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Localization of Symbiotic Mutations in *Rhizobium meliloti*

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A total of 5 Nod$^-$ and 57 Fix$^-$ symbiotic mutants of *Rhizobium meliloti* strain 41 have been isolated after either nitrosoguanidine or Tn*5* transposition mutagenesis. Chromosomal locations of mutations in 1 Nod$^-$ and 11 Fix$^-$ derivatives were ascertained by transferring the chromosome (mobilized by plasmid R68.45), in eight fragments, into symbiotically effective recipients and testing the recombinants for symbiotic phenotype. Alternatively, the kanamycin resistance marker of Tn*5* was mapped. In five mutants the *fix* alleles were localized on different chromosomal regions, but six other *fix* mutations and one *nod* mutation tested did not map onto the chromosome. It was shown that the chromosome-mobilizing ability (Cma$^+$) of R68.45 was not involved in the mobilization of genes located extrachromosomally. Moreover, Cma$^-$ derivatives of R68.45 could mobilize regions of the indigenous plasmid pRme41b but not chromosomal genes. Thus, mobilization of a marker by Cma$^-$ R68.45 indicates its extrachromosomal location. With a ^{32}P-labeled DNA fragment carrying Tn*5* as a hybridization probe, it was shown that in five extrachromosomally located Tn*5*-induced *fix* mutants and one *nod* mutant Tn*5* was localized on plasmid pRme41b. This is in agreement with the genetic mapping data.

Under appropriate environmental conditions the interaction of *Rhizobium* bacterial species with *Leguminosae* plants results in nitrogen-fixing root nodules. The development and functional maintenance of this beneficial symbiotic organ necessitates the coordinate interaction and function of certain bacterial and plant genes and gene products. Accumulating experimental data have elucidated some details of symbiotic dinitrogen fixation, but still very little is known about the exact number, location, and regulation of the genes involved in this biological process (1, 5, 21, 36).

For the bacterial partner, isolation of symbiotically defective mutants is a very powerful approach for identification and localization of genes responsible for nodule formation and function. Recently, symbiotic mutants have been isolated from several *Rhizobium* species, using different mutagenic treatments (9, 24, 25). Genetic and DNA-DNA hybridization mapping revealed that symbiotic genes may be located either on the bacterial chromosome or on extrachromosomally inherited plasmids (2, 8, 26, 31, 32, 34).

In this paper we report the isolation of symbiotically defective mutants from *Rhizobium meliloti* strain 41 after chemical (nitrosoguanidine) and transposon (Tn*5*) mutagenesis. Plasmid R68.45 with chromosomal mobilization ability (8, 14, 17, 20) was used to locate the symbiotic mutations having no selectable phenotype. Transposon-induced mutations were mapped according to the kanamycin resistance marker (Km^r) of Tn*5*. In addition, Tn*5* insertion on an indigenous plasmid of *R. meliloti*, pRme41b (2), was localized by hybridization, using ^{32}P-labeled Tn*5*-specific probes.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids are shown in Table 1. For *R. meliloti* strains, growth conditions and the complete medium YTB were described by Orosz et al. (28); complete medium GTA, minimal medium GTS, and the minimal medium fumarate-nitrate (FNO_3) were as described by Kiss et al. (18).

Mutagenesis. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; Koch-Light) mutagenesis was carried out as described previously by Kondorosi et al. (19). Care was taken to avoid the isolation of siblings. The Tn*5*-carrying plasmid pJB4JI and the technique of Beringer et al. (4) were used. Strain AK1282 was constructed by site-specific mutagenesis, according to Ruvkun and Ausubel (33).

Bacterial matings. Bacterial matings were performed according to Kondorosi et al. (20).

Genetic mapping of nonselectable mutations. To map symbiotic (nonselectable) mutations, plasmid R68.45 was introduced into the mutant strains and mated with seven recipients, and the selected double recombinants (prototrophs) were tested for their symbiotic properties. In matings with seven doubly marked strains in which the two markers showed linkage, the

