Analysis of the expression from *Rhizobium meliloti* fix-promoters in other *Rhizobium* backgrounds

Angel Cebolla, Francisco Ruiz-Berraquero and Antonio J. Palomares

Author for correspondence: Antonio J. Palomares. Tel: +34 5 455 67 66. Fax: +34 5 462 81 62.

Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Sevilla, c/o Profesor García González s/n, 41012 Sevilla, Spain

Using translational fusions to *lacZ*, we have measured expression from the promoters of *Rhizobium meliloti* regulatory genes, *nifA* and *fixK*, and structural genes, *nifH* and *fixA*, in other fast-growing rhizobia whose nitrogen fixation regulation is less known. Neither *nifA* nor *fixK* promoters were activated under both free-living microaerobic and symbiotic conditions, except in *R. tropici*, where clear symbiotic activation of either *nifA* or *fixK* expression could be observed. Both *nifH* and *fixA* promoters showed strong heterologous activation during symbiosis and weak activation under free-living nitrogen starvation conditions. Only when the *nifH* promoter was in *R. tropici* and *R. leguminosarum* bv. *phaseoli*, was clear induction observed in the microaerobic free-living state. Deletion analysis of these promoters suggested that a NifA binding site (UAS) was needed for full heterologous activation of *nifHp*, either in microaerobiosis or symbiosis. In contrast, the UAS region seemed to be unnecessary for *fixA* activation. However, a region containing a potential integration host factor (IHF) binding site was observed to be needed for complete heterologous symbiotic induction from *fixAp*. The moderate induction observed in nitrogen-free medium only required the σ^{54} holoenzyme recognition sequence; this may be indicative of the existence of non-specific activation by NtrC-like proteins. Our results suggest possible common and different features in the control mechanisms of the nitrogen fixation gene expression among *Rhizobium* species.

Keywords: *Rhizobium meliloti*, promoters, nitrogen fixation genes, gene expression, heterologous expression

INTRODUCTION

Rhizobium bacteria infect legume plant roots and cause cortical cell division leading to the formation of nitrogen-fixing nodules. The root nodule provides the appropriate biochemical environment for the conversion of atmospheric dinitrogen to ammonia.

The ability of rhizobia to induce Fix⁺ nitrogen nodules requires the specific expression of certain bacterial genes. The regulation of the expression of *R. meliloti* genes needed for symbiotic nitrogen fixation has been the subject of intense study. As a consequence, it is known that a two-component regulatory system, *fixLJ* (David *et al.*, 1988), in response to low oxygen tension activates

expression of the regulators *nifA* and *fixK* (Batut *et al.*, 1989), which in turn induce the expression of structural genes needed for nitrogen fixation.

Although expression of both *fixK* and *nifA* is under direct control of FixJ, sequence analysis of the *R. meliloti* *fixK* and *nifA* promoters did not reveal any extensive block of identical nucleotides. Nevertheless, it seems likely that there is a common sequence motif in the –54 to –39 region of both promoters that is required for *fixLJ*-dependent microaerobic induction (Agron *et al.*, 1992; Waelkens *et al.*, 1992). Since *fixK* expression is dependent on RpoD (Batut *et al.*, 1991), it is then expected that *nifAp* may also be recognized by RpoD (σ^{70}).

The regulation of symbiotic nitrogen-fixation gene expression in *Rhizobium* species other than *R. meliloti* is poorly known. As an exception, Colonna-Romano *et al.* (1990) observed heterologous microaerobic induction of a

Abbreviations: IHF, integration host factor; UAS, upstream activator sequence.

R. meliloti *fixN-lacZ* fusion in *R. leguminosarum* bv. *viciae*, and identified its activator as the product of an ORF that is homologous to *R. meliloti* *fixK*. However, attempts to find a *fixJ*-like gene in *R. leguminosarum* bv. *viciae* were unsuccessful. Symbiotic nitrogen-fixing bacteria related to *Rhizobium* and whose *fix*-regulation is also being elucidated, *Bradyrhizobium japonicum* (Anthamatten *et al.*, 1992; Anthamatten & Hennecke, 1991) and *Azorhizobium caulinodans* (Kaminski & Elmerich, 1991; Kaminski *et al.*, 1991), share some common regulatory elements with those of *R. meliloti* but differ in multiple aspects with regard to their regulatory models. Thus, in *A. caulinodans*, FixLJ activate *fixK* expression in microaerobiosis which in turn induces NifA expression (Kaminski & Elmerich, 1991; Kaminski *et al.*, 1991). *A. caulinodans* *fixKp* shows homology with that of *R. meliloti*, but NifA expression is activated via FixK, as indicated by the fact that *A. caulinodans* *nifAp* has an anaerobox found in FnrN-dependent promoters (Ratet *et al.*, 1989). In *B. japonicum*, NifA activity is regulated by oxygen (Fisher *et al.*, 1988). *nifA* is expressed from the *fixRnifA* operon and under microaerobiosis, NifA positively regulates *nif* and *fix* gene expression (Fisher & Hennecke, 1987). The *B. japonicum* *fixRnifA* operon has a RpoN consensus sequence promoter (Thöny *et al.*, 1987), differing from that of *R. meliloti*, which is expected to be RpoD-dependent (Batut *et al.*, 1991). *fixLJ* and *fixK* are present in *B. japonicum*, but *fixLJ* are not involved in *nifA* expression (Anthamatten *et al.*, 1992; Anthamatten & Hennecke, 1991).

Many of the structural genes needed for *R. meliloti* nitrogen fixation are clustered in two operons: *nifHDKE*, which encodes the structural genes of the nitrogenase complex (Ruvkun *et al.*, 1982); and *fixABCX*, encoding as yet undetermined functions essential for nitrogen fixation (Corbin *et al.*, 1983). The expression of each operon requires both the *rpoN* (*ntrA*) product, which is the σ^{54} factor (Ronson *et al.*, 1987), and the positive regulatory protein, NifA (Szeto *et al.*, 1984). The activity of NifA is regulated post-translationally by oxygen sensitivity of the protein (Fisher *et al.*, 1988). The *R. meliloti* promoters activated by NifA, *nifHp* and *fixAp*, have the conserved -24 (GG)/-12 (GC) consensus sequence of RpoN (Better *et al.*, 1983; Kustu *et al.*, 1989; Thöny & Hennecke, 1989), the putative NifA-binding site (upstream activator sequence, UAS; Buck *et al.*, 1988) and a potential site where an integration host factor may bind (IHF; Santero *et al.*, 1989; Hoover *et al.*, 1990) which stimulates NifA-mediated activation of *nif* transcription in *Klebsiella pneumoniae*. Both promoters share extensive sequence homology for more than 160 bp upstream of the RNA start point. However, *fixAp* contains a 9 bp insertion at position -27 and significant variation is apparent in the region between the transcriptional and translational start sites (Better *et al.*, 1983). These divergences seem to lead to differences in expression in *Escherichia coli* by either the *Klebsiella pneumoniae* NifA or the *E. coli* NtrC (*nifHp* is activated but not *fixAp*, Better *et al.*, 1985), and in *R. meliloti* under specific physiological conditions (Birkenhead *et al.*, 1990; Ditta *et al.*, 1987). Details of this *nif/fix* regulation in other species of

Rhizobium are poorly understood. To test for evidence of common regulatory elements that control activation of *R. meliloti* *nifAp*, *fixKp*, *nifHp* and *fixAp* in other fast-growing *Rhizobium* species, we have studied heterologous expression from those promoters under physiological conditions that allow their activation in *R. meliloti*.

METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are described in Table 1.

Media. *E. coli* was grown in LB (l^{-1} : 10 g tryptone, 5 g yeast extract and 10 g NaCl) and all rhizobia in TY (l^{-1} : 5 g tryptone, 3 g yeast extract, 0.84 g $CaCl_2$). Nitrogen-free medium contained 7.4 mM KH_2PO_4 , 5.8 mM K_2HPO_4 , 2.5 mM $MgSO_4$, 0.6 mM $CaCl_2$ and 10 mM sodium succinate. Tetracycline was used at 10 mg l^{-1} , kanamycin at 20 mg l^{-1} and nalidixic acid at 10 mg l^{-1} . Nutritive nitrogen-free solution for irrigating plant tests had the following components (mg l^{-1}): $CaSO_4$, 200; KH_2PO_4 , 200; KCl, 500; $MgSO_4$, 200; H_3BO_3 , 0.1; $ZnSO_4 \cdot 7H_2O$, 1; $CuSO_4$, 0.5; $MnCl_2 \cdot 4H_2O$, 0.5; $Na_2MoO_4 \cdot 2H_2O$, 0.1; ferric-sodium EDTA, 0.93.

Matings. Plasmids were transferred into *Rhizobium* strains with helper plasmid pRK2013 by a triparental conjugation technique (Ditta *et al.*, 1985). Transconjugants were selected in TY solid media supplemented with tetracycline and nalidixic acid.

Free-living assays. For free-living gene expression quantifications, saturated cultures were diluted with fresh medium and grown overnight under aerobic or microaerobic conditions at 29 °C to an A_{600} between 0.3 and 0.7. For aerobic conditions, 5 ml cultures were aerated by rotary shaker at 180 r.p.m. Microaerobic conditions were achieved as described by Ditta *et al.* (1987), exposing 2 ml cultures to 1% oxygen/99% nitrogen in 50 ml stoppered flasks. For nitrogen-limiting conditions, exponential-phase cultures in TY medium were washed twice and resuspended in nitrogen-free medium containing succinate as the carbon source. β -Galactosidase activity was determined as previously described by Miller (1972). Specific activities are means of at least two measurements made on independent cultures.

Symbiotic assays. Alfalfa and clover seeds were surface-sterilized by immersion in 96% ethanol for 1 min, washed with water and then incubated in 1% $HgCl_2$ solution in 10% (v/v) ethanol/water for 3 min. After washing 8–10 times in sterile water, seeds were placed on 0.8% agar plates and incubated at 29 °C. About 6–10 germinated seeds were selected and placed in jars containing vermiculite and irrigated with nitrogen-free medium. Bean, soybean and pea seeds were sterilized by washing with water, immersion in 96% ethanol for 3–5 min, washing once with water, immersion in 12% sodium hypochlorite for 5–15 min and washing at least 10 times with sterile water. Subsequent steps were identical to those described above for alfalfa and clover, but only one or two seeds were placed in each jar. Inoculations were performed as follows: alfalfa with *R. meliloti*, clover with *Rhizobium leguminosarum* bv. *trifolii*, pea with *R. leguminosarum* bv. *viciae*, bean with *R. tropici* and *R. leguminosarum* bv. *phaseoli*, and *Phaseolus aureus* with *Rhizobium* sp. NGR234.

Bacteroids were isolated from 1–5 g of nodules picked from duplicate groups of plants grown for 4–5 weeks. Nodules were crushed, their bacteroids purified by differential centrifugation and β -galactosidase activity of bacteroids was determined as previously described by Better *et al.* (1985). Data are the average of at least two independent assays. To estimate plasmid stability

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Description	Reference
Bacteria		
<i>E. coli</i> HB101	F ⁻ <i>hsdS hsdM pro leu thi gal lacY recA</i> Str ^r	Boyer & Roulland-Dussoix (1969)
<i>R. meliloti</i> 102F34	Wild-type, Nal ^r Nod ⁺ Fix ⁺ on alfalfa	Better <i>et al.</i> (1983)
<i>R. leguminosarum</i> bv. <i>viciae</i> 300	Wild-type, Nal ^r Nod ⁺ Fix ⁺ on pea	Johnston & Beringer (1975)
<i>R. leguminosarum</i> bv. <i>trifolii</i> RS800	Wild-type, Nal ^r Nod ⁺ Fix ⁺ on white and subterranean clover	Megías <i>et al.</i> (1989)
<i>R. leguminosarum</i> bv. <i>phaseoli</i> 8002	Wild-type, Nal ^r Nod ⁺ Fix ⁺ on bean	Lamb <i>et al.</i> (1982)
<i>R. tropici</i> CIAT899	Wild-type, Nal ^r Nod ⁺ Fix ⁺ on bean and leucaena	Martinez-Romero <i>et al.</i> (1991)
<i>R. fredii</i> HH003	Wild-type, Nal ^r Nod ⁺ Fix ⁺ on soybean	Dowdle & Bohlool (1985)
<i>Rhizobium</i> sp. NGR234	Wild-type, Nal ^r Nod ⁺ Fix ⁺ on a broad range of legumes, i.e. leucaena, soybean, etc.	Trinick (1980)
Plasmids		
pRK2013	Km ^r <i>tra</i> ColE1	Ditta <i>et al.</i> (1980)
pCHK57	Tc ^r , pGD926 derivative Rm <i>nifAp::lacZ</i>	Ditta <i>et al.</i> (1987)
pJJ5	Tc ^r , pIJ363 derivative Rm <i>fixKp::lacZ</i>	Batut <i>et al.</i> (1989)
pMB210	Tc ^r , pGD926 derivative Rm <i>nifHp::lacZ</i>	Better <i>et al.</i> (1985)
pMB211	Tc ^r , pGD926 derivative Rm <i>fixAp::lacZ</i>	Better <i>et al.</i> (1985)
Promoter deletion plasmids		
pMB1164	As pMB210 but with 175 bp of <i>nifHp</i> *	Better <i>et al.</i> (1985)
pMB1136	As pMB210 but with 150 bp of <i>nifHp</i>	
pMB1124	As pMB210 but with 135 bp of <i>nifHp</i>	
pMB1246	As pMB210 but with 95 bp of <i>nifHp</i>	
pMB1156	As pMB210 but with 35 bp of <i>nifHp</i>	
pMB1097	As pMB210 but with 10 bp of <i>nifHp</i>	
pMB1107	As pMB210 but with -37 bp of <i>nifHp</i>	
pMB2346	As pMB211 but with 178 bp of <i>fixAp</i>	
pMB2303	As pMB211 but with 168 bp of <i>fixAp</i>	
pMB2272	As pMB211 but with 88 bp of <i>fixAp</i>	
pMB2284	As pMB211 but with 73 bp of <i>fixAp</i>	
pMB2363	As pMB211 but with 33 bp of <i>fixAp</i>	
pMB2256	As pMB211 but with 3 bp of <i>fixAp</i>	

* Number of base pairs upstream of the transcription initiation point.

in nodules, the percentage of Tc^r cells isolated from nodules was measured in eight independent experiments testing 100 colonies each time. Permanence of the plasmids ranged from 60 to 98 %.

