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NifA-NtrA regulatory system activates transcription of *nfe*, a gene locus involved in nodulation competitiveness of *Rhizobium meliloti*

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Abstract. We have previously demonstrated that the *Rhizobium meliloti* large plasmid pRmeGR4b carries the gene locus nodule formation efficiency (*nfe*) which is responsible for nodulation efficiency and competitive ability of strain GR4 on alfalfa roots. In this study we report that expression of *nfe-lacZ fusions* in *Escherichia coli* is activated in the presence of the cloned *nifA* gene of *R. meliloti*. This activation was found to be oxygen sensitive and to require the *E. coli ntrA* gene product. In contrast to the *R. meliloti nifA*, the cloned *nifA* gene of *Klebsiella pneumoniae* was able to activate expression of *nfe* in aerobically grown cells of both *E. coli* and *R. meliloti*. Hybridization experiments did not show homology to *nfe* in four *R. meliloti* wild-type strains tested. These strains were uncompetitive when coinoculated with a GR4 derivative carrying plasmid pRmeGR4b, but were competitive when coinoculated with a GR4 derivative carrying a single transposon mutation into the *nfe* region. When *nfe* DNA was introduced into the four wild-type strains, a significant increase in the competitive ability of two of them was observed, as deduced from their respective percentages of alfalfa root nodule occupancy in two-strains coinoculation experiments.

Key words: Alfalfa – Nodulation competitiveness – *Rhizobium meliloti* – NiFA – NtrA regulation

Studies on nitrogen fixation in the soil bacteria *Rhizobium* and *Bradyrhizobium*, which fix atmospheric nitrogen in association with legumes, have been facilitated by the identification and characterization of *nif* genes in *Klebsiella pneumoniae* (Gussin et al. 1986). In the alfalfa endosymbiont *R. meliloti nif* genes map on a large plasmid (megaplasmid), called pSym, along with other genes involved in nodulation (*nod*) and nitrogen fixation (*fix*) (Long et al. 1982; Batut et al. 1985). Like in *K. pneumoniae*, positive regulation of *nif* and some *fix* genes in *R. meliloti* requires *nifA* and the sigma factor encoded by *ntrA* (*rpoN*) (Szeto et al. 1984; Weber et al. 1985; Gussin et al. 1986; Ronson et al. 1987). However, while in *K. pneumoniae nifA* expression is controlled via *ntrC* by the level of fixed nitrogen available to the cell, expression of *nifA* in *R. meliloti* is regulated by oxygen via the regulatory gene pair *fixLJ* (Ditta et al. 1987; David et al. 1988).

In addition to the Nif$^-$ phenotype shown by *nifA* mutants of *R. meliloti*, nodules induced by these mutants are subjected to severe bacteroid degradation, i.e., bacteroids rarely mature to a fully differentiated state (Hirsch and Smith 1987). A similar bacteroid phenotype has been found for *B. japonicum nifA* mutants (Fischer et al. 1986) suggesting that there may be other symbiotic genes involved in bacteroid differentiation and persistence which are regulated by *nifA*.

We have recently demonstrated that a 5 kb DNA region, named *nfe* (nodule formation efficiency), located on the large plasmid pRmeGR4b contains genes involved in nodulation efficiency and competitiveness of *R. meliloti* strain GR4 on alfalfa roots (Sanjuan and Olivares 1989). Expression of the *nfe* locus was found to be activated in microaerobically free-living cells and in alfalfa nodules of the wild-type strain, but neither in *nifA* nor in *ntrA* mutants of *R. meliloti*. Supporting the hypothesis that *nifA* could be the positive regulator of *nfe* expression, a 206 bp DNA sequence (*Pnfe*) highly homologous to *nif* promoters was characterized upstream of *nfe*.

In this study, we report that expression of *nfe* genes is activated, in the absence of other *R. meliloti* genes, by the Nifa-NtrA regulatory system that controls *nif* trancription in *Rhizobium*. The possible spread of *nfe* DNA among strains of *R. meliloti* was investigated. The efficiency of nodulation and competitive ability of other well-characterized *R. meliloti* wild-type strains were compared with GR4 derivatives carrying or not carrying functional *nfe* genes. A role of the *nfe* region in the competitive nodulation of alfalfa was established.