TABLE 1. Bacterial strains and plasmids used

Strain	Characteristics	Source/ reference
Derivatives of *R. meliloti* 41		
AK631	Nod$^+$ Fix$^+$ wild type, with compact colony morphology	This lab
AK684	Str derivative of AK631	This lab
ZB201	*cys-46 rif-3 fur-2* derivative of AK631	This work
GY889, GY891, GY900, and GY890	*fix-5*, *fix-6*, *fix-7*, and *fix-8* derivatives of AK684 obtained after NTG mutagenesis	This work
TF178, JA5, and JA7	*fix-2*, *fix-3*, and *fix-4* derivatives of AK684 obtained after Tn*5* mutagenesis	This work
ZB273, ZB274, ZB277, ZB278, and ZB313	*fix-10*, *fix-11*, *fix-12*, *fix-13*, and *fix-14* derivatives of AK631 obtained after Tn*5* mutagenesis	This work
AK1282	*fix-15* AK631 *nifH*::Tn*5*, constructed by site-specific mutagenesis	This work
GY561	*nod-1*, derivative of *R. meliloti* 41 obtained by freezing and thawing	2
AK1123	Rif^r derivative of GY561	This work
ZB306	*nod-2*, derivative of AK631 obtained after Tn*5* mutagenesis	This work
Plasmids		
R68.45	Cb^r Km^r Tc^r Cma$^+$ IncP1	14
pJB3JI	Km^s derivative of R68.45	8
pAK8	Cma$^-$ Km^s derivative of R68.45	This work
pJB4JI	Gm^r Sm^r::Mu::Tn*5* IncP1	4
pHM5	pJC307::Tn*5*	25

entire chromosome could be transferred in eight fragments (23).

Plant nodulation tests. Plant nodulation experiments were carried out with alfalfa (*Medicago sativa* L.) seedlings as described by Kondorosi et al. (22).

Root hair curling. The Fåhraeus slide technique (13, 27) was used to examine root hair curling (38). *M. sativa* L. seeds were sterilized by immersion in 70% ethanol for 10 min, 0.1% $HgCl_2$ for 3 min, and 10% sodium hypochlorite for 10 min. After the seeds were washed with sterile distilled water, they were germinated on 1.5% water-agar. When the radicles were 3 to 6 mm long, the seedlings were transferred into the slide chambers and inoculated with rhizobia in Fåhraeus solution (13).

Detection of plasmids on agarose gels. The method of Eckhardt (12) as applied by Bánfalvi et al. (2) was used for plasmid detection, except that the concentration of sodium dodecyl sulfate in solution B (12) was increased to 0.5%.

DNA isolation. For isolation of pHM5 plasmid DNA, the method of Birnboim and Doly (7) was adapted for large-scale plasmid preparation, starting from a 1-liter culture of bacterium in stationary phase. The covalently closed circular form of plasmid DNA was purified by CsCl-ethidium bromide density gradient ultracentrifugation, according to Clewell and Helinski (10). Mu phage DNA was kindly provided by V. Sakanyan.

Hybridization. DNA probes were labeled to a specific activity of 5×10^6 to 1×10^7 cpm/μg (29). Hybridization of the ^{32}P-labeled probes to DNA entrapped into dry gels was performed as described originally by Shinnick et al. (35) and modified by Tsao et al. (S. G. S. Tsao, C. F. Brunk, and R. E. Pearlman, submitted for publication). Before hybridization the dried gels were incubated for 5 h at 42°C in a solution containing 750 mM NaCl, 75 mM trisodium citrate, pH 7.0 (5× SSC [1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate]), 50% formamide, and 0.02% bovine serum albumin, polyvinylpyrollidone, and Ficoll (11). DNA probes were prepared in the same solution (1 μg of DNA per 20 to 30 ml), and the hybridizations were performed for 24 h at 42°C. After hybridization, the gels were washed at 65°C: two times in 1 liter of 2 × SSC containing 0.5% sodium dodecyl sulfate and six times without sodium dodecyl sulfate and decreasing the SSC concentration to 1 × SSC. Gels were then dried and exposed to Kodak X-Omat R film with an Ilford tungstate intensifying screen at −70°C for several days.

RESULTS

Isolation of symbiotic mutants of *R. meliloti*. To find mutants defective at any stage of the symbiotic development, a general screening approach was taken. *R. meliloti* strain 41 was mutagenized by NTG or Tn*5*, plated for single colonies, and then each clone was individually tested for its symbiotic ability. Symbiotic mutants were classified into two groups: mutants unable to form nodules on *M. sativa* L. roots were classified as Nod$^-$ mutants; those that nodulated the host plant but did not fix nitrogen were classified as Fix$^-$.

Since mutations in a number of genes can produce a symbiotically defective phenotype (e.g., some base or amino acid auxotrophs are ineffective in nitrogen fixation [5]), colonies

TABLE 2. Isolation of auxotrophic and symbiotic mutants of *R. meliloti* 41

Mutant[a]	No. screened	No. of auxotrophs[b]	Fix^- (no.)	Nod^- (no.)
NTG	3,900	26	42	2
Tn*5*	4,550	20	15	3

[a] NTG-induced mutants were obtained from one and Tn*5*-induced mutants were from three mutagenic treatments.

