Applied and Environmental Microbiology, Apr. 1998, p. 1541–1544 Vol. 64, No. 4
0099-2240/98/$04.00+0
Copyright © 1998, American Society for Microbiology

An Origin of Transfer (*oriT*) on the Conjugative Element pRS01 from *Lactococcus lactis* subsp. *lactis* ML3†

DAVID A. MILLS,[1]‡ TREVOR G. PHISTER,[1]§ GARY M. DUNNY,[1] AND LARRY L. McKAY[2]*

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]

Received 27 August 1997/Accepted 9 January 1998

Previous analysis of the Tra1 region of the conjugative element pRS01 from *Lactococcus lactis* subsp. *lactis* ML3 suggested that an origin of transfer (*oriT*) was present. Deletion derivatives of this cloned Tra1 region were assayed for mobilization in the presence of the wild-type pRS01 element in *trans*. The pRS01 *oriT* was localized to a 446-nucleotide segment in the intergenic region between open reading frames *ltrD* and *ltrE*. Sequence analysis of this region revealed a cluster of direct and inverted repeat structures characteristic of *oriT* regions associated with other conjugative systems.

Bacterial conjugation is a common mechanism for genetic exchange in nature. Studies on a variety of enterobacterial conjugative elements (11, 29) have resulted in a model in which bacterial conjugation is considered a two-part process; the first component involves bringing donor and recipient cells together to form an effective mating pair, and the second step involves enzymatic transfer of a single strand of the conjugative plasmid into the recipient cell. Initiation of single-stranded transfer involves the action of a specialized nucleoprotein complex called the relaxosome to produce single-stranded cleavage at a specific site (*nic*) within the origin of transfer (*oriT*) of the conjugative element (11, 29).

While our understanding of conjugative elements from gram-positive bacteria has increased, only recently have studies begun to define *oriT* and other relaxosome components. A specific nick site has recently been identified on the broad-host-range conjugative plasmid pIP501 from *Streptococcus agalactiae* and on the conjugative plasmid pGO1 from *Staphylococcus aureus* (5, 27). Surprisingly, the sequences of the *nic* regions of both pGO1 and pIP501 were shown to be quite similar to *nic* region sequences of a family of IncQ type conjugative elements from gram-negative bacteria (5, 11, 27). Transfer origins have also been identified for several other conjugative systems from gram-positive bacteria, including pheromone-inducible conjugative plasmid pAD1 (1, 4), non-self-transmissible staphylococcal plasmid pC221 mobilized by the conjugative vector pGO1 (17), and non-self-transmissible plasmids pUB110 and pBC16 from *Bacillus subtilis*, both of which are mobilized by the conjugative plasmid pLS20 (22). In addition, *oriT* sequences have recently been identified on the broad-host-range conjugative transposon Tn*916* (10) and the streptococcal transposon Tn*5252* (24).

The conjugative element pRS01 has been found in several *Lactococcus lactis* subsp. *lactis* genomes (7). pRS01 has been shown to mediate high-frequency transfer of genes encoding lactose utilization among lactococci and possesses the gene(s) responsible for a cell aggregation phenotype. Mapping of pRS01 identified four distinct regions (Tra1, Tra2, Tra3, and Tra4) involved in conjugative transfer (13). Sequence analysis of the Tra1 region revealed a gene, *ltrB*, with extensive homology to the genes encoding other plasmid and conjugative relaxases. In this work the *oriT* of pRS01 was localized and was shown to reside within the Tra1 region upstream of the *ltrB* gene.

Bacterial strains, media, and matings. *Escherichia coli* DH5α, which was used as a cloning host, was grown in Luria-Bertani medium (20). The selective media used for *E. coli* strains contained 30 μg of chloramphenicol per ml. *L. lactis* subsp. *lactis* strains were grown in GM17 (M17 medium [25] containing 0.5% glucose) at 30°C without agitation. The selective media used for *L. lactis* strains contained antibiotics at the following concentrations: erythromycin, 10 μg/ml; chloramphenicol, 5 μg/ml; rifampin, 50 μg/ml; and spectinomycin, 300 μg/ml. All plating media contained 1.5% Bacto Agar (Difco Laboratories).

