Sequence and structural organization of a *nifA*-like gene and part of a *nifB*-like gene of *Herbaspirillum seropedicae* strain Z78

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The deduced amino acid sequence derived from the sequence of a fragment of DNA from the free-living diazotroph *Herbaspirillum seropedicae* was aligned to the homologous protein sequences encoded by the *nifA* genes from *Azorhizobium caulinodans*, *Rhizobium leguminosarum*, *Rhizobium meliloti* and *Klebsiella pneumoniae*. High similarity was found in the central domain and in the C-terminal region. The *H. seropedicae* putative NifA sequence was also found to contain an interdomain linker similar to that conserved among rhizobial NifA proteins, but not *K. pneumoniae* or *Azotobacter vinelandii*. Analysis of the regulatory sequences found 5' from *nifA* indicated that the expression of this gene in *H. seropedicae* is likely to be controlled by NifA, NtrC and RpoN, as judged by the presence of specific NifA- and NtrC-binding sites and characteristic –24/–12 promoters. Possible additional regulatory features included an 'anaerobox' and a site for integration host factor. The N-terminus of another open reading frame was found 3' from *nifA* and tentatively identified as *nifB* by amino acid sequence comparison. The putative *nifB* promoter sequence suggests that expression of *H. seropedicae nifB* may be activated by NifA and dependent on RpoN.

Introduction

Herbaspirillum seropedicae is a free-living diazotroph found in soils and in the rhizosphere of several grasses, and has been classified as a member of the Spirillaceae (Baldani *et al.*, 1986). It shows a broader pH range for N₂-dependent growth and higher nitrogenase tolerance to oxygen than *Azospirillum* spp. It also shows low (<20%) rRNA homology to *Azospirillum* spp. (Falk *et al.*, 1986).

In *Azospirillum brasilense*, regulation of *nif* expression may be analogous to that of *Klebsiella pneumoniae* (Pedrosa & Yates, 1984). Potential *nifA* and *ntrC* mutants of *Azospirillum brasilense* were complemented for nitrogen fixation by homologous genes from *K. pneumoniae*. However, the organization of these genes and the control of nitrogen assimilation in *Azospirillum brasilense* may be different from that in *K. pneumoniae* (Bozouklian & Elmerich, 1986; Bozouklian *et al.*, 1986).

Abbreviations: IHF, integration host factor; ORF, open reading frame.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M60319.

The dependence of *nif* genes on RpoN and NifA for expression has also been demonstrated in *Rhizobium meliloti* (Ronson *et al.*, 1987) and *Azotobacter vinelandii* (Toukdarian & Kennedy, 1986; Santero *et al.*, 1988). Activation by NifA also occurs in *Rhizobium* and *Bradyrhizobium* spp. (Gussin *et al.*, 1986) and in *Azorhizobium caulinodans* (de Bruijn *et al.*, 1988), and the presence of a –24/–12 promoter sequence suggests a dependence on RpoN in the majority of *nif* promoters sequenced to date.

The *nifA* genes from several organisms have been cloned and sequenced. The NifA amino acid sequences inferred from the determined nucleic acid sequences share a high degree of homology in domains D and E, as defined by Drummond *et al.* (1986). Domain D, the central domain, appears to contain sequences homologous to ATP-binding proteins (Walker *et al.*, 1982). Domain E, located in the C-terminal region, contains a helix-turn-helix motif characteristic of DNA-binding proteins; NifA also shares high homology with NtrC in domains D and E (Drummond *et al.*, 1986).

A recombinant plasmid capable of complementing a *nifA* mutant of *A. brasilense* was identified from a gene library of *H. seropedicae* strain Z78 (Souza *et al.*, 1991). In this paper we present the nucleotide sequence of a *nifA*-

Fig. 1. Physical map and plasmids derived from pEMS101. pVK102 was the vector plasmid of pEMS101; pTZ18R was the vector plasmid of pEMS300A, pEMS300B and pEMS303; pTZ19R was the vector plasmid of pEMS301A, pEMS301B and pEMS302. The arrows indicate the direction of sequencing. Restriction sites are: B, *Bgl*II; R, *Eco*RI; P, *Pst*I; S, *Sal*I. Not all *Eco*RI sites are shown.

like gene from *H. seropedicae*. This sequence shows a high level of homology to *nifA* genes from other organisms, especially to that of *A. caulinodans*. The presence of -24/-12 promoter sequences and binding-sites for NtrC and NifA in the promoter of *H. seropedicae nifA* suggests a requirement for these proteins and RpoN for the expression of this gene. Additional regulatory factors may include an 'anaerobox' sequence and a site for integration host factor proteins.

Methods

Bacterial strains and plasmids. *Escherichia coli* strains were usually grown in LB (Maniatis *et al.*, 1982) or 2 × YT broth (Bankier *et al.*, 1987) supplemented with antibiotics as required. *E. coli* 71-18 was kept in minimal medium supplemented with thiamin (5 mg l⁻¹) (Maniatis *et al.*, 1982). The phagemids pTZ18R and pTZ19R were used as sequencing vectors and the helper phage M13KO7 was grown in liquid culture and titrated on a lawn of *E. coli* 71-18 (Mead *et al.*, 1986).

