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Homing of a Group II Intron from *Lactococcus lactis* subsp. *lactis* ML3

DAVID A. MILLS,[1]† DAWN A. MANIAS,[1] LARRY L. McKAY,[2] AND GARY M. DUNNY[1]*

Department of Microbiology, University of Minnesota, Minneapolis, Minnesota 55455-0312,[1] and Department of Food Science and Nutrition, University of Minnesota, St. Paul, Minnesota 55108[2]

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Ll.ltrB is a functional group II intron located within a gene (*ltrB*) encoding a conjugative relaxase essential for transfer of the lactococcal element pRS01. In this work, the Ll.ltrB intron was shown to be an independent mobile element capable of inserting into an intronless allele of the *ltrB* gene. Ll.ltrB was not observed to insert into a deletion derivative of the *ltrB* gene in which the intron splice site was removed. In contrast, a second vector containing a 271-nucleotide segment of *ltrB* spanning the Ll.ltrB splice site was shown to be a proficient recipient of intron insertion. Efficient homing was observed in the absence of a functional host homologous recombination system. This work demonstrates that the Ll.ltrB intron is a novel site-specific mobile element in lactococci and that group II intron self-transfer is a mechanism for intron dissemination among bacteria.

Group II introns are a unique class of introns common to fungal and plant mitochondria and chloroplasts and recently discovered in bacteria (3, 17). These introns are catalytic RNAs which splice via a lariat mechanism similar to the splicing of pre-mRNAs in the nucleus of eukaryotes (17). Some group II introns are also mobile genetic elements which possess the ability to efficiently insert themselves into cognate alleles that lack the intron (a process called homing) and, at a low frequency, to transpose to external sites (5).

While group II introns have only recently been discovered in bacteria, their distribution appears to be widespread. Group II introns have been identified within proteobacteria (*Azotobacter vinelandii* and *Escherichia coli*) (7, 8, 11), cyanobacteria (*Calothrix* sp.) (7), and gram-positive bacteria (*Lactococcus lactis* and *Clostridium difficile*) (20, 23, 29). Several of these bacterial group II introns were found to have flanking exons involved, or potentially involved, in DNA mobility, suggesting horizontal genetic transfer as a mode of intron dissemination in bacteria (8, 11, 20, 23, 29). Indeed, all group II introns identified in gram-positive bacteria were localized within conjugative elements possessing a broad host range of functional transfer (4, 18). While group II introns are clearly disseminated among bacteria by linkage to conjugal elements, independent mobility of group II introns by homing or by random transposition has not been reported.

pRS01 is a broad-host-range conjugative element common to many strains of *L. lactis* subsp. *lactis* (9). Previous work on pRS01 identified a group II intron, termed Ll.ltrB, within a gene encoding a conjugative relaxase (*ltrB*) (20). Splicing of Ll.ltrB was also demonstrated in vivo. Many group II introns possess an intron-encoded protein (IEP) involved in both intron splicing and mobility (12, 17). The Ll.ltrB-encoded protein, LtrA, contains three protein domains common to group II intron IEPs: a retroelement reverse-transcriptase domain, an intron maturase motif, and a C-terminal Zn^{2+} finger-like domain associated with DNA endonuclease activity (10, 30) and recently shown to be involved in intron mobility in the yeast *Saccharomyces cerevisiae* (21, 33). Interestingly, phylogenetic analysis of eukaryotic and bacterial group II IEPs suggested that LtrA is more closely related to those IEPs from fungal and plant mitochondria than to those from other bacterial group II introns identified to date (20, 23).

The group II intron aI2 from *S. cerevisiae* has recently been shown to be an independently mobile element which resembles a site-specific retrotransposon (21, 33, 34). Given the strong similarity between LtrA and the IEP from the yeast aI2 intron, we sought to determine if the Ll.ltrB intron is a mobile genetic element within lactococci. In this work, we demonstrated that the Ll.ltrB intron is capable of homing into an intronless allele of *ltrB* in vivo. The mobility of the Ll.ltrB intron establishes self-transfer as a mechanism by which group II introns can be disseminated among bacteria.

