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Efficient Insertional Mutagenesis in Lactococci and Other Gram-Positive Bacteria

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In lactococci, the study of chromosomal genes and their regulation is limited by the lack of an efficient transposon mutagenesis system. We associated the insertion sequence IS*S1* with the thermosensitive replicon pG$^+$host to generate a mutagenic tool that can be used even in poorly transformable strains. IS*S1* transposition is random in different lactococcal strains as well as in *Enterococcus faecalis* and *Streptococcus thermophilus*. High-frequency random insertion (of about 1%) obtained with this system in *Lactococcus lactis* allows efficient mutagenesis, with typically one insertion per cell. After IS*S1* replicative transposition, the chromosome contains duplicated IS*S1* sequences flanking pG$^+$host. This structure allows cloning of the interrupted gene. In addition, efficient excision of the plasmid leaves a single IS*S1* copy at the mutated site, thus generating a stable mutant strain with no foreign markers. Mutants obtained by this transposition system are food grade and can thus be used in fermentation processes.

Lactic acid bacteria are important industrial microorganisms because of their role in food fermentations. *Lactococcus lactis* is widely used in dairy fermentations and also serves as a model organism for biological studies of lactic acid bacteria. Most genes thus far identified in *L. lactis* have been cloned by (i) complementation (2, 10, 19, 35), (ii) immunoscreening of DNA libraries (11, 34, 48), (iii) PCR amplification of conserved genes (1, 14, 15, 17, 30), and (iv) DNA sequencing of regions adjacent to genes of interest (3, 26, 36). The chromosome and its genetic regulatory networks, however, remain for the most part unknown.

In many bacteria, transposition has been a valuable genetic tool to study chromosomal genes, their functions, and their regulators (4, 46, 50). In *L. lactis*, transposition of the conjugative elements Tn*916* (41) and Tn*919* (22, 23) have been reported. However, their use is limited by a requirement for high-efficiency conjugal transfer and site-specific transposition in certain strains. The transposition of Tn*917* has recently been demonstrated in *L. lactis* MG1614 (28). The vector used in this system is pE194, whose replication is strain specific among lactococci (7), and transposition frequencies appear to be low; also, one-third of the candidates correspond to plasmid integrants. The use of heterologous transposons can be of interest for genetic analyses, but resultant strains containing antibiotic (Ab) resistance markers would be restricted from industrial use, particularly in fermentation.

Another class of transposable elements are bacterial insertion sequences (IS). IS elements are small (between 800 and 2,500 bp) and flanked by inverted repeats and generally encode their own transposition functions (16). Three families of IS elements have been defined in lactococci (44), and their host ranges, positions, and frequencies on the chromosome have been shown to vary widely among strains (39, 45). In lactococci, iso-IS*S1* elements have been thoroughly characterized (8, 18, 21, 27, 39, 40). With nonreplicative vectors, it was shown that IS*S1* and the iso-IS*S1* element IS*946* (42) transpose randomly into the chromosome of *L. lactis* strains (IL1403, MG1363, LM0330, MMS362, and NCK203 were examined). These elements undergo replicative transposition which leads to integration of the plasmid vector between duplicated IS. IS*S1* has been used in combination with nonreplicative vectors to perform integration (43), mutagenesis (12), or chromosomal mapping (32). However, in these systems, the use of IS*S1* is limited to strains with high transformation frequencies. We have addressed the problems which limit the use of IS*S1* by employing the thermosensitive plasmid pG$^+$host as the IS*S1* delivery vector. High-frequency transposition (at least 0.5% in *L. lactis*) obtained with this system allows efficient gene inactivation and direct cloning of DNA surrounding the insertion. Efficient excision of the replicon by a temperature shift gives rise to a stable food-grade mutant strain.