RESULTS

Expression of *nifA* and *fixK* in heterologous *Rhizobium* backgrounds

Similar features seem to regulate expression from either the *R. meliloti nifA* (Virts *et al.*, 1988) and *fixK* promoters (Batut *et al.*, 1989; Waelkens *et al.*, 1992) not only in both free-living cells exposed to low oxygen but also in bacteroids existing in the environment of alfalfa roots. We used *in vitro* microaerobiosis as a primary model system to test if there are regulatory elements in other *Rhizobium*

backgrounds that activate both promoters. β -Galactosidase activities from *R. meliloti* 102F34 bearing *nifAp::lacZ* or *fixKp::lacZ* increased considerably under microaerobic conditions (Table 2). Similar results were obtained using other strains of *R. meliloti* in the assay (data not shown). However, under the same experimental conditions, none of the other species could induce expression substantially. We tested different oxygen concentrations (0, 0.5, 1, 2, 4 and 20 %) to attempt to activate *R. meliloti nifAp* in *R. tropici* and *R. leguminosarum* bv. *phaseoli*, but no induction of β -galactosidase activity was observed (data not shown). It was noticeable that the background levels of homologous expression from both *nifAp* and *fixKp* were considerably higher than those of the heterologous backgrounds.

Table 2. β -Galactosidase expression from *R. meliloti* *nifAp* and *fixKp* in other heterologous *Rhizobium* spp.

Data are the mean of at least two independent assays $\pm$ SD. +N+O, Aerobic cultures in TY medium; -N+O, aerobic cultures in nitrogen-free medium; +N-O, microaerobic cultures in TY medium. NT, Not tested.

Species	β -Galactosidase expression (Miller Units)							
	<i>nifAp::lacZ</i>				<i>fixKp::lacZ</i>			
	+N+O	-N+O	+N-O	Symbiotic	+N+O	-N+O	+N-O	Symbiotic
<i>R. meliloti</i>	119 $\pm$ 34	501 $\pm$ 300	1430 $\pm$ 194	1400 $\pm$ 300	221 $\pm$ 89	386 $\pm$ 159	1680 $\pm$ 318	1610 $\pm$ 370
<i>R. tropici</i>	39 $\pm$ 6	538 $\pm$ 209	57 $\pm$ 8	1360 $\pm$ 322	15 $\pm$ 7	12 $\pm$ 6	10 $\pm$ 4	1690 $\pm$ 220
<i>R. leguminosarum</i> bv. <i>phaseoli</i>	36 $\pm$ 3	328 $\pm$ 179	50 $\pm$ 4	18 $\pm$ 2	11 $\pm$ 6	14 $\pm$ 10	16 $\pm$ 9	NT
<i>R. leguminosarum</i> bv. <i>trifolii</i>	48 $\pm$ 3	118 $\pm$ 30	46 $\pm$ 2	11 $\pm$ 4	25 $\pm$ 10	32 $\pm$ 2	20 $\pm$ 11	NT
<i>R. leguminosarum</i> bv. <i>viciae</i>	24 $\pm$ 4	318 $\pm$ 8	25 $\pm$ 3	10 $\pm$ 4	12 $\pm$ 4	10 $\pm$ 4	17 $\pm$ 3	NT
<i>R. sp.</i> NGR234	66 $\pm$ 8	839 $\pm$ 17	50 $\pm$ 6	17 $\pm$ 1	10 $\pm$ 2	10 $\pm$ 3	13 $\pm$ 3	NT
<i>R. fredii</i>	44 $\pm$ 2	428 $\pm$ 90	34 $\pm$ 3	30 $\pm$ 11	14 $\pm$ 5	27 $\pm$ 4	59 $\pm$ 1	5 $\pm$ 2

Ditta *et al.* (1987) reported that aerobic induction of *nifA* expression could also occur during nitrogen starvation in *R. meliloti*. Under these conditions, a significant increase in expression from *nifAp* was observed in every *Rhizobium* studied (Table 2). High variation in the levels of induction was observed. Relative to the basal level of β -galactosidase seen for pCHK57 (*nifAp::lacZ*) in TY medium, there was a 14-fold increase in *R. tropici* and a 3-fold increase in *R. leguminosarum* bv. *trifolii*. In contrast, bacteria in nitrogen-free medium failed to activate transcription from the *R. meliloti* *fixK* promoter. In addition, significantly higher heterologous *nifAp::lacZ* expression was not observed when microaerobic and nitrogen starvation conditions were combined; instead, expression was slightly repressed in most of the cases (data not shown). In contrast, under these conditions, *nifAp::lacZ* expression showed little derepression in the homologous background (2600 $\pm$ 375 Miller Units).

To test if activation could occur in heterologous bacteroids, we studied *nifAp::lacZ* and *fixKp::lacZ* expression in symbiotic conditions. Surprisingly, although it failed to show microaerobic induction, levels of expression comparable to that of symbiotic *R. meliloti* could be measured from *R. tropici* bacteroids bearing either *nifAp::lacZ* or *fixKp::lacZ* fusions (Table 2). Furthermore, regulation of the expression from both promoters was stricter for *R. tropici* (34-fold increase from *nifAp* and 169-fold from *fixKp*) than for *R. meliloti* (about 12-fold highest increase). Other *Rhizobium* backgrounds did not show this type of activation.

Heterologous expression from both *R. meliloti* *nifHp* and *fixAp* in other *Rhizobium* species

Both *nifHp* and *fixAp* promoters are moderately activated by NtrC in free-living *R. meliloti*, but an *ntrC* mutant was not affected in its ability to fix nitrogen in symbiosis (Szeto *et al.*, 1987). Three- to 11-fold increases in expression from *nifHp* and *fixAp* were observed when the heterologous rhizobia were under conditions of nitrogen starvation

(Table 3). We found that β -galactosidase activity from each promoter increased approximately proportionately, except in *R. leguminosarum* bv. *phaseoli* 8002 where a weak 3-fold increase was found for pMB211 (*fixAp::lacZ*) compared to a 10-fold increase for pMB210 (*nifHp::lacZ*). When glucose was used as the carbon source instead of succinate in the nitrogen-free medium, similar levels of induction were found in the heterologous *Rhizobium* tested (data not shown).

The behaviour observed for both promoters in every rhizobia was much more varied in microaerobic conditions. In the case of *nifHp*, *R. tropici* CIAT899 and *R. leguminosarum* bv. *phaseoli* 8002 exposed to low oxygen showed activation equivalent to that seen in *R. meliloti*. The initial oxygen concentration for maximal *nifHp::lacZ* microaerobic induction was about 0.75% O₂ for *R. meliloti*, 0.5% for *R. tropici* and 1.25–2% for *R. leguminosarum* bv. *phaseoli* (data not shown). For *fixAp*, some increase of microaerobic β -galactosidase expression could be detected in both *R. tropici* (6-fold) and *R. leguminosarum* bv. *phaseoli* 8002 (3-fold). However, neither *nifHp* nor *fixAp* were activated by microaerobic conditions in the other heterologous *Rhizobium* backgrounds.

