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Symbiotic Autoregulation of *nifA* Expression in *Rhizobium leguminosarum* bv. viciae

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NifA is the general transcriptional activator of nitrogen fixation genes in diazotrophic bacteria. In *Rhizobium leguminosarum* bv. viciae UPM791, the *nifA* gene is part of a gene cluster (*orf71 orf79 fixW orf5 fixABCX nifAB*) separated by 896 bp from an upstream and divergent truncated duplication of *nifH* (Δ*nifH*). Symbiotic expression analysis of genomic *nifA*::*lacZ* fusions revealed that in strain UPM791 *nifA* is expressed mainly from a σ^{54}-dependent promoter (P_{nifA1}) located upstream of *orf71*. This promoter contains canonical NifA upstream activating sequences located 91 bp from the transcription initiation site. The transcript initiated in P_{nifA1} spans 5.1 kb and includes *nifA* and *nifB* genes. NifA from *Klebsiella pneumoniae* was able to activate transcription from P_{nifA1} in a heterologous *Escherichia coli* system. In *R. leguminosarum*, the P_{nifA1} promoter is essential for effective nitrogen fixation in symbiosis with peas. In its absence, partially efficient nitrogen-fixing nodules were produced, and the corresponding bacteroids exhibited only low levels of *nifA* gene expression. The basal level of *nifA* expression resulted from a promoter activity originating upstream of the *fixX-nifA* intergenic region and probably from an incomplete duplication of P_{nifA1} located immediately upstream of *fixA*.

NifA belongs to the bacterial enhancer-binding protein family of transcriptional regulators that activate gene expression in concert with RNA polymerase containing the specialized σ^{54} factor, which allows the polymerase core to recognize the −24/−12-type promoters (8, 15, 21). NifA normally binds to promoter sites designated upstream activating sequences (UAS) and interacts with the RNA polymerase holoenzyme via loop formation in the intervening DNA (25, 26). DNA loop formation is facilitated in many, but not all, cases by binding of the integration host factor to a site located between the UAS and the core promoter region (12, 25, 26). Transcription is promoted by interaction of NifA with the holoenzyme and subsequent catalysis of open complex formation through an ATP-dependent reaction.

In diazotrophic bacteria, NifA activates transcription of nitrogenase genes (*nif* genes) and, particularly in the legume endosymbiotic bacteria, also *fix* genes. Although *nifA* genes are conserved in diazotrophic bacteria, their organization within *nif* gene clusters differs (for a review see reference 8). In fast-growing rhizobia, such as *Sinorhizobium meliloti*, *Rhizobium leguminosarum* bv. trifolii, and *R. leguminosarum* bv. viciae, *nifA* is located in the symbiotic plasmid, between the *fixABC* operon and the *nifB* gene. In *Bradyrhizobium japonicum*, *nifA* is located in the chromosome as part of the *fixR nifA* operon.

Globally, NifA is regulated at both the transcriptional and activity levels mainly in response to oxygen or combined-nitrogen availability. In rhizobia, *nifA* gene expression is basically controlled by O_2 availability, and it is maximal at low O_2 concentrations, such as those available to the bacteroid inside the legume nodules. However, there are different mechanisms for regulation of *nifA* expression in root nodule bacteria. *S. meliloti* *nifA* promoter activity is regulated by the FixLJ cascade in free-living microaerobic cells and in symbiosis (7). In this bacterium, *nifA* expression is increased by feedback from the *fixABCX* promoter (5, 14). The *nifA* promoter is negatively controlled by FixK, a member of the Fnr/Crp family of transcriptional regulators, by an unknown mechanism (1). In *B. japonicum*, *nifA* is significantly expressed in aerobic conditions from the promoter of the *fixR nifA* operon, which is regulated by a two-component system, RegS/RegR (2). Expression of the *fixR nifA* operon is increased in microaerobic, anaerobic, and symbiotic conditions by the action of NifA in response to the redox state of the cell (33). Finally, the regulation of *nifA* expression in *Azorhizobium caulinodans* is complex and is controlled from overlapping promoter regions responding to both the nitrogen and oxygen status of the cell (18). The regulation of *nifA* expression in *R. leguminosarum* bv. viciae has not been studied in detail. Nitrogenase is not expressed in free-living cells, probably because NifA is not functional under these conditions. In symbiosis, NifA is essential for nitrogen fixation. In *R. leguminosarum* bv. viciae strain PRE, *nifA* mutants exhibit a Nif^- Fix^- phenotype (30). In this strain, a transcription start site was identified just upstream of the *nifA* gene (27), but no evidence for a *nifA* promoter was obtained from sequence analysis of this region in strain 3855 (9). In this work we found that *R. leguminosarum* bv. viciae *nifA* is expressed in symbiosis with peas from a σ^{54}-dependent NifA-autoregulated promoter located upstream of the *orf71 orf79 fixW orf5 fixABCX nifA nifB* operon, although basal levels of symbiotic NifA expression were obtained from a second promoter located upstream of the *fixX-nifA* intergenic region.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this work are listed in Table 1. *R. leguminosarum* strains were routinely grown in tryptone-yeast extract (3) or yeast extract-mannitol (YMB) (34) media at 28°C. *Escherichia coli* strains were grown in Luria-Bertani medium.