MATERIALS AND METHODS

Microorganisms and culture conditions. *E. coli* DH5α was grown in LB medium (26). Mobility assays were performed with the Rec$^-$ strain *L. lactis* subsp. *lactis* MMS370 (1) or the Rec$^+$ strain *L. lactis* subsp. *lactis* LM0230 (16). *L. lactis* strains were grown in GM17 (M17 medium [31] containing 0.5% glucose) at 30°C. Selective media contained the following antibiotics at the noted concentrations: erythromycin, 10 μg/ml; chloramphenicol, 5 μg/ml for *L. lactis* and 20 μg/ml for *E. coli*; and spectinomycin, 300 μg/ml for *L. lactis* and 50 μg/ml for *E. coli*. All plating media contained 1.5% Bacto Agar.

DNA manipulation and analysis. General molecular biology techniques were performed as indicated in reference 26. Plasmid isolation, electroporation, PCR, and reverse transcription-PCR were performed as described previously (20). Plasmid pLE23-IN, pLE23ΔH, and pMN1343-IN intron-exon junctions were sequenced via fluorescent sequencing with the Applied Biosystems (Foster City, Calif.) Prism DyeDeoxy Terminator Cycle Sequencing Kit and analyzed on the Applied Biosystems model 373 DNA sequencer. Southern transfers to MSI Magnagraph nylon membranes (Micron Separations, Inc., Westboro, Mass.) were conducted as described elsewhere (2). Hybridization and detection were performed with the Genius nonradioactive DNA labeling and detection kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) as indicated by the manufacturer. The digoxigenin-labeled, intron-specific probe (5′-CGCT ATCATTGCCATTTCCCATTTTTCTTTGCCTTTAAGATTTTTGACCT-3′) was purchased from Genoysis Biotechnologies, Inc. (The Woodlands, Tex.). Densitometric scanning of ethidium bromide-stained agarose gels containing lactococcal plasmid DNA was carried out with an Alpha Imager 2000 analysis and documentation system (Alpha Innotech, San Leandro, Calif.) equipped with the Alpha-Ease image analysis software package. The amount of DNA for each plasmid species was estimated by comparison with lanes loaded with known quantities of plasmid DNA of a size similar to that of the unknown. The molar ratios of various plasmid species in each preparation were calculated based on the amount of DNA in each band and the differences in size between the two plasmid species analyzed.

* Corresponding author. Mailing address: Department of Microbiology, University of Minnesota, 1460 Mayo Memorial Building, 420 Delaware St., S.E., Minneapolis, MN 55455-0312. Phone: (612) 625-9930. Fax: (612) 626-0623. E-mail: gary-d@biosci.cbs.umn.edu.

† Present address: Department of Food Science, North Carolina State University, Raleigh, NC 27695-7624.

