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IS*946*-Mediated Integration of Heterologous DNA into the Genome of *Lactococcus lactis* subsp. *lactis*†

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The lactococcal insertion sequence IS*946* was used to construct suicide vectors for insertion of heterologous DNA into chromosomal and plasmid sequences of *Lactococcus lactis* subsp. *lactis*. Electroporation of *L. lactis* strains, including the recombination-deficient strain MMS362, with the suicide vector pTRK145 yielded 10^1 to 10^3 transformants per μg of DNA. pTRK145 insertions occurred primarily in the chromosome, with one insertion detected in a resident plasmid. Vector-specific probes identified junction fragments that varied among transformants, indicating random insertions of pTRK145.

Members of the genus *Lactococcus* are used widely in the manufacture of fermented dairy products. Genetic analyses of lactococci have focused primarily on plasmid biology and the use of plasmid-derived cloning vectors for potential starter culture improvement. Although relatively little attention has been directed towards the chromosome of these organisms, there has been increasing interest in applying direct insertional mutagenesis and integration techniques for genetic analyses. Transposable elements have been particularly useful for this purpose (4, 5). The heterologous transposon Tn*917* and conjugative elements Tn*916*/Tn*919* and Tn*1545* have demonstrated chromosomal integration and gene inactivation in lactococci (8, 9, 11, 12, 19, 24). Tn*919* has been studied extensively in lactococci (8, 9, 11, 12). Its utility requires the use of a high-frequency conjugal element that increased transfer frequencies up to 10^{-4} per recipient (11). Random or site-specific insertions occurred depending on the host strain. Although Tn*917* has been used successfully in other gram-positive bacteria, there is only one report of Tn*917* insertion in lactococci (24). Attempts in this laboratory to conjugally deliver Tn*917* by using a pVA797::Tn*917* plasmid were unsuccessful (26). To date, there have been no reports on the use of lactococcal transposable elements as tools for genetic engineering.

Recently, integration vectors that utilize homologous recombination have been constructed by Leenhouts et al. (15–17) by cloning a lactococcal chromosomal fragment into a variety of plasmids unable to replicate in lactococci. Chromosomal integration of the vector markers and inactivation of X-prolyl dipeptidyl aminopeptidase via Campbell-like and double-crossover recombination were demonstrated by using this approach. Similarly, Chopin et al. (6) cloned bacteriophage sequences into pE194 to integrate the vector via Campbell-like or double-crossover recombination with prophage sequences in the *Lactococcus lactis* chromosome. These integration vectors will be useful for insertions into specific chromosomal locations. However, insertional mutagenesis via this approach would first require cloning the gene of interest.

As stated previously, transposable elements have proven extremely useful for generating random insertion events. In lactococci, the iso-ISS*1* elements have been most thoroughly characterized (9, 10, 18, 20). It has been demonstrated that iso-ISS*1* elements insert randomly into a target plasmid during conjugative mobilization (18, 20). This study describes the use of the iso-ISS*1* element IS*946* (GenBank M33868) for insertion of heterologous DNA into the *L. lactis* genome that is independent of resident homology. IS*946* was isolated from the self-transmissible plasmid pTR2030 following conjugal mobilization of cloning vectors pGK12 and pSA3 (20). Resolution of cointegrate plasmids resulted in vector derivatives containing a copy of IS*946* that were used in the construction of model integration vectors based on IS*946*-mediated transpositional recombination. The preliminary results of this study were reported at the Third International ASM Conference on Streptococcal Genetics (21).

Genetic manipulations were performed as described previously (20). Lactococcal genomic DNA was isolated as outlined by Hill et al. (13) with minor modifications that include using 3 ml of mid-log-phase cells, 25 μl of lysozyme solution (10 mg/ml) with incubation at 37°C for 20 min, and extraction with 500 μl of phenol saturated with 3% NaCl.

Plasmid pTRK28 (pSA3::IS*946*; Fig. 1) was isolated as a resolution product of a pTR2030::pSA3 cointegrate (20). The gram-positive origin of replication (*ori*) was removed following digestion with *Hin*dIII and self-ligation (Fig. 1). The resulting plasmid, designated pTRK145 (5.7 kb), is made up of the *Escherichia coli* p15A *ori*, a functional IS*946* (Tnp^+ [transposase]), and an erythromycin (Em^r) and chloramphenicol (Cm^r) resistance marker selectable in *L. lactis* and *E. coli*, respectively. A second plasmid (pTRK146), containing a nonfunctional copy of IS*946* (Tnp^-), was constructed as a control for IS function. pTRK28 was digested with *Eco*RV and self-ligated to create pTRK146, a 5.2-kb plasmid containing the Em^r and Cm^r markers and a truncated copy of IS*946* (Fig. 1).

