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Genetic Manipulation of *Lactococcus lactis* by Using Targeted Group II Introns: Generation of Stable Insertions without Selection

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Despite their commercial importance, there are relatively few facile methods for genomic manipulation of the lactic acid bacteria. Here, the lactococcal group II intron, Ll.ltrB, was targeted to insert efficiently into genes encoding malate decarboxylase (*mleS*) and tetracycline resistance (*tetM*) within the *Lactococcus lactis* genome. Integrants were readily identified and maintained in the absence of a selectable marker. Since splicing of the Ll.ltrB intron depends on the intron-encoded protein, targeted invasion with an intron lacking the intron open reading frame disrupted TetM and MleS function, and MleS activity could be partially restored by expressing the intron-encoded protein in *trans*. Restoration of splicing from intron variants lacking the intron-encoded protein illustrates how targeted group II introns could be used for conditional expression of any gene. Furthermore, the modified Ll.ltrB intron was used to separately deliver a phage resistance gene (*abiD*) and a tetracycline resistance marker (*tetM*) into *mleS*, without the need for selection to drive the integration or to maintain the integrant. Our findings demonstrate the utility of targeted group II introns as a potential food-grade mechanism for delivery of industrially important traits into the genomes of lactococci.

The lactic acid bacteria (LAB) are a broad group of gram-positive bacteria that possess similar morphological, metabolic, and physiological characteristics (33). The LAB have been the subject of considerable research and commercial development, given their significance in fermentation, bioprocessing, agriculture, food, and medicine (12, 13). An impediment to fundamental studies on the LAB is the lack of facile genetic tools for manipulation of chromosomal genes (19). Moreover, public concern over the use of genetically engineered cultures for food production has prompted a search for "self-cloning" methods, whereby genetic manipulation is achieved using DNA solely from food-grade microorganisms, preferably from within the same genus (6).

Mobile group II introns are catalytic RNA elements present in a wide range of prokaryotic and eukaryotic organisms (18). Some of these introns can mobilize autonomously at a high frequency to allelic sites in a process known as homing (3). Mobile group II introns possess an intron-encoded protein (IEP) that has reverse transcriptase, RNA splicing ("maturase"), and DNA endonuclease activities (3). Mobility initiates when the IEP helps the intron RNA fold into the catalytically active RNA structure to promote splicing, resulting in ligated exons and an intron lariat-IEP ribonucleoprotein (RNP) complex. The RNP complex recognizes specific DNA target sites and promotes integration by reverse splicing of the intron RNA directly into one strand of the target DNA. The IEP then cleaves the opposite strand and uses it as a primer for target DNA-primed reverse transcription of the inserted intron RNA (16, 34, 36, 37). The resulting cDNA copy of the intron is integrated into genomic DNA by cellular recombination or repair mechanisms (5, 7, 8).

DNA target site recognition by the RNP complex involves the base pairing of intron sequences denoted EBS1 and -2 (exon binding sites 1 and 2) and δ to sequences denoted IBS1 and -2 (intron binding sites 1 and 2) and δ′ in the DNA target site (Fig. 1 and 2) (9–11). In the case of the *Lactococcus lactis* Ll.ltrB intron, the IBS and δ′ sequences are located between target site positions −12 and +3. The IEP recognizes a small number of additional nucleotide residues in the 5′ and 3′ flanking regions of the target site (positions −26 to +9) and promotes local DNA unwinding, enabling the intron RNA to base pair to the IBS and δ′ sequences for reverse splicing (22, 27).

Because intron specificity is determined largely by base pairing, the intron's EBS and δ determinants can be rescripted to permit intron homing into novel DNA sites. Previous work has shown that the lactococcal Ll.ltrB intron can be retargeted to insert efficiently into plasmid and chromosomal targets within *Escherichia coli* and other enteric bacteria (9, 11). In addition, biochemical and genetic data have elucidated the target site recognition rules, thereby enabling rational design of introns targeted to any gene (9, 11, 22). Notably, retargeted Ll.ltrB variants in which the IEP has been deleted (Ll.ltrBΔIEP) could home efficiently when the IEP (termed LtrA) was expressed in *cis* downstream of the intron (9, 11). Since Ll.ltrBΔIEP introns are nonsplicing in the absence of the IEP (16), integration within a novel target site disrupts target gene function.

Given that Ll.ltrB is originally from *L. lactis*, a bacterium employed in dairy fermentations, we examined Ll.ltrB as a potential food-grade means for targeted insertion and regulation of lactococcal genes. In this work, we demonstrate that Ll.ltrB can be readily targeted to invade the *L. lactis mleS* gene, which encodes malate decarboxylase, an enzyme that catalyzes