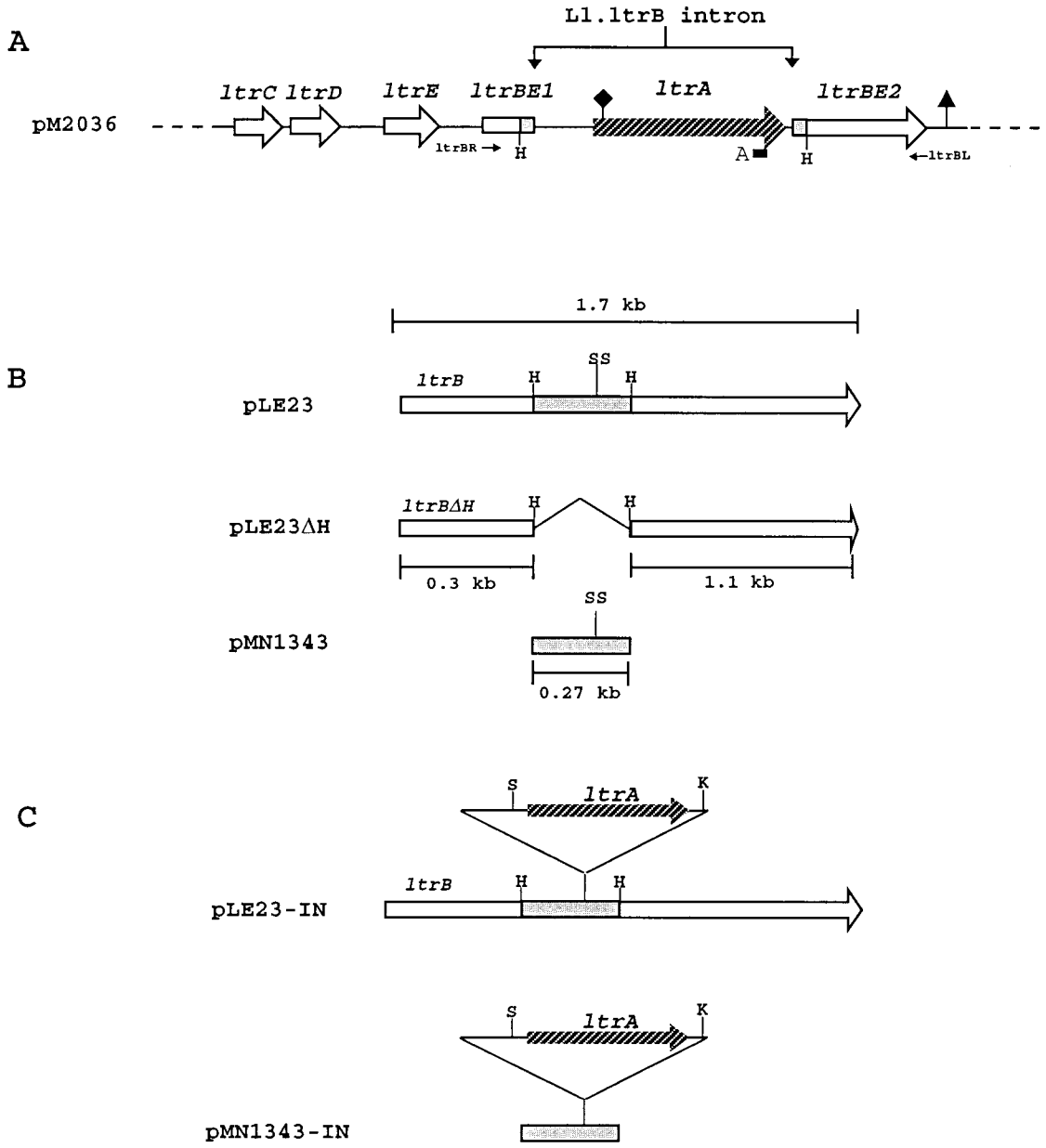

FIG. 1. Genetic constructs used for Ll.ltrB intron mobility assays. (A) Organization and insertion map of the pRS01 Tra1 region. The Ll.ltrB intron is contained within the *ltrBE1* and *ltrBE2* exons. ltrBR and ltrBL designate primers used for amplification of the *ltrB* cDNA. The solid rectangle designated by the letter A indicates the region of the intron against which the intron-specific probe was synthesized. ▲ and ◆, sites of cointegrate plasmid insertion within plasmids pM2036 and pM1014, respectively (for a more complete description of these insertions, see references 19 and 20). (B) Target site plasmid constructs. Plasmid pLE23 contains the intact *ltrB* gene with the Ll.ltrB intron splice site (SS). pLE23ΔH is deleted of a 271-nt segment internal to *ltrB* and thus lacks the intron splice site. pMN1343 is a pDL278 derivative (14) containing the 271-nt *ltrB Hin*dIII fragment which harbors the intron splice site. (C) pLE23-IN and pMN1343-IN represent target site plasmids into which the Ll.ltrB intron has inserted. Restriction site abbreviations: H, *Hin*dIII; S, *Sca*I; K, *Kpn*I.

