Expression of the *nifA* gene of *Herbaspirillum seropedicae*: role of the NtrC and NifA binding sites and of the –24/–12 promoter element

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The *nifA* promoter of *Herbaspirillum seropedicae* contains potential NtrC, NifA and IHF binding sites together with a –12/–24 σ^N -dependent promoter. This region has now been investigated by deletion mutagenesis for the effect of NtrC and NifA on the expression of a *nifA::lacZ* fusion. A 5' end to the RNA was identified at position 641, 12 bp downstream from the –12/–24 promoter. Footprinting experiments showed that the G residues at positions –26 and –9 are hypermethylated, and that the region from –10 to +10 is partially melted under nitrogen-fixing conditions, confirming that this is the active *nifA* promoter. In *H. seropedicae* *nifA* expression from the σ^N -dependent promoter is repressed by fixed nitrogen but not by oxygen and is probably activated by the NtrC protein. NifA protein is apparently not essential for *nifA* expression but it can still bind the NifA upstream activating sequence.

Keywords: *Herbaspirillum seropedicae*, nitrogen fixation, *nifA* gene

INTRODUCTION

Herbaspirillum seropedicae is an endophytic nitrogen-fixing bacterium found associated with several grasses, which fixes nitrogen under micro-oxic conditions and in the absence of a fixed nitrogen source (Baldani *et al.*, 1986; Boddey *et al.*, 1995). Initially classified as an *Azospirillum* species, it is now placed in the rRNA superfamily III or β -subdivision of the *Proteobacteria* (Young, 1992). This subdivision, which is the least studied genetically of nitrogen-fixing families, includes species of *Rhodocyclus*, *Alcaligenes*, *Derxia* and *Azoarcus*.

The NifA protein is a specific activator of the expression of *nif* gene promoters by interaction with σ^N (σ^{54} , RpoN)-containing RNA polymerase, which recognizes promoters with the dinucleotides GG and GC conserved at positions –25/–24 and –12/–13, respectively (Dixon, 1988). The NifA protein binds to a specific upstream activating sequence (UAS) and interacts with the σ^N -containing RNA polymerase bound to the promoter to induce open complex formation (Morett &

Buck, 1989). This mechanism is common to all nitrogen-fixing proteobacteria to date.

The regulation of *nifA* expression, on the other hand, differs, depending on the organism. In *Klebsiella pneumoniae*, the phosphorylated NtrC protein activates *nifA* gene expression under nitrogen limitation. NtrC is phosphorylated by NtrB, whose activity is controlled by the cellular nitrogen status via the proteins GlnB and GlnD (see Merrick & Edwards, 1995). In *Rhizobium meliloti* (now *Sinorhizobium meliloti*), *nifA* and *fixK* expression (Batut *et al.*, 1989; David *et al.*, 1988) is activated by the FixJ protein, which is inactivated under high oxygen by the FixL protein, while the FixK protein negatively regulates *nifA* expression. The NifA protein also activates its own expression (Batut *et al.*, 1989). In *Azorhizobium caulinodans* full expression of the *nifA* promoter occurs only under micro-oxic and low fixed nitrogen conditions (Loroch *et al.*, 1995) when FixJ activates *fixK* expression and FixK, in turn, activates *nifA* expression, in apparent contrast to the situation in *S. meliloti*. A NifA UAS is also present in the *A. caulinodans* *nifA* promoter and is possibly involved in negative auto-regulation (Nees *et al.*, 1988; Stigter *et al.*, 1992). Finally, the NrfA protein has also been implicated in translational *nifA* regulation (Kaminski & Elmerich, 1998). The *nifA* gene of *Bradyrhizobium japonicum* is in the operon *fixRnifA*, which is positively regulated by an

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Abbreviations: DMS, dimethylsulphate; UAS, upstream activating sequence.

oxygen-sensitive NifA protein (Fischer & Hennecke, 1987). In this organism, however, *nifA* is expressed primarily from another promoter activated by the RegR protein (Bauer *et al.*, 1998). The *nifA* gene expression in *Azospirillum brasilense* is not tightly regulated by either fixed nitrogen or oxygen, although a significant decrease of expression occurs in the presence of high levels of both ammonia and oxygen together (Liang *et al.*, 1991). Both ammonia and oxygen, on the other hand, apparently regulate NifA activity since there is no activation of *nifH* under such conditions. The PII protein probably participates in the regulation of NifA protein activity since a *glnB* null mutant of *A. brasilense* is Nif⁻ even when the *nifA* gene is expressed constitutively (de Zamaroczy *et al.*, 1993; Arsene *et al.*, 1996).

The promoter region of the *nifA* gene of *H. seropedicae* was found to contain sequences homologous to a -24/-12 type promoter, together with NifA and NtrC binding sites (Souza *et al.*, 1991a), suggesting participation of these proteins in the expression of the *nifA* gene. The activity of the NifA protein from *H. seropedicae* is controlled by ammonium ions and oxygen (Souza *et al.*, 1999). *nif* gene expression in *H. seropedicae* apparently requires a functional *glnB*-like gene (Benelli *et al.*, 1997).

Here we report the identification of a major transcriptional-start site of the *nifA* gene of *H. seropedicae* under nitrogen-fixing conditions, verification of a -24/-12 transcriptionally active promoter and an analysis of the roles of NifA and NtrC proteins in the activation of *nifA* expression.

