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Genetic manipulations in *Rhizobium meliloti* utilizing two new transposon Tn*5* derivatives

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Summary. Two derivatives of the prokaryotic transposon Tn*5* were constructed in vitro. In Tn*5*-233, the central area of Tn*5*, which carries resistance to kanamycin/neomycin, bleomycin and streptomycin, is replaced by a fragment carrying resistance to the aminocyclitol antibiotics gentamycin/kanamycin and streptomycin/spectinomycin. In Tn*5*-235, the *Escherichia coli* β-galactosidase gene is inserted within the streptomycin resistance gene of Tn*5*, and constitutively expressed from a Tn*5* promoter. Both constructs transpose with about the same frequency as Tn*5* in *Escherichia coli* and *Rhizobium meliloti*. When a Tn*5*-derivative is introduced into an *R. meliloti* strain which already contains a different Tn*5*-derivative, in situ transposon replacement is obtained at high frequency, presumably by a pair of crossovers between the IS*50* sequences at the ends of the incoming and resident transposons. In this way we converted a previously isolated *recA*::Tn*5* mutant into the corresponding *recA*::Tn*5*-233 strain, which can now be used as a genetic background in the study of complementation of other Tn*5*-induced mutations. We also replaced the drug markers of several Tn*5*-induced *exo* mutants, which we were then able to map relative to each other by transduction with phage φ M12. In a strain carrying Tn*5*-235 located near Tn*5*-233, we were able to isolate deletions of the intervening markers, presumably resulting from general recombination between the two transposons, by screening for loss of the Lac$^+$ phenotype. Unlike Tn*5* itself, resident Tn*5*-233 does not appear to suppress transposition of another incoming Tn*5*-derivative.

Key words: *Rhizobium meliloti* – Tn*5* derivatives – in situ transposon replacement – Suppression of Tn*5* transposition – Site directed deletion formation

Introduction

The procaryotic transposon Tn*5* has been used extensively for insertion mutagenesis in a large number of bacteria (Berg and Berg 1983). In *Rhizobium* its advantages are the strong expression of neomycin and streptomycin resistance, and a high degree of randomness of insertion (Beringer et al. 1978; Meade et al. 1982; Putnoky et al. 1984). Several derivatives of Tn*5* have been constructed in vitro and used for a variety of applications, namely Tn*5*-132 (Tc^r, Berg et al. 1980), Tn*5*-410 (*trp*$^+$, ibid.), Tn*5*-VB32 (Nm^r promoter probe, Bellofatto et al. 1984), Tn*5.7* (Sp^r, Zsebo et al. 1984), Tn*5*-Mob (*mob* of RP4, Simon 1984), Tn*5*-*lac* (promoter probe, Kroos and Kaiser 1984), Tn*5*-oriT (*oriT* of RP4, Yakobson and Guiney 1984), and Tn*5*-751 (Tp^r, Rella et al. 1985).

In this paper we describe the construction of two new Tn*5* derivatives. Instead of resistance to kanamycin/neomycin (Berg et al. 1975), bleomycin/phleomycins (Genilloud et al. 1984; Collins and Hall 1985), and streptomycin (Mazodier et al. 1982), Tn*5*-233 carries resistance to gentamycin/kanamycin and streptomycin/spectinomycin which can also be easily selected in *R. meliloti*. Tn*5*-235 still carries kanamycin/neomycin resistance, but also expresses constitutively the *E. coli lacZ* (β-galactosidase) gene, allowing strains that carry it to be scored on lactose indicator plates. We describe several examples illustrating the usefulness of these transposons for genetic manipulations in *R. meliloti*.

Materials and methods

Strains and plasmids. The bacterial strains, phage and plasmids used in this work are listed in Table 1.

Media, antibiotics and growth conditions. Rich medium was LB, and minimal medium was M9 (Miller 1972). Liquid LB medium was supplemented with 2 mM $CaCl_2$ and 2 mM $MgSO_4$ for *R. meliloti*. For *E. coli*, concentrations of antibiotics were: 2 μg/ml gentamycin, 10 μg/ml kanamycin, 25 μg/ml spectinomycin, 25 μg/ml chloramphenicol and 50 μg/ml neomycin; for *R. meliloti* 200 μg/ml neomycin, 25 μg/ml gentamycin, 200 μg/ml spectinomycin, 50 μg/ml rifampicin, and 0.25 μg/ml oxytetracycline (which was more effective than tetracycline for Tn*5*-132), unless otherwise indicated.

DNA isolation and manipulation. Plasmid DNA isolation, cloning of restriction fragments and restriction analysis were performed essentially as described before (De Vos et al. 1984). All fragments used for cloning experiments were isolated from agarose gels. Bacterial chromosomal DNA was isolated according to Ruvkun and Ausubel (1981), and Southern hybridization was performed as described by Maniatis et al. (1982).

