Interaction between NifL and NifA in the nitrogen-fixing *Pseudomonas stutzeri* A1501

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Pseudomonas stutzeri strain A1501 isolated from rice fixes nitrogen under microaerobic conditions in the free-living state. This paper describes the properties of *nifL* and *nifA* mutants as well as the physical interaction between NifL and NifA proteins. A *nifL* mutant strain that carried a mutation non-polar on *nifA* expression retained nitrogenase activity. Complementation with a plasmid containing only *nifL* led to a decrease in nitrogenase activity in both the wild-type and the *nifL* mutant, suggesting that NifL acts as an antiactivator of NifA activity. Using the yeast two-hybrid system and purified protein domains of NifA and NifL, an interaction was shown between the C-terminal domain of NifL and the central domain of NifA, suggesting that NifL antiactivator activity is mediated by direct protein interaction with NifA.

INTRODUCTION

Regulation of nitrogen fixation (*nif*) gene expression, in response to the environmental signals ammonium and O₂, depends on the transcriptional activator, NifA, a member of the σ^{54} -dependent family of bacterial activators (Morett & Segovia, 1993). The mode of activation of *nif* gene transcription by NifA appears to be common to a large number of diazotrophs, whereas the regulation of NifA synthesis and activity differs from one organism to another (Merrick, 2004). NifA activity in the gamma subgroup of proteobacteria is controlled by the antiactivator NifL. Indeed, NifA and NifL form an atypical two-component sensor–regulator system, and NifL modulates the activity of NifA by direct protein–protein interaction (Martínez-Argudo *et al.*, 2004). NifA proteins are structurally similar to each other. This enabled the description of NifA as a multidomain protein (Studholme & Dixon, 2003 and references therein). The catalytic domain of NifA, which interacts with the RNA polymerase, shares a high degree of identity with an AAA + -type ATPase. The C-terminal part is the DNA-binding domain and contains an HTH motif. The N-terminal domain carries a GAF domain. The NifL protein resembles histidine kinase protein (HPK), but NifL is not subject to autophosphorylation. The amino-terminal region

contains a PAS domain, and it has been proposed that this region may be involved in O₂ sensing (Zhulin *et al.*, 1997). The C-terminal region shows similarity to the GHKL superfamily of ATPases.

The nitrogen fixation ability within the genus *Pseudomonas* has been questioned for a long time (for reviews see Chan *et al.*, 1994; Lalucat *et al.*, 2006). It is now established that several strains of *Pseudomonas stutzeri* can fix nitrogen (Vermeiren *et al.*, 1999; Rediers *et al.*, 2004). *P. stutzeri* A1501, isolated from rice, fixes nitrogen in the free-living state, under microaerobic conditions in media devoid of ammonia (You *et al.*, 1991; Lin *et al.*, 2000; Desnoues *et al.*, 2003). A 30 kb DNA region containing the nitrogen fixation (*nif* and *rnf*) genes has been previously characterized and the regulatory *nifLA* region mapped within the main *nif* cluster. By using different *lacZ* fusions, it was observed that *nifA* controlled the expression of other *nif* (and *rnf*) operons and that chromosomal *nifLA*–*lacZ* fusion expression was strongly reduced in the presence of oxygen and ammonia (Desnoues *et al.*, 2003). As NtrC and RpoN were also found to control *nifLA* expression (Desnoues *et al.*, 2003), this suggested that the regulation circuitry in *P. stutzeri* resembled more the situation in *Klebsiella pneumoniae* than in *Azotobacter* species, where *nifLA* expression is not impaired in the presence of ammonia (Blanco *et al.*, 1993), although *P. stutzeri* is phylogenetically closer to *Azotobacter* (Rediers *et al.*, 2004) than to *Klebsiella*. The objective of this work was to further document the role of NifLA in *P. stutzeri* A1501. In particular, the properties of a *nifL* mutant strain non-polar on *nifA* expression as well as the physical interaction between NifL and NifA were investigated.