DNA labelling and hybridization. DNA probes were labelled with [α -³²P]dCTP by the hexadeoxynucleotide-primed method (Feinberg & Vogelstein, 1984). Restriction fragments from pEMS101 were separated by electrophoresis and transferred to a nylon membrane as described by Maniatis *et al.* (1982). Hybridizations were done under low stringency conditions (35%, v/v, formamide, 42 °C) and were washed twice with 2 × SSC (1 × SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate, pH 7.0) at room temperature and twice with 2 × SSC at 65 °C (Maniatis *et al.*, 1982).

Subcloning and sequencing. Two proximal, non-overlapping DNA fragments from pEMS101 (Fig. 1), namely the 2.0 kb *Sal*I fragment and the 1.7 *Eco*RI fragment, were subcloned into pTZ18R and pTZ19R respectively, in both orientations, yielding pEMS300A, pEMS300B, pEMS301A and pEMS301B. Sets of ordered deletions were created for each plasmid using Exonuclease III and S1 nuclease, followed by treatment with Klenow enzyme and blunt-end ligation with T4-DNA ligase, according to the Pharmacia protocol. The region between the *Sal*I and the *Eco*RI fragments was sequenced using a clone containing the 0.7 kb *Eco*RI fragment, which overlaps the 2.0 kb *Sal*I fragment

(Fig. 1). This fragment was cloned into pTZ19R. The 5.2 kb *Sal*I fragment (Fig. 1), overlapping the 1.7 kb *Eco*RI fragment, was cloned into pTZ18R and sequenced in the opposite direction to the smaller *Eco*RI fragment. A total of 56 clones covering both directions were sequenced by the chain termination method using deoxyadenosine 5'- α -[³⁵S]thiotriphosphate ([α -³⁵S]dATP) (Bankier *et al.*, 1987). Regions with severe band compression (high GC) were resolved using 7-deazaguanosine triphosphate instead of dGTP. Primary sequences were fed into a VAX computer, edited using the DButil program and analysed using the Analyseq (Staden, 1982, 1984) and UWGCG programs (Devereux *et al.*, 1984). The best alignment of the amino acid sequences was determined using the Clustal program (Higgins & Sharp, 1988).

Cloning and molecular biology methods. All enzymes were obtained from commercial sources and used according to the manufacturers' instructions. Transformation, DNA purification from agarose gels, electrophoresis and single-strand DNA purification for enzymic sequencing were done as described by Maniatis *et al.* (1982) and Bankier *et al.* (1987).

Results

A nifA-like gene is present in H. seropedicae

Plasmid pEMS1 contains a 30 kb DNA fragment, and was isolated from a genomic library of *H. seropedicae* strain Z78 constructed in the cosmid pVK102 (Souza *et al.*, 1991). This recombinant plasmid was capable of complementing the *nifA*-like mutant (FP10) of *Azospirillum brasilense*. Plasmid pEMS101 was constructed by deleting the 21 kb *Bam*HI fragment from pEMS1 and is also capable of complementing FP10 (Souza *et al.*, 1991).

To locate and confirm the presence of the *nifA*-like gene on plasmid pEMS101, hybridization experiments were performed using the following two probes derived from pMC71A (Buchanan-Wollaston *et al.*, 1981): (a) a 3 kb *Sal*I fragment containing the complete *nifA* and

Fig. 2. Location of the *nifA*-like gene of *H. seropedicae* in pEMS101. (a) Restriction pattern of pEMS101 digested with: (1) *Sal*I, (2) *Pst*I, (3) *Bgl*II, and (4) *Pst*I plus *Bgl*II. (b) Autoradiogram showing hybridization of restricted pEMS101 to the 3 kb *Sal*I fragment from pMC71A containing *nifA* and part of the *nifB* of *K. pneumoniae* (probe A). (c) Autoradiogram showing hybridization of pEMS101 to the 1.4 kb *Nru*I fragment from pMC71A containing only the *nifA* of *K. pneumoniae* (probe B).

part of *nifB* and *nifL* from *K. pneumoniae* (probe A); and (b) a 1.4 kb *Nru*I fragment containing 69 bp of *nifL* and most of the *nifA* from *K. pneumoniae* (probe B). The restriction pattern of pEMS101 cleaved with *Sal*I, *Pst*I, *Bgl*II and *Pst*I/*Bgl*II is shown in Fig. 2(a). Hybridization of these restriction fragments with probe A and probe B are shown in Fig. 2(b) and 2(c), respectively. The strong hybridization signals observed with both probes, although under low stringency conditions, indicated the presence of a *nifA*-like gene in *H. seropedicae*. The *nifA*-specific probe (probe B) did not hybridize to the 1.7 kb *Pst*I, 2.3 kb *Bgl*II or 1.6 kb *Pst*I/*Bgl*II fragments. This negative result suggested the additional presence of *nifB* or *nifL* on pEMS101, adjacent to the putative *nifA* gene in *H. seropedicae*. Hybridization of probes A and B to a single 2.0 kb *Sal*I fragment (Fig. 2b, c) positioned the majority of the *nifA*-like gene of *H. seropedicae* in this fragment. The absence of hybridization of the 1.7 kb *Pst*I fragment to probe B (Fig. 2c) suggested that the *nifA*-like

gene is restricted to the 1.1 kb *Sal*I/*Pst*I fragment (compare Fig. 1 with Fig. 2).

