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Multicopy Plasmids Carrying the *Klebsiella pneumoniae nifA* Gene Enhance *Rhizobium meliloti* Nodulation Competitiveness on Alfalfa

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Three regulatory mutants of *Rhizobium meliloti* (NifA$^-$, NtrA$^-$, or NtrC$^-$) showed delayed nodulation and were less competitive when coinoculated with the wild-type strain 2011 on alfalfa roots. Whereas the defect for the NtrA$^-$ and NtrC$^-$ strains might be due to a reduced level of *nod* gene expression under nitrogen-limiting conditions, we found that the NifA$^-$ strain expressed a *nodC-lacZ* fusion at wild-type levels under all growing conditions tested. However, the reduced nodulation competitiveness shown by this NifA$^-$ strain does not seem to be due to its Fix$^-$ phenotype. When plasmid pCK3, carrying the *Klebsiella pneumoniae nifA* gene, was transferred into the three regulatory mutants, wild-type nodulation competitiveness was only restored in the case of the *nifA* mutant strain. However, such complementation did not restore symbiotic nitrogen-fixing ability. Furthermore, the introduction of multicopy plasmids containing the cloned *nifA* gene from *K. pneumoniae* into four wild-type *R. meliloti* strains resulted in a generalized increase of their respective competitive abilities.

Additional keywords: competition, *Medicago sativa*, nodule occupancy.

Symbiotic nitrogen fixation is a complex process that requires the expression of specific plant and bacterial genes. The molecular genetics of the *Rhizobium meliloti* alfalfa symbiosis, particularly of the bacterial partner, has been relatively well characterized. Among the bacterial genes involved in late stages of the symbiosis are those that regulate and encode for polypeptides required for nitrogen fixation (*nif* and *fix* genes) (Ruvkun *et al.* 1982; Szeto *et al.* 1984; Buikema *et al.* 1987; Earl *et al.* 1987). Positive regulation of transcription of *nif* and some *fix* genes under symbiotic conditions requires the product of *nifA* (Szeto *et al.* 1984; Weber *et al.* 1985), the positive regulator of *nif* genes in aerobic and facultative anaerobic diazotrophic organisms (Gussin *et al.* 1986). Expression of *nifA* in *R. meliloti* is activated in response to oxygen limitation via the regulatory gene pair *fixLJ* (Ditta *et al.* 1987; David *et al.* 1988), and this activation is independent of the fixed-nitrogen status of the cell. Activation of *nif* transcription by *nifA* also requires the sigma factor encoded by *ntrA* (*rpoN*) (Ronson *et al.* 1987).

Recent reports have suggested that *nifA* controls not only nitrogenase and related genes, but also other genes involved in nodule maturation and bacteroid persistence in symbiotic bacteria (Fischer *et al.* 1986; Hirsch and Smith 1987). In *R. leguminosarum* expression of nonsymbiotic genes (e.g., *melA*) have been demonstrated to be *nifA*-regulated (Hawkins and Johnston 1988). Synthesis of a rhizopine by *R. meliloti* in alfalfa nodules has been reported to be *nifA*-dependent (Murphy *et al.* 1988). We have also reported that expression of *nfe* genes (nodule formation efficiency) involved in nodulation competitiveness of a particular *R. meliloti* strain GR4, are under control of *nifA* (Sanjuan and Olivares 1989). The expression of *nfe-lacZ* fusions has been demonstrated to be activated by *nifA* from *R. meliloti* or *Klebsiella pneumoniae* (Sanjuan and Olivares 1991). Because *nfe* homologous genes were not detected in other wild-type strains of *R. meliloti* (Sanjuan and Olivares 1991), we hypothesized that these strains might possess different *nifA*-regulated genes required for nodulation competitiveness. In this report we present evidence supporting a generalized role of *nifA* in the nodulation competitiveness of *R. meliloti*.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *R. meliloti* strains 1354 (*nifA*::Tn*5*), 1681 (*ntrA*::Tn*5*), and 5002 (*ntrC*::Tn*5*) were provided by F. M. Ausubel, and strain RS81 (*nifH*::Tn*5*) was provided by A. Pühler. All mutant strains are derivatives of the well-characterized wild-type strain 2011 (obtained from J. Denarié). Wild-type strains L5.30 and 41 were provided by M. Kowalski and A. Kondorosi, respectively. Strain GRO13 is a GR4 derivative carrying *nfe* genes on plasmid pRmeGR4b (Toro and Olivares 1986). Plasmid pCK3 is a pRK290 derivative carrying the *Bam*HI fragment from plasmid pMC73A, which contains the *K. pneumoniae nifA* gene (Buchanan-Wollaston *et al.* 1981) cloned into the *Bgl*II site of pRK290. pCK1 is a pKT230 derivative carrying the *Sal*I fragment from pMC71A, also containing *K. pneumoniae nifA*, cloned into the *Xho*I site of pKT230 (Kennedy and Robson 1983). pCK3 encodes resistance to tetracycline and pCK1 encodes resistance to streptomycin and kanamycin. *nifA* is constitutively expressed from the kanamycin gene promoter in both

