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Regulatory Proteins and *cis*-Acting Elements Involved in the Transcriptional Control of *Rhizobium etli* Reiterated *nifH* Genes

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In *Rhizobium etli* the nitrogenase reductase genes are reiterated. Strain CE3 has three copies; *nifHa* and *nifHb* form part of *nifHDK* operons with the nitrogenase structural genes, while *nifHc* is linked to a truncated *nifD* homolog. Their sequences are identical up to 6 residues upstream from a σ^{54}-dependent promoter. A remarkable difference among them is the absence of canonical NifA binding sites upstream of *nifHc* while a canonical binding site is located 200 bp upstream of *nifHa* and *nifHb*. To evaluate the transcriptional regulation of the reiterated *nifH* genes, we constructed fusions of *nifHa* and *nifHc* with the *lacZ* gene of *Escherichia coli*. Both genes were expressed at maximum levels under 1% oxygen in free-living cultures, and their expression declined as the oxygen concentration was increased. This expression was dependent on the integrity of *nifA*, and *nifHc* was expressed at higher levels than *nifHa*. The same pattern was observed with root nodule bacteroids. Expression of both genes in *E. coli* required σ^{54} in addition to NifA bound to the upstream activator sequence. In vivo dimethyl sulfate footprinting analyses showed that NifA binds to the canonical site upstream of *nifHa* and to a TGT half-site 6 nucleotides further upstream. NifA protected an imperfect binding site upstream of *nifHc* at position 85 from the promoter. The integration host factor stimulated each gene differently, *nifHa* being more dependent on this protein. The above results correlate the asymmetric arrangement of *cis*-acting elements with a differential expression of the reiterated *nifH* genes, both in culture and during symbiosis with bean plants.

Eubacterial nitrogen fixation genes (*nif* and *fix*) are generally transcribed from σ^{54}-dependent promoters located between positions −26 and −11 relative to the transcription start site and have the consensus sequence 5′-TGGCACN_5TTGCA/T-3′ (37). σ^{54} (also known as RpoN, s^N, or NtrA) assists in the assembly of a preinitiation closed complex with RNA polymerase and promoter DNA ($E\sigma^{54}$). Isomerization of this stable closed complex is dependent on the NifA regulatory protein and hydrolysis of a nucleoside triphosphate (37). NifA binds to a conserved sequence motif (5′-TGTN_{10}ACA-3′) located at a distance ranging from 80 to 150 nucleotides upstream from the transcriptional start site (known as the upstream activator sequence [UAS]) (36). It has been proposed that the function of the UAS is to increase the local concentration of NifA and to correctly orientate it in the vicinity of a given promoter to facilitate a productive interaction with $E\sigma^{54}$ (8, 25). Once NifA is bound to its UAS, it approximates the preinitiation closed complex by looping the intervening DNA (8, 25). In this regard, expression of several σ^{54}-dependent promoters is enhanced by the integration host factor (IHF), a DNA-bending dimeric heteroprotein that binds specifically between the UAS and the promoter (25, 29). Although remote activation is a general mechanism for the expression of σ^{54}-dependent genes, certain strong promoters, whose sequences strongly resemble the consensus sequence, can be partially activated even in the absence of the UAS by NifA proteins impaired in their DNA binding ability (4, 7, 26, 38), indicating that the DNA binding and the activation of transcription are different functions of the same regulatory protein. Gubler (23) proposed that the presence and number of UASs in a NifA-dependent promoter provide a fine-tuning mechanism for the regulation of gene expression.

Oxygen concentration is the major environmental signal regulating *nif* gene expression. This regulation is enacted by controlling both the expression and the activity of NifA. Autoactivation has been observed in several species (19). In *Rhizobium meliloti*, *nifA* expression is additionally controlled by the oxygen-sensing, two-component FixLJ system (19) while its activity is directly controlled by the oxygen concentration, probably through the oxidation of a metal cofactor linked to a conserved set of cysteine residues (19).

All *Rhizobium etli* (formerly *Rhizobium phaseoli* [53]) strains characterized to date have three copies of the nitrogenase reductase (*nifH*) gene (32, 46). As in most of the nitrogen-fixing eubacteria, in the type strain CE3, *nifHa* and *nifHb* are transcriptionally coupled with the nitrogenase structural genes, forming *nifHDK* operons, while no homologs of the latter genes have been found downstream of *nifHc* (47). The three *nifH* genes are actively expressed during symbiosis with bean plants, but it seems that only the integrity of both *nifHDK* operons is required to achieve full nitrogenase activity (39, 50). The sequences of the three genes are identical up to 6 nucleotides upstream from a putative σ^{54}-dependent promoter (5′-ATGGCACGGGTTTTGAA-3′). The identity between *nifHa*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Rhizobium etli		
CE3	Wild-type CFN42, Sm^r	41
CFNX247	CE3, *nifA*Δ::Sp/Sm	20
DEM233	CE3, *nifHc-lacZ*	This study
DEM233-1	CFNX247, *nifHc-lacZ*	This study
DEM153	CE3, *nifHa-lacZ*	This study
DEM153-1	CFNX247, *nifHa-lacZ*	This study
Escherichia coli		
ET8000	*rbs gyrA hut lacZ*::IS*1* Mu *cts62*	31
ET8045	*rpoN208*::Tn*10* derivative of ET8000	31
SE100	*himD*::Cm^r derivative of ET8000	35
Plasmids		
pSUP205	Mobilizable derivative of pBR325 (Tc^r)	54
pKOK6	*lacZ*-Km^r interposon in pKOK4 (Km^r Cb^r)	28
pDEM233	*R. etli nifHc-lacZ*-Km^r in pSUP205 (Km^r Tc^r)	This study
pDEM153	*R. etli nifHa-lacZ*-Km^r in pSUP205 (Km^r Tc^r)	This study
pMB210	*R. meliloti nifH-lacZ* in pGC926 (Tc^r)	5
pMB2101	pMB210 but in pMC1403 (Cb^r)	7
pJMW6	*K. pneumoniae nifH-lacZ* in pJEL126 (Cb^r)	6
pMJ220	*K. pneumoniae nifA* expressed from p*lac* in pEMBL8 (Cm^r)	36
pMJ221	pMJ220 but *tyr-512* to *phe* (Cm^r)	36
pMJ220tc	pMJ220 but Tc^r	This study