Nucleotide sequence of the nifA-like gene from H. seropedicae

The DNA region most likely to contain the entire *nifA*-like gene from *H. seropedicae*, as determined by hybridization comprises a 2.0 kb *Sal*I fragment, part of a 1.7 kb *Eco*RI fragment and an interconnecting region (Fig. 1). These fragments were sequenced in both directions and the final sequence was found to be 4037 bp long, 3287 bp of which are shown in Fig. 3. Analysis of this sequence by a base positional method (Staden, 1984) indicated the presence of two potential coding regions: a complete open reading frame (ORF) and the N-terminus of a 3' ORF. Hybridization and genetic complementation studies (Souza *et al.*, 1991) suggest that the complete ORF corresponds to the *H.*

CGAAGTCGGCGATGCGGAGCTGACGCAATCAAAACAATTGTGCGAACGTGCGAACAGCGGGGAATGGCGCAGGCGCGCATATGATGCA
 10 20 30 40 50 60 70 80 90

GCACCAAGTTTGCAGCAAGCGGATTTCATCACACAGCGCCAGCAACCGGTGCGCGGTTGAGGTAATTGTGGCGGCTCACATATCACCTGTTT
 100 110 120 130 140 150 160 170 180

GCCTGCGCGCGCCATCGCGGGCGGGCGACCGGCCAGTTTCAGGGTCAGATAACATGGCTGGGAGCAGGGCGCGCAGCACGCGAGTGGTG
 190 200 210 220 230 240 250 260 270 ^{NtrC binding}

site CACCGGGTTTCGGAAGTCGAAGGCACATTTCATAGTGGGCGATGGTGGATGTTGGAGGTCTGTATATCTCGCTACATAACGCTGGCTGGGCT ^{-24/}
 280 290 300 310 320 330 340 350 360

^{-12 promoter} ACCGGCTTGCTCTGCTTTGTCGTTTCGCTTGCGCGGTGAGCGGCCGATGCAGGTATGAATAGCCAAAACCATGCCATACAATATTTTCT
 370 380 390 400 410 420 430 440 450

TTTAAATCAATGGGATAGCGACGATTTCGATTGCTGCGCCAGTGGCGATGATTGTCGTAATCCGACAAAGAGGACCGGTTGTCGCCA
 460 470 480 490 500 510 520 530 540 ^{NifA binding site}

ACCGGCCAAGGCGACATCGAGGAAAGCGGCCATTGTGTGGGGTTGCTTGGCGAGATCGATTTCATGCATCTGCTTGGGCATGAAGTTTGCT
 550 560 570 580 590 600 610 620 630 ^{-24/-12 promoter}

TGACCAGCAAGGTAAGTATTGGTAATTACACGGCGCTTTTCTGCGGTTTTTCAGGATCTCGGATCTCAGGACAGAGTAGCAAGGGGG
 640 650 660 670 680 690 700 710 720 ^{-24/-12 promoters}

TTCTAGCGCTCTCAGGCAGCGCATGGGGTTCCAGTATTCACGTTAGGAGAGTCAGATGGCCACTATTCTCGAGGACCGCAGTGTGAATTT
 730 740 750 760 770 780 790 800 810 ^{S-D M A T I L D D R S V N L}

E L V T I Y E I S K I L G S S L D L S K T L R E V L N V L S
 AGAAGTTCGTAACGATTTACGAGATCAGCAAGATATTGGGTTTCATCGCTGGATCTATCCAAGACCTTGGCGGAAGTCCTCAACGTCCTCTC
 820 830 840 850 860 870 880 890 900

A H L E T K R V L L S L M Q D S G E L Q L V S A I G L S Y E
 CGCCACCTGGAAACGAAACGGGTATTGCTGAGTCTGATGCAGGACTCCGGTGAATTGCAACTGGTCAGCGCCATCGGCCCTGAGTTACGA
 910 920 930 940 950 960 970 980 990

E F Q S G R Y R V G E G I T G K I F Q T E T P I V V R D L A
 AGAATTCAGAGTGGCAGATACCGGGTGGCGAGGGGCATCACGGCAAGATCTTCAGACGGAAACTCCCATCGTGGTGGCGGACTTGCC
 1000 1010 1020 1030 1040 1050 1060 1070 1080

Q E P L F L A R T S P R Q S Q D G E V I S F V G V P I K A A
 CCAGGAGCGGCTGTTCTGGCTCGCACCAGCCCGGCCAGTCCGAAGACGGGGAAGTCATTTCTTTGTTGGGGTGGCGATCAAGGCTGC
 1090 1100 1110 1120 1130 1140 1150 1160 1170

R E M L G V L C V F R D G Q S P S R S V D H E V R L L T M V
 CGGTGAAATGCTGGGTGTGTTGTGCGTTTTTCGGGATGGACAATCGCCCTCGCGTAGTGTGCGACCATGAGGTGCGCCTGCTGACCATGGT
 1180 1190 1200 1210 1220 1230 1240 1250 1260

A N L I G Q T V R L Y R S V A A E R Q Q L Q E E K R Q L S R
 GGCCAACCTGATCGGACAAACCGTCCGGCTGTATCGCAGCGTAGCGGCGGAGCGCCAGCAGCTGCAAGAGGAAAAGCGCCAGCTGTCCCG
 1270 1280 1290 1300 1310 1320 1330 1340 1350

Fig. 3 (continued on the following two pages). Nucleotide sequence of the *nifA*-like region of *H. seropedicae*. The putative *nif* regulatory sequences and the potential ribosome binding sites are underlined. The sequence encoding NifA starts at position 776 and stops at position 2404 while the sequence encoding NifB starts at 2797. The stop codon of the *nifA* gene is indicated by an asterisk.