[b] Some auxotrophs were also Fix^-, but these mutants were not included in the Fix^- group.

were tested for their growth properties on glucose-ammonium and fumarate-nitrate media. Only those symbiotic mutants which grew well on both media are listed in Table 2 as Nod^- or Fix^- mutants. Both NTG and Tn*5* mutagenesis produced Nod^- and Fix^- derivatives.

The effectiveness of the mutagenic treatments is also indicated by the relatively high percentage of auxotrophs obtained among the clones screened (Table 2). From the mutagenized population, 0.7% (26 of 3,900) of the colonies showed growth requirements according to the Holliday test (16). These mutants were assigned into 11 phenotypic groups (2 His^-, 5 Cys^-, 3 Pur^-, 4 Pyr^-, 2 Met^-, 2 Leu^-, 1 Gly^-, 1 Tyr^-, 4 Trp^-, 1 Cit^-, 1 Arg^-). The frequency of appearance of auxotrophs after Tn*5* mutagenesis was 0.4% (20 of 4,530). Eighteen of 20 auxotrophs were determined by the Holliday test (16). These auxotrophs belonged to nine phenotypic groups (3 Cys^-, 2 Pur^-, 1 Pyr^-, 3 Met^-, 1 Tyr^-, 2 Trp^-, 1 Arg^-, 3 Thi^-, 2 Lys^-). Both results suggest relatively random mutations. In both mutagenic treatments the appearance of sulfur metabolism mutants (Cys^- Met^-) among auxotrophic derivatives was rather high (7 Cys^- Met^- of 26 in NTG treatment; 6 Cys^- Met^- of 18 in Tn*5* mutagenesis). The majority of auxotrophs isolated by either NTG or Tn*5* mutagenesis could revert to prototrophy. Most prototrophic derivatives of the Tn*5*-induced auxotrophs became Km^s at a frequency of 10^{-8}, indicating precise excision of Tn*5*.

Preliminary characterization of mutants with respect to their symbiotic ability indicated that mutants defective in different developmental steps had been obtained. For instance, four of five Nod^- mutants were able to evoke root hair curling (Hac^+; Fig. 1). Microscopic examination of the nodules after infection with different Fix^- mutants also showed differences in nodule structure (not shown).

Genetic analysis of Nod^- and Fix^- mutations. The linkage map of the *R. meliloti* 41 chromosome (20, 23, 39) contains auxotrophic and resistance markers. These markers provide a frame for the localization of any other chromosomal mutations. Since the NTG-induced symbiotic mutations have no selectable phenotype under

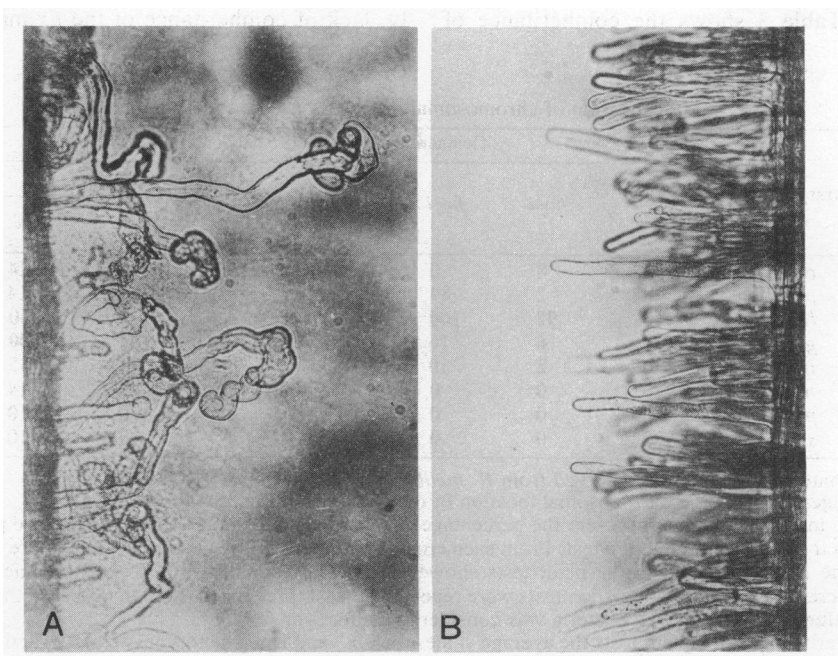

FIG. 1. Alfalfa root hair curling (Hac) with wild-type and Nod^- *R. meliloti* 41. (A) Curled root hairs infected with the wild-type strain; (B) root hairs infected with Nod^- Hac^- mutant ZB306.

