

## Molecular analysis of the chromosomal region encoding the *nifA* and *nifB* genes of *Acetobacter diazotrophicus*

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### Abstract

The *Acetobacter diazotrophicus nifA* gene was isolated by its ability to restore a Nif<sup>+</sup> phenotype to a *nifA* mutant of *Azotobacter vinelandii*. Sequencing revealed that the *nifA* gene was upstream and adjacent to the *nifB* gene and both are transcribed in the same direction but independently from different promoters. The 3' end of the *nifB* gene was located approximately 2.5 kb upstream of the nitrogenase structural gene cluster, *nifHDK*. The deduced amino acid sequences of the *A. diazotrophicus nifA* and *nifB* gene products were most similar to the NifA and NifB proteins of *Azorhizobium caulinodans* and *Rhodobacter capsulatus*, respectively. *A. diazotrophicus nifA* expression was repressed in cultures exposed to high levels of ammonium while oxygen apparently had no influence. Both oxygen and ammonium prevented expression of a *nifB*-reporter strain, consistent with the observation that ammonium repressed *nifA* expression, and indicating that *A. diazotrophicus* NifA activity is inhibited by oxygen as in other Proteobacterial  $\alpha$  group diazotrophs. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

**Keywords:** *nif* gene; Amino acid sequence; *Acetobacter diazotrophicus*

### 1. Introduction

Biological fixation of atmospheric dinitrogen by its reduction and protonation to yield ammonia is achieved in diazotrophic bacteria by the enzyme nitrogenase, the synthesis and activity of which in-

volves the expression of up to 21 different and specific *nif* genes [1]. While in all Proteobacterial diazotrophic species examined the *nifA* gene encodes the transcriptional activator required for expression of other *nif* genes (reviewed in [2]), expression of the *nifA* gene and/or activity of the NifA protein can be influenced by the environmental conditions of oxygen and fixed N (ammonium) supply.

*nifA* gene expression may require NtrC-P as a transcriptional activator under low ammonium con-

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ditions, as in *Klebsiella pneumoniae* [2] and *Rhodobacter capsulatus* [3]. In contrast, *nifA* expression in *Azotobacter vinelandii* and *Azospirillum brasilense* is NtrC-independent and not influenced by ammonium [4,5]. Expression of *nifA* may also be prevented in cultures exposed to excessive oxygen, as occurs in several symbiotic rhizobial species such as *Rhizobium meliloti*, in which FixL, an oxygen-sensitive heme-containing protein, modulates phosphorylation of FixJ which in turn activates *nifA* transcription [6]. Furthermore in all species examined, activity of the NifA protein is inhibited by exposure of cells to high levels of oxygen, and in some, by ammonium [7–9]. In diazotrophic members of the  $\alpha$ -group of Proteobacteria, to which *Acetobacter diazotrophicus* belongs, and in *Herbaspirillum seropedicae*, a  $\beta$ -group member, inactivation of NifA by oxygen occurs by inherent sensitivity of NifA protein to oxygen damage apparently via a Cys-aa<sub>4</sub>-Cys motif in the interdomain linker region [10]. Mechanisms for inactivation of NifA in ammonium-grown cells of some diazotrophs of the  $\alpha$ -group have not been well-characterized, but in the case of *A. brasilense* and *H. seropedicae*, this occurs through the N-terminal domain of NifA, the most variable of the regions of NifA proteins [11,12]. The other NifA domains include a highly conserved central region involved in ATP hydrolysis essential for conversion of closed to open complex of RNA polymerase bound to the promoter region of NifA-activated *nifH* genes, an interdomain linker, involved in the oxygen-dependent regulation of NifA activity [10], and a C-terminal domain, essential for the specific NifA interaction with susceptible promoter sequences (reviewed in [2]). NifA proteins recognize and bind to the consensus sequence TGT-N10-ACA located approximately 100 bp upstream of promoters of *nifH* genes, characterized also by a  $\sigma^{54}$  (RpoN) recognition sequence, GG-N9-tGCa, located –12 (GC), –24 (GG) upstream of the transcription start.