Q L Q G K Y K L D N V I G I S K A M Q E V F A Q V H Q S A P
GCAACTCCAGGGCAAGTACAAGCTCGATAACGTAATCGGCATTTCAGGCGATGCAGGAAGTGTTCGGCGAGGTACACCAAGTCCGCCCC
1360 1370 1380 1390 1400 1410 1420 1430 1440

S R S T M L L R G E S G T G K E V I A R A I H Y L S P R K D
CTCGCGCTCGACCATGCTGTTGCGGGGCGAGAGCGGCGACCGGCAAGGAAGTCATCGGCGCGGCCATCCATTACCTCAGTCCGGCGCAAGGA
1450 1460 1470 1480 1490 1500 1510 1520 1530

G P F I K V N C A A L S E T L L E S E L F G H E K G A F T G
TGGGCCATTTCATCAAGGTCAACTGTGCGGCCTTGTGGGAAAGCTTGTGGAATCCGAATTGTTTGGCCACGAGAAGGGCGCCTTCACCGG
1540 1550 1560 1570 1580 1590 1600 1610 1620

A Q G E R K G R F E L A H G G T L F L D E I G E I S P A F Q
GGCCAGGGTGAACGCAAGGGGCGTTTCGAAGTCCGCCATGGCGGCGACCTTGTTCCTCGACGAGATCGGCGAGATCTCGCCGGCCTTCGA
1630 1640 1650 1660 1670 1680 1690 1700 1710

A K L L R V L Q E R E F E R V G G S R S I K V D V R L V T A
GGCCAAAGCTGCTGCGGGTATTGCAGGAGCGTGAATTCGAGCGGGTGGGTGGGTGCGGTTTCGATCAAGGTGGACGTGCGTCTGGTCACCGG
1720 1730 1740 1750 1760 1770 1780 1790 1800

T N R D L E K A V A K G E F R A D L Y Y R I N V V S I F I P
CAGCAACCGCGACCTGGAGAAGGGCGTGGCCAAGGGCGAGTTCCGGGCGGACCTGTATTACCGTATCAATGTGGTCAGCATCTTCATTCC
1810 1820 1830 1840 1850 1860 1870 1880 1890

P L R E R R E D I P Y L V E H F L E K F R V E N Q R A M V A
TCCGCTGCGCGAACGGCGGAGGATATTCCTATCTGGTGGAAACACTTCTCGGAAAAATTCGGTGTGGAGAACCAGCGGCCATGGTGGC
1900 1910 1920 1930 1940 1950 1960 1970 1980

M S P Q A M K V M M N C Y W P G N V R E L E N C V E R T A T
CATGTACCGCGAGGCCATGAAGGTCATGATGAATTGCTACTGGCGGGGCAATGTACGGGAACCTGGAAAATTGTGTCGAGCGTACTGCCAC
1990 2000 2010 2020 2030 2040 2050 2060 2070

M M R G D L I T E V H F S C Q Q N K C L T K V L H E P G Q Q
CATGATGCGCGGCGATCTGATCACCGAGGTGCATTTCTCCTGTGAGCAGAACAATGCCGTGACCAAGGTCTTGATGAACCGGGCCAGCA
2080 2090 2100 2110 2120 2130 2140 2150 2160

Q P V V V V P L E R I S A P Y G A I F A E W D G Q G Q A T G
GCAACGGGTGCTGGTGGTGGCGCTGGAGCGCATATCCGCGCCATACGGCGCCATCTTCGCGGAGTGGGATGGCCAAGGACAAGCCACTGG
2170 2180 2190 2200 2210 2220 2230 2240 2250

A A P P T S E R E R L I W A M E Q C G W V Q A K A A R A L N
CGCCGCGCCCCGACCTCCGAACGCGAAGGTCTTATCTGGGCCATGGAGCAATGTGGCTGGGTGCAAGCCAAGGCAGCCCCGCGCACTCAA
2260 2270 2280 2290 2300 2310 2320 2330 2340

I S P R Q M G Y A L Q K F N I E V K K F *
CATCTCGCGCGCGCAGATGGGCTATGCGCTGCAGAAATTCAATATCGAGGTCAAGAAGTTCTGAAACTCATCTGCGAGCGTGACGTAATA
2350 2360 2370 2380 2390 2400 2410 2420 2430

CGCTGCCAGATCCAAAAGTCTTTTTCTCATTGCCGTCTCGCCCTGCGCGCGCGGTCTCTTCGGCGTCGCGCCAGCCGGTTGTATTGATGG
2440 2450 2460 2470 2480 2490 2500 2510 2520

ATTCCCGGGCTCGGCGAGTCTCTCGCATTCCTCTCCTTCTCCACTGCGTCAAGGCTCATTGGGGCGCGCTCAATTGCAGGCGCGCGGCC
2530 2540 2550 2560 2570 2580 2590 2600 2610

NifA binding site NifA binding site
GTGCTGCATTGTCGTGTTTCATGACAAGGGCCACCCACAATGTATCGCGGATTGTCGCTATCCTCAGATCGCGGTACGACGCAATTCTCGG
2620 2630 2640 2650 2660 2670 2680 2690 2700