METHODS

Strains and plasmids. The bacterial strains and plasmids used are listed in Table 1. The *Herbaspirillum seropedicae* strain SmR1 is a spontaneous streptomycin-resistant (100 µg ml⁻¹) mutant isolated from *H. seropedicae* Z78 (Baldani *et al.*, 1986).

Media and growth conditions. *H. seropedicae* was grown in JNFbP medium (Baldani *et al.*, 1986) supplemented with 20 mM NH₄Cl (JNFbPN). *Escherichia coli* was grown in LB or 2 × YT medium (Sambrook *et al.*, 1989). The minimal medium for *E. coli* was NFDM (Dixon *et al.*, 1977) supplemented with 20 mM NH₄Cl. The concentrations of antibiotics used were as follows: carbenicillin, 200 µg ml⁻¹; tetracycline, 10 µg ml⁻¹; streptomycin, 20 µg ml⁻¹ (*E. coli*) or 100 µg ml⁻¹ (*H. seropedicae*); kanamycin, 50 µg ml⁻¹ (*E. coli*) or 250 µg ml⁻¹ (*H. seropedicae*); chloramphenicol, 30 µg ml⁻¹ (*E. coli*) or 150 µg ml⁻¹ (*H. seropedicae*); nalidixic acid, 20 µg ml⁻¹.

Conjugation. Plasmids were introduced into *H. seropedicae* by triparental matings (Pedrosa & Yates, 1984), using pRK2013 as the helper plasmid.

Construction of *nifA* mutants. A 6.5 kb *Bam*HI–*Hind*III fragment from pEMS101 containing *H. seropedicae* DNA was cloned in pSUP202, producing pEMS108. This plasmid was mutagenized using the Tn5–*lacZ* construct Tn5–B21 (Simon *et al.*, 1989) to obtain a transposon insertion approximately 300 bp downstream from the *nifA* translation start. The orientation of *lacZ* in this plasmid, named pEMS109, was the same as that of the *nifA* gene. A 2 kb *Sal*I fragment from pEMS101, containing part of the *nifA* gene, was also cloned

into pSUP202 and the kanamycin-resistance cassette from pKIXX was inserted into the *Bgl*II site in the *nifA* gene to give the plasmid pEMS109.1. *H. seropedicae nifA* mutants were then obtained by introducing these mutated plasmids pEMS109 and pEMS109.1 into *H. seropedicae* by conjugation, producing strains SmR2532 and SmR54, respectively. Recombinant colonies resistant to the transposon or cassette markers were screened for a combination of the vector marker (chloramphenicol sensitivity), β-galactosidase and nitrogenase activities. Only 1–5 % of the recombinant colonies were products of a double crossover, as judged by the above parameters. The transposon or cassette insertion in the *nifA* gene was confirmed by chromosomal DNA hybridization following restriction and electrophoresis (not shown).

Construction of *nifA::lacZ* fusions. The 6.5 kb *Pst*I–*Xho*I fragment from plasmid pEMS109 (*nifA::Tn5*–B21), containing the whole *nifA* promoter region and the *lacZ* gene, was cloned into *Eco*RI/*Pst*I-digested pBR322, yielding pEMS111. pEMS114 was constructed by cutting pEMS111 with *Dra*I and religating. This construction contains the NifA UAS and the -24/-12 promoter sequence.

Another set of *nifA::lacZ* fusions was constructed using the IncP fusion vectors pPW452 and pMP220 (Fig. 1a, b). A 1.7 kb *Eco*RI fragment containing part of the N-terminal of the *nifA* gene and the whole promoter region was cloned into the transcriptional fusion vector pPW452, yielding the plasmid pEMS120, in which *lacZ* expression was under control of the *nifA* promoter. Deletions in the promoter region were prepared by cloning the 0.4 kb *Eco*RI–*Nsi*I, the 0.5 kb *Eco*RI–*Dra*I and the 0.5 kb *Eco*RI–*Ssp*I subfragments from the 1.7 kb *Eco*RI fragment into pPW452 producing pEMS121, pEMS122 and pEMS123, and the 0.7 kb *Eco*RI–*Pml*I fragment into pMP220 yielding pEMS124. The 1.3 kb *Eco*RI–*Nsi*I and 1.2 kb *Eco*RI–*Dra*I fragments were cloned into pMP220 to produce pEMS125 and pEMS126, and the 1.0 kb *Eco*RI–*Pml*I fragment into pPW452 to produce pEMS127. The *Eco*RI–*Dra*I and *Eco*RI–*Ssp*I fragments were cloned first into pTZ18 digested with *Eco*RI/*Sma*I and then transferred as *Eco*RI–*Pst*I fragments to pMP220 and pPW452 digested with *Eco*RI/*Pst*I (Table 1). The *Eco*RI–*Pml*I fragments were also cloned into pTZ18 digested with *Eco*RI/*Sma*I and cloned as *Eco*RI–*Bam*HI fragments into pPW452 and pMP220 digested with *Eco*RI/*Bgl*II. The *Eco*RI–*Nsi*I fragments were cloned in pMP220 and pPW452 digested with *Eco*RI/*Pst*I (see Table 1).

β-Galactosidase activity. Studies of the expression of the *H. seropedicae nifA* gene in *E. coli* were as follows: NFDM (2 ml) in a bijou bottle containing glutamine (100 µg ml⁻¹) plus antibiotics was inoculated with 50 µl of an overnight culture in 2 × YT in air and shaken for 18–20 h at 30 °C. NH₄Cl (20 mM) was added when necessary.