Strains of *A. diazotrophicus*, an endophytic diazotroph, have been found associated with cultivars of sugarcane in Brazil, Mexico, Argentina, Australia, and Cuba, and were more prevalent in plants from unfertilized soils than in plants where N-containing fertilizer was applied (reviewed in [13]). *A. diazotrophicus* is thought to be responsible for the high level of biologically fixed nitrogen apparently incorpo-

rated into this crop. Recent experiments with sterile sugarcane plantlets inoculated with Nif<sup>+</sup> and Nif<sup>–</sup> *A. diazotrophicus* strains, in which growth of young plants inoculated with the Nif<sup>+</sup> strain grew significantly better than plants inoculated with the Nif<sup>–</sup> mutant or uninoculated plants, supports this supposition, although other plant growth-promoting factors are indicated [13]. Understanding the mechanisms by which *nif* gene expression is regulated in *A. diazotrophicus* is important for studies of the benefit of biological nitrogen fixation to sugarcane nutrition and its eventual improvement with this host and possibly other monocot plants. In this paper, the *nifA* gene of *A. diazotrophicus* is characterized. In addition, the *nifB* gene, the product of which is required for biosynthesis of the FeMo cofactor, an important component of nitrogenase holoenzyme, was found to be located adjacent to *nifA* and is transcribed from a NifA-dependent promoter.

## 2. Materials and methods

### 2.1. Bacterial strains and plasmids

The bacterial strains and plasmids used in this work are listed in Table 1 and are schematically depicted in Fig. 1.

### 2.2. Media and growth conditions

*A. diazotrophicus* was grown at 30°C on C2 medium (10 g peptone, 15 g glucose, 5 g NaCl, 5 g yeast, per liter, pH 6.0) or LGI medium [26] supplemented with ammonium chloride at final concentrations of 1 mM or 20 mM. *Escherichia coli* was grown in LB medium as described previously [27]. Burk's medium (BS) [15] supplemented with 15 mM ammonium acetate (BSN) or 10 mM NaNO<sub>3</sub> (BSNO<sub>3</sub>), when required, was used to grow *A. vinelandii* strains and transconjugants at 30°C. For triparental matings strains were plated on BSN medium supplemented with 5% LB medium (v/v).

### 2.3. Construction of a genomic library

Chromosomal DNA of *A. diazotrophicus* PAL5 (ATCC 49037) from CNPAB (Culture Collection

number BR11281) was used for the construction for the genomic library. Fragments generated by partial digestion with *Sau3A* were inserted into the *Bam*HI site of cosmid pLAFR3 (broad host range, Inc-P vector) as described by Staskawicz et al. [28] with *E. coli* strain DH5 $\alpha$  as host. In vitro Packaging Kits were obtained commercially from Stratagene (La Jolla, CA, USA).

#### 2.4. Bacterial crosses

Conjugation between *E. coli* strains carrying the genomic library and *A. vinelandii* mutant strains was performed as described previously [29] using pRK2013 as a helper. Selection against the donor and helper was achieved by plating on nitrogen-free BS medium supplemented with tetracycline at a final concentration of 2 mg ml<sup>-1</sup>. Exconjugants in which the *nifA* phenotype of strain *A. vinelandii* MV521 was complemented were detected after 3 days of growth at 30°C.

#### 2.5. Molecular biology techniques

##### 2.5.1. Nucleotide sequencing techniques

The DNA sequence of both strands was determined by the dideoxy chain termination method with the T7 DNA sequencing kit (USB, Cleveland, OH, USA) according to the manufacturer's protocol using M13 universal, T7, T3 or M13 reverse primers and [ $\alpha^{35}$ S]dATP. Electrophoresis was performed using SeaQuateR 6% DNA sequencing gel solution (Sooner Scientific, Inc., Garvin, OK, USA) in a Gibco BRL sequencing apparatus (Gibco BRL, Grand Island, NY, USA).