-24/-12 promoter

CTATGTCATTGAATTGATTGATTATTTTGTCTGGCAGGGTTTTGGCTATGTCCTCAGGAGAAGGCCAGGCATCACTTTCTGAAAGGGA S-D
 2710 2720 2730 2740 2750 2760 2770 2780 2790

M Q P T Q Y V G I Q D I K S L G T L L D K V A E H K G C
 CTATCCATGCCAGCCACACAATACGTCGGTATCCAGGACATCAAATCGCTAGGCACGTTGCTGGACAAGGTAGCCGAACACAAAGGCTGT
 2800 2810 2820 2830 2840 2850 2860 2870 2880

G T S S E G G K A S C G S S D G P A D M A P E V W E K V K N
 GGCACCAGCAGTGAAGGGCGGCAAGCCAGTTGCGGTTTCATCCGATGGTCCGGCGGACATGGCGCCGAGGTTTGGGAAAAGGTCAAGAAC
 2890 2900 2910 2920 2930 2940 2950 2960 2970

H P C Y S E E A H H H Y A R M H V A V A P A C N I Q C N Y C
 CATCCCTGCTACAGCGAAGAGGCTCATCACCAGTATGCGGTATGCAGTGGCGGTGGCACCGGCCTGTAACATCCAGTGGCAACTACTGC
 2980 2990 3000 3010 3020 3030 3040 3050 3060

N R K Y D C A N E S R P G V V S E K L T P E Q A A K K V F A
 AACCGCAAATACGATTGGGCCAAGCAGTCCGCTCCAGGCGTGGTCAGCGAAAAGCTCAGGCCCGAACAAGCGGCCAAGAAGGTCTTTGCG
 3070 3080 3090 3100 3110 3120 3130 3140 3150

V A S T I P Q M T V L G I A G P G D P L A N P A K T F K T F
 GTGGCTCGACCATTCGGCAGATGACGGTGGTGGGATTGCGGGTGCTGGCGACCGCTGGCCAATCCGGGCCAAGACCTTCAAGACCTTC
 3160 3170 3180 3190 3200 3210 3220 3230 3240

E L I S Q T A P D I K L C L S
 GAGCTGATTTTCGACAGCCGCGCCGATATCAAGCTGTGCTGTGCGAC
 3250 3260 3270 3280

seropedicae *nifA*-like gene. This ORF comprises 1629 nucleotides, contains only one in-frame methionine initiation codon at position 776, terminates at position 2404 and codes for a protein of 542 amino acid residues (M_r 60880). A putative Shine-Dalgarno sequence was found 4 bp from the translation initiation codon (AUG) (Fig. 3).

The *H. seropedicae* NifA-like protein deduced from the nucleotide sequence was compared to the inferred amino acid sequences of NifA proteins from *K. pneumoniae* (Buikema *et al.*, 1985; Drummond *et al.*, 1986), *R. meliloti* (Buikema *et al.*, 1985), *R. leguminosarum* (Grönger *et al.*, 1987) and *Azorhizobium caulinodans* (Nees *et al.*, 1988) (Fig. 4). The homology in the N-terminal region of all proteins, domains A and C as defined by Drummond *et al.* (1986), was very poor: there were only seven identical amino acid residues and several gaps had to be introduced to reveal similarity. However, in the central domain (domain D) and in the C-terminal domain (domain E) extensive similarity was found. Overall, the NifA protein of *H. seropedicae* showed the highest degree of homology with that of *Azorhizobium caulinodans* (47%) and the lowest with that of *K. pneumoniae* (38%).

Finally, the *H. seropedicae* amino acid sequence also

contains a conserved interdomain, between domain D and domain E (Fig. 4), similar to that present in NifA proteins of *Rhizobium* species (Buikema *et al.*, 1985; Grönger *et al.*, 1987), *Bradyrhizobium japonicum* (Fischer *et al.*, 1988), *Azorhizobium caulinodans* (Nees *et al.*, 1988) and *Rhodobacter capsulatus* (Masepohl *et al.*, 1988), but which is absent from *K. pneumoniae* or *Azotobacter vinelandii* (Bennett *et al.*, 1988). Although these linking regions are not highly homologous, two cysteine residues, separated by four residues, are conserved and there are also several conservative substitutions.

Regulatory sequences

The promoter region of the *nifA*-like gene of *H. seropedicae* is complex. Three potential -24/-12 promoters occur at about 110 bp 5' from the translation initiation site: GGGCATGAAGTTTGCT, at position 615; CGGCGCTTTTCTGCC, at position 662; and CGGTAATTACACGGCG, at position 651. Of these three sequences, the first is the best match to the consensus sequence and the third is the poorest match and least likely to constitute a functional -24/-12 promoter (Fig. 3 and Fig. 5b). Furthermore, a putative NifA-binding site (TGTCGTAAATCCGACA, at

Fig. 4 (continued on the following page). Alignment of the deduced amino acid sequence from the *H. seropedicae* NifA-like protein relative to other homologous NifA proteins. HsNifA, *H. seropedicae* NifA; AcNifA, *Azorhizobium caulinodans* NifA; RINifA, *R. leguminosarum* NifA; RmNifA, *R. meliloti* NifA; and KpNifA, *K. pneumoniae* NifA. The consensus sequence shown required the simultaneous matching of amino acids in all five proteins. Conservative substitutions (Higgins & Sharp, 1988) are indicated by asterisks, and mismatches are indicated by dots. Domains A, C, D and E are boxed as originally proposed by Drummond *et al.* (1986), although the homology in the N-terminus shared among the five proteins is not sufficiently high to define domains A and C. The interdomain linker (I) is denoted by a horizontal bar and the conserved cysteine residues by the symbol +.

