Over-production and Characterization of the *nifA* Gene Product of *Klebsiella pneumoniae* – the Transcriptional Activator of *nif* Gene Expression

By R. TULI† AND M. J. MERRICK*

AFRC Institute of Plant Science Research, Nitrogen Fixation Laboratory, University of Sussex,
Brighton BN1 9RQ, UK

(Received 14 September 1987)

The *nifA* gene of *Klebsiella pneumoniae*, which encodes the transcriptional activator of *nif* gene expression, was cloned into a number of plasmid vectors to obtain high-level synthesis of *nifA* product (NifA). When over-produced, NifA was very insoluble and it precipitated with the cell debris after cell lysis. Localization of β -galactosidase activity from a *nifA*–*lacZ* translational fusion confirmed the insoluble nature of NifA. Analysis of two translational fusions in which the last six C-terminal amino acids of NifA were deleted suggests that these residues are required for activity.

INTRODUCTION

The nitrogen fixation (*nif*) genes of *Klebsiella pneumoniae* comprise a cluster of at least 18 genes organized in eight operons. One of these operons consists of two genes, *nifL* and *nifA*, whose products are *nif*-specific regulatory proteins. The promoters of all the *nif* operons do not contain consensus –35 and –10 sequences characteristic of most prokaryotic promoters but conform instead to a GG–N₁₀–GC consensus located between –24 and –11 (Beynon *et al.*, 1983). These promoters are all subject to positive control, and activation of transcription initiation from *nif* promoters requires the products of two genes, *nifA* and *ntrA*, together with RNA polymerase core enzyme (Buchanan-Wollaston *et al.*, 1981; Merrick, 1983). The *ntrA* product (NtrA) acts as a specific sigma factor (Hirschman *et al.*, 1985; Hunt & Magasanik, 1985; Austin *et al.*, 1987) and the *nifA* product (NifA) is a specific activator protein.

The method by which NifA activates transcription from the –24, –11 region is not known but recent studies have demonstrated that most *nif* promoters contain an upstream activator sequence (UAS) which significantly enhances activation of transcription (Buck *et al.*, 1986, 1987). This UAS is characterized by a sequence TGT–N₁₀–ACA typical of DNA-binding sites for regulatory proteins and it has been proposed that these sites may be binding sites for NifA (Buck *et al.*, 1986, 1987). The *nifA* gene of *K. pneumoniae* has been sequenced (Buikema *et al.*, 1985; Drummond *et al.*, 1986) and this has allowed prediction of the domain structure of the NifA protein and identification of a potential DNA-binding domain in the C-terminus of the protein (Drummond *et al.*, 1986).

As a result of an increase in the levels of intracellular fixed nitrogen, or of an increase in the extracellular oxygen tension, NifA loses its ability to activate transcription; this has been attributed to inactivation of NifA by the *nifL* product (NifL). Hence NifL appears to act as a sensor to changes in the nitrogen or oxygen status of the cell and to modulate NifA activity accordingly (Hill *et al.*, 1981; Merrick *et al.*, 1982).

† Present address: Biology and Agriculture Division, Bhabha Atomic Research Centre, Trombay, Bombay 400085, India.

Abbreviations: dNTP, deoxynucleoside triphosphate; X-gal, 5-bromo-4-chloro-3-indolyl β -D-galactoside.

In order to study the molecular details of NifA function it is necessary to purify NifA and study its action in a cell-free system. In working towards this goal we have constructed a number of NifA-overproducing plasmids and *nifA-lacZ* fusions which have been used to examine various aspects of NifA function.

METHODS

Bacterial strains. These are listed in Table 1.

