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STRUCTURAL ORGANIZATION AND REGULATION OF THE *NIF* GENES OF *HERBASPIRILLUM SEROPEDICAE*

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Summary—The promoter regions of *nifA* and *nifH* genes of *Herbaspirillom seropedicae* have been isolated and investigated, and the *nifA* promoter has been characterized in detail. Both regions contain a -24/-12 type promoter and NifA-binding sites; the *nifA* promoter also has an NtrC-binding site. The *nifA* expression is activated by NtrC and repressed by ammonium ions but not by oxygen. When the NtrC-binding site was deleted *nifA* expression was NifA-dependent. The native NifA protein of *H. seropedicae* was unable to activate *nifH* expression in *Escherichia coli* whereas a truncated protein, lacking the N-terminal domain was able to activate *nifH* expression in the absence of oxygen and presence of iron, indicating that the N-terminal domain regulates *NifA* activity. The truncated protein also activates *nifH* in *Azospirillum brasilense* in the presence or absence of ammonium ions. This suggests that the N-terminal domain senses the nitrogen status of the cell. © 1997 Elsevier Science Ltd

INTRODUCTION

Herbaspirillum seropedicae is a free-living diazotroph originally isolated from the roots of grasses and, more recently, from leaves and stem interiors of sugar cane and rice (Baldani *et al.*, 1986, 1991). It is a Gram-negative, vibrioid, sometimes helical and very motile bacterium capable of fixing nitrogen under free-living microaerobic conditions (Baldani *et al.*, 1986). *H. seropedicae* tolerates a broader pH range and a higher oxygen concentration than *Azospirillum* species (Baldani *et al.*, 1986; Fu and Burris, 1989 G. Klassen, unpublished MSc thesis, Federal University of parana). The obligately endophytic character of this organism has been recently revealed (Boddey *et al.*, 1995), and is classified, on the basis of rRNA sequence analysis, as being a member of the β-subgroup of proteobacteria (Young, 1992).

The structural organization and regulation of the *nif* genes of *H. seropedicae* have been studied in our laboratory since 1986. The first *nif* genes to be cloned and sequenced were the *nifA* and the contiguous gene *nifB*, isolated by genetic complementation of the *Azospirillum brasilense nifA* mutant FP10 (Pedrosa and Yates, 1984; Souza *et al.*, 1991a, 1991b). Nucleotide sequence analysis of the *nifA* gene promoter region revealed the presence of potential binding sites for RNA polymerase σ^{54} (σ^{N}) factor, NtrC and, also, for the NifA protein. A typical NifA-, σ^{N} -dependent promoter was also found upstream from the *nifB* gene (Souza *et al.*, 1991b). These results suggested that transcriptional activation of at least some *nif* genes was dependent on the NifA protein and also that *nifA* gene transcription itself was dependent upon the σ^{N} factor and NtrC proteins as proposed by Pedrosa *et al.* (1989). A sequence (TTCATCAAGGTCAA) reminiscent of the consensus "anaerobox" sequence (TTGATNNNNATCAA) (Nees *et al.*, 1988), was found inside the *nifA* open reading frame. The "anaerobox" sequence or Fnr binding sequence binds to transcriptional regulators related to the *E. coli* Fnr protein, which are involved in activation or repression under different O_2 levels (Spiro, 1994). An O_2-dependent repressive function for an "anaerobox" located inside a gene has been suggested (Nees *et al.*, 1988). Furthermore, a putative integration host factor (IHF) sequence was found 183 nucleotides downstream from the putative NtrC-binding site in the *nifA* promoter (Souza *et al.*, 1991b).

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RESULTS AND DISCUSSION

Regulation of the nifA *gene expression*

Analysis of *nifA–lacZ* fusions revealed that expression of the native promoter was repressed (80%) by NH_4Cl (20 mM) in both the wild type

Table 1. Effect of NH_4^+ and O_2 on the expression of β-galactosidase under control of the *nifA* promoter of *H. seropedicae*

Strain	Plasmid	β-galactosidase acitivity (Miller Units)			
		−N/1.5%O_2	+N/1.5%O_2	−N/air	+N/air
Smr1	pEMS120	579	100	510	49
Smr1	pEMS122	943	122	202	65
Smr1	pEMS124	317	204	209	85
Smr54	pEMS120	418	75	492	29
Smr54	pEMS122	148	127	191	50
Smr54	pEMS124	151	126	196	79
Smr2132	none	233	51	260	41