Three strains of *L. lactis* were electroporated with pTRK28, pTRK145, and pTRK146: *L. lactis* MMS362 (recombination deficient [Rec^-] [1]) and *L. lactis* LM0330 (recombination proficient [Rec^+] [27]), both of which were derived from *L. lactis* ML3; and *L. lactis* NCK203 (14), which was derived from an industrial starter culture and can be electroporated at high efficiency. The results are shown in Table 1. pTRK28 (Ori^+) transformed MMS362 and LM0330

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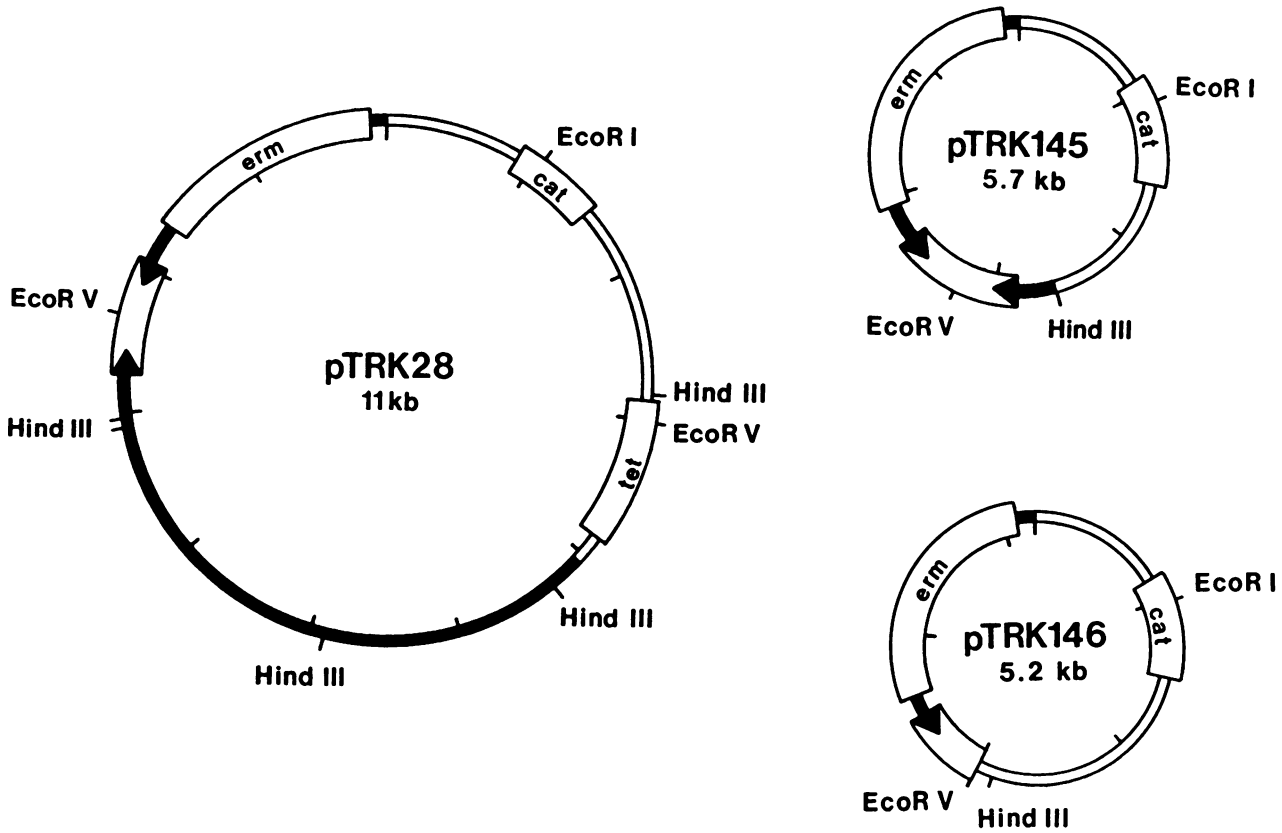

FIG. 1. Plasmid maps showing the derivation of pTRK145 and pTRK146 from resolution plasmid pTRK28. IS*946* is shown as the open box flanked by triangles that represent the inverted terminal repeats. erm, erythromycin resistance; cat, chloramphenicol resistance; tet, tetracycline resistance.