Cloning *nifA* for over-production. The *nifA* gene was cloned from plasmid pX901, which comprises a 2.9 kb *SalI* fragment, carrying the 3' end of *nifL*, the complete *nifA* gene and the 5' end of *nifB*, cloned in pEMBL8(+) (M. Drummond, personal communication) (Fig. 1). Most of *nifL* was removed by partial digestion with *NruI* and ligation with the 'filled' *BamHI* site of the vector so as to leave only 68 nucleotides at the 3' end of *nifL*. The resultant plasmid, pRT902, carries a 2.2 kb *EcoRI-HindIII* fragment which could then be cloned adjacent to a number of strong promoters in order to achieve high-level expression of NifA. Three expression vectors, pKK223-3 (Pharmacia), pHL200 (Kröger & Hobom, 1982) and pLc236 (Remaut *et al.*, 1981) were used in order to obtain expression of *nifA* from the *tac*, *lacUV5* and λp_L promoters respectively. The resultant plasmids were designated pRT903 (*tac*), pRT201 (*lacUV5*) and pRT237 (λp_L).

Construction of pRT22 – a 'compatible' *nifH-lacZ* translational fusion. In order to provide a simple assay for NifA overproduction, a *nifH-lacZ* fusion plasmid, pWVC22 (Buck & Cannon, 1987), was modified by opening the molecule at the *PstI* site in the *bla* gene, 'filling-in' the restriction site using Klenow polymerase in the presence of dNTPs and religating, thereby mutating the *bla* gene. The resultant plasmid pRT22 does not confer ampicillin or carbenicillin resistance (C^b) but retains the chloramphenicol resistance (C^m) marker of the parent plasmid. pRT22 is compatible with all three pBR322-based expression vectors used in these experiments and both the *nifA* and *nifH-lacZ* plasmids could be independently selected using C^b and C^m respectively.

Translational fusions with *nifA*. Using the nucleotide sequence of the *nifA* gene (Drummond *et al.*, 1986) it was possible to construct both out-of-frame and in-frame translational fusions using vectors pNM481 and pNM480 (Minton, 1984) respectively. The restriction enzyme *EcoRV* cleaves *nifA* between codons 518 and 519 (see Fig. 2) and therefore partial digestion of the 2.2 kb *EcoRI-HindIII* fragment from pRT903 allowed isolation of a 1.6 kb *EcoRI-EcoRV* fragment in which seven codons at the 3' end of *nifA* were deleted. This fragment was cloned into *EcoRI-SmaI*-cut pNM481 to give pRT482 in which *nifA* was 'out-of-frame' with *lacZ*. The resultant fusion extends 17 codons beyond the *EcoRV* site in *nifA* and then terminates (Fig. 2). This hybrid gene was placed under

Fig. 1. A map of the *nifA* region of *K. pneumoniae* showing restriction endonuclease sites used in this study. The extent of *nifL*, *nifA* and *nifB* in the 2.9 kb *SalI* fragment flanked by the restriction site nest of the vector pEMBL8(+) is shown. As described in Methods, the *BamHI-NruI*(2) segment was deleted in order to remove most of *nifL* prior to construction of the transcriptional fusions (pRT903, pRT201 and pRT237). The *EcoRI-EcoRV*(2) fragment from pRT903 was used to obtain the translational fusion in pRT482.

Table 1. List of strains

Strain	Genotype	Reference/source
<i>E. coli</i> K12		
71-18	$\Delta(lac-pro)$ <i>supE</i> <i>thi</i> [F' <i>proAB lacI^s lacZ</i> Δ M15]	J. Messing, University of Minnesota, St Paul, USA
MC1061	<i>araD139</i> $\Delta(ara-leu)$ 7697 <i>lac</i> Δ X74 <i>galU</i> <i>galK</i> <i>hsr</i> <i>hsm⁺</i> <i>strA</i>	Casadaban & Cohen (1980)
M5219	M72 <i>lacZam</i> <i>trpam</i> Sm ^R (<i>bio252</i> <i>ci857</i> Δ H1)	Remaut <i>et al.</i> (1981)
ET8894	<i>rbs lacZ</i> :: IS1 <i>gyrA</i> <i>hutC</i> ₆ $\Delta(rha$ <i>glnA</i> <i>ntfBC</i>)1703 :: Mucts62	T. MacNeil, Merck, Sharp & Dohme, Rahway, NJ, USA
ET8556	<i>rbs lacZ</i> :: IS1 <i>gyrA</i> <i>hutC</i> ₆ <i>ntfC1488</i> <i>gyrA</i>	
5K	<i>thr</i> <i>leu</i> <i>thi</i> <i>resK</i> <i>modK⁺</i> <i>tonA</i> <i>supE</i> λ^s	
5KR	<i>rec</i> derivative of 5K by P1 transduction from DB6659	This laboratory
<i>K. pneumoniae</i> M5a1		
UNF714	<i>nifA2263</i> <i>hisD2</i> <i>recA56</i> <i>sbl300</i> :: Tn10 <i>rpsL4</i> <i>hsdR1</i>	Buchanan-Wollaston <i>et al.</i> (1981)
UNF931	$\Delta lac2002$ <i>hisD2</i> <i>recA56</i> <i>sbl300</i> :: Tn10 <i>hsdR1</i>	This laboratory