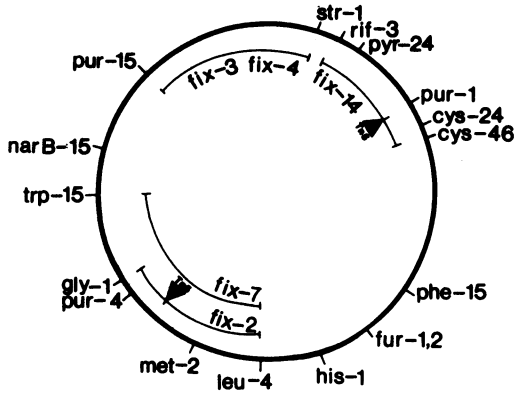

FIG. 2. Location of symbiotic mutations in *R. meliloti* 41. Location of auxotrophic and resistance markers was determined previously (20, 23, 39). Only those markers which were used in this study are presented.

free-living conditions, a mapping strategy was developed for the localization of any mutation on the linkage map. During R68.45-promoted chromosome mobilization in *R. meliloti* 41, very large chromosomal segments were transferred and incorporated into the recipient DNA. Moreover, the occurrence of multiple crossovers on the transferred chromosomal region was relatively infrequent (21). When two donor markers were coinherited, other markers located between them were cotransferred into at least 90% of the double recombinants whereas the percentage of their coinheritance with distant regions was low. Table 3 shows the coinheritance of three markers (*pur-1*, *his-1*, and *fur-1*) with eight chromosomal regions. The coinheritance values are in agreement with the map positions of these markers (Fig. 2). It is worth noting that, with these eight marker pairs in seven recipient strains, the entire chromosome could be transferred in eight fragments.

To map a symbiotic mutation, plasmid R68.45 was introduced into the Nod$^-$ and Fix$^-$ mutants. These donors were then individually mated with the seven recipients, and the selected double recombinants were tested for their Nod$^-$ or Fix$^-$ phenotypes. The seven recipient strains were all Nod$^+$, but some showed decreased efficiency in nitrogen fixation. When a wild-type donor was used, the symbiotic effectiveness of the different recipients was restored (data not shown). When several *nod* mutations were tested for chromosomal locations, no coinheritance of *nod* markers with any of the chromosomal recombinants was obtained, suggesting the extrachromosomal location of these mutations (Table 3). The symbiotic effectivity of the double recombinants originating from the cross with Fix$^-$ mutants as donors was estimated by their acetylene reduction ability in the nodule. This evaluation was somewhat more difficult than that of the Nod$^-$ phenotype due to the higher degree of variability of the acetylene reduction data. From the results of this experiment we located *fix-2* and *fix-7* markers in the *leu-4 gly-1* region of the chromosome (Table 3). *fix-5* did not map onto the chromosome. Similarly, lack of coinheritance of the *fix* marker with

TABLE 3. Localization of chromosomal markers, based on their coinheritance

Marker pairs transferred[a]		Coinheritance of marker from donor strain (%)[b]							
		pur-1	*his-1*	*fur-1*	*nod-1*	*fix-5*[c]	*fix-7*[c]	*fix-2*[c,d]	
								Fix$^-$	Km^r
str-1$^+$	*cys-46*	**98**	8	0	0	0	0	14	0[e]
cys-46	*phe-15*	8	2	84	0	0	0	14	0
phe-15	*leu-4*	0	**92**	**100**	0	4	0	60	4
leu-4	*gly-1*	0	6	16	0	0	**91**	**100**	**100**
gly-1	*trp-15*	0	2	10	0	4	76	35	
trp-15	*narB-15*	0	0	0	0	4	27	14	20
narB-15	*pur-15*	0	0	0	0	8	0	10	
pur-15	*str-1*$^+$	0	0	0	0	4	29	10	0[e]

[a] Appropriate recipient strains, derived from *R. meliloti* 41, were used (23).

[b] Percentages showing the chromosomal location of donor markers are in boldface.

[c] The data in these columns represent the percentage of recombinant colonies having ineffective phenotypes evaluated by the acetylene reduction test. From each cross five independently purified colonies were tested in 10 parallels. The Fix phenotypes in the plant tests showed higher variations than the values obtained for other markers. Therefore, the plant nodulation tests were repeated at least three times. Less than 20% of the acetylene reduction values of the wild-type bacteria was considered ineffective.