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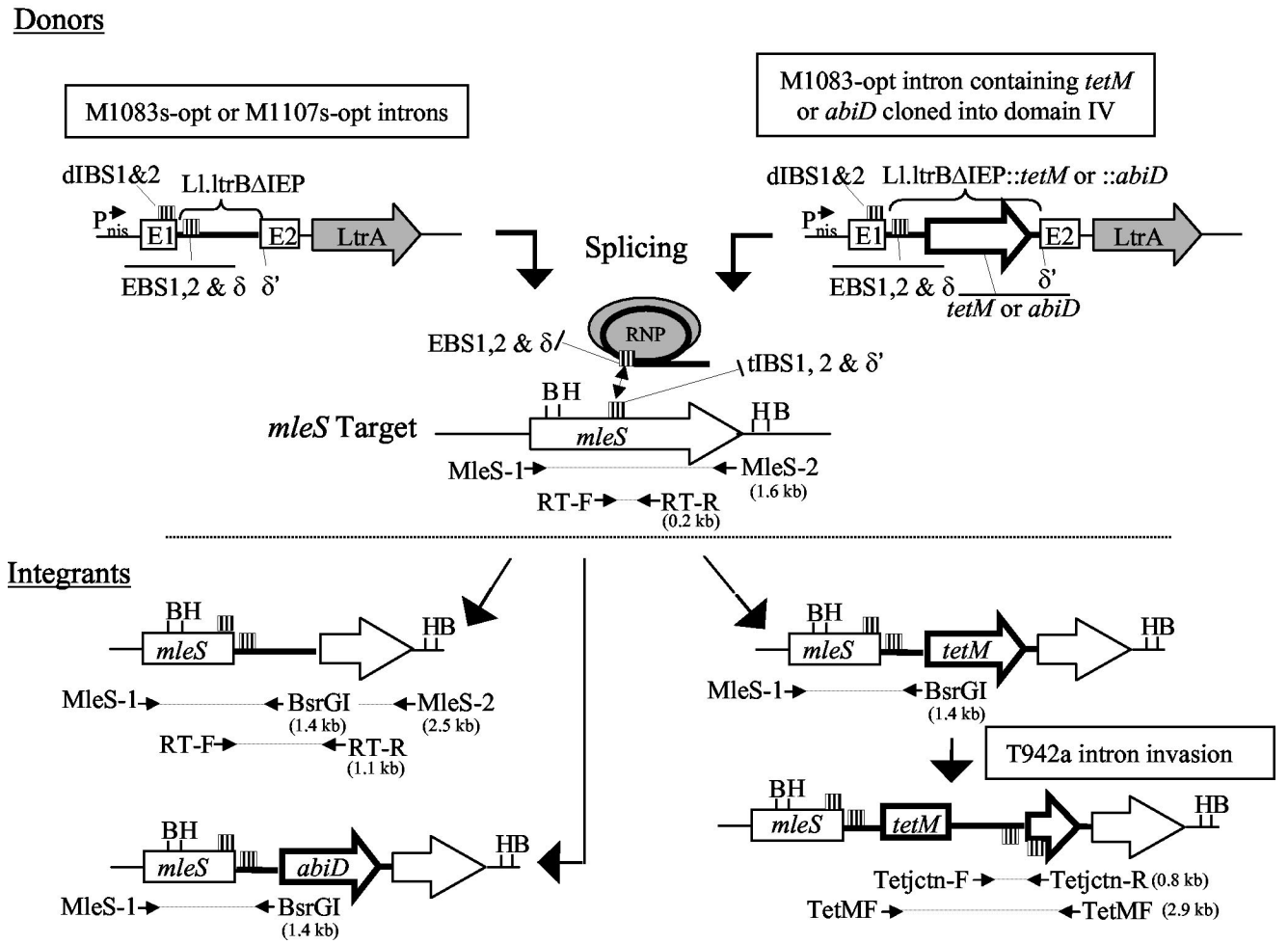

FIG. 1. Schematic of intron homing into chromosomal *mleS* and *tetM* genes. Intron DNA and RNA are represented as thick lines. Introns flanked by donor exons E1 and E2 are expressed from a nisin-inducible promoter (P_{nis}). The IEP, denoted LtrA, cloned downstream of E2 is cotranscribed, allowing splicing and homing to occur. Intron and exon binding sequences (dIBS1 and -2, EBS1 and -2, and tIBS1 and -2) as well as δ and δ′ are shown. EBS1, -2, & δ are designated by striped boxes above or below the intron to indicate sense or antisense strand orientation. Expression of donor M1083s-opt, M1107s-opt, M1083s-opt::*tetM*, or M1083s-opt::*abiD* introns results in formation of an RNP containing the IEP and excised intron lariat RNA. Invasion of the *mleS* is mediated, in part, by base pairing between RNA EBS1, -2, and δ and target IBS1, -2, and δ′ sequences. M1083s-opt, M1107s-opt, M1083s-opt::*tetM*, or M1083s-opt::*abiD* introns integrated within *mleS* are shown. The antisense strand of the *tetM* gene within the M1083s-opt::*tetM* integrant was targeted for disruption by a second intron, T942a. PCR primers used to assay intron invasion or splicing are indicated by small arrows, with sizes of expected PCR products shown in parentheses. *Bsa*JI (B) and *Hin*dIII (H) restriction sites that flank the intron insertion site are shown.

a conversion resulting in deacidification in fruit and vegetable fermentations (14). In addition, we delivered a *tetM* gene within this intron and subsequently invaded *tetM* with a second retargeted intron. Finally, we were able to confer a phage resistance phenotype to *L. lactis* by delivering an intron containing an abortive infection gene, *abiD* (17). Homing is extremely efficient, obviating the need for genetic selection using antibiotic resistance markers. Moreover, integration is highly specific and the integrants are genetically stable. Finally, invasion of a Ll.ltrBΔIEP variant results in a conditional mutant whereby target gene function is partially restored by expression of the IEP in *trans*.

MATERIALS AND METHODS

Bacterial strains, plasmids, transformation, and growth media. *E. coli* DH5α (Gibco BRL, Rockville, Md.) and MC1016 (New England Biolabs, Beverly, Mass.) were used for plasmid construction and were grown as described previously (2). Lactococcal strains were maintained in GM17 (30) with chloramphenicol and erythromycin added at 5 μg/ml and tetracycline at 10 μg/ml. Nisin induction was as described elsewhere (4). In order to determine stability of the chromosomally integrated introns, cells were cured of plasmid DNA by using ascorbic acid (25) (the percentage of cured cells was 55%) to ensure no additional homing occurred during the stability assessment. Intron stability was determined by serially passaging integrant cultures for 80 generations in GM17 supplemented with 0.8% malate, followed by plating on GM17-malate and PCR of 100 colonies using primers that flank the *mleS* or *tetM* intron-exon junction (described below). *E. coli* and *L. lactis* were transformed as described previously (2, 20).