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Abbreviations: bp, base pairs; Nm, neomycin; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Gm, gentamycin; Tc, tetracycline; Tp, trimethoprim; Ot, oxytetracycline; Rf, rifampicin, Xgal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside

Table 1. Bacterial strains, plasmids and phages

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
MM294A	*pro-82 thi-1 endA1 hsdR17 supE44*	S. Klein
YMC9	*pro-82 thi-1 endA1 hsdR17 supE44 Δlac-pro*	L. Guarente
GS294	MM294A λ^r Rf^r	S. Klein
MT604	MM294A *malE*::Tn*5*	T. Finan
R. meliloti		
Rm1021	SU47 *str-21* Nod^+ Fix^+	F. Ausubel
Rm5000	SU47 *rif-5* Nod^+ Fix^+	E. Johansen
Rm5083	Rm5000 *Ω5011*::Tn*5*-233	Finan et al. (1985)
Rm6093	Rm5000 *Ω5011*::Tn*5*-235[a]	This work
Rm6080	Rm5000 *Ω5006*::Tn*5*-132[b]	T. Finan
Rm5003	Rm5000 *met1023*::Tn*5*	T. Finan
Rm5004	Rm1021 *recA*::Tn*5*	Finan et al. (1985)
Rm6004	Rm5000 *recA*::Tn*5*	This work
Rm6026	Rm5000 *recA*::Tn*5*-233[a]	This work
Rm6011	Rm5000 *pyr*::Tn*5*-233	This work
Rm6012	Rm5000 *ura*::Tn*5*-233	This work
Rm6013	Rm5000 *ade*::Tn*5*-233	This work
Rm6014	Rm5000 *ara*::Tn*5*-233	This work
Rm6015	Rm5000 *gln*::Tn*5*-233	This work
Rm5071	Rm5000 *nifH*::Tn*5*	T. Finan
Rm6020	Rm5000 *nifH*::Tn*5*-233[a]	This work
Rm7013	Rm1021 *exoB13*::Tn*5*	Leigh et al. (1985)
Rm6085	Rm1021 *exoB13*::Tn*5*-233[a]	This work
Rm7031	Rm1021 *exoA31*::Tn*5*	Leigh et al. (1985)
Rm7032	Rm1021 *exoA32*::Tn*5*	Leigh et al. (1985)
Rm6086	Rm1021 *exoA32*::Tn*5*-233[a]	This work
Rm7055	Rm1021 *exoF55*::Tn*5*	Leigh et al. (1985)
Rm6087	Rm1021 *exoF55*::Tn*5*-233[a]	This work
Rm6094	Rm6085 *Ω5011*::Tn*5*-235	This work
Rm6095	Rm6086 *Ω5011*::Tn*5*-235	This work
Rm6096	Rm6087 *Ω5011*::Tn*5*-235	This work
Rm6097	*ΔexoB13-Ω5011*	This work
Rm6098	*ΔexoA32-Ω5011*	This work
Rm6099	*ΔexoF55-Ω5011*	This work
Plasmids		
pBR322	cloning vector, Ap^r, Tc^r	Bolivar et al. (1977)
pGS330	Ap^r deletion derivative of pBR322	This work
pGS220	pGS330 *Ω*::Tn*5*	This work
pGS232	pGS330 *Ω*::Tn*5*-233	This work
pGS235	pGS330 *Ω*::Tn*5*-235	This work
pGS236	pGS330 *Ω*::Tn*5*-236 *LacZ* in opposite orientation as compared to Tn*5*-235	This work
pRK290.34	pRK290 with cloned *recA* region of *R. meliloti*	Better et al. (1983)
pRK2013	ColE1 containing transfer region of RK2	Figurski and Helsinki (1979)
pRK600	pRK2013 *npt*::Tn*9*	T. Finan
pRK602	pRK2013 *npt*::Tn*9* *Ω*::Tn*5*	Leigh et al. (1984)
pRK607	pRK2013 *Ω*::Tn*5*-233	This work
pRK608	pRK2013 *npt*::Tn*9* *Ω*::Tn*5*-235	This work
Phages		
ϕ M12	wild type, transducing phage	Finan et al. (1984)
λ cI857 b515 b519		R. Weisberg
λ cI857 b515 b519	*Ω*::Tn*5*-233	This work
λ cI857 b515 b519	*Ω*::Tn*5*-235	This work

[a] These mutants were obtained by replacement of the corresponding Tn*5*-mutant with Tn*5*-233, except for Rm6093 which is a Tn*5*-235 replacement of the Tn*5*-233 mutant. Each of these mutants was verified by Southern blot analysis as shown in Fig. 3 for three of the mutants

[b] This is an independently isolated insert at a nearly identical site as insert *Ω5007*::Tn*5* (Finan et al. 1985), the loci of inserts *Ω5006*::Tn*5*-132 and *Ω5007*::Tn*5* are cotransduced at 100%

Construction of Tn5-233 and Tn5-235. The new transposons derivatives were constructed from Tn*5* by in vitro manipulation of a Tn*5* insert in pGS330, which is a deletion derivative of pBR322 (Bolivar et al. 1977) lacking the entire Tc^r area including the single *Eco*RI, *Hin*dIII, *Bam*HI and *Sal*I restriction sites. This plasmid was isolated after limited *Bal*31 exonuclease digestion of *Sal*I restricted-pBR322 and is about 2,400 bp in size. pGS330 was then transformed into *E. coli* strain MT604 (MM294A *malE*::Tn*5*), and Tn*5* inserts in this high copy number plasmid were isolated after selection for high Nm^r (200 μg/ml). Plasmid DNA of ten different colonies was analysed and, apart from one insert in the *amp* area, all plasmids were found to be dimers of pGS330 carrying only one insert of Tn*5*. All these Tn*5* inserts seem to have inactivated a function of pGS330 essential for plasmid stability, so that only single inserts in dimers can replicate, because Nm^r was lost at high frequency when selective pressure was released; but pGS330 (Ap^r) was retained. However, we managed to enrich for inserts in a monomer pGS330 by growing a pool of high Nm^r *E. coli* under non-selective conditions, and then preparing plasmid DNA, transforming and selecting for low Nm^r. Several of these monomer plasmid derivatives were isolated and one of them, pGS220, was retained for further work. Restriction analysis of this plasmid revealed that Tn*5* had inserted at about 600 bp from the *Pst*I site of pGS330, downstream from the *amp* gene, with IS*50*L located next to this gene.