and *nifHb* extends at least up to 110 nucleotides further upstream, and this sequence resembles a NifA binding site (UAS). In contrast, no UAS has been found upstream of *nifHc* (47). Although there is no functional evidence for any of these *cis*-acting regulatory elements, their asymmetric arrangement suggests that the reiterated *nifH* genes are differentially expressed.

In this study we analyzed the expression of the reiterated *nifH* genes of *R. etli*. We show that *nifHa* and *nifHc* are differentially transcribed both during symbiosis and in free-living conditions, in which they are dependent on NifA, σ^{54}, and low oxygen concentrations. Expression and in vivo dimethyl sulfate (DMS) footprinting analyses showed that the DNA binding ability of NifA is essential to activate both genes, although NifA protected different sites upstream of each gene. We also observed a distinct dependence on the IHF, with *nifHa* being more dependent than *nifHc*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Plasmids and strains used in this study are described in Table 1. *Rhizobium* strains were grown at 30°C in PY (peptone-yeast) complex medium or minimal medium containing succinate and ammonium chloride as the sole carbon and nitrogen sources (18). *Escherichia coli* strains were grown at 37°C in Luria-Bertani complex medium or at 30°C in minimal medium containing 10 mM glucose, 10 mM ammonium chloride or glutamine as the nitrogen source, 11 mM K_2HPO_4, 51 mM KH_2PO_4, and 4 mM $MgSO_4 \cdot 7H_2O$. Antibiotics for *E. coli* strains were added in the following final concentrations: 10 μg of tetracycline per ml, 100 μg of ampicillin per ml, 15 μg of chloramphenicol per ml, and 30 μg of kanamycin per ml.

Construction of *nifH-lacZ* transcriptional fusions. *Eco*RI fragments carrying the *nifHa* and *nifHc* regions from *R. etli* CE3 (47) were subcloned into pSUP205 (54) to generate plasmids pEM15 (39) and pDEM23, respectively. An interposon containing the promoterless structural gene for β-galactosidase and a kanamycin resistance gene (28) was cloned in the *nifH* internal *Bgl*II site, and the orientation was checked, resulting in plasmids pDEM153 (*nifHa-lacZ*) and pDEM233 (*nifHc-lacZ*). *R. etli* CE3 derivatives DEM153 and DEM233 were obtained as one-step double recombinants of their respective plasmids (pDEM153 or pDEM233) by recombination into the symbiotic plasmid by the suicide vector method (24). Selection was carried out with 15 μg of kanamycin per ml and 20 μg of nalidixic acid per ml. All strains were regularly analyzed for genomic stability (i.e., the absence of rearrangements) by Southern analysis (51).

Construction of pMJ220tc. Plasmid pMJ220 carries the *nifA* gene from *Klebsiella pneumoniae* cloned in the pEMBL8 expression vector (36). An omega interposon carrying a tetracycline resistance gene (45) was cloned in the *Eco*RI site internal to the chloramphenicol resistance gene of the vector. This construction, plasmid pMJ220tc, preserved the activation ability of the pMJ220.

Ex planta induction system. Cells from exponential-phase cultures of *R. etli* strains grown in PY medium were collected and washed with minimal medium. Flasks containing minimal medium were inoculated at an initial A_{540} of 0.05. Aliquots (20 ml) were injected into sealed 150-ml bottles previously flushed with several volumes of the appropriate oxygen-argon mixture (analytical grade; Linde, Mexico City, México). After incubation for 8 h, samples for β-galactosidase and protein estimations were withdrawn with syringes and assayed as described below.

Heterologous expression system in *E. coli*. *E. coli* strains were transformed by the calcium chloride method (51). Once the presence of the desired plasmid(s) was confirmed by restriction analysis, the strains were grown in Luria-Bertani complex medium with antibiotics until saturation. Minimal medium containing either ammonium chloride or glutamine, as needed, was inoculated at an initial A_{600} of 0.05. Samples (0.1 ml) were removed from exponentially growing cultures and assayed for β-galactosidase activity as described below.

Plant growth conditions. Surface-sterilized *Phaseolus vulgaris* L. cv. Negro Jamapa seeds were germinated under sterile conditions for 3 days in darkness. The seedlings were transferred to plastic pots containing sterile vermiculite as a support and inoculated with 1 ml of an overnight culture grown in PY medium. Plant growth and watering were carried out under aseptic conditions in a greenhouse.

