Identification of a NifL-like protein in a diazotroph of the β -subgroup of the *Proteobacteria*, *Azoarcus* sp. strain BH72

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NifA, the transcriptional activator of nitrogenase (*nif*) genes, has up to now been described to be regulated in its activity via the sensor NifL only for members of the γ -subgroup of the *Proteobacteria*. This paper reports a functionally similar NifL-like protein outside this group in *Azoarcus* sp. strain BH72, a diazotrophic grass endophyte belonging to the β -subgroup of the *Proteobacteria*. Its structural genes for nitrogenase (*nifHDK*) are regulated in response to combined nitrogen and O₂ and expressed endophytically inside rice roots. In order to characterize nitrogen-regulatory genes, an *Azoarcus* sp. BH72 genomic library was used to select cosmids that complemented a *nifA* mutation in *Azotobacter vinelandii*. Sequence analysis of the 3.4 kb genomic region complementing *nifA* showed two ORFs with sequence identities of 44% to NifL and 61% to NifA of *Azotobacter vinelandii*. According to Northern blot and reverse transcriptase PCR analysis, the *nifLA* transcript was more abundant at low combined nitrogen and O₂ levels, results which were corroborated by GUS (β -glucuronidase) assays using a transcriptional *nifL::gusA* fusion. N₂ fixation was abolished in a NifL[−] and a NifA[−] mutant, wild-type fixation being restored by *nifLA* *in trans*. The NifL[−] mutant also failed to activate *nifH::gus* expression, indicating that NifA is the obligate transcriptional activator for *nifHDK*. A *nifL* mutant was diazotrophic and did not show repression of *nifH::gusA* by ammonium or O₂, suggesting that NifL of *Azoarcus* sp. strain BH72 has a similar role in inactivating NifA in response to O₂ and combined nitrogen as NifL in bacteria of the γ -*Proteobacteria*.

Keywords: nitrogenase, gene expression, transcriptional activator, NifA, NifL

INTRODUCTION

Biological N₂ fixation is a highly energy-consuming process which is tightly controlled in free-living bacteria. Commonly, the structural genes of the nitrogenase complex, *nifHDK*, as well as other *nif* genes necessary for maturation and function of nitrogenase, are con-

trolled by the σ^{54} -dependent transcriptional activator NifA in diazotrophic proteobacteria. Its activity is affected by the cellular nitrogen status and the O₂ concentration; however, bacteria differ in the mechanisms mediating these responses.

In diazotrophs such as proteobacteria of the α -subgroup or *Herbaspirillum seropedicae* belonging to the β -subgroup (Souza *et al.*, 1991), the NifA proteins are apparently directly responsive to O₂. These proteins show a conserved cysteine motif in the central domain, not present in NifA proteins of the γ -*Proteobacteria*, which is probably the site of a redox-sensitive Fe–S cluster (Dixon, 1998; Fischer *et al.*, 1988). Diazotrophs belonging to the γ -*Proteobacteria* such as *Azotobacter vinelandii* and *Klebsiella pneumoniae* are characterized

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Abbreviation: GUS, β -glucuronidase.

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by the NifL/NifA two-component regulatory system, with NifL being the sensor inhibiting NifA activity in response to O₂ (Dixon, 1998). Stoichiometric amounts of both proteins are needed to ensure proper transcriptional regulation (Dixon, 1998; Govantes *et al.*, 1996). For O₂ sensing, the flavoprotein NifL inhibits NifA activity in the oxidized form (Dixon, 1998; Hill *et al.*, 1996).

The mechanism by which the cellular nitrogen status is sensed and the signal is transmitted is more complex and may vary considerably in different diazotrophs. One level of control is the transcriptional regulation of *nifA* itself, which may be nitrogen regulated via the two-component regulatory system NtrBC as in *K. pneumoniae* (Drummond *et al.*, 1983) or *H. seropedicae* (Souza *et al.*, 2000). At another level, the activity of NifA is modulated, P_{II}-like proteins being central signal-transmitter proteins. Depending on the internal nitrogen status of the cell, a bifunctional uridylyl-transferase/hydrolase covalently modifies or demodifies the P_{II}-like protein. Under conditions of nitrogen deficiency, the P_{II}-like proteins in enteric bacteria occur mainly in the uridylylated form. They act as molecular switches depending on their state of modification (for a review, see Arcondéguy *et al.*, 2001). Most proteobacteria harbour two paralogous gene copies of P_{II}-like proteins (*glnB/glnK*) (Ninfa & Atkinson, 2000). In *H. seropedicae* and *Azospirillum brasilense*, GlnB is required to maintain the active form of NifA, either by direct interaction or by involvement of an as yet unknown protein (Arsène *et al.*, 1996, 1999; Souza *et al.*, 1999). In γ -Proteobacteria, NifA activity is mediated by NifL in response to combined nitrogen. Under nitrogen-limiting conditions, GlnK is required to relieve the inhibitory effect of NifL on NifA in *K. pneumoniae* (He *et al.*, 1998; Jack *et al.*, 1999). Interaction of P_{II}-like proteins of *Escherichia coli* with *Azotobacter vinelandii* NifL was demonstrated in an *in vitro* system; however, *E. coli* GlnB (and *Azotobacter vinelandii* P_{II}) rather than GlnK stimulated the inhibitory function of NifL in the non-uridylylated form (Little *et al.*, 2000).