General features of the transposition system. The pG$^+$host replicon is a replication thermosensitive derivative of pWV01 (38) isolated after mutagenesis of pGK12 (31, 33). In *L. lactis*, pG$^+$host replicates at 28°C but is lost above 37°C. In this study, pG$^+$host derivatives were used as delivery vectors to establish IS*S1* in the lactococcal strain at the permissive temperature and to test for IS*S1* transposition at the nonpermissive temperature. Derivatives are here referred to as pGh:IS*S1*. Replicative transposition of IS*S1* into the chromosome of *L. lactis* leads to the integration of the plasmid vector (pGh) between duplicated IS*S1* sequences. Transposition is thus revealed by selection for Ab-resistant clones able to grow at a temperature restrictive for plasmid replication.

Methods. Transposition was tested as follows. A strain containing one of the pGh:IS*S1* plasmids was grown overnight in M17 medium (containing glucose) with the appropriate Ab for plasmid selection, either 5 μg of erythromycin (Em) or tetracycline (Tc) per ml. The saturated culture was diluted 100-fold in M17 medium without the Ab and incubated 150 min at 28°C to allow exponential growth to resume. The culture was then shifted to 37.5°C for 150 min to decrease plasmid copy number.

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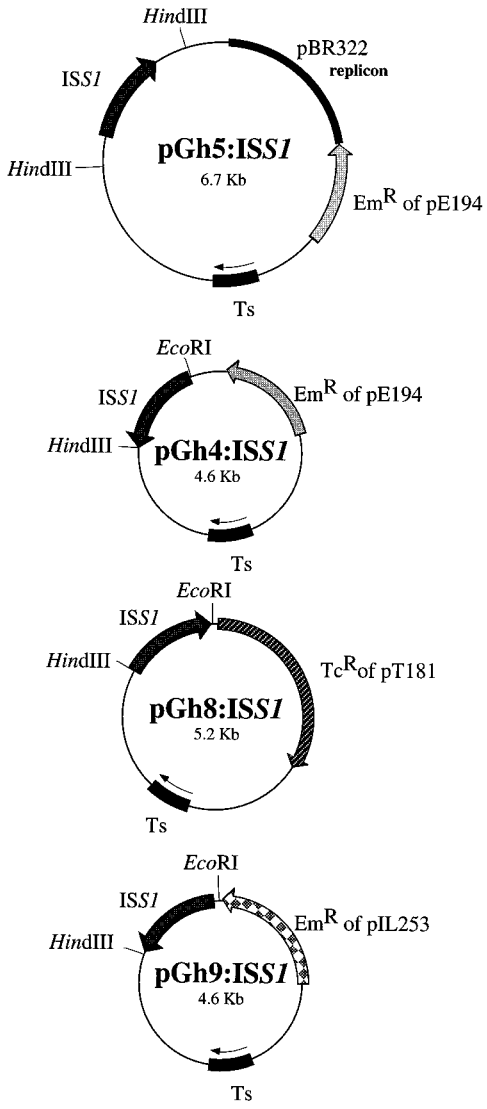

FIG. 1. Schematic representation of the IS*S1*RS delivery vectors used in this work. Only relevant restriction enzyme sites are shown. For all the pGh:IS*S1* plasmids except for pGh5:IS*S1*, the IS*S1*RS element was cloned after PCR amplification with the coding-strand primer 5′-GGAGAGAATGGGTTCTGTTGC AAAGTTTTTCTGATAAGTCTA-3′ and the complementary-strand primer 5′-GCTCTAGAGCATTCTCTGGTTCTGTTGCAAAGTTTAAAAATCAAA-3′. The tetracycline resistance gene of pT181 was PCR amplified with the coding-strand primer 5′-GCTTCACAGAAATTCTAGAACA-3′ and the complementary-strand primer 5′-GTTAATACGTGAGCTCTGCGAGGC-3′ (25). The erythromycin resistance gene corresponds to the *Hha*I fragment of pIL253 (47). Symbols: black boxes with superimposed arrows, thermosensitive (Ts) pG$^+$host replicon; thick black line, pBR322 replicon (*Ava*I-*Alw*NI fragment); black arrows, IS*S1* element; other arrows, named resistance genes.