at ca. 10^4 and NCK203 at 10^6 per μg of DNA. Transformants invariably contained free pTRK28 (data not shown). With pTRK145 (Ori$^-$ Tnp$^+$), Em^r transformants were recovered from all three strains at ca. 10^1 to 10^3 per μg of DNA. No Em^r clones were recovered with pTRK146 (Ori$^-$ Tnp$^-$), indicating that, in the absence of a gram-positive *ori*, a functional IS*946* was required for acquisition of Em^r. The lack of a gram-positive *ori* requires that pTRK145 insert into the genome (plasmid or chromosome) for stable inheritance. The recovery of Em^r transformants in the Rec$^-$ strain MMS362 indicated that the IS*946*-mediated insertions were Rec independent.

Plasmid and total genomic DNAs from Em^r transformants were analyzed to determine the location of pTRK145 within *L. lactis* MMS362. Representative isolates possessed the parental MMS362 plasmid profile. ^{32}P-labeled pSA34 (Ori$^-$pSA3 derivative [25]) hybridized only to the chromosomal DNA of Em^r transformants and not to the genomic DNA of parental MMS362 (data not shown). Total genomic DNA from Em^r transformants was digested with *Eco*RI and probed with ^{32}P-labeled pSA34. pTRK145 contains a single site for *Eco*RI. Junction fragments were identified that varied among five transformants examined. This indicated random insertions by pTRK145 in the chromosome of Rec$^-$ strain MMS362 (Fig. 2, left, lanes C to G). Probing the same membrane with IS*946*-specific sequences demonstrated that MMS362 contained at least two *Eco*RI fragments that hybridized to IS*946* (Fig. 2, right, lane B). Both *Eco*RI fragments were also present in the Em^r transformants (Fig. 2, right, lanes C to G). Homologous recombination with resident insertion sequence elements would interrupt one of these fragments. Since both fragments remained intact within the Em^r transformants, pTRK145 integration in MMS362 occurred at different sites through an apparent transpositional mechanism.

TABLE 1. Electroporation efficiency of *L. lactis* MMS362, LM0330, and NCK203 with pTRK28, pTRK145, and pTRK146

Strain	Relevant phenotype	No. of transformants per μg of DNA		
		pTRK28	pTRK145	pTRK146
MMS362	Rec$^-$	2×10^4	6×10^1	ND[a]
LM0330	Rec$^+$	2×10^4	2×10^2	ND
NCK203	Rec$^+$	3×10^6	4×10^3	ND

[a] ND, none detected at lowest dilution (<1).

The majority of pTRK145 integrants examined in MMS362 and LM0330 were chromosomal insertions. One confirmed insertion into a resident plasmid was observed in LM0330. pTRK145 had inserted into pSK07 (8.5 kb), resulting in the appearance of a new 15-kb plasmid and loss of pSK07 (data not shown). This was analogous to the insertion of the lactose plasmid pSK08 into pSK07 reported by Anderson and McKay (2).

The presence of IS*946* within the genome of MMS362 implied that resident IS*946* sequences could also be used as sites for homologous recombination similar to results re-

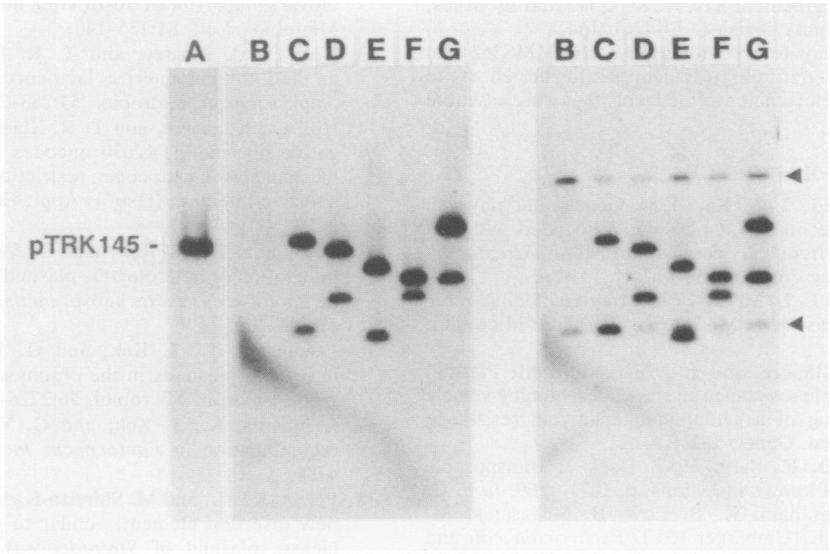

FIG. 2. Autoradiogram of *Eco*RI-digested pTRK28 (lane A) and total DNA from *L. lactis* MMS362 (lane B) and five pTRK145-Em^r transformants (lanes C to G) hybridized with ^{32}P-labeled pSA34 (left) and overlaid with an IS*946*-specific probe (right). The arrowheads indicate the MMS362 genomic *Eco*RI fragments hybridizing to IS*946*. Note that the pTRK145 (5.7 kb) hybridization pattern is identical for both probes and is shown only once.

