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Physical and Genetic Characterization of Symbiotic and Auxotrophic Mutants of *Rhizobium meliloti* Induced by Transposon Tn*5* Mutagenesis

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We have physically and genetically characterized 20 symbiotic and 20 auxotrophic mutants of *Rhizobium meliloti*, the nitrogen-fixing symbiont of alfalfa (*Medicago sativa*), isolated by transposon Tn*5* mutagenesis. A "suicide plasmid" mutagenesis procedure was used to generate Tn*5*-induced mutants, and both auxotrophic and symbiotic mutants were found at a frequency of 0.3% among strains containing random Tn*5* insertions. Two classes of symbiotic mutants were isolated: 4 of the 20 formed no nodules at all (Nod$^-$), and 16 formed nodules which failed to fix nitrogen (Fix$^-$). We used a combination of physical and genetic criteria to determine that in most cases the auxotrophic and symbiotic phenotypes could be correlated with the insertion of a single Tn*5* element. Once the Tn*5* element was inserted into the *R. meliloti* genome, the frequency of its transposition to a new site was approximately 10^{-8} and the frequency of precise excision was less than 10^{-9}. In approximately 25% of the mutant strains, phage Mu DNA sequences, which originated from the suicide plasmid used to generate the Tn*5* transpositions, were also found in the *R. meliloti* genome contiguous with Tn*5*. These latter strains exhibited anomalous conjugation properties, and therefore we could not correlate the symbiotic phenotype with a Tn*5* insertion. In general, we found that both physical and genetic tests were required to fully characterize transposon-induced mutations.

Van Vliet et al. (21) and Beringer et al. (2) have recently described a general method for introducing transposons into the genomes of a wide variety of gram-negative bacteria. A broad host-range plasmid carrying both phage Mu and a transposon is conjugated from *Escherichia coli* into the recipient gram-negative bacterium. Many nonenteric recipients are not killed by zygotic induction of prophage Mu. Instead, the Mu-containing plasmid fails to replicate stably, thus permitting direct selection for the transposition of the transposon from the Mu-containing "suicide plasmid" to a recipient genome. Using this procedure, Beringer et al. (2) obtained and genetically characterized a variety of auxotrophic and symbiotic mutants of *Rhizobium leguminosarum*, *R. trifolii*, and *R. phaseoli* caused by the insertion of the kanamycin/neomycin resistance-conferring transposon Tn*5*. This technique has also recently been successfully used by Duncan (6) to isolate carbohydrate metabolism mutants of *R. meliloti* L5-30 and by Rolfe et al. (16, 17) to obtain symbiotic mutants of *R. trifolii* strains SU329 and SU843.

We have adopted the Tn*5* suicide plasmid technique of Beringer et al. (2) to isolate neomycin-resistant mutants of *R. meliloti* which are defective in nodulation or nitrogen fixation or both, or which are auxotrophic. In this paper we describe the physical analysis of these mutants by using the Southern (20) gel transfer and hybridization technique. In combination with classical genetic analysis, the physical analysis demonstrates that some, but not all, of the symbiotic and auxotrophic mutant phenotypes can be correlated with a newly acquired transposon insertion. In approximately 25% of the cases, phage Mu sequences were found to transpose in concert with Tn*5*, and in other cases, the mutant phenotypes could not be correlated with a transposon insertion event.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in (but not constructed during the course of) this study are listed in Table 1. Some of the auxotrophic and symbiotically defective mutants of *R. meliloti* isolated during the course of this study are listed in Table 2. *R. meliloti* 1021, the symbiotic effective parent strain in which Tn*5* transpositions

TABLE 1. Bacterial strains and plasmids

Strain	Genotype	Relevant phenotype	Source or reference
E. coli			
1830	*nal pro met* (pJB4JI)	Str^{r} Ge^{r}, pJB4JI donor	(2)
W3115	*thi* (pGM102)	pGM102 donor	This paper
EC102	*thi pro leu arg hspR hspM rif*	Rif^{r}	This paper
R. meliloti:			
2011	Wild type	Prototrophic, symbiotically effective	J. Denarie
1021	*str*	Streptomycin-resistant derivative of 2011	This paper
3359	*trp his pyr rif str spc nov*	Same as genotype	(12)
3390	*trp his pan rif str spc nov*	Same as genotype	(12)
1408	*trp cys str spc rif*	Same as genotype	This paper
Plasmids			
pJB4JI	pPH1::Mu::Tn5	Tn5 donor	(2)
pHM5	pJC307::Tn5	Source of Tn5 DNA	H. Meade
pGM102	*tet bla*	RP4 derivative which does not confer Nm^{r}	J. Denarie
pHM4021	pGM102::Tn5	Carries Tn5	This paper
pHM4022	pGM102*tet*::Tn5	Does not confer Tc^{r}	This paper

were isolated, is a spontaneous streptomycin-resistant mutant of *R. meliloti* 2011.