plasmids. Plasmid pRmM57 is a pRmSL26 derivative carrying a *R. meliloti nodC-lacZ* fusion (Mulligan and Long 1985).

Media, growth conditions, and bacterial matings. *Escherichia coli* was cultured on Luria-Bertani medium and rhizobial strains on TY (tryptone-yeast extract-$CaCl_2$) medium. Tetracycline (10 μg/ml), kanamycin (25 μg/ml for *E. coli* and 200 μg/ml for *R. meliloti*), and streptomycin (50 μg/ml for *E. coli* and 250 μg/ml for *R. meliloti*) were added as required. Transfer of plasmids pCK3, pCK1, and pRmM57 into *R. meliloti* was performed by using the triparental mating protocol as described by Ditta *et al.* (1980). Transconjugants were selected on *Rhizobium* minimal medium (Robertsen *et al.* 1981) supplemented with the indicated antibiotics.

Symbiotic assays. *Medicago sativa* L. 'Aragon' plants were grown under axenic conditions in 20- × 200-mm test tubes containing nitrogen-free plant nutrient solution according to Olivares *et al.* (1980). Time course nodulation and competition experiments were performed according to Sanjuan and Olivares (1989). For competition assays 2-wk-old alfalfa plants were inoculated with mixtures (2×10^7 cells) of two strains in a 1:1 ratio of viable cells. After 12–15 days, nodules were collected, surface sterilized for 5 min in 0.25% $HgCl_2$, crushed, and streaked on TY agar with or without the appropiate antibiotics. Plates were incubated at 28° C for 3–4 days, and the identity of strains was determined. Nitrogenase activity of nodulated plants was measured by the acetylene reduction assay as previously described by Bedmar and Olivares (1980).

β-Galactosidase assays. *R. meliloti* strains carrying plasmid pRmM57 were precultured at 28° C on TY medium. Bacteria were pelleted and washed with nitrogen-free minimal medium (Dusha *et al.* 1989). After resuspension, samples were diluted with the same medium and nitrogen sources were added (ammonium sulfate, 3 or 100 mM). Samples were induced by luteolin (10 μM final concentration) for 8 hr. β-Galactosidase activity was determined as described by Miller (1972), except that chlorophenol red-β-D-galactopyranoside (Boehringer Mannheim, Indianapolis, IN) was used as a substrate instead of 2-nitrophenyl-β-D-galactopyranoside.

RESULTS

Nodulation efficiency and competition phenotype of *R. meliloti* mutants. The level of nodulation of alfalfa roots inoculated with *R. meliloti* strains 1354 ($NifA^-$), 1681 ($NtrA^-$), 5002 ($NtrC^-$), and RS81 ($NifH^-$, used as a Fix^- control) was measured with respect to the wild-type strain 2011. At day 9 after inoculation, strains 2011 and RS81 had consistently nodulated 100% of plants, whereas strains 1354, 1682, and 5002 exhibited a slight delay of 1 day (Fig. 1). The number of nodules elicited by each strain was similar until 12 days after inoculation (Fig. 2). At first the $NtrA^-$ and the $NtrC^-$ mutants formed fewer nodules than the wild-type strain; however, by day 12 they had the same values. Beyond day 12, the number of nodules formed by the Fix^+ strains 5002 and 2011 remained virtually constant, while the number of nodules formed by the Fix^- strains 1354, 1681, and RS81 continued to increase to twice the wild-type value (Fig. 3). These data suggested that the Fix character of the bacteria is important in controlling the nodule number, in contrast to those by Fischer *et al.* (1986), who reported that only inoculation with *Bradyrhizobium japonicum* $NifA^-$ strains lead to overnodulation of soybean roots.