Bacteriophage C2 resistance of an M1083s-opt::*abiD* integrant was determined by an efficiency of plating (EOP) assay on cells grown in GM17 supplemented with 0.8% malate to induce expression of *abiD* (located within the *mleS* gene). EOP was calculated by dividing the C2 phage PFU per milliliter obtained from the M1083s-opt::*abiD* integrant by the PFU per milliliter generated from the parental IL-1403 host strain. Preparation of C2 phage stock lysates and EOP determinations were carried out as described previously (17).

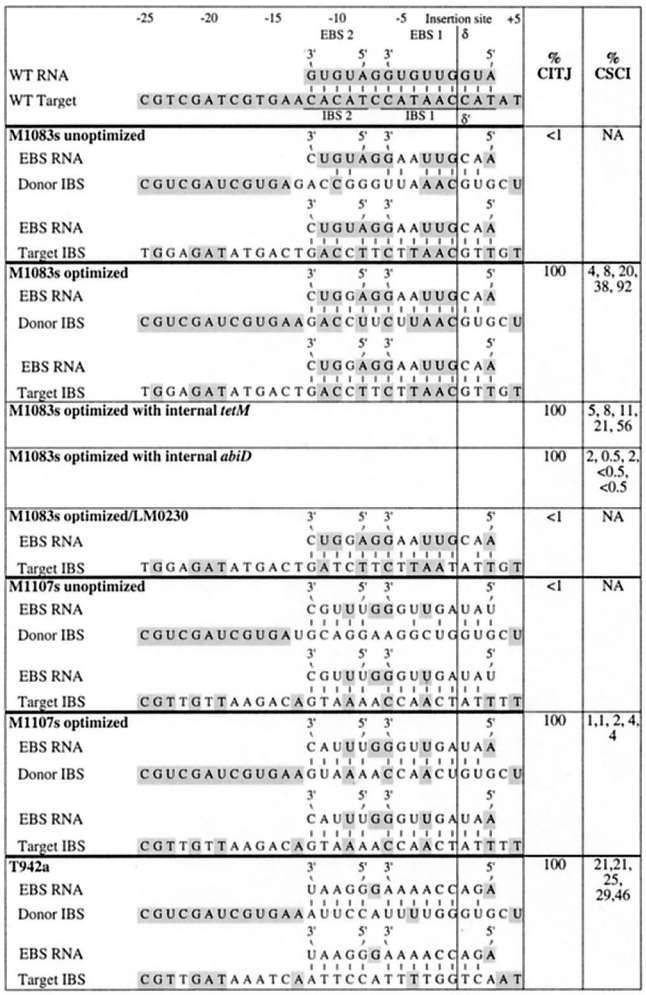

FIG. 2. Retargeted Ll.ltrB introns showing donor intron and *mleS* target sequences and base-pairing interactions. Nucleotide residues matching the WT *ltrB* sequence are shaded. Potential base pairs between the intron RNA and DNA target site are indicated by vertical lines. %CITJ, percentage of colonies that possess intron-target junctions, as determined by colony PCR using the MleS-1 and BsrGI primers and Tetjctn-F and Tetjctn-R primers, respectively. %CSCI, percentage of colony segregants that contain the intron. Five randomly selected colonies that exhibited *mleS*-intron or *tetM*-intron 5′ junctions were washed and replated. The presence of the M1083s-opt and M1107s-opt introns in *mleS* was then scored by using a 2.4-kb amplicon with primers MleS-1 and MleS-2 flanking the *mleS* gene and a lack of the WT 1.6-kb amplicon (Fig. 1). The M1083s-opt::*tetM* and M1083s-opt::*abiD* introns were scored by amplification of the *mleS*-intron junction in addition to lack of amplification of the WT 1.6-kb amplicon when using primers MleS-1 and MleS-2. The *tetM* intron was scored by the lack of the WT 2.9-kb *tetM* amplicon with primers TetM-F and TetM-R (Fig. 1). dIBS and tIBS sequences refer to IBS sequences in the donor plasmid and DNA target site, respectively. Base pairing of the intron RNA to the donor IBS sequences is required for efficient splicing. NA, not applicable.

Nucleic acid manipulations, cloning, and hybridization. Plasmid and chromosomal DNAs were isolated from *L. lactis* and *E. coli* as described elsewhere (2, 24, 32). RNA was isolated using the Fast Blue RNA kit (Bio-Rad Laboratories, Hercules, Calif.). DNA sequencing was performed by Davis Sequencing (Davis, Calif.). The selection system used to generate retargeted introns is based on a two-plasmid genetic assay developed previously (9, 11). *E. coli* intron donor chloramphenicol-resistant (Cam^r) plasmid pACD2 (previously denoted pACD-ΔORF+ORF) (9, 11) expresses a library of Ll.ltrBΔIEP introns with randomized IBS and EBS sequences from a T7lac promoter. These randomized introns also have a phage T7 promoter cloned near their 3′ end. A second, ampicillin-resistant (Amp^r) recipient plasmid contains the target gene of interest cloned upstream of a promoterless tetracycline resistance gene (*tetR*). Both plasmids are cotransformed into *E. coli* strain HMS174(DE3) with an isopropyl-β-D-thiogalactopyranoside-inducible T7 RNA polymerase (9). The introns that are able to home into the target site of interest on the recipient plasmid activate expression of the downstream *tetR* gene. Thus, colonies containing functional retargeted introns are selected by growing on Amp-and Tet-supplemented media. Introns were optimized by modifying EBS2, EBS1, and δ sequences to base pair with *mleS* target site positions −12 to −8, −6 to −1, and +1 to +3, respectively, using previously described procedures (9, 11). The IBS sequences in the 5′ exon were altered to base pair with the modified intron EBS sequences to ensure efficient forward splicing of the intron in *L. lactis* (9). The modifications were introduced via PCR. The first PCR used EBS1 and EBS2 primers (listed below), appropriate for each target site, to generate a 106-bp product that was gel purified and used as primer for the next round of PCR with the IBS primer. The second PCR produced a 350-bp product that was gel purified, digested with *Bsr*GI and *Hin*dIII, and cloned into pACD2. The retargeted introns were recloned into the *E. coli-L. lactis* shuttle vector pMSP3535 (4) by digesting pACD2 (9, 11) with *Bsi*EI and cloning the resulting 3-kb fragment, containing the 1-kb retargeted Ll.ltrBΔIEP with *ltrA* relocated downstream of the 3′ splice site, into *Sma*I-digested pMSP3535. The LtrA expression plasmid was constructed by digesting the M1083s-opt intron donor plasmid with *Pst*I and cloning the 2-kb fragment, containing the LtrA open reading frame, into *Pst*I-cut pMSP3535. The *tetM* and *abiD* genes were cloned into the M1083s-opt intron by PCR amplifying *tetM* and *abiD* (primers listed below) and cloning into the *Mlu*I site within the M1083s-opt donor plasmid.