For nucleotide sequence determination undirected deletions were generated after thionucleotide endprotection followed by successive action of exonuclease III of *E. coli* and nuclease S1 of *Aspergillus oryzae* with the double-stranded nested deletion kit (Pharmacia, Piscataway, NJ, USA).

DNA sequence analyses were performed with the computer software packages GCG (Madison, WI, USA) and DNASTAR (DNASTAR Inc., Madison, WI, USA), respectively.

##### 2.5.2. DNA preparations

For preparation of chromosomal DNA of *A. diaz-*

*otrophicus* was grown to stationary phase in liquid C2 medium. Cell lysis and DNA preparation were carried out as described previously [30]. Plasmid DNAs were prepared by the alkaline lysis method [27]. However, for sequencing experiments, plasmid DNAs were further purified either by precipitation with 6.5% (w/v) polyethylene glycol in the presence of 0.5 M NaCl [27] or by Qiagen Mini Plasmid kits (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

#### 2.6. Plasmid constructions

Plasmid constructions are listed in Table 1 and illustrated in Fig. 1. All enzymes were obtained commercially and used according to the manufacturer's recommendations. The *nifA* and *nifB* interposon mutants were constructed in *E. coli* by insertion of the promoterless *gusA*-kanamycin cartridge originated from plasmid pWM6 [31]. Plasmids pDM611 and pDM612 carry the reporter gene cassette as a *Bam*HI restriction fragment in both orientations inserted into the unique *Bam*HI site of plasmid pDM606. The *nifA* reporter gene fusion was constructed by insertion of the *Bam*HI digested *gusA*-kan cassette into the *Bg*III recognition site located within the *nifA* gene in plasmid pDM606 with the resulting construct named pDM610. The hybrid plasmids pDM613 and pDM614 carry the same interposon inserted between the two *Bg*III recognition sites of plasmid pDM606, which leads to a deletion of the internal 1.58-kb *Bg*III restriction fragment carrying the 3' region of *nifA* and the 5' region of the *nifB* gene.

#### 2.7. Construction of chromosomal mutants by electroporation

For introduction of DNA into *A. diazotrophicus* cells were grown until exponential growth phase in liquid C2 medium, collected by centrifugation at 3000 $\times$ g and washed twice in ice-cold 10% glycerol (v/v). After the final washing step cells were resuspended in 1/20 volume of 10% glycerol (v/v) and stored on ice. Aliquots of 200  $\mu$ l of cells were used for electroporation after the addition of up to 1  $\mu$ g of plasmid DNA. After application of the desired pulse of 12.5 kV cm<sup>-1</sup> and a pulse length of 12 ms

in a Bio-Rad gene pulser apparatus (Bio-Rad Laboratories, Munich, Germany), cells were washed from the cuvette with 1 ml of C2 medium. Cells were inoculated for 2 h at 28°C, collected at 3000×g and plated on solid C2 medium with 200 µg ml<sup>-1</sup> kanamycin for primary selection of marker integration. To verify double crossover events, colonies appearing after 3–4 days were tested for the lack of the vector antibiotic resistance of pDM606 derivatives by plating on solid C2 medium with chloramphenicol at a final concentration of 150 µg ml<sup>-1</sup>.

### 2.8. β-Glucuronidase assay

Strains of *A. diazotrophicus* carrying a chromosomal *gusA* fusion in *nifA* or *nifB* were grown in LGI medium supplemented with 1 mM ammonium chloride for low N and 20 mM ammonium chloride for high N conditions. Aerobically grown cultures were inoculated in unstoppered flasks under vigorous shaking on a rotary shaker at 28°C for 2 h. Micro-aerobic growth conditions were achieved under identical conditions in stoppered flasks in 2% oxygen, 98% nitrogen (v/v), with the gas phase renewed every 10 min by flushing the flask with the gas mixture. For assay of β-glucuronidase activity 10 ml of bacterial culture, grown until mid-exponential phase and a bacterial titer of approximately 5×10<sup>8</sup> cells ml<sup>-1</sup>, were collected by centrifugation at 3000×g for 5 min and the bacterial pellet was immediately resuspended in 250 µl of ice-cold GUS extraction buffer (50 mM NaHPO<sub>4</sub>, pH 7.0, 10 mM β-mercaptoethanol, 10 mM Na<sub>2</sub>EDTA, 0.1% *N*-laurylsarcosine (v/v), 0.1%