Derepression experiments in *H. seropedicae* were conducted with overnight-grown cultures. The cells were collected by centrifugation and resuspended in either fresh JNFbP or JNFbPN to an OD₆₀₀ of 0.2. The cell suspension (2 ml) was transferred to 25 ml conical flasks containing either oxygen (1.5 % in N₂) or air and shaken for 5–6 h at 30 °C. The β-galactosidase activity was then determined, using ONPG as the substrate (Miller, 1972).

RNA 5' end mapping. RNA was purified from *H. seropedicae* grown under nitrogen-fixing conditions (Krol *et al.*, 1982). The single-stranded probe used for S1 mapping was obtained by asymmetric PCR using 2 pmol primer 1 (5'-ATGCGC-TGCCTGAGAGCGCT-3') end-labelled with [γ-³²P]ATP (Amersham). Conditions of hybridization and digestion were as in Sambrook *et al.* (1989).

Table 1. Strains and plasmids

Strain/plasmid	Genotype/phenotype	Reference/source
<i>H. seropedicae</i> strains		
SmR1	Sm ^R , Nif ⁺	This work
SmR2532	Sm ^R , <i>nifA</i> ::Tn5-B21 (<i>nifA</i> :: <i>lacZ</i> , Tc ^R)	This work
SmR54	Sm ^R , <i>nifA</i> ::Km (Km ^R)	This work
<i>E. coli</i> strains		
ET8894	$\Delta(rha-ntrC)1703::Mu$ cts62	MacNeil (1981)
ET8045	<i>rpoN208::Tn10</i>	MacNeil (1981)
Plasmids		
pRK2013	Km ^R , <i>tra</i>	Figurski & Helinski (1979)
pEMS101	Km ^R , <i>HsnifA</i> in pVK102	Souza <i>et al.</i> (1991a)
pEMS107	Tc ^R , <i>HsnifA</i> in pVK102	Souza <i>et al.</i> (1991a)
pEMS108	Cm ^R Cb ^R , <i>HsnifA</i> in pSUP202	This work
pEMS109	Tc ^R Cm ^R Cb ^R , <i>HsnifA</i> ::Tn5-B21 in pSUP202	This work
pEMS109.1	Km ^R Cm ^R Cb ^R , <i>HsnifA</i> ::Km in pSUP202	This work
pEMS111	Tc ^R , <i>nifA</i> :: <i>lacZ</i> in pBR322	This work
pEMS112	Tc ^R , <i>nifA</i> :: <i>lacZ</i> IncP	This work
pEMS114	Tc ^R , <i>nifA</i> :: <i>lacZ</i> in pBR322	This work
pEMS135	Tc ^R , <i>Mael</i> fragment in pLAFR3.18 and <i>HsnifA</i> expressed from <i>lac</i> promoter	This work
pEMS120	Tc ^R , 1.7 kb <i>EcoRI</i> fragment containing whole <i>HsnifA</i> promoter in pPW452	This work
pEMS121	Tc ^R , 0.4 kb <i>EcoRI</i> - <i>NsiI</i> fragment containing part of <i>HsnifA</i> promoter in pPW452	This work
pEMS122	Tc ^R , 0.5 kb <i>EcoRI</i> - <i>DraI</i> fragment containing part of <i>HsnifA</i> promoter in pPW452	This work
pEMS123	Tc ^R , 0.5 kb <i>EcoRI</i> - <i>SspI</i> fragment containing part of <i>HsnifA</i> promoter in pPW452	This work
pEMS124	Tc ^R , 0.7 kb <i>EcoRI</i> - <i>PmlI</i> fragment containing part of <i>HsnifA</i> promoter in pMP220	This work
pEMS125	Tc ^R , 1.3 kb <i>EcoRI</i> - <i>NsiI</i> fragment containing part of <i>HsnifA</i> promoter in pMP220	This work
pEMS126	Tc ^R , 1.2 kb <i>EcoRI</i> - <i>DraI</i> fragment containing part of <i>HsnifA</i> promoter in pMP220	This work
pEMS127	Tc ^R , 1.0 kb <i>EcoRI</i> - <i>PmlI</i> fragment containing part of <i>HsnifA</i> promoter in pPW452	This work
pSUP202	Tc ^R Cb ^R Cm ^R , Mob ⁺	Simon <i>et al.</i> (1983)
pBR322	Tc ^R Cb ^R	Sambrook <i>et al.</i> (1989)
pKIXX	Cb ^R Km ^R	Pharmacia
pVK102	Tc ^R Km ^R , Mob ⁺	Knauf & Nester (1982)
pTZ18	Cb ^R	Pharmacia
pCK1	Sm ^R , IncQ <i>KpnifA</i> ^C	Kennedy & Robson (1983)
pMC71A	Cm ^R , <i>KpnifA</i> ^C	Buchanan-Wollaston <i>et al.</i> (1981)
pNH11	Cb ^R , <i>KpnifA</i> expressed from <i>tac</i> promoter	Austin <i>et al.</i> (1990)
pMM14	Km ^R , <i>KpntrC</i> ^C	Merrick (1983)
pMP220	<i>lacZ</i> fusion vector, IncP	Spaniak <i>et al.</i> (1987)
pPW452	As pMP220, but with inverted cloning nest	P. Woodley*

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Recombinant DNA techniques. All DNA manipulations including cloning, end-labelling and transformations were performed by current techniques (Sambrook *et al.*, 1989).