ported by Leenhouts et al. (15–17) and Chopin et al. (6). A survey of 17 *L. lactis* strains used in the cheese industry and several laboratory strains of lactic acid bacteria identified IS*946* sequences in most of the organisms examined, including a strain of *Pediococcus acidilactici* and *Enterococcus faecalis* (22). Since the IS*946* sequences in these organisms likely reside in a nonessential region, they would provide a nonlethal site for integration of cloned DNA. The possibility of homologous recombination between vector and chromosomally located IS*946* sequences was therefore examined. The Rec$^+$ strain *L. lactis* LM0330 exhibited a threefold increase in transformation frequency with pTRK145 over Rec$^-$ MMS362. This suggested that homologous recombination, in addition to transposition, may be functioning as a mechanism for integration. However, examination of genomic DNA from 10 pTRK145 transformants of LM0330 found that the resident IS*946* *Eco*RI fragments were undisturbed (data not shown), suggesting transposition as the favored mechanism for insertion. pTRK146, which contains a truncated IS*946* copy (Ori$^-$ Tnp$^-$), also failed to transform Rec$^+$ *L. lactis* LM0330 or NCK203. The inability to demonstrate homologous recombination between pTRK146 and resident IS*946* sequences in LM0330 is likely due to an insufficient length of homology (only 400 bp) or low electroporation efficiencies. We also attempted to transform *L. lactis* ML3, progenitor of LM0330 and MMS362. *L. lactis* ML3 harbors the lactose plasmid pSK08 that contains 2 copies of ISS*1* (96% homologous to IS*946* [20]) and is maintained at about 20 copies per cell (2, 19). Despite the estimated 40-fold increase in potential target sites for pTRK146 integration, electroporation of ML3 with pTRK146 did not yield Em^r transformants. Our inability to detect Em^r transformants via homologous recombination between IS*946* and genomic copies, however, does not preclude the use of this approach for integration of heterologous DNA.

The first-generation, IS*946*-based suicide vector pTRK145 produced random genomic insertions, primarily into the *L. lactis* chromosome. Integrants were recovered at 10^1 to 10^3 per μg of DNA through electroporation of whole cells. The relatively small size of pTRK145 and its ability to replicate in *E. coli* simplify the cloning of genes of interest. Unlike the amplification observed with integration vectors by using the pC194 *cat* gene (15), the broad-host-range pSA3-encoded Em^r gene (originally from pIP501 [3, 7]) allowed for single-copy insertions at the levels of erythromycin employed. Additional IS*946*-based insertional vectors using the pC194 *cat* gene and the Tn*919* *tetM* gene have demonstrated chromosomal integration (22). Alternatives to the suicide vector delivery system, including plasmid incompatibility and thermal replicon destabilization, have been examined in attempts to increase the recovery of integrants. Thus far, the *erm*-based suicide vectors were the most efficient and straightforward method for recovering integration events.

The construction of IS*946*-mediated random genomic insertions provides an additional tool for the genetic analysis and manipulation of lactococci. The IS*946*-based shuttle insertion vector pTRK145 can be used for single-copy chromosomal integration of cloned genes, insertional mutagenesis, in vivo cloning of chromosomal sequences for analysis in *E. coli*, genetic marking of cryptic or difficult to monitor phenotypes, and as a portable priming site for DNA sequencing. We are assessing the utility of the IS*946* insertion vectors for the delivery of cloned genes and for insertional mutagenesis not only in lactococci, but in other gram-positive bacteria. The ISS*1*/IS*946* elements belong to an extended family of insertion sequences found in gram-positive and gram-negative bacteria (23). The elements share consensus promoter and ribosomal binding sites and have highly conserved inverted terminal repeats suggestive of common ancestry. These properties contend that IS*946*-based vectors, constructed with broad-host-range antibiotic resistance markers, should be functional in almost any bacteria. We are currently addressing this question.

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