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Abbreviation: GST, glutathione S-transferase.

METHODS

Strains and plasmids. Plasmids and bacterial strains are listed in Table 1. *P. stutzeri* was grown in minimal lactate medium or in LB medium at 30 °C as described by Desnoues *et al.* (2003). The yeast strain used for the two-hybrid analysis was *Saccharomyces cerevisiae* PJ69-4A harbouring three reporter genes, *lacZ*, *ADE2* and *HIS3*, under the control of the *GAL* promoter (James *et al.*, 1996). Yeast cells were grown in YPD rich medium or in SD minimal medium supplemented with appropriate amino acids, at 30 °C, as described in the manual supplied with the Matchmaker Two-Hybrid System (Clontech). Antibiotics were used at the following concentrations (µg ml⁻¹): ampicillin (Amp), 100; kanamycin (Km), 50; tetracycline (Tc), 10.

Nitrogenase activity. Nitrogenase activity was determined with bacterial suspensions incubated at an OD₆₀₀ of 0·1, in N-free minimal lactate medium, at 30 °C, under an argon atmosphere containing 1 % oxygen and 10 % acetylene, according to the derepression protocol described by Desnoues *et al.* (2003). Nitrogenase specific activity is expressed as nmol ethylene min⁻¹ (mg protein)⁻¹. Protein concentrations were determined by the Bio-Rad protein assay (BSA protein standard). Each experiment was repeated at least three times.

Molecular techniques. Plasmid isolation, genomic DNA extraction, gel electrophoresis, restriction mapping, transformation and molecular cloning, Western blotting and amplification by PCR (Amersham kit) were performed by standard methods (Sambrook &

Russell, 2001) or as recommended by the manufacturers of the products used. Restriction enzymes were purchased from Promega and oligonucleotides from Shanghai Biotech Company. Nucleotide sequencing was performed by the Takara Company.

RT-PCR. Total RNA was isolated by acid-phenol extraction and the single-stranded cDNA synthesis was performed using the ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs). RNA (1 µg) isolated from bacteria grown under nitrogen fixation conditions was used for RT-PCR to amplify a 415 bp fragment with primers specific for *nifA*, RT-PCRnifAF and RT-PCRnifAR (Table 2). After 35 cycles of PCR amplification (94 °C for 1 min; 58 °C for 1 min; 72 °C for 1 min), PCR products were separated on 0·8 % agarose gels and visualized by ethidium bromide staining.

Construction of *nifL* mutants. Construction of strain 1507B, a *nifL* deletion non-polar mutant, was similar to strain 1507 previously described (Desnoues *et al.*, 2003), except that it carries the KIXX cassette in the same orientation as the *nifA* gene. A *nifL* polar mutant, designated 1507A, was constructed for this work, as follows. Oligonucleotide primers pL-F and pL-R (see Table 2) were designed to amplify a 1647 bp *Bam*HI–*Hind*III fragment that was cloned at the unique *Bam*HI–*Hind*III sites of the pSUP202 vector. Then, the KIXX cassette was inserted, as a *Sma*I fragment, into the unique *Apa*I site within the *nifL* coding sequence, previously treated with the Klenow fragment of DNA polymerase I to fill in ends. The orientation of the Km resistance gene was the opposite of that of the *nifA* gene. Plasmids were transferred into *P. stutzeri* recipients by conjugation, using *Escherichia coli* S17-1 as the donor, as previously

