Suicide Plasmid Vehicles for Insertion Mutagenesis in *Rhizobium meliloti* and Related Bacteria

G. SELVARAJ AND V. N. IYER*

Department of Biology and Institute of Biochemistry, Carleton University, Ottawa, Ontario, Canada K1S 5B6

We describe the construction and use of a set of plasmid vectors of the transposons Tn*1*, Tn*5*, and Tn*9* that are suicidal in *Rhizobium* species and therefore suitable for mutagenesis with these three transposons. The vectors are composed of the p15A replicon which functions in *Escherichia coli* but not in *Rhizobium* species and a region encoding the N type of bacterial conjugation system which is very efficient in matings between *E. coli* and *Rhizobium* species. The usefulness of the vectors has been most extensively assessed in *Rhizobium meliloti*. It is likely that they will be useful for mutagenesis and genome manipulation in other bacteria as well.

Transposons are discrete genetic elements that ensure their own maintenance by inserting into other autonomously maintained genetic elements of bacteria (20, 39). Such insertions lead to mutations. An added and very useful feature of transposons is that they often encode phenotypes, such as antibiotic resistance, by which their presence may be recognized. Furthermore, they can and have also been used for genetic manipulations such as deletions, inversions, and fusions. These applications have been reviewed previously (21). Although the use of transposons was pioneered in *Escherichia coli* and closely related bacteria, they can be used in other bacteria as well (for example, see Kuner et al. [24]). We describe a procedure for doing this in the symbiotic nitrogen-fixing bacteria *Rhizobium meliloti* and *Rhizobium leguminosarum*.

Since transposons themselves are not autonomous replicons, it is reasonable to expect that if they are delivered efficiently into a cell by means of a plasmid vehicle that will not itself be maintained (a suicide vehicle), this would allow the selection of the rare transposon-insertion derivatives by using the antibiotic resistance marker contained within the transposon. In practice, the efficiency of delivery of the vehicle into cells of interest, the efficiency with which it is lost from the cells, and an assurance that the transposon-insertion is the only genetic event that has occurred in a large majority of the selected colonies are all important considerations. For members of the *Rhizobiaceae* family, the suicide vehicles that have been used so far are cointegrates of a conjugative, P incompatibility group plasmid and bacteriophage Mu, with the Mu genome conferring the suicidal property to the plasmid by mechanisms that are not well under-

stood (3, 6, 43). Our experience and the results of other groups studying *R. meliloti* have shown that such Mu-containing vehicles induce secondary genetic changes incited by Mu itself (12, 26; J. J. Patel, R. M. Behki, G. Selvaraj, and V. N. Iyer, Proc. VIII N. Am. *Rhizobium* Conf., University of Manitoba, Canada p. 129–149, 1982). These observations suggested that it would be useful to develop a suicide vehicle that did not contain Mu DNA. We sought to identify or construct a plasmid which had a wide host range of efficient conjugal transmission, but with a capacity of replication and maintenance limited to *E. coli* and closely related bacteria. Such a plasmid carrying a transposon would be suicidal on entry into *R. meliloti*. Selection for a transposon-associated marker would then yield derivatives carrying transposon insertions. The plasmids constructed in this study contained one of the transposons Tn*1*, Tn*5*, or Tn*9* and were composed of a p15A-type of replicon and an N type of bacterial mating system. They have proved useful for *R. meliloti* and *R. leguminosarum* and are potentially useful for other gram-negative bacteria. We describe and discuss the construction, use and efficiency of these vehicles.

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MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophages, and transposons. The bacterial strains used are listed in Table 1. The plasmids are described in Table 2 and Fig. 1, 2, 3, and 4. Bacteriophage λ *b221 cI857 Pam Oam rex*::Tn*5* (37) was from G. C. Walker and is