To determine transfer frequencies, plate matings were performed on GM17 as described previously (13). The donor strains (*L. lactis* subsp. *lactis* DM2036 [13] containing various pLE12 deletion derivatives) and the recipient strain (*L. lactis* subsp. *lactis* LM2345 [2]) were generated by using a 2% inoculum and GM17, followed by growth at the appropriate temperature without shaking until exponential growth was achieved. The donor and recipient cells were then centrifuged, and the pellet was resuspended in a 0.02 to 0.01 volume of GM17. Typically, 50 μl of donor cells and 50 μl of recipient cells were mixed, spread onto a GM17 plate, and incubated for 12 h at 30°C. The mating mixture was then washed off the plate with 1 ml of sterile phosphate-buffered saline, diluted appropriately, and plated onto selective medium. To determine pLE12 series plasmid transfer, transconjugants were selected on medium containing 5 μg of chloramphenicol per ml. Transfer frequencies were calculated by determining the number of transconjugants recovered per input donor in at least three independent trials.

DNA manipulation and analysis. General molecular biology techniques were performed as described previously (20). Plasmid isolation and electroporation were performed as described

* Corresponding author. Mailing address: Department of Food Science and Nutrition, University of Minnesota, 1334 Eckles Avenue, St. Paul, MN 55108. Phone: (612) 624-3090. Fax: (612) 625-5272. E-mail: lmckay@che2.che.umn.edu.

† Paper no. 97-1-18-0024 of the contribution series of the Minnesota Agricultural Experiment Station based on research conducted under Project 18-62.

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

§ Present address: Department of Food Science and Nutrition, University of Minnesota, St. Paul, MN 55108.

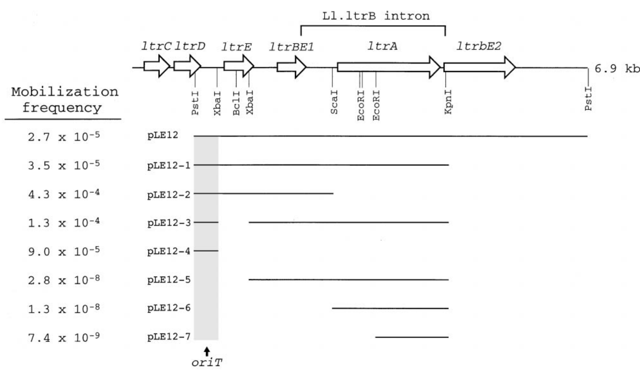

FIG. 1. Genetic map of the Tra1 region of pRS01 and results of a pLE12 derivative mobilization analysis. Plasmid pLE12 contains the complete Tra1 region. Plasmids pLE12-1, pLE12-2, and pLE12-4 are deletion derivatives of pLE12. Plasmids pLE12-3, pLE12-5, pLE12-6, and pLE12-7 are deletion derivatives of pLE12-1. The mobilization frequency was calculated by determining the number of transconjugants per input donor.

previously (16). A sequence analysis was performed with the Genetics Computer Group (Madison, Wis.) sequence analysis software.

Characterization of the pRS01 *oriT* region. Previous complementation analysis of pRS01 Tra1 region insertions with Tra1 DNA resulted in mobilization of the complementing vector, pLE12, into lactococcal recipients (14, 16). The results suggested that the pRS01 *oriT* was on the 7.5-kb *Pst*I fragment cloned into pLE12. In an effort to localize this *oriT*, we analyzed a series of pLE12 deletion derivatives for mobilization in the presence of the Tra^+ cointegrate plasmid pM2036 (13). pM2036 is a cointegrate plasmid composed of conjugal element pRS01 and the *L. lactis-E. coli* shuttle vector pTRK28 (19). All derivatives possessing a 446-nucleotide *Pst*I-*Xba*I fragment, which contained the intergenic region between *ltrD* and *ltrE*, were mobilized by pM2036 at a frequency that was 3 to 4 orders of magnitude higher than the frequencies obtained for subclones lacking the *Pst*I-*Xba*I fragment (Fig. 1). In addition, derivatives containing the 446-nucleotide *Pst*I-*Xba*I fragment were mobilized at frequencies ranging from 10^{-4} to 10^{-5} transconjugant per donor, values which were similar to the mobilization frequencies previously observed for pLE12 when it was used to complement various Tra^- Tra1 region insertions (16).

