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Development of High-Frequency Delivery System for Transposon Tn*919* in Lactic Streptococci: Random Insertion in *Streptococcus lactis* subsp. *diacetylactis* 18-16

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The conjugative transposon Tn*919*, originally isolated in *Streptococcus sanguis* FC1, is capable of low-frequency transfer (10^{-7} and 10^{-8} per recipient) on membrane filters to a wide number of streptococcal recipients including the industrially important lactic streptococci. The introduction of pMG600 (Lac^+ Lax^-; a lactose plasmid capable of conjugative transfer at high frequencies and which, in certain hosts, confers an unusual clumping phenotype) into a *Streptococcus lactis* CH919 donor, generating *S. lactis* CH001, resulted in a significant improvement in the transfer frequency of Tn*919* to *S. lactis* CK50 (1.25×10^{-4} per recipient). In addition, these matings could be performed on agar surfaces, allowing the recovery of a greater number of recipients than with filter matings. Tn*919* also transferred at high frequency to *S. lactis* subsp. *diacetylactis* 18-16S but not to *Streptococcus cremoris* strains. Insertion in 18-16S transconjugants generated from filter matings with an *S. lactis* CH919 donor was random, occurring at different sites on the chromosome and also in plasmid DNA. Thus, the conditions necessary for the practical exploitation of Tn*919* in the targeting and cloning of genes from a member of the lactic streptococci, namely, high-frequency delivery and random insertion in host DNA, were achieved.

Tn*919* is one of a number of transposons identified in the genus *Streptococcus* (2, 3, 7, 8, 14, 24). This element, from *Streptococcus sanguis* FC1, is similar but not identical to Tn*916* from *Streptococcus faecalis* DS16 (7, 8, 12). These transposons are between 15 and 17 kilobases in size, encode tetracycline resistance (Tet^r) (7, 8, 12), and share the property of conjugative transfer in the absence of plasmid DNA at frequencies ranging between 10^{-5} and 10^{-8} per donor (3, 7, 8, 12, 15). Both elements have been transferred to a number of streptococcal species by the filter mating technique (7, 8, 15) and have also been cloned in *Escherichia coli* in which tetracycline resistance is expressed (7, 13). Significantly, in this background growth of the clones in the absence of tetracycline results in high-frequency excision of the element from host DNA.

In this laboratory interest in Tn*919* concerns its potential as a tool in advancing genetic studies in the industrially important lactic acid bacteria, particularly the lactic streptococci. These bacteria are used as starter cultures in the manufacture of fermented milk products and have been the subject of intensive research, particularly at a genetic level, in recent years (11, 16, 17). While attention has focused on plasmid-coded functions of the lactic streptococci, the chromosome remains relatively unexplored. We have recently reported the conjugative transfer of Tn*919* to lactic acid bacteria in filter matings with an *S. faecalis* donor (15). Transfer within lactic streptococci from a *Streptococcus lactis* donor could also be detected, although at lower frequencies. The wide host range of Tn*919* among these bacteria may provide a means of applying the strategy developed for Tn*916* by Gawron-Burke and Clewell (13) for the targeting and cloning of streptococcal genes, including those of chromosomal origin, in *E. coli*. However, in our previous study (15), the low number of transconjugants obtained in filter matings and the site-specific insertion of Tn*919* in the *S. lactis* CK50 chromosome did not allow the application of this strategy in this strain. We now report the development of an improved delivery system for the transposon which exploits the high-frequency conjugation properties of pMG600, one of a class of plasmids derived from the pLP712 plasmid of *S. lactis* 712 (10). In addition, we describe the random insertion of Tn*919* in the *S. lactis* subsp. *diacetylactis* 18-16S chromosome and also show insertion into a native plasmid.