(v/v) Triton X-100). Samples of 50 µl were added to 450 µl GUS extraction buffer with 4-methylumbelliferyl-β-D-glucuronide (MUG) (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) as fluorometric substrate at a final concentration of 1 mM MUG and incubated at 37°C for 5–20 min. The reactions are stopped by the addition of 1.4 ml of 0.2 M Na<sub>2</sub>CO<sub>3</sub>. The enzyme activities were determined in a Hoefer DyNA Quant 200 fluorometer (Pharmacia Biotech Europe, Freiburg, Germany) and 4-methylumbelliferone (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) as standard. Activities were calculated as hydrolyzed MUG (nM) per min and ml sample with the bacterial titer standardized at a OD of 1.0.

## 3. Results

### 3.1. Identification and cloning of the chromosomal region encoding the *nifA* gene of *A. diazotrophicus*

The strategy for isolation of the *A. diazotrophicus* *nifA* gene was to attempt complementation of a *nifA* mutant of another species, which was previously successful for the isolation of *nifA* from *H. seropedicae* [14]. *A. vinelandii* strains MV300 (*nifA*) and MV521 (*nifA ntrC*) were used for this purpose [15]. The double mutant strain MV521 is unable to grow with either dinitrogen or nitrate as N source; the *nifA* mutation results in the former phenotype while the *ntrC* mutation causes the latter. A pLAFR3 cosmid

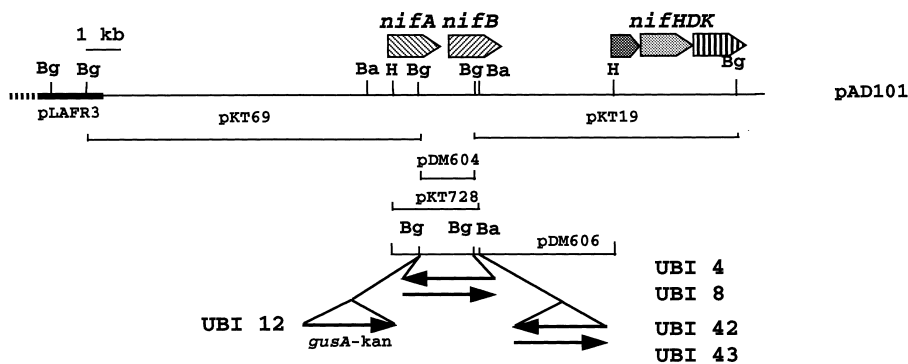


Fig. 1. Physical map of the *nifA* and *nifB* gene region of *A. diazotrophicus*. Interposon mutations constructed by insertional mutagenesis with the *gusA-kan* cassette in the recombinant plasmid pDM606 and gene replacement mutants of *A. diazotrophicus* are shown. Arrows indicate the orientation of the *gusA-kan* cassette. Abbreviations: Ba, *Bam*HI; Bg, *Bgl*II; H, *Hind*III.