Estimation of β-galactosidase activity. Nodules from single plants were crushed in cold Z buffer (51), 2 drops of chloroform were added to the mixture, and the preparation was vortexed and centrifuged at 4°C. β-Galactosidase activity was measured as an increase in sample A_{420} in the presence of 0.8 mM *o*-nitrophenyl-β-D-galactopyranoside. Activities in culture were measured as recommended previously (51). Aliquots of nodule extracts (0.05 ml) or bacterial cultures (1.5 ml) were precipitated with 1 volume of 10% trichloroacetic acid, and the protein contents were estimated by the Lowry method. Specific activities are reported as nanomoles of *o*-nitrophenol produced per minute per milligram of protein. Miller units were calculated as described previously (51).

DMS footprinting. *E. coli* cells were inoculated in minimal medium including 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and grown to an A_{600} of 0.6. At this point, β-galactosidase activity was estimated to ensure that induction had occurred. DMS was added (to a 0.1% final concentration) from a fresh 2% solution in the same medium. Cells were incubated for 1 min, diluted in cold saline phosphate, collected by centrifugation, and washed once with saline phosphate. Plasmid DNA prepared from a 100-ml culture was obtained by the alkaline lysis method (51) and resuspended in 0.1 ml of water. Piperidine was added to a 10% concentration and heated at 90°C for 30 min to cleave the DNA at methylated guanine residues. Piperidine was vacuum evaporated, and the plasmid DNA was washed and resuspended in 0.1 ml of water. DNA was annealed with 0.5 pmol of 5′-end-labelled oligonucleotide (5′-GTAAAATGCG ATTTGACGC-3′) and extended with Klenow DNA polymerase (Amersham, Little Chalfont, Buckinghamshire, United Kingdom) for 10 min at 50°C. The reaction mixture was precipitated with ethanol, and the pellet was resuspended in formamide dye mix.

DNA sequencing and analysis. Double-stranded templates were sequenced by the dideoxy method (51) with a Sequenase kit (Amersham). Sequencing reaction mixtures were electrophoresed in 6% polyacrylamide–8 M urea gels in a Bio-Rad Sequi-gen sequencing apparatus. Computer analysis of the sequences was accomplished with the Genetics Computer Group package programs (15) and the Seqscan program, kindly provided by Tracy Nixon, and now available at the universal resource locator (URL) (http://www.bmb.psu.edu/seqscan).

Nucleotide sequence accession numbers. The *R. etli* DNA sequences mentioned here have been deposited in GenBank and assigned accession numbers L14671, L14672, U38244, and U38245.

RESULTS

Free-living expression of the *nifH-lacZ* fusions. Multiple identical nitrogenase genes in *R. etli* preclude the appropriate identification of messenger RNAs coming from individual genes. In order to characterize the mechanism involved in their transcriptional regulation, we constructed locus-specific fusions with the *E. coli lacZ* gene by gene replacement. This approach has been utilized previously, but the high frequency of DNA amplifications in the *R. etli* symbiotic plasmid generated a second copy of the *nifH-lacZ* fusion by homologous recombination, hampering the accurate determination of the expression of the individual fusions (39, 49). *R. etli* strains

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TABLE 2. Expression of *nifH* genes in *R. etli* under different oxygen concentrations

Strain	Relevant characteristic(s)	Mean β-galactosidase sp act ± SD at oxygen concentration[a]:			Induction ratio (1%/20%)
		1%	5%	20%	
CE3	Wild type	10 ± 0.2	10 ± 0.9	10 ± 0.5	1.00
DEM153	*nifHa-lacZ*	141 ± 3	54 ± 7	25 ± 1	5.64
DEM 153-1	*nifHa-lacZ nifA*Δ::Sp	50 ± 9	73 ± 1	35 ± 1	1.42
DEM233	*nifHc-lacZ*	327 ± 9	74 ± 8	45 ± 9	7.26
DEM233-1	*nifHc-lacZ nifA*Δ::Sp	57 ± 4	76 ± 7	36 ± 6	1.58
CE3/pMB210	Rm[b] *nifH-lacZ*	553 ± 38	142 ± 49	134 ± 57	4.12
CFNX247/pMB210	Rm *nifH-lacZ nifA*Δ::Sp	122 ± 4	98 ± 4	77 ± 2	1.58

[a] β-Galactosidase activities of different *nifH-lacZ* fusions in the *R. etli* wild type and a *nifA* mutant derivative under different oxygen concentrations. Values are mean β-galactosidase specific activity units from two different cultures in duplicate.
[b] Rm, *R. meliloti.*

containing either the *nifHa-lacZ* (DEM153) or the *nifHc-lacZ* (DEM233) gene fusion were constructed, and their free-living expression was estimated under different oxygen concentrations. Strains DEM153 and DEM233 were cultured in liquid minimal medium under 1, 5, and 20% oxygen concentrations (Table 2). For both strains, maximal β-galactosidase activities were always observed when the strains were grown under 1% oxygen. Levels of activity declined as the oxygen concentration was increased. Interestingly, at the maximal induction point, strain DEM233 presented a level of β-galactosidase-specific activity 2.3-fold higher than that of strain DEM153. This oxygen-sensitive expression suggested a positive regulation of the reiterated *nifH* genes by NifA, as has been observed for other *nifH* genes (19).