As previously reported (Better *et al.*, 1985; Ditta *et al.*, 1987), there was strong induction of expression from both *R. meliloti* *nifHp* and *fixAp* during symbiosis. Although both microaerobic and symbiotic activation of those promoters was NifA-dependent (Ditta *et al.*, 1987), and *R. meliloti* expression from *nifAp* seemed to occur at similar levels in both conditions (Table 2), β -galactosidase levels from alfalfa bacteroids were at least 4-fold and 40-fold greater than those obtained in microaerobiosis from *nifHp* and *fixAp*, respectively (Table 3). Symbiotic β -galactosidase expression from *R. tropici* and *R. leguminosarum* bv. *phaseoli* bearing either *nifHp::lacZ* or *fixAp::lacZ* fusions was also several-fold greater under microaerobic conditions. Differences between symbiotic and microaerobic expression were also much greater in the case of *fixAp::lacZ*. Clear activation of expression in

Table 3. Free-living expression of β -galactosidase by *nifHp::lacZ* and *fixAp::lacZ* in different *Rhizobium* backgrounds

Data are the mean of at least two independent assays $\pm$ SD. +N+O, Aerobic cultures in TY medium; -N+O, aerobic cultures in nitrogen-free medium; +N-O, microaerobic cultures in TY medium. ND, Not determined.

Species	β -Galactosidase expression (Miller Units)							
	<i>nifHp::lacZ</i>				<i>fixAp::lacZ</i>			
	+N+O	-N+O	+N-O	Symbiotic	+N+O	-N+O	+N-O	Symbiotic
<i>R. meliloti</i>	36 $\pm$ 6	365 $\pm$ 69	1130 $\pm$ 147	4985 $\pm$ 1863	20 $\pm$ 4	161 $\pm$ 33	89 $\pm$ 22	3673 $\pm$ 1783
<i>R. tropici</i>	32 $\pm$ 4	210 $\pm$ 47	1590 $\pm$ 374	4451 $\pm$ 870	14 $\pm$ 2	81 $\pm$ 28	64 $\pm$ 14	770 $\pm$ 146
<i>R. leguminosarum</i> bv. <i>phaseoli</i>	36 $\pm$ 8	378 $\pm$ 35	974 $\pm$ 106	3072 $\pm$ 43	46 $\pm$ 3	132 $\pm$ 11	154 $\pm$ 45	513 $\pm$ 10
<i>R. leguminosarum</i> bv. <i>trifolii</i>	42 $\pm$ 6	257 $\pm$ 6	36 $\pm$ 1	419 $\pm$ 35	24 $\pm$ 2	147 $\pm$ 10	24 $\pm$ 3	121 $\pm$ 34
<i>R. leguminosarum</i> bv. <i>viciae</i>	30 $\pm$ 5	308 $\pm$ 28	25 $\pm$ 2	ND	14 $\pm$ 2	174 $\pm$ 33	12 $\pm$ 2	648 $\pm$ 100
<i>R. sp.</i> NGR234	45 $\pm$ 5	182 $\pm$ 56	25 $\pm$ 10	5062 $\pm$ 58	22 $\pm$ 3	81 $\pm$ 23	21 $\pm$ 2	1547 $\pm$ 48
<i>R. fredii</i>	33 $\pm$ 5	273 $\pm$ 46	20 $\pm$ 2	2585 $\pm$ 635	20 $\pm$ 5	169 $\pm$ 14	12 $\pm$ 5	363 $\pm$ 63

R. meliloti nifHp

-140   ↓pMB1124   -120   -100   ↓pMB1246 -80
 CGCCCATACGACACTGTCCGTAGCCCTTGTTCGGCTTAGCGACACGAGTTGTTTCGCTCAACCATCTGGTCAATTTCAGA
 -60   -40   ↓pMB1156   -20   ↓pMB1097 +1
 TCTAACTATCTGAAAGAAAGCCGAGTAGTTTATTTCAGACGGCTGGCAGCACTTTTGCACGATCAGCCCT.N67.ATG

R. meliloti fixAp

-150   -100   ↓pMB2272
 CGCAGCCATATGACATTGCCGTCGCCTCTGTTCGGCCCCCTCGACAGATTGTTTCCTTCAAGCATGCGGCCAATTTCCCGAT
 ↓pMB2284   -50   ↓pMB2363   pMB2256↓ +1
 CTAATCTATTGAAAGAAAGCAATTAGCATTATTTTCAGTCACCTCTGCGACCTGGCAGCACTTTTGCACGATCATCCCC.N120.ATG

Fig. 1. Sequence of the upstream region of *nifHp* and *fixAp* promoters (from Better *et al.*, 1983). Minimal formulations of σ^{54} consensus sequences are underlined. Sequences complementary and reverse to the IHF binding consensus described by Hoover *et al.* (1990) are overlined. Consensus upstream activator sequences are in bold. The TGT motif of *nifHp* whose deletion may affect NifA-dependent expression is in italics and placed 10 bases upstream from the TGT-N₁₀-ACA motif. Names of deletion plasmids are presented adjacent to the arrow showing each deletion end point. Only deletions that may affect promoter activity are shown.

symbiosis was found from both promoters in all the rhizobia studied. Absolute values of β -galactosidase expression found from *nifHp* in the heterologous *Rhizobium* were similar to that of *R. meliloti*. However, heterologous activation in *R. leguminosarum* bv. *trifolii* was at least 6-fold weaker than that obtained in the other backgrounds. Although also showing clear induction in every symbiotic background, absolute β -galactosidase levels from *fixAp::lacZ* were from 2.5-fold (*Rhizobium* sp. NGR234) to 31-fold (*R. leguminosarum* bv. *trifolii*) lower in heterologous bacteroids than in *R. meliloti*.

Deletion analysis of *nifHp* and *fixAp* functions

Transcription regulatory elements interact with promoters during transcription activation. To determine which regions of the *nifHp* and *fixAp* promoters are required for heterologous activation, two series of deletion plasmids extending from the 5' end of the *nifH* and *fixA* promoters were employed. The DNA sequence of

these promoters is shown in Fig. 1. At least three regions may be involved in the activity of these promoters: the σ^{54} recognition sequences located between -28 and -10 of each promoter (Better *et al.*, 1983); a potential IHF-binding site between -75 and -46 in the case of *nifHp* (Hoover *et al.*, 1990), and -80 and -56 in *fixAp*; and an upstream activator sequence (UAS) originally characterized as a highly conserved TGT-N₁₀-ACA (Buck *et al.*, 1988) NifA binding site (Morett & Buck, 1988).

In *R. meliloti*, nitrogen-starved activation only required the -24/-12 consensus sequences for promoter activation of each promoter (Figs 2a and 3a). Similar results were obtained when heterologous expression was examined under nitrogen starvation conditions. Therefore, induction of β -galactosidase expression in every genetic background tested under these conditions did not need other regulatory elements upstream of the σ^{54} promoter recognition sequence.