TABLE 1. Bacterial strains

Strain	Relevant characteristics[a]	Derivation or source and reference
E. coli		
WA803	Met⁻ Thi⁻	(44)
HB101	Pro⁻ Leu⁻ Thy⁻ Thi⁻ Sm^r	(4)
GS500	Pro⁻ Leu⁻ Thy⁻ Thi⁻ Sm^r Rif^r	Spontaneous Rif^r derivative of HB101; this study.
CR34	Thr⁻ Leu⁻ Thy⁻ Thi⁻ P1^r	F. Bonhoeffer
PB3	His⁻ Thi⁻ λ^r	(30)
1830	Nal^r; carried pJB4J1 encoding resistance to Gm and Nm (Tn5)	(3)
R. meliloti[b]		
JJ1	Nod⁺ Fix⁺ Rif^r; carries two indigenous plasmids (500 and 200 kb)[c]	Spontaneous Rif^r derivative of a natural isolate (IZ450) from Brazil
GS1002	Nod⁺ Fix⁺ Rif^r; carries two indigenous plasmid (500 and 200 kb)	Spontaneous Rif^r derivative of Rhodesia 304 from Zimbabwe
GS1003	Nod⁺ Fix⁺ Rif^r; carries two indigenous plasmids (500 and 300 kb)	Spontaneous Rif^r derivative of 2043 from England
R. leguminosarum		
GS1400	Nod⁺ Fix⁺ Rifrd	Spontaneous Rif^r derivative of I28C52; (29)
GS1401	Nod⁺ Fix⁺ Rif^r	Spontaneous Rif^r derivative of I28C53; (5)
GS1402	Nod⁺ Fix⁺ Rif^r	Spontaneous Rif^r derivative of I75G16; (29)

[a] His, histidine; Leu, leucine; Met, methionine; Nal, nalidixic acid; Thr, threonine; Thi, thiamine; Thy, thymine.

[b] The natural isolates of *R. meliloti* were provided by R. W. Miller and E. A. Peterson of the Canada Department of Agriculture, Ottawa.

[c] The symbols Nod⁺ and Fix⁺ denote nodulation and nitrogen fixation in alfalfa roots. The sizes of the indigenous plasmids were estimated in Eckhardt-type gels (11).

[d] Nod⁺ and Fix⁺ denote nodulation and nitrogen fixation in pea roots.

referred to as λ::Tn5. λb515 b519 int am219 cI857 nin5::Tn1, abbreviated as λ::Tn1, was from J. Way and N. Kleckner. P1Cm, carrying Tn9 (32) was provided by J. L. Rosner.

Media, chemicals, and biochemicals. For the growth of *E. coli* strains, tryptone-yeast extract-sodium chloride (TYS) medium (35) was used at 37°C. *Rhizobium* spp. strains were grown at 30°C in tryptone-yeast

extract (TY) medium containing CaCl₂ (2). Plasmid-carrying strains were grown in medium containing an antibiotic to which the plasmid specified resistance. For solidifying media, Difco agar was used at 1.5% (wt/vol). The concentration of antibiotics used in solid media was as follows (μg/ml): carbenicillin (Cb), 40; chloramphenicol (Cm), 50; gentamicin (Gm), 10; neomycin (Nm), 100; spectinomycin (Sp), 50; streptomy-

TABLE 2. Plasmids[a]

Plasmid	Relevant characteristics[b]	Derivation or source and reference
pCU1	Tra⁺ (N type), N group replicon, Sp^r Sm^r Cb^r	This laboratory (22)
pCU29	Tra⁺ (N type), N group replicon, Nm^r (Tn5) Sp^r Sm^r Cb^r	Tn5-carrying derivative of pCU1 (42)
pCU51	N group replicon, Nm^r	Deletion derivative of pCU29 (V. Thatte, unpublished construct)
pACYC177	p15A replicon, Nm^r Cb^r	(7)
pACYC184	p15A replicon, Cm^r Tc^r	(7)
pRK248	P group replicon, Tc^r	(19)
pJB4JI	P group replicon, Nm^r (Tn5) Gm^r; carries bacteriophage Mu	(3)
pCU101	Tra⁺ (N type), p15A replicon Cm^r Nm^r (Tn5)	(42)
pGS33	p15A replicon, Cm^r	Tc^s derivative of pACYC184 (unpublished construct)
pGS38	p15A replicon, Cm^r Nm^r (Tn5)	Tn5-carrying derivative of pGS33
ColE1::Tn5	ColE1 replicon, Nm^r (Tn5)	E. W. Nester

[a] Other plasmids derived in this study are described in Fig. 1, 2, and 3.

[b] Tra, Conjugal transfer.

cin (Sm), 50; and rifampin (Rif), 150. In liquid media, half of these antibiotic concentrations were used. The antibiotics were from Bristol Myers Canada Ltd. and Sigma Chemical Co.

Bacteriophage λ DNA, restriction endonucleases, and T4 DNA ligase were purchased from New England Biolabs. DNA polymerase I was from Boehringer-Mannheim. ^{32}P-labeled ATP was from New England Nuclear Corp. The remainder of the chemicals and biochemicals were from Sigma Chemical Co. and British Drug House and were of reagent grade.