Azoarcus sp. strain BH72 is an endophyte of grasses (Hurek *et al.*, 1994b) which belongs to the β -Proteobacteria (Reinhold-Hurek *et al.*, 1993b). N₂ fixation and *nifHDK* transcription occur only under microaerobic and nitrogen-limiting conditions (Egner *et al.*, 1999; Huwrek *et al.*, 1987). This diazotroph shows the capacity of endophytic N₂ fixation: the structural genes of nitrogenase *nifHDK* were found to be expressed and translated in the aerenchyma of rice seedlings (Egner *et al.*, 1999). Under certain culture conditions including very low O₂ concentrations, the cells can shift into a state of very high and efficient N₂ fixation called hyperinduction (Hurek *et al.*, 1994a), forming novel intracytoplasmic membrane stacks (diazosomes) with which the iron protein of nitrogenase is associated (Hurek *et al.*, 1995). Therefore, we are interested in unravelling the signal transduction cascade for *nif* gene regulation of strain BH72. We report here the occurrence

of a functionally similar NifL-like protein outside the γ -Proteobacteria.

METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. Additional subclones are shown in Fig. 1. *Azotobacter vinelandii* strains were kindly provided by C. Kennedy, University of Arizona, Tuscon.

Culture media and growth conditions. Unless stated otherwise, *Azoarcus* sp. BH72 was grown at 37 °C in VM medium supplemented with ethanol (Reinhold-Hurek *et al.*, 1993a, b). N₂-fixing cells were grown on N-free SM medium (Reinhold *et al.*, 1985) under microaerobic conditions in Erlenmeyer flasks (Karg & Reinhold-Hurek, 1996). To study the effect of O₂ on gene expression, cells precultured on SM medium with combined nitrogen (0.05 % NH₄Cl, 0.01 % yeast extract) were washed in N-free medium and incubated under different O₂ concentrations at an OD₅₇₈ of 0.2 as described previously (Egner *et al.*, 1999). To test the influence of ammonium on nitrogenase activity and *nif* gene expression, the following modifications were applied: cells were incubated aerobically at OD₅₇₈ 0.5 in N-free medium for 1 h followed by an incubation for 3 h under a headspace of 1 % O₂ and 10 % acetylene in N₂. Before cells were harvested to measure GUS activity (see below), the ethylene formed was measured gas chromatographically. For strain BHNLK the nitrogen-starvation phase was omitted but the incubation time was prolonged to 4 h.

Gas chromatography. To determine O₂ concentrations and ethylene formation, an HRGC-4000A (Konik, Barcelona, Spain) gas chromatograph was used as described previously (Egner *et al.*, 1999).

Techniques for DNA and RNA manipulation. DNA and RNA analysis was carried out according to standard procedures (Ausubel *et al.*, 1987; Hurek *et al.*, 1993; Reinhold-Hurek *et al.*, 1993a). Homologous DNA gene probes for Southern and Northern blot analysis were digoxigenin (DIG)-labelled in a PCR reaction using the DIG Labelling and Detection Kit (Boehringer Mannheim). The *nifA* probe was amplified with T3/T7 primers using a 0.5 kb *Pst*I subclone (pP05) of pTEA24 as template. Primers for *nifH* were TH25/TH26 (Hurek *et al.*, 1997b), and for 16S rDNA TH3/TH5 (Hurek *et al.*, 1993). RNA was isolated from exponentially growing cells, and Northern blot analysis carried out as previously described (Reinhold-Hurek *et al.*, 1993a). Reverse transcription PCR (RT-PCR) was carried out on RNA extracted by peqGOLD TriFast (peqLab) (Egner *et al.*, 2001), with 1 µg RNA using primer *nifA*rev3RT (TCGTCCAGGTGCTCGCGCTG) and Ready-to-go-beads (AmershamPharmacia Biotech) for reverse transcription at 42 °C for 30 min. Amplification of *nifA* cDNA was carried out using primers *nifA*for1RT (ATGAGCGCGCCGGTCCGATG) and *nifA*rev2RT (CACGGTTTCGTGCCCGGCGCG) for 18–28 cycles of 1 min 95 °C, 1 min 65 °C, 1 min 72 °C; samples were taken after different cycle numbers. For RT-PCR amplification of *nifLA*, primers RTnifLAfor (GAGAACGGCCAGGTGACGTGGA, positions 1772–1792) and RTnifArev (GTTGAAGCCGCACTCCTCGTCCGAGCA, positions 2194–2170) were used for 35 cycles of 95 °C for 1 min, 62 °C for 1 min and 72 °C for 1 min. Products were separated on 1.2 % agarose gels. RT-PCR of 16S rRNA was carried out from 10 ng RNA with primers 1401rev (CGGTGTGTACAAGACCC) for reverse