HsNifA
 AcNifA
 RINifA
 RmNifA
 KpNifA
 Consensus

INVVSIFIPPLRERREDIPYLVEHFLEKFRVENQRAMVAMSPQAMKVMNCYWPGENVREL
 IHVVPLILPPLRERPGDIPKLAKNFLDRFNKENKLHMM-LSAPAI DVLRRCYFPGENVREL
 INVVPIILPPLRQRDGDISLLAQVFLEQFNNANDRNC-DFGPSAIDILSKCAFPGENVREL
 ISGVPLILPPLRHRDGDIPLLARAFLORFNEENGRDL-HFAPSALDHLKCKFPGENVREL
 LNVMPIALPPLRERQEDIAELAHFLVRKIAHSQGR-LRISDGAIRLLMEYSWPGENVREL
 *.**.*PPLR*R.*DI*.L*.*.*.*.*.*.*.*.*.*.*PGENVREL

+ + +
 HsNifA
 AcNifA
 RINifA
 RmNifA
 KpNifA
 Consensus

ENCVERTATMMRGDLITEVHFSCQQNKCLTKVLHEPGQQQPVVV-----VPLERIS-AP
 ENGIRRTATLAHDAVITPHDFACDSGQCLSA MLWKGSA PKPVM PHVPPAPTPLTPLSPAP
 DNGVQRTATLASSNTITSSDFACQQDQCSSALLRKADGDIGNDAMN-GLNSRDTMSGGL
 ENCVRRATLARSKITSSDFACQTDQCFSSRLWKG VHGSHGHIEID-APAGTTPLL GAP
 ENGLERSAVLSESGLI-----
 NG.R*A**.*.*I.....

(I)

E
 HsNifA
 AcNifA
 RINifA
 RmNifA
 KpNifA
 Consensus

YGAIFAEDWGQGQATGAAPPT-----SERERLIWAMEQCGWVQAKAARALN
 LATAAPAAASPAPAADSLPVTCPGTEACPAVPPRQSEKEQLLQAMERSGWVQAKAARLLN
 CAHAGT-----PSGAAATIEAAGLTERDRLIKAMERAGWVQAKAARILG
 -ANDVP-----PKEPGSAGVASNLERDRLISALEEAGWNQAKAARILE
 -DRDVLFNHRDNPPKALASSGPAEDGW--LONSLEERQRLIAALEKAGWVQAKAARLLG
 *.*****E***L*.A*E..GW.QAKAAR.L*

HsNifA
 AcNifA
 RINifA
 RmNifA
 KpNifA
 Consensus

ISPRQMGYALQKFNIEVKKF
 LTPRQVG YALRKYDIDIKRF
 KTPRQVG YALRRHRIDVKKE
 KTPRQVG YALRRHGV DVRKL
 MTPRQVAYRIQIMDITMPRL
 *.PRQ**Y.*.*..***.*.

position 504) about 110 bp 5' from the $-24/-12$ motif at 615, suggests this region as a likely promoter (Fig. 3 and Fig. 5*b*). Another $-24/-12$ promoter sequence (GGGCTACCGGCTTGCT, at position 356) and a NtrC-binding site (GCACGCACGTGGTGCAC, at 257) are located further 5' to the initiation site (Fig. 3 and Fig. 5*a*).

The 'anaerobox' and the integration host factor (IHF) site

There are two additional features which may have a role in the control of the expression of *H. seropedicae nifA*. First, a potential 'anaerobox' sequence was identified at position 1538 (TTCATCAAGGTCAA), 3' from the translation initiation site. This structure has two mismatches (underlined) with respect to the 'anaerobox' consensus sequence (TTGATNNNNATCAA) (Nees *et al.*, 1988). A putative repressive function has been ascribed to an 'anaerobox' in a coding region (Nees *et al.*, 1988).

Secondly, starting 49 bp 5' from the putative NifA-binding site in the *H. seropedicae* *nifA*-like promoter

(position 455) is the sequence AATCAATGGGATAT which differs from the IHF consensus sequence (WATCAANNNTTTR, where W = A, T, and R = A or G) in only one conserved thymine residue (underlined) (Yang & Nash, 1989; Goodrich *et al.*, 1990). This factor has the ability to introduce bends in DNA (Yang & Nash, 1989), presumably to assist contact between upstream activators and the sigma factor and is normally located between the 5' activator binding site and the promoter.