Bacterial matings and transformation. Spot matings between 10^6 donors and recipients were done on appropriate selective plates. Alternatively, 10^8 cells of donors and recipients were mixed on membrane filters and incubated on TY or TYS agar plates for 12 to 16 h, followed by plating on appropriate selective plates.

Genetic transformation of *E. coli* was carried out by the method of Cohen et al. (8) with the following change. The cells were washed with 0.03 M CaCl$_2$ instead of 0.01 M NaCl. This prevented the clumping of cells.

R. meliloti strains were transformed with freshly isolated plasmid DNA as described earlier (36).

Screening for plasmids. The procedure of Eckhardt (11) was used as described by Behki et al. (1a).

Plasmid DNA manipulations. The plasmid DNA preparations used in all cloning and transformation experiments were isolated and purified by the procedure of Davis et al. (10) with some minor modifications. A 600-ml batch of an overnight culture was centrifuged at 4°C at 7,000 rpm in a Sorvall GSA rotor, and the pellet was suspended in 4 ml of chilled STE (15% sucrose, 0.05 M Tris-hydrochloride [pH 8.3], 0.05 M EDTA); 0.5 ml of freshly prepared lysozyme solution (Sigma; 10 mg/ml in STE) was added. The cell suspension was held at room temperature for 5 min and on ice for a further 5 min. A 5-ml amount of Triton X-100 solution (0.1% in 0.05 M Tris-hydrochloride [pH 8.3], 0.005 M EDTA) was added, and the contents were gently mixed once. If there was no increment in viscosity of the cell suspension, a few drops of sodium dodecyl sulfate (10% in distilled water) was added, and the lysate was spun at 15,000 rpm for 1 h in a Sorvall SS34 rotor at 4°C. Cesium chloride was added to the supernatant at a final concentration of 45% (wt/wt); once it was dissolved, 1 ml of ethidium bromide (10 mg/ml in distilled water) was added. The contents were held at 4°C in the dark for about 30 min, and the precipitated debris was removed either by centrifugation at 10,000 rpm in a SS34 rotor or by filtration through siliconized glass wool. The DNA was banded by spinning at 40,000 rpm in a type 50 rotor (Beckman Instruments, Inc.) at 20°C for 36 to 40 h, and the plasmid DNA was rerun for 4 h in a 50% CsCl (wt/wt) solution at 65,000 rpm in a VTi80 rotor (Beckman) at 20°C. The plasmid DNA was extracted three times with CsCl-saturated isopropanol, precipitated two times with 95% ethanol, and redissolved in 0.1 ml of distilled water.

Plasmid DNA used in restriction mapping was isolated by the rapid method of Holmes and Quigley (16). Restriction enzymes were purchased from New England Biolabs or Boehringer-Mannheim and were used as recommended by Davis et al. (10). Gel electrophoresis was carried out in 0.9% agarose gels in Tris-acetate buffer (40) at 2 V/cm for 12 h. *Hind*III frag-

ments of bacteriophage λ DNA were used as size standards (28). DNA ligation reactions were performed at 17°C for 12 to 20 h with T4 DNA ligase in a buffer recommended by the supplier.

Colony hybridization. ^{32}P-labeled DNA of pACYC184, pCU1 and ColE1::Tn5 were prepared by nick translation (31) and were used as probes in colony hybridization (13). The nitrocellulose sheets (Schleicher and Schuell; BA85) carrying the colony blots were hybridized in 50% formamide at 42°C. Strains of *E. coli* carrying pACYC184, pCU1, ColE1::Tn5, pGS12, or shortened derivatives of pCU1 were used as controls.

RESULTS AND DISCUSSION

Attempts to use IncN group plasmids as suicide vehicles in *R. meliloti*. Of the different bacterial conjugation systems that have been studied in *E. coli*, N, P, and W systems have a wide host range (2, 9, 17, 41). Leemans et al. (25) have reported that N plasmids could be used as suicide vehicles for insertion mutagenesis in *Agrobacterium tumefaciens*, a member of the *Rhizobiaceae* family that is closely related to *R. meliloti*. pCU1 is a prototype N group plasmid whose structural and functional organization is known (22). pCU29 is a derivative of this plasmid carrying Tn5 at a nonessential site (42). The results shown in Fig. 1 suggest that this plasmid could be transmitted to *R. meliloti* but that putative Tn5-carrying derivatives of *R. meliloti* also inherit some of the other antibiotic resistance markers of the plasmid.