Table 1  
Bacterial strains and plasmids

Strain	Phenotype/genotype/feature	Source
<i>A. diazotrophicus</i>		
PAL5	wild-type	ATCC 49037, CNPAB Culture Collection BR11281
UBI 1	<i>rif</i> -resistant, spontaneous	this work
UBI 4	<i>rif</i> , $\Delta nifAB::gusA$ -kan, $\beta$ -orientation	this work
UBI 8	<i>rif</i> , $\Delta nifAB::gusA$ -kan, $\alpha$ -orientation	this work
UBI 12	<i>rif</i> , <i>nifA::gusA</i> -kan	this work
UBI 42	<i>rif</i> , <i>nifB::gusA</i> -kan, $\beta$ -orientation	this work
UBI 43	<i>rif</i> , <i>nifB::gusA</i> -kan, $\alpha$ -orientation	this work
<i>E. coli</i>		
DH5 $\alpha$	F' <i>endA1 hsdR17</i> ( $r_k^- m_k^+$ ) <i>supE44 thi-1 recA1 gyrA</i> (Nalr) <i>relA1</i> $\Delta(lacIZYA-argF)$ <i>U169 deoR</i> ( $\phi 80dlac\Delta(lacZ)M15$ )	Life Technologies, Inc., Gaithersburg, MD, USA
<i>A. vinelandii</i>		
MV300	<i>nifA1</i>	[15]
MV521	<i>nifA1</i> , <i>ntrC</i> :K1XX	[15]
Plasmids		
pAD101	<i>nifA</i> -complementing cosmid clone of <i>A. diazotrophicus</i> , pLAFR3 derivative	this work
pBluescript II KS <sup>+</sup>	<i>lacZ</i> $\alpha$ -complementing vector, Ap <sup>R</sup>	Stratagene, La Jolla, CA, USA
pDM604	1.58-kb <i>Bgl</i> III fragment in pSVB30, <i>nifA'</i> , <i>nifB'</i>	this work
pDM606	6.6-kb <i>Hind</i> III fragment in pSUP401, <i>nifA'</i> , <i>nifB'</i>	this work
pDM607	6.6-kb <i>Hind</i> III fragment in pSVB30, <i>nifA'</i> , <i>nifB'</i>	this work
pDM610	<i>nifA::gusA</i> -kan, $\alpha$ -orientation	this work
pDM611	<i>nifB::gusA</i> -kan, $\alpha$ -orientation	this work
pDM612	<i>nifB::gusA</i> -kan, $\beta$ -orientation	this work
pDM613	$\Delta nifAB::gusA$ -kan, $\alpha$ -orientation	this work
pDM614	$\Delta nifAB::gusA$ -kan, $\beta$ -orientation	this work
pKT19	8.0-kb <i>Bgl</i> III fragment in pBluescript II	this work
pKT63	10.0-kb <i>Bgl</i> III fragment in pBluescript II	this work
pKT728	2.9-kb <i>Hind</i> III- <i>Bgl</i> III fragment in pBluescript II	this work
pLAFR3	broad host range cosmid vector, Inc-P, Tc <sup>R</sup> , <i>lacZ</i> $\alpha$ -complementation	[28]
pRK2013	helper plasmid, Tra <sup>+</sup> , kan <sup>R</sup>	[32]
pSVB30	<i>lacZ</i> $\alpha$ -complementing vector, Ap <sup>R</sup>	[16]
pSUP401	Kan <sup>R</sup> , Cm <sup>R</sup> , <i>mob</i> <sup>+</sup>	[33]
pWM6	promoterless <i>gusA</i> -Kan gene cartridge	[31]

library of *A. diazotrophicus* genomic fragments of 20–30 kb in size was constructed and conjugated en masse from the *E. coli* host strain DH5 $\alpha$  to *A. vinelandii* MV300 and MV521 using helper plasmid pRK2013 in triparental matings. Nif<sup>+</sup> Tet<sup>r</sup> transconjugants were selected. Several colonies arose after conjugation with either MV300 or MV521. Among the MV521 Nif<sup>+</sup> transconjugants, none was able to grow using nitrate as N source indicating that the *ntrC* mutation of MV521 was not complemented. Several independent complementing cosmids were isolated from both *A. vinelandii* mutant strains and were found to share common *Bgl*III and *Eco*RI re-

striction fragments. One of these cosmids, pAD101, carried a chromosomal insert of approximately 22 kb and was presumed to carry the *nifA* gene of *A. diazotrophicus*. In order to locate the gene on this fragment, several restriction fragments were cloned in *E. coli* using the vector pSVB30 [16] and partially sequenced from both ends. One of the constructed hybrid plasmids, pDM604, carried a 1.58-kb *Bgl*III restriction fragment with sequences at one end encoding a NifA-like protein and at the other end encoding a NifB-like gene product (Fig. 1). DNA regions overlapping the insert in pDM604 were located by restriction mapping (Fig. 1). Southern hy-