Samples were diluted and plated at 37°C on M17 agar containing the Ab (to detect transposition) and without the Ab (to determine the viable cell count). pGh5:IS*S1* transposants were selected and grown at 37°C in the presence of 5 μg of erythromycin per ml. To avoid tandem transposition (see below), transposants of pGh4:IS*S1*, pGh9:IS*S1*, and pGh8:IS*S1* were selected with the appropriate Ab at 2 μg/ml. For further analysis, transposants were grown at 37°C in M17 medium without the Ab.

To excise the transposed vector, transposants were grown overnight in M17 medium at 37°C. The culture was then diluted 10^6-fold in M17 medium and incubated at 28°C until saturation (about 18 h); this step stimulates recombination by allowing plasmid replication. Cultures were then diluted and plated without Ab at 37°C to allow loss of the excised plasmid. Colonies were transferred with toothpicks to selective and nonselective plates. Colonies in which excision had occurred were phenotypically Ab sensitive. In all cases, the structure of the transposant DNA was analyzed by enzyme restriction and Southern hybridization using an IS*S1*-containing fragment as the probe.

High-frequency transposition of pG$^+$host5::IS*S1*. In initial experiments, a 1.4-kb fragment containing IS*S1* (defined by Huang et al. [27] as IS*S1*RS) was excised as a *Hin*dIII fragment from pRL1 (32) and joined to pG$^+$host5 (5). This plasmid, named pGh5:IS*S1*, is thermosensitive in *L. lactis* and confers erythromycin resistance (Fig. 1).

Transposition (estimated by the number of Em^r clones at 37°C) is highly efficient in both *L. lactis* IL1403 and MG1363 (Table 1). Southern hybridization analysis of the Em^r clones demonstrates that transposition is random in both strains. The hybridizing profile of the preexisting chromosomal copy of IS*S1* in MG1363 is unaltered in transposants (data not shown), indicating that the transposition process is unaffected by the presence of another copy of the IS element in the cell. For both strains, transposition resulted in the integration of multiple tandem copies of pGh5:IS*S1* (Fig. 2 and 3). We refer to this as tandem transposition. This system could be used to generate random insertions in the chromosome. However, the presence of tandem plasmid repeats can favor rearrangements and thus generate a nonhomogeneous population and also complicate cloning of the interrupted gene.

We consider two factors that could provoke tandem transposition. First, poor expression of the plasmid marker in single copy could result in the selection of tandem transposants. We have evidence that the Em^r gene present on pGh5:IS*S1* (derived from pE194 [25]) may be poorly expressed in single copy (data not shown). Second, the pGh5:IS*S1* plasmid generates linear plasmid multimers (20) at the permissive temperature in *L. lactis* which is correlated with the presence of Chi sites (6, 9). The pBR322 fragment present on pGh5:IS*S1* contains three sequences related to the identified *L. lactis* Chi site (6), which may provoke the accumulation of linear plasmid multimers. The sequences surrounding the IS*S1* element are unknown and could also contain Chi-like sequences.

Monocopy transposition. To overcome the problem of tandem transposition, we designed new plasmids; sequences which might provoke the accumulation of linear plasmid multimers

TABLE 1. Transposition frequencies[a]

Plasmid	Transposition frequency of strain:	
	IL1403	MG1363
pGh5[b]	10^{-7}	10^{-6}
pGh5:IS*S1*	1.2×10^{-2}	5×10^{-3}
pGh4[b]	1.3×10^{-6}	ND[c]
pGh4:IS*S1*	6.4×10^{-3}	4.4×10^{-2}
pGh8[b]	1.1×10^{-8}	5.7×10^{-4}
pGh8:IS*S1*	2.4×10^{-2}	3.9×10^{-2}
pGh9[b]	10^{-6}	6×10^{-5}
pGh9:IS*S1*	4.9×10^{-3}	5.5×10^{-2}

[a] Transposition frequencies correspond to the number of Ab-resistant cells (obtained on M17 medium with the Ab at 37°C) divided by the total cell count (measured on M17 medium at 37°C).
[b] Frequencies correspond to nonspecific plasmid integration.
[c] ND, not determined.