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TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Reference or source
R. leguminosarum strains		
UPM791	128C53 Str^r	16
UPM791.5	UPM791 cured of the symbiotic plasmid	17
GLF1	UPM791::pVIKM1, *nifA*::*lacZ* fusion, Fix$^+$ Km^r	This study
GLF2	UPM791::pVIKM2, *nifA*::*lacZ* fusion, Fix$^+$ Km^r	This study
GLF3	UPM791::pVIKM3, *nifA*::*lacZ* fusion, Fix$^+$ Km^r	This study
GLF4	UPM791::pVIKM4, *nifA*::*lacZ* fusion, Fix$^-$ Km^r	This study
GLF8	UPM791::pVIKM8, *nifA*::*lacZ* fusion, Fix$^-$ Km^r	This study
GLF12	UPM791::pVIKM12, *orf79*::*lacZ* fusion, Fix$^+$ Km^r	This study
GLF18	UPM791::pVIKM18, *nifA*::*lacZ* fusion, Fix$^-$ Km^r	This study
E. coli strains		
ET8000	*rbs lacZ*::IS*1 gyrA hutC$_{ka}$*	19
S17.1	*thi pro hsdR hsdM$^+$ recA RP4 2*-Tc::*Mu*-Km::Tn*7*	31
S17.1 λpir	*294*::[RP4 2-Tc::*Mu*-Km::Tn*7*]*pro res* Δ*recA mod$^+$* λ*pir$^+$*	6
Plasmids		
pALM31	UPM791 *fixW* upstream region in pLAFR1 cosmid; Tc^r	This study
pALM35	UPM791 *nifA* cluster in pLAFR1 cosmid; Tc^r	This study
pCR 2.1-TOPO	PCR product cloning vector; Ap^r	Invitrogen
pCRnifA	UPM791 *nifA* gene in pCR2.1-TOPO; Ap^r	This study
pSKM2	*orf79-nifA* fragment in pBluescript(SK); Ap^r	This study
pSKM3	pSKM2 with a deletion of *fixX-nifA* intergenic fragment; Ap^r	This study
pSKM4	Δ*nifH-orf79* fragment in pBluescript(SK); Ap^r	This study
pVIK112	R6K *oriV lacZY*; Km^r	13
pVIKM1	Δ*nifH orf71 orf79 fixW orf5 fixABCX nifA′*::*lacZ* fusion in pVIK112; Km^r	This study
pVIKM2	*orf79 fixW orf5 fixABCX nifA′*::*lacZ* fusion in pVIK112; Km^r	This study
pVIKM3	*orf79 fixW orf5 fixABCX nifA′*::*lacZ* fusion in pVIK112; intergenic *fixX-nifA* fragment deleted; Km^r	This study
pVIKM4	*′fixX nifA′*::*lacZ* fusion in pVIK112; Km^r	This study
pVIKM8	*′nifA*::*lacZ* fusion in pVIK112; Km^r	This study
pVIKM12	*orf79 fixW orf5 fixABCX nifA′* fragment in opposite orientation with respect to *lacZ* in pVIK112; Km^r	This study
pVIKM18	*nifA* fragment in opposite orientation with respect to *lacZ* in pVIK112; Km^r	This study
pSPV4	pMP220 derivative; Tc^r	24
pSPM9	*orf79*::*lacZ* fusion in pSPV4; Tc^r	This study
pSPM10	Δ*nifH*::*lacZ* fusion in pSPV4; Tc^r	This study
pMJ220	*K. pneumoniae* NifA in pACYC184; Cm^r	22

Antibiotics were added at the following concentrations: tetracycline, 12 μg ml^{-1} (6 μg ml^{-1} for *Rhizobium*); kanamycin, 50 μg ml^{-1}; and ampicillin, 100 μg ml^{-1}. *Klebsiella pneumoniae nifA* was aerobically expressed from plasmid pMJ220 in *E. coli* strain ET8000 at 28°C as previously described. Nonsuicide plasmids pSPM9 and pSPM10 were introduced into *R. leguminosarum* by conjugation by using *E. coli* strain S17.1, and transconjugants were selected in *Rhizobium* minimal medium (23) supplemented with tetracycline. Suicide plasmids derived from pVIK112 were introduced into *R. leguminosarum* by conjugation by using *E. coli* strain S17.1 λpir, and plasmid integration into the genome was selected in *Rhizobium* minimal medium supplemented with kanamycin.

DNA manipulation and analysis. Plasmid DNA preparation, restriction enzyme digestion, agarose and polyacrylamide gel electrophoresis, DNA cloning, and transformation of DNA into *E. coli* cells were carried out by standard protocols (29). *R. leguminosarum* genomic DNA was extracted as previously described (16). For Southern hybridization, DNA probes were labeled with digoxigenin (Roche Molecular Biochemicals, Mannheim, Germany), and hybridization bands were visualized by luminography. DNA sequencing was carried out by using a rhodamine terminator cycle sequencing Ready Reaction kit and an ABI377 automatic sequencer (PE Biosystems, Foster City, Calif.) or by using a Sequenase kit (Sequenase, version 2.0; United States Biochemicals, Cleveland, Ohio).

Transcription start mapping. The location of the 5′ end of the mRNA from the *nifA* gene was determined by primer extension analysis as described by Hidalgo et al. (10). For this assay, two synthetic oligonucleotides, RT-LP1 (5′-CCCGACGAAGACGATACCAG-3′) and RT-LP2 (5′-GCACGAGGCCATTCCAGAGC-3′), were used. These primers were complementary to the sequence corresponding to codons 16 to 22 and 52 to 58 of *orf71*, respectively. Total RNA for this analysis was isolated from microaerobic cells and bacteroids of *R. leguminosarum* UPM791 by using an RNeasy mini kit (QIAGEN, Hilden, Germany). The synthetic primers were labeled with [γ-^{32}P]dATP, and the corresponding DNA extension products obtained by a reverse transcriptase reaction from RNA were visualized by autoradiography.

RT-PCR. Reverse transcription (RT)-PCRs were performed with a GeneAmp PCR System 2400 DNA thermal cycler (PE Biosystems) by using the Titan One Tube RT-PCR system with avian myeloblastosis virus and Expand High Fidelity (Roche Molecular Biochemicals) as specified by the manufacturer. Total RNA from aerobic cells and bacteroids of *R. leguminosarum* UPM791 was isolated as described above and treated with RNase-free DNase (Roche Molecular Biochemicals) to remove contaminating chromosomal DNA. As a negative control, PCRs were carried out in parallel with each RT-PCR by using the Expand High Fidelity PCR system (Roche Molecular Biochemicals). The primers used are described in Table 2.

Generation of *R. leguminosarum* UPM791 *nifA* and *fixW* upstream region probes. Two oligonucleotides (primer NIFA1 [5′-CGGAATTCGGCATGATTAAACCAGAGGC-3′] and primer NIFA2 [5′-CGGGATCCTGACTCCTTCTTCACATCGATA-3′]) derived from the *R. leguminosarum nifA* sequence (EMBL accession no. X05049 [9]) were used as primers for PCR amplification of total DNA from strain UPM791. Two other oligonucleotides (primer UPFixW [5′-GTCGGCAACCATACAAACCC-3′] and primer LPFixW [5′-CTGCGGGTGCGTGACATTGC-3′]), which were derived from another *R. leguminosarum* sequence (EMBL accession no. X16521 [11]), were used as primers for PCR amplification of total DNA from strain UPM791. The amplified 1.6- and 0.4-kb DNA fragments were sequenced to confirm that they corresponded to the *nifA* and *fixW* region sequences, respectively.