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
<i>Azoarcus</i> sp.		
BH72	Wild-type	Reinhold <i>et al.</i> (1986)
BHLAO	Sp ^r /Sm ^r , BH72 <i>nifL</i> ::Ω	This study
BHNLK	Km ^r , <i>nifL</i> :: <i>aphII</i>	This study
BHLAONG	Sp ^r /Sm ^r Ap ^r , BHLAO::pEGN3.1, chromosomally integrated transcriptional <i>nifH</i> :: <i>gusA</i> fusion	This study
BHNLKnifH::gusA	Km ^r Ap ^r , BHNLK::pEGN3.1, chromosomally integrated transcriptional <i>nifH</i> :: <i>gusA</i> fusion	This study
<i>Azotobacter vinelandii</i>		
UW1	<i>nifA</i>	Shah <i>et al.</i> (1973)
<i>E. coli</i>		
DH5αF'	F' <i>endA1 hsdR17</i> (r _k ⁻ m _k ⁻) <i>supE44 thi-1 recA1 gyrA relA1</i> φ80lacZΔM15 Δ(<i>lacZYA-argF</i>)U169	Hanahan (1983)
Plasmids		
pUC18/19	Ap ^r , ColE1 replicon	Yanisch-Perron <i>et al.</i> (1985)
pBSKII/pBKSII	Ap ^r , ColE1 replicon	Stratagene
pHP45Ω	Sp/Sm ^r , Sp/Sm-resistance cartridge	Prentki & Krisch (1984)
pUC4K	Ap ^r Km ^r , Km-resistance cartridge	Stratagene
pLAFR3	Tet ^r , cosmid vector	Staskawicz <i>et al.</i> (1987)
pTEA1	Tet ^r , cosmid from genomic library (<i>nifA</i>)	This study
pTEA2	Tet ^r , cosmid from genomic library (<i>nifA</i>)	This study
pLAFR24	Tet ^r , 9 kb <i>EcoRI</i> / <i>HindIII</i> subclone from pTEA2 in pLAFR3	This study
pTEA24	Ap ^r , 9 kb <i>EcoRI</i> / <i>HindIII</i> fragment from pLAFR24 in pBSKII	This study
pTEA24Ω	Ap ^r Sp/Sm ^r , pTEA24 with insertion of Sp/Sm ^r cartridge of pHP45Ω in <i>EcoRV</i> site	This study
pTEA24Km	Ap ^r Km ^r , pTEA24 with insertion of Km cartridge of pUC4K in <i>EcoRV</i> site	This study
pEGN3.1	Ap ^r , transcriptional <i>nifH</i> :: <i>gusA</i> fusion of <i>Azoarcus</i> sp. BH72 <i>nifH</i> on pUC18	Egener <i>et al.</i> (1999)
pGusKS	Ap ^r , <i>gusA</i> on <i>EcoRI</i> fragment from pEGN3.1 in pBKSII	This study
pLAFRL	Tet ^r , <i>HindIII</i> / <i>BsrBI</i> fragment of <i>nifL</i> and upstream region in pLAFR3	This study
pLGus	Tet ^r , <i>nifL</i> :: <i>gusA</i> fusion in pLAFR3	This study

Fig. 1. Restriction map and subclones of pTEA24. The position of the ORFs are indicated. pTEA24 is a *HindIII*/*EcoRI* subclone in pBSKII of pTE2 derived from the cosmid library. Depicted below are subclones of 0.5–2 kb inserts generated by *Pst*I, *Xho*I and *Sal*I and cloned into pBluescript SK. Sites for important and cloning-relevant sites are shown. A, *Apal*; E, *EcoRI*; EV, *EcoRV*; HIII, *HindIII*; P, *Pst*I; K, *Kpn*I; S, *Sal*I; X, *Xho*I.

transcription and 104f (GGCGAACGGGTGMGTAAAYGCACTGG) and 1346rev (TAGCGATTCCGACTTCA) for PCR amplification: 1 min 95 °C, 2 min 65 °C, 2 min 72 °C.

For primer extension analysis (Egener *et al.*, 2001), 15 µg RNA extracted with the peqGOLD TriFast Kit (peqLab) was used as starting template, along with the Cy5 labelled primer (Cy5PEnifLArev: GATCGCCGACTGCTCCACGG). The reaction mix was denatured at 72 °C for 3 min followed by gradual cooling to 42 °C, then the dNTP mix and AMV reverse transcriptase were added and incubated for 30 min. The reaction mix was then extracted with phenol/chloroform followed by ethanol precipitation of the single-stranded cDNA. The product was dissolved in TE (10 mM Tris, 1 mM EDTA) and analysed on an automated sequencer (ALFexpress; AmershamPharmacia Biotech) in parallel with a sequencing reaction carried out with the same primer and plasmid template pS08.

DNA sequencing and computational analysis. Plasmid DNA was sequenced with Cy5 labelled primers (T3 and T7) using an automated sequencer (ALFexpress, AmershamPharmacia Biotech) as described by Hurek *et al.* (1997a). Sequences determined for both strands were aligned using the DNASTar

software and compared to databases using BLAST (Altschul *et al.*, 1990). Domain searches were carried out with the SMART (Schultz *et al.*, 1998) and Pfam (Sonnhammer *et al.*, 1997) programs. The sequence of *nifL*A was submitted to GenBank (accession no. AF518560).

