Isolation and characterization of *Rhizobium meliloti* Tn5 mutants showing enhanced symbiotic effectiveness

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A series of Tn5-induced mutants which showed enhanced symbiotic effectiveness (Eff⁺⁺) was isolated from *Rhizobium meliloti* strains CXM1-105 and CXM1-188. Alfalfa plants inoculated by the Eff⁺⁺ mutants had significantly higher shoot dry mass than plants inoculated by the parental strains. In the greenhouse, the most effective mutants increased the shoot dry mass of the host plants by 23–26% and plant total nitrogen by 23–27%. Interestingly, the frequency of the Eff⁺⁺ mutants in strain CXM1-188 was higher than in the strain CXM1-105 (1.1% versus 0.4%); this was also the case for the auxotrophic mutants (1.2% in CXM1-188 versus 0.3% in CXM1-105). Genetic analysis of the mutants showed that the enhanced symbiotic effectiveness was cotransducible with Tn5. By the use of Southern hybridization and plasmid transfer, it was found that ten Tn5 insertions were located in the chromosome, five in megaplasmid 1, and six in megaplasmid 2.

Keywords: *Rhizobium meliloti*, symbiotic effectiveness, nitrogen fixation, Tn5 mutants

INTRODUCTION

The study of the nodule bacterium *Rhizobium meliloti* has proved to be very helpful in elucidating the *Rhizobium*–legume symbiotic interaction. The efforts of various laboratories, focussing mainly on two *R. meliloti* strains, have revealed numerous genes encoding the formation and functioning of nitrogen-fixing nodules (for a review, see Martinez *et al.*, 1990). However, it remains unclear which genes are responsible for the practically important trait of symbiotic effectiveness. The symbiotic effectiveness is usually measured by the increase of the host plant's growth, at the expense of nitrogen fixation in the root nodules.

It is well documented that random mutation and genetic recombination give rise to isolates with improved symbiotic properties (Feodorov & Simarov, 1987; Maier & Brill, 1978; Shukla *et al.*, 1989; Williams, 1981). To our knowledge, only one attempt has been made to identify genes which, when mutated, enhance the symbiotic effectiveness of *R. meliloti* (Plazinski, 1981). Unfortunately, the author could not correlate a mutation which led to this phenotype with a Tn5 insertion, and failed to locate either in the *R. meliloti* chromosome. It was proposed that

the target gene was in one of the indigenous plasmids of strain 41.

At the biochemical and physiological level, the symbiotic effectiveness of *Rhizobium* has been presumed to be determined by the concerted expression of nitrogenase and complementary functions, such as hydrogen recycling (Evans *et al.*, 1987), transport of C₄-dicarboxylates into bacteroids (Birkenhead *et al.*, 1988; Engelke *et al.*, 1987), bacteroid respiration (Soberon *et al.*, 1989), constitutive *nod* gene expression (Spaink *et al.*, 1989), etc. Therefore, some researchers believe that *Rhizobium* strains could be improved through the manipulation of genes involved in the control of these functions, e.g. gene *nifA*, which encodes a positive regulator for the expression of *nif/fix* (nitrogen fixation) genes (Ronson *et al.*, 1990).