Nodules induced by Fix^- strains were white, generally smaller than those formed by Fix^+ strains, and clustered along the root. Nodules elicited by the *nifA* strain 1354 were variable in size, some being as elongate as Fix^+ nodules. A detailed description on the ultrastructure of these nodules has been reported previously (Hirsch and Smith 1987). Coinoculation experiments showed that the three regulatory mutants were less competitive relative to strain 2011. Strains 1354, 1681, and 5002 occupied less than 10% of the nodules when coinoculated with the wild type (Table 1). In contrast, the $NifH^-$ strain RS81 occupied approximately 50% of the nodules and therefore was equally competitive as the wild type (Table 1). These results suggested that *nifA, ntrA,* and *ntrC* genes are important for the expression of the competitive ability of strain 2011. On the other hand, the competition phenotype was independent of the Fix^+ or Fix^- character of the strains.

Dusha *et al.* (1989) reported a reduced level of *nod* gene expression in *R. meliloti* $NtrA^-$ or $NtrC^-$ strains grown under nitrogen-limiting conditions and suggested that this might be the cause of their delayed nodulation phenotype. We investigated whether the nodulation phenotype of the $NifA^-$ strain 1354 might also be due to a reduced expression

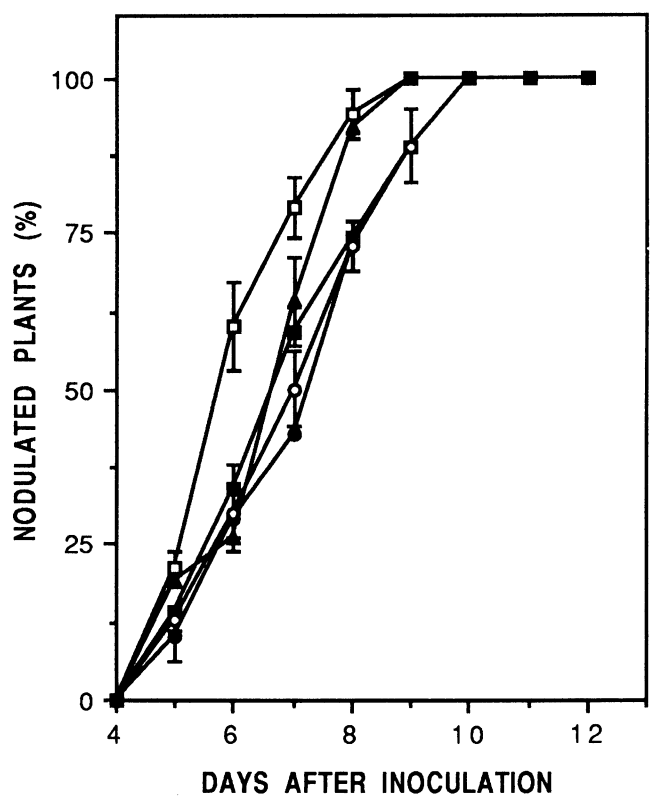

Fig. 1. Nodulation kinetics of alfalfa plants inoculated with *Rhizobium meliloti* mutant or wild-type strains. (■) 1354, (●) 1681, (○) 5002, (▲) RS81, and (□) wild-type strain 2011. Each point represents the mean ± standard error (bars) of three independent experiments (15 plants each).

of *nod* genes and found that this strain expressed a *nodC-lacZ* fusion (encoded in plasmid pRmM57) at wild-type levels under all growing conditions tested, unlike the *ntrA* or *ntrC* mutant strains, which showed a reduced level of *nod* gene expression under nitrogen-limiting conditions (data not shown).