Construction of a cosmid library of *Azoarcus* sp. BH72 and triparental mating. Genomic DNA of *Azoarcus* sp. BH72 was partially digested with *Sau*3AI and cloned into the *Bam*HI site of the cosmid vector pLAFR3 (Staskawicz *et al.*, 1987). The ligation mix was packaged into lambda phages using the DNA Packaging Kit (Boehringer Mannheim) and transfected into *E. coli* DH5 α . A total of 1440 colonies were picked and stored as glycerol stocks at -80°C . Triparental mating in *Azotobacter vinelandii* was carried out according to Page & Sadoff (1976) using a helper plasmid [*E. coli*(pRK2013)] with a donor:helper:recipient ratio of 1:1:100. When the donor carried different inserts (i.e. a library) the ratio was altered to 5:1:100. The selection medium, containing tetracycline ($12\text{ }\mu\text{g ml}^{-1}$), was N-free BS medium (Newton *et al.*, 1953) for *Azotobacter vinelandii* UW1. For conjugative plasmid transfer into *Azoarcus* sp., recipients were grown in liquid VM medium, mixed in a ratio of 1:1:500 and spread as a thin liquid layer on KON agar plates (SM medium supplemented with 5 g yeast extract l^{-1} and 1 g NaCl l^{-1}). After 5–7 h incubation at 37°C , cells were scraped off and plated in serial dilutions on SEL medium (SM medium containing 6 ml ethanol l^{-1} instead of potassium malate and 1 g $\text{KNO}_3\text{ l}^{-1}$ as sole nitrogen source) with $12\text{ }\mu\text{g}$ tetracycline ml^{-1} .

Construction of *Azoarcus* sp. BH72 NifLA mutants and plasmids. A *nifL*A knock-out mutant was constructed by interrupting the ORF of *nifL* by insertion of a Sp/Sm-resistance cartridge excised by *Sma*I from pHP45Q (Prentki & Krisch, 1984) into the *Eco*RV site (Fig. 1) of pTEA24, resulting in pTEA24Q. pTEA24Q was introduced into *Azoarcus* sp. BH72 by electroporation, yielding double recombinants that had exchanged the interrupted allele with the wild-type allele. In Southern blot analysis, the wild-type showed hybridization of a 14 kb fragment of *Kpn*I-digested genomic DNA with a probe against *nifA*, while the *nifL*A mutant, named BHLAO, showed a shift of 2 kb to 16 kb corresponding to the inserted cassette (data not shown). For a NifA $^{-}$ phenotype, strain BHLAO was complemented with pLAFRL, carrying the *nifL* gene including upstream sequences. pLAFRL was constructed by excising a 2 kb *Hind*III/*Bsr*BI fragment from pTEA24, and cloning it into *Hind*III/*Eco*RV of pBKSII. From this vector the fragment was excised using *Hind*III/*Eco*RI and cloned into pLAFR3, yielding pLAFRL. This plasmid was introduced into *Azoarcus* BHLAO by triparental mating and the resulting strain named BHLAO(pLAFRL). The *nifL* mutant (BHNLK) was constructed by insertion of a non-polar Km-resistance (*aph*II) cartridge from pUC4K (AmershamPharmacia Biotech) into the *nifL* gene. The cartridge was excised by *Sal*I, the overhanging ends blunted using T4 DNA polymerase, and the fragment inserted into the *Eco*RV site of *nifL* on pTEA24, yielding pTE24Km. Integration in the correct orientation was confirmed, and the construct was used for electroporation into *Azoarcus* sp. BH72. A double recombinant (*nifL::aph*II) confirmed by Southern hybridization (see above) was named BHNLK. For expression studies of *nifL*A, *gusA* was transcriptionally fused to the *nifL* gene by cloning a 1.3 kb *Pst*I fragment from pP13 into the *Pst*I site of pGusKS. This *nifL::gusA* fusion was excised by *Hind*III and partial digestion with *Bam*HI and inserted into pLAFR3, yielding pLGus.

β -Glucuronidase (GUS) assay. GUS assays were carried out according to Jefferson *et al.* (1986). Glucuronidase activity

was calculated as $(A_{420} \times 1000)/(t \text{ (min)} \times \text{OD}_{600}) = \text{Miller units}$.

RESULTS AND DISCUSSION

Azoarcus sp. BH72 genomic library clones can complement a *nifA* mutation in *Azotobacter vinelandii* and contain genes encoding a NifL and a NifA homologue