In vivo footprinting. For dimethylsulphate (DMS) footprinting, an overnight culture of *E. coli* ET8894 carrying the relevant plasmids was diluted to an OD₆₀₀ of 0.1 in 2 × YT and shaken at 30 °C until the OD₆₀₀ was 0.5–0.7. The culture was treated with DMS (0.05 %, v/v) for 2 min; the cells were then collected by centrifugation and washed in Tris/HCl (50 mM, pH 8.0), EDTA (10 mM) solution. The methylated plasmids were purified by alkaline lysis (Sambrook *et al.*, 1989).

For *in vivo* DMS footprinting in *H. seropedicae*, an overnight culture of a strain carrying pEMS120 (*nifA*::*lacZ* fusion) was centrifuged, diluted to an OD₆₀₀ of 1.1 in 20 ml JNFBP or JNFBPN in a 65 ml bottle and incubated in a rotary shaker at 120 r.p.m. for 5 h. The culture was treated with freshly prepared DMS (0.05 %, v/v, final concn) for 2 min and plasmid purification was done as above. When used, rifampicin (100 µg ml⁻¹) was added 7 min before the addition of DMS. To cleave the DNA at the methylated G residues, the plasmid DNA isolated after DMS treatment was dissolved in 30 µl 1 M piperidine, incubated at 90 °C for 30 min and submitted to 3 cycles of lyophilization followed by dissolution

(a)

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201 GGGCCGGCGA CCGGCCAGTT CAGGGTCAGA TAACATGCCT GGGAGCAGGG
      NtrC UAS
251 CGCCCAGCAC GCACGTGGTG CACCGGGTTC GGAAGTCGAA GGCACATTCA
      PmlI
301 TAGTGGGCGA TGGTGGATGT TGGAGGTCTG TATATCTCGC TACATAACGC
351 TGGCTGGGCT ACCGGCTTGC TCTGCTTTGT CGTTTCGCTT GGCCGGTGAG
401 CGGCCGATGC AGGTATGAAT AGCCAAAACC ATGCCATACA ATATTTTTCT
      SspI
451 TTTAAATCAA TGGGATAGCG ACGATTCTTG ATTGCTGCGC CAGTGGCGAT
      DraI NifA UAS
501 GATTGTCGTA AATCCGACAA AGACGACACC GTTGTGCCA ACCCGCCAAG
      -24/-12 promoter
551 GCGACATCGA GGAAGCCGG CATTGTGTGG GGTGCTTGG CGAGATCGAT
      NsiI
601 TCATGCATCT GCTTGGGCAT GAAGTTTGCT TGACCAGCAA GGTAACATAT
651 CGGTAATTAC ACGGCGCTTT TTTCGCCGTT TTTCAGGATC TCGGATCTCA
  
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Fig. 1. Construction of *nifA::lacZ* fusions. (a) Nucleotide sequence of the promoter region of *H. seropedicae nifA*. The NtrC and NifA UASs are underlined, the -24/-12 promoter element is double underlined and the arrow indicates the RNA start site. (b) Scheme of the *nifA::lacZ* fusions. Restriction enzymes are: E, *EcoRI*; P, *PmlI*; S, *SspI*; D, *DraI*; N, *NsiI*.

in 20 µl water. The cleaved DNA was used as the template for primer extension with *Taq* DNA polymerase (Sasse-Dwight & Gralla, 1991).

For *in vivo* KMnO₄ footprinting, an overnight culture of a *H. seropedicae* strain carrying the *nifA::lacZ* fusion was derepressed as above. Freshly prepared potassium permanganate (15 mM, final concn) was added and the cultures incubated for 5 min. When required, rifampicin (100 µg ml⁻¹) was added to trap open complexes 7 min before addition of KMnO₄. Plasmid purification, cleavage with piperidine and extension was as above.

Autoradiograms were analysed densitometrically in a Molecular Dynamics PSI Densitometer and differences in the level of band reactivity were calculated as described by Moret & Buck (1988).

RESULTS

Construction of *nifA* mutants of *H. seropedicae*

Plasmid pEMS109.1 was used to produce the mutant strain SmR54 that carries a kanamycin-resistance cassette inserted into the coding region of the *nifA* gene corresponding to the central domain of the NifA protein. This strain failed to reduce acetylene and was complemented by the plasmids pEMS107 (which expresses *H. seropedicae nifA* from the *nifA* promoter) and pEMS135 (which expresses *nifA* constitutively from the *lac* promoter). pEMS109 was used to insert Tn5-B21 in the *H. seropedicae nifA* gene, producing strain SmR2532 carrying the *lacZ* gene in the same orientation as the *nifA* gene. This strain was also Nif⁻ and was complemented by plasmid pEMS101, which carries the *nifA* gene from *H. seropedicae*.

Expression of the *H. seropedicae nifA* gene in *E. coli*

The *nifA::lacZ* fusion in plasmid pEMS120 carrying the whole promoter region of the *nifA* gene of *H. seropedicae* in the low-copy-number vector pPW452 exhibited a very low expression rate in *E. coli* ET8894, which increased 50-fold when the NifA protein from *K. pneumoniae* was provided by the plasmid pMC71A (Table 2). Deletion of the NifA UAS (pEMS121) reduced the activity of the *nifA* promoter by 90% in the presence of *K. pneumoniae* NifA. Deletion of DNA sequences upstream from base 453 (pEMS122) resulted in a twofold increase in the NifA-dependent β-galactosidase activity. The NtrC protein of *K. pneumoniae* expressed from pMM14 also activated expression of the *nifA* gene (pEMS120). This activation was reduced (65%) when the NtrC binding site was completely (pEMS122) or partially (pEMS124) deleted.