Media. LB medium is 1% tryptone (Difco Laboratories, Detroit, Mich.), 0.5% yeast extract, and 0.5% NaCl (pH 7.2). The minimal basal salts medium (M9) used for the screening of *R. meliloti* auxotrophs has been described (13). LB and M9 media were supplemented with neomycin (Nm) (50 μg/ml), streptomycin (Sm) (500 μg/ml), novobiocin (Nov) (50 μg/ml), spectinomycin (Spc) (50 μg/ml), rifamycin (Rif) (100 μg/ml), gentamicin (Gm) (50 μg/ml), or tetracycline (Tc) (10 μg/ml), or any combination of these, when appropriate. LB and M9 media were solidified with 1.5% agar (Difco).

Genetic techniques. Bacterial conjugations were performed as described (12), except that pGM102 was used to mobilize the donor *R. meliloti* chromosome instead of RP4. Derivatives of *R. meliloti* 1021 which contained Tn5 transpositions and which failed to grow on unsupplemented M9 medium (see text) were screened by the method of Holliday (9) on M9 medium to identify specific auxotrophic requirements. The reversion frequencies of presumptive Tn5-induced auxotrophs were determined by growing cells in LB to saturation (approximately 5 × 10^{9} cells per ml), washing, concentrating 10-fold, and then plating on minimal medium (M9).

Nodulation and nitrogenase assays. *R. meliloti* strains with Tn5 insertions were screened for symbiotic phenotype by inoculation onto alfalfa seedlings. Plants were prepared by sterilizing seeds of alfalfa (variety "Iroquois") in ethanol and sodium hypochlorite, soaking in water overnight, and planting on nitrogen-free agar (22) slants in 18-by-150-mm tubes. Individual Tn5-containing strains were suspended in sterile water and added to duplicate tubes containing 1-week-old germinated seedlings; approximately 1 ml of suspension, containing at least 10^{6} bacteria, was added to each tube. Inoculated plants were grown for 4 to 6 weeks in a growth chamber (25°C, 16-h light at 200 ft-c [ca. 2,150 lx]). They were examined for the presence or absence of nodules and for symptoms of nitrogen starvation, which indicate ineffective symbiosis. Nitrogenase activity was measured in the nodules by capping each tube with a serum stopper, injecting 1 ml of acetylene, and withdrawing a 0.5-ml sample from the tube after 12 h to measure reduction of acetylene to ethylene by gas chromatographic analysis (8).

Isolation of DNA. Total DNA was isolated from *R. meliloti* strains according to the following procedure. A 5-ml saturated culture grown in LB at 30°C was suspended in 25 ml of 1.0 M NaCl, shaken on a wrist-action shaker for 1 h at 4°C, suspended in 25 ml of cold TES buffer (Tris, 0.01 M, pH 8.0; 0.025 M EDTA; 0.15 M NaCl), centrifuged, and suspended in 5 ml of cold TE buffer (TES without NaCl). A 0.5-ml sample of lysozyme solution (2 mg of lsozyme per ml in TE) was added to the resuspended cells. After 15 min of

TABLE 2. Mutants obtained by Tn5 mutagenesis

Strain[a]	Phenotype	Genotype	Reversion frequency
1023	Met^{-} Nm^{r}	*met-1023*::Tn5 *str*	2.8 × 10^{-8}
1033	Gly^{-} Nm^{r}	*gly-1033* (Tn5)[b] *str*	10^{-10}
1102	Arg^{-} Nm^{r}	*arg-1102*::Tn5 *str*	10^{-10}
1126	Nod^{-} Nm^{r}	*nod-1126*::Mu Tn5 *str*	NT[c]
1029	Nod^{+} Fix^{-} Nm^{r}	*fix-1029*::Tn5 *str*	NT[c]

[a] All strains are derived from *R. meliloti* 1021.

[b] Tn5 and Gly^{-} not linked; see Table 3.

[c] NT, Not tested. Revertants cannot be selected directly.

incubation at 37°C, 0.6 ml of a Sarkosyl-pronase (10% Sarkosyl and 5-mg/ml pronase in TE) solution was added, and the mixture was incubated for an additional 1 h at 37°C. The lysed cells were extracted at least twice with 5 ml of phenol (saturated with 0.01 M Tris, pH 8.0) and then with 5 ml of chloroform. The aqueous phase was brought to 0.3 M ammonium acetate, and the DNA was precipitated with 0.54 volumes of isopropanol at room temperature. The precipitated DNA was removed with a glass rod and then dissolved in 2 ml of Tris (0.01 M, pH 8.0)-EDTA (0.001 M).