In contrast to nitrogen starvation, more than 85% of

Fig. 2. Homologous and heterologous β -galactosidase expression from *nifHp* deletions under nitrogen starvation (▲), microaerobic (○) and symbiotic (●) conditions. Results are plotted as the percentage β -galactosidase activity from pMB210, showing relative expression from *nifHp* deletion plasmids. (a) *R. meliloti*, (b) *R. tropici*, (c) *R. leguminosarum* bv. *phaseoli*, (d) *R. leguminosarum* bv. *trifolii*, (e) *R. fredii* and (f) *Rhizobium* sp. NGR234. Absolute values corresponding to 100% β -galactosidase activity for every case are shown in Table 3. A small scheme of the promoter is presented in the upper left of the figure to facilitate identification of the deletion end points. I, RpoN consensus; II, IHF binding consensus; III, UAS.

nifHp activity during microaerobiosis was lost when only the UAS was deleted (pMB1246) in both homologous and heterologous backgrounds (*R. tropici* and *R. leguminosarum* bv. *phaseoli*) (Fig. 2a–c). Thus, the UAS was required for full *nifHp* microaerobic activation. Activation was not affected by the *nifHp* deletion extending to –95 (pMB1246), nor by a deletion extending to –35 (pMB1156), indicating that when the UAS was deleted, the potential integration host factor (IHF) binding site did not seem to affect positively microaerobic expression. A moderate (40–50%) reduction of expression under low oxygen conditions was observed for the three genetic backgrounds containing pMB1124 (deletion to –135).

Furthermore, something of a decrease of expression was observed for every heterologous strain bearing pMB1124 during symbiotic conditions (Fig. 2b–f). Since this plasmid contains an intact UAS (Fig. 1), removal of sequences upstream of this motif negatively affected expression from *nifHp*.

As previous work in *R. meliloti* suggested (Better *et al.*, 1985; Ditta *et al.*, 1988), only the –24/–12 region of both promoters was indispensable for complete induction of β -galactosidase expression during symbiosis. When we observed heterologous symbiotic expression from *nifHp* deletions (Fig. 2), the patterns were usually comparable to

Fig. 3. Homologous and heterologous β -galactosidase expression from *fixAp* deletions under nitrogen starvation (▲) and symbiotic (●) conditions. Results are plotted as the percentage β -galactosidase activity from pMB211, showing relative expression from *fixAp* deletion plasmids. (a) *R. meliloti*, (b) *R. tropici*, (c) *R. leguminosarum* bv. *phaseoli*, (d) *R. leguminosarum* bv. *trifolii*, (e) *R. fredii* and (f) *Rhizobium* sp. NGR234. Absolute values corresponding to 100% β -galactosidase activity for every case are shown in Table 3. A small scheme of the promoter is presented in the upper left of the figure to facilitate identification of the deletion end points. I, RpoN consensus; II, IHF binding consensus; III, UAS.

that of microaerobic *R. meliloti*. Thus, as seen with *R. meliloti* under low oxygen, a 10–50% loss of *nifH*p activity was observed in the heterologous *Rhizobium* bearing pMB1124 under symbiotic conditions (Fig. 2b–f). When the UAS was deleted (pMB1246), β -galactosidase activity decreased about 50–80% in the heterologous bacteroids. In *R. leguminosarum* bv. *phaseoli*, UAS deletion was most drastic (loss of 95% activity, Fig. 2c). On the other hand, as seen in microaerobic *R. meliloti*, the heterologous rhizobia containing pMB1246 (deletion to –95) never showed higher values than when they contained pMB1156 (deletion to –35), during either microaerobic or symbiotic conditions. Therefore, when the UAS was deleted the supposed IHF-binding sequence did not seem to

affect promoter activity either in microaerobic or symbiotic conditions.

In contrast, a moderate (approx. 50%) loss of β -galactosidase expression in *R. meliloti* during symbiosis was observed when *fixAp* was deleted upstream of the σ^{54} recognition sequence (Fig. 3a). The same extent of deletion resulted in an even more drastic loss of heterologous symbiotic expression, showing the importance of upstream elements for activation (Fig. 3b–f). Deletion of the *fixAp* region up to –88 (pMB2272) had little effect on β -galactosidase expression during symbiosis. Since the *R. meliloti fixAp* UAS lies upstream of this deletion endpoint, the UAS sequences may be unnecessary for symbiotic

fixAp activation in either the homologous or most of the heterologous backgrounds. Only *fixAp* activation in *R. leguminosarum* bv. *phaseoli* seemed to be slightly affected by the presence of the UAS, showing about 30 % loss of expression when the UAS is deleted (Fig. 3c). In contrast, deletion of the *fixAp* region from -88 (pMB2272) to -72 (pMB2284) resulted in the loss of a great portion of the observable symbiotic stimulation in the heterologous rhizobia, but almost no loss (only 6 %) in *R. meliloti*. That loss was considerable (60–95 %) for most of the genetic backgrounds, but only 25 % for *R. leguminosarum* bv. *trifolii*. A DNA sequence similar to the IHF-binding site consensus sequence was identified between -77 and -63 of *fixAp* (Fig. 1); it was 1 bp closer to the IHF-binding site consensus sequence (A/T)ATCAA-N₄-TT(A/G) (Hoover *et al.*, 1990) than the similar sequence seen in *nifHp*. When this region was completely deleted (-33, pMB2363), an additional decrease in heterologous expression was observed. Nevertheless, in most of the cases there was still a residual regulation activity of the promoter some fold higher than the basal level.

DISCUSSION

Neither *R. meliloti nifAp* nor *fixKp* is activated either in free-living microaerobic or in symbiotic heterologous rhizobia (except in symbiotic *R. tropici*). This fact makes it difficult to extrapolate the regulatory cascade of *R. meliloti* symbiotic nitrogen fixation (David *et al.*, 1988; Batut *et al.*, 1989) to other *Rhizobium* species. This cascade starts with the oxygen sensor and kinase FixL which catalyses its own phosphorylation under low oxygen tension (Gilles-Gonzalez *et al.*, 1991). Then, the transcriptional activator FixJ is also phosphorylated catalysing *nifAp* and *fixKp* activity (Batut *et al.*, 1989; Gilles-Gonzalez *et al.*, 1991; Kahn & Ditta, 1991). Some genes have been described whose expression is oxygen-regulated via a mechanism involving *fixLJ*: a *fixK*-like gene in *A. caulinodans* (Kaminski & Elmerich, 1991), and *fixK* (Anthamatten *et al.*, 1992) in *B. japonicum*. Each of these symbiotic bacteria are expected to be genetically more distanced from *R. meliloti* than the species used in this study. It is thus surprising that neither microaerobic nor symbiotic conditions were able to activate transcription from *R. meliloti nifAp* and *fixKp* in most of the heterologous rhizobia. The simplest hypothesis would be that there are no functional homologous *fixLJ* in those strains. This is consistent with the fact that only weak homology has been found between the *fixLJ* region of *R. meliloti* and other *Rhizobium* species (Ditta *et al.*, 1988), and that no significant hybridization signals could be detected in *R. leguminosarum* bv. *viciae* (Colonna-Romano *et al.*, 1990). In *R. leguminosarum* bv. *viciae*, the *furN* gene, homologous to *R. meliloti fixK*, is capable of promoting microaerobic and symbiotic induction of *fixN* independently of *fixLJ* (Colonna-Romano *et al.*, 1990). Alternatively, *nif* regulation of the heterologous rhizobia tested here may occur via *fixLJ*, but result in production of an inactive FixJ or one without affinity for the *R. meliloti nifAp* and *fixKp* used in this study. In *A. caulinodans*, while *fixKp* shows sequence homology to that of *R. meliloti*,

NifA expression is activated via FixK. Despite the fact that *A. caulinodans fixLJ* are structurally very similar to *R. meliloti fixLJ*, *R. meliloti nifAp* (β -galactosidase) expression was not significantly induced in microaerobic cultures of *A. caulinodans* (Kaminski & Elmerich, 1991). This is consistent with the idea that the presence of *fixLJ* alone in other *Rhizobium* species may not guarantee microaerobic or symbiotic activation of *R. meliloti nifAp*.