It is of interest to ascertain which genes are randomly mutated when *R. meliloti* strains are selected for their improved symbiotic effectiveness, and to determine which of these genes is associated with already-known symbiotic functions. The work described here may be considered as the first step in this task. We isolated a series of Tn5 mutants which showed an enhanced symbiotic effectiveness (Eff⁺⁺), analysed the mutants by transduction and localized the Tn5 insertions in the *R. meliloti* genome.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
<i>Rhizobium meliloti</i>		
CXM1	Spontaneous Sm ^r mutant of commercial strain 425a	Zaretskaya (1976)
CXM1-105	Eff ⁺⁺ mutant of CXM1, induced by UV light	Feodorov & Simarov (1987)
CXM1-188	Eff ⁺⁺ mutant of CXM1, induced by UV light	Feodorov & Simarov (1987)
ZB121	Exo ⁻ Nod ⁻ deletion mutant of AK631, Rf ^r	Banfalvi <i>et al.</i> (1981)
L5-30	Non-lysogenic strain, used for propagation of phage ϕ M12	Kowalski (1970)
<i>Agrobacterium tumefaciens</i>		
UBAPF2	Plasmid-free derivative of strain LBA275	Hynes <i>et al.</i> (1986)
<i>Escherichia coli</i>		
S17-1	<i>pro res mod</i> pRP4-2 (Tc::Mu) (Km::Tn7) integrated into the chromosome, Tp ^r Sm ^r	Simon <i>et al.</i> (1983)
Plasmids		
pSUP2021	pSUP202::Tn5, Ap ^r Cm ^r Km ^r	Simon <i>et al.</i> (1983)
pSUP5011	pBR325::Tn5- <i>mob</i> , Ap ^r Cm ^r Km ^r	Simon (1984)
pSUP102-21	pSUP102-Gm carrying Tn5-B21, Cm ^r Gm ^r Tc ^r	Simon <i>et al.</i> (1989)

Parts of this work were presented at the 8th and 9th International Congresses on Nitrogen Fixation (Sharypova *et al.*, 1990; Chesnokova *et al.*, 1993).

METHODS

Bacterial strains and culture conditions. The strains used in this work are listed in Table 1. *R. meliloti* and *Agrobacterium tumefaciens* strains were cultured in either TY medium (Beringer, 1974) or yeast mannitol medium (Allen, 1959). The *Escherichia coli* strains were grown in LB medium (Miller, 1972). The antibiotics used for selection were added to the media at the following concentrations. For *R. meliloti* and *A. tumefaciens*: neomycin (Nm) 200 µg ml⁻¹, tetracycline (Tc) 15 µg ml⁻¹, gentamicin (Gm) 40 µg ml⁻¹, streptomycin (Sm) 500 µg ml⁻¹, rifampicin (Rf) 50 µg ml⁻¹. For *E. coli*: Nm 50 µg ml⁻¹, Tc 5 µg ml⁻¹, Gm 10 µg ml⁻¹, Sm 100 µg ml⁻¹, Rf 50 µg ml⁻¹. *R. meliloti* and *A. tumefaciens* strains were cultured at 30 °C, *E. coli* at 37 °C.

Genetic manipulations. Tn5 mutagenesis and bacterial crosses were carried out as described by Simon *et al.* (1983). Only one mutant clone was taken from each cross. The conjugal transfer of plasmids was performed by mixing donor and recipient cultures (ratio 1:5) on non-selective TY or LB plates. After overnight incubation, the transconjugants were selected on the appropriate medium. Transduction was done according to Novikova & Simarov (1983) using phage ϕ M12, which transduces approximately 130 kb DNA (Finan *et al.*, 1984). Briefly, an exponentially growing culture of the donor strain was infected by the phage suspension with a multiplicity of infection of about 10 phage particles per cell. This mixture was grown for 12 h. Then 0.1 vol. chloroform was added. The suspension was shaken at 200 r.p.m. for 30 min at room temperature. After centrifugation for 10 min at 5000 r.p.m. the water phase was transferred into sterile tubes and 0.1 vol. chloroform was added. This phage lysate, with a titre of about 10⁸–10¹⁰ p.f.u. ml⁻¹, was stored at 4 °C. The sterility of the phage lysate was checked on rich and selective agar medium. The frequency of transduction, scored as the ratio of recom-

binant cells to the total number of phage-infected recipient cells, was 10⁷–10⁸ on average.

Agarose gel electrophoresis of plasmids. The method of Eckhardt (1978) as modified by Hynes *et al.* (1985) was used for the detection of plasmids by agarose gel electrophoresis.

Plasmid DNA isolation, hybridization probe preparation and Southern blotting. These were performed using standard protocols (Maniatis *et al.*, 1982).