When a *nifA::lacZ* fusion was cloned into a medium-copy-number vector (pBR322) however, the effect of the NtrC binding site was clearer: deletion of the NtrC UAS in pEMS114 reduced activity fivefold compared to that of pEMS111 (Table 2). In *E. coli* ET8045 (*rpoN*⁻) neither NifA nor NtrC activated the *nifA* fusion carried by pEMS109 whereas in ET8894 (*rpoN*⁺) a 10-fold or 20-fold increase in β-galactosidase activity was observed when NifA or NtrC, respectively, was expressed constitutively (Table 2). This result indicates that the *nifA* promoter is dependent on the presence of the *rpoN* gene product. Addition of NH₄Cl (20 mM) did not alter *nifA* expression in *E. coli* (not shown).

Expression of the *nifA* gene in *H. seropedicae*

Expression of the chromosomal *nifA* gene in *H. seropedicae nifA::lacZ*. Fixed nitrogen (20 mM NH₄Cl) decreased the expression of the *H. seropedicae* chromosomal

Table 2. β -Galactosidase activity of *nifA::lacZ* fusions in *E. coli*

Data are the means of three independent assays. Numbers in parentheses are standard deviations. ND, Not determined. *E. coli* strains ET8894 and ET8045 are *ntrC* and *rpoN* mutants, respectively. The plasmids are described in the text and in Table 1, and were constructed as described in Methods. Plasmids pEMS120, pEMS111 and pEMS109 contain the whole promoter region of the *nifA* gene of *H. seropedicae*. Plasmid pEMS114 is a derivative of pEMS111 and contains the same region of the promoter as plasmid pEMS122.

Strain	<i>lacZ</i> fusion plasmid	β -Galactosidase activity with additional plasmid (Miller units)		
		None	pMC71A	pMM14
ET8894	pEMS111	20 (3)	ND	381 (83)
ET8894	pEMS114	34 (9)	ND	84 (14)
ET8894	pEMS120	2.9 (2.9)	138 (4.7)	66 (6.4)
ET8894	pEMS121	5.0 (1.6)	16.7 (7.8)	ND
ET8894	pEMS122	2.3 (2.5)	315 (74.8)	24.5 (11.7)
ET8894	pEMS124	5.1 (2.3)	106 (7.1)	23.5 (3.7)
			pCK1	pMM14
ET8894	pEMS109	75 (26)	1471 (123)	771 (10)
ET8045	pEMS109	143 (95)	132 (107)	77 (10)

Table 3. β -Galactosidase activity of *nifA::lacZ* fusions in *H. seropedicae*

Data are the mean of three independent assays. Numbers in parentheses are standard deviations. The β -galactosidase activity of *H. seropedicae* strains without any fusion (SmR1 and SmR54) was less than 5 Miller units. The plasmids are described in the text and in Table 1 and were constructed as described in Methods. *H. seropedicae* SmR1 and SmR54 strains are wild-type and a *nifA* mutant, respectively. *H. seropedicae* SmR2532 carries a chromosomal *nifA::lacZ* fusion.

Strain	<i>lacZ</i> fusion plasmid	β -Galactosidase activity (Miller units)			
		–NH ₄ ⁺ /1.5% O ₂	+NH ₄ ⁺ /1.5% O ₂	–NH ₄ ⁺ /air	+NH ₄ ⁺ /air
SmR2532	None	233 (46)	51 (17)	260 (30)	41 (7)
SmR1	pEMS120	579 (78)	100 (22)	510 (107)	49 (16)
SmR1	pEMS121	93 (22)	63 (15)	155 (16)	56 (16)
SmR1	pEMS122	943 (85)	123 (27)	202 (36)	66 (10)
SmR1	pEMS123	440 (36)	116 (66)	113 (45)	57 (26)
SmR1	pEMS124	317 (85)	204 (78)	209 (39)	85 (10)
SmR1	pEMS125	10.2 (5)	22 (9)	17 (7)	17 (10)
SmR1	pEMS126	36 (17)	66 (13)	32 (9)	38 (10)
SmR1	pEMS127	129 (59)	235 (10)	139 (83)	184 (50)
SmR54	pEMS120	418 (19)	75 (22)	492 (78)	29 (3)
SmR54	pEMS122	148 (25)	127 (59)	191 (86)	50 (9)
SmR54	pEMS123	178 (44)	107 (28)	207 (24)	64 (23)
SmR54	pEMS124	151 (3)	126 (9)	196 (35)	79 (5)

nifA::lacZ fusion (strain SmR2532) by 80 %, but oxygen did not repress expression (Table 3).

Expression of plasmid-borne *nifA::lacZ* fusions in *H. seropedicae*. As in the chromosomal mutant, the β -galactosidase expression of the fusion carried by plasmid

pEMS120 (comprising the whole promoter region of the *nifA* gene) in *H. seropedicae* strain SmR1 was also depressed 4–5-fold in the presence of 20 mM NH₄Cl but was not substantially affected by oxygen (Table 3). Similar levels of expression were observed in strain SmR54 (*nifA* mutant) carrying pEMS120. Deletion of

the sequence upstream from base 264 (pEMS124), including half of the NtrC binding site, decreased the β -galactosidase activity in SmR1 under normal N_2 -fixing conditions by 50%; this activity was only depressed 30% by ammonia. In strain SmR54, expression from this mutant promoter was lower under N_2 -fixing conditions than in the wild-type (SmR1) with the same deletion and it was insensitive to either added ammonium or air but was depressed further by 50% when both were added together. Deletion of 179 bp (pEMS123) and 190 bp (pEMS122) from base 264 caused an increase in β -galactosidase activity under derepressing conditions and marked repression by either ammonium ions or oxygen in the wild-type strain, while in the *nifA* mutant there was no response to the absence of fixed nitrogen and low oxygen. The β -galactosidase activity of pEMS122 was twice that of pEMS123 in the derepressing wild-type strain.