Plasmid construction. The 1.6-kb DNA fragment containing *nifA* mentioned above was cloned in plasmid pCR 2.1-TOPO, generating plasmid pCRnifA. The *nifA* probe was used to isolate cosmid pALM35 from a previously constructed *R. leguminosarum* UPM791 genomic library (16).

Plasmid pSKM2 was generated by cloning a 1.1-kb SmaI/EcoRI fragment, which contained the *fixX-nifA* intergenic region, from the pALM35 cosmid in pBluescript. The *fixX-nifA* intergenic region in pSKM2 was deleted by sequentially introducing two AvrII cut sites, the first one immediately downstream of the *fixX* gene and the second one immediately upstream of the *nifA* gene. AvrII cut sites were introduced by site-directed mutagenesis by using a Quick Change site-directed mutagenesis kit (Stratagene) and following the manufacturer's protocol. Two synthetic, complementary oligonucleotides carrying the corresponding mutations were used to synthesize the entire plasmid. Oligonucleotide 5′-G

TABLE 2. Primers used in RT-PCR experiments

RT-PCR product length (bp)	Upper primer		Lower primer	
	Designation (sequence)	5′ Position	Designation (sequence)	5′ Position
782	RT-UP1 (5′-GGGCGGATTGCGACACGACA-3′)	939[a]	RT-LP4 (5′-ACGCCCGTGTCGCTGAACTT-3′)	1720[a]
1,472	RT-UP1 (5′-GGGCGGATTGCGACACGACA-3′)	939[a]	RT-LP5 (5′-GCCCCCATAGCGGTTACGAA-3′)	2410[a]
1,270	RT-UP2 (5′-GCTTCGGACGCCTGGTATCG-3′)	1141[a]	RT-LP5 (5′-GCCCCCATAGCGGTTACGAA-3′)	2410[a]
2,108	RT-UP6 (5′-CAAGGCGTACCGACCATCAT-3′)	2330[a]	RT-LP8 (5′-GCGTCATCTCGGAAGTTTGG-3′)	4437[a]
1,874	RT-UP8 (5′-GCAACCGCACAACTCTATCA-3′)	4197[a]	PD-Inter2 (5′-TGGAGCCGCGCCTCTGGTTT-3′)	995[b]
954	IDH549 (5′-GAAATGACGCGCATCATCTGG-3′)	421[c]	IDHA982 (5′-CCAGGCGAAGATCGAGGCGAT-3′)	1354[c]

[a] EMBL accession no. AJ431175 sequence.
[b] EMBL accession no. X05049 sequence (9).
[c] GenBank accession no. AY535005 sequence.

GATGAGGAGTCCCTAGGTCCGGCGG-3′ and the complementary primer were used to create the AvrII cut site downstream of *fixX*. The AvrII cut site upstream of *nifA* was created by using primer 5′-CACCCTCCCCTGTTCCTA GGCATGATTAAA-3′ and the complementary primer. Introduction of the correct mutations into the plasmid was confirmed by sequencing of the *fixX-nifA* intergenic region. The resulting plasmid was cut with the AvrII enzyme and religated, giving plasmid pSKM3. Plasmid pSKM4 was generated by cloning in pBluescript a 1.3-kb SalI/EcoRI fragment from the pALM35 cosmid containing the P_{nifA1} promoter.

Generation of *lacZ* fusions. A 4.8-kb EcoRI/XbaI fragment and the upstream 1.3-kb SalI/EcoRI fragment were sequentially cloned in pVIK112 to generate plasmid pVIKM1 containing a *nifA*::*lacZ* fusion. A 5.6-kb EcoRI fragment containing the 5′ end of *nifA* from cosmid pALM35 was cloned in the pVIK112 vector in both orientations, generating plasmids pVIKM2 and pVIKM12 containing *nifA*::*lacZ* and *orf79*::*lacZ* fusions, respectively. The 4.5-kb EcoRI/SmaI fragment from cosmid pALM35 and the 939-bp SmaI/EcoRI fragment from plasmid pSKM3 were sequentially cloned in pVIK112, giving plasmid pVIKM3. The 1.1-kb SmaI/EcoRI fragment from plasmid pSKM2 containing the 5′ end of *nifA* was cloned in the pVIK112 vector, generating plasmid pVIKM4. The 0.66-kb EcoRI fragment from plasmid pCRnifA was cloned in pVIK112 in both orientations, generating plasmids pVIKM8 and pVIKM18. The 1.3-kb SalI/EcoRI fragment from cosmid pALM35 was cloned in vector pSPV4, generating plasmids pSPM9 and pSPM10 containing *orf79*::*lacZ* and Δ*nifH*::*lacZ* fusions, respectively.

Plant tests and enzyme assays. Pea (*Pisum sativum* L. cv. Frisson) plants were used as hosts for *R. leguminosarum* bv. viciae strains. The conditions used for plant inoculation and growth have been described previously (16). β-Galactosidase activities in *E. coli* and *Rhizobium* cell cultures and pea bacteroid suspensions were determined as described by Miller (20). Whole-root acetylene reduction was determined as described by Ruiz-Argüeso et al. (28) by using 120-ml flasks containing the whole pea plant root system. The protein contents of bacteroid suspensions and cell cultures were measured by the bicinchoninic acid method (32) after alkaline digestion in 1 N NaOH at 90°C for 10 min; bovine serum albumin was used as a standard.