bridization using the heterologous *nifA* and *nifB* genes of both *A. brasilense* and *A. vinelandii* as probes confirmed the location of these genes (data not shown). The physical map of the *nifAB* region of *A. diazotrophicus* deduced from restriction mapping, partial sequencing, and Southern hybridization was further refined by complete DNA sequencing of the recombinant plasmids and their deletion derivatives (Fig. 1).

### 3.2. Analysis of the nucleotide sequence of the DNA region encoding *nifA* and *nifB*

The 4862-bp DNA sequence of this region, derived from sequencing both strands, is on deposit in GenBank with the accession number AF072689. It includes two open reading frames of 1746 and 1422 bp, corresponding to the *nifA* and *nifB* genes, respectively, on the basis of the high degree of similarity of their translated gene products to NifA and NifB proteins of other diazotrophs (see below). In addition, the 3' end of the *nifK* gene was found by sequencing from the end of pKT19 opposite to that lying within the *nifB* gene. Because the size and sequence of the *nifHDK* operon are known (Sevilla et al., in preparation), the 5' end of the *nifH* gene must be located approximately 2.5 kb downstream of the 3' end of *nifB*.

The *A. diazotrophicus* NifA amino acid sequence of 581 amino acids, deduced from the DNA sequence, was 60–80% identical to NifA sequences in other  $\alpha$ -group Proteobacterial diazotrophs. It was overall most similar to NifA of *Azorhizobium caulinodans* [17]. *A. diazotrophicus* NifA contains the motif Cys-aa<sub>4</sub>-Cys located within the 85 amino acid long interdomain linker region between the central and C-terminal domains which, as previously discussed [10], is associated with oxygen sensitivity of NifA proteins of the  $\alpha$ - and  $\beta$ -groups of diazotrophic Proteobacteria. The degree of identity of the distinct *A. diazotrophicus* NifA protein domains to those known among members of the proteobacterial  $\alpha$ -group is as follows: NifA of *Bradyrhizobium japonicum* [18] is most similar in the highly variable N-terminal domain (32% identity and 48% similarity), *A. caulinodans* NifA [19] is most related in the central domain (77% identity and 87% similarity) and NifA of *Rhizobium leguminosarum* [20] is most ho-

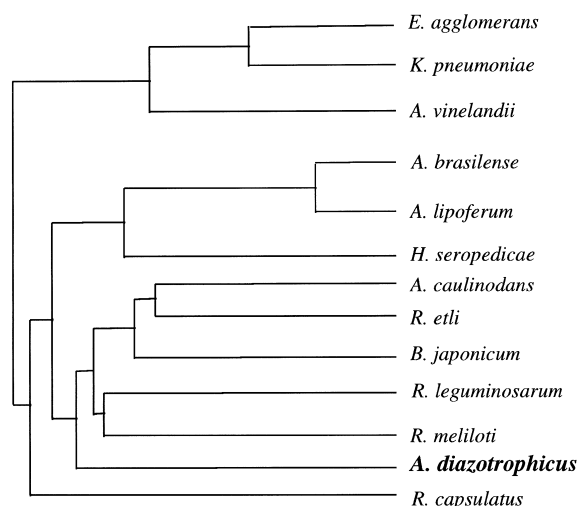


Fig. 2. Phylogenetic tree of NifA proteins. The GCG program PILEUP was used to perform the analysis.

mologous in the C-terminal domain (78% identity and 89% similarity). A dendrogram using the PILEUP program of the GCG sequence analysis package showing the phylogenetic relatedness of the entire *A. diazotrophicus* NifA sequence to those of other diazotrophs is shown in Fig. 2.