Finally, elimination of a region upstream from position 604 (pEMS121), leaving only the $-24/-12$ promoter sequence, produced a fusion with a low level of expression not regulated by either ammonium ions or oxygen (Table 3). Clearly, the upstream sequences are important for promoter activity.

A set of progressive deletions from the 3' end of the *nifA* promoter was also tested. Deleting the $-24/-12$ promoter (pEMS125) caused a large decrease of β -galactosidase activity under all conditions. With pEMS126, where both the NifA UAS and the promoter region were eliminated, there was a weak non-regulated expression. That level was increased 3–4-fold in pEMS127, where a further 220 bp were deleted (Table 3).

Mapping of the 5' end of *nifA* RNA

To identify the 5' end of the *nifA* RNA, a single-stranded probe was synthesized by unidirectional PCR using an oligonucleotide primer labelled with ^{32}P . This probe was used for S1 mapping, revealing position 641 as the *nifA* RNA 5' end (Fig. 2). No signal was detected with RNA isolated from cultures under repressing conditions (20 mM NH_4Cl ; not shown).

In vivo footprinting

In vivo footprinting in *E. coli*. To determine if NifA could bind to the NifA UAS (position 504 to 519) in the promoter region of the *H. seropedicae nifA*, the plasmids pEMS111 (carrying the *nifA* promoter) and pNH11 (expressing *K. pneumoniae nifA* from the *tac* promoter) were transformed into *E. coli* and the culture treated with DMS. In this experiment, the binding of the protein to the DNA is assessed by a relative decrease in methylation (protection) of the G residues and/or by an enhancement of methylation if the base is more exposed to the chemical modification upon protein binding. The G residue of the TGT motif at positions 505 and 518 of the top and bottom strands, respectively, were strongly

Fig. 2. Mapping of the transcriptional-start site of the *nifA* gene of *H. seropedicae*. The lanes labelled 5U and 50U show the S1 nuclease digestion products with 5 and 50 units of S1 nuclease, respectively, from the hybridization reaction between total RNA and the single-stranded probe synthesized using primer 1 (Methods). The arrow indicates the transcription-start site.

protected in the presence of *K. pneumoniae* NifA (Fig. 3a, b, lanes 4 and 5), indicating NifA binding to the UAS *in vivo*. The residues at positions 522 and 525 of the top strand and 527 of the bottom strand were weakly protected by *K. pneumoniae* NifA. These residues are outside of the NifA UAS but the sequence 522-GACGACA may constitute a half NifA UAS.

In vivo footprinting in *H. seropedicae*. To identify the interaction of the $-24/-12$ promoter sequence with the σ^N -containing RNA polymerase, cultures of *H. seropedicae* harbouring pEMS120 in the absence or presence of NH_4Cl with and without rifampicin, were chemically treated with DMS or permanganate. In the DMS footprinting, methylation of the residue at position 615 (-26) of the top strand was enhanced in the absence of fixed nitrogen and that of residue 632 (-9) when rifampicin was also added (Figs 4 and 5). In the bottom strand there was no substantial difference between the four conditions (not shown). Enhancement of methylation of residue -26 might reflect a higher occupancy of the promoter by σ^N -containing RNA polymerase under nitrogen-fixing conditions, whereas the altered reactivity of residue -9 might be due to formation of an open

Fig. 3. Interaction of NifA UAS of *H. seropedicae* *nifA* promoter with the NifA protein. Bands protected from methylation by DMS in the presence of NifA expressed constitutively in *E. coli* or under nitrogen-fixing conditions in *H. seropedicae* are indicated by ○. (a) Top strand. (b) Bottom strand. Lanes: 1, SmR1(pEMS120) under nitrogen-fixing conditions; 2, SmR1(pEMS120) plus 20 mM NH_4Cl ; 3, SmR54(pEMS120) under nitrogen-fixing conditions; 4, ET8894(pEMS111) with *K. pneumoniae* NifA provided by pNH11; 5, ET8894(pEMS111).

Fig. 4. DMS footprinting of the top strand of the $-24/-12$ element of *H. seropedicae* *nifA*. Hypermethylation is indicated by ●. Lanes: 1, SmR1(pEMS120) under nitrogen-fixing conditions; 2, SmR1(pEMS120) plus 20 mM NH_4Cl ; 3, SmR1(pEMS120) under nitrogen-fixing conditions plus rifampicin; 4, SmR1(pEMS120) plus 20 mM NH_4Cl and rifampicin. Rifampicin was added to block RNA synthesis and trap the *nifA* promoter in the open-complex form. For comparison, the C sequencing track (corresponding to the G position of the top strand) is shown. No obvious protection was detected.

complex. DMS footprinting of the promoter region of the *nifA* in an *E. coli* *rpoN*⁺ (ET8894) strain revealed that the G residue at position 616 (−25) in the top strand was protected in the presence of the *rpoN* gene (not shown), also suggesting interaction of the σ^{N} -containing RNA polymerase with this sequence. In the bottom strand no protection was observed.