Tn*5*-233 was constructed by replacement of the central *Bgl*II-*Bgl*II fragment of Tn*5* in pGS220 by a 3,500 bp *Bam*HI-*Bgl*II fragment of plasmid Sa (Ward and Grinsted 1982) which carried the Gm/Km and Sp/Sm resistance markers. Both the 3,500 bp Sa fragment and the vector without the Tn*5*-core were isolated from an agarose gel, and after ligation and transformation into MM294A, recombinant plasmids were selected on LB-Sp agar. Restriction analysis of the plasmids confirmed the predicted structure for the transposon Tn*5*-233 (Fig. 1), and one plasmid, pGS232, was used for further study. We grew phage *λcI857 b515 b519* on MM294A(pGS232) and used the ly-

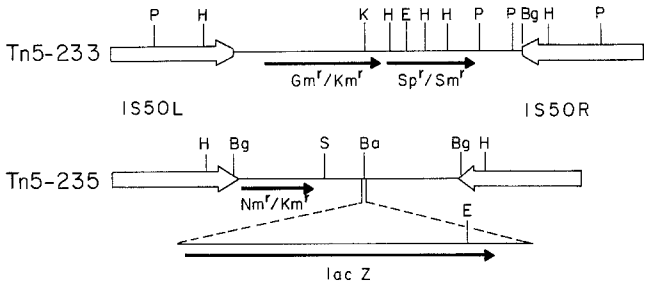

Fig. 1. Restriction endonuclease maps of the transposons Tn*5*-233 and Tn*5*-235. The areas for Gm^r/Km^r and Sp^r/Sm^r are only approximations (Ward and Grinsted 1982). Restriction sites are: Ba, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI, H, *Hin*dIII; K, *Kpn*I; PstI; and S, *Sal*I. No *Pst*I sites are shown on the Tn*5*-235 map, as the only sites are in the Tn*5* area of the transposon (Jorgensen et al. 1979)

sate to lysogenize *E. coli* MM294A (M.O.I. = 5). Km^r (10 μg/ml) lysogens were obtained at a frequency of 10^{-5}, similar to the frequency obtained with Tn*5* in a parallel experiment with a lysate grown on MM294A(pGS220). pRK2013 was then transferred into the λ(Ω::Tn*5*-233) lysogen and after mating with GS294 (MM294 λ^r, Rf^r), we isolated several Tn*5*-233 inserts in pRK2013. One of these inserts, pRK607, was used as transposition vector for *R. meliloti* in all subsequent work.

Tn*5*-235 was obtained in essentially the same way by insertion of a 4,300 bp *Bam*HI restriction fragment carrying the entire *E. coli lacZ* and *lacY* gene without a promoter into the single *Bam*HI site of Tn*5* in plasmid pGS220, inactivating the Sm^r gene. This insert is a deletion derivative of the *Bam*HI fragment of plasmid pMC903 (Casadaban et al. 1980), lacking part of the region downstream from the *lacY* gene (L. Marsh, unpublished). Hybrid plasmids containing the insert in each orientation were isolated in the Δ(lac-pro) *E. coli* strain YMC9 by scoring for the visible Lac^+ phenotype. One of them, pGS235, which contained the insert with the translational start towards IS*50*L, was used to transpose the *lacZ* containing Tn*5*-derivative, Tn*5*-235 (Fig. 1), into λ as above. A Km^r lysogen was isolated, and the new transposon was subsequently transposed into the pRK2013 derivative, pRK600, as above, to give the transposition vector pRK608.

Conjugative crosses and transductions. Transposons Tn*5*, Tn*5*-233 and Tn*5*-235 were introduced in *R. meliloti* strain Rm5000 by biparental mating with *E. coli* MM294A harboring, respectively, pRK602 (pRK600 Ω::Tn*5*), pRK607 (pRK2013 Ω::Tn*5*-233) or pRK608 (pRK600 Ω::Tn*5*-235), followed by selection on rifampicin and the appropriate antibiotics for the transposon. Mobilization of pRK290.34 into *R. meliloti* was done in a tri-parental mating with pRK2013 as mobilizing plasmid. Transduction of the transposon induced mutants was done with phage φ M12 as described by Finan et al. (1984) except that selection for transductants was done on LB/M9 medium (1:1).

In situ transposon replacement. Replacement of one Tn*5*-derived transposon by another was done by mating of *R. meliloti* containing the original insert with MM294A harboring either pRK602, pRK607 or pRK608. Colonies resistant to the antibiotic marker of the incoming transposon (either Nm, 200 μg/ml or Gm, 25 μg/ml and Sp, 100 μg/ml) were obtained at a frequency of 10^{-3} to 10^{-4} and screening of these colonies showed that about 2% of them had lost the original transposon marker. Further analysis showed that exact replacement of the transposon had occurred. In this way we also replaced the Tn*5* inserts (Nm^r) of RM7031 (*exoA31*::Tn*5*), Rm7032 (*exoA32*::Tn*5*), Rm7013 (*exoB13*::Tn*5*), and Rm7055 (*exoF55*::Tn*5*) with Tn*5*-233 (Gm^r/Sp^r). A ten-fold higher replacement frequency of Tn*5* by Tn*5*-233 could be obtained by plating the mating mixture on LB-agar containing high concentration of antibiotics (Gm, 50 μg/ml; Sp, 250 μg/ml). We did not investigate whether an increase in frequency can be obtained for replacements of Tn*5*-233 by Tn*5*.