In an attempt to find genes homologous to *nifA* by heterologous complementation of a *nifA* mutant of *Azotobacter vinelandii*, a genomic library of *Azoarcus* sp. strain BH72 was constructed in the cosmid vector pLAFR3 (Tet r); 1440 Tet r clones of *E. coli* DH5 α were used as cosmid library (insert size approx. 20 kb). After plasmid transfer by triparental mating into *Azotobacter vinelandii* mutant strain UW1, Nif $^{+}$ Tet r colonies were selected on N-free BS medium. Out of 10 colonies tested, two types of cosmids with similar restriction fragments were isolated. The cosmids pTEA1 (16 kb insert) and pTEA2 (11 kb insert) allowed N $_2$ fixation of mutant UW1, as did a subcloned 9 kb *Eco*RI/*Hind*III fragment (pLAFR24). Sequence analysis of subclones (Fig. 1) revealed two putative ORFs; the deduced amino acid sequence of the first ORF showed 44% and 28% identity to the *Azotobacter vinelandii* and *K. pneumoniae* NifL protein, respectively, that of the second ORF showed 61% and 54% identity to *Azotobacter vinelandii* and *K. pneumoniae* NifA, respectively. The N-terminal 'input domains' of the few known NifL sequences show a significant similarity (amino acid positions 14–130, 67% and 65% with *Azotobacter vinelandii* and *K. pneumoniae*, respectively). A PAS domain was detected in this region with statistical significance in SMART ($E = 1.7 \times 10^{-4}$, amino acids 14–84) or PFAM ($E = 5.4 \times 10^{-7}$, amino acids 14–56), as well as an adjacent PAC domain (SMART: amino acids 86–130, $E = 2.1 \times 10^{-1}$; PFAM: amino acids 86–130, $E = 7.4 \times 10^{-4}$) which is proposed to contribute to the PAS domain fold. PAS domains are found in a large family of sensory proteins from all kingdoms including *Archaea* (Zhulin *et al.*, 1997). They are proposed to be the site of FAD-cofactor binding in NifL and may therefore be involved in O $_2$ sensing (Dixon, 1998). The C-terminal part of *Azoarcus* NifL contains five conserved regions in common with other members of the histidine protein kinase family (termed H, N, G1, F and G2 domains). These regions are also present in the *Azotobacter vinelandii* sequence but are missing (except for G2) in the enterobacterial counterparts. Moreover, *Azotobacter vinelandii* NifL shows the highly conserved histidine residue of orthodox histidine autokinase transmitter domains (Parkinson & Kofoid, 1992) at position 304, which is missing in the *Azoarcus* and the enterobacterial sequences. However, mutational analysis of *Azotobacter vinelandii* NifL revealed that phosphorylation of His304 is not essential for any of the known functions of NifL (Woodley & Drummond, 1994). Moreover, all attempts to demonstrate NifL autophosphorylation or phosphotransfer to NifA *in vitro* failed (Austin *et al.*, 1994), suggesting that NifL–NifA-mediated signal transduction differs from the

Fig. 2. Analysis of *Azoarcus* sp. BH72 *nifLA* transcription. (a) Sequence of the putative promoter region. Putative binding motifs for transcriptional activators (NifA dotted, NtrC dashed), RBS and start codon are depicted. Asterisks mark the bases that are conserved in the promoter. (b) Primer extension analysis localizing the transcriptional start at minute 145:16, corresponding to nucleotide 453 (marked with an arrow). Top, sequencing reaction; bottom, primer extension. (c) Northern blot analysis of 12 µg RNA extracted from cells of *Azoarcus* sp. BH72 grown aerobically on combined nitrogen (+) or diazotrophically (-). A digoxigenin-labelled probe directed against the *nifA* gene was used which had been amplified with T3/T7 primers from a 0.5 kb *Pst*I subclone (pP05) of pTEA24 as

classical two-component system paradigm (Dixon, 1998).

In *Azoarcus* NifA, the domain putatively interacting with σ^{54} and the DNA-binding domains showed a much higher similarity to other NifA proteins than the N-terminal regulatory (or receiver) domains, which are highly variable, possibly indicating various modes of signal transduction in different bacteria. NifA proteins occurring outside the γ -*Proteobacteria* reveal a characteristic motif of conserved cysteine residues located between the central catalytic domain and the C-terminal DNA-binding domain (Fischer *et al.*, 1988) which leads to O₂ sensitivity of the protein. This motif is absent in the *Azoarcus* sequence, which further supports the notion that NifA proteins which operate in concert with NifL proteins are not intrinsically O₂ sensitive (Dixon, 1998).

Expression of *nifLA* is affected by O₂ and ammonium

The ORFs of *nifL* and *nifA* of strain BH72 were closely adjacent (intergenic region 78 bp) (Fig. 1). Only upstream of *nifL* were motifs characteristic of σ^{54} -dependent promoters (Fig. 2a) corresponding well to the consensus (Merrick, 1992). Primer extension analysis with an RNA preparation of an N₂-fixing culture of strain BH72 corroborated the transcriptional start site. Using plasmid pS08 as a template for the parallel sequencing reaction, the transcriptional start was localized at minute 145:16, corresponding to the nucleotide marked by an arrow in Fig. 2(a, b) (minute 145:11), which was at position +1 with respect to the -12/-24 motif of the σ^{54} promoter. Further upstream, motifs similar to the consensus of the NtrC-binding site (GCAC-N₇-GTGC) and a motif identical to the consensus of the NifA-binding site (TGT-N₁₀-ACA) were found (Fig. 2a). However, data on *nifLA* expression in a NifLA⁻ mutant (see below) suggest that NifA is not involved in the transcriptional control.