The *A. diazotrophicus* NifB protein is 473 amino acids in length and is most related to the NifB proteins of *R. capsulatus* [21], several *Rhizobium* species [22–24], and *A. vinelandii* [25] with amino acid identities being in the range of 70–80%. The N-terminal domain of *A. diazotrophicus* NifB is rich in serine and glutamine as observed in rhizobial species and in *A. vinelandii* and *R. meliloti*, but absent in *R. capsulatus*.

No sequences similar to known promoter region motifs associated with expression of other *nifA* genes, such as a  $\sigma^{54}$  recognition sequence or a NtrC binding site, were observed within a 500-bp region sequenced lying upstream of the most probable start codon of the *nifA* open reading frame. However, a conserved NifA-UAS sequence, TGT-N10-ACA, was located 39 bp upstream of the most likely translational start site of NifA.

The *nifA* and *nifB* genes are separated by 205 bp. This sequence contains motifs characteristic of NifA-dependent promoters, including a conserved NifA binding site at –161 bp and a  $\sigma^{54}$  recognition se-

Table 2  
 $\beta$ -glucuronidase activity of the *nifA::gusA* and *nifB::gusA* gene fusions in *A. diazotrophicus*

Strains	Target gene(s) and interposon orientation	2% O <sub>2</sub>		21% O <sub>2</sub>	
		+ 1 mM NH <sub>4</sub> <sup>+</sup>	+ 20 mM NH <sub>4</sub> <sup>+</sup>	+ 1 mM NH <sub>4</sub> <sup>+</sup>	+ 20 mM NH <sub>4</sub> <sup>+</sup>
UBI 4	← <i>nifAB</i>	11 ± 3	15 ± 4	23 ± 5	18 ± 3
UBI 8	→ <i>nifAB</i>	2374 ± 321	41 ± 15	2611 ± 310	19 ± 12
UBI 12	→ <i>nifA</i>	2906 ± 919	160 ± 100	2768 ± 344	42 ± 21
UBI 42	← <i>nifB</i>	12 ± 4	4 ± 2	7 ± 1	3 ± 0
UBI 43	→ <i>nifB</i>	3256 ± 470	11 ± 8	37 ± 14	53 ± 16

Data are averages of three independent assays. Strains UBI 4, UBI 8 and UBI 42, UBI 43 carry the same *gusA*-kan insertion, but in the opposite orientation (see Fig. 1). GUS activity given as hydrolysis of 4-methylumbelliferyl- $\beta$ -D-glucuronide (nM min<sup>-1</sup> and strain OD = 1.0). Arrows indicate the relative orientation of the *gusA*-kan interposon with respect to the relevant target gene(s).

quence at position -89 bp upstream of the *nifB* translational start codon. It was therefore expected that *nifB* expression would be activated by NifA and require  $\sigma^{54}$ .

### 3.3. Expression of *nifA::gusA* and *nifB::gusA* gene fusions

In order to investigate the mode of regulation of both the *nifA* and the *nifB* genes with respect to ammonium and oxygen supply, reporter gene fusions were constructed using a promoterless *gusA*-kan interposon as described in Section 2 and Fig. 1. Constructions included those in which the interposon cassette was inserted in two different orientations. These were introduced into *A. diazotrophicus* by electroporation. Kan<sup>r</sup> colonies arising were tested for resistance to chloramphenicol (Cm). Cm<sup>s</sup> colonies were presumed to have arisen by double crossover events between DNA of the plasmid and the chromosome on both sides of the reporter gene insert, resulting in replacement of the wild-type region. Gene replacements were confirmed by a Southern hybridization (data not shown).

The resulting strains were grown in media with 1 mM (low) or 20 mM (high) ammonium chloride and under microaerobic or aerobic conditions (see Section 2).  $\beta$ -Glucuronidase activity was measured in each culture. The data indicate that expression of *nifA::gusA* was 18–65-fold higher in cultures grown with 1 mM ammonium acetate than in cultures supplied with 20 mM, while *nifA::gusA* expression was apparently not influenced by oxygen supply. In contrast, expression of *gusA* from the *nifB* promoter, a probable target of transcriptional activation by

NifA, was about 300-fold higher when ammonium supply was low compared to high supply under microaerobic conditions. Expression of *nifB::gusA* was not significant in cultures supplied with high amounts of oxygen, indicating that oxygen inhibited expression from the *nifB* promoter (Table 2).

