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Genetic Analysis of Regions of the *Lactococcus lactis* subsp. *lactis* Plasmid pRS01 Involved in Conjugative Transfer

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The genes responsible for conjugative transfer of the 48.4-kb *Lactococcus lactis* subsp. *lactis* ML3 plasmid pRS01 were localized by insertional mutagenesis. Integration of the IS*946*-containing plasmid pTRK28 into pRS01 generated a pool of stable cointegrates, including a number of plasmids altered in conjugative proficiency. Mapping of pTRK28 insertions and phenotypic analysis of cointegrate plasmids identified four distinct regions (Tra1, Tra2, Tra3, and Tra4) involved in pRS01 conjugative transfer. Tra3 corresponds closely to a region previously identified (D. G. Anderson and L. L. McKay, J. Bacteriol. 158:954–962, 1984). Another region (Tra4) was localized within an inversion sequence shown to correlate with a cell aggregation phenotype. Tra1 and Tra2, two previously unidentified regions, were located at a distance of 9 kb from Tra3. When provided in *trans*, a cloned portion of the Tra3 region complemented Tra3 mutants.

The ability to transfer genetic material via conjugation is widespread among lactococci. Conjugative transfer of various industrially significant traits, including carbohydrate utilization, proteinase activity, bacteriophage resistance, and bacteriocin production, has been demonstrated in *Lactococcus lactis* subsp. *lactis* (*L. lactis*), *L. lactis* subsp. *cremoris*, and *L. lactis* subsp. *lactis* biovar *diacetylactis* (31). While several conjugative plasmids (10, 12, 14, 15, 21, 24, 30) and conjugative transposons (13, 26) have been identified in lactococci, only limited work has been done to characterize specific genes required for conjugative transfer. In view of the strategic role of bacterial conjugation among industrially important strains of lactococci, an understanding of the specific mechanisms involved in conjugal transfer is critical in order to optimize and expand the utility of conjugation as a means of strain development.

The conjugative plasmid pRS01 from *L. lactis* ML3 and the sex factor of *L. lactis* 712 have been shown to be similar. Both factors form large cointegrate plasmids in transconjugants, exhibit high-frequency transfer, and confer a cell aggregation (Clu) phenotype. During conjugation of ML3, the 48-kb conjugative plasmid pRS01 joins with a 55-kb nonconjugative Lac^+ plasmid, pSK08, resulting in a 104-kb cointegrate plasmid in the recipient cell. The results of Polzin and Shimizu-Kadota (25) suggested that either of two ISS*1* sequences present within pSK08 can direct the cointegration event. During conjugation of *L. lactis* 712, the chromosomally encoded sex factor (50 to 60 kb [11]) forms a cointegrate with a 55-kb Lac^+ plasmid, pLP712, resulting in a transconjugant pool containing pLP712::sex factor cointegrate plasmids of various sizes (9, 11). Gasson et al. (11) proposed that this variability in plasmid size was due to differential excision of chromosomal DNA flanking the integrated sex factor within the donor strain.

Utilizing the apparently random insertion of pSK08 into pRS01, Anderson and McKay (1) carried out preliminary mapping of the regions of pRS01 responsible for the Clu and conjugative transfer (Tra) phenotypes by analyzing the junction sites of pSK08::pRS01 cointegrate plasmids in various Clu^- and Tra^- transconjugants. Clu expression was localized to a 24-kb region. This region contains a 4.3-kb *Pvu*II-*Kpn*I fragment which was present in opposite orientations in strains exhibiting either the Clu^+ or Clu^- phenotype. The loss of the Clu^+ phenotype, accompanied by an inversion of the 4.3-kb *Pvu*II-*Kpn*I fragment, also resulted in a 10^4-fold decrease in conjugal transfer, suggesting a role for cell aggregation in high-frequency plasmid transfer (1). A 15-kb *Pvu*II-*Sal*I fragment overlapping the 24-kb Clu region appeared to carry genes for conjugative transfer. The large size, variable copy number, and relative instability of the pSK08::pRS01 cointegrate plasmids have seriously hampered further efforts to analyze the Clu and Tra regions of pRS01 genetically or physically.

Recently Romero and Klaenhammer (27) demonstrated the ability of pTRK28, a small *Escherichia coli-L. lactis* shuttle plasmid containing an iso-ISS*1* insertion sequence, IS*946*, to integrate randomly within pRS01. Like that of pSK08, cointegration of pTRK28 with pRS01 was shown to be a Rec-independent recombination event with a strict requirement for the presence of IS*946*. The pTRK28::pRS01 cointegrates appear to be stable and are maintained at a higher copy number, most likely because of the plasmid pIP501-derived (3) origin of replication contained within the pTRK28 plasmid.