Southern hybridizations were carried out on nylon membranes (Zeta-probe; Bio-Rad) as described previously (2) and probed with a digoxigenin-labeled probe specific to sequences within intron domain IV (9). Probe signal was detected using a DIG luminescence detection kit (Boehringer Roche, Indianapolis, Ind.).

Primers, probes, and PCR methods. PCRs were done using a PTC-200 thermal cycler (MJ Research, San Francisco, Calif.) under the following reaction conditions: 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 0.2 mM deoxynucleoside triphosphates, 6 mM $MgCl_2$, a 0.25 μM concentration of each primer, 2.5 U of *Taq* DNA polymerase, and 100 ng of DNA. Colony PCR was done by toothpicking a single colony into 2 μl of water in a PCR tube, adding 13 μl of GeneReleaser (Bioventures Incorporated, Murfreesboro, Tenn.), and microwaving for 5 min. Reverse transcription-PCR (RT-PCR) was performed with the Access RT-PCR system (Promega, Madison, Wis.). All primers were obtained from Operon Technologies (Alameda, Calif.). Primers MleS-1 (5′-TTG TACGATGCGTGCACATG) and MleS-2 (5′-GATATTCCCCTTACTACT CT) were used to amplify the complete *mleS* gene; BsrGI (5′-GGGGTGTACA AATGTGGTGA) was used with MleS-1 to amplify *mleS*-intron 5′ junctions; RT-F (5′-GGTTTACTTTTTGATGATATG) and RT-R (5′-GAAGATTACTG GGCGTTCTG) were used to assay M1083s-opt intron splicing from within the *mleS* gene; BsrGI and MleS-2 were used for sequencing of 5′ and 3′ *mleS*-intron junctions, respectively, from the amplicon produced with primers MleS-1 and MleS-2; Intron-1 (5′-GACGCGTTGGGAAATGGC) and Intron-2 (5′-TCACT GTCGACCCTATAGTGAGTCGTATTA) were used to create a digoxigenin-labeled probe specific to intron domain IV; and Tetjctn-F (5′-TTGTTTCGCT ATCATTGCCATTTCC) and Tetjctn-R (5′-TCGTTTCCCTCTATTACCGTA TCCC) were used to amplify the *tetM*-intron junctions. *tetM* was amplified with TetMF (5′-GGGGACGGCTGAGGAAAATC) and TetMR (5′-GGGGACGG CTCAACATAAAATAC). Amplification of *abiD* was performed with primers abiD-F (5′-GGGGACGCGTGTATATAAGGTCTAAAAT) and abiD-R (5′-G GGGACGCGTCTTATATTCTAATCATTT). Primers used for intron optimization were as follows: M1083sE1 (5′-GATTGTACAAATGTGGTGATAACA GATAAGTCTTAACGTTGACTTACCTTTC); M1083sE2 (5′-CTAATTTCG GTTAGGTCTCGATAGAGGAAAG); M1083sIBS (5′-AAAAAGCTTCGTC GATCGTGAAGACCTTCTTAACGTGCGCCCAG); M1107sE1 (5′-GATTG TACAAATGTGGTGATAACAGATAAGTCCCAACTATTTACTTACCTTT C); M1107sE2 (5′-CTAATTTCGGTTTTTACTCGATAGAGGAAAG); and M1107sIBS (5′-AAAAAGCTTCGTCGATCGTGAAGTAAAACCAACTGTG CGCCCAG). Underlined nucleotides represent modified positions.

Homing assays. An *L. lactis* culture containing an intron donor plasmid was induced overnight with nisin, then plated at different dilutions onto GM17-Erm plates containing 25 ng of nisin/ml, and incubated at 30°C for 1 to 2 days. While continued expression of the intron from the nisin promoter was not detrimental to homing or to cell growth, homing frequencies were determined after an initial induction of expression in order to more closely gauge the insertion frequencies

resulting from a defined window of induction. Chromosomal DNA isolated from the induced culture, as well as from 50 to 100 colonies from GM17-Erm-nisin plates, was tested for intron integration via PCR. To determine the percentage of *mleS* and *tetM* disruptants, select colonies that exhibited *mleS*-intron or *tetM*-intron junctions were toothpicked into 100 μl of phosphate-buffered saline, vortexed, diluted, and plated onto GM17-Erm medium without nisin. A total of 50 to 100 colonies from the second plating were tested for intron integration via colony PCR with primers flanking the *mleS* gene and primers to the *mleS*-intron junction. *tetM*-intron segregants were verified by colony PCR with primers flanking the *tetM* gene and primers to the *tetM*-intron junction.

Malate decarboxylase assay. To prepare extracts, an overnight culture was diluted 1/100 in 100 ml of GM17-Erm with or without nisin and grown until A_{600} = 0.6 to 0.8. Cells were pelleted and incubated with 500 μl of 1-mg/ml lysozyme for 30 min at 37°C and then washed by centrifugation in 10 ml of 0.1 M potassium phosphate buffer (pH 6.0). The pelleted cells were resuspended in 2 ml of potassium phosphate buffer and disrupted in a Fast Prep (Bio 101, La Jolla, Calif.) instrument at a setting of 6 for 40 s. Cell debris was pelleted for 20 min at 4°C and the supernatant was removed for assay. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories). The decarboxylase assay was performed as previously described (21), except that the reaction mixture consisted of 0.1 M potassium phosphate buffer (pH 6.0), 50 μM NAD, 87 μM $MnSO_4$, 1.5 mM malate, 10 nCi of L-1,4(2,3)-[^{14}C]malic acid (55 mCi/mmol; Amersham Pharmacia, Piscataway, N.J.).