As an exception among the rhizobia tested, it was surprising that *R. tropici* CIAT899 could strongly activate *nifAp* and *fixKp* under symbiotic conditions but not during microaerobiosis. This result suggests the existence of a FixJ-like protein in *R. tropici* whose activation signal occurs in nodules but not under microaerobic conditions *ex planta*. Since *in vitro* low oxygen was not sufficient to induce FixJ-dependent promoters, it may be considered that the regulators that caused its activation (FixLJ in *R. meliloti*) may respond to some other physiological signal *in planta*. It may also be possible that another factor is needed for FixJ-mediated activation or for activating a FixJ-like protein in *R. tropici*.

Moderate induction from *nifAp* under nitrogen starvation conditions occurred in both the homologous and heterologous *Rhizobium*. However, *fixKp* could not be significantly activated *ex planta* in all the heterologous backgrounds. Accordingly, Nooman *et al.* (1992) described that ammonia negatively regulates microaerobic expression of *nifA* but not of *fixK*. de Philip *et al.* (1990) also found differences in FixLJ-mediated regulation of *nifA* and *fixK* genes, at least in *E. coli*. The nature and meaning of these differences remain unknown.

R. meliloti nifHp and *fixAp* in the heterologous rhizobia behaved very similarly to when they were in their homologous background, under nitrogen-limiting conditions. The fact that sequences upstream of the σ^{54} recognition region are unnecessary for induction of β -galactosidase expression under nitrogen limitation in the three species tested, indicates that this activation is probably not specific. Activation of both promoters under nitrogen starvation was shown to be dependent on the general nitrogen regulatory protein NtrC in *R. meliloti* (Szeto *et al.*, 1987). None of our results contradicts the idea that activation of NifA-dependent promoters under free-living nitrogen starvation by NtrC is conserved among different *Rhizobium* species.

Rhizobium RpoN/NifA-dependent promoters are extremely well conserved (Alvarez-Morales & Hennecke, 1985; Better *et al.*, 1983). Even though both *R. meliloti* promoters were activated to some extent during symbiosis, activation of *nifHp* and *fixAp* in microaerobiosis were not observed in several *Rhizobium* species. However, microaerobic activation in both *R. tropici* and *R. leguminosarum* bv. *phaseoli* was very similar to that seen in the homologous background. These rhizobia that activate both promoters in microaerobic conditions may produce an active NifA *ex planta*. On the other hand, *Rhizobium* backgrounds that failed to induce expression in low oxygen may not have an active NifA, or may need other

factors, found in bacteroids, to achieve activation by NifA. Alternatively, a repression could be exerted *ex planta* to prevent free-living expression of nitrogenase. Therefore, the use of *in vitro* microaerobic conditions as a model system for studies on the mechanisms of *fix* regulation using heterologous promoters may be limited in many *Rhizobium* species.

In contrast to *R. meliloti*, the heterologous *Rhizobium* required more than the -24/-12 consensus sequence of *nifHp* and *fixAp* to achieve full symbiotic transcriptional activation. Almost all expression is lost when the NifA-binding sequences are deleted from *nifHp*. Microaerobic activation of *nifHp* required the same DNA segments in *R. meliloti*, *R. tropici* and *R. leguminosarum* bv. *phaseoli*. Furthermore, these DNA segments coincide with those required for heterologous expression during symbiosis. Heterologous microaerobic and symbiotic activation of *nifHp* seemed to be highly dependent on the UAS, suggesting activation by NifA. On the other hand, deletion analysis of *R. meliloti nifHp* suggests an alternative UAS as a TGT-N₁₀-TGT-N₁₀-ACA motif (Fig. 1). Since the minimal UAS consensus, described as a TGT-N₁₀-ACA motif, remained intact in pMB1124 (-135), and alteration of the first repeated TGT resulted in a loss of promoter activation during both microaerobiosis and symbiosis in the heterologous *Rhizobium*, this suggests that this sequence contributed to NifA-*nifHp* affinity.

NifA activation of *nif* promoters that lack a NifA binding site could be accomplished if there is a high concentration of active NifA and appropriate RNA polymerase recognition sequences (Albright *et al.*, 1988; Buck & Cannon, 1989). One might expect that the level and/or the activity of *R. meliloti* NifA in mature nodules is higher than exists either in microaerobic conditions or in the heterologous *Rhizobium* bacteroids. This expectation is compatible with the results with *fixAp* in *R. meliloti*. Deletion analysis of this promoter indicated that the UAS was not necessary for full heterologous activation in symbiotic rhizobia. Consistent with these results is the finding that the *B. japonicum fixA* promoter has no UAS of the TGT-N₁₀-ACA type, yet its expression is also strongly dependent on the presence of NifA (Gubler & Hennecke, 1988; Gubler, 1989). Surprisingly, although activation was UAS-independent, a sequence identified as a potential IHF binding site, seemed to be necessary for most *R. meliloti fixA* heterologous expression. IHF has the ability to bend DNA significantly (Friedman, 1988). The model of IHF function in σ^{54} -dependent promoters proposed for *K. pneumoniae nifHp* (Hoover *et al.*, 1990), corroborated for the promoters *Ps* of *xylS*, *Pu* of *xylCAB* (Holtel *et al.*, 1990; de Lorenzo *et al.*, 1991) and *glnHp* (Claverie-Martín & Magasanik, 1991), is that it helps to achieve both high efficiency and high fidelity in the control of transcription mediated by its particular activators, especially for weak promoters. According to this model, IHF binding could not stimulate transcription independent of the UAS. The behaviour of *fixAp* could have two possible explanations. Firstly, other as yet uncharacterized sequences potentially essential for activation might have been removed in the deletions that also remove the IHF binding motif.

Secondly, although expression depends on σ^{54} , it is possible that IHF function at *fixAp* differs from that of other σ^{54} -dependent promoters, because IHF bending of DNA may help to activate *fixAp* independent of the UAS. Since specific activation of *fixAp* under nitrogen starvation was independent of the IHF binding motif, IHF may act as an architectural element in the assembly of a NifA/ σ^{54} -dependent transcription initiation complex. In this complex, the *fixAp* UAS could be unnecessary. IHF binding might improve both its efficiency and specificity for symbiotic expression of the *fixABCX* operon contributing to its fine-tuning control. Further study of the role and function of *Rhizobium* IHF in symbiotic expression of nitrogen fixation genes may be required for a fuller elucidation of *fix* gene regulation.

This report indicates that there are some common features in the expression of *R. meliloti* symbiotic nitrogen fixation genes (at least those controlled by NifA) that are shared by heterologous *Rhizobium* species. We also present evidence that the control mechanisms may differ to some extent among the *Rhizobium* species. Other bacteria with a similar life style, *B. japonicum* and *A. caulinodans*, differ considerably in their *fix* regulation circuits but share many of the known regulatory elements (FixL, FixJ, FixK, NifA, *nif*-promoters, etc). There is specificity in the *Rhizobium*-legume interaction; some *Rhizobium* genes are adapted for nodulation of a specific plant (e.g. *nod* specificity genes, *nodD*-flavonoid specificity, *exo* genes; see reviews of Long, 1989; and Martínez *et al.*, 1990). It is likely that such parameters as nodule development and the physiological conditions specific to a particular plant, vary in other plants. Because of that, each specific *Rhizobium* has probably optimized nitrogen fixation during the course of evolution, adapting its regulatory mechanisms to fit the host plant conditions, resulting in diversification of *Rhizobium* symbiotic *fix* regulation circuits.