Effectiveness assays of *R. meliloti* strains. These were performed in gnotobiotic plant tests and in greenhouse trials. In the former, alfalfa plants (*Medicago varia* Mart. cv. Zaikevicha) were grown in 60 ml glass tubes containing vermiculite and 10 ml nitrogen-free nutrient solution, as described by Provorov & Simarov (1990). Each *R. meliloti* strain was used to inoculate 12 plants, two plants per tube. The shoot dry mass of 30-d-old plants was measured after 48 h at 60 °C. In the greenhouse, the alfalfa plants were grown in pots which contained 5 kg field soil supplemented with the following nutrients (g per kg soil): FeCl₃·6H₂O, 0.025; KCl, 0.075; KH₂PO₄, 0.136; MgSO₄·7H₂O, 0.123; H₃BO₃, 0.003; MnSO₄, 0.003; (NH₄)MoO₄, 0.001. The total nitrogen in the soil was 0.12%. The soil was adjusted to a pH of 6.5 by the addition of CaCO₃ (5 g per pot). Alfalfa seeds were surface-sterilized with H₂SO₄, rinsed and sown in the pots, 30 seeds in each pot. Immediately after this, the seeds were inoculated with rhizobia (10⁹ cells per pot). Eight replications were used per treatment. One week later, the seedlings were thinned out to leave the 13 healthiest ones in each pot. Plant shoots were cut at the beginning of the flowering period, 6 weeks after inoculation. The cutting was repeated 5 weeks later, when new shoots had reached the same stage of growth. The values for shoot dry mass and nitrogen content of both cuttings were added and used to compare symbiotic effectiveness of the *Rhizobium* strains.

Nodulation ability of *R. meliloti* and *A. tumefaciens* transconjugants. This was tested by inoculating alfalfa seedlings which were grown in 60 ml tubes containing nitrogen-free agar medium. The concentrations of salts in this medium were the same as described by Provorov & Simarov (1990).

Table 2. Isolation of Eff⁺⁺ mutants in *R. meliloti* CXM1-105 and CXM1-188

Mutagenesis in CXM1-105 was performed by the use of pSUP2021; mutagenesis in CXM1-188 was performed using pSUP2021 and pSUP5011 (both vectors showed similar mutagenic activity in CXM1-188).

Parental strain	Total no. of colonies tested	No. of colonies showing Eff ⁺⁺ phenotype at the		Frequency (%) of mutants		
		1st selection	2nd selection	Eff ⁺⁺	Fix ⁻ *	Aux*
CXM1-105	1056	60	4	0.4	0.2	0.3
CXM1-188	2031	431	22	1.04	0.7	1.2

*Frequencies of Fix⁻ and auxotrophic (Aux) mutants were taken from Sharypova *et al.* (1987).

Nitrogen content of plants. This was determined by the use of an automatic analyser, 'Kjeltek-Auto' (Tekator, Sweden).

RESULTS

Isolation of *Rhizobium meliloti* mutants with enhanced symbiotic effectiveness (Eff⁺⁺)

Two derivatives of a commercial *R. meliloti* strain, 425a, were used for Tn5 mutagenesis. These strains, CXM1-105 and CXM1-188, have been shown to be more effective in nitrogen fixation than the parental strain (Feodorov & Simarov, 1987). Previously, by the use of the suicide vectors pSUP2021 and pSUP5011 (Simon, 1984; Simon *et al.*, 1983), a set of auxotrophic and symbiotic mutants was generated in strains CXM1-105 and CXM1-188 (Novikova *et al.*, 1986; Sharypova *et al.*, 1987). To isolate mutants with enhanced symbiotic effectiveness, the same pool of Tn5-containing prototrophic colonies was screened in the plant test.

To screen as many colonies as possible and to reduce the influence of host plant phenotypic variation on *R. meliloti* symbiotic performance, a two-step analysis was undertaken. Initially, every Nm^r colony was assayed on four alfalfa plants. The shoot growth of the inoculated plants was assessed visually. Strains which caused a greater shoot mass production relative to the parental strains were studied further (Table 2). In the second step, each colony was tested on 12 alfalfa plants. This allowed a more stringent selection to be carried out. Only those strains which caused a statistically significant increase in shoot mass as compared to the parents were considered to be Eff⁺⁺ mutants. Twenty-six strains met this requirement (the increase in shoot dry mass ranged from 19 to 36%). Interestingly, the frequency of the Eff⁺⁺ mutants was higher than the frequency of Fix⁻ mutants and nearly coincided with the frequency of auxotrophic mutants (Table 2). None of the Eff⁺⁺ mutants showed discernible differences from the parent in their vegetative growth or in such symbiotic traits as nodule number and rate of acetylene reduction.