RESULTS

Retargeted introns can home efficiently and specifically in lactococci. We used an *E. coli*-based selection system with a combinatorial library of Ll.ltrB intron donor plasmids having randomized IBS, EBS, and δ sequences (9, 11) to obtain two Ll.ltrB introns targeted to the *mleS* gene of *L. lactis* IL-1403 (1) and a third intron targeted to a *tetM* antibiotic resistance marker. These intron variants are denoted M1107s, M1083s, and T942a, indicating that they insert in the sense (s) or antisense (a) DNA strand at positions +1107, +1083, or +942 from their respective initiation codons. *E. coli* intron donor plasmids contain Ll.ltrB ΔIEP variants with the IEP (LtrA) expressed in *cis* from a position downstream of the 3′ exon (Fig. 1). In *E. coli*, the M1107s and M1083s introns, without alterations to optimize base pairing, invaded a plasmid-borne *mleS* gene at frequencies of 17 and 47%, respectively.

The M1107s and M1083s introns, containing *ltrA* relocated downstream of the intron's 3′ splice site, were recloned under the control of a nisin-inducible promoter in the *E. coli-L. lactis* shuttle vector pMSP3535 (4) and transformed into *L. lactis* IL1403. Two versions of the introns were tested: the original introns isolated from the combinatorial library in *E. coli*, and optimized versions in which mismatches were corrected between the intron's EBS sequences and IBS sequences in the DNA target site (tIBS) as well as IBS sequences in the donor plasmid (dIBS) for efficient RNA splicing (Fig. 2). Since previous analysis indicated that positions −7 and −13 are not recognized by base pairing, these positions were left unchanged (9) (Fig. 2). After induction with nisin, intron invasion was scored by PCR amplification across the 5′ *mleS*-intron junction within the chromosomal *mleS* gene (primers MleS-1 and BsrGI [Fig. 1]). Nisin-induced expression of the optimized introns in broth culture gave *mleS*-intron junctions indicative of a homing event (data not shown), whereas the unoptimized introns gave no detectable *mleS*-intron junctions, even though these introns homed efficiently in the plasmid assay in *E. coli*.

To score invasion frequency, the induced cultures were plated onto GM17 medium in the presence of nisin and the resulting colonies were analyzed directly by PCR. One hundred percent of colonies derived from M1083s-opt- and M1107s-opt-induced cultures possessed the *mleS*-intron junction (Fig. 2 and 3). A similar plating analysis of unoptimized M1083s and M1107s variants did not reveal any *mleS*-intron junctions (<1% invasion). The necessity of using optimized introns could reflect that base pairing requirements are more stringent in *L. lactis* than in *E. coli* (see Discussion).

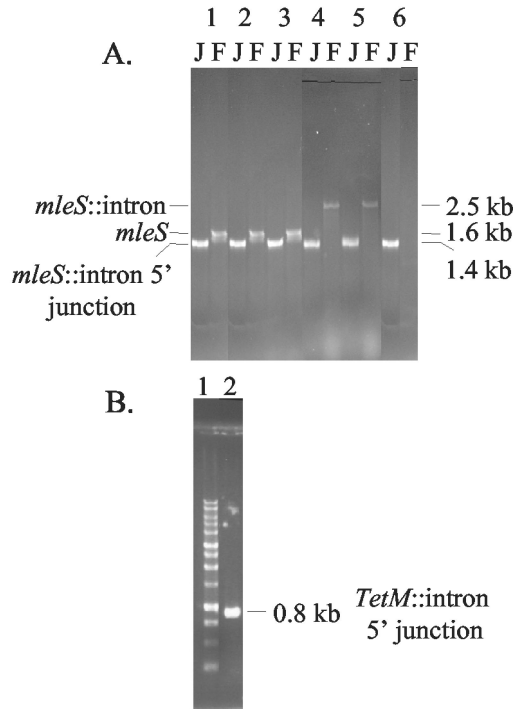

FIG. 3. (A) Agarose gel showing PCR products from homing assays and clonal segregants containing the inserted intron. J indicates PCR with primers that detect the 5′ intron-exon junction (MleS-1 and BsrGI), and F indicates PCR with primers flanking the *mleS* gene (MleS-1 and MleS-2) (Fig. 1). Lanes: 1, a nonclonal *mleS*::M1083s-opt colony; 2, a nonclonal *mleS*::M1107s-opt colony; 3, a nonclonal *mleS*::M1083s-opt::*tetM* colony; 4, a clonal *mleS*::M1083s-opt segregant; 5, a clonal *mleS*::M1107s-opt segregant; 6, a clonal *mleS*::M1083s-opt::*tetM* segregant. (B) Agarose gel of the PCR product from homing assays in which the T942a-*tetM* junction was amplified with primers TetMjctn-F and TetMjctn-R (Fig. 1). Lane 1, molecular mass ladder; 2, 0.8-kb T942a-*tetM* junction PCR product.

Since the *mleS* integration was achieved without selection, it was essential to determine if the colonies that possessed *mleS*-intron junctions were clonal. Therefore, an additional PCR was performed using primers that flank the intron invasion site in the *mleS* gene (primers MleS-1 and MleS-2 [Fig. 1]). If intron invasion occurs, the *mleS* amplicon should be 0.9 kb larger than that derived from a wild type (WT) gene (Fig. 1). However, PCRs performed on DNA from induced broth cultures containing the retargeted introns, as well as selected colonies identified as possessing *mleS*-intron junctions, exhibited only the 1.6-kb amplicon indicative of the WT *mleS* gene (Fig. 3A). This suggested that lactococcal colonies identified as positive for *mleS*-intron junctions contained a mixture of WT and disrupted *mleS* genes, with the smaller product predominating during PCR.