Northern blot analysis using RNA extracts of strain

template. The arrow indicates a transcript of approximately 3.4 kb. (d) Analysis of *nifLA* cotranscription by RT-PCR. Primers RTnifLAfor and RTnifArev, annealing to *nifL* or *nifA*, respectively, were used for RT-PCR with 1 µg RNA isolated from N₂-fixing cells. Lane 1, size marker (*Pst*I-digested λ DNA); lane 2, no RNA added; lane 3, reverse transcriptase inactivated (95 °C, 5 min) prior to addition of RNA; lane 4, RNA added without heat inactivation; lane 5, 30 ng chromosomal DNA of strain BH72 as template. (e) Top, analysis of *nifA* expression by RT-PCR. One microgram of RNA isolated from wild-type BH72 cells grown under ammonium excess (+N) or on N₂, and from the *nifL*:: Ω mutant BHLAO grown on ammonium, was used for RT-PCR with primers specific for *nifA* (nifArev3RT, nifAfor1RT, nifArev2RT). Samples were taken after 18 (lane 1), 23 (lane 2) and 28 (lanes 3 and 4) cycles; lane 4, reverse transcriptase heat inactivated as in (d); lane 5, DNA size marker. Products of the expected size (421 bp for *nifA* and 1242 bp for 16S rRNA) were separated on 1.2% agarose gels and visualized by ethidium bromide staining. Bottom, as a quality control of the RNA, the same RNA preparations were used for 16S rRNA-directed RT-PCR using primers 1401rev, 104f and 1346rev with 10 ng RNA as template; lanes are numbered as above.

Fig. 3. Expression of *nifLA* under various growth conditions: GUS activity of a *nifL::gusA* fusion in *Azoarcus* sp. Black columns, wild-type BH72(pLGus); white columns, *nifL::Ω* mutant BHLAO(pLGus). Cells were analysed after 6 h incubation aerobically in complex (VM, 0.1% NH_4Cl and yeast extract, 0.3% peptone) (1) or minimal medium (SM+N containing 0.05% NH_4Cl , 0.01% yeast extract) (2), microaerobically in minimal medium (3), aerobically in nitrogen-free minimal medium (SM) (4) and microaerobically in N-free minimal medium (5). Error bars show the standard deviation of three independent experiments with 3–5 replicates.

Fig. 4. Role of *Azoarcus* sp. *nifLA* for growth on N_2 . Growth on N_2 of wild-type *Azoarcus* sp. BH72 (■), of wild-type carrying the vector control [BH72(pLAFR3), ▼], of the *nifL::Ω* mutant BHLAO (□) and of BHLAO complemented with *nifLA* [BHLAO(pTEA2), ○]. Error bars show the standard deviations from two replicates in three independent experiments.

BH72 revealed only one transcript, approximately 3.4 kb in size, when hybridized with a *nifA* probe of strain BH72, corresponding to the approximate length of the *nifLA* coding sequence (Fig. 2c). Cotranscription of *nifL* and *nifA* was confirmed by RT-PCR using a primer annealing to *nifL* and a reverse primer annealing to *nifA* (Fig. 2d): RT-PCR using DNase-treated RNA of strain BH72 revealed an amplification product of the expected size (422 bp, lane 4) which was obtained also by PCR using chromosomal DNA as a template (lane 5), but not in an RNA-free control (lane 2) or after heat-inactivation of reverse transcriptase (lane 3). In the *nifL::Ω* insertional mutant BHLAO (see below), transcription of *nifA* was no longer detectable by RT-PCR (Fig. 2e), demonstrating a polar effect on *nifA* transcription. Cotranscription of *nifLA* was also found in *Enterobacter agglomerans* (Siddavattam *et al.*, 1995), *Azotobacter vinelandii* and *K. pneumoniae* (Dixon, 1998). In contrast, in the β -subgroup member *H. seropedicae* *nifA* is not preceded by *nifL*, is followed by *nifB*, and is

controlled by its own promoter (Souza *et al.*, 1991). The latter gene organization is more similar to the organization in α -Proteobacteria such as *Azospirillum* (Liang *et al.*, 1991).

The *nifLA* transcript was detectable in aerobically grown cells on combined nitrogen, but was more abundant during N_2 fixation (Fig. 2c). This differential expression was confirmed by RT-PCR. RT-PCR amplification products of *nifA* were more abundant in RNA extracts from N_2 -fixing cells than in extracts from ammonium-grown cells (Fig. 2e). The use of equal amounts of RNA in both extracts was confirmed by RT-PCR using primers for 16S rRNA (Fig. 2e).