On further growth under nonselective conditions, there was a continual segregation of these markers. Thus, it seemed that N plasmids might not be completely unstable in *R. meliloti* and that some stability might be achieved by continuous selection for their markers. Recently, it has been shown that pCU1 DNA has inverted repeats, structures that are often associated with transposable elements (23). The regions of pCU1 (and its derivative pCU29) encoding resistance to Sp and Sm are on such structures. Perhaps the continual instability of pCU29-associated markers reflects this feature of the N plasmid.

To determine whether a simpler N plasmid would be completely unstable in *R. meliloti*, pCU51, a derivative of pCU29, was introduced into strain JJ1 by genetic transformation. This plasmid is a deletion derivative of pCU29 generated by *Bam*HI cleavage and ligation of the fragment that carries the maintenance region and the Nm^r marker of pCU29 (V. Thatte, unpublished results; see Fig. 1 for a map of pCU29). Nm^r transformants of strain JJ1 were obtained at a frequency of 400 transformants per μg of DNA, a frequency that is comparable to that observed with IncP1 group plasmids (36; see Fig. 2). However, pCU51 was lost in all of the 100 transformants when they were grown on TY agar lacking Nm. In contrast, the plasmid
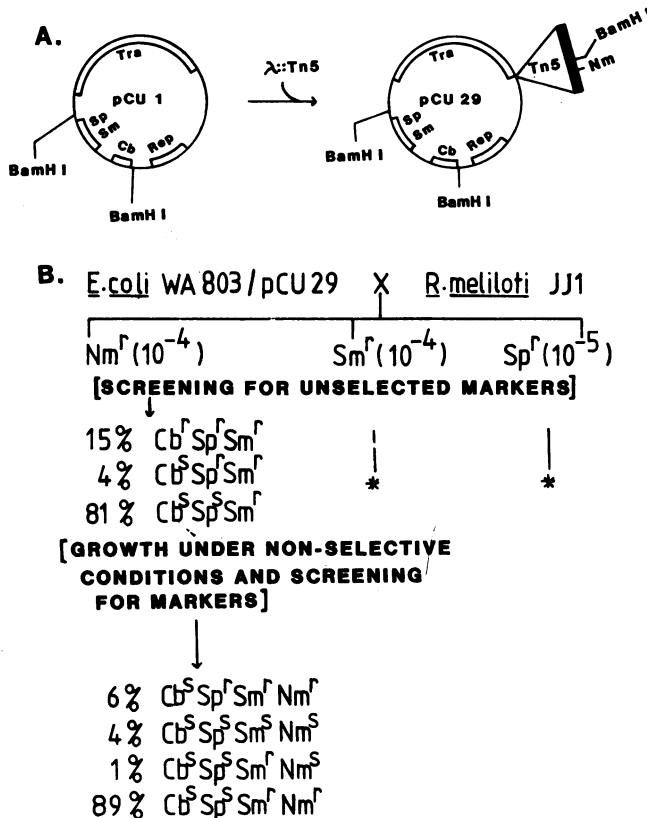
FIG. 1. (A) Derivation of an N group plasmid pCU29 carrying transposon Tn5 (42). Rep, replication and maintenance; Tra, conjugal transfer. (B) Transfer of pCU29-associated antibiotic resistance markers to *R. meliloti*. The matings were carried out on membrane filters. The transconjugants of *R. meliloti* from TY agar containing Rif and a selective antibiotic (Nm, Sm, or Sp) were screened for unselected markers by replica plating on TY agar containing an appropriate antibiotic. Patches of growth from TY-Nm plates were further streaked on TY agar (nonselective conditions) and one colony from each streak was examined for inheritance of the different plasmid markers. The asterisk indicates that transconjugants selected on TY-Rif-Sm or TY-Rif-Sp also underwent a segregation. It is likely that in the majority of the Nm^r Sm^r transconjugants, Sm resistance is due to the presence of *str*, a gene of transposon Tn5, conferring resistance to Sm in *Rhizobium* spp. (manuscript submitted).

was maintained when the transformants were grown in media containing Nm. These results indicate that N replicons are not suitable as suicide vehicles for *R. meliloti*, unlike *A. tumefaciens* for which an N group plasmid, RN3, has been reported to be suicidal (25).