To determine the fraction of cells possessing WT or intron-

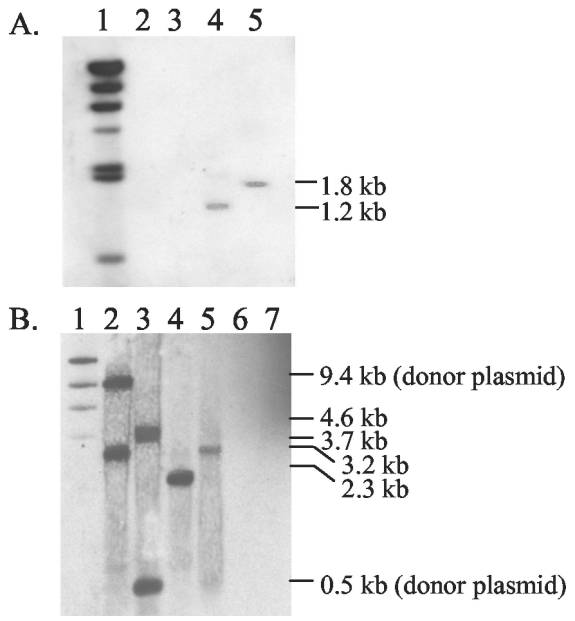

FIG. 4. Southern hybridization analysis. *Bsa*JI- or *Hin*dIII-cut DNAs from intron-containing *L. lactis* IL-1403 integrants were hybridized to a sequence in intron domain IV (see Materials and Methods). A single, randomly chosen integrant was examined for each intron delivered. (A) M1083s-opt integrant cured of donor plasmid. Lane 1, λ-*Hin*dIII molecular mass markers; 2, *Hin*dIII-cut IL-1403 DNA; 3, *Bsa*JI-cut IL-1403 DNA; 4, *Hin*dIII-cut IL-1403 M1083s-opt integrant DNA; 5, *Bsa*JI-cut IL-1403 M1083s-opt integrant DNA. The probe detects a single band of the expected sizes (1.8 and 1.2 kb, respectively, for *Bsa*JI and *Hin*dIII cleavage) for the M1083s-opt or M1107s-opt intron-invaded *mleS* genes and no band for the control WT IL-1403 DNA. (B) M1083s-opt::*tetM* and T942a integrants. Lane 1, λ-*Hin*dIII molecular mass markers; 2, *Hin*dIII-cut T942a integrant DNA; 3, *Bsa*JI-cut T942a integrant DNA; 4, *Hin*dIII-cut M1083s-opt::*tetM* integrant DNA; 5, *Bsa*JI-cut M1083s-opt::*tetM* integrant DNA; 6, *Hin*dIII-cut *L. lactis* IL-1403 WT DNA; 7, *Bsa*JI-cut *L. lactis* IL-1403 WT DNA. The probe detects bands of the expected sizes for the M1083s-opt::*tetM* intron in *mleS* (3.7 and 2.3 kb, respectively, for *Bsa*JI and *Hin*dIII cleavage) and the T942a intron in *tetM* (4.6 and 3.2 kb, respectively, for *Bsa*JI and *Hin*dIII cleavage) and no band for the control *L. lactis* IL-1403 WT DNA. Bands noted as donor plasmid result from the probe hybridizing to intron DNA present on donor plasmid within uncured T942a integrants.

invaded *mleS* genes, selected colonies were washed with phosphate-buffered saline and replated on GM17 medium without nisin to eliminate further intron expression. The resulting colonies were then directly assayed by PCR using the same primers flanking the intron target site within the *mleS* gene. Analysis of five individual colonies that contained M1083s-opt integrants indicated the fractions of cells possessing an intron-invaded *mleS* gene were 4 to 92% (Fig. 2). M1107s-opt integrants had lower percentages, 1 to 4% (Fig. 2), consistent with the relative insertion frequencies of these introns (1 and 5% for M1107s-opt and M1083s-opt, respectively) in *E. coli*.

Intron-mediated delivery and inactivation of *tetM*. To determine the impact of heterologous DNA carriage on targeted invasion of *mleS*, the 2-kb *tetM* gene was cloned into domain IV within M1083s-opt. The resultant M1083s-opt::*tetM* intron gave a somewhat lower range of homing frequencies, comparing five of these colonies (5 to 56% [Fig. 2]) to five colonies with M1083s-opt (4 to 92%). A second intron, T942a, was then targeted to the antisense strand of *tetM*. T942a expressed in an M1083s-opt::*tetM* integrant homed at frequencies of 21 to 46% (Fig. 2), as judged by PCR across the *tetM*-intron junction (Fig. 3B) and an inability to amplify the 2.0-kb WT *tetM* with primers TetMF and TetMR (Fig. 1). This demonstrates that group II introns can be used to deliver, and subsequently disrupt, selectable markers used for the manipulation of lactococcal strains.

Food-grade delivery of *abiD* into the lactococcal chromosome. The ability of Ll.ltrB to deliver genetic information into the lactococcal chromosome without selection suggests that introns could be used to stabilize industrially significant traits within lactococcal starter cultures. To demonstrate this, we cloned the 1.4-kb *abiD* gene, which encodes resistance to common lactococcal bacteriophages (17), into domain IV of the M1083s-opt intron. The resultant M1083s-opt::*abiD* intron homed into *mleS* at frequencies between <0.5 and 2%, slightly lower that that observed for the M1083s-opt::*tetM* intron. EOP assays on the M1083s-opt::*abiD* integrants demonstrated resistance to bacteriophage C2 at levels similar to those previously shown for *abiD* (EOP = 0.09) (17).

