ltrA* (U50902). The B.m. *rbcL* I1 ORF was compiled as indicated previously (35). All deduced amino acid sequences were aligned by use of the Pileup program (GCG package) and manually refined over 213 sites encompassing the conserved polymerase-like domains I to VII (55). Phylogenetic analysis was obtained by use of the maximum parsimony method with the PROTPARS program in the PHYLIP 3.5c package (12) with bootstrap analyses obtained by using the SEQBOOT, PROTPARS, and CONSENSE programs in PHYLIP 3.5c. An analysis of the data using a distance matrix generated by PROTDIST and the Neighbor-Joining algorithm (NEIGHBOR) produced a similar consensus tree (34).

initiator proteins with conservation of Mob protein domains 2 and 3 (23). Interestingly, the group II intron splice junction falls between the two conserved histidines in domain 2 proposed to function as ligands for a metal ion cofactor (23). This suggests that translation of the unspliced exon *ltrBE1* would result in a nonfunctional relaxase and supports the hypothesis that splicing of Ll.ltrB is necessary for conjugative transfer of pRS01.

## DISCUSSION

Group II introns are self-splicing RNA enzymes commonly found in fungal and plant mitochondria and in chloroplasts (30). Several groups have recently identified group II introns within the genomes of select proteobacteria and cyanobacteria (13, 14, 25). Molecular analysis of group II introns in *S. cerevisiae* has revealed a retroelement capability which enables the introns to integrate into specific sites of the genome (36, 57). In addition, group II introns have also been shown to transpose independently to novel sites (37, 47). While retrotransposition of bacterial group II introns has not been demonstrated, the few introns characterized revealed flanking exons potentially involved in DNA mobility (13, 25), suggesting horizontal genetic transfer as an additional mode of intron dissemination in bacteria. The identification of Ll.ltrB within the conjugative relaxase of pRS01 extends the association of bacterial group II introns with mobile elements in prokaryotes by demonstrating the involvement of intron splicing in the pRS01 conjugative process. Since splicing-dependent conjugative transfer events can be readily detected at frequencies ranging from $10^{-2}$ to $10^{-9}$ transconjugants per donor in pRS01 (Table 1) (33), this system is ideal for genetic analysis of group II intron structure and function.

The conjugative element pRS01 and the homologous *L. lactis* subsp. *lactis* 712 sex factor have been shown to reside in