Table 1. Strains and plasmids

Strain/plasmid	Relevant characteristics	Source/reference
<i>P. stutzeri</i>		
A1501	Wild-type, Chinese Culture Collection: CGMCC 0351	Lin <i>et al.</i> (2000); Desnoues <i>et al.</i> (2003)
1506	<i>nifA</i> - <i>km</i> deletion mutant, Nif ⁻ Km ^r	Desnoues <i>et al.</i> (2003)
1507B	<i>nifL</i> - <i>km</i> deletion mutant, non-polar on <i>nifA</i> expression, Nif ⁺ Km ^r	Nicole Desnoues
1507A	<i>nifL</i> - <i>km</i> mutant, polar on <i>nifA</i> expression, Nif ⁻ Km ^r	This study
<i>S. cerevisiae</i>		
PJ69-4A	<i>MATa trp1-109 leu2-3,112 ura3-52 his3-200 gal4Δ gal80 LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ</i>	James <i>et al.</i> (1996)
Plasmids		
pET-28a(+))	Cloning vector carrying a His ₆ linker for expression and purification of proteins, Km ^r	Novagen
pGAD-C ₁ *	Expression vector for yeast two-hybrid system, <i>GAL4</i> (768–881), <i>LEU2</i> , Amp ^r	James <i>et al.</i> (1996)
pGBD-C ₁ *	Expression vector for yeast two-hybrid system, <i>GAL4</i> (1–147), <i>TRP2</i> , Amp ^r	James <i>et al.</i> (1996)
pGAD-SIP and pBDU-SNF I	Positive control for yeast two-hybrid system, Amp ^r	James <i>et al.</i> (1996)
pGEX-2T	Cloning vector which expresses GST protein, Amp ^r	Pharmacia
pGST-NifLc	pGEX-2T derivative carrying a 1145 bp fragment encoding the C-terminal portion of <i>nifL</i> , Amp ^r	This study
pHis ₆ -NifA	pET28a(+) derivative carrying 1587 bp PCR product of <i>nifA</i> , Km ^r	This study
pSUP202	Suicide vehicle, Ap ^r Tc ^r Cm ^r	Simon <i>et al.</i> (1983)
pUC4-KIXX	Source of <i>km</i> cassette	Pharmacia
pVA1	pVK100 derivative carrying a <i>nifA</i> promoterless gene in opposite orientation of the <i>km</i> gene, Tc ^r	This study
pVA3	As pVA1, but with <i>nifA</i> under the control of the <i>km</i> promoter, Tc ^r	This study
pVK100	RK2 replicon, Tra ⁻ Km ^r Tc ^r	Allen & Hanson (1985)
pVL	pVK100 derivative carrying <i>nifL</i>	This study

*For pGAD and pGBD derivatives containing entire or portions of *nifA* and *nifL*, constructed in this work, see Fig. 2, Table 2 and Table 3.