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Table 1. Bacterial strains and plasmids

	Genotype	Source or reference
Strains and plasmids		
E. coli		
ET8000	*rbs lacZ::ISl gyrA hutC*^{c}	MacNeil et al.
ET8045	ET8000 *ntrA*::Tn*10*	MacNeil et al.
HB101	*pro leu thi lacY endA recA hsdR hsdM Str*^{r}	Boyer and Roulland-Dussoix
R. meliloti		
GR4	Wild-type, Nod$^+$ Fix$^+$	This laboratory
GRO13	GR4 derivative, harbouring pRmeGR4b	Toro and Olivares
GRO48	GRO13 derivative, Str^r Rif^r	Sanjuan and Olivares
GRO48-30	GRO48 derivative with TN*3*HoHo1 into *nfe*	Sanjuan and Olivares
2011	Wild-type, Nod$^+$ Fix$^+$	J. Denarié
L5.30	Wild-type, Nod$^+$ Fix$^+$	M. Kowalski
41	Wild-type, Nod$^+$ Fix$^+$	A. Kondorosi
104A14	Wild-type, Nod$^+$ Fix$^+$	G. Ditta
Plasmids		
pRK2013	Helper plasmid, Kan^r	Figurski and Helinski
pRmNT40	21 kb of pRmeGR4b, cloned in pRK290, Tc^r	Toro and Olivares
pCK1	*nifA* of *K. pneumoniae* cloned in pKT230, Sm^r	Kennedy and Robson
pRmW54.10	*nifA* of *R. meliloti* cloned in pACYC177-C, Cm^r	Weber et al.
pRmNT40-30, -25, -5	pRmNT40 with *lacZ*(Tn*3*HoHo1) fusions into *nfe*	Sanjuan and Olivares
pMB210	*R. meliloti nifH-lacZ* fusion in pGD926, Tet^r	Better et al.

Materials and methods

Strains and plasmids

The bacterial strains and plasmids used are listed in Table 1.

Microbiological techniques

Routine growth of *E. coli* strains was performed at 37°C in LB medium. *R. meliloti* strains were grown in TY medium (tryptone-yeast extract-$CaCl_2$) at 28°C. *E. coli* cultures used for β-galactosidase tests were grown in NFDM medium (Cannon et al. 1974) supplemented with L-glutamine (2 mg · ml^{-1}) at 28°C. Microaerobic cultures were done by the STA (stoppered-tube assay) technique (Ditta et al. 1987) with an initial oxygen tension of 2%. β-Galactosidase activity was determined according to Miller (1972). Plasmids were transferred into *Rhizobium* by conjugation in triparental matings using pRK2013 as helper plasmid (Ditta et al. 1980).

Hybridization procedures

Plasmid pRmNT40 was *Eco*RI digested and a 2.1 kb DNA fragment internal to the *nfe* region was isolated from low-melting agarose by the method of Langridge et al. (1980). In vitro α-^{32}P labelling of purified DNA was accomplished by the nick translation procedure of Rigby et al. (1977). Total DNA from Eckhardt agarose gels (Eckhardt 1978) or previously-isolated *Eco*RI-digested total DNAs were transferred to nitrocellulose filters by the method of Southern (1975). Conditions for hybridization were as described by Maniatis et al. (1982).

Nodulation tests and competition assays

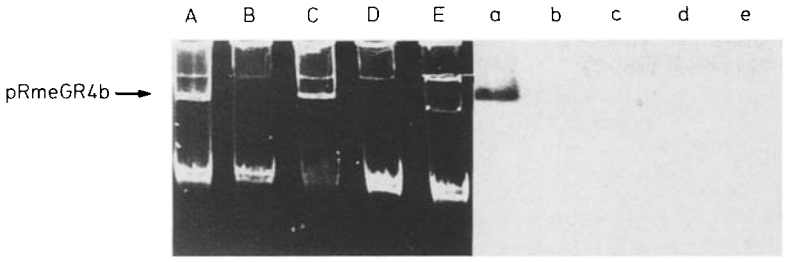
The technique of Olivares et al. (1980) was followed for axenic culture of alfalfa (*Medicago sativa* L., ecotype Aragon) in 20 × 200 mm test tubes. For nodulation tests fifteen individually cultured plants were inoculated with 10^7 cells of each strain. After inoculation, the number of nodulated plants and the number of nodules per plant were recorded daily.

