Expression and functional analysis of an N-truncated NifA protein of *Herbaspirillum seropedicae*

R.A. Monteiro, E.M. Souza, S. Funayama[1], M.G. Yates, F.O. Pedrosa, L.S. Chubatsu*

Department of Biochemistry, Universidade Federal do Paraná, CP 19046, Curitiba PR 81531-990, Brazil

Received 17 February 1999

Abstract **In *Herbaspirillum seropedicae*, an endophytic diazotroph, *nif* gene expression is under the control of the transcriptional activator NifA. We have over-expressed and purified a protein containing the central and C-terminal domains of the *H. seropedicae* NifA protein, N-truncated NifA, fused to a His-Tag sequence. This fusion protein was found to be partially soluble and was purified by affinity chromatography. Band shift and footprinting assays showed that the N-truncated NifA protein was able to bind specifically to the *H. seropedicae nifB* promoter region. In vivo analysis showed that this protein activated the *nifH* promoter of *Klebsiella pneumoniae* in *Escherichia coli* only in the absence of oxygen and this activation was not negatively controlled by ammonium ions.**

Key words: NifA protein; Transcriptional activator; Nitrogen fixation; *Herbaspirillum seropedicae*

1. Introduction

Herbaspirillum seropedicae is an endophytic diazotroph found in association with important agricultural crops [1]. It is classified by rDNA analysis as a member of the β-subclass of Proteobacteria [2]. Nitrogen fixation in this organism occurs under microaerobic conditions and the expression of the nitrogenase structural genes *nifHDK* as well as the *nifB* gene is under control of the transcriptional activator NifA [3–5].

The NifA protein activates the transcription of σ^N-dependent *nif* and other promoters [6] and it also activates the *Rhizobium meliloti* and *Bradyrhizobium japonicum fixABCX* genes [7,8]. The NifA proteins have a modular structure typical of σ^N-dependent promoter-activating proteins. The N-terminal domain is involved in regulatory functions and has a low similarity among NifA proteins. The central domain has two potential nucleotide triphosphate-binding sites as revealed by sequence analysis and is responsible for interaction with the σ^N-RNA polymerase complex [6]. The C-terminal domain contains a helix-turn-helix motif probably involved in DNA-binding. These domains are usually linked by the short interlinker sequences Q (N-terminal to central) and ID (central to C-terminal).

Klebsiella pneumoniae and *Azotobacter vinelandii* NifA proteins are insensitive to oxygen and ammonia and they are regulated by the NifL protein. In *H. seropedicae* however, the *nifL* gene has not been detected and NifA apparently responds directly to oxygen and ammonia. The control of the NifA protein activity may involve the PII protein [9]. Oxygen sensitivity may be related to a cysteine motif (Cys-X_{11}CysX$_{19}$CysX$_4$Cys) present in the central domain and the ID linker [6].

Studies of the NifA protein in vitro have been made difficult because of the insolubility of the over-expressed proteins. So far, only the NifA proteins of *K. pneumoniae* and *A. vinelandii*, organisms of the γ-subclass of Proteobacteria, have been purified and analyzed in vitro. Both proteins are insensitive to oxygen and regulated by NifL [10–13]. The *K. pneumoniae* NifA protein was expressed in separate domains as fusions to the maltose-binding protein. These fusion proteins were more soluble than the native protein or the single domains. The expressed proteins were capable of in vitro DNA-binding and transcriptional activation [10–12]. The *A. vinelandii* NifA, however, was purified in its native form and showed DNA-binding, transcriptional activation and inhibition by purified NifL in vitro [13].

In this work, we describe the expression, purification and both in vivo and in vitro characterization of the linked central and C-terminal domains of the NifA protein, an oxygen-sensitive protein of *H. seropedicae*, a member of the β-subclass of Proteobacteria.

2. Materials and methods

2.1. Expression and purification of H. seropedicae His-tagged N-truncated NifA

The sequence encoding the central and C-terminal domains of the NifA protein was PCR-amplified using the primers HSNIFA4 (5′-TCGGATCCGTAATCGGC) and HSNIFA2A (5′-AGAGATCCTA-GAACTTCTTGACCTCG) corresponding to positions 1374 and 2386 of the *nifA* gene [3]. These primers introduced *Bam*HI restriction sites flanking the sequence. The amplified fragment corresponds to an N-truncated NifA protein lacking the first 203 amino acid residues.