Fig. 2. Structure of *nifA-lacZ* translational fusions. At the *EcoRV-SmaI* junction, the 518th codon (GAT) of *nifA* was joined through the cloning nest of pNM481 or pNM480 to the 8th codon (CCC) of *lacZ*. In the resultant plasmid pRT904, *lacZ* was out-of-frame, leading to the termination of *nifA* at TGA after an extension of 17 codons. In pRT905 the linker region of pNM480 restored the reading frame of *lacZ*.

control of the *tac* promoter by cloning a 4.7 kb *EcoRI-AhaIII* fragment from pRT482 between the *EcoRI* and *SmaI* sites of pKK223-3 to construct pRT904. The fusion junction in pRT904 was confirmed by sequencing.

An in-frame translational fusion (pRT905) was constructed by cloning a 2 kb *SalI* fragment from pRT904 into pNM480, which contains two extra bases in the linker region (Fig. 2). The correct orientation for the *SalI* fragment was identified by selection of blue colonies on media containing X-gal.

Purification of *nifA* gene product. The *nifA* over-producing strain ET8894(pRT903) was grown to late-exponential phase in L-broth containing 200 µg carbenicillin ml⁻¹ and induced overnight in the presence of 0.2 mM-IPTG before harvest. The cell pellet was washed in buffer A (50 mM-Tris pH 8.0, magnesium acetate 5 mM, NaCl 50 mM, 2-mercaptoethanol 2 mM, EDTA 0.1 mM), resuspended in the same buffer, using six times the pellet volume, and the cells were disrupted by sonication or French press. The suspension was centrifuged at 15000 *g* for 30 min to obtain a NifA-enriched pellet. The pellet was solubilized in buffer A containing 0.2% SDS by stirring at room temperature in 500 times the pellet volume for 30 min. Ammonium sulphate was added to the suspension to 30% saturation and the precipitated material was discarded. The proteins precipitated between 50 and 80% saturated ammonium sulphate were enriched in NifA, as followed by SDS-PAGE (see Fig. 5).

In vitro coupled transcription/translation. An S-30 extract was prepared from *E. coli* strain ET8556 and plasmid-encoded gene products were labelled using the method of Collins (1979). The [³⁵S]methionine-labelled polypeptides were visualized by autoradiography after separation by SDS-PAGE and treatment of the acrylamide gel with Amplify (Amersham).

Other methods. All enzymes were obtained from commercial sources and used according to the manufacturers' instructions. Sequencing was done by dideoxy sequencing reactions using clones prepared in M13mp8 and mp11. β-Galactosidase units were determined by the method of Putnam & Koch (1975).

RESULTS

Over-production of NifA

All three plasmids, pRT903, pRT201 and pRT237, carrying transcriptional fusions of *nifA* with *tac*, *lacUV5* and *λp_L* promoters respectively, were able to activate the expression of β-galactosidase from the *nifH* promoter in pRT22. Expression from the *tac* and *lacUV5* promoters, unlike that from *λp_L*, was not tightly regulated (Table 2).