Sequence analysis of the intergenic region between *ltrD* and *ltrE* revealed a series of inverted repeat and direct repeat structures (IR1 through IR7, DR1, and DR2) (Fig. 2). Clusters of inverted and direct repeats and a general AT-rich nucleotide composition are characteristics of other bacterial *oriT* regions (11, 29). Surprisingly, the AT ratio of the pRS01 *oriT* region (60%) was lower than the AT ratio of the flanking Tra1 region sequence (65%) or the general AT ratio for lactococci (64%) (21).

In recent years biochemical and sequence data from a variety of conjugal elements have revealed that most *nic* region sequences fall into one of three consensus sequence classes named for prototype *nic* regions of each group, namely, IncQ, IncP, and F-like *nic* sites (11). In addition, all three classes of *nic* regions (IncQ, IncP, and F) are known to possess an upstream inverted repeat involved in termination of DNA transfer into the recipient cell (11). A search of the *ltrD-ltrE* intergenic region for these consensus sequences revealed three potential IncP family *nic* sites (P1 through P3) (Fig. 2), one F family site (F1), and one IncQ family site (Q1) (Fig. 2). One of the three IncP class sequences, P2 exhibited the highest similarity to the consensus IncP *nic* region sequence, matching it at seven of eight nucleotide positions (88% identity). While the sequences of sites P1 and P3 were less homologous to the consensus IncP *nic* region sequence (level of identity for each site, 75%), P3 did possess an upstream inverted repeat (IR5) (Fig. 2). The sequence of the single IncQ family homologous site, Q1, matched the consensus sequence at 10 of 12 nucleotide positions (83% identity). In addition, the Q1 site contained an upstream inverted repeat similar in structure to the inverted repeats found in the *nic* regions of the staphylococcal conjugal plasmid pGO1, the streptococcal plasmid pIP501, and other IncQ family elements (5, 11). Finally, the F family site, F1, exhibited minimal homology to the F family consensus *nic* region (13 of 17 nucleotides) and overlapped the upstream C-terminal end of the *ltrD* reading frame.

Discussion. The conjugal element pRS01 is present in certain lactococci (7). A previous analysis of this element identified four distinct transfer regions (13). Complementation of the Tra1 region resulted in mobilization of the complementing plasmid, suggesting that a *cis*-acting *oriT* was present (14, 16). A sequence analysis of the Tra1 region revealed six potential open reading frames, including a putative conjugative relaxase *ltrB* open reading frame interrupted by the group II intron (15, 16). In this work we localized the pRS01 *oriT* to a 446-nucleotide segment of the Tra1 region between *ltrD* and *ltrE*. Like other bacterial *oriT* regions, this segment possessed multiple inverted and direct repeats. Recently, a specific *nic* site within an *oriT* region was identified for the streptococcal conjugative plasmid pIP501 (27) and the staphylococcal plasmid pGO1 (5). These *nic* site regions exhibited strong sequence similarity to the IncQ family of *nic* regions (11), suggesting that there is a possible link between gram-positive and gram-negative bacterial conjugal systems. Analysis of the pRS01 *oriT* region identified a sequence segment possessing 10 of 12 nucleotides present in a consensus IncQ *nic* region (11). In addition, three putative IncP family *nic* region sites and an F class *nic* region were also identified. The precise location of the pRS01 nick site remains to be determined.