To quantify the expression of the *nifLA* operon, a transcriptional *nifL::gusA* fusion was constructed on the broad-host-range vector pLAFR3 (pLGus) which was conjugated into *Azoarcus* strains. GUS activity of this fusion was tested in the wild-type background as well as the *NifLA*[−] background of BHLAO (see below) under various growth conditions 2, 4, 6, 8 and 16 h after incubation (data not shown). Maximum GUS activity was observed at 6 h. During aerobic incubation in both complex (VM) and minimal (SM) medium containing combined nitrogen (including 0.05% NH_4Cl) the *nifLA* operon was expressed (Fig. 3, bars 1 and 2), in accordance with the observed expression of *nifLA* in the presence of combined nitrogen in Northern blot experiments. O_2 limitation increased the expression threefold (Fig. 3, bar 3), while nitrogen limitation (absence of combined nitrogen in N-free SM medium) led to a fivefold increase (Fig. 3, bar 4). Under culture conditions favourable for N_2 fixation (1% O_2 , no combined nitrogen), a six- to sevenfold increase was measured (Fig. 3, bar 5). The expression level (except for N_2 -fixing conditions) was equal for both genetic backgrounds, suggesting that autoregulation of *nifLA* does not occur in *Azoarcus* sp. BH72. Surprisingly, under N_2 -fixing conditions the *nifLA* mutant BHLAO reached lower expression levels than the wild-type, which might in part be due to severe growth limitations (the mutant stopped growing after 2–3 h of incubation and is not able to grow on N_2 at all; see below).

While in *Azotobacter vinelandii* transcription of *nifLA* is not repressed by ammonium and is not dependent on NtrC or RpoN (Blanco *et al.*, 1993), it is NtrC-dependent in *K. pneumoniae* (Minchin *et al.*, 1988) and *Enterobacter agglomerans* (Siddavattam *et al.*, 1995). In *Klebsiella* and *Enterobacter cloacae* expression is also strongly enhanced (e.g. approx. tenfold) under anaerobic conditions, mediated by negative supercoiling depending on a DNA gyrase (Dixon *et al.*, 1988; Hu *et al.*, 2000). In *E. cloacae*, FNR is additionally involved in this regulation (Hu *et al.*, 2000). *Azoarcus* sp. BH72 appears to be slightly different but shows some similarities to *K. pneumoniae* in *nifLA* regulation, since the expression is enhanced both by ammonium deficiency and by microaerobiosis (without an obvious involvement of an FNR box). However, the expression level increased only moderately (three- to fivefold) in strain BH72. Strain BH72 showed no evidence for autoregulation.

Fig. 5. Nitrogenase gene expression and acetylene reduction of a *nifL* mutant. (a) Top, comparison of *nifA* mRNA abundance by RT-PCR (*nifA*) in wild-type BH72 and the non-polar *nifL::aphII* mutant BHLNK grown on ammonium under microaerobic conditions. Reverse transcription was carried out on 1 µg RNA, followed by PCR with 18–26 cycles; samples were taken after 18 (lane 1), 20 (lane 2), 22 (lane 3), 24 (lane 4) and 26 (lane 5) PCR cycles; lane 6, control reaction (26 cycles) without reverse transcription (heat inactivation). Products of the expected size (421 bp for *nifA* and 1242 bp for 16S rRNA) were separated on 1.2% agarose gels and visualized by ethidium bromide staining. Bottom, as a control, 16S rRNA-specific RT-PCR was carried out on 10 ng RNA. Lanes as for *nifA*, except for lane 6 (28 cycles) and lane 7 (control reaction at 28 cycles without reverse transcription). Middle lane, *Pst*I-digested lambda DNA as size marker. All RT-PCR reactions were independently performed

lation of *nifA* expression by NifA in a NifLA[−] mutant, although putative NifA-binding sites are present. This was also reported for *H. seropedicae*, whose expression through a σ^{54} -dependent promoter is repressed by nitrogen but not by O₂ (Souza *et al.*, 2000).

The *nifA* gene product is essential for N₂ fixation of *Azoarcus* sp. strain BH72

To confirm the function of NifLA, a *nifL::Ω* strain (BHLAO) was constructed by marker-exchange mutagenesis, carrying a polar mutation in the *nifL* gene by insertion of a Sp/Sm-cartridge which also abolished *nifA* transcription (see above). While growth rates under aerobic conditions in both full (VM) and minimal (SM) medium were not affected in the mutant (not shown), BHLAO was not able to grow on N₂ in the absence of combined nitrogen under microaerobic conditions (Fig. 4). This inability to fix N₂ was restored when pTEA2 provided the *nifLA* operon *in trans* in the mutant BHLAO(pTEA2). Growth of the complemented mutant was comparable to that of the wild-type and a control strain carrying a pLAFR3 vector devoid of insert (Fig. 4). This suggested that a product of the *nifLA* operon was essential for N₂ fixation in *Azoarcus* sp. strain BH72. A transcriptional fusion of *Azoarcus* sp. *nifH* with *gusA* (pEGN3.1; Egner *et al.*, 1999) was integrated into the chromosome of mutant BHLAO by single recombination, resulting in strain BHLAONG. In comparison to the wild-type BHNG3.1 (Egner *et al.*, 1999), transcriptional activation of the *nifH::gusA* fusion under conditions for N₂ fixation was not observed in BHLAONG (745 ± 69 or 20 ± 9 Miller units, respectively), implying that the *nifLA* operon encodes the transcriptional activator of *nifHDK* genes in *Azoarcus* sp. BH72. To obtain a NifA[−] phenotype, the double mutant BHLAO was complemented with a 2 kb *Hind*III/*Bsr*I fragment of pTEA24 carrying only *nifL* including upstream regions (plasmid pLAFRL). This strain BHLAO(pLAFRL) was not capable of N₂ fixation (data not shown), similar to mutant BHLAO, indicating that NifA and not NifL was essential for diazotrophy as a transcriptional activator. These results also confirmed that *nifLA* are cotranscribed in strain BH72.