The nifB-like sequence

The N-terminal of another ORF was found 3' from the *nifA* gene. The protein deduced from this sequenced region spans the first 162 amino acid residues, starting at position 2797 (Fig. 3). Hybridization studies had previously indicated the presence of sequences homologous to either *nifB* or *nifL* in this region of pEMS101 (Fig. 2). Comparison of the partial amino acid sequence, deduced from this ORF, with the NifB proteins from *Azotobacter vinelandii* (Joerger & Bishop,

(a) *NtrC*-activated promoters

	UAS	-24	-12
KpglnA	TGCACTATATTGGTGCA-----	TTGGCACAGATTTCGGT	
KpnifL	TGCACTACCGCGGCCCA-----	AGGGCGCAGGTTTGCA	
HsnifA	AGCAGGCACGTGGTGCA-----	TGGGCTACCGGCTTGCT	
Consensus†	GCACY-N ₅ -GGTGCA-----	NTGGCRGR-N ₄ -TTGC ^A _T	

(b) Putative *NifA* upstream activating sequence (UAS) in *nifA* promoters

	UAS	-24	-12
AcnifA	GGTTTCCATTATAACA----	AGGCTGATCCCTCGCA	
KpnifL‡		GGGCGCACGGTTTGCA----	TGT-N ₁₀ -ACA
HsnifA	TGTCGTAAATCCGACA----	GGGCATGAAGTTTGCT	
Consensus†	TGT - N ₁₀ - ACA----	TGGYAYR-N ₄ -TTGC ^A _T	

(c) Putative *NifA* upstream activating sequence (UAS) in *nifB* promoters

	UAS	UAS	-24	-12
KpnifB		TGTGAGATTCAGGACA----	CTGGTACAGCATTGCA	
RmnifB	TGTCGGAAGTCCGACA----	TGTGAGCAAAGAGACA----	TTGGCATAGCTGTTGCT	
AvnifB	TGTCGCTTGTCTGACAAAA	TGTGCGTTGCGCGACA----	TGGGCAGGGGTATTGCT	
HsnifB	TGTCGTGTTTATGACAAAA	TGTGCGCTATGCTCACA----	CTGGCACGGTTTTGGCT	
Consensus†	TGT - N ₁₀ - ACA----	TGT - N ₁₀ - ACA----	NTGGYAYR-N ₄ -TTGC ^A _T	

Fig. 5. Comparison of *H. seropedicae* *nifA* and *nifB* promoters with *NtrC*- and *NifA*-activating promoters of *K. pneumoniae* (Kp), *R. meliloti* (Rm), *Azotobacter vinelandii* (Av) and *Azorhizobium caulinodans* (Ac).

† Consensus sequences are according to Dixon (1988).

‡ The *NifA* binding site in the *nifL* promoter of *K. pneumoniae* is located downstream from the transcription initiation site (Drummond *et al.*, 1983).

HsNifB	MQPTQYVG IQD I KSLGTL LDKVAEHKGGTSSEGGKAS-CGSSDGP-ADMAPEVWEKVKN
BjNifB	MQ-----S I TEHKGCRA SAKTGRARL--RSQAGRGLPVEIWERVKN
AvNifB	ME-----LSVLGQNNGGQHSAGGSSSSCGSTHDQLSHLPENIRAKVQN
Consensus	M*.....*****G...S*.*.***.*****.*****V*N
HsNifB	HPCYSEEAHHHYARMHVAVAPACNIQCNYCNRYDCANESRPGVVSEKLTPEQA AKKVF A
BjNifB	HPCYSEDAHHHYARMHVAVAPACNIQCNYCNRYDCANESRPGVVSEKLTPEQAVRKV I A
AvNifB	HPCYSEEAHHYFARMHVAVAPACNIQCHYCNRYDCANESRPGVVSEVLTPAQAVKKVKA
Consensus	HPCYSE*AHH**ARMHVAVAPACNIQC*YCNRYDCANESRPGVVSE.LTPEQA**KV.A
HsNifB	VASTIPQMTVLG I AGPGDPLANPAKTFKTFELISQTAPDIKLCLS-----
BjNifB	VATTIPQMTVLG I AGPADALANPAKTFKTLALVTEAAPDIKLC LSTNGLALPDYVD I VR
AvNifB	VAAAI PQMSVLG I AGPGDPLANPKRTLDTFRMLSEQAPDMKLCVSTNGLALPECVEELAK
Consensus	VA**IPQM*VLG I AGP*D*LANP.*T**T*.****.APD*KLG*S.....

Fig. 6. Alignment of the deduced N-terminus of the NifB protein according to amino acid homology. HsNifB, *H. seropedicae* NifB; AvNifB, *Azotobacter vinelandii* NifB; and BjNifB, *B. japonicum* NifB. The consensus sequence shown required the simultaneous matching of amino acids in all three proteins. Conservative substitutions (Higgins & Sharp, 1988) are indicated by asterisks, and mismatches are indicated by dots. The conserved cysteine cluster is boxed.

1988) and *Bradyrhizobium japonicum* (Noti *et al.*, 1986) showed extensive homology (Fig. 6). A cluster of cysteine residues (cys-X₃-cys-X₂-cys-X₅-cys), characteristic of NifB proteins (Buikema *et al.*, 1987) was found between

residues 80 and 94, and most (approximately 90%) of the 20 amino acid residues spanning each side of this cluster were found to be conserved in the *H. seropedicae* NifB-like protein (Fig. 6). A possible -24/-12 pro-

moter and two potential NifA-binding sites (TGTCGTGTTCATGACA, at position 2620 and TGTCGCTATCCTCACA, at position 2661) were identified 5' from the initiation codon of the *H. seropedicae* *nifB*-like gene (Fig. 3 and Fig. 5c). A possible IHF-binding motif occurs at position 2708–2720 (Fig. 3).