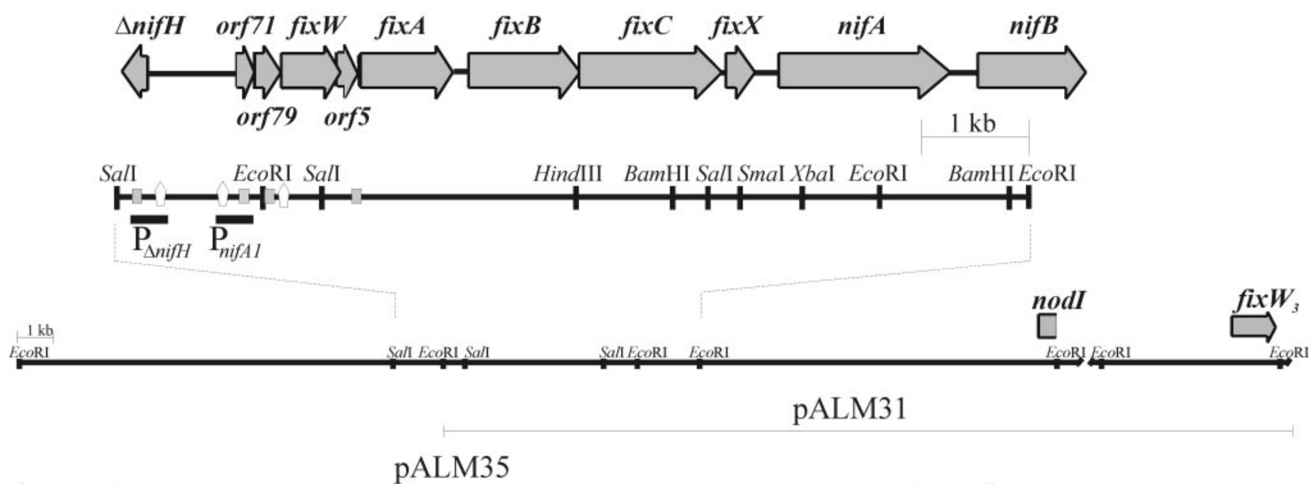
RESULTS

Analysis of the *nifA* DNA region. The *nifA* DNA region of *R. leguminosarum* bv. viciae strain UPM791 was located and sequenced as a first step towards identifying the regulatory DNA elements involved in NifA expression in this symbiotic bacterium. A gene library was screened with *nifA* and *orf71 orf79* probes (see Materials and Methods) that were generated from available *nifA* (9) and *fixW* region (11) sequences from strains 3855 and PRE, respectively. Two overlapping cosmids, pALM31 and pALM35, spanning a 50-kb region that included the *nifA* region, *nodI*, and *fixW3*, were identified by using these probes (Fig. 1A). PCR and Southern hybridization analyses with strains UPM791 and UPM791.5 (cured of the symbiotic plasmid) indicated that only one copy of *nifA* was located in the symbiotic plasmid (data not shown). From this region, a 9.3-kb DNA fragment containing *nifA* was sequenced (EMBL accession no. AJ431175 and AJ520101), and the sequence analysis revealed a gene cluster (*orf71 orf79 fixW orf5 fixA fixB fixC fixX nifA nifB*) separated by 896 bp from a truncated duplication of *nifH* (Δ*nifH*). Genes and open reading frames in this cluster were designated on the basis of their similarity to genes and open reading frames previously described in *R. leguminosarum* bv. viciae 3855 (9) and PRE (11) and in other rhizobia. This is the first report of a complete *fixABCX* gene sequence from *R. leguminosarum* bv. viciae. The gene products FixA, FixB, FixC, and FixX were homologous (with overall levels of identity ranging from 53 to 97% [data not shown]) to the same proteins from other diazotrophic bacteria present in databanks. Δ*nifH*, *orf71*, *orf79*, and *fixW* were highly conserved with respect to corresponding genes and coding sequences from strain PRE (11). The Δ*nifH-orf71* intergenic region contained two potential σ^{54}-binding sequences preceded by two consensus NifA-binding sequences, suggesting the existence of two divergent NifA-dependent promoters that we designated $P_{\Delta nifH}$ and P_{nifA1} (Fig. 1A). An imperfect repetition of the P_{nifA1} region was found at the 5′ end of *fixA*. This region included the σ^{54}-binding motif but not the NifA-binding site (Fig. 1B).

Expression analysis of *nifA*. Expression analysis of *R. leguminosarum* UPM791 *nifA* was accomplished by generating genomic *lacZ* fusions to *nifA* and to other target genes within a 6.9-kb SalI/EcoRI fragment, including the potential *nifA* promoter region. The suicide vector pVIK112 (13) was used to clone fragments of the *nifA* region upstream of the *lacZ* reporter gene, and the genomic fusions were created in a single step by integrative homologous recombination, which resulted in UPM791 derivative strains belonging to the GLF series (Fig. 2 and 3A). This process is illustrated in Fig. 2 for strain GLF1. Except for GLF8 and GLF18, cloned fragments contained the intact 5′ end of the *nifA* gene with or without the P_{nifA1} promoter.

Only plants inoculated with *R. leguminosarum* strain GLF1 exhibited a wild-type phenotype (green plants and normal red nodules) (Fig. 3B), suggesting that normal amounts of NifA were present. Plants inoculated with the GLF2, GLF3, and GLF12 strains exhibited an intermediate yellow-green color and smaller red nodules (Fig. 3B), as well as a 65% decrease in the acetylene reduction rate and a 50% decrease in the dry weight (Table 3). Finally, plants inoculated with strains GLF4, GLF8, and GLF18 contained tiny, white nodules and could not be distinguished from noninoculated control plants on the basis of color (Fig. 3B), dry weight, and nitrogenase activity (Table 3).