Isolation of deletion mutants with defined end points. In the same way as above, the Gm^r marker of the insert *Ω5011*::Tn*5*-233 (Finan et al. 1985, linked to *exoB355*) was replaced by Nm^r of Tn*5*-235, which also carries the *E. coli lacZ* gene. Double insertions carrying both *Ω5011*::Tn*5*-235 and each of the *exo*::Tn*5*-233 described above (Gm^r/Sp^r) were constructed by φ M12 transduction. Deletion mutants which had lost both *E. coli lacZ* and the DNA between the two transposons were isolated with a frequency of about 10^{-4} on agar containing Gm (25 μg/ml) and Xgal (75 μg/ml), by scoring for the resulting Lac^- phenotype. On this medium, colonies of *R. meliloti* wild type are nearly white, whereas colonies of *R. meliloti* carrying Tn*5*-235 are dark blue.

Results

Construction of Tn5-233 and Tn5-235

Tn*5*-233 was constructed from Tn*5* by replacement of the central area with a restriction fragment of plasmid Sa which carries resistance to the aminocyclitol antibiotics gentamycin/kanamycin and spectinomycin/streptomycin (Materials and methods). Although both Tn*5* and Tn*5*-233 carry resistance to kanamycin and streptomycin, one can easily differentiate between the two transposons, using the neomycin marker of Tn*5* and gentamycin and spectinomycin of Tn*5*-233. Tn*5*-235 has the *E. coli* β-galactosidase gene inserted in the unique *Bam*HI site of Tn*5*, thereby abolishing the Tn*5* encoded streptomycin resistance (De Vos et al. 1984; Mazodier et al. 1985), but introducing a blue phenotype on Xgal-agar. Since the insert itself has no promoter, it is apparently transcribed from the promoter in IS*50*L. The construct which has the β-galactosidase insert in the opposite orientation (pGS236) also has a blue phenotype in *E. coli* and *R. meliloti*, confirming that the reciprocal promoter in IS*50*R is functional in both backgrounds (Rothstein et al. 1980; De Vos et al. 1984). The construct pGS236 was not used for further work. Both Tn*5*-233 and Tn*5*-235 were subsequently transposed into λcI857 b515 b519 and then into pRK2013 or pRK600 (pRK2013 *npt*::Tn*9*) respectively (Materials and methods), yielding the plasmids pRK607 (pRK2013 Ω::Tn*5*-233) and pRK608 (pRK600 Ω::Tn*5*-235). These plasmids were subsequently used to introduce the two Tn*5*-derivatives into *R. meliloti*.

Random insertion of the new Tn5-derivatives in R. meliloti

When MM294A(pRK607) or YMC9(pRK608) were used as donors in biparental matings with Rm5000, Gm^r or Nm^r rhizobia respectively were obtained at a frequency between

Table 2. Auxotrophs obtained by Tn*5*, Tn*5*-233 and Tn*5*-235 insertions in Rm5000 and Rm5000(*recA*)

Transposon	Recipient	Colonies screened	Auxotrophs	% auxotrophs
Tn*5*	Rm5000	529	4	0.76
Tn*5*	Rm5000(*recA*::Tn*5*-233)	499	4	0.80
Tn*5*-233	Rm5000	523	6	1.15
Tn*5*-233	Rm5000(*recA*::Tn*5*)	533	5	0.94
Tn*5*-235	Rm5000	745	7	0.94

10^{-4} and 10^{-5} per recipient. Subsequent screening showed that about 1% of these insert strains were auxotrophs, comparable to results obtained with Tn*5* (Table 2). These results indicate that like Tn*5*, both Tn*5*-derivatives still transpose in a random fashion in *R. meliloti*. In addition, for the Tn*5*-233 induced mutants Rm6011, Rm6012, Rm6013, Rm6014 and Rm6015, in each case the auxotrophic phenotype cotransducted with the Tn*5*-233 resistance markers (data not shown).

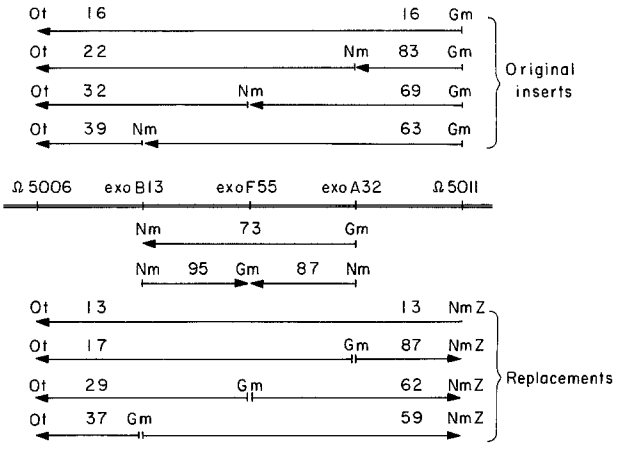

Fig. 2. Schematic linkage map of the three *exo* mutants *exoA32, exoB13, exoF55*, and the linked inserts *Ω5006* and *Ω5011*. Linkage frequencies (in %) were determined by two-point crosses as described in Materials and methods, and are shown above the arrows identifying the crosses. Abbreviations for the drug markers are Gm, gentamycin resistance (Tn*5*-233); Nm, neomycin resistance (Tn*5*); Nm Z; neomycin resistance, lacZ expression (Tn*5*-235); and Ot, oxytetracycline resistance (Tn*5*-132)

In situ transposon replacement

Berg et al. (1980) reported that Tn*5* can be replaced by Tn*5*-410 (*trp*$^+$) during λ transduction experiments in *E. coli*. Also, Avery and Kaiser (1983) reported replacements of Tn*5* by Tn*5*-132 in *M. Xanthus* during P1 transduction experiments. We observed a similar type of replacement in *R. meliloti* when one Tn*5*-type transposon, inserted in pRK2013, was introduced into a strain carrying a different Tn*5*-type transposon.