ACKNOWLEDGEMENTS

We thank Gary Ditta for providing most of the plasmids. We also thank Dr Boistard for providing pJJ5 plasmid, Dr E. Santero for his valuable comments and criticism, Dr D. N. Rodríguez and Dr F. Temprano for their help in plant tests. A.C. was an FPI fellowship recipient. Research support from the Spanish Ministry of Education and Science through CYCYT projects PB87-0918 and BIO90-0521, and from Fundación Ramón Areces is gratefully acknowledged.

REFERENCES

- Agron, P., Ditta, G. & Helinski, D. R. (1992). Mutational analysis of the *Rhizobium meliloti nifA* promoter. *J. Bacteriol.* **174**, 4120-4129.
- Albright, L. M., Huala, E., Gu, Q. & Ausubel, F. E. (1988). Regulation of *R. meliloti* NifA function. In *Nitrogen Fixation: Hundred Years After*, pp. 345-349. Edited by H. Bothe, F. J. de Bruijn & W. E. Newton. Stuttgart: Gustav Fischer.
- Alvarez-Morales, A. & Hennecke, H. (1985). Expression of *Rhizobium japonicum nifH* and *nifDK* operons can be activated by the *Klebsiella pneumoniae nifA* protein but not by the product of *ntrC*. *Mol. & Gen. Genet.* **199**, 306-314.

- Anthamatten, D. & Hennecke, H. (1991).** The regulatory status of the *fixL* and *fixJ* genes in *Bradyrhizobium japonicum* may be different from that in *Rhizobium meliloti*. *Mol & Gen Genet* **225**, 38–48.
- Anthamatten, D., Scherb, B. & Hennecke, H. (1992).** Characterization of a *fixLJ*-regulated *Bradyrhizobium japonicum* gene sharing similarity with the *Escherichia coli* *fnr* and *Rhizobium meliloti* *fixK* genes. *J Bacteriol* **174**, 2111–2120.
- Batut, J., Daveran, M. L., David, M., Jacobs, J., Garnerone, A. M. & Kahn, D. (1989).** *fixK*, a gene homologous with *fnr* and *crp* from *Escherichia coli*, regulates nitrogen fixation genes both positively and negatively in *Rhizobium meliloti*. *EMBO J* **8**, 1279–1286.
- Batut, J., Santero, E. & Kustu, S. (1991).** *In vitro* activity of the nitrogen fixation regulatory protein FixJ from *Rhizobium meliloti*. *J Bacteriol* **173**, 5914–5917.
- Better, M., Lewis, B., Corbin, D., Ditta, G. & Helinski, D. R. (1983).** Structural relationships among *Rhizobium meliloti* symbiotic promoters. *Cell* **35**, 379–385.
- Better, M., Ditta, G. & Helinski, D. R. (1985).** Deletion analysis of *Rhizobium meliloti* symbiotic promoters. *EMBO J* **4**, 2419–2424.
- Birkenhead, K., Nooman, B., Reville, W. J., Boesten, B., Manian, S. S. & O'Gara, F. (1990).** Carbon utilization and regulation of nitrogen fixation genes in *Rhizobium meliloti*. *Mol Plant-Microbe Interact* **3**, 167–173.
- Boyer, H. W. & Roulland-Dussoix, D. (1969).** A complementation analysis of restriction and modification in *Escherichia coli*. *J Mol Biol* **41**, 459–472.
- Buck, M. & Cannon, W. (1989).** Mutation in the RNA polymerase recognition sequence of *Klebsiella pneumoniae* *nifH* promoter permitting transcriptional activation in the absence of NifA binding to upstream activator sequences. *Nucleic Acids Res* **17**, 2597–2612.
- Buck, M., Miller, S., Drummond, M. & Dixon, R. (1988).** Upstream activator sequences are present in the promoters of nitrogen fixation genes. *Nature* **320**, 374–378.
- Claverie-Martín, F. & Magasanik, B. (1991).** Role of integration host factor in the regulation of the *glnHp2* promoter of *Escherichia coli*. *Proc Natl Acad Sci USA* **88**, 1631–1635.
- Colonna-Romano, S., Arnold, W., Schlüter, A., Boistard, P., Pühler, A. & Preifer, U. B. (1990).** An Fnr-like protein encoded in *Rhizobium leguminosarum* biovar *viciae* shows structural and functional homology to *Rhizobium meliloti* FixK. *Mol & Gen Genet* **223**, 138–147.
- Corbin, D., Barran, L. & Ditta, G. (1983).** Organization and expression of *Rhizobium meliloti* nitrogen fixation genes. *Proc Natl Acad Sci USA* **80**, 3005–3009.
- David, M., Daveran, M. L., Batut, J., Declien, A., Domergue, O., Ghai, J., Hertig, C., Boistard, P. & Kahn, D. (1988).** Cascade regulation of *nif* gene expression in *Rhizobium meliloti*. *Cell* **54**, 671–683.
- Ditta, G., Stanfield, S., Corbin, D. & Helinski, D. R. (1980).** Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc Natl Acad Sci USA* **77**, 7347–7351.
- Ditta, G., Schmidhauser, T., Yakobson, E., Lu, P., Liang, X.-W., Finlay, D. R., Guiney, D. & Helinski, D. R. (1985).** Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. *Plasmid* **13**, 149–153.
- Ditta, G., Virts, E., Palomares, A. & Kim, C. K. (1987).** The *nifA* gene of *Rhizobium meliloti* is oxygen regulated. *J Bacteriol* **169**, 3217–3223.
- Ditta, G., Virts, E. & Helinski, D. R. (1988).** Oxygen regulation of *nif* genes in *Rhizobium meliloti*. In *Molecular Genetics of Plant-Microbe Interactions*, pp. 109–110. Edited by D. P. S. Verma & R. Palacios. Saint Paul, MN: APS Press.
- Dowdle, S. F. & Bohlool, B. B. (1985).** Predominance of fast-growing *Rhizobium japonicum* in a soybean field in the People's Republic of China. *Appl Environ Microbiol* **50**, 1171–1176.
- Fisher, H. M. & Hennecke, H. (1987).** Direct response of *Bradyrhizobium japonicum* *nifA*-mediated *nif* gene regulation to cellular oxygen status. *Mol & Gen Genet* **209**, 621–626.
- Fisher, H. M., Bruderer, T. & Hennecke, H. (1988).** Essential and non-essential domains in the *Bradyrhizobium japonicum* NifA protein: identification of indispensable cysteine residues potentially involved in redox reactivity and/or metal binding. *Nucleic Acids Res* **16**, 2207–2224.
- Friedman, D. I. (1988).** Integration host factor: a protein for all reasons. *Cell* **55**, 545–554.
- Gilles-Gonzalez, M. A., Ditta, G. & Helinski, D. R. (1991).** A haemoprotein with quinase activity encoded by the oxygen sensor of *Rhizobium meliloti*. *Nature* **350**, 170–172.
- Gubler, M. (1989).** Fine-tuning of *nif* and *fix* gene expression by upstream activator sequences in *Bradyrhizobium japonicum*. *Mol Microbiol* **3**, 149–159.
- Gubler, M. & Hennecke, H. (1988).** Regulation of the *fixA* gene and *fixBC* operon in *Bradyrhizobium japonicum*. *J Bacteriol* **170**, 1205–1214.
- Holtel, H., Abril, M.-A., Marques, S., Timmis, K. N. & Ramos, J. L. (1990).** Promoter-upstream activator sequences are required for expression of the *xylS* gene and upper-pathway operon on the *Pseudomonas* TOL plasmid. *Mol Microbiol* **4**, 1551–1556.
- Hoover, T. R., Santero, E., Porter, S. & Kustu, S. (1990).** The Integration Host Factor (IHF) stimulates interaction of RNA polymerase with NifA, the transcriptional activator for nitrogen fixation operons. *Cell* **63**, 11–22.
- Johnston, A. W. B. & Beringer, J. E. (1975).** Identification of the *Rhizobium* strains in pea root nodule using genetic markers. *J Gen Microbiol* **87**, 343–350.
- Kahn, D. & Ditta, G. (1991).** Modular structure of FixJ: homology of the transcriptional activator domain with the -35 binding domain of sigma factors. *Mol Microbiol* **5**, 987–997.
- Kaminski, P. A. & Elmerich, C. (1991).** Involvement of *fixLJ* in the regulation of nitrogen fixation in *Azorhizobium caulinodans*. *Mol Microbiol* **5**, 665–673.
- Kaminski, P. A., Mandon, K., Arigoni, F., Desnoues, N. & Elmerich, C. (1991).** Regulation of nitrogen fixation in *Azorhizobium caulinodans*: identification of a *fixK*-like gene, a positive regulator of *nifA*. *Mol Microbiol* **5**, 1983–1991.
- Kustu, S., Santero, E., Keener, J., Popham, D. & Weiss, D. (1989).** Expression of the σ^{54} (*ntfA*)-dependent genes is probably united by a common mechanism. *Microbiol Rev* **53**, 367–376.
- Lamb, J. M., Hombrecher, G. & Johnston, A. W. B. (1982).** Plasmid determined nodulation and nitrogen fixation abilities in *Rhizobium phaseoli*. *Mol & Gen Genet* **186**, 449–452.
- Long, S. (1989).** *Rhizobium*-legume nodulation: life together in the underground. *Cell* **56**, 203–214.
- de Lorenzo, V., Herrero, M., Metzke, M. & Timmis, K. N. (1991).** An upstream XylR- and IHF-induced nucleoprotein complex regulates the σ^{54} -dependent Pu promoter of TOL plasmid. *EMBO J* **10**, 1159–1167.
- Martínez, E., Romero, D. & Palacios, R. (1990).** *Rhizobium* genome. *Crit Rev Plant Sci* **9**, 59–87.
- Martínez-Romero, E., Segovia, L., Mercante, F. M., Franco, A. A., Graham, P. & Pardo, M. A. (1991).** *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. *Int J Syst Bacteriol* **41**, 417–426.
- Megías, M., Caviedes, M. A., Andres, M., Sousa, C., Ruiz-**