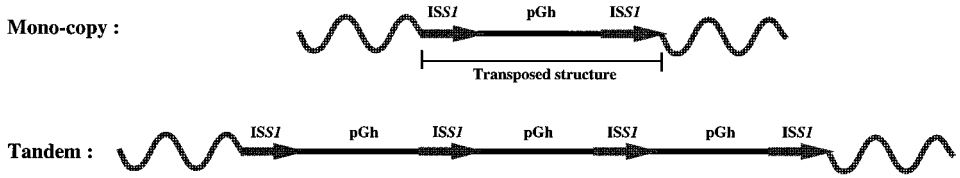

FIG. 2. Representation of IS*S1* transposition products. IS*S1* replicative transposition is expected to generate monocopy transposition, i.e., integration of the plasmid vector between duplicated IS*S1* sequences. Transposition may also give rise to a clone containing multiple copies of the transposed structure (referred to here as tandem transposition). Symbols: wavy lines, chromosomal DNA; arrows, IS*S1*RS; solid line, vector DNA (pGh).

were eliminated, and selection markers were changed (in two cases). In addition, Ab concentrations used to select transposition events were reduced (see Methods). Three plasmids (Fig. 1) were generated: pGh4:IS*S1* (Em^r marker of pE194), pGh8:IS*S1* (Tc^r marker of pT181 [29]), and pGh9:IS*S1* (Em^r marker of pIL253 [47]). Transposition frequencies of these three plasmids in *L. lactis* were comparable to those of pGh5:IS*S1* (Table 1). Southern hybridization confirmed that IS*S1* integrated randomly in the chromosome. Monocopy transpositions were predominant, and represent 80%, 80%, and 70% of transposition events of pGh4:IS*S1*, pGh9:IS*S1*, and pGh8:IS*S1*, respectively (Fig. 4). Transposants in which tandem transposition occurred contain a reduced number of repeats compared with what was previously observed with pGh5:IS*S1*. These results confirm that different characteristics of the transposition vector can influence the final chromosomal structure and demonstrate the use of these vectors to perform transposition in *L. lactis.*

Isolation of mutants and cloning of interrupted genes. The transposition system has been used in MG1363 to identify genes involved in DNA repair pathways. Transposants were obtained with pGh9:IS*S1*, and 1,000 clones were screened for mitomycin sensitivity. Five sensitive clones were obtained and confirmed to be independent by their unique hybridization patterns (13). The high frequency with which the mutants were obtained (0.5%) in *L. lactis* suggests that numerous genes are involved in DNA repair pathways, as has already been observed for *Bacillus subtilis* (49).

The pGh:IS*S1* plasmids contain unique sites adjacent to the IS*S1* element, which can be used after transposition to clone chromosomal DNA flanking the pGh:IS*S1* insertion site. Chromosomal DNAs of pGh9:IS*S1*-mutated strains were digested with *Hin*dIII or *Eco*RI, treated with ligase, and transformed into *Escherichia coli*. The recipient strain was either TG1, with selection at the pG^+host permissive temperature, or TG1, which contains a chromosomal copy of the pWV01 *rep* gene (kindly provided by K. Leenhouts), with selection at 37°C. This procedure allowed us to recover pG^+host plasmids containing one IS*S1*-chromosome junction. The plasmids containing chromosomal DNA could then be sequenced to identify the mutated genes. It should be noted that the Tc^r marker is efficiently expressed in *L. lactis* (as well as in other gram-positive bacteria) but does not confer resistance to tetracycline in *E. coli*. Therefore, the use of pGh9:IS*S1* is recommended if the experimental objective is to clone the gene in *E. coli.*

The chromosomal targets of 29 IS*S1*-generated mutants were sequenced. The transposition target of IS*S1* is defined as an 8-bp sequence which is duplicated during the transposition process and which flanks the transposed structure as a direct repeat (27, 44). Target sequence comparisons (Table 2) indicate some preference for an adenine at position 5 and a cytosine at position 8. Guanines are underrepresented at positions 2, 3, 5, and 8, and cytosines are underrepresented at positions 5 and 6. Nevertheless, the targets show no overall homologies, confirming that IS*S1* transposition is not site specific.