Few transfer origins from conjugative elements in lactococci have been identified. Lucey et al. (12) characterized a region of plasmid pCI528 involved in mobilization by the ML3-712 class of lactococcal strains, which possess the homologous pRS01 or 712 sex factor elements. In that work, a minimal mobilization region was shown to contain a series of inverted repeat structures upstream of a putative mobilization protein (ORF1) (12). Surprisingly, the transfer origin from pRS01 identified in this work is not related to the mobilization region identified in pCI528 even though both regions, when placed in *trans*, are mobilized by the same ML3-712 class sex factor elements. The mobilization protein encoded on pCI528 and the predicted LtrB relaxase from pRS01 do exhibit some homology (22%), however (16). The difference in putative transfer origins which are mobilized by the same conjugal elements suggests that either (i) the pRS01 and 712 sex factor elements can mobilize different transfer origins or (ii) the pCI528 mobilization region is a site for recombination with conjugative elements, resulting in mobilization by a cointegrate mechanism (18).

In the past decade electroporation has become the method of choice for transforming various lactic acid bacteria, and consequently numerous electroporation protocols have been developed (3, 6, 8, 9, 26, 28). Unfortunately, many lactic acid bacteria are still difficult to transform by electroporation, which results in tedious and sometimes unsuccessful optimization of electroporation conditions. In addition, larger plasmid constructs are generally less effectively transformed by electroporation (23). In contrast to electroporation, conjugative mo-

```
                    PstI
     AAACAGTAAAAGAAATTA CTGCAGAGATTCAAACAACTTCTTCAGATGACCTCGAAAAAC
     TTTGTCATTTTCTTTAATGACGTCTCTAAGTTTGTTGAAGAAGTCTACTGGAGCTTTTTG
ltrD ⇒ T  V  K  E  I  T  A  E  I  Q  T  T  S  S  D  D  L  E  K  R

     GAGAGGGCAAAGAACAAAAAGCCAAAAGTATGTTGGCTGATTTTTCTTGGTTACAAGGAA
     CTCTCCCGTTTCTTGTTTTTCGGTTTTCATACAACCGACTAAAAAGAACCAATGTTCCTT
       E  G  K  E  Q  K  A  K  S  M  L  A  D  F  S  W  L  Q  G  K

     DR1  DR1  IR2  IR2
     F1  IR1  IR1
     AAAGAAAAAATGCGAAGTAGAAACTACTTTTCATAAAAAATGGGGATTGGGGACACCCCC
     TTTCTTTTTTACGCTTCATCTTTGATGAAAAGTATTTTTTACCCCTAACCCCTGTGGGGG
       R  K  N  A  K  *

     IR3  IR3  IR4
     AAAAATTAAGTAAATGGATAAGGAAGAAACCTTATCCATTTCTTAAATTCCGTAAGATGC
     TTTTTAATTCATTTACCTATTCCTTCTTTGGAATAGGTAAAGAATTTAAGGCATTCTACG

     IR4  P1  DR2
     TATCATCTTACTATGCTTGCAAAAGGTCAAGGAAGGGATAGGCTCTTCCTCCCAAAGGGG
     ATAGTAGAATGATACGAACGTTTTCCAGTTCCTTCCCTATCCGAGAAGGAGGGTTTCCCC

     DR2  P2
     GTGTAGGGGGTAATGGAATGACCTCCTGCCTGTCAGGGATGTTTGCTTTTTCTGTTATAA
     CACATCCCCCATTACCTTACTGGAGGACGGACAGTCCCTACAAACGAAAAAGACAATATT

     TTGTATTAGGTTGAAGGAGGGGCGCAATGAGCTTGTTTACAATGTTAAAAAAAAGAAACC
     AACATAATCCAACTTCCTCCCCGCGTTACTCGAACAAATGTTACAATTTTTTTTCTTTGG
     P3  Q1  IR5  IR5

     IR6  XbaI  IR6
     AGATGTGGAAACGACTACGGAAGTTTGTCCTAGATGTCAT TCTAGACTGACACTCAGGGA
     TCTACACCTTTGCTGATGCCTTCAAACAGGATCTACAGTAAGATCTGACTGTGAGTCCCT

     IR7  IR7
     AGGAAAATATGGTAAATTTTGGGGCTGCAAAAATTTCCCCAAATGCGTAGGAACTCTTCC
     TCCTTTTATACCATTTAAAACCCCGACGTTTTTAAAGGGGTTTACGCATCCTTGAGAAGG

     TTATGATAGATATATGAGTGAACAAGAACTAGCTGACTACTTTGCGAACCGAAGTAAACC
     AATACTATCTATATACTCACTTGTTCTTGATCGACTGATGAAACGCTTGGCTTCATTTGG
               ltrE ⇒ M  S  E  Q  E  L  A  D  Y  F  A  N  R  S  K  P
```