To determine the role of NifA in microaerobic induction, *nifHa-lacZ* and *nifHc-lacZ* gene fusions were also introduced into the *nifA* mutant derivative CFNX247 to generate strains DEM153-1 and DEM233-1, respectively. Both strains failed to induce significant β-galactosidase activity even when grown under 1% oxygen (Table 2). This result indicates that NifA is required for the free-living induction of the *nifH* promoters tested. In support of this, expression of the *R. meliloti nifH* gene, whose activation has been shown to be dependent on NifA (5, 17), was also triggered under low oxygen concentrations in *R. etli* in a NifA-dependent manner (Table 2).

Symbiotic phenotype of the *nifH-lacZ* fusion strains. After thorough verification of the genomic stability of our fusion strains (data not shown), we evaluated the symbiotic transcriptional activities of the *nifHa* and *nifHc* promoters. In planta β-galactosidase activity was measured in extracts of root nodules collected 13 and 23 days after inoculation (Fig. 1). As observed in free-living cultures, the level of β-galactosidase activity of nodules induced by strain DEM233 was fourfold higher than that induced by strain DEM153. Symbiotic effectiveness was also evaluated, and as previously reported (39, 50), disruption of *nifHa* diminished nitrogenase-specific activity to approximately 50% by abating the expression of the transcriptionally linked *nifDKa* genes while disruption of *nifHc*, which does not form part of a *nifHDK* operon, had no effect (data not shown).

Heterologous expression analysis of the *nifH-lacZ* fusions. To further analyze the role of the putative *cis*- and *trans*-acting elements that control reiterated *nifH* gene expression, we made use of a heterologous system in *E. coli*. We evaluated the role of NifA in the expression of the different *nifH-lacZ* fusions by estimating the β-galactosidase activities of strains carrying a plasmid that harbors the *K. pneumoniae nifA* gene. All of the four fusions tested were expressed only in the presence of *nifA* (Table 3). The expression of *nifHa* was at a level similar to those of the *K. pneumoniae* and *R. meliloti nifH* genes. Interestingly, *nifHc* was expressed at a level lower than that of *nifHa*, in contrast to the twofold ratio obtained in the *R. etli* genetic background. A plausible explanation of this result is discussed below.

Sequences strongly resembling that of σ^{54}-dependent promoters are located upstream of the three reiterated *nifH* genes (5′-TGGCACGGGTTTTGAA-3′) (47). In order to evaluate the requirement of σ^{54} for the expression of the *nifHa*- and *nifHc-lacZ* gene fusions, we determined their β-galactosidase activities in strain ET8045 (an *rpoN* mutant derivative of ET8000) harboring plasmid pDEM153 or pDEM233, respectively. These strains failed to induce β-galactosidase activity even in the presence of the *nifA* gene, showing that σ^{54} is essential for the expression of both *nifHa* and *nifHc* and supporting the functionality of the putative promoter sequences (Table 3).

It has been shown that certain σ^{54}-dependent promoters whose sequences greatly resemble the consensus sequence, like the *R. meliloti nifH* promoter, are prone to be partially activated by other proteins of the NifA family, such as NtrC (30, 33, 55). This results from the strong binding of Eσ^{54} to the promoter. The nucleotide sequences of the reiterated *nifH* gene promoters differ from the consensus sequence at the

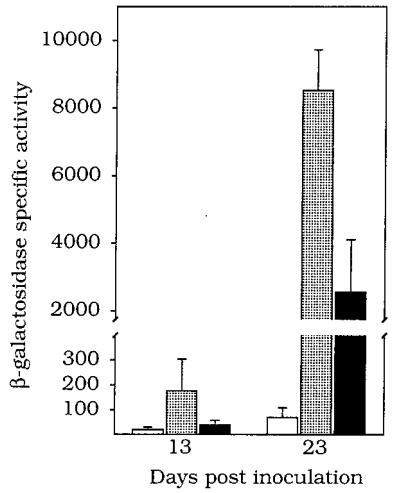

FIG. 1. Symbiotic expression of the *R. etli nifHa* and *nifHc* genes. Root nodules from *P. vulgaris* inoculated with *R. etli* strains CE3 (□), DEM233 (▨), or DEM153 (■), were assayed for β-galactosidase activities as described in Materials and Methods. Values are the means from experiments using 10 different plants.

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TABLE 3. Heterologous expression in *E. coli* of *nifH* genes from *R. etli*, *R. meliloti*, and *K. pneumoniae*