[d] Due to variations in the plant tests the average values for Fix$^-$ and Km^r phenotypes are not exactly the same. The Km^r clones, however, were always Fix$^-$.

[e] The genetic linkage was tested to the *cys-46* and *pur-15* markers only instead of regions *str-1*$^+$ *cys-46* and *pur-15 str-1*$^+$.

TABLE 4. Transfer of chromosomal and plasmid markers by Cma$^-$ and Cma$^+$ Km^s derivatives of R68.45[a]

Donor strain	Genotype (location)	Transfer frequency of:	
		cys-46	Tn*5*
ZB273	*fix-10*::Tn*5* (plasmid)	$<3 \times 10^{-9}$	$<3 \times 10^{-9}$
ZB273(pAK8)		$<7 \times 10^{-9}$	2×10^{-6}
ZB273(pJB3JI)		3×10^{-4}	4×10^{-6}
ZB277	*fix-12*::Tn*5* (plasmid)	$<7 \times 10^{-9}$	$<7 \times 10^{-9}$
ZB277(pAK8)		$<1 \times 10^{-9}$	3×10^{-6}
ZB277(pJB3JI)		3×10^{-4}	3×10^{-6}
AK1282	*fix-15*::Tn*5* (plasmid)	$<6 \times 10^{-9}$	$<6 \times 10^{-9}$
AK1282(pAK8)		$<1 \times 10^{-8}$	4×10^{-6}
AK1282(pJB3JI)		5×10^{-4}	2×10^{-6}
ZB313	*fix-14*::Tn*5* (chromosomal)	$<6 \times 10^{-9}$	$<6 \times 10^{-9}$
ZB313(pAK8)		$<4 \times 10^{-9}$	$<4 \times 10^{-9}$
ZB313(pJB3JI)		2×10^{-4}	2×10^{-4}

[a] Strain ZB201 was used as recipient. Cys$^+$ recombinants were selected by using Rif^r and Fur^r as counterselection markers. Using the same mating mixture, transfer of Tn*5* into the recipient was determined by selecting for Km^r Nm^r Rif^r Fur^r derivatives. Five to 10 recombinants from each cross were checked in plant tests and all showed the Fix$^-$ phenotype. The Tn*5* recombinants from crosses with the plasmid-carrying derivatives of ZB273, ZB277, and AK1282 were all Cys$^-$ and those with ZB313 donors (about 60%) were Cys$^+$, indicating linkage of Tn*5* to *cys-46* (Fig. 2). Transfer frequency was related to the number of recipients.

any of the eight chromosomal regions for two other NTG-induced mutations (*fix-6* and *fix-8*) was found (data not shown).

During genetic analysis of the Tn*5*-induced mutants, we observed that transposition of Tn*5* from the *R. meliloti* 41 DNA to new locations was not detectable. In these experiments the ability of Tn*5* to transpose from the chromosome into a Cma$^-$ Km^s derivative of R68.45 (pAK8) was tested. Three different Tn*5*-induced auxotrophic mutants (Ura$^-$ Trp$^-$, and Met$^-$), each carrying pAK8, were mated with a Rif^r derivative of *R. meliloti* 41 (AK1123). With the Rif^r marker for selection against the donor, Tc^r transconjugants appeared at frequencies of 10^{-1} to 1, indicating very efficient plasmid transfer. However, no Km^r transconjugants were obtained (frequency, $<10^{-9}$). As controls, it was demonstrated that pJB3JI was able to mobilize Km^r from these strains, and in an *Escherichia coli* host Tn*5* could jump into pAK8 at a frequency 10^{-5}.

This finding allowed us to manipulate Tn*5* in *R. meliloti* 41 as a simple kanamycin resistance marker. Before mapping the location of Tn*5* in these mutants, the genetic linkage of Tn*5*-coded kanamycin resistance and the symbiotic defect was tested. After introducing a Km^s derivative of plasmid R68.45 (pJB3JI) into the strains to be mapped, these transconjugants were used as donors in matings with a Nod$^+$ Fix$^+$ strain carrying the marker *cys-46*. This test has been performed with 3 Nod$^-$ and 10 Fix$^-$ mutants obtained after Tn*5* mutagenesis. In only eight cases did Tn*5* show 100% linkage to the symbiotically defective phenotype, indicating that the mutation is due to the insertion of Tn*5* into a symbiotic gene. These eight mutants (TF178, ZB273, ZB274, ZB277, ZB278, ZB313, ZB306, and AK1282) were further analyzed (see below).