Permanganate footprinting probes for open-complex formation, since T residues of single-stranded DNA become more reactive. The T residue at position 650 (+10) in the top strand was slightly more reactive towards permanganate in the absence of ammonium ions than in their presence (Fig. 6a, lanes 3 and 4; Fig. 7). When rifampicin was added to trap open complexes, T residues at positions 631, 643, 647, 649 and 650 (−10, +3, +7, +9 and +10) were more reactive in the absence than in the presence of fixed nitrogen (Fig. 6a, lanes 1 and 2; Fig. 7). These results suggest melting of the region between residues −10 and +10 during open-complex formation under nitrogen-fixing conditions. The only residue in the bottom strand to show an increased reactivity towards permanganate was T 633 (position −8) in the presence of rifampicin (Fig. 6b). Permanganate footprinting of the *nifA* promoter was determined in ET8894 (*rpoN*⁺) and ET8045 (*rpoN*[−]) carrying pEMS109 (*HsnifA* promoter) and pCK1 (*K. pneumoniae* *nifA*^c) in the presence of rifampicin. In the *rpoN*⁺ strain, the residues at positions 631, 643 and 647 in the top strand (−10, +3 and +7 in relation to the 5' end of *nifA* RNA) showed an increase in reactivity towards KMnO_4 (not shown). No increase in reactivity was identified in the bottom strand.

To determine whether there was a change in methylation by DMS in the NifA UAS (positions 504–519), plasmid DNA was purified from *H. seropedicae* strain SmR1

Fig. 5. Densitometric analysis of the DMS footprint at top strand of the *H. seropedicae* *nifA* promoter. (a) SmR1(pEMS120) under nitrogen-fixing conditions. (b) SmR1(pEMS120) plus 20 mM NH_4Cl . (c) SmR1(pEMS120) under nitrogen-fixing conditions plus rifampicin. (d) SmR1(pEMS120) plus 20 mM NH_4Cl and rifampicin. (e) The autoradiographs were scanned and the peak heights determined. The normalized height of each peak under nitrogen-fixing conditions was divided by the value in the presence 20 mM NH_4Cl and expressed as the logarithm of the quotient. Peaks were normalized against the value of residues 598. Rifampicin was added (black bars) or not (white bars) to trap open complexes. Positive values indicate hypermethylation and negative values indicate protection. Guanine residue -26 is hyper-reactive under derepressing conditions while residue -9 is hypermethylated only when rifampicin was added to derepressed cells.

(pEMS120) derepressed in either the absence or presence of 20 mM NH_4Cl and from *H. seropedicae* strain SmR54 (pEMS120) derepressed in the absence of fixed nitrogen. The G residue at position 505 of the top strand and the residue 518 of the bottom strand were protected from methylation by DMS in the absence of NH_4Cl , indicating binding of the NifA protein under nitrogen-fixing conditions (Fig. 3a, b, compare lane 1 with lanes 2 and 3).

DISCUSSION

The results of the expression of the *nifA* gene of *H. seropedicae* in an enteric background (Table 2) indicated that the transcription of this gene was activated by either the NifA or NtrC proteins and is dependent on the σ^N factor. Indeed, the *nifA* RNA 5' end was mapped under derepressing conditions at position 641 and, 11 bp upstream from this position, a sequence homologous to a -24/-12 promoter element (*nifAp*) is located (Fig. 1a). The relatively high activity of the fusion with the

NtrC UAS deleted may be due to interaction between non-bound NtrC and the closed promoter/ σ^N -containing RNA polymerase complex.

The expression of *nifA* in *H. seropedicae* is regulated by fixed nitrogen (4–5-fold reduction by NH_4Cl) but not by oxygen (Table 3). The expression levels of *nifA* in the *H. seropedicae* *nifA* mutant carrying pEMS120 were similar to those in the wild-type carrying the same plasmid, which suggests that, contrary to results in *E. coli*, the NifA protein is not essential for the expression of the *nifA* gene in *H. seropedicae*. Partial deletion of the NtrC UAS (pEMS124) abolished the regulation by ammonium ions but decreased by half the β -galactosidase activity in the wild-type carrying pEMS120, indicating that the putative NtrC UAS is necessary for activation of *nifA* gene expression in *H. seropedicae*. These results, together with transcription-start-site mapping, indicate that the increase in *nifA* expression under nitrogen-fixing conditions is primarily dependent on the NtrC activation of the -24/-12 promoter element.

Fig. 6. Probing open complexes at the *H. seropedicae nifA* promoter with KMnO₄. (a) Top strand. (b) Bottom strand. Lanes: 1, SmR1(pEMS120) under nitrogen-fixing conditions plus rifampicin; 2, SmR1(pEMS120) plus 20 mM NH₄Cl and rifampicin; 3, SmR1(pEMS120) under nitrogen-fixing conditions; 4, SmR1(pEMS120) plus 20 mM NH₄Cl. Rifampicin was added to block RNA synthesis and trap the *nifA* promoter in the open complex form. For comparison, the A sequencing track (corresponding to the T positions) is shown.

Fig. 7. Densitometric analysis of potassium permanganate footprinting of the top strand at the *H. seropedicae nifA* promoter. (a) SmR1(pEMS120) under nitrogen-fixing conditions plus rifampicin. (b) SmR1(pEMS120) plus 20 mM NH₄Cl and rifampicin. (c) SmR1(pEMS120) under nitrogen-fixing conditions. (d) SmR1(pEMS120) plus 20 mM NH₄Cl. (e) The autoradiographs were scanned and the peak heights determined. The normalized height of each peak under nitrogen-fixing conditions was divided by the value in the presence of 20 mM NH₄Cl and expressed as the logarithm of the quotient. Peaks were normalized against the value of residues 610. Rifampicin was added (black bars) or not (white bars) to trap open complexes. Positive values indicate hyper-reactivity towards permanganate.