Table 2. Oligonucleotides primers

Primer	Sequence*	Amplicon size (bp)	Use†
RT-PCRnifA F	5'-ACCGGAAACCTGCTCGAATCGGA-3'	415	<i>nifA</i> transcription
RT-PCRnifA R	5'-GCTTGAGTTTGCAGCCCTGCT-3'		
pL F	5'-CGGGATCCCAACAGCCCCCTAACCGTATG-3'	1647	Construction of 1507A
pL R	5'-CTAAGCTTATCAGCTGGCCGAGAAGGGCA-3'		
pVA F	5'-CCGAAGCTTTCAGATCTTGCATATGA-3'	1634	Construction of pVA1 and pVA3
pVA R	5'-CCGAAGCTTACGGTGCATATCGATAGC-3'		
pVL F	5'-GAATCTCGAGATAGCGCAAC-3'	1899	Construction of pVL
pVL R	5'-ATGCAAGCTTCCCCTGTCA-3'		
pnifA F	5'-CGGGATCCGAAGCTCGCATGAACG-3'	1587	Entire <i>nifA</i> , Y2H and pHis ₆ -NifA
pnifA R	5'-CCATCGATGATCTTGCATATGAATG-3'		
pnifAn F	5'-ATCGAATTCATGAACGCCACATTCGCCG-3'	617	N-terminal portion of <i>nifA</i> , Y2H
pnifAn R	5'-GATAGATCTTTCGCGGCGTAGCTCGTC-3'		
pnifAm F	5'-GAAGGATCCATAGAGGACGGCCAGGAAG-3'	829	Central portion of <i>nifA</i> , Y2H
pnifAm R	5'-CATATCGATACCGGTGAGGGAGACCAC-3'		
pnifAc F	5'-GAAGGATCCAGCAGGGTCGCAAACTC-3'	374	C-terminal portion of <i>nifA</i> , Y2H
pnifAc R	5'-CCATCGATGATCTTGCATATGAATG-3'		
pnifL F	5'-CGGAATTCCCAACAGCCCCCTAACCGT-3'	1644	Entire <i>nifL</i> , Y2H
pnifL R	5'-CGGGATCCGCTGGCCGAGAAGGGCA-3'		
pnifLn F	5'-ATCGAATTCGCTTTGCAACGGATACGG-3'	545	N-terminal portion of <i>nifL</i> , Y2H
pnifLn R	5'-GATAGATCTGCTGTGACCAACCGCCT-3'		
pnifLc F	5'-ATCGAATTCAGCGCGTCAGCAACCAG-3'	1145	C-terminal portion of <i>nifL</i> , Y2H and pGST-NifLc
pnifLc R	5'-GATAGATCTGCTGGCCGAGAAGGGCAGTTC-3'		

*Restriction sites are underlined.

†Y2H: amplicons were cloned in pGAD and pGBD vectors for the yeast two-hybrid experiments; see Table 3 and Fig. 2.

described (Desnoves *et al.*, 2003). Recombination in the host genome at the correct location was checked for by PCR amplification with appropriate oligonucleotide primers.

Construction of *nifL*- and *nifA*-containing plasmids.

Oligonucleotides flanking the coding region of *nifA* and *nifL* were designed (see Table 2) to amplify the corresponding genes by PCR. For *nifA*, a 1634 bp promoterless DNA fragment was amplified and cloned as a *Hind*III fragment into the Km resistance gene of pVK100 under the control of the *km* promoter for pVA3 and in the opposite orientation for pVA1. For *nifL*, the amplified 1899 bp fragment contained 239 bp of the non-coding sequence; it was cloned as a *Hind*III-*Xho*I fragment into the Km resistance gene of pVK100 in the same orientation as the Km resistance gene, to yield pVL.

Yeast two-hybrid analysis. The oligonucleotides designed to amplify the fragments encoding the desired domains of NifA and NifL are listed in Table 2. PCR amplification of strain A1501 genomic DNA was performed with the Proof Start DNA polymerase (Qiagen) and the resulting amplicons were cloned in-frame into pGBD-C₁ or pGAD-C₁ vectors (Table 1). The fusion junctions were verified in all the constructed plasmids by nucleotide sequence analyses. *S. cerevisiae* competent cells were prepared using the method described in the Matchmaker II protocol and pGAD and pGBD derivatives were co-transformed into the yeast recipient. The interaction between NifA and NifL domains was screened for by growth on SD medium lacking Leu, His, Trp and Ade (Table 3). The filter and quantitative liquid β -galactosidase assay were done according to the protocol described by the Matchmaker II system (Clontech). Activity is expressed as Miller units (Miller, 1972).

Expression and purification of NifL and NifA fusion proteins.

A1501 *nifA* was amplified by PCR using oligonucleotides pnifA F and R (Table 2) and the amplicon was cloned into pET28a(+) vector (Novagen) to generate a His₆-tagged NifA fusion protein. Similarly, oligonucleotides pnifL-C, F and R were used to amplify the C-terminal portion of *nifL*, which was constructed to fuse in-frame with GST (glutathione S-transferase) in the pGEX-2T vector (Pharmacia). Cultures containing the recombinant plasmids were grown aerobically in LB broth and expression from the T7 promoter was induced by addition of 1 mM IPTG. His₆-NifA fusion protein was purified by nickel affinity chromatography on Hi-trap chelating columns (Pharmacia) and GST-NifLc fusion protein was purified by batch elution from affinity glutathione-Sepharose 4B beads (Pharmacia) as recommended by the manufacturer. SDS-PAGE of protein samples was performed by standard methods (Sambrook & Russell, 2001).