Phage Mu DNA was a gift from G. Riedel. Supercoiled plasmid DNA was prepared by the cleared lysate technique followed by ethidium bromide-cesium chloride equilibrium centrifugation (5).

Restriction endonucleases. Restriction endonuclease *Eco*RI was purified according to the procedure of P. Myers, Cold Spring Harbor Laboratories (personal communication). *Xho*I was purchased from Bethesda Research Laboratories, Rockville, Md., and used according to the manufacturer's specifications.

Southern hybridizations. Restriction endonuclease-digested DNAs were subjected to agarose gel electrophoresis as described (14) and transferred to nitrocellulose as described by Southern (20) with modifications by Botchan et al. (3). Purified DNAs were labeled with ^{32}P by the nick translation method (11, 15) as described (14). To remove hybridized DNA, nitrocellulose filters were washed at room temperature for 15 min in 0.02 M NaOH and then neutralized with 0.05 M Tris-hydrochloride, pH 7.0.

RESULTS

Transposon Tn*5* confers neomycin resistance to *R. meliloti*. Transposon Tn*5* (1) confers neomycin and kanamycin resistance and was chosen for these studies because the *R. meliloti* strains we study are naturally sensitive to relatively low levels of neomycin (20 μg/ml) and because Tn*5* exhibits little site specificity and in general causes polar mutations (19). To utilize Tn*5* to generate insertion mutations in *R. meliloti*, it was first necessary to establish that Tn*5* confers neomycin/kanamycin resistance to *R. meliloti*.

E. coli W3115 carrying plasmid pHM4021 (Tc^r; carrying Tn*5*) was conjugated with *R. meliloti* 1021 (Sm^r). Tc^r Sm^r *R. meliloti* exconjugants were selected and then tested for resistance to neomycin. All Tc^r exconjugants tested were resistant to 100 μg of neomycin per ml. In comparison, growth of strain 1021 is inhibited by 20 μg of neomycin per ml, at which level spontaneous resistant mutants arise at a frequency of 10^{-7}. pHM4021 replicates stably in *R. meliloti* 1021 and can be conjugated from this strain to appropriate *E. coli* recipients at a frequency of 10^{-4} per 1021 donor.

Suicide plasmid mutagenesis of *R. meliloti*. To obtain Tn*5* transpositions to *R. meliloti* replicons (chromosome and indigenous plasmid[s]) we adopted the suicide plasmid techniques described by Van Vliet et al. (21) and Beringer et al. (2) to mutagenize *Agrobacterium tumefaciens* and *R. leguminosarum*, respectively. In particular, we used the P-type plasmid pJB4JI, constructed by Beringer et al. (2), which confers gentamicin resistance and which carries Tn*5* inserted into prophage Mu. Because plasmid pJB4JI does not replicate when transferred to *R. meliloti*, neomycin-resistant exconjugants should contain Tn*5* transpositions to the *Rhizobium* genome.

E. coli 1830 (containing pJB4JI) was conjugated with *R. meliloti* 1021 (see Materials and Methods for details), and Nm^r Sm^r exconjugants were obtained at frequencies ranging from 10^{-6} to 2×10^{-5} in different experiments. On the average, 99% of the Nm^r exconjugants were sensitive to gentamicin, and these were candidates for strains containing Tn*5* transpositions to *R. meliloti* replicons.

Physical analysis of presumptive Tn*5* transposition strains for phage Mu DNA sequences. Utilizing the Southern gel transfer and hybridization procedure (20), we constructed a partial restriction map of pJB4JI surrounding the site of Tn*5* insertion and corroborated genetic evidence presented by Beringer et al. (2) that Tn*5* is inserted into prophage Mu (data not shown). If, after transfer of pJB4JI to *R. meliloti*, Nm^r Gm^s exconjugants were due to the transposition of Tn*5* to *R. meliloti* replicons and the subsequent loss of pJB4JI, then Nm^r exconjugants should not contain Mu DNA sequences. To test this prediction, we isolated total DNA from 24 Nm^r Gm^s exconjugants selected at random, digested the DNAs with the restriction endonuclease *Xho*I, and hybridized the restricted DNAs with ^{32}P-labeled Mu DNA by the Southern blotting and hybridization procedure. Eleven of the 24 DNAs contained significant amounts of Mu DNA sequences as illustrated in Fig. 1A, which shows strong hybridization to six of nine tested DNAs. (The weak hybridization seen in lane 6 may not be due to Mu sequences because this strain did not contain Tn*5*; see discussion of Fig. 1B below.) Examination of this figure reveals that among the strains containing Mu sequences, different-sized DNA fragments containing Mu sequences were present. When the ^{32}P-labeled Mu DNA was melted off the filter (see Materials and Methods for details) and the filter was rehybridized with ^{32}P-labeled Tn*5* DNA, it was found in the case of each strain containing Mu DNA that Mu DNA and Tn*5* DNA hybridized to at least one DNA fragment in common (Fig. 1B). In the five other cases (not shown) in which Mu DNA was found in the Nm^r Gm^s exconjugants, both Mu DNA and Tn*5* also hybridized to at least one DNA fragment in common. These results indicate that in those strains containing Mu sequences, Tn*5* and Mu sequences are still contiguous.