Instability of p15A-replicons in *R. meliloti*. Plasmid p15A is a cryptic, nonconjugative plasmid of *E. coli* from which cloning vectors have been derived that are extensively used in this host (7). These plasmids have not been used in hosts other than *E. coli* and closely related bacteria, presumably owing to their limited host range. We wished to take advantage of their narrow host range in constructing a suicide plasmid for the delivery of transposons into *R. meliloti*. Initially, the instability of plasmid

pACYC177, a derivative of p15A, in this host was assessed. pACYC177 was introduced into *R. meliloti* JJ1 by transformation. Transformants of *R. meliloti* JJ1 could not be obtained with this plasmid (Fig. 2), in contrast to results with pRK248, an IncP group plasmid. When pACYC177 was fused to pRK248 by ligation of the linear molecules generated by *Bam*HI cleavage of pACYC177 and *Bgl*II cleavage of pRK248, a composite plasmid specifying the resistance phenotypes of both plasmids was obtained. This plasmid, pGS12, could be transformed into *R. meliloti* JJ1, and the transformants maintained the plasmid stably (Fig. 2). Restriction analysis of the plasmid confirmed the physical stability of the composite plasmid (data not shown). These results suggested that

p15A derivatives might be unstable in *R. meliloti*, unless fused to a stable replicon. Our unsuccessful attempts to transform *R. meliloti* with another p15A derivative, pACYC184, and the results of Ruvkun et al. (34), who could transfer a fusion derivative of pACYC184 and an IncP1 group plasmid into *R. meliloti*, provide additional evidence for the instability of p15A derivatives in *R. meliloti*. Therefore, pACYC184 and pACYC177 were chosen for the construction of suicide vehicles.

Construction of a p15A group plasmid with N transfer system and transposon Tn5. Although plasmids ranging in size from 9.6 to 56 kilobases (kb) could be introduced into *R. meliloti* by transformation (36), this procedure would be relatively less efficient than mating to derive strains carrying transposon insertions from a suicide vehicle. Transformation of *R. meliloti* strain JJ1 with pGS38, a derivative of pACYC184 carrying Tn5, yielded only three Nm^r transformants per 15 μg of DNA (10^{-10} per viable cell). Therefore, we developed a suicide vehicle that would also be conjugative. Recently, Thatte and Iyer (42) have cloned a region of the N group plasmid pCU1, specifying conjugal transfer (*tra* genes) into pACYC184. This region

of pCU1 does not include any transposon-like structures (23). It was reasoned that this recombinant plasmid pCU101 would be conjugative in interspecific matings between *E. coli* and *R. meliloti*, but unstable in the latter host. *E. coli* HB101(pCU101) was infected with λ cI857 b221 Pam Oam rex::Tn5 (37) and the resultant Nm-resistant colonies were mated en masse with *E. coli* PB3. The Nm-resistant transconjugants of PB3 were further tested for conjugal transmission of Nm^r and Cm^r (pCU101-associated marker). Plasmid DNA from one such derivative that transferred both Nm^r and Cm^r at a frequency of one (per donor cell) was analyzed by restriction analysis for the presence and location of the transposon. This plasmid was designated as pGS9 (Fig. 3). Its characteristics with respect to conjugal transfer and instability are shown in Table 3. These results show that the yield of Nm^r transconjugants of *R. meliloti* (potential Tn5 insertion derivatives) is much higher compared to Tn mutagenesis with the nonconjugative vehicle pGS38. Thus, in pGS9 we seem to have combined the advantageous features of pACYC184 and pCU1, namely replicon instability and transfer proficiency.

Protocol for insertion mutagenesis of *Rhizobium*

Plasmid	Transformants		Co-inheritance of unselected marker	
	Nm^r	Tc^r	Nm^r	Tc^r
pACYC 177	$<5 \times 10^{-2}$	–	–	–
pRK 248	$<2 \times 10^{-1}$	350	$<1\%$	–
pGS12	400	350	100%	100%