Construction of plasmids pLE23, pLE23ΔH, and pMN1343. Plasmid pLE23 was constructed by cloning the *ltrB* gene generated via reverse transcription-PCR, using the oligonucleotide primers ltrBR (5′-GGGGCATATGATG GTTTACACAAAACACATTATTG-3′) and ltrBL (5′-GGGGAATTCTGA TAGCGTAAGAAATTATAGTATTTTTC-3′) and RNA purified from *L. lactis* subsp. *lactis* DM2036 as described previously (20). The amplified *ltrB* product was cleaved with restriction enzymes *Nde*I and *Eco*RI and subcloned into similarly cut plasmid pET-28b (Novogen Inc., Madison, Wis.), resulting in plasmid pLtrBH. Plasmid pLtrBH was then cleaved by *Xba*I-*Sal*I double digestion, and the *ltrB* gene was cloned into similarly cut plasmid pLE1 (19), resulting in plasmid pLE23. Plasmid pLE23ΔH was generated by cleaving plasmid pLE23 with *Hin*dIII followed by self-ligation to remove the *Hin*dIII fragment internal to the *ltrB* gene. Plasmid pMN1343 was generated by cloning the 271-nucleotide (nt) *Hin*dIII fragment into shuttle plasmid pDL278 (14).

RESULTS

The Ll.ltrB intron is a mobile element. The most prevalent form of group II intron mobility is homing, or invasion into an intronless allele (3, 12, 17). To assay for homing of the Ll.ltrB intron, it was necessary to construct an intronless allele of the *ltrB* gene in the *E. coli-L. lactis* shuttle plasmid pLE1 (19). Plasmid pLE23 contains the complete *ltrB* gene, including the intron splice site junction (Fig. 1B). This plasmid was transformed into the Rec$^-$ *L. lactis* strains DM2036 and DM1014 (19). Strain DM2036 harbors the transfer-proficient (Tra$^+$) cointegrate plasmid pM2036, which has been shown to possess

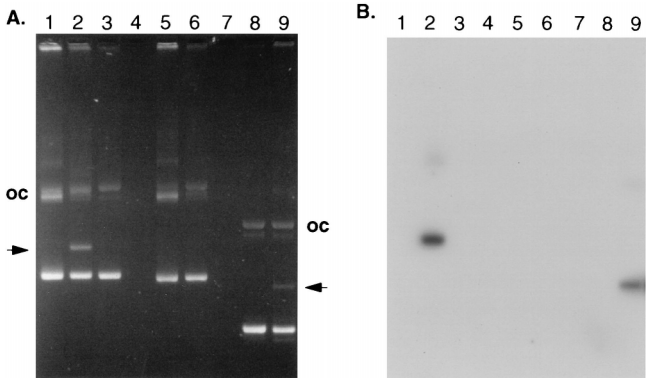

FIG. 2. Gel (A) and Southern blot (B) analysis of plasmids isolated from various lactococci used in the Ll.ltrB mobility assay. The procedures used for extraction of plasmid DNA and the gel electrophoresis conditions used in this experiment were designed for analysis of small plasmids; thus the larger pM2036 and pM1014 plasmids (both ~60 kb) are not visible in these gels. Lanes: 1, pLE23; 2, pM2036 and pLE23; 3, pM1014 and pLE23; 4, blank; 5, pLE23ΔH; 6, pLE23ΔH and pM2036; 7, blank; 8, pMN1343; 9, pMN1343 and pM2036. Southern analysis was performed with the intron-specific probe depicted in Fig. 1. OC, open circular forms of the target plasmids. The arrows indicate the positions of the novel forms of the target plasmids pLE23 and pMN1343. These new plasmid bands (pLE23-IN, left arrow; pMN1343-IN, right arrow) migrate to a region 2.5 kb above the cognate target plasmids.