We have utilized the plasmid pTRK28 for insertional mutagenesis to further localize regions of pRS01 involved in conjugative transfer. Mapping of the site of pTRK28 integration within 34 pTRK28::pRS01 cointegrates exhibiting altered conjugative transfer phenotypes has identified four regions of pRS01 involved in cointegrate plasmid transfer (Tra1, Tra2, Tra3, and Tra4). Two of these regions correspond to those previously identified by Anderson and McKay (1). Two novel unlinked Tra regions were also identified. Complementation studies of one region (Tra3) were also carried out.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source, description, and/or reference
E. coli DH5αF$^-$	F′ *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA-1 gyrA96* (Nal^r) *relA1* Δ(*lacZYα-argF*)U169 (ϕ80 *lacZ*ΔM15)	Bethesda Research Laboratories
L. lactis subsp. *lactis* strains		
NCK168	*str-1* Rec$^-$, pRS01, pTRK28	MMS362 transformed with pTRK28 (27); conjugal donor
LM0230	Plasmid free	Conjugation host (22)
LM2345	*spc-4 rif-5*, plasmid free	Conjugation host (1)
MMS370	*str-1 neo* Rec$^-$, plasmid free	Conjugation host (1)
DM1001	Em^r, pM1001	LM0230 transformed with pM1001
DM1004 to DM6134	*str-1 neo* Em^r Rec$^-$, pM1004 to pM6134 (respectively)	NCK168 × MMS370 transconjugants
Plasmids		
pRS01	Tra$^+$ Clu$^{-/+}$, 48.4 kb	Resident plasmid of NCK168 (27)
pTRK28	Em^r Cm^r, 9 kb	pSA3::IS*946* (27)
pTRK24	Em^r Cm^r, 5.2 kb	pGK12::IS*946* (27)
pSKH1-B	Em^r Cm^r, 7.7 kb	Shuttle vector[b]
pE231	Tet^r, 24 kb	Subclone from pM1001
pLE1	Cm^r, 8.7 kb	pSKH1-B with Cm^r gene cloned from pTRK24 and deleted of Em^r
pLE33	Cm^r, 16.2 kb	pLE1 containing 6.8-kb *Xba*I-*Sst*I insert from pE231; complete Tra3A and Tra3B
pM1001 to pM6134	Em^r Tra$^{+/-}$ Clu$^{+/-}$, ca. 60 kb	pTRK28::pRS01 cointegrates

[a] Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant; Nal^r, nalidixic acid resistant; Tet^r, tetracycline resistant; *spc*, spectinomycin gene; *rif*, rifampin gene; *str*, streptomycin gene; *neo*, neomycin gene; Rec, host recombination; Tra, conjugative proficiency; Clu, cell aggregation; −, negative; +, positive.
[b] pSKH1-B was generously provided by G. Stoddard and S. Harlander.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are shown in Table 1. *E. coli* strains were grown in LB medium (20). Selective media for *E. coli* strains contained various antibiotics at the following concentrations: tetracycline, 15 μg/ml; chloramphenicol, 30 μg/ml; and erythromycin, 150 μg/ml. *L. lactis* strains were grown in GM17 medium (M17 medium [32] containing 0.5% glucose) at 30°C without agitation. Selective medium for *L. lactis* strains contained antibiotics at the following concentrations: erythromycin, 10 μg/ml; chloramphenicol, 5 μg/ml; rifampin, 50 μg/ml; streptomycin, 600 μg/ml; spectinomycin, 300 μg/ml; and neomycin, 1,000 μg/ml. All plating media contained 1.5% Bacto-agar.

Cloning, transformation, and DNA manipulation. Restriction endonucleases, T4 DNA ligase, the Klenow fragment of DNA polymerase I, and calf intestinal phosphatase were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., from New England BioLabs, Inc., Beverly, Mass., or from Promega Corp., Madison, Wis., and were used as described by the manufacturers.

Isolation of plasmids from *L. lactis* was performed as described by Anderson and McKay (2). Rapid plasmid isolation from *E. coli* was accomplished by the alkaline lysis method (20). Large-scale plasmid isolation from *E. coli* was performed by to the polyethylene glycol precipitation procedure of Krieg and Melton (18).

DNA fragments were analyzed by horizontal electrophoresis in agarose gels with TBE buffer (20). Lambda DNA cut with *Hin*dIII or a kilobase ladder (Bethesda Research Laboratories) was used as a molecular weight standard and as a concentration reference. DNA fragments were isolated from agarose gels by using the GeneClean II kit (BIO 101, La Jolla, Calif.) as indicated by the manufacturer.

Southern transfers to MSI Mangnagraph nylon membranes (Micron Separations, Inc., Westboro, Mass.) were conducted as described elsewhere (20). DNA labeling, hybridization, and detection were performed by using the Genius nonradioactive DNA labeling and detection kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) as indicated by the manufacturer.

Electroporation of *E. coli* or *L. lactis* was performed by using a Bio-Rad Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.). *L. lactis* cells grown in GM17 to an optical density of 0.5 (λ = 600 nm) were harvested at 4,000 × *g* and washed twice in an equal volume of sterile cold water. Cells were suspended in 1/50th the original volume, and electroporation was conducted in a 0.1-cm cuvette at a voltage of 1.3 kV/cm and a capacitance setting of 100 Ω.

Matings. As a rapid screen for transfer phenotype, *L. lactis* MMS370 strains containing pTRK28::pRS01 cointegrates were cross-streaked with *L. lactis* LM2345 as described by Steele and McKay (31). Strains possessing the Tra^{high} phenotype (see Results) typically produced confluent growth on selection plates, whereas stains defective in transfer ability produced few or no colonies. For determination of transfer frequencies, plate matings were performed on milk-agar plates (5% nonfat dry milk, 1% glucose, and 1.5% agar) as described by Anderson and McKay (1). For a typical mating, actively growing cultures of donor and recipient cells were generated from a 2% inoculation of GM17 followed by growth at 30°C, without shaking, for 6 to 7 h. Donor and recipient cells were then centrifuged, and the pellet was resuspended in a 1/50th to 1/100th volume of M17. Typically, 50 μl of donor and 50 μl of recipient were mixed, spread onto a milk-agar plate, and allowed to incubate for 12 h 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 pTRK28::pRS01 cointegrate plasmid transfer, transconjugants were selected on GM17 medium containing 10 μg of erythromycin per ml. For determination of pLE1 or pLE33 plasmid transfer, transcon-

jugants were selected on GM17 medium containing 5 μg of chloramphenicol per ml. Transfer frequencies were calculated as the numbers of transconjugants recovered per input donor from at least two independent trials.