Rm5003 carries *met1023*::Tn*5* (Finan et al. 1984). Mating of pRK607 into this strain gave Gm/Sp *R. meliloti* exconjugants at a frequency of 10^{-3} to 10^{-4}, which is about 10-fold higher than the transposition frequency in its parent Rm5000. Presumably a single crossover between the IS*50* homologues of the incoming and resident transposons resulted in the formation of a cointegrate of pRK607 with the chromosome, because most of the colonies were still Nm^r. However, about 2% of these Gm^r colonies were no longer Nm^r but still auxotrophic, suggesting replacement of the resident transposon. Consistent with this interpretation, the methionine auxotrophic marker cotransduced with Gm^r/Sp^r at 100%. Conversely, when one such strain was transduced with a lysate grown on Rm5003, 100% of the selected Nm^r colonies had now lost Gm^r/Sp^r. Because replacements could not be obtained in a *recA* background (see next section), we infer that homologous recombination between the IS*50* sequences at either end of the two tranposons is required both for cointegrate formation (single crossover) and for replacement ('swapping') of the transposon (double crossover).

Several other strains carrying replacements made in this way are listed in Table 1. Replacements have the same Tn*5* border fragments as the original mutants in genomic Southern blot hybridization (Fig. 3), and the same linkage frequency to an outside marker in transduction (Fig. 2).

Construction of a recA::Tn5-233 mutant of R. meliloti

No Tn*5*-233 replacements could be obtained for Rm6004 (*recA*::Tn*5*) indicating that a functional *recA* gene is required for transposon replacement. However, a replacement of Tn*5* in the *recA* gene was obtained in the following way. Plasmid pRK290.34 (Better and Helinski 1983) was first introduced into Rm6004 to complement the RecA phenotype; Tn*5* was then replaced with Tn*5*-233 as above; finally, the replaced insert was transduced into Rm5000. The resulting strain, Rm6026, has the same RecA phenotype as Rm6004. Because this strain is Gm^r/Sp^r rather than Nm^r, the *recA* mutation can now be readily introduced into strains carrying Tn*5* mutations in loci of choice, for example, to test complementation.

Suppression of Tn5-233 transposition by Tn5

When the Tn*5*-233 transposition vector, pRK607, was mated into the *recA*::Tn*5* strain Rm6004, Gm^r/Sp^r exconjugants were obtained at a frequency of 10^{-6} to 10^{-7}. This is about 50-fold lower than that obtained with recipient Rm5000 and 1000-fold lower than that obtained with recipient Rm5003 (*met1023*::Tn*5*). Further analysis showed that these colonies were due to transposition of Tn*5*-233 at a lower frequency. After screening, auxotrophic mutants were found at a frequency of 0.9% (Table 2), similar to the value obtained for transposition of Tn*5* and Tn*5*-233 in Rm5000 in a parallel experiment (Table 2). In addition, five independent Gm^r/Sp^r exconjugants were purified, and their inserts were then transduced into Rm5000; complete segregation of the Nm^r (Tn*5*) and Gm^r/Sp^r (Tn*5*-233) markers was observed, suggesting that recombination between the two transposons had not occurred; moreover, the Nm transductants still retained the RecA phenotype and suppressed transposition of an incoming Tn*5*-233. Suppression of Tn*5* transposition by a resident Tn*5* has been observed before (see Discussion), and therefore these data indicate that Tn*5* also suppresses Tn*5*-233 transposition in *R. meliloti*.

In the converse experiment, we examined the transposition frequency of Tn*5* into Rm6026 (*recA*::Tn*5*-233). In this experiment, however, the transposition frequency was found to be identical to that with recipient Rm5000. Screen-