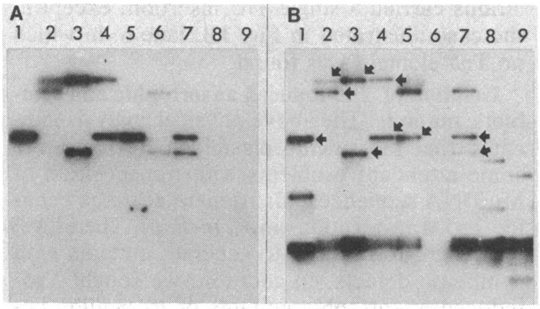

FIG. 1. Cotransposition of phage Mu and Tn*5* sequences from the suicide plasmid to the *R. meliloti* genome. Total DNA was isolated from *R. meliloti* strains chosen at random from neomycin-resistant, gentamicin-sensitive exconjugants of a suicide plasmid mating between *E. coli* 1830(pJB4JI) and *R. meliloti* 1021. Purified DNA was digested with *Xho*I, and the resulting fragments were subjected to agarose gel electrophoresis and transfer to nitrocellulose sheets as described in the text. The filter was successively hybridized with ^{32}P-labeled Mu and then ^{32}P-labeled ColE1::Tn*5* DNA. Each lane contains DNA from a different presumptive Tn*5*-containing strain. (A) Autoradiogram of the filter hybridized with ^{32}P-labeled Mu DNA. (B) Autoradiogram of the same nitrocellulose sheet, washed with NaOH (see Methods) and rehybridized with ^{32}P-labeled ColE1::Tn*5* DNA. Before rehybridization, an autoradiogram of the sheet was made to verify that the Mu^{32}P-DNA had been removed (data not shown). One strain (lane 6) does not appear to contain Tn*5* (see Fig. 2 and 3) and is probably a spontaneous Nm^r mutant. The remaining strains (except lane 1) contain two internal *Xho*I fragments of Tn*5* (labeled A and B in Fig. 2). Lane 1 appears to contain a deleted form of Tn*5*. Lanes 1 through 5 and 7 contain at least one band which also hybridized to Mu DNA; these bands are indicated with small arrows. Lane 7 contains an "extra" boundary fragment (see Fig. 2 and text) between Tn*5* and adjacent DNA sequences which cannot be readily explained.

The 11 Mu-containing strains were analyzed for the presence of plasmids by using a rapid screening method (7), and in all cases no plasmids could be detected, in contrast to strain 1021(RP4), in which RP4 was readily observed. This result indicates that these strains did not arise as the result of deletions of Mu and gentamicin resistance genes to produce derivatives of pJB4JI capable of replication in *R. meliloti*.

One of the 24 Nm^r exconjugant DNAs tested in this experiment did not appear to contain Tn*5* (Fig. 1B, lane 6). This strain is probably a spontaneous Nm^r mutant. Examination of lane 6 in Fig. 1A reveals weak hybridization to the Mu DNA probe. This latter result is difficult to explain although it may be due to the transposition of a very limited amount of Mu DNA from the suicide plasmid, independent of Tn*5* transposition.

The results presented in this section indicate that in the 11 Mu-containing strains one of two events had occurred: (i) concerted transpositions of Tn*5* with Mu sequences, or (ii) replicon fusion of a partially deleted pJB4JI plasmid with the *R. meliloti* genome. If Mu and Tn*5* are transposing together in these strains, different Mu sequences appear to have transposed in different strains because we could not detect any obvious pattern in the particular Mu sequences which did transpose.

The results presented in this section also indicate that approximately half of the presumptive Tn*5* transposition strains contain no detectable Mu sequences and probably contain genuine Tn*5* transpositions. The strains carrying bona fide Tn*5* insertions are examined in more detail in the next section.

Physical analysis of presumptive bona fide Tn*5* transpositions. In the experiment described in the previous section, 12 of 24 Nm^r Gm^s *R. meliloti* exconjugants from a cross between *E. coli* 1830(pJB4JI) and *R. meliloti* 1021 did not contain Mu DNA sequences and were candidates for bona fide Tn*5* transpositions. If Tn*5* transposed from pJB4JI to new locations on the *R. meliloti* genome, it should have acquired new restriction sites on both sides which are different in independent transposition strains. Therefore, the DNA sequences surrounding Tn*5* in 12 strains with symbiotic defects were characterized using the Southern gel transfer and hybridization method. The strategy employed in this experiment is illustrated in Fig. 2.