FIG. 2. Transformation of *R. meliloti* JJ1 with plasmid DNA. pACYC177 is a derivative of *E. coli* plasmid p15A (7), and pRK248 is a wide host range plasmid derived from the IncP1-group RK2 (19). The recombinant plasmid pGS12 was constructed by cleaving the parental plasmids with *Bgl*II and *Bam*HI and ligating the protruding complementary cohesive ends. The composite plasmid was maintained stably by *E. coli* and *R. meliloti* on media containing any one of the three antibiotics (Tc, Nm, or Cb). Transformants, The number of transformants are per microgram of plasmid DNA. Co-inheritance of unselected marker, The transformants were replica plated on TY agar containing an appropriate antibiotic.
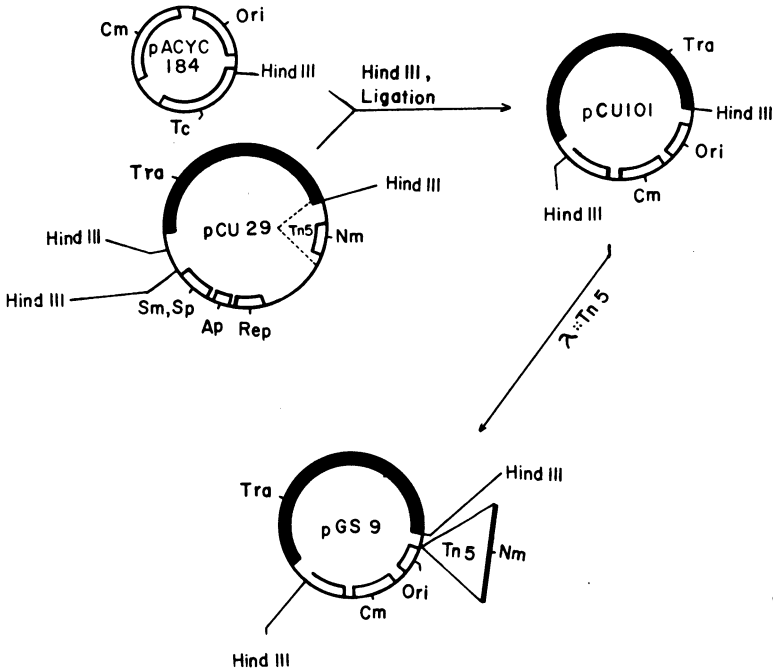
FIG. 3. Construction of a conjugative plasmid (pGS9) composed of p15A replicon, N transfer system, and transposon Tn5. The sizes of pGS9 and its parental plasmids as determined by restriction analysis are (kb): pACYC184, 4.0; pCU29, 44.2; pCU101, 24.5; and pGS9, 30.5. Ap, ampicillin; Ori, origin of replication; other symbols have been decribed in the legend to Fig. 1.

spp. with pGS9. The protocol recommended for the isolation of Tn5 insertion mutants of *R. meliloti* and *R. leguminosarum* is as follows. *E. coli* WA803(pGS9) is mated with the recipient at an equal cell density of donors and recipients (10^8 to 10^9 cells) on sterile membrane filters placed on TY agar. The optimal duration of mating varies with the recipient. For *R. meliloti* JJ1, this was 4 h, and for other strains it varied from 12 to 24 h. After mating, dilutions of cell suspensions in TY broth were plated on TY-Rif-Nm. The Nm-resistant colonies that arose after 4 to 5 days of incubation at 30°C were purified on plates of the same composition and screened for Cm resistance or sensitivity and auxotrophy. The latter screening was done on M9 minimal agar (27) by the method of Holliday (15).

Analysis of putative Tn5 insertions. 20 Nm^r colonies chosen at random were analyzed for plasmids and for conjugal transfer of Nm resistance. None of these displayed any plasmids other than their indigenous plasmids (ca. 500 and 200 kb) in Eckhardt-type gels (11). Transfer of Nm resistance to a tetracycline (Tc)-resistant derivative of *E. coli* WA803 could not be detected in spot matings. These results suggest that the suicide vehicle did not establish itself as an independent plasmid in these *R. meliloti* trans-

conjugants. However, of a total of 800 independent Nm^r colonies, 3% were Cm resistant, indicating the presence of pGS9 DNA sequences in addition to Tn5 in this class of transconjugants. Five randomly chosen Nm^r Cm^r colonies also

TABLE 3. Conjugal transfer of pGS9[a]

Recipient strain	Transfer frequency		Co-inheritance of unselected marker[b]	
	Nm	Cm	Nm (%)	Cm (%)
R. meliloti				
JJ1	4×10^{-3}	$<1 \times 10^{-7}$		3
GS1002	4×10^{-4}	$<1 \times 10^{-7}$		3
GS1003	1×10^{-4}	$<1 \times 10^{-7}$		2
E. coli GS500	1	1	100	100

[a] Matings between 10^8 cells of *E. coli* WA803(pGS9) and the recipients were done on membrane filters placed on TY or TYS agar at 30°C. The duration of matings was 2 h for GS500, 4 h for JJ1, and 12 to 16 h for GS1002 and GS1003. Transconjugants were sought on TY or TYS agar containing Rif and appropriate antibiotics.