MATERIALS AND METHODS

Bacteria, media, and chemical reagents. Bacterial strains and their plasmid contents, where relevant, are described in Table 1. All streptococcal strains and *E. coli* V517 were routinely grown in M17 medium (23) in which glucose (0.5%) replaced lactose when necessary (GM17 medium). *E. coli* DH1 (pAM554) was grown in LB medium (6). Solid media contained 1.5% agar (Oxoid no. 3). Antibiotics present in selective media were added at the following levels: rifampin (Rif), 25 μg/ml; streptomycin (Str), 500 μg/ml; and tetracycline, 10 or 4 μg/ml when either streptococci or *E. coli*, respectively, were involved. The bromocresol purple lactose indicator agar of McKay et al. (21) was used to determine the ability of streptococcal strains to ferment lactose. Restriction endonuclease *Hin*dIII was obtained from the Boehringer Corp., Dublin, and used according to their specifications. RNase and lysozyme were obtained from the Sigma Chemical Co., Poole, Dorset, England.

Conjugation experiments. The filter mating technique has been described previously (5, 8). Solid surface agar matings were done essentially as described by McKay et al. (20). Briefly, this involved inoculating (2%) GM17 medium with overnight cultures of donor and recipient and incubating at 30°C for 4 h. These were then mixed in a 2:1 recipient:donor ratio, and 0.2-ml samples were plated on skim milk agar plates (5% nonfat dry milk plus 1% glucose) which were incubated for 16 h at 30°C. The cells were then removed from

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TABLE 1. Bacterial strains and phenotypes

Strain	Relevant phenotype	Plasmid content (MDa)	Remarks
Streptococcus lactis			
MG1363		None	9
CK50	Str^r	None	Spontaneous Str^r mutant of MG1363
CH919	Rif^r Tet^r	None	Rif^r MG1363 harboring Tn*919* on the chromosome (15)
MG4600	Rif^r Str^r Lac$^+$ Lax$^-$	pMG600 (52)	MG1363 Rif^r Str^r harboring a high-frequency Lac$^+$ Lax$^-$ plasmid (15)
CH001	Rif^r Tet^r Lac$^+$ Lax$^-$	pMG600 (52)	Transconjugant from MG4600 × CH919 mating (this study)
SK3S	Str^r	NR[a]	University College, Cork (UCC) Culture Collection
Streptococcus cremoris 17S	Str^r	NR	UCC Culture Collection
Streptococcuc lactis subsp. *diacetylactis*			
18-16S	Str^r	46, 32, 5.7, 3.8, 3.4	UCC Culture Collection
18-16S2	Str^r Lac$^-$	32, 5.7, 3.8, 3.4	UCC Culture Collection
DRC3S	Str^r	NR	UCC Culture Collection
Escherichia coli			
DH1(pAM554)		pAM554 (13)	7
V517		35.8, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8, 1.4	Standard reference plasmids (18)

[a] NR, Not relevant to this study.

the skim milk agar in 1.0 ml of Ringer solution and diluted if necessary, and 0.1-ml samples were plated on selective plates which were incubated at 30°C for 24 to 48 h. Broth matings were done exactly as outlined by Gasson and Davies (10). Transconjugants displaying the Lax$^-$ or Lax$^+$ phenotype were identified by examining their colony morphology and their ability to clump when grown in broth (10).

DNA isolation. Chromosomal DNA from streptococci was isolated and purified as previously described (7, 8). The method of Clewell and Helinski (4) was used to isolate pAM554 (Table 1) from *E. coli*. Plasmid DNA was isolated from streptococcal strains by the procedure of Anderson and McKay (1). Chromosomal DNA fragments generated after restriction endonuclease digestion were fractionated by agarose gel (1%) electrophoresis in TAE buffer (0.04 M Tris acetate, 0.002 M EDTA, pH 8.0) with a horizontal gel unit. Plasmid DNA was separated by electrophoresis through 0.7% agarose gels in TAE buffer with a vertical gel apparatus. After staining in an ethidium bromide (1 μg/ml) solution, DNA was visualized with shortwave UV light and photographed with a Polaroid MP4 Land camera with Polaroid type 52 film. Plasmid sizes were estimated by comparing mobilities with those of the standard reference plasmids in *E. coli* V517 (Table 1) (18).