The amplified DNA fragment was subcloned into the *Bam*HI site of the expression vector pET28-a (Novagen). The cloned fragment was completely sequenced to confirm that no mutation was introduced during the amplification procedure. The recombinant plasmid obtained, named pRAM2, was introduced into *E. coli* strain BL21(DE3)pLysS and, upon induction, produced an N-truncated NifA protein with a His-tag sequence in its N-terminal portion. The His-tagged N-truncated NifA was over-expressed after induction with 0.5 mol/l IPTG or 0.5% (w/v) lactose for 3 h at 30°C in LB medium. The cells were harvested by centrifugation and the pellet was resuspended in 1 ml of TP1 buffer (50 mmol/l Tris-Cl pH 8.0, 500 mmol/l NaCl, 5% glycerol, 1 mmol/l DTT, 20 mg/ml of lysozyme) per gram of wet cells. The cells were then lysed by sonication and the crude extract was centrifuged at 13 000 × *g* for 10 min at 4°C. The supernatant, containing the His-Tag-N-truncated-NifA, was loaded onto a Hi-Trap-Chelating-Ni^{2+} affinity column (Pharmacia). The column was

*Corresponding author. Fax: (55) (41) 266 2042.
E-mail: chubatsu@bio.ufpr.br

[1]present address: Universidade Tuiuti do Paraná, Curitiba, PR, Brazil.

Abbreviations: DTT, dithiothreitol; ONPG, *o*-nitrophenyl-galactoside; IPTG, isopropyl β-D-thiogalactopyranoside; DMS, dimethyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MW, molecular weight markers

PII: S0014-5793(99)00314-2

washed by a stepwise increase in imidazole concentration (10, 20, 30, 40, 50 mmols/l) in TP2 buffer (50 mmol/l Tris-Cl pH 6.3, 500 mmol/l NaCl) using 10 volumes of 10 mmol/l imidazole and 5 volumes of the other concentrations. The column was then washed with five column volumes of TP3 buffer (50 mmol/l Tris-Cl pH 8.0, 100 mmol/l KCl, 10% glycerol, 1 mmol/l DTT) containing 50 mmol/l of imidazole. The His-Tag-N-truncated protein was eluted with one–two volumes of TP3 buffer containing 100 mmol/l of imidazole.

2.2. Protein analysis

The protein quantification was by the Bradford method [14] using bovine serum albumin as the standard. The protein purity was analyzed by SDS-PAGE [15] and the proteins were identified by staining with Coomassie blue R-250. The β-galactosidase activity was determined using ONPG as described by Miller [16].

2.3. Band shift assay

Binding of the N-truncated NifA to the *H. seropedicae nifB* promoter region was assayed in vitro as described [17]. A 345 bp DNA fragment corresponding to region 2510 and 2855 of the *H. seropedicae nifB* promoter [3] was end-labelled with ^{32}P using T4 polynucleotide kinase [18].

Each reaction mixture contained 6×10^{-14} mol of ^{32}P-labelled *nifB* promoter region, 2×10^{-12} mol of the N-truncated NifA protein and the indicated amounts of calf thymus DNA or unlabelled *nifB* promoter in a DNA-binding buffer (10 mmol/l Tris-Acetate pH 8.0, 8 mmol/l $MgCl_2$, 10 mmol/l potassium acetate, 1 mmol/l DTT and 3.5% (w/v) PEG 8000) in a total volume of 15 μl. The reaction was incubated for 10 min at room temperature (approximately 20°C). After incubation, Ficoll (final concentration of 6%, w/v) was added and the whole reaction was loaded onto a 4% non-denaturing polyacrylamide gel in TAE buffer [18] with 8 mmol/l $MgCl_2$. The electrophoresis was at 60 V for 2 h at 4°C. Autoradiographs were developed on X-ray films. Densitometer analyses were carried out using Personal Densitometry SI of Molecular Dynamics.