The amount of NifA synthesized from a single chromosomal *nifA* gene or from *nifA* expressed constitutively from pMC73A (Buchanan-Wollaston *et al.*, 1981) was too low to be visualized by staining of whole-cell proteins separated by one-dimensional SDS-PAGE. However, all the three plasmids made in this study directed synthesis of a distinct protein band (see pRT903 in Fig. 3a) with an estimated molecular mass of 56000 Da, which agrees well with the molecular mass of 58632 Da calculated from the *nifA* DNA sequence (Drummond *et al.*, 1986). The position of the presumptive NifA band was confirmed by comparison with the mobility of [³⁵S]methionine-labelled NifA synthesized from pRT903 in a cell-free coupled transcription/translation system (Fig. 3b).

Fig. 3. Expression of *nifA* and the *nifA-lacZ* in-frame fusion from the *tac* promoter. (a) SDS-PAGE of cell-free extracts from 5KR(pKK223-3) (lanes 2 and 3), 5KR(pRT903) (lanes 4 and 5), 5KR(pMC73A) (lanes 6 and 7) and UNF931(pRT905) (lanes 8 and 9), 2 h (lanes 2, 4, 6 and 8) and overnight (lanes 3, 5, 7 and 9) after induction with IPTG. The positions of NifA and hybrid NifA- β -galactosidase are marked by arrows. Lane 1 contains molecular mass standards. (b) Autoradiograms of SDS-PAGE of *in vitro* transcription/translation products from pBR322 (lane 2) pRT903 (lane 4) and pRT905 (lane 5). Lane 3 shows the translation products of pRD572 (Hawkes *et al.*, 1985), which contains genes for β -lactamase and β -galactosidase. Lane 1 contains molecular mass standards.

Table 2. Activation of *nifH* promoter on pRT22 (*pnifH-lacZ*) by various *nifA* plasmids

Cells were grown in LB to exponential phase at 28 °C and induced with IPTG (0.2 mM) or temperature (42 °C for 15 min). β -Galactosidase activity is expressed as nmol ONPG hydrolysed per ml cells ($OD_{600} = 1$) min^{-1} . Induced cells were assayed 2 h after induction. ND, Not done.

Strain	Genotype of <i>nifA</i> plasmid	Promoter induction	β -Galactosidase
71-18(pRT22)	—	– IPTG	ND
		+ IPTG	94
71-18(pRT22) (pRT903)	(<i>ptac-nifA</i>)	– IPTG	4978
		+ IPTG	7714
71-18(pRT22) (pRT904)	(<i>ptac-nifA'</i>)	– IPTG	ND
		+ IPTG	100
MC1061(pRT22) (pRT201)	(<i>placUV5-nifA</i>)	– IPTG	12046
		+ IPTG	15314
M5219(pRT22) (pRT237)	(<i>lp_L-nifA</i>)	28 °C	195
		42 °C	6726

Fig. 4

Fig. 5

Fig. 4. Reversible temperature-sensitivity of NifA function. The time course of appearance of β -galactosidase activity is shown for M5219(pRT237)(pRT22). After induction of λp_L at 45 °C for 15 min, the cultures were incubated at 28 °C immediately (○) or 2 h after incubation at 40 °C (●). Samples were taken to estimate the enzyme activity at various intervals (Putnam & Koch, 1975). The time of transfer to 28 °C is indicated by vertical arrows.

Fig. 5. Purification of NifA from ET8894(pRT903): SDS-PAGE of denatured samples. Lane 1, molecular mass standards; lane 2, cell-free extract; lane 3, pellet; lane 4, pellet after a wash with buffer A; lane 5, partially purified NifA preparation. The position of NifA is indicated by the arrow.

Reversibility of temperature-inactivation of NifA function

K. pneumoniae NifA does not activate transcription from *nif* promoters at temperatures above 37 °C (Buchanan-Wollaston *et al.*, 1981; Brooks *et al.*, 1984). The reversibility of this temperature-sensitivity was studied by comparing the kinetics of appearance of β -galactosidase activity from the *nifH-lacZ* fusion in pRT22 after different periods of heat induction of λp_L -*nifA* in pRT237. Duplicate exponential-phase cultures of *E. coli* strain M5219 carrying pRT237 and pRT22 were incubated at 45 °C for 15 min in order to induce NifA synthesis from pRT237 and incubation was then continued at either 28 °C or 40 °C. In the 28 °C culture induction of NifA synthesis was followed by an increase in *nifH-lacZ* expression after a lag of about 20 min (Fig. 4). However in the 40 °C culture no significant increase in *nifH-lacZ* expression was observed for 2 h. After 2 h at 40 °C this culture was shifted to 28 °C and an immediate increase in *nifH-lacZ* expression was observed (Fig. 4).