DNA-DNA hybridization. The conditions used in the Southern blot (22) and hybridization experiments have been described previously (15). The biotinylated probe DNA was prepared with a nick translation kit (Bethesda Research Laboratories, Inc., Paisley, Scotland), and the components of the DNA detection reactions were obtained from the same source.

RESULTS

Effects of pMG600 on frequency of Tn*919* transfer in *S. lactis* derivatives. Transfer of Tn*919* from an *S. lactis* CH919 donor to an *S. lactis* CK50 recipient occurred at a frequency of 7.0×10^{-8} when the filter mating technique was used (Table 2). However, under these conditions only between 50 and 500 transconjugants per ml of mating mix were obtained. *S. lactis* CH919 did not transfer Tn*919* by the alternative agar surface mating protocol which would allow the recovery of a greater number of recipients (Table 2) (15). When the high-frequency conjugative plasmid pMG600, conferring a Lac$^+$ Lax$^-$ (i.e., clumping) phenotype, was introduced into CH919, a selected transconjugant designated CH001 was capable of donating Tn*919* in agar surface matings. In addition, the transfer frequency was significantly higher than that obtained by filter matings (Table 2), and the number of transconjugants generated increased to between 100,000 and 500,000 per ml of mating mix. Analysis of the transconjugants revealed that 80% exhibited the Lac$^+$ Lax$^-$ Tet^r phenotype, suggesting transfer of both pMG600 and Tn*919*. The remaining 20% were Lac$^-$ and Lax$^+$ Tet^r, indicating transfer of Tn*919* only. Interestingly, high-frequency Tn*919* transfer was also observed when pMG600 was present in the *S. lactis* CK50 recipient rather than the CH919 donor (Table 2).

pMG600-aided transfer of Tn*919* to *S. lactis* subsp. *diacetylactis* 18-16S recipient. Agar surface matings between *S. lactis* CH001 and an *S. lactis* subsp. *diacetylactis* 18-16S recipient resulted in transfer of Tn*919* at frequencies greater than those obtained from a CH919 donor by the filter mating technique (Table 3). The 18-16S recipient used in these experiments was Lac$^+$, harboring a native 46-megadalton (MDa) Lac plasmid. However, when a Lac$^-$ derivative of this strain (designated 18-16S2) missing this 46-MDa molecule was used as a recipient in identical mating experiments, CH001 was consistently capable of donating Tn*919* at a fivefold-elevated frequency. In addition, Lac$^+$ transconjugants, suggesting transfer of pMG600, were also obtained at high frequency (Table 3). In the 80% of the transconjugants which were Lac$^+$ Tet^r, pMG600 could be detected on agarose gels (data not shown). However, these transconjugants were not Lax$^-$ (i.e., were unable to clump) suggesting that this phenotype cannot be detected in 18-16S2. This was confirmed when these 18-16S2 Lac$^+$ isolates could subsequently transfer both the Lac$^+$ and Lax$^-$ phenotypes to an MG1363 recipient at high frequency (5×10^{-1} per recipient). The frequency with which the remaining 18-16S2 transcon-

TABLE 2. Conjugative transfer of Tn*919* or pMG600 or both between *S. lactis* derivatives

Donor (phenotype)	Recipient (phenotype)	DNA transferred	Relevant transconjugant phenotype	Frequency/ recipient
CH919 (Lac^-)	CK50 (Lac^-)	Tn*919*	Tet^r	7.0×10^{-8a}
CH919 (Lac^-)	CK50 (Lac^-)	Tn*919*	Tet^r	$<5.0 \times 10^{-9b}$
MG4600 (Lac^+)	CH919 (Lac^-)	pMG600	Lac^+ Lax^-	5.0×10^{-1c}
CH001 (Lac^+)	CK50 (Lac^-)	pMG600	Lac^+ Lax^-	5.0×10^{-1b}
CH001 (Lac^+)	CK50 (Lac^-)	pMG600, Tn*919*	Lac^+ Lax^- Tet^r	1.25×10^{-4b}
CH001 (Lac^+)	CK50 (Lac^-)	Tn*919*	Lac^- Lax^+ Tet^r	2.0×10^{-5b}
CH919 (Lac^-)	MG4600 (Lac^+)	Tn*919*	Lac^+ Lax^- Tet^r	1.0×10^{-4b}

[a] Filter matings.
[b] Agar surface matings.
[c] Broth matings.

jugants (Lac^- Tet^r) occurred was identical to the frequency of transfer of Tn*919* from CH001 to an 18-16S (Lac^+) recipient (Table 3).