Isolation of a stable IS*S1*-generated mutant by excision of the transposed vector. Replicative transposition leads to integration of the plasmid vector between duplicated IS*S1* sequences. This structure is maintained if bacteria are grown above 37°C, the nonpermissive temperature for pG^+host replication in *L. lactis*. Recombination between the duplicated sequences would give rise to a stable structure in which a single IS*S1* element remains in the chromosome. Excision of pG^+host would obviate the temperature restriction and generate a stable, food-grade mutated strain.

It was previously shown that pG^+host excision by homologous recombination between flanking duplicated regions is stimulated by rolling-circle replication (5, 37). We made use of this property to favor plasmid excision. Two random integrants resulting from pGh8:IS*S1* (Tc^r) transposition, one monocopy and one tandem transposant, were submitted to a temperature shift to 28°C prior to plating at 37°C. Plasmid excision is estimated by the number of Tc^s cells at 37°C (see Methods for the experimental procedure). The monocopy transposant gave rise to 85% Tc^s bacteria (170 of 200 colonies tested were Tc^s). Analysis of the chromosomal DNA of Tc^s clones confirmed that the plasmid excised precisely in 10 of 16 Tc^s clones (data not shown), although base-pair modifications during excision

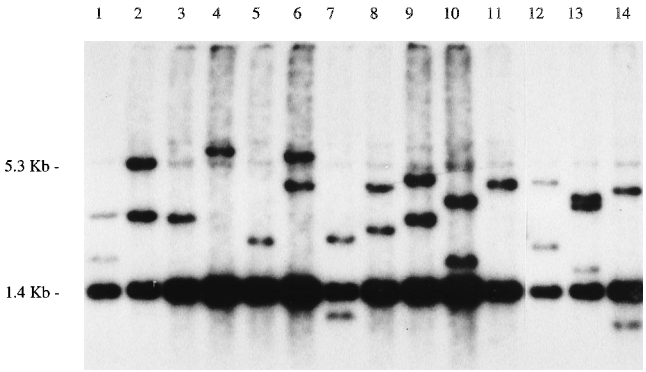

FIG. 3. Southern analysis of 14 independent IL1403 pGh5:IS*S1* integrants. Chromosomal DNA from integrants (selected as Em^r at 37°C) was digested by *Hin*dIII and probed with a ^{32}P-labelled IS*S1* probe (a 1.4-kb *Hin*dIII IS*S1* fragment of pGh5:IS*S1*). The pGh5:IS*S1* plasmid contains two *Hin*dIII sites flanking the IS*S1* element (Fig. 1). In most cases, three bands are observed. Two fragments that vary in size from one clone to another correspond to the junctions between the transposed structure and the chromosome. The third fragment is common to all the clones and corresponds to the 1.4-kb fragment typical of tandem transposition. In lanes 3, 4, 5, and 11 two bands are observed; we suppose that one of the junctions comigrates with the 1.4-kb fragment. The faint band of 5.3 kb corresponds to low-level hybridization between the plasmid backbone and our probe. The Raoul marker (Appligene) was used as a size reference (data not shown).