2.4. Footprinting assays

These were conducted as described [19,20]. The reaction mixtures had the same composition as those for the band shift experiments. After 10 min incubation, DMS (0.05% final concentration) was added, incubated for a further 5 min and the reaction was stopped by adding 5 mmol/l ammonium acetate, 1 mol/l β-mercaptoethanol and 10 μg/ml yeast tRNA (final concentrations). The reaction mixtures were precipitated with ethanol, washed with 95% ethanol and dissolved in 1 mol/l piperidine (30 μl). The DNA was cleaved at methylated guanine residues by heating at 90°C for 20 min, lyophilized twice and dissolved in formamide dye. The products were analyzed in 6% polyacrylamide sequencing gels [18]. Autoradiographs were developed on X-ray films.

2.5. Transcriptional activation of a nifH::lacZ fusion

E. coli JM109(DE3) transformants carrying plasmids pRT22 (*K. pneumoniae nifH::lacZ* [21]) and/or pET28-a (Novagen), pRAM1 (His-tag NifA protein) or pRAM2 (His-tag N-truncated NifA protein) were grown overnight in NFDM medium [22] containing 20 mmol/l NH_4Cl, 5% LB medium and antibiotics as indicated, at 30°C. The cultures were diluted to an OD_{600} of 0.13 in NFDM medium plus 5 μg/ml thiamine, antibiotics and 0.5 mmol/l IPTG. The cell suspensions were incubated for 8 h at 30°C in the presence or absence of 20 mmol/l NH_4Cl, under air or N_2, and assayed for β-galactosidase activity.

3. Results and discussion

Attempts to purify NifA proteins from several microorganisms have been unsuccessful due to the insolubility of the over-expressed protein [21,6]. The *H. seropedicae* NifA protein also aggregated when over-expressed in *E. coli* as a fusion protein either with glutathione S-transferase or a His-Tag sequence (Monteiro and Chubatsu, unpublished results). Since *H. seropedicae* NifA has domains structurally similar to those of *K. pneumoniae* NifA which were partially soluble when expressed separately as fusion proteins [10–12], we constructed a recombinant plasmid capable of expressing a N-truncated form of the NifA containing the central domain, the ID linker and the C-terminal domain of *H. seropedicae* fused to a hexahistidine tag at its N-terminus.

The N-truncated NifA protein was tested in vivo for the capacity to activate the expression of the *K. pneumoniae nifH* promoter (*nifH::lacZ* fusion). *E. coli* strain JM109(DE3) transformants, hosting plasmids pET28-a (vector), pRAM1 (His-Tag-NifA), pRAM2 (His-Tag-N-truncated NifA) or pRT22 (*nifH::lacZ*) alone or in combinations were analyzed for their β-galactosidase activity after induction in the presence or absence of oxygen or NH_4Cl (Table 1). The His-tagged NifA protein (pRAM1) was unable to activate the *K. pneumoniae nifH::lacZ* fusion under the assay condition, confirming results by Souza et al. [23]. A β-galactosidase activity was only detected when pRAM2 was present, indicating that transcriptional activation was due to the expressed N-truncated NifA. SDS-PAGE analysis confirmed the expression of the proteins in all conditions tested (not shown). Promoter activation as revealed by β-galactosidase activity was detected only when cells were induced under a nitrogen gas phase, confirming the oxygen-sensitivity of *H. seropedicae* NifA [23]. These results are also consistent with the hypothesis that the oxygen-sensitivity does not involve the N-terminal domain of NifA but rather the central domain and the ID linker, probably involving the putative metal-binding and redox status-sensing cysteine motif, Cys-X_{11}-Cys-X_{19}-Cys-X_4-Cys [6]. On the other hand, *nifH* expression was not inhibited by ammonia. This result also agrees with the previous observation [23] that the N-terminal domain of *H. seropedicae* NifA is involved in ammonia-sensing. The N-terminal domain of the NifA protein may interact with a nitrogen-sensing protein

Table 1
Transcriptional activation of *K. pneumoniae nifH*::*lacZ* by *H. seropedicae* N-truncated NifA