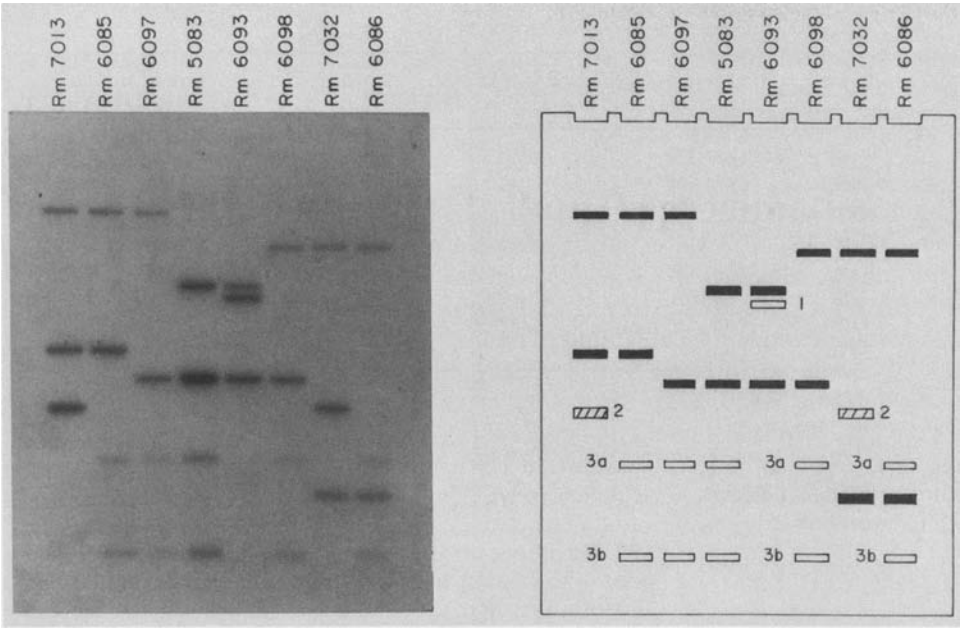

Fig. 3. Hybridization data for *R. meliloti* mutants. Genomic DNA of several mutants was restricted with *Hin*dIII and after Southern transfer probed with pGS220 *Ω*::Tn*5*. The different mutants are: Rm7013 (*exoB13*::Tn*5*), Rm7032 (*exoA32*::Tn*5*), Rm5083 (*Ω5011*::Tn*5*-233); and their replacements: Rm6085 (*exoB13*::Tn*5*-233), Rm6086 (*exoA32*::Tn*5*-233), Rm6093 (*Ω5011*::Tn*5*-235); and the deletion mutants Rm6097 (*ΔexoB13-Ω5011*), and Rm6098 (*ΔexoA32-Ω5011*). The tracing at the *left* shows (1) the internal *Hin*dIII fragment of Tn*5*-235, (2) the internal *Hin*dIII fragment of Tn*5*, and (3a, b) the two internal *Hin*dIII border fragments of Tn*5*-233; other fragments are external borders

ing for auxotrophs gave about the same frequency as obtained for wild type (Table 2). Ten independent Tn*5*-233 replacements of the *recA*::Tn*5* were subsequently tested and all were found to lack suppression of Tn*5* transposition. This result suggests that unlike its parent Tn*5*, resident Tn*5*-233 does not suppress transposition of an incoming Tn*5* derivative.

Consistent with this interpretation, we found that in *E. coli* transposition of Tn*5*-233 from the chromosome into plasmid pGS330 is also 50-fold more frequent than for Tn*5* and Tn*5*-235 (data not shown). We interpret this as due to lack of autosuppression by Tn*5*-233, as contrasted with Tn*5* (Biek and Roth 1980).

Cotransductional linkage mapping of R. meliloti exo mutants

Finan et al. (1985) and Leigh et al. (1985) recently described new mutants of *R. meliloti* that are defective in exopolysaccharide synthesis (*exo*$^-$) and also induce 'empty' nodules on alfalfa. Tn*5* inserts in three of these mutant loci, *exoA*, *exoB* and *exoF*, were shown to be linked to the same randomly generated *Ω5011*::Tn*5*-233 and *Ω5006*::Tn*5*-132 inserts in transduction (Finan et al. 1985; J. Leigh, personal communication). By replacing the original transposon inserts with Tn*5* derivatives in various combinations, we have been able to map the mutations. Two point cotransductional linkage frequencies were determined between each of the linked inserts *Ω5011*::Tn*5*-233 (Rm5083) and *Ω5006*::Tn*5*-132 (Rm6080), and the mutant inserts *exoA32*::Tn*5* (Rm7032), *exoB13*::Tn*5* (Rm7013) and *exoF55*::Tn*5* (Rm7055) (Fig. 2). The Nm^r marker of each of the three *exo* mutants was then replaced by Gm^r/Sp^r as above, and Tn*5*-233 of Rm5083 was replaced by Tn*5*-235. Cotransduction frequencies were then found to be essentially identical to the original values (Fig. 2). Using the replacements of *exoA31*, *exoA32* and *exoF55*, we also determined cotransduction frequencies for pairs of the three *exo* mutants. The tight linkage (95%) found for the *exoB13*::Tn*5* and *exoF55*::Tn*5*-233 inserts is consistent with the fact that both mutations can be complemented by the same recombinant plasmid (Leigh et al. 1985).

In further experiments the order of the *exo* mutants was unambiguously determined by three factor crosses. The results (Table 3) are consistent with both the order deduced from the two point crosses (Fig. 2), and the position of an *exoB* point mutation (Finan et al. 1985).

Isolation of deletion mutants with defined end points

Hughes and Roth (1984) have shown that the DNA sequences located between two adjacent copies of Mud(Ap, *lac*) can be deleted at a low frequency via general recombination between the two transposons. Similarly, we found that deletions could be generated spontaneously by recombination between two Tn*5*-derivatives. To simplify screening we used Tn*5*-235, the loss of which can easily be detected on Xgal agar (see Materials and methods).

Having determined the position of the inserts *exoB13*, *exoF55*, *exoA32* and *Ω5011* (Fig. 2), we used these mutations to generate deletions in this area. For each of the double insertion strains Rm6094, Rm6095, and Rm6096 (Table 1), putative deletions were isolated (Materials and methods) at a frequency of about 10^{-4}. One derivative of each double insert (Rm6097, Rm6098, and Rm6099 respectively) was confirmed to have the predicted deletion by the following criteria. (1) When the putative deletion (Gm^r) was transduced into strain Rm6093 (Nm^r, right border) or the original *exo* mutant (Nm^r, left border) from which it was derived before replacement, the Nm^r marker was lost at 100%. (2) Each of the putative deletions had approximately the same linkage frequency to *Ω5006*::Tn*5*-132 as had the parental *exo* mutant, namely 46% for Rm6097, 38% for Rm6099 and 22% for Rm6098. (3) Southern blot hybridization (Fig. 3) showed that in each putative deletion one border fragment of the transposon was missing, compared with the double insert from which it was derived. For the right border this was always the same fragment.