R. leguminosarum GLF strains were tested for β-galacto-



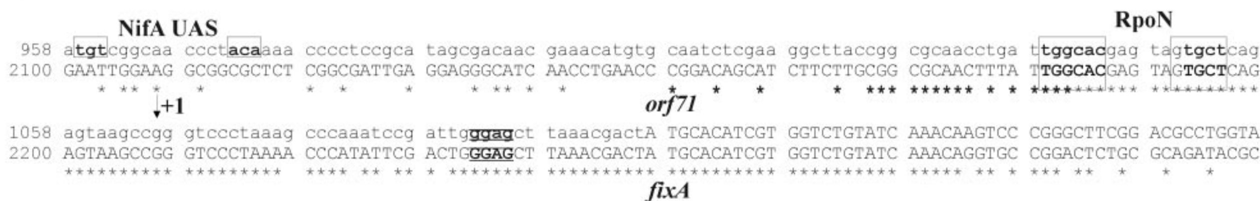


FIG. 1. Structure of *nifA* DNA region from *R. leguminosarum* bv. viciae UPM791. (A) Gene organization. The grey arrows correspond to genes or open reading frames identified in a 6.9-kb SalI/EcoRI fragment containing the *nifA* gene from *R. leguminosarum* and adjacent DNA (EMBL accession no. AJ431175 and AJ520101). The pattern of restriction enzyme cutting sites is indicated below the genetic map. The positions of NifA upstream binding sequences (UAS) and σ^{54}-binding sites are indicated by open vertical arrows and grey boxes, respectively. $P_{\Delta nifH}$ and P_{nifA1}, indicated by horizontal lines below the restriction map, correspond to potential NifA and σ^{54}-dependent promoters located upstream of Δ*nifH* and *orf71*, respectively. The DNA bordering the NifA region and the cosmids used in its identification are shown at the bottom. (B) Comparison of nucleotide sequences of the region upstream of *orf71* plus the 5′ end of *orf71* (top line) with the 3′ end of *orf5*, the intergenic fragment *orf5-fixA*, and the 5′ end of *fixA* (bottom line). The numbers on the left indicate the positions of the first nucleotide according to the EMBL accession no. AJ431175 sequence. Nucleotides conserved in both sequences are indicated by asterisks. NifA- and σ^{54}-binding sites are enclosed in boxes. The transcription initiation site (+1) of the P_{nifA1} promoter is indicated by a vertical arrow.

sidase activity in free-living cells grown under aerobic or microaerobic conditions and in bacteroids from peas. No significant β-galactosidase activity was detected in free-living cultures of any of the GLF strains grown under either aerobic or microaerobic conditions (data not shown). In strain GLF1 bacteroids, in which high levels of *nifA* transcription were observed (Table 3), the *nifA* promoter must be included in the region upstream of *nifA* located in the fragment integrated in the GLF1 strain. This region contains $P_{\Delta nifH}$ and P_{nifA1} promoters and any other potential promoter located upstream of the *nifA* gene (Fig. 3A). The plant phenotypes suggested that the intact copy of the *nifA* gene was not transcribed at wild-type levels in bacteroids from the GLF2, GLF3, and GLF12 strains. These strains did not contain either $P_{\Delta nifH}$ or P_{nifA1} upstream of the intact copy of the *nifA* gene. Comparison of these results with those obtained with strain GLF1 indicated that *nifA* is mainly transcribed from P_{nifA1} and also that low levels of expression originated from the activity of a basal promoter located between *orf79* and *nifA*. This potential promoter was designated P_{nifA2}. The β-galactosidase activities observed in bacteroids from *nifA*::*lacZ* fusions (Table 3) demonstrated that expression of *nifA* in bacteroids from strain GLF2 was only 25% of the expression in bacteroids from strain GLF1. Given that P_{nifA1} has the structure of a NifA-dependent promoter (NifA UAS and properly spaced σ^{54} box), these results suggest that NifA is the activator of the P_{nifA1} promoter. The fact that GLF3, a strain lacking the intergenic region between *fixX* and *nifA* in the cloned fragment upstream of *lacZ*, exhibited β-galactosidase activities similar to those of strain GLF2 (Fig. 3) suggests that P_{nifA2} is located upstream of the intergenic *fixX-nifA* region.

β-Galactosidase activities could not be determined in strains GLF4, GLF8, and GLF18 since these strains initiated only incipient, inefficient nodules in pea plants. The plant phenotype obtained with strain GLF4 suggested that its intact copy of the *nifA* gene was not transcribed in bacteroids. Since the upstream region of this *nifA* gene contained only the 3′ end of *fixX* and the intergenic region between *fixX* and *nifA*, we concluded that there was no promoter activity in this intergenic region. This result implies that the basal promoter P_{nifA2} may

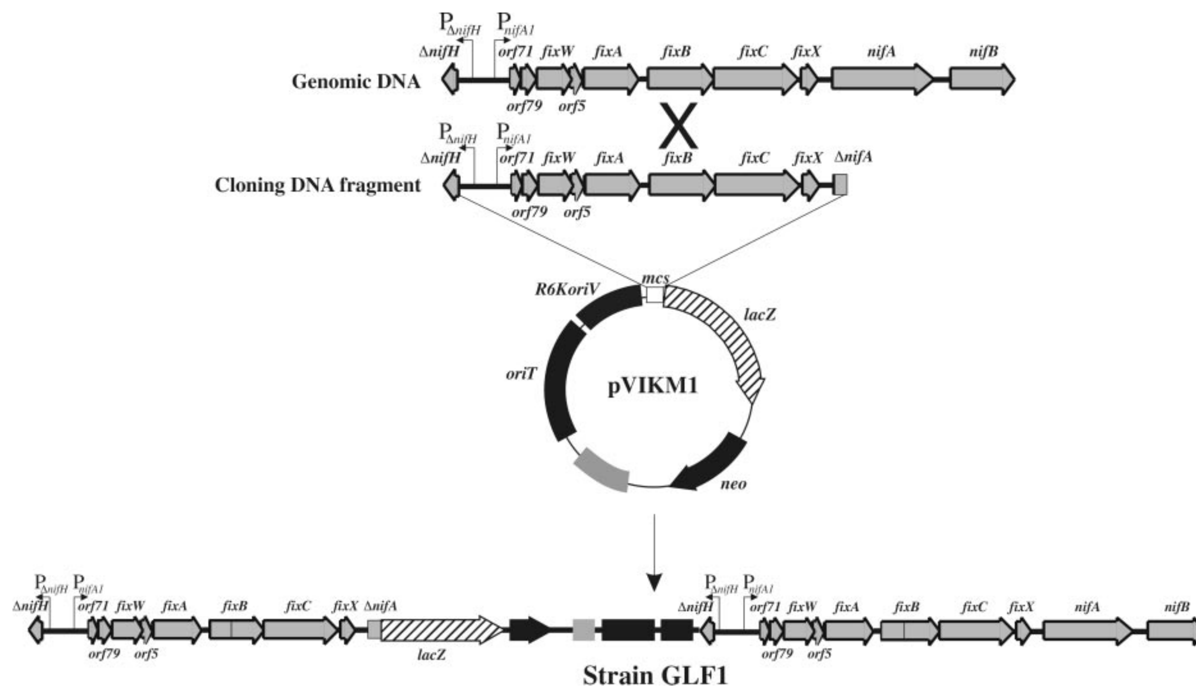


FIG. 2. Construction of *R. leguminosarum* GLF1 genomic *nifA*::*lacZ* fusion. A 6.1-kb SalI/XbaI fragment spanning the region from Δ*nifH* to *nifA* was cloned in suicide vector pVIK112 upstream of a *lacZ* reporter gene, generating plasmid pVIKM1. Single homologous recombination between the cloned fragment and genomic DNA resulted in integration of the entire pVIKM1 plasmid in the genome. The resulting GLF1 strain harbored a duplication of the cloned DNA fragment and a genomic *nifA*::*lacZ* fusion.