Because Tn*5* (5.7 kilobases) contains no recognition site for *Eco*RI (10), an *Eco*RI digest of total cellular DNA from a strain carrying a single Tn*5* element should contain a single *Eco*RI fragment that will hybridize with a ^{32}P-labeled Tn*5* DNA probe. Figure 3A shows an autoradiogram

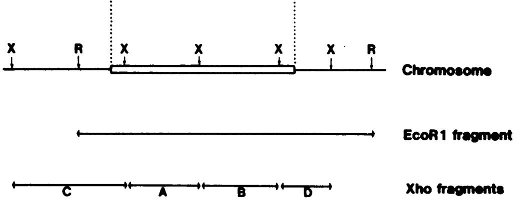

FIG. 2. Representational map of Tn*5* and region of insertion. Sites for the restriction enzymes *Eco*RI (R) and *Xho*I (X) are shown for Tn*5* and for a hypothetical genomic site where Tn*5* has inserted. Cleavage of total DNA with *Eco*RI will yield one fragment containing Tn*5* for each independent insertion. Digestion with *Xho*I will produce for each insertion two fragments internal to Tn*5* (A and B) and two border fragments (C and D) containing an end of Tn*5* and part of the adjacent genome.

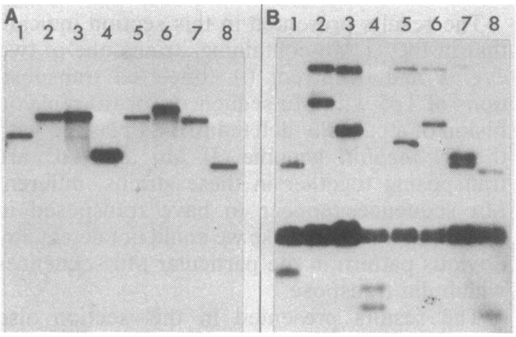

FIG. 3. Restriction and hybridization analysis of nine non-Mu-containing strains with Tn*5* inserts demonstrating a single Tn*5* insertion. (A) Hybridization of ^{32}P-labeled ColE1::Tn*5* to *Eco*RI digests of DNA from mutant strains. See the text and legend to Fig. 1 for details. (B) Hybridization of labeled Tn*5* to *Xho*I digests of DNA from the same mutant strains. Each strain contains a doublet of internal fragments (designated A and B in Fig. 2). A different set of border fragments (C and D in Fig. 2) is apparent in each strain. In lane 7, an extra band is visible which is most likely due to a partial digest.

of a Southern filter blot of eight *Eco*RI-digested DNAs from presumptive bona fide Tn*5* transposition strains hybridized with ^{32}P-labeled ColE1::Tn*5* DNA. Because each lane appears to contain a single hybridization band, it is likely that each strain carries a single Tn*5* element and that the Tn*5* element is inserted into different *Eco*RI fragments in different strains.

To corroborate and extend the results obtained with *Eco*RI, total cellular DNA from the same Tn*5* transposition strains shown in Fig. 3A was cleaved with *Xho*I. *Xho*I cleaves Tn*5* three times, once near the center and once within each of the terminally repeated DNA sequences at the ends of the element (10). This results in four fragments containing Tn*5* sequences. Two of the fragments (labeled A and B in Fig. 2) are internal to Tn*5* and will be the same wherever Tn*5* is inserted. The other two fragments contain two identical 0.5-kilobase Tn*5* segments attached to the "left" and "right" portions of the particular *Xho*I fragment in which the Tn*5* is inserted. These two boundary fragments (C and D in Fig. 2) will vary in size, depending on the size of the particular *Xho*I fragment into which Tn*5* is inserted and on the location of Tn*5* within this fragment, and are likely to be different for each independent Tn*5* insertion. Comparison of the hybridization patterns in the different lanes in Fig. 3B clearly shows that Tn*5* is inserted at different locations in the eight different strains. Using the hybridization procedure described in the legend of Fig. 3, we have examined a total of 80 independent presumptive Tn*5* insertion strains, and in all cases we concluded that the strains carried a single Tn*5* insertion, except in the case illustrated in Fig. 1B, lane 6, in which no Tn*5* element was found.

Isolation of Tn*5*-induced auxotrophic and symbiotic mutants. The above physical analysis indicated that the suicide plasmid pJB4JI, despite some attendant problems with transposition of Mu DNA sequences, also generated some genuine Tn*5* transpositions in *R. meliloti*. Therefore, we sought to use Tn*5* to generate mutants with symbiotic defects. In addition, we sought Tn*5*-induced auxotrophic mutants of *R. meliloti* because their more precisely defined phenotype would allow us to answer some technical questions about the Tn*5* mutagenesis procedure as described below.