FIG. 2. Nucleotide sequence of the pRS01 *ltrD-ltrE* intergenic region. Inverted repeats and direct repeats are indicated by solid arrows and dashed arrows, respectively. Regions homologous to the IncQ (Q1), IncP (P1 through P3), and F (F1) family *nic* regions are enclosed in boxes.

bilization of pRS01 *oriT*-containing derivatives may offer an alternative for genetic delivery into lactococci and, potentially, other lactic acid bacteria. Since the transfer is based on conjugal delivery, the size of the mobilizable element probably does not influence the transfer frequency. Moreover, the technical ease with which *oriT*-containing derivatives can be mobilized, via simple cross-streak matings, makes pRS01-dependent mobilization an attractive alternative to electroporation. Given that pRS01 is an indigenous conjugal element in lactococci, the discovery of the pRS01 *oriT* should also allow development of food grade mobilizable delivery systems.

This work was supported by grants from the Minnesota-South Dakota Dairy Foods Research Center (to G.M.D.) and the Kraft General Foods Chair (to L.L.M.). D.A.M. was supported by a NIGMS Predoctoral Biotechnology Training Grant.

REFERENCES

1. **An, F. Y., and D. B. Clewell.** 1997. The origin of transfer (*oriT*) of the enterococcal, pheromone-responding, cytolysin plasmid pAD1 is located within the *repA* determinant. Plasmid **37:**87–94.
2. **Anderson, D. G., and L. L. McKay.** 1984. Genetic and physical characterization of recombinant plasmids associated with cell aggregation and high-frequency conjugal transfer in *Streptococcus lactis* ML3. J. Bacteriol. **158:** 954–962.
3. **Aukrust, T. W., M. B. Brurberg, and I. F. Nes.** 1995. Transformation of *Lactobacillus* by electroporation. Methods Mol. Biol. **47:**201–208.
4. **Clewell, D. B.** 1993. Sex pheromones and the plasmid-encoded mating response in *Enterococcus faecalis*, p. 349–364. *In* D. B. Clewell (ed.), Bacterial conjugation. Plenum Press, New York, N.Y.
5. **Climo, M. W., V. K. Sharma, and G. L. Archer.** 1996. Identification and characterization of the origin of conjugative transfer (*oriT*) and a gene (*nes*) encoding a single-stranded endonuclease of the staphylococcal plasmid pGO1. J. Bacteriol. **178:**4975–4983.
6. **Dicks, L. M. T.** 1994. Transformation of *Leuconostoc oenos* by electroporation. Biotechnol. Techniques **8:**901–904.