Plasmid	Genotype[a]	Mean β-galactosidase activity (Miller units) ± SD with strain[b]:								
		ET8000[e] (NrC$^-$ NifA$^-$ NifAY512F$^-$ IHF$^+$ σ^{54+})	ET8000/pMJ220[e] (NrC$^-$ NifA$^+$ NifAY512F$^-$ IHF$^+$ σ^{54+})	ET8000/pMJ221[c] (NrC$^-$ NifA$^-$ NifAY512F$^+$ IHF$^+$ σ^{54+})	ET8000[d] (NrC$^+$ NifA$^-$ NifAY512F$^-$ IHF$^+$ σ^{54+})	ET8045/pMJ220[d] (NrC$^+$ NifA$^+$ NifAY512F$^-$ IHF$^+$ σ^{54-})	SE100[e] (NrC$^-$ NifA$^-$ NifAY512F$^-$ IHF$^-$ σ^{54+})	SE100[d] (NrC$^+$ NifA$^-$ NifAY512F$^-$ IHF$^-$ σ^{54+})	SE100/pMJ220tc[c] (NrC$^-$ NifA$^+$ NifAY512F$^-$ IHF$^-$ σ^{54+})
pDEM153	Re *nifHa-lacZ*	22 ± 0.7	16,622 ± 2,220	52 ± 4	30 ± 2	21 ± 3	40 ± 4	37 ± 9	624 ± 7
pDEM233	Re *nifHc-lacZ*	94 ± 10	10,713 ± 872	166 ± 17	57 ± 3	101 ± 40	203 ± 44	159 ± 45	3,543 ± 746
pJMW6	Kp *nifH-lacZ*	170 ± 6	22,561 ± 2,281	188 ± 17	142 ± 31	NT[e]	104 ± 39	104 ± 63	543 ± 142
pMB2101	Rm *nifH-lacZ*	215 ± 48	19,199 ± 1,679	1,317 ± 270	1,268 ± 121	317 ± 128	93 ± 32	3,624 ± 546	3,844 ± 754

[a] Abbreviations: Re, *R. etli*; Rm, *R. meliloti*; Kp, *K. pneumoniae*.

[b] ET8000, ET8045, and SE100 are isogenic *lacZ*::IS*1* *E. coli* strains. They carry different *nifH-lacZ* fusions whose expression is activated by the *K. pneumoniae* NifA proteins contained either in the pMJ220 (wild-type), the pMJ221 (unable to bind DNA derivative), or the pMJ220tc (pMJ220 but tetracycline-resistant) plasmid. ET8045 and SE100 are derivatives of ET800 carrying the *rpoN*::Tc and *himD*::Cm insertions, respectively. For induction conditions see Materials and Methods. Values are mean β-galactosidase Miller units from at least two independent cultures. The sources of the regulatory proteins (where present) are as follows: NtrC is encoded by the chromosomal *ntrC* gene; NifA is encoded in the pMJ220 or pMJ220tc plasmid; NifAY512F is encoded in the pMJ221 plasmid; IHF is encoded by the chromosomal *himA* and *himD* genes; and σ^{54} is encoded by the chromosomal *rpoN* gene.

[c] Culture was grown in minimal medium with ammonium chloride as the nitrogen source.

[d] Culture was grown in minimal medium with glutamine as the nitrogen source.

[e] NT, not tested.

conserved −12 position, where they have an A instead of a C. We measured the proficiency of these promoters to be cross-activated by NtrC in strain ET8000 by growing ET8000 on glutamine (in which NtrC is active) or on ammonium chloride (in which NtrC is inactive). In contrast to the *R. meliloti nifH* promoter (which was cross-activated by NtrC in the absence of NifA [Table 3]), the β-galactosidase activities of strains carrying the *nifHa* and *nifHc* promoters were negligible and very similar in both conditions (Table 3). As previously shown, the same result was obtained for the strain carrying the weak *K. pneumoniae nifH* promoter (7, 9). Thus, it seems that the *R. etli nifH* promoters are weak Eσ^{54} binding sites, despite having a sequence greatly resembling the consensus sequence.

Influence of the DNA binding function of NifA on the activation of the *nifH* genes. The specificities in the activation observed for both *R. etli nifH* genes suggest that NifA must act in *cis*, interacting with the DNA in the vicinity of the *nifH* promoters. The *nifHa* gene is preceded by a consensus UAS (47); however, in spite of being highly expressed, no canonical NifA binding sites have been detected up to 200 nucleotides upstream of *nifHc* (47). To assess the dependence of the *R. etli nifH* genes on the DNA-binding function of NifA, we made use of a *K. pneumoniae* NifA mutant protein that carries a single amino acid substitution in the recognition helix of the helix-turn-helix motif (Tyr-512 to Phe) (36). This mutation abolishes the ability of NifA to bind to DNA, but it does not affect its positive control properties (36). As shown in Table 3, the mutant *K. pneumoniae nifA* gene failed to induce the expression of the *nifHa* and *nifHc* promoters. A similar result was obtained for the weak *K. pneumoniae nifH* promoter, while the *R. meliloti nifH* promoter, which has been shown to be less dependent on the UAS, was partially activated by this DNA-binding-defective protein.

The expression results shown above strongly suggested the presence of NifA binding sites upstream of *nifHc*. In the search for these predicted sites, we extended the nucleotide sequence up to 500 nucleotides upstream of this gene. As shown in Fig. 2A, two elements resembling the UAS were identified at positions 85 (5′-TGGN$_{10}$ACA-3′) and 167 (5′-TGTN$_{10}$ACG-3′) upstream from the promoter. The former resulted from the correction of the previously reported sequence (47).