It was of interest to ascertain whether Eff⁺⁺ mutants could

enhance shoot mass under greenhouse conditions, which are less artificial than gnotobiotic ones. Fourteen mutants derived from strain CXM1-188 were chosen for the analysis (Table 3). Because of the longer period of plant growth and greater influence of environmental factors (including the soil environment, which contained combined nitrogen and indigenous *Rhizobium* strains), the variation in the mutants' effectiveness was higher in the greenhouse than in the gnotobiotic tests. Nevertheless, the Eff⁺⁺ phenotype of 10 mutants was confirmed: one mutant caused statistically significant ($P = 0.05$) enhancement in shoot mass production, another one was significantly better than the parent in nitrogen accumulation, and eight were superior to the parent in both characteristics.

Transductional analysis of the Tn5-mutants which showed enhanced symbiotic effectiveness

Since it is known that upon introduction of suicide plasmids in the *R. meliloti* genome, mutations might arise not only because of Tn5 transpositions (Dusha *et al.*, 1987; Ruvkun *et al.*, 1982), but also due to genomic rearrangements, it was necessary to analyse the genetic linkage between Tn5 and the mutation which caused the Eff⁺⁺ phenotype. By the use of bacteriophage ϕ M12, the Km^r marker encoded by Tn5 was transduced from seven Eff⁺⁺ mutants to their parental strains. The frequency of the transduction was 10^{-6} – 10^{-7} per recipient cell. The examination of 87 Km^r transductants in gnotobiotic plant tests revealed that a large proportion of them were superior to the recipients in their ability to increase shoot dry mass. Thus, transductants which inherited the donor phenotype (they were significantly more effective than the recipient) comprised 52.9%; those which showed intermediate effectiveness (no significant difference from either donor or recipient) comprised 36.8%; and those which retained the recipient's phenotype (significantly less effective than the donor) comprised only 10.3% (Table 4). None of the transductants was inferior to the recipient. In all transductional crosses, except T707 $\times$ CXM1-188, the number of donor-class recombinants was higher than the number of recipient-class recombinants.

Table 3. Symbiotic effectiveness of *R. meliloti* Tn5 mutants under greenhouse conditions

Strain†	Shoot dry mass		Total nitrogen	
	g	Difference from parent strain (%)	mg	Difference from parent strain (%)
CXM1-188	9.35	0	326.9	0
T47	9.54	2.0	322.2	-1.4
T129	9.73	4.1	327.0	0
T55	10.06	7.6	348.1	6.5
T91	10.19	9.0	341.1	4.3
T868 (Chr)	10.35	10.7	356.3*	9.9
T54 (Chr)	10.54*	12.7	347.9	6.4
T707 (Chr)	10.75*	15.0	355.8*	8.0
T624 (Chr)	10.89*	16.5	373.0*	14.1
T828 (Chr)	10.93*	16.9	373.0*	14.1
T748 (M1)	11.03*	18.0	374.1*	14.4
T810 (M2)	11.06*	18.3	373.3*	14.2
T841 (M1)	11.30*	20.9	372.7*	14.0
T798 (M2)	11.54*	23.4	416.0*	27.3
T716 (M1)	11.64*	25.6	402.0*	23.0
Uninoculated	8.05	—	260.1	—
LSD (0.05)‡	1.07		25.44	

* Significantly greater than the value for CXM1-188.

† The location of Tn5 is shown in parentheses: Chr, chromosome; M1, megaplasmid 1; M2, megaplasmid 2.

‡ LSD (least significant difference) was calculated not only for the 16 variants presented here, but also for 59 variants which were tested in the same greenhouse experiment.