The procedure outlined above was used to obtain Tn*5* transpositions in *R. meliloti* 1021; a total of 6,000 Nm^r exconjugants were purified by streaking for single colonies on LB-SmNm agar and were then test-streaked for residual gentamicin resistance (on LB-Gm agar) and auxotrophy (on M9-Nm agar). To identify Tn*5*-induced symbiotic mutants, the Nm^r Gm^s prototrophic exconjugants (approximately 5,900) were screened on individual alfalfa plants (see Materials and Methods).

Of the 6,000 presumptive Tn*5*-containing strains tested, 20 were auxotrophs, a frequency of 0.3%. Four of the auxotrophs were Met$^-$, five were deficient in more general sulfur metabolism functions (supplemented by thiosulfate or cysteine, or both, as well as methionine), two were Arg$^-$, and the other nine were deficient in different functions. The distributions of auxotrophs may indicate nonrandomness of Tn*5* insertion or may reflect a high number of genes involving sulfur metabolism in *R. meliloti*; this is currently being investigated further. The properties of a few of the auxotrophs are listed in Table 2.

We also found at least 50 symbiotic mutants among the 6,000 presumptive Tn*5* insertions. The symbiotic mutants were grouped into two categories, Nod$^-$ and Fix$^-$: in the first case nodules were not formed (although in some cases some abnormal swellings or other root reactions seemed to occur); the Fix$^-$ phenotype is defined by the formation of nodules which fail to reduce acetylene. In both cases, nitrogen starvation symptoms in the host plant were apparent. The number of Nod$^-$ mutants found was 4 (0.07%); the number of Fix$^-$ mutants found was 46 (0.08%).

The 50 symbiotic mutants were subjected to a series of genetic and physical tests which are described in detail below. On the basis of these tests, the mutations causing the lesions in the 4 Nod$^-$ and 16 of the 46 Fix$^-$ mutants appear to correlate with the insertion of Tn*5*. Although the remaining 30 Fix$^-$ mutants contained a single Tn5 insertion, we were able to demonstrate that the Fix$^-$ phenotype of these strains was not due to the insertion of Tn5 but rather to the site-specific integration of an endogenous *R. meliloti* insertion sequence into a cluster of essential "Fix" genes closely linked to the structural genes for nitrogenase. We were able to demonstrate the existence of this endogenous *R. meliloti* insertion sequence because of the previous cloning of the *R. meliloti* structural genes for nitrogenase (18). Characterization of this endogenous insertion sequence and the Fix$^-$ mutations caused by its site-specific transposition will be the subject of a separate publication. The properties of a representative Fix$^-$ mutant and a representative Nod$^-$ mutant are listed in Table 3.

The 20 auxotrophic, 4 Nod$^-$, and 16 Fix$^-$ mutants which, in our initial screen, did not correlate with the transposition of an endogenous insertion sequence were tested for the presence of Mu DNA sequences by using the procedures described for Fig. 1. Three of the auxotrophs, two of the Nod$^-$ mutants, and three of the Fix$^-$ mutants contained residual Mu DNA sequences, a significant proportion, although not as high as in the Nm^r exconjugants as a whole. Hybridization experiments similar to those illustrated in Fig. 1 indicated that the residual Mu sequences in these strains are still contiguous with Tn5 (data not shown).

All 40 of these auxotrophic and symbiotic mutants were also tested for the number of Tn5 elements present in the genome of each strain by using the hybridization procedures outlined in Fig. 3. All 40 mutants contained a single Tn5 element (data not shown).

Genetic linkage of Tn5 to auxotrophic and symbiotic mutations. To verify that the insertion of Tn5 was indeed the cause of the symbiotic and auxotrophic mutations, we determined the linkage of the Tn5-conferred neomycin resistance phenotype to the auxotrophic or symbiotic phenotype. This was done by conjugating the mutant Tn5-containing strains with appropriate recipient strains, selecting or screening for Nm^r exconjugants, and then testing these for auxotrophy or symbiotic deficiency, according to the donor's mutant phenotype. In these experiments, genome mobilization was mediated by the promiscuous P-type plasmid, pGM102. The results of some of these experiments are listed in Table 3. It was found that in all but one case transfer of Nm^r to the recipient strain was accompanied by transfer of the auxotrophic or symbiotic phenotype. This was found both when Nm^r exconjugants were selected directly and when they were obtained by first selecting another marker and then screening for Nm^r. This establishes that the Tn5 insert and the mutant phenotype are at least in close genetic linkage and suggests that the Tn5 insertion is the cause of mutation. In one case (*R. meliloti* 1033 Gly$^-$), however, the Nm^r exconjugants were not Gly$^-$. Moreover, in those cases in which the donor strains contained Mu DNA sequences, no Nm^r exconjugants were recovered. At present, we have no explanation for this finding. This points out the desirability of conducting thorough genetic tests on putative Tn5-caused mutations before concluding that the mutant phenotype is caused by the transposon insertion.