Analysis of protein complexes.

The pull-down assay was performed according to the method of Hasan *et al.* (2004). Purified GST-NifLc (10 ng) was immobilized on glutathione-Sepharose 4B beads and washed thoroughly with wash buffer (20 mM Tris/HCl pH 7.5, 2 mM EDTA, 100 mM NaCl, 12 mM β -mercaptoethanol and 0.1% Triton X-100). Immobilized GST-NifLc was then incubated with the purified His₆-NifA for 1 h at 4 °C. After washing, bound proteins were eluted in SDS-PAGE sample buffer and separated by 8% SDS-PAGE. Western blotting with anti-histidine or anti-GST antibodies (Invitrogen) was performed by standard methods (Sambrook & Russell, 2001).

Table 3. Interaction between NifL and NifA detected by the yeast two-hybrid system

Plasmid combination	Growth on selective media*	β-Galactosidase activity† (Miller units)	Interacting peptides
pGAD-SIP and pGBDU-SNF I	+ + + +	57.6 ± 1.5	Positive control
pGAD-C ₁ and pGBD-C ₁	—	2.7 ± 0.3	Negative control
pGAD- <i>nifA</i> and pGBD- <i>nifL</i>	+ +	23.6 ± 1.1	NifL and NifA
pGAD- <i>nifL</i> and pGBD- <i>nifA</i>	+ +	25.3 ± 2.3	NifL and NifA
pGAD- <i>nifA</i> and pGBD- <i>nifLn</i> (N-terminal domain)	—	4.1 ± 0.3	None
pGAD- <i>nifA</i> and pGBD- <i>nifLc</i> (C-terminal domain)	+ +	22.9 ± 1.3	NifL C-terminal domain and NifA
pGAD- <i>nifL</i> and pGBD- <i>nifAn</i> (N-terminal domain)	—	5.3 ± 0.5	None
pGAD- <i>nifL</i> and pGBD- <i>nifAm</i> (central domain)	+ +	18 ± 2.7	NifL and NifA central domain
pGAD- <i>nifL</i> and pGBD- <i>nifAc</i> (C-terminal domain)	—	3.15 ± 0.7	None
pGAD- <i>nifLc</i> (C-terminal domain) and pGBD- <i>nifAm</i> (central domain)	+ +	12.0 ± 2.4	NifL C-terminal domain and NifA central domain
pGAD- <i>nifAm</i> (central domain) and pGBD- <i>nifLc</i> (C-terminal domain)	+ +	13.1 ± 1.6	NifA central domain and NifL C-terminal domain
pGAD- <i>nifA</i> and pGBDC ₁	—	4.4 ± 0.4	None
pGAD- <i>nifL</i> and pGBDC ₁	—	3 ± 0.2	None

*Growth was assayed on three different media: SD lacking Leu, Trp and His; SD lacking Leu, Trp and Ade; and SD lacking Leu, Trp, His and Ade and containing X-Gal. + indicates that growth was observed on all three media and that colonies were blue on the X-Gal plates; — indicates that growth was not observed on any of the three media tested.

†β-Galactosidase activities are the means ± SD of three determinations.