Localization of the Tn*5*-induced symbiotic mutation was carried out in two ways: by mapping the *fix* alleles as performed with the NTG-induced mutants and by mapping the Km^r marker of Tn*5*. Table 3 shows the position of such a *fix* mutation (*fix-2* in strain TF178) by separately determining the coinheritance of the Fix$^-$ and Km^r phenotypes with the eight chromosomal regions. The symbiotic defect and Km^r were located in the same region, as expected. In addition, the precise location of *fix-2*::Tn*5* was determined in three-point crosses by mapping the Km^r marker and using other recipients with different markers in the *leu-4 gly-1* region (data not shown). Figure 2 shows the location of another Tn*5*-induced *fix* mutation (*fix-14* in strain ZB313) between *pur-1* and *cys-24*.

For some Tn*5* mutants (*fix-10*, *fix-11*, and *fix-12*), no chromosomal region could be attributed to the Km^r marker. Table 4 shows that for *fix-10*::Tn*5* and for *fix-12*::Tn*5* the pJB3JI-promoted transfer of Tn*5* occurred at a much lower frequency than that for chromosomally located *fix-14*::Tn*5*. In these matings strain ZB201 was used as recipient, allowing us to demonstrate that the chromosomal marker *cys-46*$^+$ was transferred from the different donors at about the same frequencies. Therefore, the differences in the Tn*5* transfer frequency values were significant.

Low transfer frequency of Tn*5* can be ex-

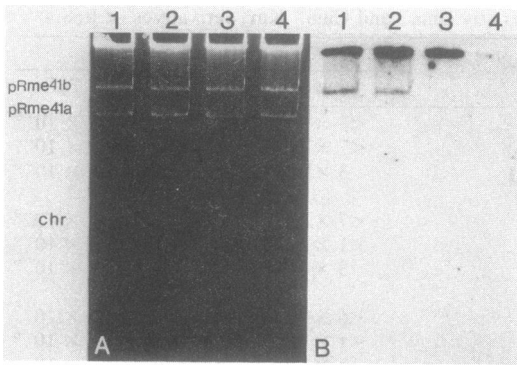

FIG. 3. Demonstration of Tn*5* insertions in the indigenous plasmids of *R. meliloti* 41. (A) Plasmids pRme41a and pRme41b were separated on 0.7% agarose gel prepared according to Eckhardt (12), stained with ethidium bromide, and visualized with UV light. Recording of gel patterns was with Planfilm NP22 (ORWO East Germany). (B) Hybridization of Tn*5* probe to the plasmid. The gel was dried onto a glass sheet (35), and hybridization was carried out with ^{32}P-labeled pHM5 DNA, using about 10^5 cpm/cm^2 and 0.5 ml of hybridization mix per cm^2. Lanes 1 to 3, Fix$^-$ mutants ZB273, ZB277, and ZB313; lane 4, AK631.

plained by assuming transposition of Mu DNA sequences together with Tn*5*, as reported earlier (2), since the presence of Mu DNA may kill the new *Rhizobium* hosts (4). The presence of Mu DNA was in fact demonstrated in one of these derivatives (see below).

Another explanation is that in strains with lower Tn*5* transfer frequency the Tn*5* is inserted into an indigenous plasmid, which implies that mobilization of a plasmid marker by pJB3JI occurs at low frequency. In fact, Brewin et al. (8) reported the lack of mobilization of nodulation genes carried on an indigenous plasmid of *Rhizobium leguminosarum* by plasmid R68.45. To test this possibility, a strain (AK1282) carrying Tn*5* in the *nifH* gene on plasmid pRme41b was constructed by a site-specific mutagenesis technique, as described by Ruvkun and Ausubel (33). With pJB3JI, the transfer frequency of Tn*5* from AK1282 was at least two orders of magnitude lower than that of *cys-46*$^+$ (Table 4), indicating low frequency of transfer and establishment of genes carried by pRme41b.

If the IS*21* element responsible for the chromosome mobilization ability (Cma$^+$) of R68.45 (40) cannot effectively interact with pRme41b, a Cma$^-$ derivative of R68.45 should be as effective in mobilizing pRme41b markers as the Cma$^+$ R68.45 plasmid. It has been observed that the Cma$^+$ property of R68.45 is easily lost during growth of *R. meliloti* (20), and Cma$^-$ Km^s derivatives have been readily obtained (unpublished data), which enabled us to test this assumption. Indeed, these derivatives (for instance, pAK8), did not mobilize the chromosome at detectable frequencies, whereas the plasmid markers were mobilized at a frequency similar to that obtained with pJB3JI (Table 4).