For competition assays, 2-weeks-old alfalfa plants (5 plants per tube) were inoculated with mixtures (2×10^7 cells) of two strains (5 tubes were mixture), in a 1:1 ratio of viable cells. In each mixture the strains were marked with different antibiotic resistances. 12–15 days after inoculation, nodules were collected, surface sterilized for 5 min in 0.25% $HgCl_2$, washed in sterile water, crushed and sequentially stabbed on TY agar with and without the appropiate antibiotics. After incubation at 28°C plates were inspected for the identity of the strains. We studied the stability of plasmid pRmNT40 in nodules. When a strain carrying this plasmid was inoculated on alfalfa, most of nodules formed (98%) contained an average of 62% of bacteria carrying plasmid pRmNT40.

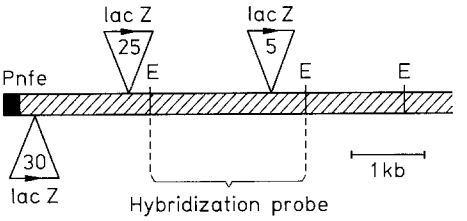
Results

Transcription of nfe is activated by nifA of R. meliloti and K. pneumoniae

The recombinant plasmid pRmNT40 contains the previously identified *nfe* region involved in nodulation competitiveness of *R. meliloti* GR4 (Toro and Olivares 1986; Sanjuan and Olivares 1989). To test if *nfe* responded to activation by *nifA*, three pRmNT40 derivative *lacZ*-fusion plasmids, obtained by Tn*3*-HoHo1 insertion into *nfe* (Sanjuan and Olivares 1989), were individually introduced by transformation into the *lacZ* strain *E. coli* ET8000 and into ET8000 derivatives containing the plasmids pCK1 or pRmW54.10. In plasmid pRmNT40-30 the *lacZ* fusion is located about 0.3 kb downstream of P*nfe*, while in plasmids pRmNT40-25 and pRmNT40-5 *lacZ* is located 1.5 and 3.3 kb downstream of P*nfe*, respectively (Fig. 1; Sanjuan and Olivares 1989). A *R.*



A



B

Fig. 1. **A** Eckhardt agarose gel of lysates of several wild-type strains of *R. meliloti* and DNA hybridization to a *nfe* DNA probe. *A* GR4; *B* 2011; *C* 41; *D* 104A14; *E* L5.30. A similar result was obtained with hybridization to isolated and *Eco*RI-digested total DNAs (not shown). **B** Location of *nfe-lacZ* fusions with respect to the *nif* promoter-like sequence P*nfe*. Numbers 30, 25 and 5 refer to *lacZ* fusions in plasmids pRmNT40-30, pRmNT40-25 and pRmNT40-5, respectively. The hatched region represents *nfe*. The 2.1 kb *Eco*RI DNA fragment used as hybridization probe is indicated

Table 2. Activation of *nfe-lacZ* fusions by *nifA*

Plasmids	Units of β-galactosidase					
	ET8000[a]		ET8045[a]		GRO13[c]	
	$+O_2$	$-O_2^b$	$+O_2$	$-O_2^b$	$+O_2$	$-O_2^b$
pMB210	95	64	78	70	45	1353
pRmNT40-30	55	42	31	33	9	142
pRmNT40-25	69	53	29	31	7	115
pRmNT40-5	68	51	34	30	7	140
pMB210+pRmW54.10	684	6435	82	96	–	–
pRmNT40-30 + pRmW54.10	239	1914	35	51	–	–
pRmNT40-25 + pRmW54.10	251	1719	33	35	–	–
pRmNT40-5 + pRmW54.10	212	2016	35	29	–	–
pMB210 + pCK1	5101	5840	69	85	1930	ND
pRmNT40-30+pCK1	1764	2184	44	37	227	ND
pRmNT40-25+pCK1	1815	1840	37	40	208	ND
pRmNT40-5+pCK1	1860	1965	39	41	211	ND