Regulation by ammonium or oxygen, as well as dependence upon the NifA protein, was introduced when all but 70 bp upstream of the putative NifA binding site (pEMS123, Fig. 1b) was deleted. Elimination of a further 20 bp (pEMS122) had the same pattern, although the β -galactosidase activity under nitrogen-fixing conditions was higher in the wild-type strain carrying pEMS122 than that carrying pEMS123 (Table 3). Interestingly, the fusion carried by pEMS122 expressed β -galactosidase at twice the level of that of pEMS120, suggesting that the sequences upstream from the NifA binding site interfere with the ability of NifA to activate this promoter.

Although NifA is not essential for *nifA* expression (579 Miller units in the wild-type compared to 418 in the NifA⁻ background), it may contribute to the full expression at the promoter. Moreover, the β -galactosidase activities with the deletions in plasmids pEMS123 and pEMS122 indicate that the NifA UAS is functional under certain conditions in *H. seropedicae*. This conclusion is also supported by footprinting evidence showing that NifA does interact with its putative UAS. DMS footprinting in *E. coli* showed that the G residues of TGT motifs in both top and bottom strands were strongly protected by NifA (Fig. 3a, b); two positions in the NifA UAS of *K. pneumoniae nif* genes that are characteristically protected by bound NifA (Morett & Buck, 1988). The half NifA UAS located at position 522–528 was also protected, possibly due to the high concentration of the NifA protein produced by pNH11 allowing for the binding to a lower-affinity DNA sequence. In *H. seropedicae* the G residues of the TGT motif of the NifA UAS were protected in both strands in the absence of fixed nitrogen. Since *nifA* expression is activated under this condition, this result suggests that the NifA protein is able to bind to the NifA UAS in *H. seropedicae*. Plasmids pEMS122 and pEMS123, which lack the sequences at the 5' end of the NifA UAS, show higher activation dependent on NifA. It is possible that the sequences close to the 5' end of the NifA UAS play a regulatory role in NifA activation of the -24/-12 promoter. The sequence ~50 bp upstream from the NifA UAS may constitute an IHF binding site (Souza *et al.*, 1991a). Presumably the IHF could inhibit NifA binding and hence transcription in the absence of a NtrC UAS. This possibility parallels the observation by Austin *et al.* (1994) that NifA-dependent *in vitro* transcription of the *nifH* promoter was inhibited by the IHF if the NifA UAS was deleted. IHF and NifA may compete for binding in this DNA region and regulate *nifA* expression by a feedback mechanism. Only when the NifA concentration is high enough can it out-compete the IHF and activate the -24/-12 promoter. Alternatively, a high concentration of NifA might activate the promoter with binding to the UAS.

DMS footprinting of the putative -24/-12 promoter of the *nifA* gene confirmed that this region was in contact with the σ^N -containing RNA polymerase. The -25 G residue (in relation to the RNA 5' end) alone was protected in the top strand but no base was protected in the bottom strand in *E. coli* (not shown) indicating weak

contact, since the residues -25, -24 and -12 are characteristically protected by σ^N -containing RNA polymerase in *E. coli* (Buck & Cannon, 1989). In *H. seropedicae*, when the promoter was trapped in the open-complex form by the addition of rifampicin, residues -26 and -9 were hypermethylated under nitrogen-fixing conditions, suggesting contact with the σ^N -containing RNA polymerase. Hypermethylation of residue -9 by DMS also occurs in the open complex of the *nifU* promoter of *K. pneumoniae* (Buck & Cannon, 1992). Finally, the permanganate footprinting (Fig. 6) showed that the region between -10 and +10 of the *H. seropedicae nifA* promoter was more reactive towards permanganate, and probably engaged in an open complex under nitrogen-fixing conditions. These results indicated that the identified -24/-12 promoter sequence of *H. seropedicae nifA* is transcriptionally active in the absence of fixed nitrogen.

In pEMS127, where the -24/-12 promoter, the NifA and NtrC UASs were deleted, *nifA* expression was low and not regulated by ammonium ions or oxygen, neither in *E. coli* nor in *H. seropedicae*. In addition, a background level of *nifA* expression was present under repressing conditions, suggesting the presence of an upstream promoter. This promoter organization could maintain a constitutive low level of *nifA* RNA synthesis, allowing a low level of NifA protein to activate the expression of *nif* genes as soon as conditions become favourable for nitrogen fixation. On the other hand, smaller deletions of the 3' region, which leave the NtrC and NifA binding sites present, decreased the level of expression from this putative promoter in pEMS125 and pEMS126 in *H. seropedicae*. In *E. coli* pEMS125 also had a very low level of expression. It is possible that the region between bases 264 and 442 may destabilize the RNA or that binding of regulatory proteins diminishes transcription.

The *nifA* promoter active under nitrogen-fixing conditions is of the type -24/-12. This promoter is transcriptionally active in both *E. coli* and *H. seropedicae*. In *H. seropedicae* this promoter is repressed by fixed nitrogen but not by oxygen and is probably activated by the NtrC protein. NifA is apparently not essential for *nifA* expression but it can still bind the NifA UAS and may activate transcription when present in sufficient amounts to out-compete the IHF.

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