In vivo footprinting analysis of the *nifH* upstream promoter regions. The resemblance to the UAS of the elements located upstream of *nifHa* and *nifHc* suggested that they function as NifA binding sites. In order to identify residues contacted by this protein in the regulatory regions of both genes, we performed in vivo methylation protection experiments with DMS. Figure 3 shows the patterns of cleavage products obtained from these regulatory regions after exposure of ET8000 cells harboring plasmid pDEM153 or pDEM233 to DMS, both in the presence and in the absence of plasmid pMJ220. In the presence of NifA, residues 95, 96, 98, and 99 upstream from the *nifHc* promoter were protected from methylation. These residues constitute the promoter-proximal putative NifA binding site depicted in Fig. 2A. No consistent protection was observed at any other position, not even around the putative promoter-distal UAS (data not shown). In the regulatory region of *nifHa*, residues 100, 104, and 113 upstream from the promoter were protected in the presence of NifA (Fig. 2B). The first two positions correspond to the putative canonical site, while the latter is within a NifA binding half-site (TGT) located just 6 nucleotides upstream (Fig. 2B).

Influence of the IHF in the expression of the reiterated *nifH* genes. It is well known that the IHF has a major role in the activation of several σ^{54}-dependent promoters, including those of *K. pneumoniae* and *R. meliloti nifH* (11, 25, 52). A computer-

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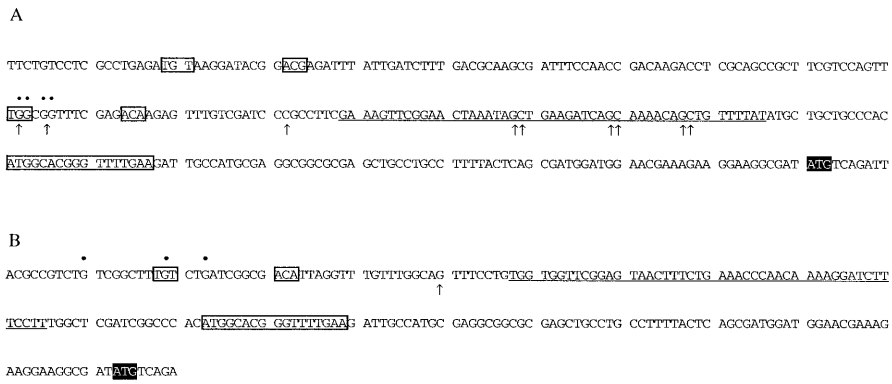

FIG. 2. Nucleotide sequences of *nifHc* (A) and *nifHa* (B) upstream regions. The UAS and promoter sequences are enclosed by open boxes. Shaded boxes enclose the putative translation initiation codons. Putative IHF binding sites are underlined. Arrows indicate differences from the nucleotide sequences previously reported by Quinto et al. (47). Residues protected from cleavage in NifA-dependent in vivo methylation protection analyses are marked by dots.

aided search for IHF binding sites of the regulatory regions of both *R. etli nifH* genes based on the algorithm of Goodrich et al. (22) identified putative sites between the UAS and the promoter at 15 and 18 nucleotides upstream from the *nifHc* and *nifHa* promoters, respectively (Fig. 2).

In order to evaluate the role of the IHF in the expression of the reiterated *nifH* genes, we determined the β-galactosidase activity of strain SE100 (a *himD* mutant derivative of ET8000) carrying the different *nifH-lacZ* gene fusions. In spite of the pleiotropic effect of the *himD* mutation, the β-galactosidase activities observed in the absence of *nifA* were equivalent to the values obtained with the isogenic ET8000 wild-type strain (Table 3). When *nifA* was present in the cells, induction of *nifH-lacZ* fusion expression was observed; however, the level of expression was lower than in the wild-type strain (Table 3), in which *nifHa* is much more dependent on the IHF. As previously shown, *K. pneumoniae nifH* was highly dependent on the IHF while *R. meliloti nif* was only partially dependent (52). These results suggest that the NifA-dependent transcription of the reiterated *nifH* promoters is differently stimulated by the IHF, despite having a sequence that greatly resembles the consensus sequence. It is noteworthy that this is the only condition of *E. coli* under which *nifHc* was expressed at levels higher than those of *nifHa*, as observed with *R. etli* (Table 2).

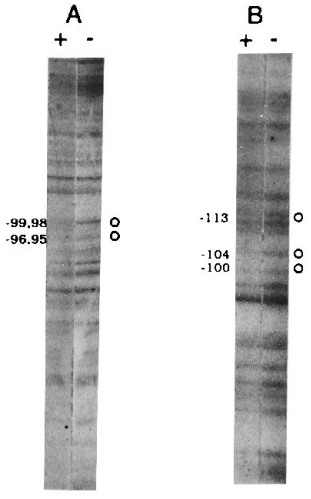

FIG. 3. NifA-dependent methylation protection of the *nifHc* (A) and *nifHa* (B) promoter elements. Methylation was performed in vivo in the presence (+) or absence (−) of NifA. The open circles indicate guanine residues protected when NifA was present in the cells. Numbers to the left of the gels indicate nucleotide positions relative to the promoter (Fig. 2).

It has been shown that the sharp bend induced by the IHF on the promoter region may restrict access of nonbound activators to the $E\sigma^{54}$ promoter complex (43). The absence of cross-activation by NtrC observed with the *nifHa* and *nifHc* promoters (Table 3) could be due either to the lack of a strong $E\sigma^{54}$ binding site or to a hindrance produced by the IHF. As shown in Table 3, neither *nifHa* nor *nifHc* was cross-activated by NtrC in the absence of the IHF. In contrast, expression of the strong *R. meliloti nifH* promoter was increased about threefold in the absence of the IHF. These observations support our proposition that the *nifHa* and *nifHc* promoters are low-affinity $E\sigma^{54}$ binding sites.