Moreover, a *thi*$^-$ insert has been located near

Table 3. Results of three point crosses between different transposon mutants

Selection	Screening	Donor strains		
		exoA31::Tn*5*-*Ω5011*::Tn*5*-233	*exoA32*::Tn*5*-*Ω5011*::Tn*5*-233	*exoF55*::Tn*5*-*exoB13*::Tn*5*-233
Nn^r	Gm^r, Ot^r	68	234	317
Nm^r	Gm^r, Ot^s	5	48	192
Nm^r	Gm^s, Ot^r	7	24	16
Nm^r	Gm^s, Ot^s	7	18	1
Gm^r	Nm^r, Ot^r	52	210	319
Gm^r	Nm^r, Ot^s	14	52	196
Gm^r	Nm^s, Ot^r	22	65	6
Gm^r	Nm^s, Ot^s	0	0	6

The double mutants (donor strains) were transduced into strain Rm6080 (*Ω5006*::Tn*5*-132) and selection was for Nm^r or Gm^r. Colonies obtained were subsequently screened for Gm^r and Ot^r, or Nm^r and Ot^r respectively

exoA32::Tn*5* (TM Finan, B Kunkel, G De Vos and ER Signer, J Bacteriol, in press). All three putative deletion mutants were found to be Thi^-, which would locate the *thi* locus between *exoA32* and *Ω5011* (Fig. 2).

Discussion

Tn*5* insertion mutants of *R. meliloti* are easily obtained, and are stable, as secondary transposition has not been reported. The present results show that the drug marker associated with the insert can readily be exchanged, as shown earlier in *E. coli* (Berg et al. 1980) and *M. xanthus* (Avery and Kaiser 1983). Introducing the second transposon on a plasmid like pRK2013, which has a broad host range for transfer and a narrow host range for replication, provides a more general method for marker exchange, than does transduction as used by the authors cited. The exchange mechanism appears to be $recA^+$ dependent and presumably occurs via a pair of crossovers between the homologous IS*50* sequences of the two transposons. In this way, insertion mutations generated with one Tn*5*-derivative can be tagged with a different drug marker, allowing the construction of strains carrying multiple insertion mutations.

This procedure has also allowed us to map by transduction closely linked inserts in an area of interest. In addition, scoring for the Lac phenotype of Tn*5*-235 on indicator plates has allowed us to isolate deletion mutants with defined end points. With this type of analysis, which should be generally applicable, we have established the order *Ω5006*—*exoB*—*exoF*—*exoA*—*Ω5011*, and also located a previously isolated *thi* mutant between *exoA* and *Ω5011*. A similar type of deletion in this area has also been isolated independently, from a strain carrying Tn*5*-oriT adjacent to *Ω5011*::Tn*5*-233 (T. Finan, personal communication).

In *S. typhimurium*, a resident Tn*5* suppresses transposition of an incoming Tn*5* transposon approximately ten-fold (Biek and Roth 1980). Similarly, in *E. coli*, pBR322 constructs which express high levels of protein 2 from IS*50*R also suppress the transposition of an incoming Tn*5* transposon about fifty-fold (Isberg et al. 1982; Johnson et al. 1982; Lowe and Berg 1983). Recently, another Tn*5* derivative carrying Gm^r/Sp^r has also been reported to suppress transposition (Wang et al. 1985). In *R. meliloti* suppression of transposition was tested in a RecA background, because otherwise efficient rescue of the incoming transposon occurs via a crossover between the homologous IS*50* sequences. A 50-fold suppression of Tn*5*-233 transposition by Tn*5* in Rm6004 (*recA*::Tn*5*) was observed. However, no suppression of Tn*5* transposition was observed in strain Rm6026 (*recA*::Tn*5*-233, obtained by replacement of the *recA*::Tn*5*).

Depending on the relative orientation of both transposons with respect to the pair of crossovers, a replacement might in principle generate a transposon carrying the ochre mutation, normally present in IS*50*L only, (Rothstein et al. 1980; Auerswald et al. 1980) in IS*50*R as well, which would inactivate both transposition and suppression of transposition (Isberg et al. 1982; Johnson et al. 1982). However, because we obtained the same result with ten independent replacements, and because a 50-fold higher frequency of secondary transposition was also obtained in *E. coli*, we speculate that this reduction in suppression is instead an intrinsic property of Tn*5*-233. In IS*50*R, protein 1 and protein 2 are encoded by the same open reading frame, but protein 2 lacks the amino-terminal end of protein 1 (Rothstein et al. 1980; Auerswald et al. 1980). Protein 1 promotes transposition but protein 2 promotes suppression only, (Isberg et al. 1982; Johnson et al. 1982) which locates the suppressive function to the carboxy terminal end of the reading frame. However, that carboxy-terminus spans the *BglII* site used to construct Tn*5*-233. Thus, in Tn*5*-233, the hybrid equivalent of protein 1 might still promote transposition, whereas in contrast the hybrid equivalent of protein 2 might no longer promote suppression.

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References

Avery L, Kaiser D (1983) In situ transposon replacement and isolation of a spontaneous tandem genetic duplication. Mol Gen Genet 191:99–109

Auerswald EA, Ludwig G, Schaller H (1980) Structural analysis of Tn*5*. Cold Spring Harbor Symp Quant Biol 45:107–113

Bellofatto V, Shapiro L, Hodgson DA (1984) Generation of a Tn*5* promoter probe and its use in the study of *Caulobacter crescentus*. Proc Natl Acad Sci USA 81:1035–1039