RESULTS

Properties of a *nifL* mutant strain non-polar on *nifA* expression and effect of overexpression of NifA and NifL

A *nifA* mutant, as well as a *nifL* mutant polar on *nifA* expression, was previously shown to be devoid of nitrogenase activity (Desnoues *et al.*, 2003). To further investigate the role of NifL in the regulation of *nif* gene expression in *P. stutzeri*, we used strain 1507B, in which most of the *nifL* coding sequence (1506 bp *Clal* fragment) was substituted by a KIXX cassette cloned in the same orientation as the *nifA* gene. The non-polar effect of the *nifL* mutation on *nifA* expression was first checked by RT-PCR. A *nifA* transcript was indeed detected, both in the wild-type and in the 1507B *nifL* mutant, while no *nifA* transcript was produced in the *nifL* polar mutant constructed for this work, 1507A (Fig. 1a). The lack of *nifA* transcript in the *nifL* mutant 1507A strongly suggested that, as in other nitrogen fixers containing a *nifL* gene, *nifA* and *nifL* were transcribed from the same promoter in strain A1501. In addition, in strain 1507B the presence of *nifA* transcript was consistent with *nifA* transcription from the *km* promoter of the cassette.

The *nifL* non-polar mutant strain 1507B displayed residual nitrogenase activity, up to 30 % of that of the wild-type strain (Fig. 1b). It was then possible to investigate the effect of the increased production of NifA and NifL on nitrogenase activity by complementation analysis with plasmids expressing *nifA* or *nifL*. Expression of *nifA* in pVA3 was monitored

from the *km* promoter of the vector. Indeed, pVA3 restored 30 % nitrogenase activity to the *nifA-km* mutant (1506) and *nifL-km* polar (1507A) strains (Fig. 1b), but the complementation reached up to 60 % when kanamycin was added to the culture medium (not shown). As expected, pVA1, in which *nifA* was cloned in the opposite orientation, did not affect nitrogen fixation (Fig. 1b). Plasmid pVA3 also increased nitrogenase activity of the wild-type and of the *nifL* non-polar mutant 1507B (Fig. 1b). In contrast, plasmid pVL, which carries the *nifL* gene, decreased nitrogenase activity of the wild-type and of the *nifL* non-polar mutant (Fig. 1b). Thus, NifL negatively modulates NifA activity. This is consistent with a role for NifL as NifA antiactivator.

Physical interaction between NifL and NifA *in vivo*

Deduced translation products of A1501 NifA and *Azotobacter* NifA share 82 % identity, suggesting that the domain structure of the *P. stutzeri* protein is very similar to that defined in *Azotobacter* NifA, and hence in other NifAs (Studholme & Dixon, 2003). This enabled us to design appropriate oligonucleotides that could amplify portions encoding the N-terminal (NifAn), central (NifAm) and C-terminal (NifAc) domains, respectively, as schematized in Fig. 2. The same was applied to NifL, which shared 72 % identity with *Azotobacter* NifL. In that case, the *nifL* gene was divided into only two regions, encompassing the N-terminal PAS (NifLn) and the C-terminal (NifLc) domains, respectively (Fig. 2). Each of the domains was cloned into the yeast pGAD vector, which carries the activating domain of the yeast *GAL4* transcriptional activator and into the pGBD

Fig. 1. Expression of *nifA* and nitrogenase activity in A1501 wild-type and mutant strains. (a) Amplification of *nifA* transcript by RT-PCR. Lanes: L, DNA size marker; 1, wild-type; 2, 1507B non-polar *nifL* mutant; 3, 1507A *nifL* polar mutant. The arrow indicates the 415 bp amplification product of *nifA*. (b) Nitrogenase activity in wild-type and mutant strains complemented with plasmids carrying *nifA* and *nifL* and incubated under nitrogen-fixation conditions: A1501, wild-type; 1507B, *nifL*-*km* non-polar mutant; 1507A, *nifL*-*km* polar mutant; 1506, *nifA*-*km* mutant. pVA3 contains *nifA* under the control of the *km* promoter; pVA1 contains *nifA* in the opposite orientation; pVL contains *nifL*.

vector, which carries the DNA-binding domain of *GAL4*. After co-transformation into yeast strain PJ69-4A, an interaction between the peptides fused to the pGAD and pGBD vectors is required so that the transcription from the promoters under the control of *GAL4* can proceed (Table 3). As one of the reporter genes is *lacZ*, an enzymic assay for β -galactosidase gives an estimation of the relative strength of the interaction (James *et al.*, 1996). Fig. 3 shows an example of the growth on selective media and Table 3 summarizes the data obtained for growth and β -galactosidase activity. From these data it is concluded that an interaction is detected between the entire NifA and NifL,

and that the binding is limited to the C-terminal part of NifL and the central domain of NifA.