Physical localization of Tn*5* and Mu DNA in symbiotic mutants. To demonstrate the presence of Tn*5* on the indigenous plasmids of *R. meliloti* 41, plasmid DNAs of symbiotic mutants isolated after Tn*5* mutagenesis were separated by agarose gel electrophoresis (Fig. 3A). The gels were dried onto a glass sheet (35) and hybridized with a ^{32}P-labeled, nick-translated Tn*5* probe (pHM5). The autoradiogram obtained for three mutants is shown in Fig. 3B. In two strains hybridization of the Tn*5* probe to the DNA band corresponding to plasmid pRme41b was detected, indicating that Tn*5* was inserted into this plasmid. It was found that in six of eight symbiotic mutants the Tn*5* was located on pRme41b (Table 5), in agreement with the genetic mapping data.

As reported earlier (2), after Tn*5* mutagenesis of *R. meliloti* 41 by pJB4JI a significant portion of the Tn*5* insertion mutants contained some Mu sequences. Therefore, the presence and the location of Mu in the Tn*5*-containing symbiotic mutants were tested by hybridization of a Mu

TABLE 5. Location of Tn*5* and Mu sequences[a] in mutants

Strain	Genotype	Location of Tn*5*		Presence of Mu sequences
		Genetic mapping	Hybridization	
TF178	*fix-2*	Between *pur-4 met-2* on the chromosome	Chromosome	Chromosome
ZB273	*fix-10*	Nonchromosomal	pRme41*b*	—
ZB274	*fix-11*	Nonchromosomal	pRme41*b*	pRme41*b*
ZB277	*fix-12*	Nonchromosomal	pRme41*b*	—
ZB278	*fix-13*	NT	pRme41*b*	—
ZB313	*fix-14*	Between *cys-24 pur-1* on the chromosome	Chromosome	—
AK1282	*fix-15*	NT	pRme41*b*	NT
ZB306	*nod-2*	NT	pRme41*b*	pRme41*b*

[a] —, Not present; NT, Not tested.

DNA probe to the separated plasmids of *R. meliloti* 41, as was done with the Tn*5* probe. In three of seven mutants tested Mu DNA sequences hybridized to pRme41b or to the fragmented chromosomal DNA on the agarose gel (data not shown). Table 5 summarizes the hybridizations with the Tn*5* and Mu DNA probes. It is noticeable that in the three Mu DNA-containing strains both probes hybridized to the same band in a particular strain, suggesting that during the transposition event Tn*5* and Mu transposed together.

DISCUSSION

Since NTG mutagenesis induces mainly reversible random point mutations whereas Tn*5* insertions have a strong polar (though random) effect (3), the two sets of mutants produced in this way may complement each other in future studies on the identification of symbiotic nitrogen fixation genes. Recently, a technique allowing site-directed mutagenesis of the nitrogen fixation genes has been developed (33) and is based on the mutagenesis of cloned DNA fragments carrying the structural genes of nitrogenase. This procedure allows efficient mutagenesis of genes cloned previously, whereas random mutagenesis may detect symbiotic genes residing on other DNA regions. The number of Fix^- mutants isolated was about 10 times higher than the number of Nod^- mutations. Earlier screenings of NTG-mutagenized cultures of *R. japonicum* (24) and *R. leguminosarum* (6) also demonstrated differences in the number of Nod^- and Fix^- mutants. As pointed out by Beringer et al. (5), these data do not necessarily reflect the differences in the number of *nod* and *fix* genes in rhizobia. It is probable that in plant tests Nod^+ revertants are more likely to mask the Nod^- phenotype than are Fix^+ revertants to mask the Fix^- phenotype. On the other hand, our studies on recombinant plasmids carrying sections of plasmid pRme41b indicate that genes specifying nodulation of alfalfa are clustered on a relatively small region of pRme41b (2; A. Kondorosi, Z. Banfalvi, W. J. Broughton, T. Forrai, G. B. Kiss, E. Kondorosi, C. Pankhurst, G. Randhawa, Z. Svab, and E. Vincze, *in* O. Ciferri and L. Dure, ed., *Structure and Function of Plant Genomes*, in press), suggesting the existence of only a few *nod* genes.