Genetic characterization of Tn5 insertions. To determine whether Tn5 introduced into the *R. meliloti* genome via the suicide plasmid pJB4JI could function subsequently as a transposon in *R. meliloti*, we tested the ability of several bona fide Tn5 elements in *R. meliloti* to excise precisely and to transpose to new locations. In *E. coli*, precise excision occurs at frequencies ranging from 10^{-5} to 10^{-6} (1), and transposition occurs at frequencies of 10^{-3} (1).

We tested for precise excision by selecting for prototrophic revertants of the 20 Tn5-induced *R. meliloti* auxotrophic mutants. The reversion fre-

TABLE 3. Genetic linkage of Tn5 with auxotrophic or symbiotic phenotypes

Donor[a]	Relevant donor phenotype	Recipient[a]	Selected phenotype[d]	% of selected colonies which were Nm^r [b]	% of Nm^r exconjugants with donor phenotype[c]
1023(pGM102)	Met$^-$	3390	Pan$^+$	74	100
1023(pGM102)	Met$^-$	3359	Nm^r	100	99
1033(pGM102)	Gly$^-$	3390	Pan$^+$	12	0
1033(pGM102)	Gly$^-$	3359	Nm^r	100	0
1102(pGM102)	Arg$^-$	3359	Trp$^+$	1	100
1102(pGM102)	Arg$^-$	3359	Nm^r	100	100
1029(pGM102)	Nod$^+$ Fix$^-$	3390	Pan$^+$	60	NT
1029(pGM102)	Nod$^+$ Fix$^-$	1048	Nm^r	100	100

[a] All strains were *R. meliloti*.

[b] At least 96 were tested in each case.

[c] All Nm^r exconjugants were tested. NT, Not tested.

[d] Pan$^+$ and Trp$^+$ are growth without pantothenate and tryptophan, respectively.

quencies ranged from 10^{-8} to less than 10^{-10}, and in the case of all strains except one, all of the revertants obtained (if any) were still Nm^r. Analysis of several revertants by the Southern transfer and hybridization technique showed complex patterns of DNA rearrangements and deletions in different revertants (data not shown). We concluded from these results that when Tn*5* transposes from pJB4JI to the *R. meliloti* genome, subsequent precise excision of Tn*5* does not occur or is extremely rare. (A detailed study of prototrophic revertants of Tn*5*-induced auxotrophs will be published separately.)

In contrast to the failure to obtain precise excision of Tn*5* when the original Tn*5* transposition event occurred in *R. meliloti*, when Tn*5* was inserted into the *tet* gene of pGM102 when resident in *E. coli* and the pGM102*tet*::Tn*5* (pHM4022) plasmid was subsequently transferred to *R. meliloti*, precise excision, as measured by the frequency of Tc^r colonies, occurred at a frequency of 10^{-7}. This result may indicate that Tn*5* transposition events in *R. meliloti* are fundamentally different from those in *E. coli*.

We also tested the transposition frequency of Tn*5* from a locus in the *R. meliloti* genome to plasmid pGM102. *R. meliloti* 1023(pGM102) (Met::Tn*5*) was conjugated with *E. coli* EC102. The ratio of Nm^r Tc^r *E. coli* exconjugants to Tc^r exconjugants was 2×10^{-8}, indicating a transposition frequency of Tn*5* to pGM102 of approximately 10^{-8} in *R. meliloti*. This rate of transposition is four to five orders of magnitude less than that observed in *E. coli* (1). The apparent low rate of transposition of Tn*5* from a locus in the *R. meliloti* genome is consistent with our finding that among approximately 100 *R. meliloti* strains containing Tn*5* that we tested, each one contained a single Tn*5* element.

The results reported in this section indicate that Tn*5* mutagenesis in *R. meliloti* mediated by the suicide plasmid pJB4JI results in very stable mutants which should prove useful in many genetic manipulations.

DISCUSSION

We have successfully used a suicide plasmid technique to isolate a series of Tn*5*-induced symbiotic and auxotrophic mutants of *R. meliloti* Rm1021. This was accomplished by screening individual clones of *R. meliloti* containing presumptive Tn*5* transpositions on individual alfalfa plants or on appropriate media. However, physical analysis of the genomes of these presumptive mutants revealed that a significant fraction of the mutants were not the result of simple Tn*5* transposition events. In one class of mutants, representing 20% of those tested, Tn*5* and phage Mu sequences appear to have transposed in a concerted fashion from the suicide plasmid to the *R. meliloti* genome. Another class of mutants, representing 65% of the Fix$^-$ mutants tested (not discussed in this paper), are the result of the transposition of an endogenous *R. meliloti* insertion sequence into symbiotic genes. We were able to distinguish these latter two classes of mutants from genuine Tn*5* transposition mutants by using both genetic and physical tests, neither of which alone would have been sufficient for fully characterizing the mutants.