Physical interaction between NifLc and NifA *in vitro*

To further confirm the interaction between NifLc and NifA, the corresponding DNA fragments were cloned into

Fig. 2. Schematic representation of the domains of NifL (top) and NifA (bottom). See text for explanations. The oligonucleotides designed to amplify the gene portions encoding the different domains are listed in Table 3.

Fig. 3. Specificity of interaction between NifL and NifA. *S. cerevisiae* PJ69-4A strains carrying different plasmid combinations were grown on SD medium lacking Leu, Trp, His and Ade and containing X-Gal, and the colony filter-lift assay in the presence of X-Gal was performed according to the protocol of the Matchmaker manual: 1, positive control; 2, pGAD-*nifAc* (C-terminal)+pGBD-*nifL* (entire *nifL* coding region); 3, pGAD-*nifA* (entire *nifA* coding region)+pGBD-*nifL* (entire *nifL* coding region); 4, pGBD-*nifAn* (N-terminal)+pGBD-*nifL* (entire *nifL* coding region); 5, pGBD-*nifAm* (central domain)+pGBD-*nifLc* (C-terminal); 6, negative control (pGAD-C1, pGBD-C1).

expression vectors to obtain a GST-NifLc and a His₆-NifA fusion protein as described in Methods. After purification and SDS-PAGE analysis, the GST-NifLc product had an apparent molecular mass in the range 67 kDa, consistent with the size of the NifL C-terminal part, 40·7 kDa (from residues 162–537; Fig. 2), and the GST moiety of 27 kDa. The migration of the His₆-NifA product, estimated at 64·2 kDa, was similar to that of the GST-NifLc fusion (Fig. 4). However, the two fusion proteins could be easily differentiated using anti-histidine or anti-GST antibodies after Western blotting, as shown below.

The result of NifLA complex formation and elution using the pull-down assay is shown in Fig. 4. Lanes 1 and 3 correspond to control experiments performed only with NifA (lane 1) or with NifLc (lane 3), while lane 2 contained the elution product after the complex formation between NifLc and NifA. Fig. 4(a) shows protein staining. Anti-GST antibody, shown in Fig. 4(b), revealed material containing the GST-NifLc in lane 3 but not in lane 1, whereas anti-His antibody, in panel (c), revealed material containing His₆-NifA in lane 1 but not in lane 3. Both antibodies revealed a protein product in lane 2 (Fig. 4b, c), suggesting that it corresponded to a mixture of NifA and NifLc. This is strongly in favour of complex formation between NifL and NifA, suggesting a specific binding between NifA and NifLc.

DISCUSSION

It was previously established that a *nifL* mutant polar on *nifA* expression led to a Nif[−] phenotype in *P. stutzeri* A1501 (Desnoues *et al.*, 2003). In this report, assay for nitrogenase activity of the *nifL* non-polar mutant 1507B showed it displayed a significant residual activity, suggesting that NifL is not an essential product for nitrogen fixation in strain A1501. This result is similar to that reported first in the case of *K. pneumoniae* (see Filser *et al.*, 1983) and subsequently found in other strains containing a *nifL* gene, such as *Azotobacter vinelandii* (Blanco *et al.*, 1993) and *Azoarcus* sp. (Egener *et al.*, 2002). In addition, the decrease of nitrogenase activity observed both in the wild-type and in the *nifL* non-polar mutant in the presence of NifL excess produced by plasmid pVL (Fig. 1) is consistent with previous observations in *K. pneumoniae* (Buchanan-Wollaston *et al.*, 1981). Thus, NifL most likely acts in modulating NifA activity in

A1501, and this suggests that NifL is an antiactivator of NifA activity.