Competitiveness of *R. meliloti* carrying *nifA*-expressing plasmids. Several reports have demonstrated that the *K. pneumoniae nifA* gene cannot restore nitrogen-fixing ability to *Rhizobium nifA* mutants. (Better *et al.* 1985; Hawkins and Jonhston 1988). In contrast, multicopy plasmids carrying the *R. meliloti nifA* gene have been reported to influence, both negatively and positively, nitrogen fixation in alfalfa nodules (Cannon *et al.* 1988). We investigated whether *nifA* from *K. pneumoniae* might be able to increase the nodulating competitiveness of the NifA$^-$ strain 1354. Plasmid pCK3, which carries the *K. pneumoniae nifA* gene, was transferred into strain 1354 and strains 1681, 5002, and RS81, and the nodulation and competitive abilities of the corresponding transconjugants were studied in relation to the parental or wild-type strains. Strains 1354(pCK3) and 5002(pCK3) showed no nodulation delay when compared with the wild type. In addition, no changes from the parental phenotype were observed in the 1681 and RS81 derivatives carrying plasmid pCK3 (data not shown). Strains carrying plasmid pCK3 elicited a similar number of nodules to that by their respective parental strains (data not shown). Two-strain coinoculation experiments showed that strain 1354(pCK3) exhibited wild-type competitive ability (Table 1, lines 5 and 6), but no changes were detected in strain 1681(pCK3) relative to the behavior of the parental strain (Table 1, lines 7 and 8). Nodules induced by strain 1354(pCK3) were still Fix$^-$ (data not shown). Strain 5002(pCK3), in coinoculation with the parental 5002, occupied 80–90% of nodules (Table 1, line 9). However, this enhancement of competitiveness was not apparent when coinoculated with the wild-type strain 2011 (Table 1, line 10). Strain RS81(pCK3) was more competitive

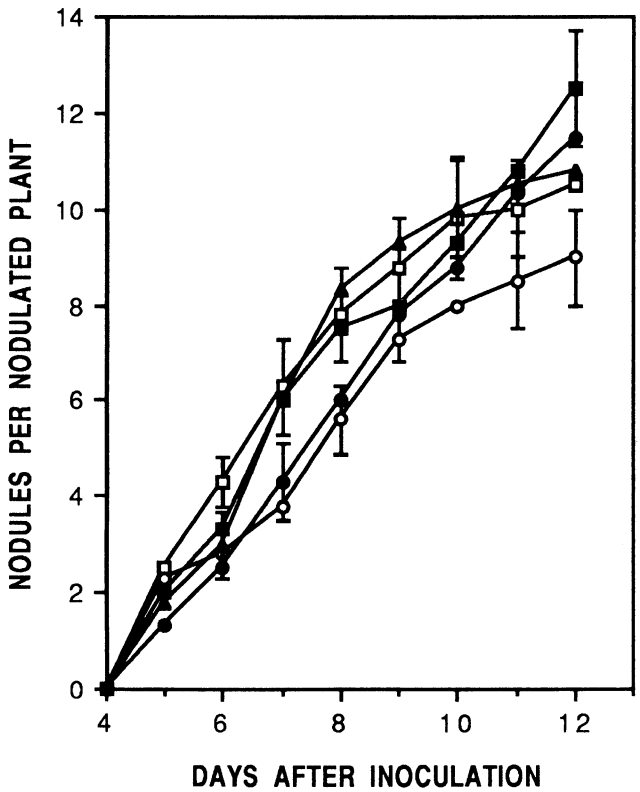

Fig. 2. Kinetics of nodule formation on alfalfa plants inoculated with *Rhizobium meliloti* mutant or wild-type strains. (■) 1354, (●) 1681, (○) 5002, (▲) RS81, and (□) wild-type strain 2011. Each point represents the mean ± standard error of three independent experiments (15 plants each).