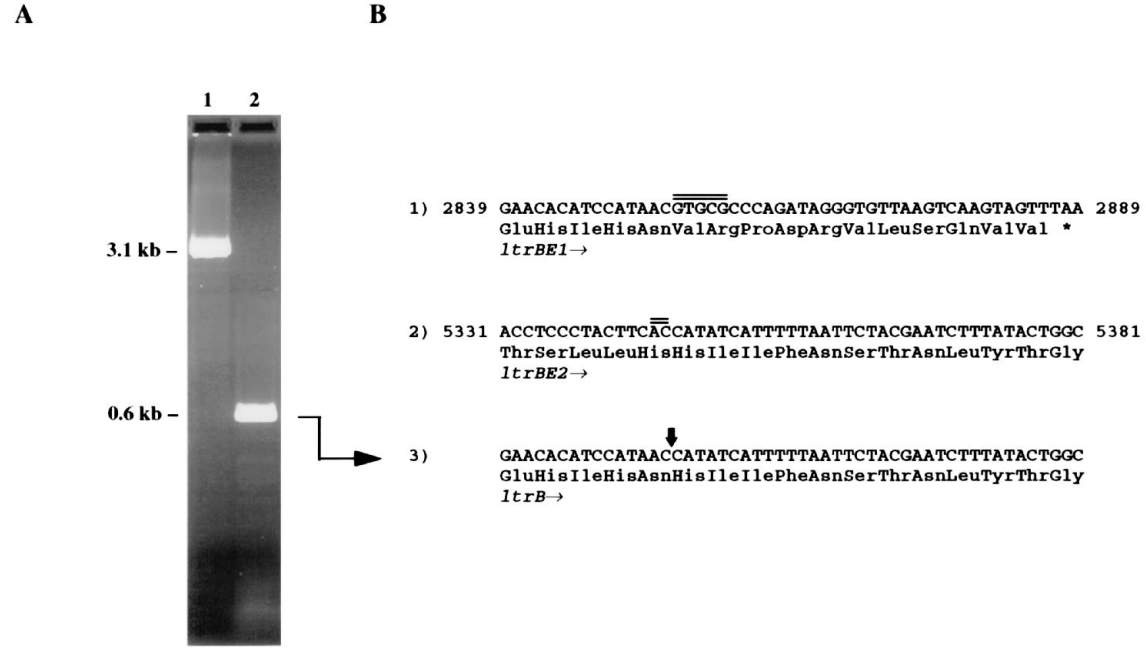


FIG. 4. Splicing of Ll.ltrB in vivo. (A) Identification of spliced *ltrB* product. Electrophoretic analysis of the PCR product obtained by employing primers A21230 and LtrBRT1 (see Fig. 1) on plasmid pM2036 as a template (lane 1) and the reverse transcription-PCR product from *L. lactis* subsp. *lactis* DM2036 (strain which contains pM2036) by using the same primers (lane 2). (B) DNA sequence of the 5′ (sequence 1) and 3′ (sequence 2) junction sites of the Ll.ltrB intron. Double lines above the sequence correspond to group II intron splice sites consensus sequences (26, 31, 44). Sequence 3 is the sequence of spliced *ltrB* reverse transcription-PCR product. The thick arrow indicates the splice junction of *ltrB*.