Smr1 is the wild-type strain; Smr54 is a *nifA*-minus mutant where a Km cassette was inserted into the *nifA* gene; and, Smr2132 has a transposon Tn5-21 inserted into the *nifA*, with the *lacZ* gene in the same orientation as *nifA*;. pEMS120 has a 1.7 Kb *Eco*RI fragment containing the native *nifA* promoter region; pEMS124 has a *Eco*RI/*Pml*I fragment where the promoter sequences upstream from base 264, including half of the NtrC site, were deleted; pEMS122 has a *Dra*I/*Eco*RI fragment where the promoter sequence upstream from base 179, including the NtrC and IHF binding sites were deleted. The *lacZ* transcriptional fusion vector was pPW452 which is similar to pMP220 (Spaniak *et al.*, 1987), but with the cloning nest inverted.

and in a *nifA*-minus mutant of *H. seropedicae* (Table 1). However, its expression was not affected by 20% O_2 in these backgrounds. Deletion of sequences upstream from base 264 (Souza *et al.*, 1991b), including half of the NtrC-binding site (pEMS124), decreased promoter activity by 40%, suggesting that NtrC and its binding site are involved in the expression of the *nifA* promoter and in the response to NH_4Cl. Deletion of an additional 190 nucleotides (pEMS122), which eliminated the NtrC-binding site, restored promoter activity under N_2-fixing conditions and repression by ammonium ions and, in addition, resulted in apparent repression by oxygen. These deleted promoters failed to express in a *nifA*-minus mutant of *H. seropedicae*. The native *nifA* promoter is, therefore, primarily under NtrC regulation being affected by ammonium ions but not by oxygen. Deletion of sequences immediately upstream from the NifA-binding site apparently transfers regulation of the *nifA* gene expression from the NtrC to the NifA protein.

Control of the NifA *protein activity*

The wild-type NifA protein of *H. seropedicae* failed to activate the *nifH* promoter of *Klebsiella pneumoniae* in an *Escherichia coli* background; a N-terminal truncated-(202 aminoacids deleted)-β-galactosidase-fused NifA (*lacZ-ΔnifA*), however, successfully activated this promoter under anaerobiosis (Table 2). Both the native and the truncated NifA proteins were inactive in the presence of oxygen. In an *A. brasilense* background (FP10 *nifA nifH:lacZ*) the truncated NifA protein, in contrast to the native NifA, was active in the presence of high NH_4Cl concentrations.These results suggest that the NifA protein of *H. seropedicae* is inhibited by ammonium ions and oxygen. The N-terminal region appears to be involved in the sensitivity of the protein to inhibition by ammonium (manuscript in preparation). Oxygen-sensitivity of the NifA protein appears to be related to the interdomain linker as in *B. japonicum* (Fischer *et al.*, 1988).

*Cloning and sequencing of the nitrogenase structural genes (*nifHDK*) of Herbaspirillum seropedicae*

Two contiguous 2.0 kb and 1.6 kb *SalI* fragments from phage λIM02, isolated from a genomic λEMBL3 library of *H. seropedicae* strain SMR1, which hybridized strongly with a *nifHDK* genes probe of *A. brasilense*, were isolated and sequenced (EMBL/GENEBANK Accession number Z54207 HSNIFHDK, full manuscript submitted). Analysis of the data showed the presence of two complete and one incomplete open reading frames (ORFs), which were identified as the *nifH*, *nifD*, and a partial *nifK* gene, respectively. The predicted NifH and NifD polypeptide chains showed highest levels of similarity (>90%) and identicality (>71%) to homologous proteins from *Bradyrhizobium japonicum* (Fuhrmann and Hennecke, 1984; Kaluza and Hennecke, 1984), *A. brasilense* (Passaglia *et al.*, 1991; Fani *et al.*, 1989), and *Thiobacillus ferrooxidans* (Pretorius *et al.*, 1987; Rawlings, 1988). The partial *H. seropedicae nifK* gene product showed

Table 2. Effect of native and N-truncated NifA protein on the expression of the *nifH* promoter

Strains	Plasmids	β-galactosidase activity		
		−N/−O[a]	−N/+O[b]	+N/−O[c]
ET8894	pRT22/pNH11	19582	20904	20800
ET8894	pRT22/pEMS130	21	15	19
ET8894	pRT22/pEMS131	2884	31	3016

Plasmid pRT22 carries a *K. pneumoniae nifH-lacZ* fusion; pNH11 expresses *K. pneumoniae nifA* constitutively from a tac promoter; pEMS130 expresses the intact *H. seropedicae nifA* gene from the *lacZ* promoter; plasmid pEMS131 expresses the N-terminal truncated *H. seropedicae nifA* gene.