[a] Strains were grown overnight in NFDM plus glutamine at 28° C
[b] Bacteria grown in tightly closed screw capped tubes with initial 2% O_2, 98% N_2 atmosphere
[c] Strains were grown overnight at 28° C in TY; ND, not determined

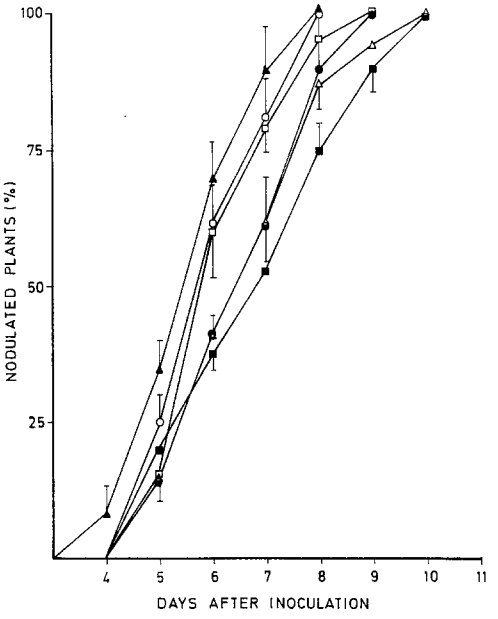


Fig. 2. Nodulation kinetics of *R. meliloti* strains on alfalfa roots. (○) GRO48; (●) GRO48-30; (□) 2011; (△) L5.30; (■) 104A14; (▲) 41. Each point represents the mean ± standard error (*bars*) of three independent experiments (15 plants each)

meliloti NifH-lacZ fusion (plasmid pMB210) was used as a control of *nifA* activity. The three *nfe-lacZ* fusions expressed an increased β-galactosidase activity when plasmid pCK1 or pRmW54.10 were present, as did the *nifH-lacZ* fusion (Table 2). While *nifA* from *K. pneumoniae* could activate β-galactosidase expression in the presence and in the absence of oxygen, activation of the four fusions by *R. meliloti nifA* in *E. coli* was found to be oxygen sensitive. However, it remained a low level of β-galactosidase expression in the presence of high level of oxygen (Table 2). This could be due to amplification of residual NiFa activity due to the multi-copy character of the *nifA* plasmid pRmW54.10.

To test if the *nifA*-mediated activation of *nfe* in *E. coli* also requires *ntrA*, induction of β-galactosidase from the three *nfe-lacZ* and the *nifH-lacZ* fusion plasmids was assayed in the *E. coli ntrA* mutant strain ET8045 containing pCK1 or pRmW54.10. Results indicated that transcription of *nfe* requires the *ntrA* gene product of *E. coli*, since β-galactosidase activities were at background levels in all the cases (Table 2).

Activation of *nfe-* and *nifH-lacZ* fusions also was detected in aerobic cultures of a GRO13 derivative carrying the *K. pneumoniae nifA* gene cloned in pCK1 (Table 2). In this case the level of activation was significantly lower than in *E. coli*.

Role of nfe in the nodulation competitiveness of R. meliloti

We investigated whether *nfe* homologous genes exist in other strains of *R. meliloti*. *Eco*RI-digested total DNAs and Eckhardt lysates DNAs of the wild type strains 2011, L5.30, 41 and 104A14 were transferred to nitrocellulose filters and hybridized to a 2.1 kb *Eco*RI DNA fragment internal to the *nfe* region (Fig. 1). No hybridizing DNA was detected in any of the strains investigated (Fig. 1),