S. lactis CH001 was also capable of transferring Tn*919* to *S. lactis* SK3S and *S. lactis* subsp. *diacetylactis* DRC3S in agar surface matings but at frequencies similar to those previously obtained in filter matings (15). Significantly, no transfer to *Streptococcus cremoris* strains could be detected even after repeated experiments (data not shown).

Determination of insertion specificity of Tn*919* in *S. lactis* subsp. *diacetylactis* 18-16S host DNA. Tetracycline-resistant transconjugants of *S. lactis* subsp. *diacetylactis* 18-16S were obtained at a frequency of 7.0×10^{-8} per recipient with an *S. lactis* CH919 donor in filter matings (Table 3). Five transconjugants, obtained from independent mating experiments to eliminate the possibility of transconjugant relatedness, were examined in detail to determine whether insertion of Tn*919* in host DNA was site specific or random. When *Hin*dIII-digested chromosomal DNA was probed with biotin-labeled pAM554 (a 13-MDa recombinant plasmid into which Tn*919* has been cloned [7]) the results revealed that in strain 18-16S insertion of the tetracycline resistance element was random (Fig. 1). Since Tn*919* contains only one *Hin*dIII recognition site, two chromosomal fragments would be expected to harbor transposon DNA after restriction with this enzyme. The proximity of this internal *Hin*dIII site to the nearest sites on the chromosome is an indication of the specificity of insertion of the element in the host DNA. When insertion is site specific, digestion of the chromosome with *Hin*dIII will always yield two fragments which will be identical in size in all transconjugants examined. However, for strain 18-16S this clearly is not the case. In four of the five transconjugants, transposon-chromosome junction fragments of different sizes were apparent after the hybridization experiment (Fig. 1, lanes 3 to 7). Although the banding patterns generated for two of the isolates appear similar (lanes 3 and 4), closer examination revealed that the fragments in lane 3 are larger in size than the corresponding fragments in lane 4. In three transconjugants (lanes 5 to 7) multiple bands were evident, probably indicating the insertion of more than one copy of Tn*919*.

The plasmid profiles of the five transconjugants were also examined to determine whether insertion of the element occurred in any of the five resident plasmids of strain 18-16S. In the case of one transconjugant, which was designated 18-16SA, comparison with the 18-16S parent (Fig. 2, lane 1) showed that its 46-MDa plasmid (encoding lactose metabolism [unpublished data]) was no longer detectable (lane 2). However, a larger plasmid of 56 MDa was evident. The difference in size between these plasmids suggested the insertion of the 10-MDa Tn*919*. Confirmation was obtained when the pAM554 probe hybridized to this 56-MDa molecule after Southern hybridization experiments (lane 2′) but not to the 46-MDa plasmid in the parent (lane 1′). The plasmid profiles of the four other transconjugants were identical to that of the 18-16S parent culture (data not shown).