The yeast two-hybrid system is a useful technique to detect protein–protein interaction (James *et al.*, 1996). It has been successfully used to explore binding between several components of the *ntr* and *nif* regulatory systems (Lei *et al.*, 1999; Martínez-Argudo *et al.*, 2002; Rudnick *et al.*, 2002; Pawlowski *et al.*, 2003; Chen *et al.*, 2005). Thus, a direct protein–protein binding was detected between NifA and NifL in *K. pneumoniae*, *Azotobacter vinelandii* and *Enterobacter cloacae* (Lei *et al.*, 1999; Martínez-Argudo *et al.*, 2002; Liao *et al.*, 2002). Results reported in *P. stutzeri* reveal also a direct protein–protein interaction between NifA and NifL and more precisely between the NifL GHKL (NifLc) and NifA AAA+ (NifAm) domains. Indeed, NifL PAS domain (NifLn) does not display binding activity to NifA (Table 3).

A prerequisite to further study of the mechanisms of interaction between NifL and NifA is to obtain the protein products in a soluble form (Lee *et al.*, 1993). In this work we used convenient expression vectors to overproduce His-tagged NifA and GST fusion to NifLc. This enabled a rapid purification of both proteins and allowed us to demonstrate *in vitro* complex formation between NifLc and NifA (Fig. 4). This is consistent with the *in vivo* binding observed. It also suggests that under conditions not compatible with nitrogen fixation, inactivation of NifA is probably due to a protein complex between NifL and NifA so that *nif* genes cannot be transcribed and hence nitrogenase cannot be synthesized.

Mechanisms by which NifL and NifA modulate *nif* gene expression have been mainly studied in *K. pneumoniae* and *A. vinelandii*. The activity of NifLA complexes is modulated by GlnK (a PII paralogue protein), ATP/ADP ratio and 2-oxoglutarate, but it is clear that mechanisms of signal communication between NifA and NifL are different in these two species (Martínez-Argudo *et al.*, 2004). In both systems, GlnK, which senses the nitrogen status of the cell, was shown to interact with the NifLA via direct protein–protein interaction (Stips *et al.*, 2004; Martínez-Argudo *et al.*, 2004). In the case of *Azotobacter*, when nitrogen is limiting, GlnK, in its uridylylated form, does not interact with NifL, and thus NifL does not antagonize NifA activity

Fig. 4. Analysis of protein complexes after elution from the Sepharose column. (a) SDS-PAGE. (b) Western blot with GST-Tag antibody. (c) Western blot with His-Tag antibody. Lanes: 1, control His₆-NifA; 2, His₆-NifA + GST-NifLc; 3, control GST-NifLc.

(Martínez-Argudo *et al.*, 2004). In contrast, GlnK is required for the relief of NifL inhibition in *K. pneumoniae* (He *et al.*, 1998; Stips *et al.*, 2004). In the present report, we have limited our investigation to the demonstration of an interaction between NifL and NifA, using both *in vivo* and *in vitro* techniques. It is not known whether a PII protein is involved in the modulation of NifLA activity. *P. stutzeri* A1501 carries a single copy of a *glnB*-like gene (this laboratory, unpublished observation), as in *Azotobacter*. The inactivation of the *glnB*-like gene and the purification of its protein product is in progress. Although *P. stutzeri nifL* is not an essential gene for nitrogen fixation, the NifL protein plays a regulatory role as antiactivator of NifA activity. The purification of both NifA and NifL proteins opens interesting perspectives to further study the mechanisms of interaction of NifL and NifA in this species.

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