Purification of NifA

Initial attempts to purify NifA showed that it migrated on one-dimensional SDS-PAGE very close to certain other proteins including glutamine synthetase (GS) and NtrC, as expected from previous estimates of the molecular masses of these proteins (NtrC 52.3 kDa, NifA 58.6 kDa, GS 51.8 kDa) (Espin *et al.*, 1982; Merrick, 1983). Consequently strain ET8894(pRT903), which has a deletion of the *glnA ntrBC* region, was used for all subsequent experiments. The NifA

Table 3. *Solubilization of hybrid NifA- β -galactosidase protein*

A 2 ml culture of ET8894(pRT905) induced overnight with IPTG was sonicated in buffer A and centrifuged in a microfuge for 15 min. The pellet was resuspended in 20 μ l buffer A and 10 μ l of this suspension was incubated in 50 μ l buffer A made up to give the treatments indicated. After 15 min at room temperature, the suspension was centrifuged and β -galactosidase activity estimated in the two fractions.

Treatment	β -Galactosidase activity* per assay	
	Supernatant	Pellet
Control	0.13	1.81
50% glycerol	0.22	2.16
1% Triton X-100	0.06	1.7
2 M-NaCl	0.22	2.0
25% ethylene glycol	0.11	1.8
pH 6.5	0.23	1.96
pH 9.0	0.2	1.77
0.2% SDS	1.06	0.69

* Expressed as A_{420} per assay for the colour developed after 15 min incubation at 28 °C.

synthesized in ET8894(pRT903) sedimented with the pellet obtained on centrifuging the cell-free extract at 15000 g (Fig. 5). This protein co-migrated in SDS-PAGE with the [35 S]methionine-labelled NifA obtained from the *in vitro* transcription/translation of pRT903 and was absent from the pellet obtained from cells containing vector alone (data not shown).

Co-sedimentation of NifA with the pellet of cellular debris was further substantiated by the behaviour of the hybrid NifA- β -galactosidase protein synthesized from the in-frame fusion in pRT905. β -Galactosidase is a soluble enzyme and more than 90% of the enzyme activity was present in the supernatant of cell extracts from strain 5K(pRT903), which carries a chromosomal *lacZ* together with a *nifA* plasmid, pRT903. However, in comparable cells containing a *nifA-lacZ* in-frame translational fusion (pRT905), nearly 90% of β -galactosidase activity sedimented with the pellet of cellular debris (data not shown). Therefore the tendency of NifA to sediment was retained in the hybrid NifA- β -galactosidase protein. The β -galactosidase activity in the hybrid protein served as a convenient marker to examine the solubilization of NifA from the pellet.

Inclusion of 50% (v/v) glycerol, 1% (v/v) Triton X-100, 2 M-NaCl or 25% (v/v) ethylene glycol in buffer A, or adjustment of the pH to 6.5 or 9.0, failed to solubilize any appreciable amount of β -galactosidase activity from the pellet of ET8894(pRT905). However, SDS at 0.05% released nearly 25% of the β -galactosidase activity from the pellet and 0.2% SDS released nearly 60% (Table 3). Higher levels of SDS inactivated the β -galactosidase activity of the hybrid protein and even 0.2% SDS made the protein thermolabile. The fusion protein was completely inactivated by a 15 min preincubation in the SDS buffer at 37 °C, whereas in the absence of SDS, the fusion protein, like the native enzyme, was fairly stable (data not shown).

The ability of SDS to solubilize the native NifA protein partially from the pellet was utilized to develop a simple procedure for its purification. The pellet was solubilized by stirring in a 500-fold excess of buffer A containing 0.2% SDS. Ammonium sulphate precipitation (50–80% fraction) of the solubilized pellet gave a highly enriched NifA preparation that contained one major and a few minor contaminating proteins (Fig. 5). Resolubilization of this fraction required continued presence of SDS.