The suicide plasmid we used (pJB4JI) to generate Tn*5* transpositions carries Tn*5* inserted into prophage Mu. It is possible that the location of Tn*5* inside of Mu is responsible for the concerted transposition of Mu and Tn*5* sequences, and that a Mu-containing suicide plasmid in which Tn*5* is inserted outside of Mu may eliminate the cotransposition events. Also, it may be that the behavior of Mu depends on the recipient strain into which the suicide plasmid is conjugated.

The results presented in this paper indicate that once Tn*5* has transposed in *R. meliloti* it does not readily transpose to a new location, nor does is excise precisely. These defects could be due to the fact that a Tn*5*-encoded transposition repressor is more effective in *R. meliloti* than in *E. coli* and that initial transposition events occur in a repressor-free cytoplasm analogous to zygotic induction. Alternatively, these defects could be due to the lack of host-specific transposition factors(s) in *R. meliloti* which are supplied in trans by the suicide plasmid vector or by prophage Mu. This latter possibility is unlikely because a plasmid containing a ColE1 replicon, the *tra* genes of RP4, and Tn*5* can be conjugated into *R. meliloti* and used to generate Tn*5* transpositions at a comparable frequency to pJB4JI (H. Meade, unpublished data). A missing transposition factor in *R. meliloti* could result in a structural defect in Tn*5* elements which had transposed within *R. meliloti* such that subsequent transposition and excision events were impaired. A different type of explanation for the transposition defects of Tn*5* in *R. meliloti* is that a host factor essential for an early step in transposition is missing in *R. meliloti*, resulting in a situation in which early transposition events occur in *E. coli*, a transposition intermediate survives conjugation into *R. meliloti*, and the transposition event is completed in *R. meliloti*. This latter model would allow transposition events to occur just once in the primary *R. meliloti* exconjugant cells.

One major consequence of the cotransposition of Mu + Tn*5* sequences was that Mu-containing strains could not be used as Tn*5* donors in conjugation experiments because no Nm^r exconjugants could be obtained. In fact, before the physical analysis was performed indicating the presence of Mu sequences, these strains were considered to be an anomalous class, resistant to genetic analysis. Explanations for the failure to obtain Nm^r exconjugants from these strains include the possibilities that Mu sequences are not readily mobilized or that they kill the recipient cells.

Another disadvantage resulting from cotransposition of Mu + Tn5 is that the mutant gene containing the Mu-Tn5 transposition cannot be readily cloned. In the case of bona fide Tn5 transpositions, the *Eco*RI fragment into which Tn5 has inserted can be easily cloned in *E. coli* because Tn5 does not contain an *Eco*RI site and because Tn5-containing fragments can be selected for directly. Mu, however, contains two *Eco*RI sites, and when Tn5 and Mu have transposed together, the *Eco*RI fragment containing Tn5 may not contain host DNA flanking Tn5. This prevents use of a method we have recently developed for the replacement of a wild-type gene in *R. meliloti* with a homologous cloned DNA fragment containing Tn5 (18), whose success depends on cloned host sequences flanking both sides of Tn5.

It appears that pJB4JI behaves differently in various species of *Rhizobium*. For example, when three strains of *R. trifolii* were used as recipients for pJB4JI, one yielded no Tn5-containing exconjugants, whereas Tn5-containing symbiotic mutants were found in the other two (17). Such differences have also been observed in *R. meliloti*: strain 102F51 (Nitragin Co., Milwaukee, Wis.) was found not to yield Tn5 mutants from a pJB4JI conjugation (H. Meade, unpublished data). Another example of variation in Tn5 behavior after a suicide plasmid mutagenesis is in *R. leguminosarum*, where a chromosomal Tn5 insert (*ade-92*::Tn5) was successfully used as a source of Tn5 for new transpositions to an indigenous plasmid (4). The apparent frequency of transposition was between 10^{-4} and 3×10^{-6}, within one or two orders of magnitude of the frequency found in *E. coli*. Thus in *R. leguminosarum*, Tn5 insertions generated by pJB4JI do not appear to have a pronounced defect for subsequent transpositions.

Although it is apparent from our results that there are problems in using suicide plasmid pJB4JI to generate Tn5 transpositions in *R. meliloti*, approximately half of the presumptive auxotrophic and symbiotic mutants isolated appear to be the result of legitimate Tn5 transposition events. Thus, pJB4JI can be used successfully in *R. meliloti* given that the mutations are subjected to the physical and genetic tests described here. Moreover, the legitimate mutants obtained are unusually stable for transposon-induced mutations and should prove to be very useful in the study of the *R. meliloti*-alfalfa symbiosis.

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