NifL is required for control of *nifH* expression in response to O₂ and combined nitrogen

To construct a *nifL* mutant, a non-polar Km-resistance cartridge was inserted into the *Eco*RV site disrupting the *nifL* gene, resulting in mutant strain BHLNK after allelic exchange. This mutant strain had similar levels of *nifA* mRNA expression as the wild-type when grown microaerobically on SM medium supplemented with 10 mM

three times and gave consistently similar results. (b) Expression of a *nifH::gusA* fusion in the non-polar *nifL* mutant BHLNK*nifH::gusA* in response to O₂ concentrations on N-free medium; (c, d) acetylene reduction (c) and GUS activity (d) in response to NH₄Cl concentration under microaerobic conditions (headspace 1% O₂). Error bars indicate the standard deviation from three replicates in two independent experiments.

NH₄Cl, as shown by RT-PCR (Fig. 5a). Thus, insertion of the Km-resistance cartridge did not lead to unusually high *nifA* mRNA levels under repressing conditions.

To study the influence of NifL on *nif* gene expression, a *nifH::gusA* reporter gene fusion (Egner *et al.*, 1999) was additionally introduced into the *nifL* mutant by single homologous recombination of pEGN3.1, resulting in strain BHNLK*nifH::gusA*. Wild-type *Azoarcus* sp. strain BH72 showed an optimal *nifH::gusA* expression under microaerobic conditions of 1% O₂ in the headspace, expression being completely abolished at 4% O₂ (reduction of GUS expression by a factor of 40) (Egner *et al.*, 1999). In contrast, the *nifL* mutant did not show this response but expressed *nifH* to similar levels from 1% to 8% O₂ in the headspace (Fig. 5b), indicating that expression was not modulated in response to different O₂ levels in the absence of NifL. The influence of ammonium on *nifH* gene expression and nitrogenase activity was measured in BHNLK*nifH::gusA* incubated microaerobically with different NH₄Cl concentrations. The activity of nitrogenase decreased at ammonium concentrations above 0.5 mM in the *nifL* mutant (Fig. 5c) as in the wild-type. This was probably due to a rapid inactivation ('switch-off') of nitrogenase by ammonium, which has recently been demonstrated for strain BH72 (Egner *et al.*, 2001). In contrast, the *nifH* gene expression remained at a high level and even showed a significant increase with rising ammonium concentrations (Fig. 5d), while in the wild-type strain *nifH::gusA* expression is abolished at 0.5 mM NH₄Cl (reduction by a factor of approx. 40-fold) (Egner *et al.*, 1999). This increase might be due to a more rapid growth of the strain in the presence of ammonium. Thus, the NifA protein alone was not inactivated by ammonium in the absence of NifL. It is unlikely that differences in *nifA* transcription accounted for the observed derepression of *nifH* under permissive conditions: expression levels of *nifA* under non-permissive conditions are relatively high (albeit elevated under nitrogen-limitation) in the wild-type, and no change of *nifA* expression was detected in the nonpolar *nifL* mutant. Nitrogen-responsiveness of the NifLA regulatory system in *Azoarcus* sp. might be mediated by P_{II}-like proteins as postulated for members of the γ -Proteobacteria (He *et al.*, 1998; Jack *et al.*, 1999; Little *et al.*, 2000) (unpublished observations). Unlike in most proteobacteria, three instead of two paralogues of P_{II}-like proteins were found in *Azoarcus* sp. BH72 (Martin *et al.*, 2000).

Concluding remarks

Our results have demonstrated that with respect to the function of NifA in signal transduction, *Azoarcus* sp. strain BH72 is more similar to diazotrophs of the γ -subgroup than to the closer relative *H. seropedicae*, both genera being members of the β -subgroup. As in bacteria of the α -subgroup, in *H. seropedicae* NifA activity is self-modulated: NifA activity is sensitive to combined nitrogen and O₂ (Souza *et al.*, 1999). In contrast, in *Azoarcus* sp. BH72 NifA by itself was not able to

modulate *nifH* transcription in response to ammonium or O₂ when *nifL* was inactivated. This suggests that O₂ signalling or sensing occurs through the NifL protein as described for diazotrophs of the γ -subgroup (Dixon, 1998), reflecting the phylogenetic relationship of the β - and γ -subgroups according to 16S rDNA analysis. Interestingly, in *H. seropedicae* also the iron protein of nitrogenase is located in a clade of α -proteobacterial proteins according to phylogenetic analysis, leading to the speculation that the structural *nif* genes in *H. seropedicae* might have been gained by lateral gene transfer (Hurek *et al.*, 1997a). The similarities of details of transcriptional regulation of *nif* genes to α -subgroup diazotrophs support the view that the entire N₂-fixing apparatus in this bacterium might have been gained by lateral gene transfer.

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