Table 4. Symbiotic phenotypes of Km^r transductants isolated from crosses between Eff⁺⁺ donors and wild-type recipients

Each transductant was tested on 12 alfalfa plants. T482 was crossed with its parent CXM1-105, while the other mutants were crossed with their parent CXM1-188.

Donor	No. of transductants with effectiveness			Shoot dry mass (mg)			Pattern of inheritance*
	Of donor	Of recip.	Intermediate	Donor	Recip.	Transductants, $\bar{x}$	
T482	5	0	0	22.0	15.6	22.8	D
T54	7	0	2	19.3	14.0	19.9	D
T707	1	5	3	20.6	14.0	15.9	R
T798	19	2	21	18.3	14.0	18.7	D
T810	6	1	3	19.5	14.0	18.2	D
T828	4	0	1	19.7	14.0	20.1	D
T868	4	1	2	20.2	14.0	18.4	D
	46	9	32	LSD (0.05) = 4.10			

* The pattern of inheritance is denoted by 'D' (like donor type) if the mean value for transductants is significantly greater than the value for the recipient, and has no difference from that for the donor; 'R' (like recipient type) denotes an opposite relation between the same values.

Irrespective of the variation in shoot mass, the averaged values for the transductants from all but one cross showed unambiguously the donor type of inheritance (Table 4).

This allowed the conclusion to be drawn that Tn5 insertions can significantly increase symbiotic effectiveness of *R. meliloti*.

Fig. 1. Agarose gel electrophoresis of *R. meliloti* plasmids. Lane 1, recipient strain ZB121; lane 2, a recombinant strain which acquired megaplasmid 1 from the donor; lanes 3 and 5, recombinant strains which acquired megaplasmid 2 and pRP4-4; lane 4, a recombinant strain which acquired megaplasmid 1 and pRP4-4; lane 6, donor strain, which carries pRP4-4. M1, M2, megaplasmids 1, 2.

To exclude the possibility that the Eff^{++} phenotype of transductants was caused by a genetic event other than homologous exchange between donor and recipient DNA (e.g. integration of phage DNA into the bacterial genome, or $\text{Tn}5$ transpositions and associated rearrangements), transducing lysates were prepared from seven $\text{Tn}5$ -containing derivatives of CXM1-105 and CXM1-188 with unaltered symbiotic effectiveness. It was found that 33 out of 35 transductants showed the same symbiotic phenotype as donor and recipient. One transductant appeared to be significantly superior to the parents, another one inferior. This allowed the estimation to be made that uncontrolled genetic events contributed about 3% (1 out of 35) to the improved symbiotic phenotype.

Localization of $\text{Tn}5$ insertions in the *R. meliloti* genome

To localize the $\text{Tn}5$ insertions in the *R. meliloti* genome, cell lysates of the mutants were electrophoresed in Eckhardt gels and probed with the $\text{Tn}5$ -carrying plasmid pSUP2021, which was labelled with ^{32}P (Simarov *et al.*, 1990). The examination of 21 mutants revealed that 10 of them carried $\text{Tn}5$ in the chromosome and 11 carried it in one of the megaplasmids which comigrated in the agarose gel.

To distinguish between the megaplasmids, those which carried $\text{Tn}5$ were transferred into another genetic background using the *mob* system described by Simon *et al.* (1983). A mutant which was induced by $\text{Tn}5$ -*mob* and another one with an integrated plasmid pSUP2021 could readily be used as donors. Other mutants, whose megaplasmids did not contain a *mob* site, were conjugated with *E. coli* S17-1 carrying vector pSUP102-21 (Simon *et al.*, 1989). This vector was loaded with $\text{Tn}5$ -21, which allowed integration to occur at the site of $\text{Tn}5$ insertion. The frequency of such transconjugants was 10^{-4} , while rescue of the vector markers in $\text{Tn}5$ -free background of CXM1-105 or CXM1-188 occurred at a frequency of 10^{-8} . Following the introduction of a helper plasmid pRP4-4, the $\text{Tn}5$ mutants were conjugated with the *R. meliloti*