Construction of pLE1. Plasmid pLE1 is a derivative of pSKH1-B, a plasmid generated by the ligation of the *Ava*II-cleaved pIL253 (29) with *Ava*I-*Xba*I-digested pACYC184 (5). A chloramphenicol resistance gene encoded on a 1.6-kb *Sau*3A fragment from pTRK24 (27) (a derivative of pGK12 [16]) was subcloned into *Hpa*I-cleaved pSKH1-B to create pSKH1-BC. The erythromycin resistance gene of pSKH1-BC was then removed by an *Afl*II digestion and self-ligation, resulting in pLE1. Plasmid pLE1 is stably maintained in *L. lactis* MMS370 strains containing pTRK28::pRS01 cointegrates (designated pM1001 to pM6078 in Table 2), indicating that the three plasmid replication origins, i.e., those of pRS01, pTRK28, and pLE1, are compatible within *L. lactis*.

RESULTS

Generation of pTRK28::pRS01 cointegrates. A transconjugant pool containing pTRK28::pRS01 cointegrate plasmids was generated from six independent matings of *L. lactis* NCK168 and *L. lactis* MMS370. Five hundred fifty-eight pTRK28::pRS01-containing transconjugants were then assayed for secondary transfer in cross-streak plate matings with *L. lactis* LM2345 as a recipient. Of the 558 MMS370(pTRK28::pRS01) transconjugants, 120 strains (21%) exhibited a reduced transfer phenotype by comparison with the remaining Tra^+ transconjugant pool. Thirty-four of these strains were subjected to plate matings with LM2345 as a recipient in order to quantitate transfer frequencies. For comparison, the transfer frequencies of 13 randomly selected Tra^+ MMS370 (pTRK28::pRS01) transconjugants were also obtained. The results of these secondary matings are presented in Table 2. An examination of transfer frequencies led to an arbitrary grouping of MMS370(pTRK28::pRS01) transconjugants strains into four categories: Tra^{high}, 1 to 10^{-3} per donor cell; Tra^{med}, 10^{-4} to 10^{-6} per donor cell; Tra^{low}, 10^{-7} to 10^{-9} per donor cell; and Tra^-, $<10^{-9}$ per donor cell (no detectable transfer). The fact that Tra^- pTRK28::pRS01 cointegrate plasmids were able to transfer from the parental NCK168 strain suggests that expression of pRS01 transfer functions occurs prior to cointegration of pTRK28 and pRS01.

Mapping of pTRK28 insertion sites within pTRK28::pRS01 cointegrates. Plasmid pRS01 is cleaved into five distinct fragments by a *Pvu*II-*Sph*I restriction digestion (fragments *A*, *B*, *C*, *D*, and *E* [Fig. 1]). Disruption of one of the five pRS01 fragments by insertion of pTRK28 results in the generation of two novel junction fragments (for example, fragments C^1 and C^2 in Fig. 1) and two fragments (*F* and *G* in Fig. 1) internal to the pTRK28 plasmid. Within pTRK28 each *Pvu*II site is approximately 3.6 kb from the IS*946* sequence. Localization of pTRK28 inserts in pRS01 was obtained by subtracting the junction pTRK28 DNA (total of 4.4 kb, including the IS*946* sequence) from the novel *Pvu*II-*Pvu*II or *Pvu*II-*Sph*I junction fragments. Verification of the pTRK28 insertions was achieved by Southern hybridization analysis with pTRK28 plasmid as a DNA probe (data not shown). Confirmation of the locations of pTRK28 inserts within pRS01 fragments *C*, *D*, and *E* was accomplished by additional *Bam*HI or *Xba*I restriction digestions.

Analysis of the 34 pTRK28::pRS01 cointegrate plasmids altered in transfer proficiency indicated that four regions of pRS01 were involved in conjugation (Fig. 2). One region, Tra3, was localized within the 15-kb *Pvu*II-*Sal*I fragment previously identified as a transfer region by Anderson and McKay (1). The pattern of insertions within Tra3 suggested a further subdivision into three separate regions, Tra3A, Tra3B, and Tra3C (Fig. 2). All insertions within Tra3A completely eliminated cointegrate plasmid transfer. These insertions fell within a 1.5-kb region beginning 2.1 kb from the *Sph*I site in the *A* fragment. Tra3B is separated from Tra3A by 1.6 kb and consists of three insertions spanning 1.4 kb. All insertions within Tra3B reduced but did not eliminate plasmid transfer. Tra3C was localized approximately 2.2 kb from Tra3B, encom-