DISCUSSION

We have previously reported the low-frequency transfer of Tn*919* to lactic acid bacteria using the filter mating technique (15). The potential of pMG600 to improve the transfer frequency of the transposon was examined in this study. In addition to encoding lactose metabolism, this plasmid, when present in derivatives of *S. lactis* 712, causes

TABLE 3. Transfer of Tn*919* or pMG600 or both from *S. lactis* donors to *S. lactis* subsp. *diacetylactis* 18-16 recipients

Donor (phenotype)	Recipient (phenotype)	DNA transferred	Relevant transconjugant phenotype	Frequency/ recipient
CH919 (Lac^-)	18-16S (Lac^+)	Tn*919*	Lac^+ Tet^r	7.0×10^{-8a}
CH919 (Lac^-)	18-16S (Lac^+)	Tn*919*	Lac^+ Tet^r	$<5.0 \times 10^{-9b}$
CH001 (Lac^+)	18-16S (Lac^+)	Tn*919*	Lac^+ Tet^r	3.0×10^{-6b}
CH001 (Lac^+)	18-16S2 (Lac^-)	pMG600	Lac^+	5.0×10^{-1b}
CH001 (Lac^+)	18-16S2 (Lac^-)	pMG600, Tn*919*	Lac^+ Tet^r	1.5×10^{-5b}
CH001 (Lac^+)	18-16S2 (Lac^-)	Tn*919*	Lac^- Tet^r	3.0×10^{-6b}

[a] Filter matings.
[b] Agar surface matings.

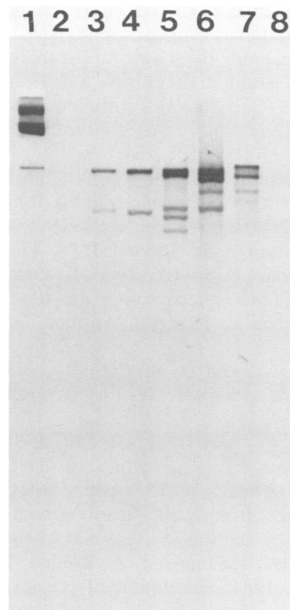

FIG. 1. Color reaction obtained from probing chromosomal DNA from *S. lactis* subsp. *diacetylactis* 18-16S Tet^r transconjugants with biotin-11-dUTP-labeled pAM554. Lanes 3 to 7 show *Hin*dIII-digested DNA from five independently isolated transconjugants. Lane 8 contains DNA from 18-16S parent strain not harboring Tn*919*. Lane 1 contains pAM554 DNA. Lane 2 contains λ DNA.

an unusual clumping phenotype (termed Lax$^-$) which is apparently responsible for subsequent high-frequency conjugation (up to 5×10^{-1} per recipient [10]). When pMG600 was introduced into *S. lactis* CH919, a derivative of *S. lactis* 712 containing Tn*919* on its chromosome, the resultant transconjugant, CH001, was then capable of transferring the transposon at a considerably elevated frequency in agar surface matings. This improved frequency, allied to higher recipient recoveries, resulted in significantly greater transconjugant numbers per mating compared with filter matings. Franke and Clewell (8) have similarily reported that the conjugative plasmids pAD1 and pAMγ1 can mobilize Tn*916*

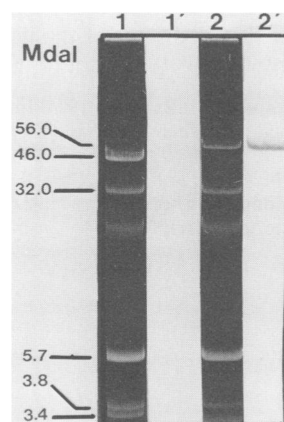

FIG. 2. Plasmid DNA profiles of strain 18-16S and 18-16SA (lanes 1 and 2, respectively) after agarose gel (0.7%) electrophoresis. Lanes 1′ and 2′ show the color reaction obtained after hybridization with the biotin-11-dUTP-labeled pAM554 probe. Mdal, Megadaltons.

and increase the frequency of transfer by between 1 and 2 orders of magnitude in filter matings between *S. faecalis* strains. The effect of the plasmids in agar surface matings was not examined.

The exact role of pMG600 in mediating higher-frequency transfer of Tn*919* in addition to facilitating its transfer on agar surfaces is not clear. However, it is significant that transfer of the plasmid itself is not required since Lac$^-$ Tet^r transconjugants could be obtained at a frequency of 2.0×10^{-5} per recipient, and, in addition, Tn*919* transferred at a similar frequency irrespective of whether pMG600 was located in the donor or the recipient (Table 2).