be located between *orf79* and the 3′ end of *fixX*. *R. leguminosarum* strains GLF8 and GLF18 were generated by integration of a pVIK112 derivative plasmid containing the 3′ end of *nifA*. This resulted in an intact copy of the *nifA* gene that was upstream of the plasmid DNA but was separated from *nifB*. Since these strains exhibited a Fix^- phenotype when they were used as inocula for peas, although the intact copy of the *nifA* gene was fully transcribed from P_{nifA1}, we suggest that *nifB*, which is normally cotranscribed with *nifA*, was not transcribed in these strains.

To confirm the functionality of the P_{nifA1} promoter, primer extension analysis of RNA from bacteroids was carried out by using primers RT-LP1 and RT-LP2 located upstream of *orf79* (Fig. 4A). A clear DNA extension band identified the G located 13 bp downstream of the TGGCAC-N_6-TGCT σ^{54}-binding signature as the P_{nifA1} transcription initiation site (Fig. 4B). No transcription initiation site was detected by using RNA from free-living microaerobic cells. These results confirmed the functionality of the σ^{54}-type promoter P_{nifA1} in bacteroids. Similarly, we attempted to show that transcription corresponding to the P_{nifA2} promoter was associated with the P_{nifA1} promoter homologous sequence located upstream of *fixA* (Fig. 1B). However, primer P3nifA (5′-ATTTGTCACCG GGTGGACGC-3′), corresponding to a *fixA* sequence, did not detect any transcription initiation site in the primer extension experiment with bacteroid RNA (data not shown). A similar, negative result was obtained with primer P2nifA (5′-AGAGCCGCTACTTAAACTAG-3′), which corresponded to a *fixW* sequence and was designed to identify promoter activity associated with the potential σ^{54}-binding site within *orf79* (data not shown).

***P_{nifA1}* controls symbiotic expression of the *nifA* gene.** To confirm that *nifA* was transcribed from the P_{nifA1} promoter under symbiotic conditions, we investigated the existence of an mRNA extending from this promoter to *nifA* in pea bacteroids from wild-type strain UPM791 by performing RT-PCR experiments (Fig. 5). RNA isolated from bacteroids from 21-day-old pea plants and from free-living aerobic cells (as a control) served as templates for the RT-PCRs and PCRs with various primer sets (Table 2). These sets, covering the *orf71 orf79 fixW orf5 fixA* (1,270-bp fragment), *fixA fixB fixC* (2,108-bp fragment), and *fixB fixC fixX nifA* (1,874-bp fragment) DNA regions, yielded the expected products (Fig. 5, lane 1), providing evidence that the *orf71 orf79 fixW orf5 fixA fixB fixC fixX nifA* gene cluster forms a transcription unit. In contrast, a primer designed to anneal with the region upstream of the P_{nifA1} transcription start site gave weak RT-PCR products (782- and 1,472-bp fragments). No PCR products were obtained in the absence of reverse transcriptase (Fig. 5, lanes 2 and 4), which excludes the possibility that the PCR products observed resulted from contaminated chromosomal DNA. RT-PCR amplification of the isocitrate dehydrogenase gene was used as an internal standard. For this gene, PCR products (954 bp) with similar intensities were obtained from bacteroids and aerobically grown free-living cells (Fig. 5, lanes 1 and 3).

Promoter *P_{nifA1}* is regulated by NifA. Since P_{nifA1} has the structure of a NifA-dependent promoter, we tested its activation in *E. coli* in the presence of *K. pneumoniae* NifA supplied by plasmid pMJ220. Reporter plasmids pSPM9 and pSPM10 containing the $P_{\Delta nifH}$ and P_{nifA1} promoters were transformed into *E. coli* strains ET8000 and ET8000(pMJ220), and the β-galactosidase activities of the resulting strains were assayed

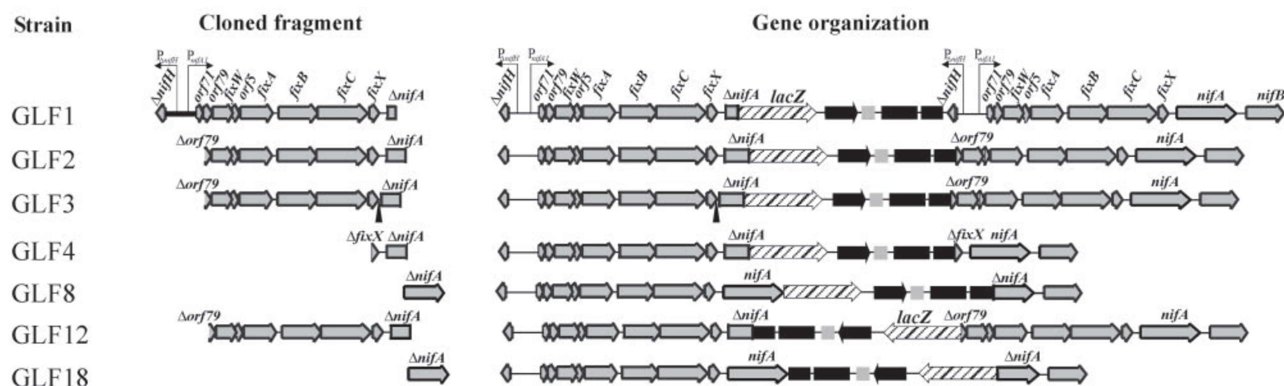


FIG. 3. Expression analysis of *nifA* region. (A) Structure of *lacZ* fusion constructs: DNA fragments cloned in suicide vector pVIK112 and the genetic organization resulting after integration of the pVIKM plasmids in the genome. The designations of the derivative GLF reporter strains are indicated on the left. (B) Appearance of 21-day-old pea plants inoculated with the *R. leguminosarum* GLF1 (plant a), GLF2 (plant b), and GLF4 (plant c) strains containing *nifA*::*lacZ* genomic fusions. Root nodules are shown below the corresponding inoculated plants.

in cells from aerobic cultures (Fig. 6). For both plasmids, β-galactosidase activity was detected only in the presence of NifA. These results demonstrated that both the P_{nifA1} and $P_{\Delta nifH}$ promoters can be activated by NifA.