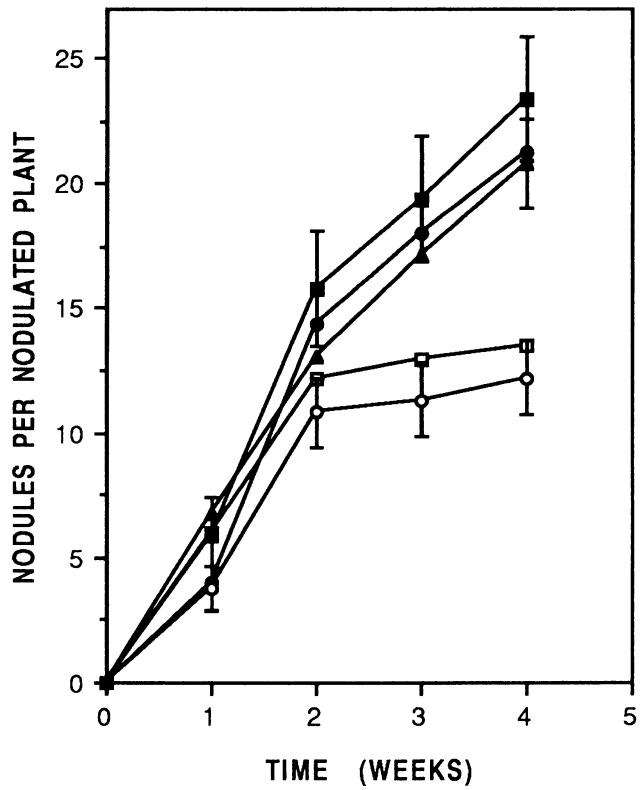

Fig. 3. Nodulation patterns of alfalfa plants inoculated with Fix$^+$ or Fix$^-$ strains of *Rhizobium meliloti*. (■) 1354, (●) 1681, (○) 5002, (▲) RS81, and (□) wild-type strain 2011. Each point represents the mean ± standard error of three independent experiments (15 plants each).

Table 1. Competition studies with *Rhizobium meliloti* mutants and complementation with plasmid pCK3

Strains in the inoculum		Nodules occupied by strain (%)[a]	
A	B	A	B
1354 *nifA*	2011	9 ± 4	91 ± 4
1681 *ntrA*	2011	9 ± 3	91 ± 3
5002 *ntrC*	2011	12 ± 5	88 ± 5
RS81 *nifH*	2011	51 ± 8	49 ± 8
1354(pCK3)	1354	86 ± 8	14 ± 8
1354(pCK3)	2011	46 ± 9	54 ± 9
1681(pCK3)	1681	53 ± 12	47 ± 12
1681(pCK3)	2011	19 ± 7	81 ± 7
5002(pCK3)	5002	89 ± 8	11 ± 8
5002(pCK3)	2011	17 ± 6	83 ± 6
RS81(pCK3)	RS81	81 ± 9	19 ± 9
RS81(pCK3)	2011	83 ± 4	17 ± 4

[a] Data are the mean of three experiments (50 nodules each) ± standard error.

than the parental or the wild-type strains (Table 1, lines 11 and 12). When plasmid pRK290 was transferred into the four mutant strains, no changes in the nodulation competitiveness were detected (data not shown).

Multicopy plasmids expressing the *K. pneumoniae nifA* gene (pCK1 or pCK3) were also introduced into several wild-type strains of *R. meliloti*, including strain 2011 and the *nfe*-carrying strain GRO13, and the nodulation competitiveness of the resulting strains was studied. As shown in Table 2, four strains tested showed a very significant enhancement of their respective competitive abilities upon acquiring the *nifA* plasmid. However, no changes were observed in the level of nitrogenase activity in nodules formed by these strains (data not shown).