- Berraquero, F. & Palomares, A. J. (1989).** Localization of *his* genes on the *Rhizobium trifolii* RS800 linkage map. *Mol & Gen Genet* **211**, 369–372.
- Miller, J. H. (1972).** *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Morett, E. & Buck, M. (1988).** NifA-dependent *in vivo* protection demonstrated that the upstream activator sequence of *nif* promoter is a protein binding site. *Proc Natl Acad Sci USA* **85**, 9401–9405.
- Nooman, B., Motherway, M. & O’Gara, F. (1992).** Ammonia regulation of the *Rhizobium meliloti* nitrogenase structural and regulatory genes under free-living conditions: involvement of the *fixL* gene product? *Mol & Gen Genet* **234**, 423–428.
- de Philip, P., Batut, J. & Boistard, P. (1990).** *Rhizobium meliloti* FixL is an oxygen sensor and regulates *R. meliloti* *nifA* and *fixK* genes differently in *Escherichia coli*. *J Bacteriol* **172**, 4255–4262.
- Ratet, P., Pawloski, K., Schell, J. & de Bruijn, F. J. (1989).** The *Azorhizobium caulinodans* nitrogen fixation regulatory gene, *nifA*, is controlled by the cellular nitrogen and oxygen status. *Mol Microbiol* **3**, 825–838.
- Ronson, C. W., Nixon, B. T., Albright, L. M. & Ausubel, F. M. (1987).** *Rhizobium meliloti* *ntfA* (*rpoN*) gene is required for diverse metabolic functions. *J Bacteriol* **169**, 2424–2431.
- Ruvkun, G. B., Sundaresan, V. & Ausubel, F. M. (1982).** Directed transposon Tn5 mutagenesis and complementation analysis of *Rhizobium meliloti* and related bacteria. *Cell* **29**, 551–559.
- Santero, E., Keener, J. & Kustu, S. (1989).** *In vitro* activity of the nitrogen fixation regulatory protein NIFA. *Proc Natl Acad Sci USA* **88**, 7346–7350.
- Szeto, W. W., Zimmerman, J. L., Sundaresan, V. & Ausubel, F. M. (1984).** A *Rhizobium meliloti* symbiotic regulatory gene. *Cell* **36**, 1035–1043.
- Szeto, W. W., Nixon, B. T., Ronson, C. W. & Ausubel, F. M. (1987).** Identification and characterization of the *Rhizobium meliloti* *ntfC* gene: *R. meliloti* has separate regulatory pathways for activation of nitrogen genes in free-living and symbiotic cells. *J Bacteriol* **169**, 1423–1432.
- Thöny, B. & Hennecke, H. (1989).** The 24/12 promoter comes from ages. *FEMS Microbiol Rev* **63**, 341–358.
- Thöny, B., Fischer, H.-M., Anthamatten, D., Bruderer, T. & Hennecke, H. (1987).** The symbiotic nitrogen fixation regulatory operon (*fixRnifA*) of *Bradyrhizobium japonicum* is expressed aerobically and is subject to a novel, *nifA*-independent type of activation. *Nucleic Acids Res* **15**, 8479–8499.
- Trinick, M. J. (1980).** Relationships amongst the fast-growing rhizobia of *Lablab purpureus*, *Leucaena leucocephala*, *Mimosa* spp., *Acacia farnesiana* and *Sesbania glandiflora* and their affinities with other rhizobial groups. *J Appl Bacteriol* **49**, 39–53.
- Virts, E. L., Stanfield, S. W., Helinski, D. R. & Ditta, G. (1988).** Common regulatory elements control symbiotic and microaerobic induction of *nifA* in *Rhizobium meliloti*. *Proc Natl Acad Sci USA* **85**, 3062–3065.
- Waelkens, F., Foglia, A., Morel, J.-B., Fourment, J., Batut, J. & Boistard, P. (1992).** Molecular genetic analysis of the *Rhizobium meliloti* *fixK* promoter: identification of sequences involved in positive and negative regulation. *Mol Microbiol* **6**, 1447–1456.

Received 21 May 1993; revised 17 September 1993; accepted 6 October 1993.