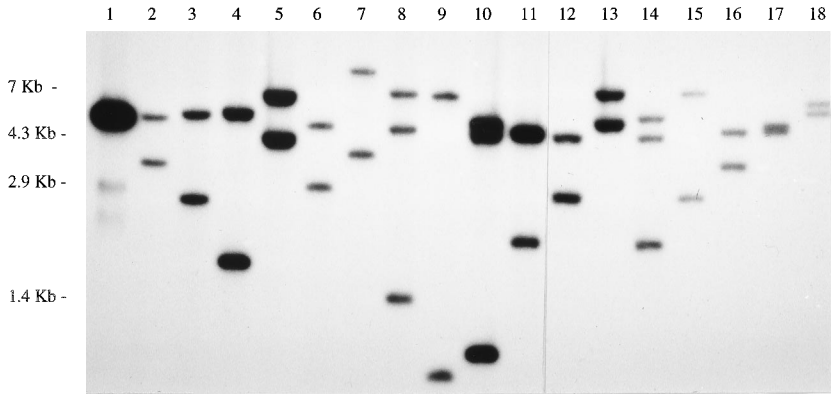

FIG. 4. Southern hybridization analysis of 17 independent IL1403 pGh4:IS*S1* integrants. Chromosomal DNAs from integrants (Em^r at 37°C) were digested by *Hin*dIII and analyzed by agarose gel electrophoresis and Southern hybridization with a ^{32}P-labelled IS*S1* probe (a 1.4-kb *Hin*dIII fragment of pGh5:IS*S1*). The pGh4:IS*S1* plasmid contains a single *Hin*dIII site. Monocopy transposition produces two IS*S1* hybridizing bands corresponding to junction fragments. Tandem transposition (lanes 8, 10, and 14) generates an additional band corresponding to linear pGh4:IS*S1* (shown in lane 1).

cannot be ruled out. Plasmid excision in the mutant containing a tandem transposition structure was less efficient than in the mutant with the monocopy structure (16 of 200 colonies tested were Tc^s). Possibly, multiple temperature shift cycles of 37 to 28°C could increase the number of clones in which excision takes place. Chromosomal DNA of 10 Tc^s strains confirmed precise plasmid excision in eight cases (data not shown).

This system can be used to generate double or complex mutants. A strain already containing a copy of IS*S1* can be mutagenized again by pGh:IS*S1* by repeating the procedure (data not shown). Alternatively, inactivation by gene replacement can be performed with pG^+host in an IS*S1*-mutated strain (5).

IS*S1* transposes randomly in *Streptococcus thermophilus* and *Enterococcus faecalis*. Transposition was tested in *S. thermophilus* IL1704 using pGh5:IS*S1* (selecting with 5 μg of erythromycin per ml). The plasmid was first established by the transformation of electrocompetent cells (24). Transposition was evaluated as described above, except that the nonpermissive temperature was 40°C instead of 37°C and plates were incubated in anaerobiosis. Em^r colonies appeared at 40°C with a frequency of about 0.01%. Southern analysis of the chromosomal DNA of 10 Em^r clones established that they result from independent transposition events (data not shown). Tandem transposition was observed in all cases.

Transposition of pGh5:IS*S1* was also tested in the *E. faecalis recA* strain UV202. The transposition frequency was around 10^{-5}. Hybridization analysis of 10 Em^r transposants showed that random tandem transposition had occurred, as expected with this construct.

TABLE 2. Frequencies of base occurrence at each position of the IS*S1* transposition site

Base	Frequency of occurrence (%) at target site position[a]:							
	1	2	3	4	5	6	7	8
A	38	31	31	38	**62**	41	41	14
T	21	41	41	31	27	31	17	31
C	17	24	21	21	*7*	4	21	**48**
G	24	*4*	*7*	10	*4*	24	21	*7*

[a] The target site is defined as the 8-bp sequence duplicated upon IS*S1* insertion which flanks the transposed structure as a direct repeat. The 8-bp direct repeat was determined for 29 mutants by sequencing both of the IS*S1*-chromosomal junctions. Sequencing data were obtained from one strand. The frequency of occurrence of a base was calculated for each position of these 29 transposition sites. Boldface and italicized numbers indicate over- and underrepresented bases, respectively.

Conclusions. In this work, we demonstrated the feasibility of using an IS*S1*- and pGh^+host-based transposon delivery vector as a mutagenic tool in at least three gram-positive organisms. In *L. lactis*, the high transposition frequency allows us to generate a large number of mutants by plating at 37°C. This system was previously used in our laboratory to identify five genes involved in DNA repair (13). The cloning of the mutated gene was facilitated by the transposition of the vector together with the IS*S1* element. By using the procedures described here, it is possible to excise the plasmid, thus generating food-grade mutant strains.

In view of the good transposition activity observed in *L. lactis*, *S. thermophilus*, and *E. faecalis* as well as the broad-host-range thermosensitivity of pG^+host, the pGh:IS*S1* transposon may have a broad application in gram-positive organisms.

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