DISCUSSION

The *nifA* region of *R. leguminosarum* bv. viciae UPM791 contains a gene cluster (*orf71 orf79 fixW orf5 fixA fixB fixC fixX nifA nifB*) separated by 896 bp from an upstream truncated duplication of *nifH* (Δ*nifH*) in the opposite orientation. The gene organization of this region was similar to that described for strain PRE (11) and was identical to the gene organization of part of this region described for strain 3855 (9). Sequences corresponding to *orf71*, *orf79*, and *fixW* sequences have been detected only in certain strains of *R. leguminosarum* (11).

UPM791 reporter strains, constructed by insertion of suicide pVIKM plasmids into the genome (Fig. 3A), clearly demonstrated that *nifA* is exclusively transcribed in pea bacteroids and that this transcription originates from a σ^{54}-dependent promoter (P_{nifA1}) located upstream of *orf71* at the expected distance (−25/−13) from the identified transcription initiation site (Fig. 4). mRNA analysis confirmed that *orf71 orf79 fixW orf5 fixABC fixX* and *nifA* form an operon in symbiotic conditions (Fig. 5). This operon might also include *nifB* since non-nitrogen-fixing plants were obtained when *nifB* was transcriptionally separated from *nifA*. Green, efficient nitrogen-fixing pea plants were obtained only in the presence of full transcription levels of *nifA nifB* that originated from P_{nifA1} (Fig. 3B).

P_{nifA1} contains canonical NifA-binding sequences ($TGTN_{10}$ ACA) located 91 bp upstream of the transcription start site. These NifA-binding sequences in a σ^{54}-type promoter suggested that there was autoregulation of P_{nifA1} by NifA, and this was confirmed by showing that *K. pneumoniae* NifA promoted the expression of an *orf79*::*lacZ* fusion in *E. coli* (Fig. 6). No

TABLE 3. Symbiotic characteristics of reporter GLF derivative strains[a]

Strain	Fix phenotype	Plants		Bacteroids	
		Color	Dry wt (g/pot)[b]	Nitrogenase activity[c]	β-Galactosidase activity (Miller units)
GLF1	Fix^+	Green (wild type)	4.85 ± 0.3	5.1 ± 1.6	1,021 ± 30
GLF2	Fix^+	Intermediate	2.65 ± 0.25	1.9 ± 0.6	267 ± 65
GLF3	Fix^+	Intermediate	2.25 ± 0.25	1.8 ± 0.45	172 ± 80
GLF4	Fix^-	Yellow	1.15 ± 0.25	<10	ND[d]
GLF8	Fix^-	Yellow	1.20 ± 0.32	<10	ND
GLF12	Fix^+	Intermediate	2.67 ± 0.35	1.9 ± 0.7	40 ± 20
GLF18	Fix^-	Yellow	1.12 ± 0.2	<10	ND

[a] The strains were used as inoculants for peas, and the Fix phenotypes, plant colors, dry weights 32 days after inoculation, and nitrogenase and β-galactosidase activities of pea bacteroids were determined. All values are averages ± standard deviations for three assays.
[b] Each pot contained five plants.
[c] Nitrogenase activity is expressed in nanomoles of C_2H_2 reduced per hour per plant.
[d] ND, not determined.

consensus sequences involved in bending the DNA (integration host factor) to facilitate contact of NifA with the RNA polymerase were identified between the UAS and the σ^{54}-binding signature in the P_{nifA1} and $P_{\Delta nifH}$ promoters. The autoregulated P_{nifA1} promoter is required for effective nitrogen fixation by pea plants inoculated with the UPM791 strain. Derivative strains lacking P_{nifA1} induced pea nodules that were symbiotically inefficient, and the bacteroids exhibited only basal, although bacteroid-specific, levels of *nifA* expression. Autoregulation of *nifA* expression in symbiosis appears to be a frequent phenomenon in endosymbiotic bacteria. In *S. meliloti*, autoregulation of NifA expression occurs from the

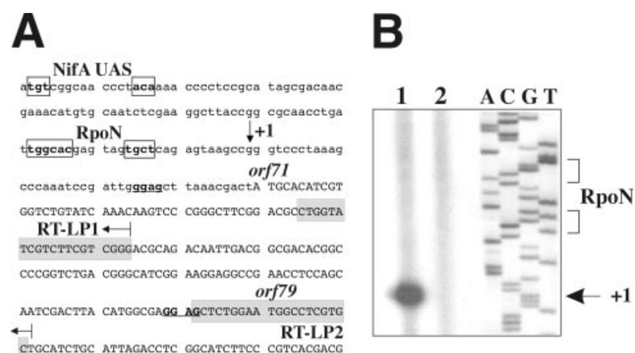


FIG. 4. Identification of the transcription initiation site of the P_{nifA1} promoter. (A) Nucleotide sequence (360 bp) that included the region upstream of *orf71*, *orf71*, and the 5′ end of *orf79*. Potential NifA- and σ^{54}-binding sites are enclosed in boxes. The potential ribosome-binding sites for *orf71* and *orf79* are underlined and in boldface type. The transcription initiation site (+1) is indicated by a vertical arrow. The sequences in grey boxes designated RT-LP1 and RT-LP2 correspond to the two primers used in primer extension reactions. (B) Determination of the transcription start site. Total-RNA samples (10 μg) isolated from pea bacteroids (lane 1) and microaerobic free-living cells (lane 2) of *R. leguminosarum* bv. viciae UPM791 were used in primer extension reactions with primer RT-LP1. Lanes A, C, G, and T, dideoxynucleotide sequencing reactions carried out with plasmid pSKM4 and the same primer. The positions of the relevant sequence for RpoN and the transcription initiation site are indicated on the right. The same results were obtained with primer RT-LP2.