TABLE 2. Summary of pTRK28::pRS01 transfer frequencies

Donor and plasmid	Insert location[a]	Conjugation frequency[b]	Frequency category[c]
DM1004, pM1004	*	2.0×10^{-3}	Tra^{high}
DM2035, pM2035	*	3.2×10^{-2}	Tra^{high}
DM2036, pM2036	*	5.7×10^{-2}	Tra^{high}
DM2038, pM2038	*	2.2×10^{-2}	Tra^{high}
DM2048, pM2048	*	1.7×10^{-3}	Tra^{high}
DM2062, pM2062	*	3.3×10^{-3}	Tra^{high}
DM6010, pM6010	*	5.0×10^{-2}	Tra^{high}
DM6043, pM6043	*	6.4×10^{-2}	Tra^{high}
DM6069, pM6069	*	2.6×10^{-2}	Tra^{high}
DM6119, pM6119	*	1.4×10^{-2}	Tra^{high}
DM6132, pM6132	*	1.9×10^{-2}	Tra^{high}
DM6134, pM6134	*	4.9×10^{-2}	Tra^{high}
DM1001, pM1001	*	4.3×10^{-4}	Tra^{med}
DM5020, pM5020	Tra2	6.0×10^{-6}	Tra^{med}
DM5076, pM5076	Tra2	5.0×10^{-6}	Tra^{med}
DM5082, pM5082	Tra3B	1.4×10^{-5}	Tra^{med}
DM5114, pM5114	Tra2	1.1×10^{-5}	Tra^{med}
DM1005, pM1005	Tra1	2.7×10^{-7}	Tra^{low}
DM2068, pM2068	Tra3B	2.1×10^{-7}	Tra^{low}
DM5008, pM5008	Tra2	1.5×10^{-6}	Tra^{low}
DM5097, pM5097	Tra3B	1.5×10^{-8}	Tra^{low}
DM1003, pM1003	Tra3C	$<2.2 \times 10^{-9}$	Tra^-
DM1014, pM1014	Tra1	$<5.7 \times 10^{-9}$	Tra^-
DM1036, pM1036	Tra3A	$<1.0 \times 10^{-9}$	Tra^-
DM2031, pM2031	Tra3A	$<1.5 \times 10^{-9}$	Tra^-
DM2032, pM2032	Tra3C	$<1.8 \times 10^{-9}$	Tra^-
DM2033, pM2033	Tra3C	$<1.8 \times 10^{-9}$	Tra^-
DM2041, pM2041	Tra4	$<2.0 \times 10^{-9}$	Tra^-
DM3016, pM3016	Tra3C	$<1.5 \times 10^{-9}$	Tra^-
DM3020, pM3020	Tra1	$<4.3 \times 10^{-9}$	Tra^-
DM3022, pM3022	Tra1	$<1.8 \times 10^{-9}$	Tra^-
DM3047, pM3047	Tra3A	$<1.2 \times 10^{-9}$	Tra^-
DM3086, pM3086	Tra1	$<1.9 \times 10^{-9}$	Tra^-
DM5015, pM5015	Tra1	$<2.1 \times 10^{-9}$	Tra^-
DM5018, pM5018	Tra3A	$<5.2 \times 10^{-9}$	Tra^-
DM5024, pM5024	Tra1	$<6.4 \times 10^{-9}$	Tra^-
DM5032, pM5032	Tra3C	$<2.7 \times 10^{-9}$	Tra^-
DM5034, pM5034	Tra4	$<1.3 \times 10^{-9}$	Tra^-
DM5054, pM5054	Tra3C	$<1.3 \times 10^{-9}$	Tra^-
DM5062, pM5062	Tra3C	$<2.5 \times 10^{-9}$	Tra^-
DM5064, pM5064	Tra1	$<4.3 \times 10^{-9}$	Tra^-
DM5066, pM5066	Tra3A	$<1.3 \times 10^{-9}$	Tra^-
DM5086, pM5086	Tra1	$<3.8 \times 10^{-9}$	Tra^-
DM5105, pM5105	Tra3A	$<2.6 \times 10^{-9}$	Tra^-
DM5113, pM5113	Tra3A	$<4.3 \times 10^{-9}$	Tra^-
DM5120, pM5120	Tra3A	$<9.7 \times 10^{-10}$	Tra^-
DM6078, pM6078	Tra3A	$<5.0 \times 10^{-9}$	Tra^-

[a] As defined in Fig. 3. *, insertion occurred outside defined pRS01 transfer regions.
[b] Number of transconjugants per input donor.
[c] Tra^{high}, 1 to 10^{-3}; Tra^{med}, 10^{-4} to 10^{-6}; Tra^{low}, 10^{-7} to 10^{-9}; Tra^-, no detectable transfer.