DISCUSSION

Competition for nodulation of legumes between indigenous soil rhizobia and introduced selected *Rhizobium* strains can limit successful improvement of legume yields. Diverse ecological and biological factors, including the host and the bacterial genomes, have been suggested to influence competitive nodulation in soil (Dowling and Broughton 1986). We have recently reported the existence of *nifA*-regulated genes (*nfe*) in *R. meliloti* strain GR4 involved in competition for nodulation (Sanjuan and Olivares 1989). Genes homologous to *nfe* have not been detected in other strains of *R. meliloti* (Sanjuan and Olivares 1991). However, we are currently working on the hypothesis that there are further *nifA*-regulated genes in all strains of *R. meliloti* involved in interstrain competition for nodulation. In accordance with this hypothesis, a *nifA* mutant derivative of *R. meliloti* 2011, similar to a *ntrA* mutant, showed a very diminished competitive ability. This defect does not appear to be due to the Fix^- phenotype of the $NifA^-$ or $NtrA^-$ strain, since the $NifH^-$ strain RS81 was as competitive as the wild type. Interestingly, another regulatory mutant, the $NtrC^-$ strain 5002, which forms nitrogen-fixing nodules, also showed a competition defective phenotype. Our results agree with those reported by Dusha *et al.* (1989) demonstrating that *R. meliloti ntrA* or *ntrC* mutants show a delayed nodulation phenotype, probably due to a reduced level of *nod* gene expression under limiting-nitrogen conditions. In addition, we now demonstrate that these *ntr* mutants also show a competition defective phenotype, probably due to the low level of induction of *nod* genes. The *nifA* mutant strain 1354 also showed delayed nodulation and decreased competitive ability, but in contrast to the *ntrA* and *ntrC* mutant strains, it exhibited wild-type levels of induction of a *nodC-lacZ* fusion. Therefore, it appears unlikely that the nodulation defective phenotype shown by this mutant is due to reduced levels of *nod* gene expression.

The *K. pneumoniae nifA*-expressing plasmid pCK3 enabled strain 1354 to recover wild-type nodulating competitiveness, but did not restore nitrogen-fixing ability in alfalfa nodules. Because plasmid vectors alone had no effect on the competitiveness of this strain, it should be considered that the effects observed are due to expression of *K. pneumoniae nifA* from plasmid pCK3. The failure to complement nitrogen fixation may be due to the inability of the *K. pneumoniae* NifA to induce *R. meliloti nif* expression to a level sufficient for nitrogen fixation. An alternative explanation is that in this *R. meliloti nifA* mutant strain the Tn*5* insertion could have polar effects on genes located downstream. Plasmid pCK3 did not complement the nodulation phenotype of the $NtrA^-$ strain 1681 and provided only partial enhancement of the competitiveness of the *ntrC* strain 5002 (i.e., strain 5002[pCK3] showed increased competitive ability when compared with the parental strain but not when compared with the wild type; Table 1, lines 9 and 10). In contrast, plasmid pCK3 conferred greater competitiveness on the *nifH* strain RS81 when coinoculated with the wild-type strain 2011 (Table 1, line 12). One possible explanation for these results is that the constitutive expression of the *K. pneumoniae nifA* led to an overexpression, or alternatively to an earlier expression, of genes involved in competition for nodulation. Our investigations on nodulation competition in *R. meliloti* indicate that 1) there is no apparent relationship between nitrogen-fixation activity and competitive ability; 2) the regulatory gene *nifA* appears to be important to the nodulation competitiveness of *R. meliloti*, and 3) multicopy plasmids expressing *nifA* gene can enhance the competitiveness of *R. meliloti*. The lack of a strong effect of multicopy NifA in a $NtrA^-$ or $NtrC^-$ mutant may be due to the reduced level of *nod* gene expression in these strains. The positive effect of *nifA*-multicopy plasmids on the competitive ability of *R. meliloti* is further supported by the results shown in Table 2. The competitive ability of four wild-type strains was significantly enhanced upon acquiring pCK3 or pCK1. The results with strain GRO13 can be explained by an overexpression of the previously identified *nfe* genes (Sanjuan and Olivares 1989, 1991). However, an alternative explanation, namely, the existence of *nfe*-analogous genes might explain the positive effects observed for the other three wild-type strains that do not carry *nfe*-homologous DNA.

Results reported here provide new evidence for the existence in *R. meliloti* of as yet undiscovered *nifA*-regulated genes, apparently involved in interstrain competition for nodulation. Identification of these genes will help further to understand the regulatory role of NifA in *R. meliloti*.

Table 2. Competition experiments with *Rhizobium meliloti* wild-type strains carrying plasmids pCK1 or pCK3

Strains in the inoculum		Nodules occupied by strain (%)[a]	
A	B	A	B
2011	2011(pCK3)	5 ± 3	95 ± 3
L5.30	L5.30(pCK3)	22 ± 4	78 ± 4
GRO13	GRO13(pCK1)	7 ± 5	93 ± 5
41	41(pCK1)	13 ± 5	87 ± 5

[a] Data are mean of three independent experiments ± standard error.

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