Specificity of intron insertion. Invasion of *mleS* by M1083s-opt and T947a is highly specific. Southern blot analysis of *Bsa*JI- or *Hin*dIII-digested DNA from clonal M1083s-opt, M1083s-opt::*tetM*, and T942a integrants confirmed that M1083s-opt and T942a invasion occurred only in single sites within *L. lactis* IL1403 (Fig. 4). As an additional check for specificity, the M1083s-opt donor plasmid was transformed into *L. lactis* LM0230, a host that harbors an *mleS* gene with sequence differences from the *mleS* gene in IL1403. These differences result in a tIBS mismatch with the M1083s-opt intron EBS at position +1 and wobble base pairs at positions −1 and −10 (Fig. 2). No other sequence polymorphisms exist between the LM0230 and IL1403 *mleS* alleles within the intron target site region. PCR analysis of DNA purified from LM0230 cultures expressing M1083s-opt did not reveal *mleS*-intron junctions, suggesting that intron homing occurred at a low frequency if at all. PCR analysis of colonies isolated from this culture also failed to identify *mleS*-intron junctions (Fig. 2).

ΔIEP-intron gene inactivation. All of the introns used in this study do not possess the IEP LtrA. Previous analysis of ΔIEP variants of Ll.ltrB suggested a severe reduction in splicing activity (16, 35). Invasion by M1083s-opt or M1107s-opt effectively disrupted malate decarboxylase activity by insertion of a 0.9-kb nonsplicing intron into *mleS* (Table 1). To determine if ΔIEP introns could splice after integration into *mleS*, an M1083s-opt integrant was assayed for intron splicing by RT-

TABLE 1. Malate decarboxylase activity

Strain	Sp act[a]	% WT activity
IL1403	9.8 ± 2.6	100
mleS::M1083s-opt	0.19 ± 0.02	1.9
mleS::M1083s-opt; LtrA uninduced	0.17 ± 0.02	1.7
mleS::M1083s-opt; LtrA induced	2.07 ± 0.15	21

[a] Average specific activity calculated as micromoles of CO_2 evolved per minute per milligram of protein.

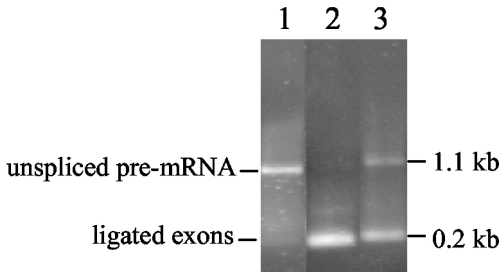

FIG. 5. Conditional splicing of the integrated M1083s-opt intron from the chromosomal *mleS* gene. Splicing was assayed by RT-PCR using RT-F and RT-R primers (Fig. 1) on strains with and without LtrA expressed in *trans*. The primers yield a 0.2-kb PCR product corresponding to ligated exons or a 1.1-kb PCR product corresponding to unspliced precursor RNA containing the inserted intron. Lanes: 1, *mleS*::M1083s-opt integrant without LtrA expression plasmid; 2, *mleS*::M1083s-opt integrant, LtrA induced; 3, *mleS*::M1083s-opt integrant, LtrA present but not induced.

PCR (Fig. 5, lane 1). No spliced product could be detected, suggesting that the M1083s-opt intron integrated into the sense strand was nonfunctional.

The T942a intron invaded the antisense strand of *tetM*. Therefore, no intron splicing is possible from that target and, as expected, all M1083s-opt::*tetM* integrants ($n = 34$) were tetracycline sensitive.

Integrated intron stability. Most methods for insertional mutagenesis require selection to drive integration into the chromosome and to maintain the mutation. Since intron-based insertion did not require selection to identify integrants, it was essential to determine if the integrated introns were genetically stable within expressed genes. To this end, clonal M1083s-opt, M1083s-opt::*tetM*, M1083s-opt::*abiD*, and T942a integrants, cured of donor intron plasmids, were serially transferred for 80 generations in GM17 broth containing malate prior to plating. Malate induces expression of *mleS* in IL1403 (26). Colony PCR demonstrated that 100% of M1083s-opt, M1083s-opt::*tetM,* and M1083s-opt::*abiD* integrants were stable after 80 generations. Moreover, 100% of M1083s-opt::*tetM* integrants continued to be resistant to tetracycline and 100% of T942a integrants (within the *tetM* gene) were sensitive to tetracycline. Similarly, resistance to bacteriophage C2 in the M1083s-opt::*abiD* integrants remained stable for 80 generations, with no change in strain EOP before and after passage (EOP = 0.09). Thus, within lactococci, mutants obtained by group II intron invasion appear to be very stable, even when residing within expressed genes.

Complementation of intron splicing partially restores *mleS* activity. Since invasion into the sense strand of *mleS* by M1083s-opt abolished gene function, restoration of intron splicing should result in restoration of *mleS* function. To examine this, the gene encoding LtrA was cloned into the nisin-inducible expression vector pMSP3535 and transformed into the M1083s-opt mutant strain, which had been cured of the original intron donor plasmid. RT-PCR on RNA purified from nisin-induced broth cultures of this transformant clearly demonstrated splicing of the M1083s-opt intron within *mleS* (Fig. 5, lane 2). Sequence analysis of the resultant RT-PCR product verified the correct ligation of *mleS* exons. Uninduced cultures also exhibited some spliced *mleS* mRNA product, indicating low-level expression of LtrA from the pMSP3535 vector in the absence of nisin (Fig. 5, lane 3). Expression of LtrA within the M1083s-opt mutant partially restored cellular malate decarboxylase levels in the induced culture to ~21% that of WT (Table 1).