Nucleotide sequence downstream of *nifHc*. The *nifHc* gene is actively expressed during symbiosis with bean plants (Fig. 1) and in cultures under low oxygen concentrations (Table 2). Unlike *nifHa* and *nifHb*, *nifHc* seemed not to form part of a *nifHDK* operon (47). Further sequencing of the downstream region of *nifHc* allowed us to identify two open reading frames (ORFs): ORF70 and ORF251. Interestingly, the deduced amino acid sequence of ORF70 bears a high level of similarity (up to 91%) to the first 70 residues of the nitrogenase NifD protein from several nitrogen-fixing bacteria (data not shown); thus, this ORF is a truncated *nifD* gene of unknown function. The deduced amino acid sequence of ORF251 is 56 to 58% similar to that of the oxygen-independent coproporphyrinogen III oxidase proteins from *Salmonella typhimurium* (60), *Rhodobacter sphaeroides* (14), and *E. coli* (57) encoded by the *hemN* gene. A sequence resembling a σ^{E}-dependent promoter is located upstream of ORF251. No expression of ORF251-*lacZ* fusions was detected in culture under any oxygen concentration tested, but it was actively transcribed during nodule development (data not shown). The nucleotide sequence determination downstream of *nifHc* allowed us to show that the identity between the *nifHc* and the *nifHb* regions extends throughout the promoter until 13 nucleotides before the ORF70 translation stop codon.

DISCUSSION

In this study we characterized the complex *cis*- and *trans*-acting regulatory elements involved in reiterated *R. etli nifH* gene expression. Strain CE3 contains three *nifH* genes, two of which are functional *nifHDK* operons separated by 120 kb in the symbiotic plasmid, while *nifHc* is located in between (21)

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and seems to be unlinked to other *nif* genes (32, 47). Here we show that *nifHc* is transcriptionally coupled to a truncated *nifD* homolog comprising the first 70 amino acids of the protein. Directly downstream, we identified ORF251, which is similar to several *hemN* genes. Restriction fragment length polymorphism analyses of other distantly related isolates of *R. etli*, previously classified and characterized (32, 44), suggest that this arrangement is typical in this species (data not shown). Although the truncated *nifD* and the *hemN* homologs were readily transcribed in nodules, we do not have any direct evidence for their functionality. As has been shown for other multimeric enzymes, like the hepatocyte growth factor (12) and the Fos-Jun transcriptional factor (40), the incorporation of truncated, nonfunctional monomers instead of full-length polypeptides can regulate their activity. The first 70 residues of the NifD protein do not form an independent domain and do not interact with the NifK protein or with any of the metal cofactors (27). Consequently, it is unlikely that the truncated NifD protein could be involved in the regulation of nitrogenase activity by this mechanism. Homologs to ORF251 have been characterized for *E. coli*, *S. typhimurium*, and *R. sphaeroides* (14, 57, 60); these *hemN* genes code for the oxygen-independent coproporphyrinogen III oxidase, an enzyme involved in the alternative biosynthesis of the heme cofactor under anaerobic conditions. In contrast to *hemA* mutations (16), gene disruption in ORF251 had no symbiotic effect (not shown).

The different nucleotide sequences of the *nifHa* and *nifHc* upstream regulatory regions and the asymmetric arrangements of their NifA binding sites suggested differential transcription of these genes. As is well known, low oxygen concentration is the most important effector in the induction of *nifH*, so we estimated the expression of the reiterated *R. etli nifH* genes under different oxygen concentrations. For both genes, maximal induction was observed under 1% oxygen concentration, with *nifHc* being transcribed at higher levels than *nifHa* (Table 2).

By using a *nifA* mutant derivative of strain CE3, we demonstrated that expression of the reiterated *nifH* genes, as reported for *Rhizobium leguminosarum* bv. phaseoli (34), was dependent on NifA in an oxygen-concentration-responsive manner. Evidence obtained by two different approaches shows that the activation is mediated by NifA bound to UAS elements: (i) induction of both *nifH* genes was strictly dependent on the DNA-binding ability of NifA, since a derivative impaired in its specific binding to the UAS was unable to activate transcription (Table 3) and (ii) in vivo methylation protection of residues located within the putative UAS was dependent on the presence of NifA (Fig. 2 and 3). NifA exerts its activation properties by binding to a minimal DNA sequence located around 100 residues upstream from the promoter and having the sequence 5′-TGTN$_{10}$ACA-3′ (36). Here we show that the NifA-dependent activation of the *nifHc* promoter is mediated by the specific binding of NifA to a divergent UAS located 85 residues upstream from the promoter (Fig. 2A and 3A). Fully functional divergent NifA-UASs upstream of the *K. pneumoniae nifJ* (10) and the *Bradyrhizobium japonicum fixRnifA* promoters have also been described (3). The protection pattern observed upstream of *nifHc* resembled the one observed at the *K. pneumoniae nifH* UAS (36), where the G of the TGT conserved motif and the internal G residues at positions 5 and 6 were protected, suggesting a similar mode of contacting the DNA (Fig. 2A and 3A). At the *nifHa* regulatory region, in addition to protection of the conserved G of the TGT motif, the G residue at position 6 and a G residue located 8 nucleotides upstream from the UAS were also protected (Fig. 2B and 3B). The latter residue forms part of a TGT UAS half-site. The same pattern was observed by Cannon et al. (10) in the upstream region of the *K. pneumoniae nifE* gene, where a canonical UAS and an additional half-site exactly 6 nucleotides upstream were protected by NifA. Protection of this half-site could be due either to the binding of an additional NifA molecule or to the DNA bending induced by NifA, allowing the establishment of an additional contact with the DNA. This half-site could help to increase the number of NifA molecules in the vicinity of the *nifHa* promoter, raising the probability of productive interactions with Eσ^{54}. In support of this enhancing role, mutations in the UAS half-site diminished the NifA-dependent activation of the *K. pneumoniae nifE* promoter (10). Protection of a TGT UAS half-site in the bottom strand has also been detected in the *K. pneumoniae nifU* regulatory region (11).