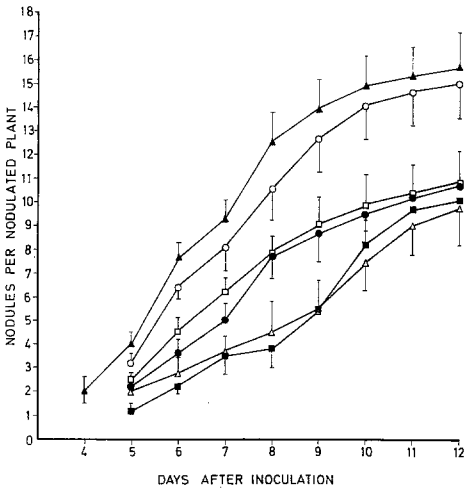


Fig. 3. Kinetics of nodule formation of *R. meliloti* strains on alfalfa roots. (○) GRO48; (●) GRO48-30; (□) 2011; (△) L5.30; (■) 104A14; (▲) 41. Each point represents the mean ± standard error of three independent experiments

Table 3. Results derived from competition studies between *R. meliloti* strains

Strains in the mixed inoculum		% nodules occupied by strain[a]	
A	B	A	B
GRO48	2011	87 ± 5	13 ± 5
GRO48	L5.30	82 ± 3	18 ± 3
GRO48	104A14	97 ± 8	3 ± 8
GRO48	41	97 ± 5	3 ± 5
GRO48	GRO48-30	92 ± 4	8 ± 4
GRO48-30	2011	51 ± 5	49 ± 5
GRO48-30	L5.30	55 ± 5	45 ± 5
GRO48-30	104A14	59 ± 6	41 ± 6
GRO48-30	41	54 ± 7	46 ± 7

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

suggesting that *nfe*-like DNA is not present in these strains.

The nodulating competitiveness of strain GRO48 with respect to these four strains lacking *nfe* was further studied. Figure 2 shows that all strains nodulated 100% of the plants within 12 days after inoculation. Strains GRO48 and 41 were the most rapid strains and nodulated 100% of plants 8 days after inoculation, whereas plants inoculated with GRO48-30 nodulated by day 9. Strain 2011 was slightly delayed and L5.30 and 104A14 were delayed by 2 days with respect to GRO48. The number of nodules elicited by each strain were closely related to their respective nodulation times as slower strains formed fewer nodules per plant during the first 12 days after inoculation (Fig. 3).

Results derived from competition for nodulation experiments showed that strain GRO48 was the most competitive of those studied (Table 3). When coinoculated with any of the wild-type strains GRO48 was recovered from most of nodules formed. Nodule occupancy ranged from 82% of nodules in the GRO48/L5.30 mixture to 97% when GRO48 was competing with strain 41. As previously reported (Sanjuan and Olivares 1989) the *nfe* mutant strain GRO48-30 was uncompetitive relative to GRO48 (Table 3) and when it was coinoculated with any of the wild-type strains of *R. meliloti*, the percentage of nodules occupied by GRO48-30 was greatly reduced in comparison to those occupied by GRO48 under similar conditions (Table 3). These results suggested that the competitive advantage shown by strain GRO48 could be due to the presence of functional *nfe* gene(s).

Table 4. Competition experiments with strains carrying plasmid pRmNT40

Strains in the mixed inoculum		% nodules occupied by strain[a]	
A	B	A	B
2011 (pRmNT40)	2011	76 ± 8	24 ± 8
2011 (pRmNT40)	GRO48	54 ± 6	46 ± 6
GRO48-30 (pRmNT40)	GRO48-30	78 ± 4	22 ± 4
GRO48-30 (pRmNT40)	GRO48	51 ± 8	49 ± 8
L5.30 (pRmNT40)	L5.30	51 ± 8	49 ± 8
L5.30 (pRmNT40)	GRO48	30 ± 9	70 ± 9
104A14 (pRmNT40)	104A14	41 ± 6	59 ± 6
104A14 (pRmNT40)	GRO48	13 ± 3	87 ± 3
41 (pRmNT40)	41	83 ± 6	17 ± 6
41 (pRmNT40)	GRO48	48 ± 6	52 ± 6

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

In a further step, plasmid pRmNT40, which carries *nfe* region, was transferred into the four wild-type strains under study and competition experiments were carried out to test whether any changes in the competitive abilities of the so complemented strains could be detected. The *nfe* mutant strain GRO48-30 complemented with plasmid pRmNT40 was used as a control. A significant increase of the percentage of nodule occupancy by strains GRO48-30, 2011 and 41 when carrying plasmid pRmNT40 was observed (Table 4). No positive effect was observed for strains L5.30 and 104A14. Individually, no changes in the speed of nodulation or the number of nodules per plant formed by strains carrying plasmid pRmNT40 were detected, except for strain GRO48-30 (pRmNT40), which nodulated as efficiently as strain GRO48 (data not shown).