DISCUSSION

This work demonstrates that retargeted group II introns can be used to generate chromosomal insertions within lactococci, bacteria employed in the production of numerous fermented dairy products (31). Three different retargeted introns were shown to efficiently invade chromosomal genes in *L. lactis* IL1403. Homing frequencies were extremely high, and integrants were readily identified by simple PCR screening, thereby eliminating the need for antibiotic marker selection to enrich for integrants. Given that no selection was employed to identify colonies with an integrated intron, the resultant colonies were nonclonal and subsequent segregation of clonal populations was necessary. The percentage of cells containing the intron varied within colonies, suggesting that the timing of the homing event during the growth of the colony influences the percentage of the population invaded. M1107s-opt appeared to have a lower homing efficiency compared to M1083s-opt and T942a, likely reflecting specific features of the target site or differences in optimal matches to the target sequence.

Cousineau et al. (5) previously demonstrated the Ll.ltrB intron can home with a 0.8-kb kanamycin resistance gene cloned into intron domain IV. In this work, *tetM* and *abiD* were separately delivered within the M1083s-opt intron. Invasion frequencies for both M1083s-opt::*tetM* and M1083s-opt::*abiD* introns were lower than that observed for the M1083s-opt intron, suggesting that heterologous DNA carriage within domain IV lowers homing frequencies. Surprisingly, different homing efficiencies were observed for M1083s-opt introns containing internal *tetM* (2-kb) or *abiD* (1.4-kb) genes, with the M1083opt::*tetM* intron integrating at a higher frequency. This suggests that the size and content of heterologous DNA delivered within domain IV of the intron can impact targeted homing efficiencies.

Directed intron homing in *Lactococcus* appears to be quite specific. M1083s-opt, its *tetM* derivative, and T942a integrated only into the specific chromosomal sites for which they had been targeted. Additionally, when expressed in *L. lactis* LM0230, which has a slightly different *mleS* target site, the M1083s-opt intron showed no detectable homing. The sequence polymorphisms in LM0230 result in intron RNA-DNA target site mismatch at position +1 and wobble base pairs (29) at positions −10 and −1, which have been shown previously to be required for efficient homing (Fig. 2) (10, 22).

We were surprised to find that the unoptimized M1107s and M1083s introns isolated in the *E. coli*-based selection could home efficiently in *E. coli* but not in *L. lactis*. Although these introns have several mismatches between their EBS sequences and IBS sequences in both the donor plasmid and DNA target site, it was observed previously that retargeted introns could function in *E. coli* and remain highly specific despite a small number of mismatches at some positions (9, 11). It is possible that expression from the T7lac promoter in the *E. coli* system is significantly higher than the cognate expression from the

nisin-inducible promoter in lactococci, so that splicing and homing can occur despite mismatches. Alternatively, conditions in *L. lactis* may require more stringent base pairing for intron function. The difference does not present a problem for gene targeting, since base pairing is easily optimized for selected introns, and optimal base pairing is incorporated into a newly developed computer program for designing efficient retargeted introns, thereby avoiding the necessity for intron selection within *E. coli* (J. Perutka and A. M. Lambowitz, unpublished data).

All introns used in this study are ΔIEP intron variants. Invasion of M1083s-opt and M1107s-opt into the *mleS* gene effectively eliminated cellular malate decarboxylase activity (1.9% of WT), and T942a invasion into the antisense strand of the *tetM* gene resulted in sensitivity to tetracycline. RT-PCR analysis of RNA expressed from the *mleS*::M1083s-opt gene did not reveal any spliced products. M1083s-opt, its *tetM* and *abiD* derivatives, and T942a integrants were stable for 80 generations, suggesting that reversion to WT status, if occurring, is infrequent.

Splicing of *mleS*::M1083s-opt was achieved by providing the IEP (LtrA) in *trans*. Cellular levels of malate decarboxylase were restored to 21% of WT upon expression of LtrA from a nisin-inducible vector. The reduced level of complementation could be due to several factors. Foremost is that changes in tIBS-intron EBS pairings as well as surrounding *mleS* exon sequences may reduce the splicing efficiency relative to WT levels. In addition, separation of the IEP from the cognate intron-containing mRNA may impact effective RNP formation and function. Zhou et al. (35) complemented mutant Ll.ltrB variants by expressing LtrA in *trans* and observed these introns to be functioning at a 20-fold-lower level than that of WT. Our results are consistent with a general reduction in splicing efficiency when the intron IEP is supplied in *trans*. Lastly, LtrA has been found to down regulate itself by binding to its own ribosome binding site (28). This regulatory mechanism may impede full complementation of intron-integrated *mleS*.

The LAB have long been employed in the preservation of foodstuffs. In general, genetic improvement of LAB strains by recombinant methods promises to greatly enhance their efficacy in various arenas (23). Methods for performing targeted gene disruptions in the LAB predominately employ temperature-sensitive vectors where homology-driven integration into the chromosome is achieved at the higher, nonpermissive temperature (19). Unlike the intron method described here, these systems require larger stretches of target site homology (>500 bp) in addition to the need for selection to drive the integration event. Such conditionally replicative vector systems are often limited if the host strain does not grow (or grows poorly) at the nonpermissive temperature needed to drive the integration. This is true for many commercial strains of *L. lactis* subsp. *cremoris*, which do not grow well at 37°C (15). Thus, targeted invasion with the lactococcal group II intron, Ll.ltrB, provides an attractive alternative for chromosomal manipulation of lactococci, since no selection is needed to drive the integration event and conditionally replicative vectors are not needed. For food-grade delivery of novel traits, or insertional mutagenesis of a target gene, appropriately modified introns simply need to be expressed in a host cell and, upon invasion, the donor plasmid is cured. Rescripting of intron EBS sequences to accommodate donor IBS and target IBS base pairing allows virtually any genomic locus to be targeted. The ability to identify stable insertions in a specific gene without genetic selection eliminates the need for antibiotic markers, or even food-grade markers (6), to stabilize genetic insertions within lactococci. Given that homing of Ll.ltrB occurs efficiently in hosts as diverse as *E. coli* and *L. lactis*, it is likely that targeted intron mutagenesis will work in other food-grade LAB as well.

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