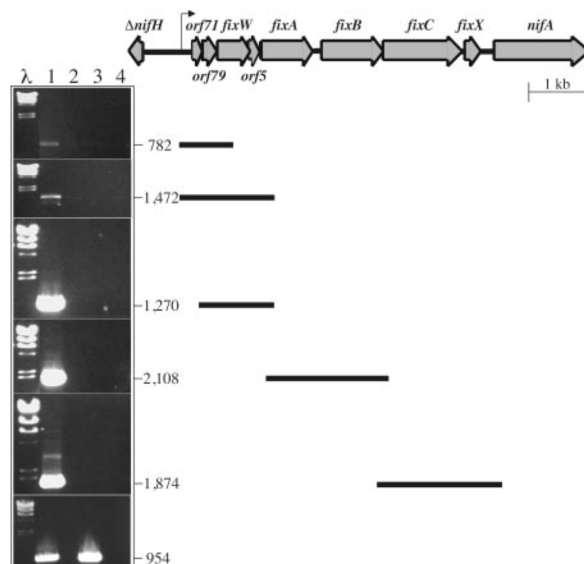


FIG. 5. RT-PCR expression analysis of *nifA* region from *R. leguminosarum* bv. viciae UPM791. The gene organization of the *nifA* region is indicated at the top. The gel DNA band profiles are the profiles for RT-PCR (lanes 1 and 3) and PCR (lanes 2 and 4) products obtained with total RNA from bacteroids (lanes 1 and 2) or from aerobic free-living cells (lanes 3 and 4). The lengths of the resulting RT-PCR products (in base pairs) and their positions within the *nifA* region are indicated on the right. The primers used for RT-PCR and PCRs are described in Table 2. Amplification of an internal fragment of the isocitrate dehydrogenase gene (954 bp) was used as a control for RNA quantitation (bottom panel).

fixABCX promoter, but basal levels of symbiotic NifA expression are dependent on the FixLJ system acting on a *nifA* promoter (5, 7). Similarly, the *fixR nifA* operon of *B. japonicum* is regulated by RegR in aerobic cells and is autoregulated by NifA in symbiosis (33).

Deletion analysis of the DNA region upstream of *nifA* clearly showed that basal, bacteroid-specific *nifA* expression was prompted by a second, unidentified promoter (P_{nifA2}) lo-

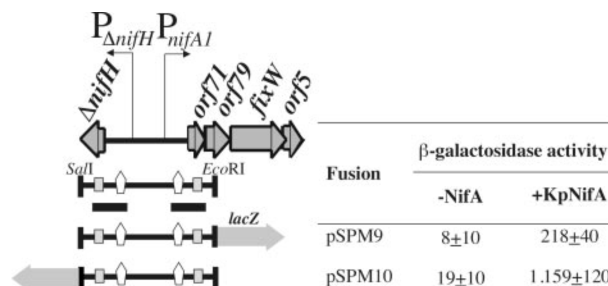


FIG. 6. NifA-dependent expression of $P_{\Delta nifH}$ and P_{nifA1} promoters in *E. coli.* The physical and genetic map of a 1.3-kb SalI/EcoRI fragment containing the $P_{\Delta nifH}$ and P_{nifA1} promoters is shown on the left. The positions of NifA upstream binding sequences (UAS) and σ^{54}-binding sites are indicated by open vertical arrows and grey boxes, respectively. The fragment fused to the *lacZ* gene in both orientations is shown at the bottom. β-Galactosidase activities associated with the *lacZ* fusions were measured in *E. coli* strain ET8000 expressing *K. pneumoniae nifA* (KpNifA) from plasmid pMJ220. The values (in Miller units) are averages ± standard deviations for three assays.

cated downstream of *orf79* and upstream of the 3′ end of *fixX*. No promoter activity associated with the *nifA-fixX* intergenic region was detected. This was confirmed by the absence of symbiotic nitrogen fixation with strains whose *nifA* expression was dependent on this intergenic DNA fragment. This conclusion is supported by the absence of recognizable promoter sequence motifs immediately upstream of *nifA*. Initially, it was hypothesized that the promoter responsible for basal levels of NifA expression (P_{nifA2}) in bacteroids was located immediately upstream of *fixA*. This assumption was based on (i) the existence of a *fixABCX* operon in other bacteria (*S. meliloti*); (ii) the absence of promoter-like sequences upstream of *fixX*; (iii) the lack of intergenic space among the *fixA, fixB*, and *fixC* genes; and overall (iv) the observation that the DNA sequence preceding *fixA* is identical to the DNA sequence of P_{nifA1} except for the absence of the NifA-binding UAS. The existence of this sequence duplication suggests that P_{nifA1} was the original promoter of the *fixABC* operon but it was interrupted and partially duplicated, together with the N-terminal end of *fixA*, after insertion of *fixW*. However, primer extension assays, directed to identify a possible transcription initiation site downstream of the σ^{54}-binding motif by using bacteroid RNA samples, were unsuccessful. It is possible that the P_{nifA2} promoter is indeed active and that the failure was due to its low activity. If this were the case, the existence of a symbiosis-specific, *trans*-acting regulatory factor should be postulated for P_{nifA2} activity, since σ^{54}-dependent promoters possess no constitutive activity in the absence of an activator protein.

In conclusion, our analysis showed that in *R. leguminosarum* bv. viciae UPM791 *nifA* is expressed only under symbiotic conditions, and this explains the previously observed inability of this organism to express NifA-dependent systems, such as nitrogenase and hydrogenase (4), under free-living conditions. Symbiotic *nifA* expression is under positive autoregulation by NifA and originates from a promoter (P_{nifA1}) located 4.7 kb upstream of *nifA*. This promoter determines transcription of the *orf71 orf79 fixW orf5 fixABC fixX nifA* operon. It is absolutely required for full *nifA* transcription and, consequently, for efficient nitrogen fixation. In its absence, basal *nifA* transcription originates from an uncharacterized promoter (P_{nifA2}) located upstream of the intergenic *fixX-nifA* region and probably upstream of *fixA*. This mode of regulation of *nifA* is characteristic of *R. leguminosarum* bv. viciae and differs from models studied in other rhizobia (8).

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