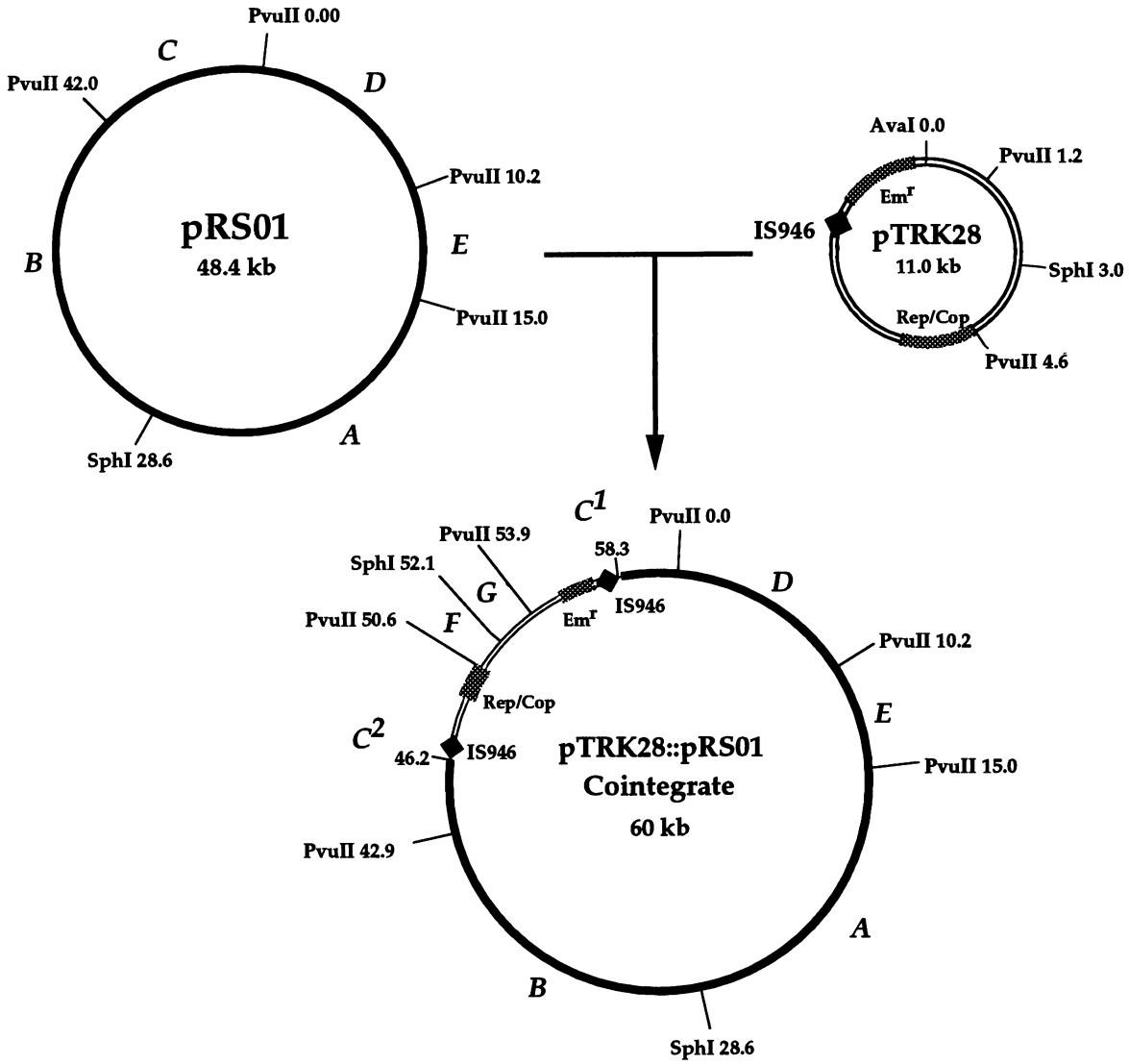

FIG. 1. IS*946*-mediated cointegration of pTRK28 and pRS01 within the donor cell. In the example shown here, pTRK28 has inserted into fragment *C* of pRS01. Localization of the pTRK28 insertion is obtained by subtraction of the pTRK28 junction DNA from the novel *Pvu*II-*Pvu*II fragments (C^1 and C^2). Abbreviations: Em^r, erythromycin resistance gene; Rep/Cop, replication and copy control region.

passing a 5.2-kb segment adjacent to the *Pvu*II site within the *A* fragment (Fig. 2). All insertions in Tra3C completely eliminated transfer.

Tra4 is defined by two insertions which mapped within a 4.3-kb *Pvu*II-*Kpn*I fragment (fragment *D*) (Fig. 2) which was previously shown to be associated with a cell aggregation phenotype (1). These insertions eliminated plasmid transfer, suggesting an essential role of cell aggregation or a linked genetic function in the pRS01 conjugative process. All cointegrate plasmids analyzed, whether phenotypically Clu^- or Clu^+, contained the *Pvu*II-*Kpn*I fragment in the Clu^+ orientation (orientation shown in Fig. 2) as defined by Anderson and McKay (1). This suggests that the orientation of the *Pvu*II-*Kpn*I fragment may not be the only mechanism for regulation of Clu expression.

Tra1 and Tra2 are two previously unidentified transfer regions located at the junction of fragments *C* and *B* (Fig. 2). These regions are divided by a single Tra^{high} insertion (pM2048) (Fig. 2). Tra2 is a small locus (~0.5 kb) approximately 9 kb away from Tra3A. Insertions in Tra2 reduced but did not eliminate conjugative transfer. Tra1 is a larger region covering a 1.8-kb segment which extends out from the *Pvu*II site in fragment *B*. With the exception of the Tra^{low} insert pM1005, all the insertions within Tra1 eliminated plasmid transfer.