7. **Gasson, M. J., S. Swindell, S. Maeda, and H. M. Dodd.** 1992. Molecular rearrangement of lactose plasmid DNA associated with high-frequency transfer and cell aggregation in *Lactococcus lactis* 712. Mol. Microbiol. **6:** 3213–3223.
8. **Gautier, M., A. Rouault, and R. Lemee.** 1995. Electrotransformation of *Propionibacterium freudenreichii* TL110. Lett. Appl. Microbiol. **20:**125–129.
9. **Holo, H., and I. F. Nes.** 1995. Transformation of *Lactococcus* by electroporation. Methods Mol. Biol. **47:**195–199.
10. **Jaworski, D. D., and D. B. Clewell.** 1995. A functional origin of transfer (*oriT*) on the conjugative transposon Tn*916*. J. Bacteriol. **177:**6644–6651.
11. **Lanka, E., and B. M. Wilkins.** 1995. DNA processing reactions in bacterial conjugation. Annu. Rev. Biochem. **64:**141–169.
12. **Lucey, M., C. Daly, and G. Fitzgerald.** 1993. Analysis of a region from the bacteriophage resistance plasmid pCI528 involved in its conjugative mobilization between *Lactococcus* strains. J. Bacteriol. **175:**6002–6009.
13. **Mills, D. A., C. K. Choi, G. M. Dunny, and L. L. McKay.** 1994. Genetic analysis of regions of the *Lactococcus lactis* subsp. *lactis* plasmid pRS01 involved in conjugative transfer. Appl. Environ. Microbiol. **60:**4413–4420.
14. **Mills, D. A., C. K. Choi, G. M. Dunny, and L. L. McKay.** 1995. Characterization of the conjugation system associated with the *Lactococcus lactis* ssp. *lactis* plasmid pRS01. Dev. Biol. Stand. **85:**543–548.
15. **Mills, D. A., D. A. Manias, L. L. McKay, and G. M. Dunny.** 1997. Homing of a group II intron from *Lactococcus lactis* subsp. *lactis* ML3. J. Bacteriol. **179:** 6107–6111.
16. **Mills, D. A., L. L. McKay, and G. M. Dunny.** 1996. Splicing of a group II intron involved in the conjugative transfer of pRS01 in lactococci. J. Bacteriol. **178:**3531–3538.
17. **Projan, S. J., and G. L. Archer.** 1989. Mobilization of the relaxable *Staphylococcus aureus* plasmid pC221 by the conjugative plasmid pGO1 involves three pC221 loci. J. Bacteriol. **171:**1841–1845.
18. **Reimmann, C., and D. Haas.** 1993. Mobilization of chromosomes and nonconjugative plasmids by cointegrative mechanisms, p. 137–188. *In* D. B. Clewell (ed.), Bacterial conjugation. Plenum Press, New York, N.Y.
19. **Romero, D. A., and T. R. Klaenhammer.** 1990. Characterization of insertion sequence IS*946*, an iso-IS*S1* element, isolated from the conjugative lactococcal plasmid pTR2030. J. Bacteriol. **172:**4151–4160.
20. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
21. **Sandine, W. E., P. C. Radich, and P. R. Elliker.** 1972. Ecology of the lactic streptococci. A review. J. Milk Food Technol. **35:**176–185.
22. **Selinger, L. B., N. F. McGregor, G. G. Khachatourians, and M. F. Hynes.** 1990. Mobilization of closely related plasmids pUB110 and pBC16 by *Bacillus* plasmid pXO503 requires *trans*-acting open reading frame beta. J. Bacteriol. **172:**3290–3297.
23. **Siguret, V., A. S. Ribba, G. Cherel, D. Meyer, and G. Peitu.** 1994. Effect of plasmid size on transformation efficiency by electroporation of *Escherichia coli* DH5α. BioTechniques **16:**422.
24. **Srinivas, P., A. O. Kilic, and M. N. Vijayakumar.** 1997. Site-specific nicking in vitro at *oriT* by the DNA relaxase of Tn*5252*. Plasmid **37:**42–50.
25. **Terzaghi, B. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. **29:**807–813.
26. **Walker, D. C., K. Aoyama, and T. R. Klaenhammer.** 1996. Electrotransformation of *Lactobacillus acidophilus* group A1. FEMS Microbiol. Lett. **138:** 233–237.
27. **Wang, A., and F. L. Macrina.** 1995. Streptococcal plasmid pIP501 has a functional *oriT* site. J. Bacteriol. **177:**4199–4206.
28. **Wei, M. Q., C. M. Rush, J. M. Norman, L. M. Hafner, R. J. Epping, and P. Timms.** 1995. An improved method for the transformation of *Lactobacillus* strains using electroporation. J. Microbiol. Methods **21:**97–109.
29. **Wilkins, B., and E. Lanka.** 1993. DNA processing and replication during plasmid transfer between Gram-negative bacteria, p. 105–129. *In* D. B. Clewell (ed.), Bacterial conjugation. Plenum Press, New York, N.Y.