a functional group II intron (Ll.ltrB [Fig. 1A]) (20). Strain DM1014 contains the cointegrate plasmid pM1014, in which the IS*946*-containing plasmid pTRK28 has inserted within Ll.ltrB (Fig. 1A). Plasmid pM1014 does not transfer conjugatively and does not produce a spliced *ltrB* mRNA product (19, 20). Examination of the plasmid pool from DM2036(pLE23) revealed the presence of a novel DNA species with an apparent size of 12.5 kb, approximately 2.5 kb larger than the pLE23 plasmid (Fig. 2A, lane 2). In addition, this novel plasmid hybridized with an intron-specific probe (Fig. 2B, lane 2). In contrast, the plasmid pool from strain DM1014(pLE23), which possesses a nonfunctional intron, lacked a similarly sized band (Fig. 2A, lane 3) and did not exhibit any hybridization to the intron-specific probe (Fig. 2B, lane 3). To determine if this novel band represented plasmid pLE23 which had acquired the 2.5-kb Ll.ltrB intron, the DNA in the band was extracted from the gel and transformed into *E. coli* DH5α. Restriction and PCR analysis of the transformants revealed the presence of the Ll.ltrB intron in the *ltrB* gene contained within pLE23 (designated pLE23-IN). Sequence analysis confirmed that the 5′ and 3′ Ll.ltrB intron-exon splice junctions on pLE23-IN were identical to those present in the wild-type Tra1 region of pRS01 (20). As a control, the region of the gel from the lane containing plasmid DNA prepared from DM1014(pLE23) (Fig. 2A, lane 3) and corresponding to the position of migration of a 12.5-kb plasmid was excised and the extracted DNA was transformed into *E. coli* DH5α. All transformants obtained (a total of four) were analyzed by restriction enzyme digestion and shown to be the 10-kb plasmid pLE23 which had migrated aberrantly to the 12.5-kb region of the gel.

The *ltrB* splice site region is a target for mobility. In fungal mitochondria, group II introns have been shown to home into intronless alleles (13, 21) and, at a lower frequency, to transpose to ectopic sites (22, 28). To assess whether the splice site region of the *ltrB* gene was required for intron insertion, a deletion construct of pLE23 was generated by *Hin*dIII digestion followed by self-ligation to generate pLE23ΔH. Plasmid pLE23ΔH contains a deletion internal to *ltrB* which extends 179 nt upstream and 92 nt downstream of the Ll.ltrB intron splice site (Fig. 1B). This 271-nt *Hin*dIII fragment was also cloned into the *L. lactis-E. coli* shuttle vector pDL278 (14) to generate an additional homing target construct, pMN1343. Analysis of plasmids from strain DM2036(pMN1343) revealed the presence of a novel 9.5-kb band (Fig. 2A, lane 9) migrating above the 7-kb pMN1343 species and hybridizing to the intron-specific probe (Fig. 2B, lane 9). DNA from this band was extracted and transformed into *E. coli* DH5α, and 10 of the 217 resultant transformants were screened for the presence of the intron. Eight of 10 transformants contained plasmid pMN1343 with the Ll.ltrB intron (designated pMN1343-IN). Sequence analysis of pMN1343-IN confirmed the presence of Ll.ltrB intron splice junctions identical to the intron junctions on pRS01 (20). In contrast, gel analysis of the plasmids isolated from DM2036(pLE23ΔH) revealed no apparent novel bands migrating 2.5 kb above pLE23ΔH (Fig. 2A, lane 6), and no intron-specific hybridization was observed (Fig. 2B, lane 6). DNA from the region 2.5 kb above pLE23ΔH was purified and transformed into *E. coli* DH5α. Eighteen transformants were obtained, and 10 of these were analyzed by restriction enzyme digestion; the intron splice site regions of two of the isolates were also sequenced. All transformants analyzed contained pLE23ΔH, which had migrated aberrantly in the original gel. These results demonstrate that, similar to fungal group II introns, the mobility of the Ll.ltrB intron is preferential for the intronless *ltrB* allele, specifically the region encompassing the intron splice site. Although the sensitivity of detection of homing cannot be stated absolutely, examination of the hybridization and transformation data presented above indicates that deletion of the 271-bp target site reduces homing into the *ltrB* allele by at least 100-fold.