Subcloning of Tra3A and Tra3B into pLE1. Romero and Klaenhammer (28) outlined a strategy to subclone Tn-EmA::pRS01 junction DNA by utilizing restriction sites that fall within the Tn-EmA composite transposon and pRS01. The Tn-EmA composite transposon, a derivative of pTRK28, contained the *E. coli* plasmid pACYC184 origin of replication and chloramphenicol resistance gene, thus allowing for selection in *E. coli*. We used a similar strategy to isolate junction DNA derived from the cointegrate pTRK28::pRS01 plasmids; the first transfer region cloned into pLE1 by this approach was the Tra3 region. To isolate the Tra3 region, pM1001 (Fig. 3) was

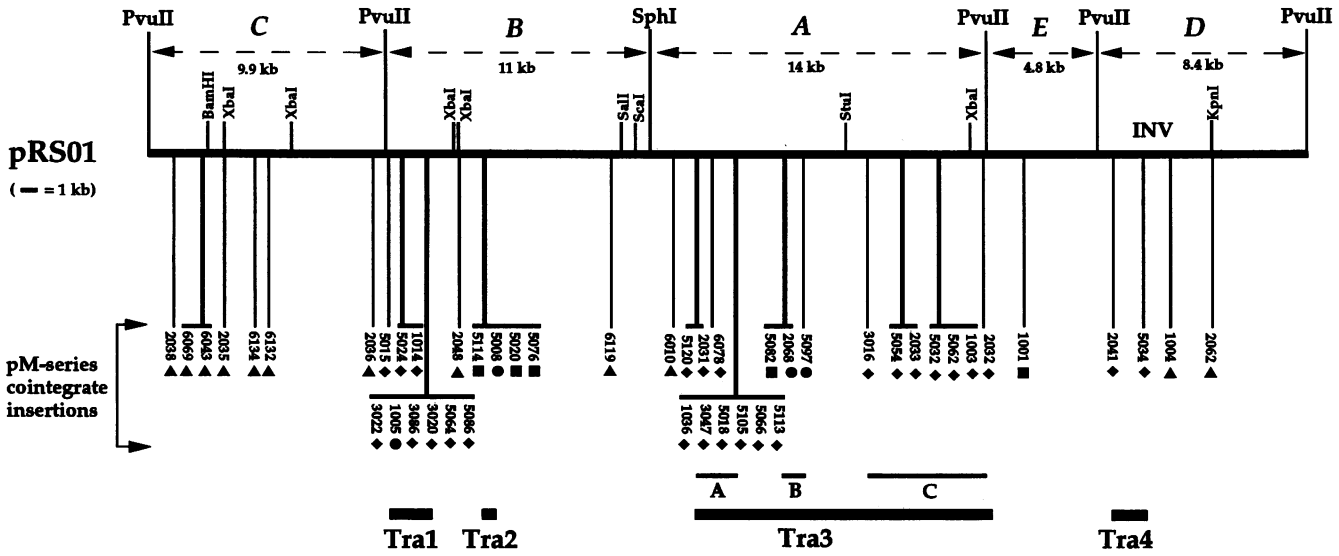

FIG. 2. Plasmid pTRK28 insertions within the 47 pTRK28::pRS01 cointegrate plasmids. Plasmid pRS01 is cleaved into five fragments (*A*, *B*, *C*, *D*, and *E*) by *Pvu*II and *Sph*I sites. Individual cointegrate plasmids are denoted at the site of pTRK28 insertion into pRS01 by the final four numbers of the designations for plasmids pM1001 to pM6078. Assignments of Tra^{high} (▲) (1 to 10^{-3} per donor cell), Tra^{med} (■) 10^{-4} to 10^{-6} per donor cell), Tra^{low} (●) (10^{-7} to 10^{-9} per donor cell), and Tra^{-} (◆) ($<10^{-9}$ per donor cell) phenotypes are based on calculated transfer frequencies in secondary matings. Tra^{high} cointegrates were selected at random for physical mapping. Insertion sites which mapped within the same 1.0-kb segment are grouped. Individual loci within Tra3 are designated by A, B, or C.

digested with *Sca*I, and the reaction products were then self-ligated and transformed into *E. coli* DHFα. The resulting plasmid, pE231, contains the *E. coli* plasmid pACYC184 origin of replication, a tetracycline resistance gene, and approximately 16 kb of pRS01 DNA (Fig. 3). A 6.8-kb portion of the Tra3 transfer region containing both Tra3A and Tra3B (bounded by *Sca*I and *Stu*I) (Fig. 3) was subcloned by a *Xba*I-*Stu*I restriction of the plasmid pE231. The resulting fragment was ligated into *Xba*I-*Sma*I-cleaved pLE1, generating the plasmid pLE33.

Complementation of pTRK28 inserts in Tra3. Plasmid pLE33 was transformed into all strains containing cointegrate plasmids with insertions within Tra3A and Tra3B. As indicated in Table 3, all of the resulting transformants demonstrated increased transfer frequencies to a Tra^{high} phenotype, suggesting complementation, in *trans*, of both the Tra3A and Tra3B insertions with the 6.8-kb *Sca*I-*Stu*I fragment. Mobilization of the complementing plasmid, pLE33, was not observed in any mating, suggesting that the 6.8-kb *Sca*I-*Stu*I fragment does not encode the pRS01 origin of transfer and verifying the lack of recombination in the Rec^{-} donor strain. Control matings with strains containing cointegrate plasmids with insertions within Tra3A or Tra3B and the vector plasmid pLE1 exhibited transfer frequencies similar to those obtained with the original strains (Table 2). Plasmid pLE33 did not complement transfer in strains DM3016.33 and DM1003.33, two strains containing cointegrates with insertions in Tra3C.