NifA function requires C-terminal residues

The translational fusion in pRT905 made a hybrid protein of approximately 170 kDa that retained catalytic activity of β -galactosidase. However, the chimaeric NifA-LacZ failed to activate the *nifH* promoter in pRT22 or to complement the *nifA2263* mutation in *K. pneumoniae* UNF714 (data not shown), suggesting that the hybrid protein was not functionally active as NifA. The out-of-frame translational fusion in pRT904 is predicted to produce a hybrid protein

which contains the native primary sequence of NifA with the exception of six C-terminal amino acids which are replaced by a new sequence of 17 amino acids joined to Asp518 of NifA (Fig. 2). In a cell-free transcription/translation system pRT904 expressed a polypeptide that migrated to the position of NifA on SDS-PAGE. However, pRT904 failed to activate the *nifH* promoter *in vivo* (Table 2), indicating that the last six amino acids of NifA are important structurally or functionally for its activity.

DISCUSSION

We have described the construction of a number of plasmids which allow over-production of NifA, together with a rapid method for partial purification of the protein. However, the sedimentation of NifA in the 15000 *g* pellet creates difficulties in its purification, and in the absence of an *in vitro* assay it is not presently possible to monitor NifA activity.

Both the NifA-LacZ hybrid encoded by pRT905 and the hybrid NifA from pRT904 lack the last six residues of NifA and therefore the proposed C-terminal helix-turn-helix (HTH) motif may be disrupted. The failure of both these proteins to activate transcription from the *nifH* promoter therefore supports the suggestion (Drummond *et al.*, 1986) that the HTH motif is required for DNA-binding by NifA. Our demonstration of the reversibility of temperature-inactivation of NifA is consistent with the results of Brooks *et al.* (1984). The temperature-sensitive component responsible for failure of *nif* expression at temperatures above 37 °C may be either the NifA protein itself, the interaction of NifA with *nif* promoters or possibly the thermolability of the *nifH-lacZ* mRNA (Collins *et al.* 1986). In the light of the proposed role of DNA looping in activation of transcription by NifA (Buck *et al.*, 1987) it is possible that the temperature-sensitive component is indeed the formation of the DNA conformation necessary for activation rather than the NifA protein *per se*.

The NifA protein is functionally very similar to the homologous activator protein NtrC and these proteins can substitute for one another in activation of NtrA-dependent promoters such as *pnifLA* or *pglnA* (Merrick, 1983; Ow & Ausubel, 1983). However, NtrC is a soluble protein and unlike NifA it does not precipitate when overproduced (Hawkes *et al.*, 1985). Comparison of the predicted amino acid sequences of NifA and NtrC led Drummond *et al.* (1986) to propose a three-domain structure for the two proteins, comprising a 120–180 amino acid N-terminal domain followed by a short linker sequence, a central domain of about 240 amino acids and a C-terminal domain of about 70 residues. Whilst the central domains of NifA and NtrC, which may be involved in interaction with RNA polymerase, are highly homologous, both the N-terminal and C-terminal domains are distinctly different. The role of the N-terminus is unknown but it has been proposed that this domain is involved in interaction with the respective partner protein (Drummond *et al.*, 1986; Drummond & Wootton, 1987; Ronson *et al.*, 1987).

The C-terminus in both NifA and NtrC contains a potential HTH motif which is distinctly different in the two proteins, and the second helix of this motif is probably responsible for the DNA-binding specificities of the two proteins (Drummond *et al.*, 1986). As the hybrid proteins synthesized from both pRT904 and pRT905 had a modified C-terminus but retained the insolubility characteristics of wild-type NifA these results suggest that the N-terminal domain of NifA might be the region responsible for the differences in solubility properties between NifA and NtrC.

We thank R. A. Dixon, S. Austin and B. E. Smith for helpful comments on the manuscript and Beryl Scutt for typing it. R. Tuli was supported by an Overseas Fellowship from the National Biotechnology Board of India.

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