[b] Transconjugants (100 to 800) were screened on TY agar containing the antibiotics.
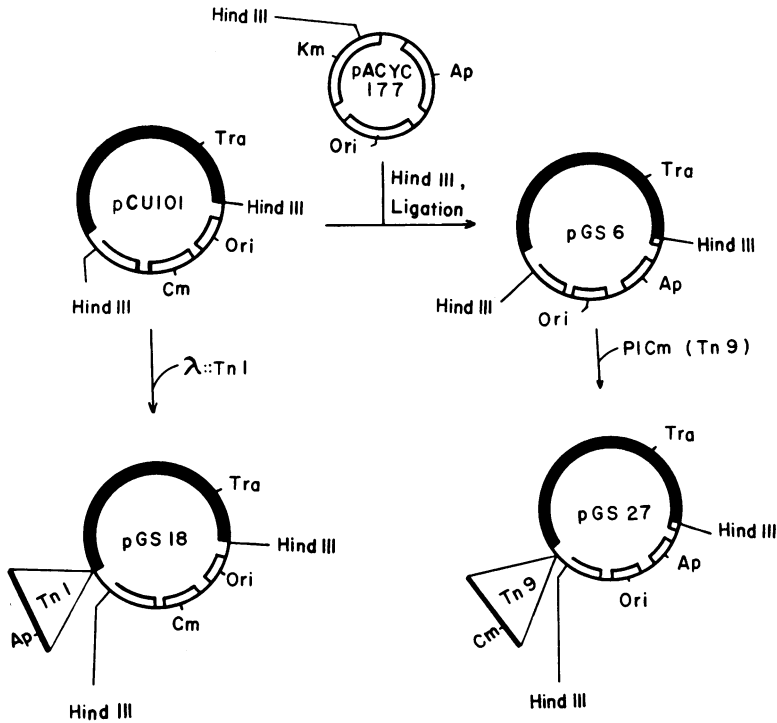
FIG. 4. Construction of self-transmissible suicide vehicles carrying Tn*1* (pGS18) and Tn*9* (pGS27). These plasmids are composed of p15A replicon and N transfer system. The addition of Tn*1* to plasmid pCU101 was done in a manner analogous to the construction of pGS9 as described in the text. pGS27 was constructed by mating P1Cm lysogens of *E. coli* HB101(pGS6) with *E. coli* CR34, followed by selection for Cm resistance (Tn9-associated marker). *E. coli* CR34 did not adsorb bacteriophage P1Cm (data not shown), and therefore, the Cm-resistant transconjugants arose owing to the transfer of pGS6 carrying Tn9. Plasmid DNA from one such transconjugant was characterized further and designated as pGS27. The sizes of pGS18, pGS27, and their parental plasmids as determined by restriction analysis are (kb): pGS18, 29.5; pGS27, 27.0; pACYC177, 3.7; pGS6, 24.2; and pCU101, 24.5. The symbols have been described in the legend to Fig. 3.

displayed the characteristics described above. It is likely that this rare class of transconjugants could have arisen by Tn*5*-promoted insertion of the entire plasmid (replicon fusion) into an indigenous genetic element of *R. meliloti*.

The remainder of the transconjugants (Nm^r Cm^s) were screened for auxotrophic mutations. About 3% of these colonies exhibited auxotrophy with different nutritional requirements (adenosine, methionine, glycine, glutamine, cysteine, serine, phenylalanine, histidine, and isoleucine-valine), suggesting that mutagenesis with pGS9 yields different mutations in *R. meliloti*. Spontaneous prototrophic revertants of these auxotrophs were sought. These should have given rise to Nm^s colonies when Tn*5* was inserted at the mutated site. The frequency of reversion was very low ($\leq 10^{-10}$). When revertants were isolated, they were of two types: those that were now Nm^s (five of six auxotrophs) and those that continued to be Nm^r (one of six). The appearance of Nm^s revertants in a