DISCUSSION

Anderson and McKay (1) first localized regions involved in conjugative transfer and expression of a cell aggregation phenotype associated with the *L. lactis* ML3 plasmid pRS01. In this study, we identified additional regions of pRS01 involved in conjugative transfer. Cointegrate pTRK28::pRS01 plasmids possess a higher copy number, increased stability, and smaller size than the pSK08::pRS01 cointegrate plasmids analyzed previously (1). The smaller size has allowed for a more definitive mapping of the junction regions of pTRK28::pRS01 cointegrate plasmids than was possible with pSK08::pRS01 cointegrate plasmids.

We identified four noncontiguous transfer regions on pRS01. Regions Tra3 and Tra4 were localized within a 24 kb segment of pRS01 (fragments *A*, *D*, and *E*; Fig. 2) previously shown to be involved in pSK08::pRS01 plasmid transfer and cell aggregation (1). Our mapping suggests that three distinct transfer loci, Tra3A, Tra3B, and Tra3C, within the 15-kb *Pvu*II-*Sal*I fragment of pRS01 are important for transfer of pSK08::pRS01 cointegrate plasmids as identified by Anderson and McKay (1). While no Tra^{high} insertions were localized between these three regions, the fact that insertions in Tra3B reduce, but do not eliminate, transfer suggests that Tra3A and Tra3C are independent loci and are not expressed on a single transcript. Complementation of Tra3A and Tra3B insertions with a subcloned 6.8-kb *Sca*I-*Stu*I fragment from pRS01 implies that a single large transcript originating within Tra3C (transcribing from Tra3C to Tra3A) is unlikely. The data do not rule out the possibility, however, that a single transcript initiating in Tra3A or Tra3B is required for Tra3C expression.

While analyzing transconjugants containing the cointegrate pSK08::pRS01, Anderson and McKay (1) detected an inversion of two restriction sites (*Pvu*II and *Kpn*I; fragment *D* in Fig. 2) coinciding with changes in expression of the Clu phenotype. Our pTRK28::pRS01 mapping has identified a region, Tra4, which falls between the *Pvu*II and *Kpn*I restriction sites. This finding suggests that the inversion locus is essential for conjugative transfer, possibly by enabling expression of the Clu phenotype and proper cell attachment for plasmid transfer. The fact that several of the Tra^{high} strains were phenotypically Clu^{-} suggests that minimal expression of the Clu gene product(s) may be sufficient for high-frequency plasmid transfer. All of the cointegrate plasmids mapped in this study contain the *Pvu*II-*Kpn*I fragment in the Clu^{+} orientation as previously defined (orientation shown in Fig. 2) (1) whether they are