## 4. Discussion

The *nifA* gene of *A. diazotrophicus* was isolated on a pLAFR3 cosmid by its ability to restore a Nif<sup>+</sup> phenotype to a *nifA* and *nifA ntrC* double mutant of *A. vinelandii*. Because the *ntrC* gene of *A. diazotrophicus* is not located on pAD101, but lies elsewhere on the chromosome (Meletzus et al., unpublished), and because the *nifA* gene in pAD101 is located more than 5 kb from the *lac* promoter adjacent to the cloning site in pLAFR3, expression of the *nifA* gene of *A. diazotrophicus* in *A. vinelandii* apparently does not require NtrC. This is consistent with the observations that no NtrC or  $\sigma^{54}$  binding sites were located upstream of the sequenced *A. diazotrophicus nifA* gene. Nevertheless, *nifA* expression is apparently regulated by fixed nitrogen as was evident from studies of a *nifA::gusA* reporter strain in which expression in 20 mM ammonium-grown cells was only about 5% of that in cultures with 1 mM ammonium. The mechanism of control of *nifA* expression by fixed nitrogen is not known but could be novel if it is  $\sigma^{54}$ -independent as suggested by the sequence upstream of the *nifA* gene.

The expression of the *nifA* gene, at least under the conditions tested here, is not influenced by oxygen concentration and takes place under microaerobic as

well as atmospheric oxygen concentrations as determined from the corresponding  $\beta$ -glucuronidase assays using strains carrying chromosomal *nifA::gusA* reporter gene fusions. This observation is in contrast to what occurs in species of Rhizobiaceae, the prominent symbiotic bacteria within the Proteobacterial  $\alpha$ -group. In these organisms the intracellular concentration of oxygen is a crucial signal for the modulation of *nifA* activity. These results suggest that no heme-containing transcriptional activator such as FixJ is involved in regulation of expression of *nifA* in *A. diazotrophicus*.

The activity of the NifA protein is, however, sensitive to oxygen. This was apparent from the observation that *nifB::gusA* expression is drastically reduced in cells grown at 20% oxygen (and 1 mM ammonium) as compared to cells at 2% oxygen (and 1 mM ammonium). This result is expected because of the interdomain linker region present in the *A. diazotrophicus* NifA protein shares features with NifAs of other  $\alpha$ -Proteobacteria which are associated with oxygen sensitivity. This interdomain linker motif metal-binding sites and has been found in NifA proteins of diazotrophs in which no homologous *nifL*-like gene has been detected. The post-translational regulation of NifA depends on its redox-sensitive nature which might involve the binding of a metal cofactor to the cysteine motif of these proteins thereby causing inactivation [10]. Whether ammonium also influences NifA activity in this organism is not known and could not be deduced from these experiments because expression of *nifA* is repressed by ammonium.

A phylogenetic comparison of the amino acid sequences of the *A. diazotrophicus nifA* gene product to other NifA proteins was in good agreement with the taxonomic status of this organism as a member of the Proteobacterial  $\alpha$ -group, most closely related to the taxa of the Rhizobiaceae and more distantly related to the free-living members of this group such as *Azospirillum* and *Rhodobacter*. The highest degree of phylogenetic divergence occurred between the NifA proteins of *A. diazotrophicus* and members of the  $\gamma$ -group of Proteobacteria: *K. pneumoniae*, *Enterobacter agglomerans* and *A. vinelandii*. This was to be expected, since these NifA proteins differ significantly with respect to the typical domain structure, such as lack of the cysteine-rich interdomain linker.

Work is in progress to determine the mechanism of ammonium regulation of NifA activity as well as the determination of the relevant promoter sequences and regulatory proteins involved in ammonium regulation of *nifA* gene expression in *A. diazotrophicus*.

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