Discussion

In this work we show data indicating that expression of the *nfe* (nodule formation efficiency) gene locus is directly regulated by the *nifA-ntrA* regulatory system that positively controls transcription of *nif* and some *fix* genes in *Rhizobium*. The pattern of activation of *nfe-lacZ* fusions in *E. coli* was similar to that of a *nifH-lacZ* fusion. They required the cloned *nifA* gene of *R. meliloti* and the *ntrA* gene of *E. coli*. Activation of the fusions by *R. meliloti nifA* was oxygen sensitive, which agrees with previous data clearly demonstrating that NifA function is oxygen-

labile (Beynon et al. 1988). The *nifH-lacZ* and *nfe-lacZ* fusions were also activated by the homologous NifA of *K. pneumoniae*. In this case activation was detected under low-oxygen conditions as well as in normal aerobiosis, as expected since it is known that NifA from this species is not oxygen sensitive in the absence of NifL, the negative regulator of NifA (Hill et al. 1981; Merrick et al. 1982). Activation of *nfe-* and *nifH-lacZ* fusions was observed in aerobically grown cells of a GRO13 derivative carrying the *K. pneumoniae nifA* gene cloned in pCK1 (Table 2). Activation of the same *nifH-lacZ* fusion in the presence of the cloned *nifA* from *K. pneumoniae* was previously reported not to occur in another strain, *R. meliloti* 102F34 (Better et al. 1985). Such a different result is difficult to explain, but it could be due to strain-specific factors. Indeed, we have found that activation of the *nifH-* and *nfe-lacZ* fusions in the presence of plasmid pCK1 is detected only in some strains of *R. meliloti* (J. Sanjuan, unpublished).

Hybridization experiments showed that DNA homologous to *nfe* is not present in other commonly-used wild-type strains of *R. meliloti*. These strains were shown to be uncompetitive in coinoculation with strain GRO48, with independence of their respective efficiency to nodulate alfalfa roots. It was demonstrated that a GRO48 *nfe* mutant derivative lost this competitive advantage. When plasmid pRmNT40 was transferred into the wild-type strains and the *nfe* mutant, changes in the speed of nodulation or the number of nodules formed per plant were observed only in the case of the *nfe* mutant strain GRO48-30. On the other hand, coinoculation experiments demonstrated a very significant increase in the competitive abilities of strains GRO48-30, 2011 and 41 when carrying *nfe* DNa. These results suggest that correct expression of the *nfe* phenotype depends on strain-specific genetic or physiological characteristics. Secondly, it would be reasonable to separate the positive effect of *nfe* on speed of nodulation by one strain from a role in competitive nodulation. More widely, *nfe* appears to influence the nodulation of alfalfa by one strain in presence of another strain. To date, the relationship between speed of nodulation and ocmpetitive ability is unclear. Some authors have reported no relation between these phenotypes (Mcloughlin et al. 1987; Zdor and Pueppke 1988) whereas some others support a strong relationship (Oliveira and Graham 1990). On the other hand, it is known that competition for nodulation of legumes is influenced by many factors, included ecological, physiological and genetic characteristics from both the bacterial and plant partners and it is generally considered that competition for nodulation is limited to early events of nodule initiation (reviewed by Dowling and Broughton 1986). In the case of *nfe*, the mechanism allowing this *nifA* activated gene(s) to confer a competitive advantage on a strain appears intricate. The regulation by *nifA* implies that maximal expression of *nfe* will be achieved in late stages of nodule development. This suggests a mechanism not involving direct bacteria-bacteria interaction but perhaps a fine competition implicating specific response(s) of the plant root to the expression of *nfe*. Finding out the precise mechanism of action and the fine structure of the *nfe* genes are the main goal of current investigation in our laboratory.

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