The absolute dependence on NifA for expression of the reiterated *nifH* genes was also demonstrated for *E. coli* with a plasmid harboring the *K. pneumoniae nifA* gene (Table 3). Under this condition, *nifHa* was transcribed at levels higher than those of *nifHc*, in contrast to the ratio observed for *R. etli* (Table 2). Although a reproducible NifA-dependent protection pattern was observed in the divergent UAS located upstream of *nifHc* under saturation conditions (Fig. 3A), we cannot rule out a lower level of affinity of *K. pneumoniae* NifA to this site.

The reiterated *nifH* genes are preceded by identical sequences greatly resembling that of consensus σ^{54}-dependent promoter sequences (47). Here we presented evidence of their functionality by showing the requirement of σ^{54} for their expression (Table 3). These promoter sequences differ from the consensus sequence only at position −12, where there is an A instead of a C. This position seems to be relevant for promoter recognition by Eσ^{54}, since the position is highly conserved and an equivalent transversion in the *K. pneumoniae nifH* promoter abolishes the promoter capacity to be cross-activated by NtrC (9). Most of the σ^{54}-dependent promoters described so far have the conserved C in position −12, but some exceptions are known among rhizobia: in the *nifN* gene of *R. meliloti* (1) and in the *nifH* genes of *R. leguminosarum* (48), *Rhizobium* sp. strain ANU240 (2), *R. leguminosarum* bv. trifolii (59), and *R. leguminosarum* bv. phaseoli (34). It is interesting that in the last three species the *nifH* genes are reiterated as in *R. etli.*

If a given promoter, like *K. pneumoniae nifH*, is a weak Eσ^{54} binding site, it is more dependent on the IHF and UAS and consequently it is less prone to cross-activation (7, 9, 37, 52). Conversely, promoters with sequences that match the consensus sequence, as with *R. meliloti nifH*, are strongly bound by Eσ^{54} and are less dependent on the IHF and UAS but are readily cross-activated (7, 26, 37, 52, 56). Although the IHF has not been characterized for any *Rhizobium* species, a homologous activity is likely to be present, since the stimulation of expression by the IHF in *E. coli* has been specifically demonstrated for several *nif* genes (11, 52). As shown above, expression of the reiterated *nifH* gene promoters is dependent on the upstream binding of NifA and they are not cross-activated by NtrC, despite having sequences that greatly resemble the consensus sequence. Therefore, they probably bind Eσ^{54} with low levels of affinity and their expression is likely to be dependent on the IHF. Results with *E. coli* showed this dependence and also that the requirements for this protein differ, with the *nifHa* promoter being more dependent than the *nifHc* (Table 3). This difference could be the result of the relative orientations of all three binding sites, which have been shown to be critical for the expression of other σ^{54}-dependent promoters, such as *K. pneumoniae nifH* (35) and *E. coli glnH* (13). Interestingly, the UAS of *nifHc* is located 8 helical turns from the promoter while in

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nifHa it is located 8.5 turns from the promoter. Furthermore, the partial independence of the *nifHc* promoter on the IHF could also be the result of an intrinsically bent DNA structure between the UAS and the promoter that brings bound NifA and $E\sigma^{54}$ in close contact. This structure is either absent in the *nifHa* regulatory region or, given the half-turn-different phasing of the UAS, does not allow productive contact of the bound proteins.

By having reiterated genes, an organism gains additional flexibility in the control of gene expression. Two identical genes can be differentially expressed in response to a changing environment or can be simultaneously transcribed to ensure functional concentrations of their product. In *Azorhizobium caulinodans* two *nifH* genes are alternatively expressed under free-living nitrogen fixation conditions and during symbiosis with *Sesbania rostrata* (42). In bacteria of the *Azotobacter* genus, three different sets of *nifHDK* gene homologs are expressed in response to the metal availability (58). In *R. etli*, it has been shown that both *nifHDK* operons are required to achieve full nitrogenase activity during symbiosis with *P. vulgaris* (39, 50), while in this study we show that *nifHa* and *nifHc* are differentially expressed. If a promoter is selectively modified to narrow its activation specificity range and avoid cross-activation, then a decrease in its transcription rate is likely to occur. Duplication of the gene would be an advantageous alternative to restore the original product level without losing the stringent control on its expression. Then, the asymmetric arrangements of the *cis*-acting factors described here, the σ^{54}-dependent promoters, the IHF binding sites, and NifA-UAS contribute to the differential expression of the reiterated *nifH* genes by creating a finely regulated architecture that assures optimal levels of the nitrogenase enzyme in *R. etli.*

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