Lactococcal Rec functions do not play a major role in Ll.ltrB homing. The fact that homing events could be detected, without selection, at a level sufficient to generate a visible product on a gel suggests that this process is efficient in lactococci. To confirm this observation, and to examine the effects of host generalized recombination function on Ll.ltrB homing, we did a comparative analysis of homing of the intron in Rec^+ and Rec^- lactococcal hosts. The experiment was designed such that homing was measured in various strains in which the donor and target plasmids were maintained together for an approximately equal number of generations. Rec^+ strain LM0230(pM2036) and Rec^- strain MMS370(pM2036) were each transformed by electroporation with pMN1343 DNA. For each transformation, 10 colonies (1 to 1.5 mm in diameter) of resultant transformants were picked, inoculated individually into 4 ml of liquid medium, and grown for 18 h. The turbidities of the cultures were measured spectrophotometrically, and each culture was diluted to an A_{600} of 0.3. Plasmid DNA was then extracted from a 3.5-ml volume of each culture and was analyzed by gel electrophoresis. Densitometric analysis of the pMN1343 and pMN1343-IN DNAs in each transformant strain was carried out, and the molar ratios of the two species were calculated. All transformants showed significant homing of the intron, with the pMN1343-IN/pMN1343 molar ratio generally exceeding 1 (Fig. 3). Interestingly, homing appeared to be more efficient in the Rec^- background, although differences in growth rates and other variables probably had some effect on the observed ratios of the two plasmids. In spite of these limitations, it is clear that homologous recombination is not required for efficient homing in this system. The lack of homing into the pLE23ΔH target, which contains 1.4 kb of homology to the intron donor plasmid pM2036 (Fig. 1), also argues against a critical role for generalized recombination in this phenomenon in lactococci. Host recombination functions have been implicated in the group II intron reverse transcriptase

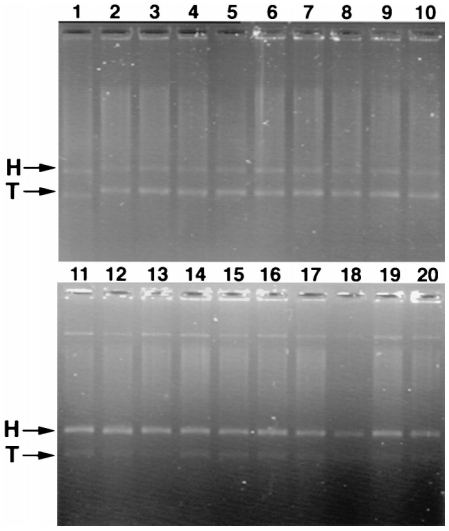

FIG. 3. Intron mobility in Rec$^+$ and Rec$^-$ Strains. Rec$^+$ strain LM0230 (pM2036) and Rec$^-$ strain MMS370(pM2036) were transformed with pMN1343 DNA. Ten transformant colonies of each strain were selected, grown to approximately equal cell numbers, and subjected to plasmid DNA isolation and analysis by agarose gel electrophoresis as described in the text. The amounts of pMN1343 target DNA (arrows designated T) and pMN1343-IN homed DNA (arrows designated H) in each lane were determined as described in Materials and Methods, and the molar ratios of the two species were calculated. The top panel shows the plasmid profiles of the Rec$^+$ transformants, and the bottom panel shows the profiles of the Rec$^-$ transformants. The pMN1343-IN/pMN1343 molar ratios for the isolates were as follows: lane 1, 1.42; lane 2, 1.36; lane 3, 1.24; lane 4, 1.24; lane 5, 1.18; lane 6, 1.30; lane 7, 1.30; lane 8, 1.36; lane 9, 1.18; lane 10, 1.30; lane 11, 1.78; lane 12, 1.95; lane 13, 1.98; lane 14, 1.86; lane 15, 1.79; lane 16, 1.78; lane 18, 1.59; lane 19, 1.86; and lane 20, 1.63.

(RT)-independent homing pathway observed in *S. cerevisiae* (6, 21). In addition, host recombination has been proposed to be involved in the later stages of the retrohoming pathway (6).