majority of the mutants that yielded revertants gave assurance that these mutations were indeed caused by Tn*5*. Since this analysis depended on the precise excision of Tn*5*, which is a rare event, it could not be extended to all of the Tn*5* insertion mutants. In one of the mutants (adenosine$^-$) that gave rise to Ade$^+$ Nm^r revertants, the reversion event could have occurred owing to activation of an alternative metabolic pathway. Alternatively, the *ade* mutation could have been caused by an indigenous insertion element similar to ISRm1 (33). Further assurance that no other part of the suicide vehicle was inserting independent of Tn*5* was obtained by colony hybridizing the Nm^r Cm^s transconjugants (including all auxotrophs) with ^{32}P-labeled probes of the parental plasmids of pGS9, pCU1, and pACYC184 (see legend to Fig. 3) that are devoid of Tn*5*. Under the experimental conditions employed, we estimate that we would have detected a sequence homology of ≥ 1 kb. Of 233 colonies thus screened, only 2 exhibited DNA

sequence homology to the probes. Thus, it appears that a very large fraction of the mutants isolated after mutagenesis with pGS9 arise by bona fide Tn*5* insertions. In summary, 97% of the Nm^r transconjugants of *R. meliloti* derived from a mating with *E. coli* carrying pGS9 were Cm^s, of which about 3% were auxotrophic mutants. Among these, >98% did not exhibit any sequence homology to the parental plasmids of the suicide vehicle.

We also found pGS9 to be effective for all of the three strains of *R. leguminosarum* (128C52, 128C53, and 175G16). Recently Simon et al. (R. Simon, G. Weber, W. Arnold, A. Puhler, Proc. VIII N. Am. *Rhizobium* Conf., University of Manitoba, Canada, p. 67–89, 1982) have described the construction of a suicide vehicle suitable for Tn*5* mutagenesis of *R. meliloti*. This plasmid, a derivative of pACYC184, is mobilizable into *R. meliloti* from an *E. coli* strain that carries RP4::Mu in its chromosome.

Suicide plasmids carrying Tn*1* and Tn*9*. It has been reported that Tn*5* exhibits some regional insertion specificity in *E. coli* (38) and in *R. meliloti* (12, 26). The proportion of different types of auxotrophic mutants of *R. meliloti* JJ1 and its derivatives after mutagenesis with pGS9 (for example, Ade$^-$, 20%; Ilv$^-$, 4%; His$^-$, 10%; Thr$^-$, 1%; and Ala$^-$, 1%) also support these observations. It is therefore clear that the use of Tn*5* alone would not saturate a given large segment of the genome of *R. meliloti*. It should be possible to overcome this limitation by insertion mutagenesis with other transposons such as Tn*1* and Tn*9*. Modelled after pGS9, we constructed pGS18 (Tn*1*) and pGS27 (Tn*9*) as shown in Fig. 4. Transposons Tn*1* and Tn*9* are well-characterized transposons that specify resistance to Cb and Cm, respectively (1, 14). The vectors carrying these transposons were also found to yield Tn*1* and Tn*9* insertion mutants in *R. meliloti* JJ1. A suicide vehicle similar to pGS9 but carrying the transposon Tn*10* (pGS16; construction not shown) was transmissible among *E. coli* strains at a frequency of ca. 10^{-2}. Assuming that the transfer frequency of the plasmid is the same in *E. coli* × *R. meliloti* JJ1 crosses (by analogy with pGS9), tetracycline resistant (Tc^r) insertions of *R. meliloti*, if they occurred, should have been detected. We did not observe them (<2 × 10^{-9}).

Other uses of suicide vehicles derived in this study. Tn*5* DNA has unique sites for *Sal*I and *Bam*HI (18). Cloning of DNA into these sites does not abolish the transposition proficiency of the resultant derivatives of Tn*5* or its Nm^r marker (unpublished data). By cloning exogenous DNA into these sites it should be possible to transpose these sequences to different targets in *R. meliloti* (and other bacteria). We have

constructed a derivative of Tn*5* that carries the *mob* (*ori*T) region of the wide host range plasmid, RK2. This transposon, Tn*5*-A1 was transposable in *R. meliloti*. Suicide vehicles carrying such modified transposons and similar vehicles described by Simon et al. (R. Simon, U. Priefer, and A. Puhler, Proc. Int. Symp. Mol. Genet. Bacteria-Plant Interactions, in press) should facilitate further genetic manipulations of *R. meliloti* and related organisms. The suicide vehicles described in this study are sufficiently small (ca. 30 kb) to permit their in vitro manipulations.

LITERATURE CITED

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