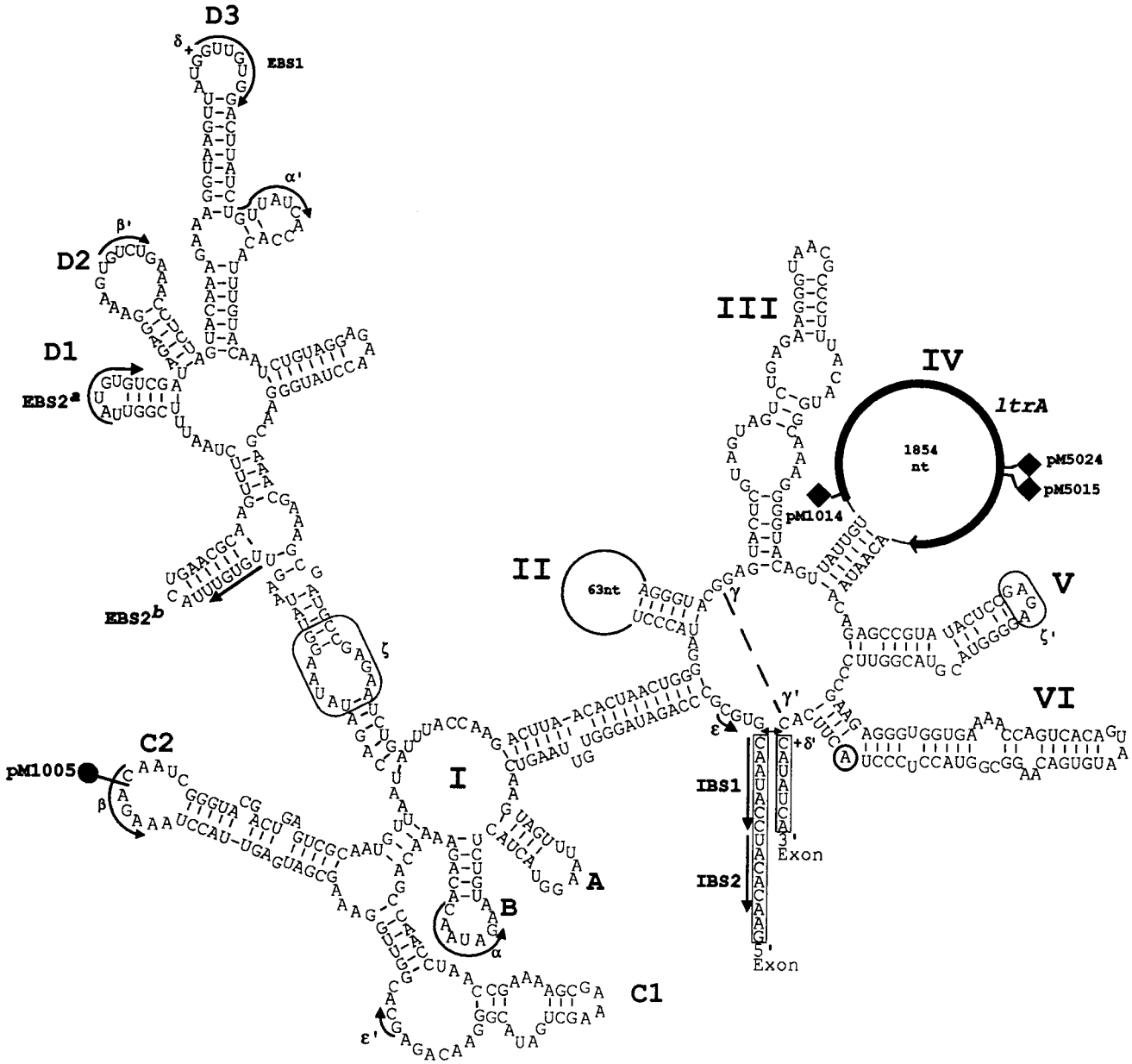


FIG. 5. Secondary-structure model of the Ll.ltrB intron. The model was constructed by comparative analysis with a consensus group II intron structure model (31) combined with RNA folding analysis by using the MFOLD program (GCG package). Bold roman numbers correspond to the six major structural domains of group II introns. Potential tertiary pairings are designated α-α′, β-β′, δ-δ′, ε-ε′, γ-γ′, ζ-ζ′, and EBS1-IBS1 (7, 24, 30, 31). EBS2$^a$ and EBS2$^b$ represent two potential tertiary pairings with IBS2. *ltrA* is encoded completely within domain VI. Insertions are indicated by the plasmid designation, and accompanying symbols indicate plasmid transfer frequency category: ●, $10^{-7}$ to $10^{-9}$ transconjugants per donor: ◆, $<10^{-9}$ transconjugants per donor (as described in reference 33). The splice site is indicated by ↔. Exon sequences are boxed. The bulging A residue (circled) in domain VI is predicted to be involved in lariat formation (30). nt, nucleotides.

several *L. lactis* subsp. *lactis* strains (19). Recently, a group II intron similar to Ll.ltrB has also been identified in the sex factor from *L. lactis* subsp. *lactis* 712 (17). Splicing of this intron has also been demonstrated in vivo (18). We have detected sequences homologous to *ltrA* in several *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, and *L. lactis* subsp. *lactis* biovar *diacetylactis* strains (34); however, in every case, the *ltrA* sequences were linked to *ltrB* sequences, suggesting the presence of the complete pRS01 element and not an independent Ll.ltrB intron.

The discovery of Ll.ltrB provides experimental support for the proposal that theories on the origin of group II introns must be considered in the context of genetic transfer as a means of intron dissemination (25). Genetic transfer between phylogenetically diverse bacteria has been demonstrated with a variety of conjugative systems (28). Recently, the plasmid pM2036 used in this study was shown to transfer to other gram-positive genera (streptococci and lactobacilli) (34), a result which indicates that the distribution of the Ll.ltrB intron may encompass a wider spectrum of gram-positive bacteria as well. Analyzing antibiotic resistance genes, Courvalin (8) proposed a natural conjugative gene flux from gram-positive to gram-negative bacteria on the basis of several lines of evidence, including the fact that gram-positive genes are common-