Table 5. Transfer of nodulation ability from Eff^{++} mutants to *A. tumefaciens* UBAPF2 and *R. meliloti* ZB121

Donor	Recipient	No. of transconjugants tested with the following phenotypes		Location*
		Nod ⁺	Nod ⁻	
T173	<i>A. tum.</i>	0	20	M2
	<i>R. mel.</i>	0	20	
T482	<i>A. tum.</i>	9	1	M1
	<i>R. mel.</i>	10	0	
T716	<i>A. tum.</i>	32	3	M1
	<i>R. mel.</i>	35	0	
T748	<i>A. tum.</i>	31	6	M1
	<i>R. mel.</i>	45	0	
T728	<i>A. tum.</i>	0	30	M2
	<i>R. mel.</i>	0	20	
T798	<i>A. tum.</i>	0	11	M2
	<i>R. mel.</i>	0	19	
T841	<i>A. tum.</i>	15	1	M1
	<i>R. mel.</i>	13	0	
T810	<i>A. tum.</i>	0	13	M2
	<i>R. mel.</i>	0	21	
T835	<i>A. tum.</i>	0	11	M2
	<i>R. mel.</i>	0	12	
T837	<i>A. tum.</i>	0	18	M2
	<i>R. mel.</i>	0	10	
T816	<i>A. tum.</i>	42	1	M1
	<i>R. mel.</i>	11	0	

* M1, megaplasmid 1; M2, megaplasmid 2.

deletion mutant ZB121 (Banfalvi *et al.*, 1981). The frequency of Nm^+ transconjugants was about 10^{-8} – 10^{-9} per recipient cell. Strain ZB121 contains three plasmids which have different electrophoretic mobilities (Banfalvi *et al.*, 1981); pRme41a, a cryptic plasmid of medium size (210 kb); pRme41b, a derivative of the symbiotic megaplasmid 1 with a deletion larger than 135 kb located in a region encoding nodulation and nitrogen fixation; and pRme41c, megaplasmid 2, which according to estimations of Burkardt and co-authors (Burkardt & Burkardt, 1984; Burkardt *et al.*, 1987), is about 1600 kb in size. Hynes *et al.* (1986) have shown that both megaplasmids of ZB121 could be replaced by the corresponding megaplasmids of other *R. meliloti* strains. When the megaplasmids of our mutants were mobilized into ZB121, the plasmid profiles of transconjugants were in agreement with those expected for the replacement of either megaplasmid (Fig. 1). Thus, the introduction of megaplasmid 1 led to the loss of a plasmid band corresponding to pRme41b, whereas the introduction of megaplasmid 2 did not alter the plasmid profile of the recipient (Fig. 1). It was found that some transconjugants acquired pRP4-4, and others did not. The

megaplasms marked with Tn5 were also mobilized to a plasmid-free *A. tumefaciens* strain UBAPF2 (Hynes *et al.*, 1985). All transconjugants showed the same plasmid pattern: a single band corresponding to one of the megaplasms.

In addition to the plasmid analysis, the transconjugants were examined for those phenotypic traits known to be determined by megaplasms 1 (nodulation ability) and megaplasms 2 (exopolysaccharide production). Both recipients ZB121 and UBAPF2 proved to be very convenient for this purpose (Hynes *et al.*, 1986), since they were unable to induce nodules on alfalfa (Nod⁻) and, in addition, ZB121 was deficient in exopolysaccharide production (Exo⁻). Following the introduction of megaplasms 1, both recipients acquired the Nod⁺ phenotype and showed the same exopolysaccharide production as the recipients. Following the transfer of megaplasms 2, the transconjugants could not nodulate alfalfa, but showed altered exopolysaccharide production: ZB121 transconjugants were Exo⁺, while UBAPF2 transconjugants were less mucoid than the recipient. The nodulation ability of transconjugants is presented in Table 5. ZB121 transconjugants containing megaplasms 1 restored the Nod⁺ phenotype in addition to the Fix⁺ phenotype. The phenotypic traits of all transconjugants tested were consistent with results obtained from the plasmid analysis of the ZB121 transconjugants. This allowed the conclusion to be made that five Eff⁺⁺ mutants donated megaplasms 1, and six of them donated megaplasms 2. It is interesting that the mutants which showed the highest symbiotic effectiveness in the greenhouse carried Tn5 in one of the megaplasms, while other mutants carried the transposon in the chromosome (Table 3).