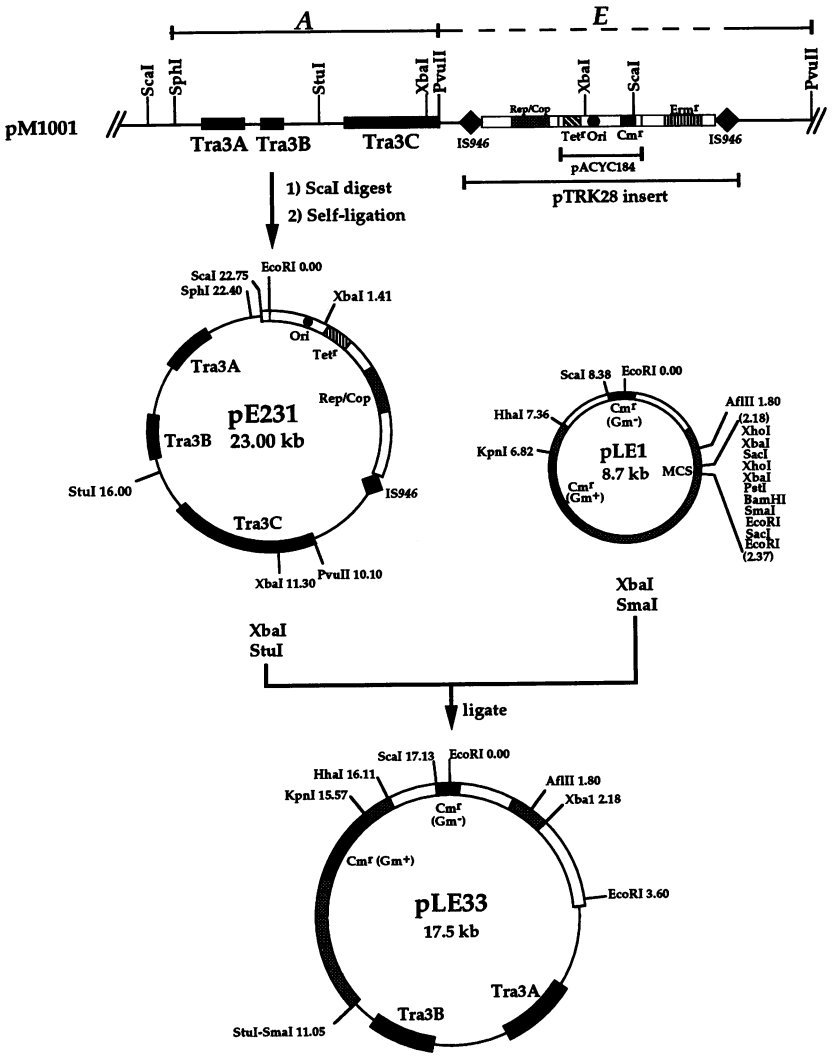

FIG. 3. Enlarged map of pM1001 showing fragments *A* and *E*. Plasmid pM1001 fragment *A* contains the complete Tra3 region (Tra3A, Tra3B, and Tra3C), while fragment *E* contains a pTRK28 insertion (dashed line). To subclone Tra3 region DNA, pM1001 was cleaved with *Sca*I and the reaction products were self-ligated, resulting in pE231. Plasmid pE231 was cleaved with *Xba*I and *Stu*I, and the resulting 6.8-kb *Xba*I-*Stu*I fragment, containing regions Tra3A and Tra3B, was ligated with *Sma*I-*Xba*I-cleaved pLE1, resulting in pLE33. Abbreviations: Cm^r, chloramphenicol resistance gene; Tet^r, tetracycline resistance gene; Erm^r, erythromycin resistance gene; Ori, plasmid replication origin derived from pACYC184; Rep/Cop, replication and copy control regions from pTRK28; MCS, multiple cloning site; Gm$^+$, resistance gene expressed in *L. lactis*; Gm$^-$, resistance gene expressed in *E. coli*.

phenotypically Clu$^+$ or Clu$^-$. This suggests that other factors, in addition to the orientation of the *Pvu*II-*Kpn*I fragment, may regulate Clu expression.

Two novel regions, Tra1 and Tra2, important for pTRK28:: pRS01 transfer were also identified approximately 9 kb from Tra3. The demonstration of genes responsible for conjugative transfer residing in disparate regions has also been reported for two staphylococcal plasmids. Using Tn*551* insertion mutagenesis, Evans and Dyke (8) identified two regions involved in conjugative transfer of the *Staphylococcus aureus* plasmid pJE1 which were separated by approximately 12 kb. Working with a similar *S. aureus* plasmid, Morton et al. (23) sequenced the conjugative transfer genes from the *S. aureus* plasmid pGO1 and found a contiguous span of 14 open reading frames covering a 13.6-kb segment. Subsequent work (19) has suggested a second transfer region of pGO1 removed from the primary transfer complex. Genetic analysis of the streptococcal conjugative plasmid pIP501 identified two regions where Tn*917lac* insertions abolished plasmid transfer (17). However, these regions were separated by less than 1 kb. The genes involved in transfer of the pheromone-responsive plasmids pCF10 and pAD1 (6) from *Enterococcus faecalis* are encoded within a contiguous span of 30 kb, with a major portion devoted to a complex regulatory mechanism.

Because of the homology between the *L. lactis* 712 sex factor and plasmid pRS01 detected by Gasson et al. (11), the transfer regions identified here are most likely similar to those encoded on the 712 sex factor. Using this homology, Gasson et al. (11) detected pRS01 within the chromosome of some, but not all, strains of ML3, indicating the factor was curable. Recently Bringel et al. (4) demonstrated transfer of a chromosomally encoded element, *laf*, within *L. lactis* MG1363 to the chromosome of a recipient *L. lactis* strain. Southern hybridization analysis indicated that this element was homologous to the 712

TABLE 3. Complementation analysis of Tra3A and Tra3B[a]

Mutant pTRK28::pRS01 cointegrate	pRS01 region interrupted by pTRK28 insert	Conjugation frequency with coresident plasmid:	
		pLE1	pLE33
pM1036	Tra3A	$<3.6 \times 10^{-9}$	1.3×10^{-3}
pM2031	Tra3A	$<2.1 \times 10^{-9}$	1.4×10^{-3}
pM3047	Tra3A	$<5.3 \times 10^{-9}$	2.5×10^{-4}
pM5018	Tra3A	$<2.0 \times 10^{-8}$	3.2×10^{-2}
pM5066	Tra3A	$<5.0 \times 10^{-9}$	4.2×10^{-4}
pM5105	Tra3A	$<2.0 \times 10^{-9}$	1.6×10^{-3}
pM5113	Tra3A	$<4.3 \times 10^{-9}$	1.1×10^{-3}
pM5120	Tra3A	$<2.5 \times 10^{-9}$	2.5×10^{-3}
pM6078	Tra3A	$<3.6 \times 10^{-9}$	1.4×10^{-3}
pM2068	Tra3B	7.9×10^{-7}	1.4×10^{-3}
pM5082	Tra3B	1.7×10^{-6}	2.2×10^{-3}
pM5097	Tra3B	4.1×10^{-8}	4.9×10^{-4}
pM1003	Tra3C	$<7.7 \times 10^{-9}$	$<5.0 \times 10^{-9}$
pM3016	Tra3C	$<3.4 \times 10^{-9}$	$<3.7 \times 10^{-9}$

[a] All matings utilized *L. lactis* subsp. *lactis* LM2345 as a recipient. Matings involving strains containing a pTRK28::pRS01 cointegrate (designated here pM1036 to pM6078) were scored for erythromycin-resistant CFU per input donor. Mobilization of either complementing plasmid, pLE1 or pLE33, was not observed. Control matings with strains containing either pLE1 or pLE33 alone exhibited no conjugal transfer ($<10^{-9}$ per donor).

sex factor, suggesting independent transfer of the sex factor without prior cointegration into a second plasmid encoding an observable phenotype. The identification and subcloning of regions important for transfer of pRS01 should allow for a more detailed analysis of pRS01 and shed light on the derivation of similar conjugative elements in lactococci. In particular, the identification and cloning of the pRS01 region which contains the conjugative origin of transfer would be beneficial in the generation of mobilizable vectors. Such vectors would be useful in the development of industrially important strains of lactococci and other lactic acid bacteria.

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