Berg DE, Davies J, Allet B, Rochaix J-D (1975) Transposition

of R factor genes to bacteriophage λ. Proc Natl Acad Sci USA 72:3628–3632
Berg DE, Egner C, Hirschel BJ, Howard J, Johnsrud L, Jorgensen RA, Tlsty TD (1980) Insertion, excision, and inversion of Tn*5*. Cold Spring Harbor Symp Quant Biol 45:115–123
Berg DE, Berg CM (1983) The procaryotic transposon Tn*5*. Biol Technol 1:417–435
Beringer JE, Beynon JL, Buchanan-Wollaston AV, Johnston AWB (1978) Transfer of the drug resistance transposon Tn*5* to *Rhizobium*. Nature 276:633–634
Better M, Helinski DR (1983) Isolation and characterization of the *recA* gene of *Rhizobium meliloti*. J Bacteriol 155:311–316
Biek D, Roth JR (1980) Regulation of Tn*5* transposition in *Salmonella typhimurium*. Proc Natl Acad Sci USA 77:6047–6051
Bolivar F, Rodrigues RL, Betlach MC, Boyer HM (1977) Construction and characterization of new cloning vehicles. I. Ampicillin resistant derivatives of the plasmid pMB9. Gene 2:75–93
Casadaban MJ, Chou J, Cohen SN (1980) In vitro gene fusions that join an enzymatically active β-galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translational initiation signals. J Bacteriol 143:971–980
Collins FG, Hall RM (1985) Identification of a Tn*5* determinant conferring resistance to phleomycins, bleomycins and tallysomycins. Plasmid 14:143–151
De Vos GF, Finan TM, Signer ER, Walker GC (1984) Host-dependent transposon Tn*5*-mediated streptomycin resistance. J Bacteriol 159:395–399
Figurski DH, Helinski DR (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. Proc Natl Acad Sci USA 76:1648–1652
Finan TM, Hartwieg E, LeMieux K, Bergman K, Walker GC, Signer ER (1984) General transduction in *Rhizobium meliloti*. J Bacteriol 159:120–124
Finan TM, Hirsch AM, Leigh JA, Johansen E, Kuldau GA, Deegan S, Walker GC, Signer ER (1985) Symbiotic mutants of *Rhizobium meliloti* that uncouple plant from bacterial differentiation. Cell 40:869–877
Genilloud O, Garrido MC, Moreno F (1984) The transposon Tn*5* carries a bleomycin resistance determinant. Gene 32:225–233
Hughes KT, Roth JR (1984) Directed formation of deletions and duplications using Mud(Ap, *lac*). Genetics 109:263–282
Isberg RR, Lazaar AL, Syvanen M (1982) Regulation of Tn*5* by the right repeat proteins: control at the level of the transposition reaction? Cell 30:883–892
Jorgensen RA, Rothstein SJ, Reznikoff WS (1979) A restriction enzyme cleaveage map of Tn*5* and location of a region encoding neomycin resistance. Mol Gen Genet 177:65–72
Johnson RC, Yin JCP, Reznikoff WS (1982) Control of Tn*5* transposition in *E. coli* is mediated by protein from the right repeat. Cell 30:873–882
Kroos L, Kaiser D (1984) Construction of Tn*5*-lac, a transposon that fuses LacZ expression to exogenous promoters, and its introduction into *Myxococcus xanthus*. Proc Natl Acad Sci USA 81:5816–5820
Leigh JA, Signer ER, Walker GC (1985) Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. Proc Natl Acad Sci USA 82:6231–6235
Lowe JB, Berg DE (1983) A product of the Tn*5* transposase gene inhibits transposition. Genetics 103:605–615
Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Mazodier P, Giraud E, Gasser F (1982) Tn*5* dependent streptomycin resistance in *Methylobacterium Organophylicum*. FEMS Microbiol Lett 13:27–30
Mazodier P, Cassart P, Giraud E, Gasser F (1985) Completion of the nucleotide sequence of the central region of Tn*5* confirms the presence of three resistance genes. Nucleic Acids Res 13:195–205
Meade HM, Long SR, Ruvkun GB, Brown SE, Ausubel FM (1982) Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J Bacteriol 149:114–122
Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
Putnoky P, Kiss GB, Ott I, Kondorosi A (1984) Tn*5* carries a streptomycin resistance determinant downstream from the kanamycin resistance gene. Mol Gen Genet 191:288–294
Rella M, Mercenier A, Haas D (1985) Transposon mutagenesis of *Pseudomonas aeruginosa* with a Tn*5* derivative: application to physical mapping of the *arc* gene cluster. Gene 33:293–303
Rothstein SR, Jorgensen RA, Yin JC-P, Yong-di Z, Johnson RC, Reznikoff WS (1980) Genetic organization of Tn*5*. Cold Spring Harbor Symp Quant Biol 45:99–105
Ruvkun GB, Ausubel FM (1981) A general method for site-directed mutagenesis in prokaryotes. Nature (London) 289:85–88
Simon R (1984) High frequency mobilization of gram-negative bacterial replicons by the *in vitro* constructed Tn*5*-Mob transposon. Mol Gen Genet 196:413–420
Wang GC, Spokes JR, Woodward MJ, Hirsch PR (1985) Transposon mutagenesis of Rhizobium using a derivative of Tn*5* conferring gentamycin and spectinomycin resistance. In: Evans HJ, Bottomley P, Newton WE (eds) Nitrogen fixation research progress. Martinus Nijhoff Publishers, Dodrecht, p 153
Ward JM, Grindsted J (1982) Physical and genetic analysis of the Inc-W group plasmids R388, Sa, and R7K. Plasmid 7:239–250
Yakobson EA, Guiney DG Jr (1984) Conjugal transfer of bacterial chromosomes mediated by the RK2 plasmid transfer origin cloned into transposon Tn*5*. J Bacteriol 160:451–453
Zsebo KM, Wu F, Hearst JE (1984) Tn*5.7* construction and physical mapping of pRPS404 containing photosynthetic genes from *Rhodopseudomonas capsulata*. Plasmid 11:182–184

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