[a]N-free minimal medium under nitrogen.

[b]N-free minimal medium under air.

[c]Minimal medium plus NH_4Cl under nitrogen.

highest homology to *B. japonicum* (83.3% similarity and 69.0% identicality) (Kaluza and Hennecke, 1984), *A. brasilense* (75.4% and 62.0% identicality) (Passaglia *et al.*, 1991), and *T. ferrooxidans* (75.0% similarity and 56.9% identicality) (Rawlings, 1988).

Structural organization of the nifH *promoter*

The DNA region preceding the *nifH* gene contains a sequence homologous to the σ^N recognition promoter consensus sequence (5'-gT*GG*gACGGCATTT*GC*A-3'). Two potential NifA-binding sites (5'-*TGT*-N_{10}-*ACA*-3') were found at −311 and −281 bp upstream from the translation initiation codon. In addition, a potential integration host factor (IHF) binding sequence (5'-A*ATCAA*tggc*TT*G-3') was found at position −213 from the start codon. Potential ribosome binding sites are presently starting at 6 bp, 3 bp and 7 bp upstream from the translation initiation codon of *nifH*, *nifD* and *nifK* genes, respectively.

Cloning and physiological properties of the glnAntrBC genes

Five recombinant plasmids (pKRT1 to pKRT5), from a genomic library of *H. seropedicae* in the cosmid pLAFR3, were isolated by genetic complementation of the *E. coli ntrC*$^-$ mutant strain ET8556. They restored the L-arginine-dependent growth of this strain, and nitrogenase activity of the *ntrC*$^-$ mutants of *A. brasilense*, FP8 and FP9. Plasmid pKRT1 contains a 22.5 Kb *H. seropedicae* DNA insert capable of hybridizing with the *ntrC* gene of *Azotobacter vinelandii*. Other hybridization experiments revealed the presence of *glnA*- and *ntrB*-like genes contiguous with the *ntrC* gene. These results indicate that in *H. seropedicae* the *glnAntrBntrC* genes are organized contiguously, as in *K. pneumoniae* (Espin *et al.*, 1982) and *A. vinelandii* (Toukdarian and Kennedy, 1986). Sequencing of these genes is also in progress.

Cloning of the glnB *and* rpoN *genes*

The *glnB* gene was isolated by genetic complementation of nitrate-dependent growth of the *glnB* mutant of *K. pneumoniae* UNF1529. Two distinct, non-overlapping recombinant cosmids were found to hybridize to a *K. pneumoniae glnB* probe, suggesting the presence of two homologous *glnB* genes in *H. seropedicae*, as recently reported for *E. coli* (Van Heeswijk *et al.*, 1995) and *A. brasilense* (De Zamaroczy *et al.*, 1995).

Similarly, the *rpoN* gene of *H. seropedicae* was isolated by genetic complementation of the NtrC-minus phenotype (L-arginine-dependent growth) of an *rpoN* mutant of *E. coli* strain ET8045. Recombinant cosmids (pEMB1 to 3) showed similar restriction patterns and, when eliminated by introducing of R68.45 of the same IncP1 group, the recipient bacteria had their RpoN phenotype restored. Sequencing of the above genes is in progress.

CONCLUSIONS

The expression of nitrogen fixation genes in *H. seropedicae* requires a NifA protein, the synthesis and activity of which is under strict regulation and control. Regulation of *nifA* expression appears to be analogous to that originally described for *K. pneumoniae*, being responsive to ammonium ions and involving the general nitrogen regulatory system (Ntr). Control of NifA activity, on the other hand, is similar to that described for rhizobial NifA proteins (see Fischer, 1994), being inactivated by oxygen. Evidence also indicates that the N-terminus of the *H. seropedicae* NifA is involved in sensing the N levels, while the interdomain linker located between the C-terminus and the central domains is probably involved in the oxygen response and Fe binding, as in species of rhizobia (Fischer, 1994). These characteristics suggest that regulation of nitrogen fixation in *H. seropedicae*, which belongs to the β-subgroup of proteobacteria, has features in common with members of both *K. pneumoniae* (γ-subgroup) and Rhizobia (α-subgroup). A third characteristic which differs from either of these latter two groups is that the NifA protein activity of *H. seropedicae* is sensitive to inhibition by ammonia. Whether this hybrid regulation system is a characteristic of obligate endophytic diazotrophs remains to be confirmed.

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