DISCUSSION

Following the introduction of Tn5 into two *R. meliloti* strains, Nm^r transposants were screened for their ability to increase shoot mass of alfalfa plants grown in the absence of combined nitrogen. This allowed the isolation of mutants with improved symbiotic effectiveness. When tested in the greenhouse, eight Eff⁺⁺ mutants increased the shoot dry mass of the host plant by 15.0–25.6% and the plant's total nitrogen by 8.0–23.0% (all these differences were statistically significant at $P = 0.05$). The rate at which Eff⁺⁺ mutants were obtained was comparable to that of the auxotrophic mutants. This finding indicates that there are quite a number of genes which, when mutated, may enhance the *R. meliloti* symbiotic effectiveness.

Addressing the functions of the tagged genes, we propose that they are involved in the negative control of gene or protein activity. Evidence for genetic factors which suppress the effectiveness of symbiosis was supplied by the work of Pankhurst *et al.* (1986). They found that such a factor (or factors) was attributable to an indigenous plasmid of *Rhizobium loti* NZP2037. The loss of the plasmid resulted in the enhanced symbiotic effectiveness (Pankhurst *et al.*, 1986). If a gene affected by Tn5 insertion was not switched off, but acquired a read-through

promoter of Tn5, which could also be the case (Rostas *et al.*, 1986), it is difficult to speculate about the function of the gene.

To prove that the Eff⁺⁺ phenotype of the mutants was caused by Tn5 insertions, we transduced Tn5 from seven mutants to the parental strains. In all but one cross, transductants showing the donor symbiotic phenotype outnumbered those which had the recipient phenotype. Thus, transductants superior to the recipient comprised 53%, and those which retained the recipient's level of the effectiveness comprised 10%. All other transductants could not be related definitely to either donor or recipient class, since they showed an intermediate level of effectiveness. It is known that Eff⁺⁺ isolates can arise fortuitously as a result of genetic exchange between two strains. All the methods of genetic transfer which account for genetic exchange in *R. meliloti* (transformation, transduction, conjugation and spheroplast fusion), were shown to result in 1.3–4.8% Eff⁺⁺ recombinants (Simarov *et al.*, 1989). In our control experiments, where transduction of Tn5 was performed from donors to recipients with equal symbiotic effectiveness, the frequency of the Eff⁺⁺ phenotype was of the same order, about 3%. This frequency is substantially lower than that obtained using Eff⁺⁺ donors. If mutations leading to the Eff⁺⁺ phenotype were caused by Tn5 insertions and were expressed in an absolute manner (such as Nod⁻ and Fix⁻), one would expect 100% of the recombinants to be of the donor class. Our mutations, however, affected symbiotic effectiveness quantitatively. Perhaps this explains why only 53% of the recombinants exhibited the Eff⁺⁺ phenotype. In addition, variation of alfalfa plants could mask the symbiotic potential of *R. meliloti* strains (Provorov & Simarov, 1990).

By the use of physical and genetic methods, we located ten Tn5 insertions in the chromosome, five in megaplasms 1 and six in megaplasms 2. According to these data, genes which may enhance symbiotic effectiveness by mutation are distributed over different replicons.

The enhanced symbiotic effectiveness shown by the Tn5 mutants was not associated with any alteration in bacterial vegetative growth, number of nodules induced or rate of acetylene reduction. However, when the plant nitrogen accumulation was measured, Tn5 mutants appeared to be superior to their parents. Therefore it can not be excluded that the mutants are better nitrogen fixers than the parents. On the other hand, the mutants could increase plant growth by optimization of nitrogen assimilation, or through hormone or vitamin production. The biochemical and molecular genetic characterization of the mutants will permit the mechanisms involved to be found. This is the objective of our current investigation.

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