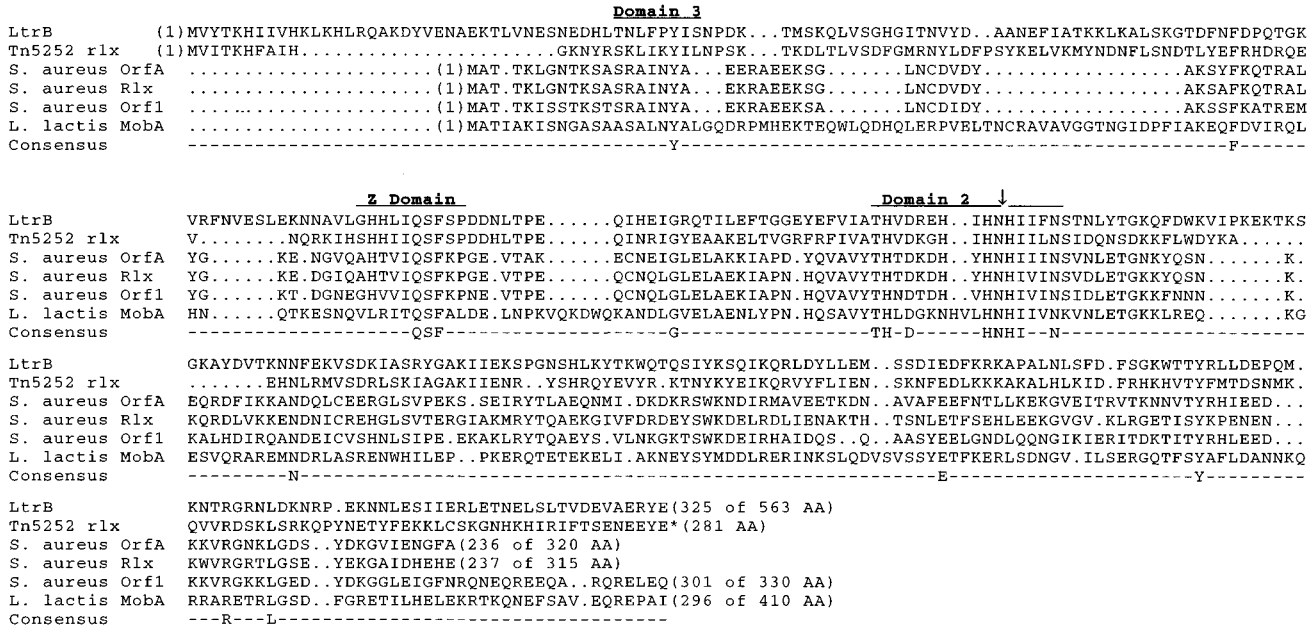


FIG. 6. Alignment of *ltrB* with several plasmid and transposon relaxases. Protein alignment of the encoded *ltrB* protein with the Tn*5252* relaxase (GenBank accession number L29324), *Staphylococcus aureus* plasmid pS194, pC221, and pC223 relaxases (11, 41, 42), and the lactococcal plasmid pCI528 mobilization protein MobA (27). Conserved domains 3, Z, and 2 (23) are denoted by the line above the alignment. The vertical arrow indicates the Ll.ltrB intron splice junction. Individual similarities to *ltrB*: for Tn*5252* relaxase, BLAST analysis score of 230, Poisson probability (*P*) of $1.2 \times 10^{-50}$, and FASTA analysis results of 38.2% identity over 259 amino acids; for pS194 Rlx1, BLAST analysis score of 104, Poisson probability (*P*) of $9.8 \times 10^{-7}$, and FASTA analysis results of 30.5% identity over 105 amino acids; for pC221 Rlx2, BLAST analysis score of 96, Poisson probability (*P*) of $1.8 \times 10^{-5}$, and FASTA analysis results of 20.9% identity over 235 amino acids; for pC223 Rlx3, BLAST analysis score of 97, Poisson probability (*P*) of $4.4 \times 10^{-5}$, and FASTA analysis results of 25.8% identity over 259 amino acids; for pCI528 MobA, FASTA analysis results of 19.6% identity over 352 amino acids. AA, amino acids.

ly expressed in gram-negative bacteria while the reverse is not generally observed. These observations coupled with the present results suggest that group II introns in gram-positive bacteria may represent a focal point for dissemination of introns to other bacteria.

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