DISCUSSION

This work demonstrates that the group II intron from the lactococcal element pRS01 is an independent mobile element capable of homing into an intronless allele of the *ltrB* gene. Recent analysis of the yeast mitochondrial group II introns has revealed that homing occurs via a complex pathway (5, 32, 33). Homing is initiated by a unique catalytic ribonucleoparticle composed of spliced lariat RNA and IEPs acting in concert to generate a double-stranded cleavage at a cognate intronless allele (32, 33). A reverse splicing reaction then integrates intron RNA into the DNA target site (6, 32, 33), and ensuing strand synthesis and repair functions are likely to complete the integration of the intron-containing allele. Group II introns from fungal mitochondria have also been shown to transpose, at low frequencies, to novel sites (22, 27, 28). This type of mobility is proposed to occur via reverse splicing of an intron into an alternate RNA species followed by RT-mediated cDNA synthesis and subsequent allelic replacement of the homologous genomic site with the newly synthesized cDNA (22, 28). Alternatively, transposition to novel sites may occur by reverse splicing of intron RNA directly into a novel DNA site and subsequent conversion to DNA via IEP-directed RT activity and ensuing synthesis and repair functions (6). Finally, an additional mechanism of group II intron mobility has been suggested based on the homing of certain aI2 intron mutants which lack RT activity. It was proposed that these mutants invade intronless alleles via a double-stranded gap repair mechanism similar to that of group I introns (6, 21, 33). The mechanism for mobility of the Ll.ltrB intron remains to be determined. However, recent experiments involving expression of cloned Ll.ltrB DNA in *E. coli* (15) indicate that maturase and RT activities are conferred by the LtrA protein and that DNA endonuclease activity is present in Ll.ltrB-derived ribonucleoprotein particles. This suggests that the splicing and mobility mechanisms of bacterial group II introns and those of the fungal elements may have many common features. Analysis of homing in the Rec$^-$ lactococcal strain MMS370 suggests that wild-type levels of host recombination activity are not essential for homing of the Ll.ltrB intron, implying that proposed group II intron mobility mechanisms which rely heavily on host-mediated recombination may not be operative in the Ll.ltrB intron. Although such mechanisms may occur in recombination-proficient hosts, our data suggest that lactococcal generalized recombination functions do not significantly increase the overall efficiency of intron homing.

Most of the bacterial group II introns identified to date have been localized within genes likely to be involved in DNA mobility, thereby implicating genetic transfer as a means of intron dissemination within bacteria (8, 11, 20, 23, 29). Evidence in *C. difficile* of the recent horizontal transfer of a group II intron was suggested when a comparison of the G/C ratio of the host genome with that of the transposon Tn*5397* revealed significant differences, suggesting that the intron-containing transposon was a recent immigrant from another host (23). This is not surprising given that Tn*5397* has been shown to transfer among strains of *C. difficile* and from *C. difficile* to *Bacillus subtilis* (24). Moreover, sequence analysis of Tn*5397* suggests that it is related to the Tn*916*-Tn*1545* family of broad-host-range conjugative transposons (4, 23). The likelihood of horizontal transfer of group II introns is also suggested by the broad host range of the lactococcal conjugal element pRS01 (25). Interestingly, in both Tn*5397* and pRS01, the group II introns were localized within genes essential for conjugative transfer, thereby coupling intron splicing activity with conjugative transfer ability (20, 23, 29).

The demonstration of homing of the lactococcal Ll.ltrB intron identifies an additional mechanism for intron dispersal in bacteria. Indeed, the dependence of conjugative transfer on functional intron splicing in pRS01 coupled with the ability of introns to self-mobilize provides an exquisite mechanism for dispersal of viable group II introns among bacterial genomes. Work with yeast group II introns demonstrated that nonsplicing intron constructs were also nonmobile (13, 21). By analogy, one would expect similar nonsplicing intron mutations within Tn*5397* or pRS01 to be nonmobile in addition to disrupting transfer of the linked conjugative element. This is evident in the case of the transfer-deficient cointegrate plasmid pM1014, which carries a 13-kb pTRK28 insertion within the Ll.ltrB intron and does not produce a spliced *ltrB* product (19, 20). Lactococcal cells containing pM1014 and the target plasmid pLE23 did not exhibit any intron-containing pLE23 species, suggesting that the mobility of the Ll.ltrB intron was also disrupted in pM1014. Thus, in the case of Tn*5397* and pRS01, there appears to be a unique synergism between intron and conjugative-element mobility mechanisms which in effect act cooperatively to enable the dispersal of introns to a wide range of bacterial genomes.

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