Since most symbiotic nitrogen fixation genes are not expressed outside the root nodule, genetic analyses of symbiotic mutations are greatly hampered by the lack of selectable phenotypes under free-living conditions. This difficulty has been partly overcome by the application of the Tn*5* mutagenesis technique for rhizobia (4).

We have found, however, that in about 40% of the presumptive transposition derivatives the Tn*5* was found not to be linked to the symbiotic mutation. These mutations might have been results of spontaneous mutations or secondary events of the transposition mutagenesis. For instance, in two of three Nod^- strains the *nif* structural genes had been deleted. This was shown by hybridizing a cloned DNA fragment carrying the *nifHD* genes of *R. meliloti* 41 (2, 37) to total DNAs from the wild type and from the Nod^- bacteria. The same approach was used earlier to demonstrate the deletion of *nif* structural genes in some spontaneous Nod^- mutants. Although transposition elements are known to promote deletion formation (3) and we have demonstrated earlier that the *nod* and *nif* genes are closely located on pRme41b (2), the involvement of Tn*5* or Mu sequences in the origin of these mutants has not been established.

Another complication, arising from the use of pJB4JI for transposition mutagenesis in *R. meliloti*, is the observed transposition of Mu sequences together with Tn*5* (2, 25). We have reported earlier (2) that in some *R. meliloti* 41 derivatives carrying pRme41b::Mu::Tn*5* the region of pRme41b containing the insertion is extremely unstable and often results in deletions of large plasmid segments. Moreover, transfer of any DNA region containing the Mu::Tn*5* insertion could be mobilized by pJB3JI only at a lower frequency, which might be explained by the killing effect of Mu in *Rhizobium* (4). Mapping of Tn*5* was facilitated by our observation that Tn*5* cannot be transposed to new locations in *R. meliloti* 41. A very low (10^{-8}) transposition frequency was recently reported for another *R. meliloti* strain (Rml021) by Meade and colleagues (25). In *R. leguminosarum*, however, Tn*5* transposes fairly effectively (9). Another transposon property, the precise excision of Tn*5*, occurred in *R. meliloti* 41 at a frequency of 10^{-8}. In *R. meliloti* 1021 this phenomenon was not observed (25). It seems that expression of Tn*5* functions varies with different bacterial species and strains. This is supported by our recent finding that Tn*5* codes for streptomycin resistance, which is expressed in *R. meliloti* but not in *E. coli* (Putnoky et al., manuscript in preparation). Recently, Rolfe and Gresshoff reported the cloning of a symbiotic gene from the Tn*5*-induced symbiotic mutant of *R. trifolii* by cloning Tn*5* with adjacent host DNA (30). This approach to the isolation of symbiotic genes is not applicable for Mu::Tn*5* insertion mutants. We found that only 4 of 15 symbiotic mutants obtained after Tn*5* mutagenesis were "true" Tn*5* insertion mutants and consequently amenable to such studies. In these four mutants further hybridization experiments revealed that Tn*5* was inserted into different *Eco*RI fragments of

R. meliloti 41, indicating that different symbiotic genes mutated.

Although we have mapped relatively few (i.e., 13) symbiotic mutations, it seems that the majority of the symbiotic genes are extrachromosomally located. In our experiments five *fix* mutations were localized on the chromosome and eight had extrachromosomal location. None of the three *nod* alleles tested previously (2) and in this study, however, mapped onto the chromosome. This is in line with our previous results (2) obtained from the analysis of pRme41b deletion mutants and from hybridization of the cloned *nif* region to pRme41b DNA, where *nod* and *fix* genes, including the *nifH* and *nifD* genes, were localized on pRme41b. This large plasmid is present in most *R. meliloti* strains, and its involvement in symbiotic nitrogen fixation seems to be a general feature of most *R. meliloti* strains (2, 31). In the course of these mapping studies we found that the transferability of chromosomal markers by R68.45 or its Cma^- derivative differs from that of the markers carried on pRme41b. On the one hand, the DNA region resulting in the Cma^+ phenotype of R68.45 cannot promote the mobilization of pRme41b markers; on the other hand, Cma^- derivatives of R68.45 can mediate a low-frequency transfer of plasmid genes but not of chromosomal genes. It follows that the transfer of a marker by Cma^- R68.45 suggests its extrachromosomal location.

We found that the chromosomally located *fix* mutations are not clustered. Moreover, the number of Fix^- mutants compared with the number of auxotrophs obtained after the two different mutagenesis techniques suggests that a relatively large number of *Rhizobium* genes are involved in the development and functioning of the nitrogen-fixing bacteroids in the nodules.

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