Plasmids	Characteristic	β-Galactosidase activity (Miller units)			
		$+NH_4^+/+O_2$[a]	$-NH_4^+/+O_2$	$+NH_4^+/-O_2$[a]	$-NH_4^+/-O_2$
pET28a	Expression vector	1	2	4	3
pRAM1	His-Tag-NifA	1	0	3	1
pRAM2	His-Tag-N-truncated NifA	0	2	0	5
pRT22	*nifH::lacZ*	6	9	0	8
pET28a/pRT22		2	0	6	0
pRAM1/pRT22		6	9	7	10
pRAM2/pRT22		3	8	867	1135

JM109 (DE3) cells with plasmids were induced with 0.5 mmol/l IPTG for 8 h at 30°C in the presence or absence of 20 mmol/l NH_4Cl, under air or N_2 and assayed for β-galactosidase activity. The data are averages of five experiments, with S.D.s of less than 10%.
[a]There was not a significant decrease in the NH_4Cl concentration during the induction time.

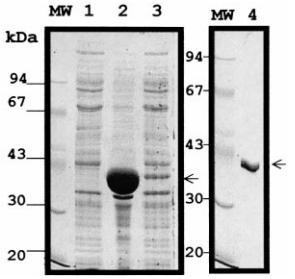

Fig. 1. His-tagged-N-truncated NifA protein in 10% SDS-PAGE. BL21(DE3)pLysS-transformed cells were induced with IPTG. Lane 1: soluble fraction from the pET28-a transformant that does not express the N-truncated NifA protein. Lanes 2 and 3: insoluble and soluble fractions from the pRAM2 transformant, respectively. Lane 4: affinity-purified HisTag-N-truncated NifA. Proteins were stained with Coomassie blue. Arrows indicate the over-expressed His-Tag-N-truncated NifA protein. Molecular weight markers are indicated in kDa.

or signal molecule which, in turn, controls the interaction between the central domain and the RNA polymerase. In *K. pneumoniae* and *A. vinelandii*, NifL senses the cellular redox and ammonia status indirectly, inhibiting the NifA activity possibly by an interaction with the central domain of the NifA protein [24,25]. In *H. seropedicae* and *Azospirillum brasilense* where NifL has not been found, regulation of the NifA activity by the ammonia may involve the PII protein, the product of the *glnB* gene [9,26] or possibly the PII paralogue, the GlnK protein.

Since an active N-truncated NifA protein was expressed in vivo, a procedure to purify and analyze it in vitro was developed. A high level of N-truncated NifA expression was obtained after induction of the promoter by IPTG (Fig. 1) and, although most of the expressed protein was in an insoluble form, a fraction (5%) remained in the supernatant after low speed centrifugation. Induction with 0.5% lactose yielded essentially the same amount of the protein as 0.5 mmol/l IPTG, however, a higher percentage of soluble protein was obtained following induction by lactose (up to 40%). Presumably when induced by IPTG, the protein does not fold correctly and aggregates, whereas lactose, producing a slower induction, allows correct folding. The soluble His-Tag-N-truncated NifA protein was purified by affinity chromatography. Changes in the buffer composition such as ionic strength, salt, presence or absence of DTT or glycerol and different concentrations of imidazole were made to eliminate contaminant proteins non-specifically bound to the Hi-Trap-Chelating-Ni^{+2} column. Heparin sepharose and ionic resins (SP-Sepharose and Q-Sepharose) were also tested but did not improve the purification. The conditions described here were essential to yield a highly purified protein as detected by SDS-PAGE (lane 4, Fig. 1).

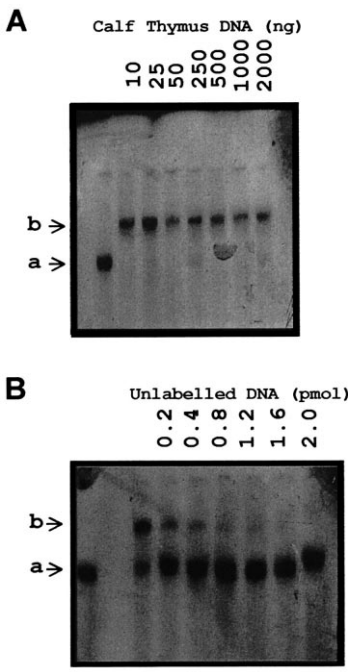

Fig. 2. Band shift assay with the purified His-Tag-N-truncated NifA protein and a 345 bp *nifB* promoter region of *H. seropedicae*. A shows the effect of increasing amounts of calf thymus DNA on the DNA-binding of N-truncated NifA protein. B shows the effect of increasing amounts of unlabelled DNA. Arrow a indicates free DNA, arrow b indicates protein bound DNA.