Discussion

Our results show that *H. seropedicae* contains *nifA*- and *nifB*-like genes which code for proteins similar in structure to NifA and NifB proteins from other species. As expected, the NifA homology was confined to the C-terminal half of the proteins. The degree of homology of domains D (central domain) and E (DNA-binding site) of *H. seropedicae* was higher with the respective domains of *Azorhizobium caulinodans* and rhizobia, than with those of *H. seropedicae* and *K. pneumoniae*. Our results also indicate that the *nifA*-like gene of *H. seropedicae* may be regulated by NtrC, NifA and RpoN, as is *nifA* in *K. pneumoniae* (Merrick, 1983; Drummond *et al.*, 1983).

A conserved interdomain is present in the NifA proteins from *R. meliloti*, *B. japonicum*, *R. leguminosarum*, *Azorhizobium caulinodans*, *Rhod. capsulatus* and *H. seropedicae*, but is absent from those of *K. pneumoniae* and *Azotobacter vinelandii*. Two highly conserved cysteine residues are found in this region which, together with two other cysteine residues located at the end of the central domain (Fig. 4), have been proposed to be part of a metal-binding site which could act as a sensor for oxygen. Under low oxygen levels the bound metal would be reduced, thus stabilizing NifA in an active form, whereas at high oxygen concentrations the bound metal would be oxidized, destabilizing NifA and preventing activation of *nif* transcription (Fischer *et al.*, 1988). In *B. japonicum* the four cysteine residues are essential for NifA function and metal chelators prevent *B. japonicum* NifA from activating *nifD* transcription (Fisher *et al.*, 1988). Metal chelators have no effect on transcription activation by NifA of *K. pneumoniae*. Recently, it has been demonstrated that the NifA from *B. japonicum* is indeed sensitive to oxygen whereas *K. pneumoniae* NifA, which lacks this interdomain, is not (Fischer & Hennecke, 1987). The presence of this interdomain in the NifA-like protein of *H. seropedicae* suggests that its activity may be controlled by the prevailing oxygen levels.

The arrangement of a $-24/-12$ promoter element and a NifA-binding site about 100 bp 5' in the *nifA*-like gene of *H. seropedicae* is typical of *nif* promoters which are activated by NifA protein and require RpoN as

σ -factor (Gussin *et al.*, 1986; Dixon, 1988). Most promoters of *nifA* genes do not contain a prominent NifA upstream activating sequence. However, a potential NifA-binding site was found 5' from the *nifA* of *Azorhizobium caulinodans* (Nees *et al.*, 1988), where it may act as a negative autoregulator under non-symbiotic N_2 -fixing conditions (de Bruijn *et al.*, 1988). Furthermore, a putative NifA-binding site was also found in the intercistronic region of the *fixRnifA* operon from *B. japonicum*, which is autoactivated by the NifA protein (Thöny *et al.*, 1989). The presence of a $-24/-12$ promoter element and a correct spacing in respect to the upstream activating sequence, favour the idea that NifA may autogenously activate transcription of the *nifA* gene in *H. seropedicae*.

An NtrC-binding site and a $-24/-12$ promoter sequence are located 505 bp and 407 bp 5' from the putative initiation codon of the *H. seropedicae* *nifA* gene, suggesting a possible role for NtrC in *nifA* transcription.

The IHF-binding site has been reported in other *nif* promoters: it occurs in the *nifH* promoter region between the NifA-binding site and the promoter (Santero *et al.*, 1989) and the IHF protein enhances NifA-mediated expression of *nifH* *in vitro* (Hoover *et al.*, 1990). The *nifU* promoter, on the other hand, contains multiple NifA-binding sites, one of which abuts the IHF-binding site at which NifA and the IHF compete for binding, thus inhibiting *nifU* transcription (Cannon *et al.*, 1990). The proximity of the NifA-binding site to the IHF-binding site could lead to competition between NifA and IHF, affecting *nifA* expression in *H. seropedicae*.

The partial N-terminal amino acid sequence of the ORF 3' from *nifA* in *H. seropedicae* showed extensive homology with those of the NifB proteins from *Azotobacter vinelandii* and *B. japonicum*. The homology of the N-terminal of the putative NifB of *H. seropedicae* is closer to that of *B. japonicum* (68%) or *Azotobacter vinelandii* (66%) than to that of *K. pneumoniae* (53%) (not shown). The cysteine cluster, postulated to bind metal during the processing of FeMo-co (Buikema *et al.*, 1987), is conserved, as well as most of the amino acid residues surrounding it. A $-24/-12$ promoter sequence and two NifA-binding sites were identified in the *nifB* promoter region (Fig. 5c), suggesting that transcription of *nif* promoters in *H. seropedicae* could be regulated by the NifA protein and dependent on the σ^{54} factor (RpoN). The presence of a doublet of NifA-binding sites in the promoter region of *nifB*-like gene has been described before in *R. meliloti* (Buikema *et al.*, 1987) and *Azotobacter vinelandii* (Joerger & Bishop, 1988) and may be involved in transcriptional control.

This is the first *nifA*-like gene of a member of the Spirillaceae to be sequenced, as well as the first evidence supporting the existence of the *nifA* in the Spirillaceae

since the work of Pedrosa & Yates (1984). Our results indicate that expression of the *nifA* gene in *H. seropedicae* may be RpoN- and NtrC-dependent, and thus analogous to that of *K. pneumoniae*, but that it may also be regulated by the NifA protein. Whether this regulation, if it occurs, is negative or positive remains to be determined. We have found no evidence for a *nifL* type gene in *H. seropedicae* and it is possible that oxygen (and nitrogen) regulation of *nif* expression in this organism is mediated by the interdomain region of the NifA protein.

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