While pMG600 is derived from pLP712 (the Lac-Prt plasmid of *S. lactis* 712 [9]) and is capable of promoting high-frequency transfer of Tn*919* between donors and recipients derived from strain 712, it is significant that pMG600-assisted transfer to *S. lactis* subsp. *diacetylactis* 18-16S was also achieved at frequencies similar to those observed with the 712 system. Interestingly, pMG600, which itself carries *lac* genes, promoted conjugative transfer of Tn*919* to both Lac$^+$ and Lac$^-$ 18-16S recipients. In the case of the 18-16S recipient, which harbors a 46-MDa Lac plasmid, transfer occurred at a frequency of 3.0×10^{-6} in agar surface matings (transfer of Tn*919* does not occur on agar surfaces in the absence of pMG600). However, when a Lac$^-$ isolate, 18-16S2, was used as a recipient, the transfer frequency of Tn*919* was elevated fivefold in repeated mating experiments, and pMG600 had transferred into 80% of the transconjugants. The remainder received only Tn*919*. The frequency of this latter event was identical to that of Tn*919* transfer to 18-16S. This suggests that pMG600 promotes transfer to both recipient types, but in the case of 18-16S (Lac$^+$), pMG600 is either unable to transfer to or replicate in this recipient, perhaps as a result of incompatibility between itself and the native Lac plasmid. When Tn*919* transfers alone, incompatibility would not be a factor, and therefore the frequency of this event is the same irrespective of whether the recipient is Lac$^+$ or Lac$^-$.

It is also noteworthy that while pMG600 transferred to strain 18-16S2 at high frequency, these transconjugants failed to exhibit the Lax$^-$ (i.e., clumping) phenotype displayed by *S. lactis* 712 and its derivatives when harboring the same plasmid. However, these 18-16S2 transconjugants could subsequently donate both Lac$^+$ and Lax$^-$ at high frequency to an *S. lactis* MG1363 recipient, suggesting that the gene(s) responsible for the clumping phenotype can only be expressed in derivatives of *S. lactis* 712. Furthermore, the inability of CH001 to transfer Tn*919* to *S. cremoris* strains suggests that the conjugal functions specified by pMG600 in the CH001 donor are incompatible with some recipient cell types. The failure to detect transfer of Tn*919* alone confirms the previous observation that transfer of the transposon will not occur on agar surfaces (15).

High-frequency delivery of Tn*919* is only one prerequisite which must be fulfilled if the transposon is to be used to target and clone specific genes in lactic acid bacteria. A further requirement is that insertion in host DNA be random. In a previous report we showed that insertion of Tn*919* in the *S. lactis* CK50 chromosome was site specific when the transposon was introduced by the filter mating technique (15). However, we now report that in *S. lactis* subsp. *diacetylactis* 18-16S transconjugants, isolated by the same protocol, insertion was random. Of five transconjugants examined, four showed insertion at obviously different sites on the chromosome. In addition, in the transconjugant designated 18-16SA, Tn*919* had also inserted into the native

46-MDa Lac plasmid, although expression of the lactose utilization genes remained unaltered. It is significant that insertion into plasmid DNA was observed since many of the key functions associated with the ability of the lactic streptococci to produce fermented milk products (e.g., lactose utilization, proteinase production) are plasmid coded (11, 17, 19). Three of the five transconjugants in which chromosomal insertion was observed contained more than one copy of Tn*919* at different locations. Gawron-Burke and Clewell (12) have made similar observations with Tn*916* in the *S. faecalis* chromosome and have suggested that secondary transposition occurs from a replicated portion of the chromosome to an unreplicated region, resulting in the generation of two copies of the transposon in one of the daughter cells.

Thus, in this report, random insertion and high-frequency transfer of Tn*919* to *S. lactis* subsp. *diacetylactis* 18-16S was described, making it an attractive system by which the transposon can be used to target and clone specific genes, experiments which are currently under way in this laboratory.

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