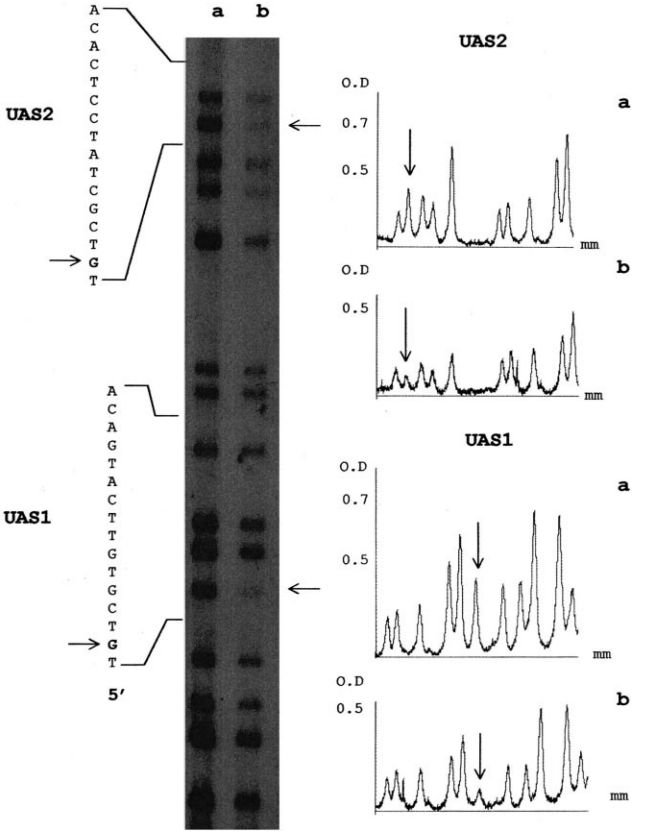

Fig. 3. DMS footprinting analysis of the N-truncated NifA protein-binding to the *H. seropedicae nifB* UAS sequences. The protection of the two UAS sequences from cleavage with piperidine are shown. Methylation was performed in vitro in the absence (lane a) or in the presence (lane b) of the purified protein. On the left, the two UAS sequences are indicated. Densitometric profiles are shown on the right. Arrows indicate the G residues protected in lane b.

The purified N-truncated NifA protein was assayed in vitro for DNA-binding activity. The protein bound to a ^{32}P-labelled 345 bp DNA fragment of the *H. seropedicae nifB* promoter region [3], as revealed by a decrease in the migration rate of the DNA fragment (Fig. 2A). In this experiment, the binding of the DNA was observed using a molar protein/

DNA ratio of about 30 and the complex was stable even in the presence of a high excess of calf thymus DNA (2 μg, about 150 times excess in mass). The protein/DNA ratio needed to observe a minimal DNA-binding varied with the protein preparation, suggesting that some preparations were partially inactive. Competition with the same unlabelled *nifB* promoter DNA fragment titrated out the radioactively labelled DNA (Fig. 2B). Densitometric analysis showed a decrease of 77% of the N-truncated-NifA protein-[^{32}P]*nifB* complex when 0.2 pmol (three times excess) unlabelled *nifB* DNA was present. These results indicate that the purified protein binds specifically to the *nifB* promoter region. The N-truncated NifA also bound the *nifH* promoter region of *R. meliloti* in the presence of calf thymus DNA (data not shown).

The *nifB* promoter region has two upstream activator sites (UAS), by sequencing analysis [3]. Both UAS sites were recognized by *H. seropedicae* N-truncated-NifA protein in vitro (Fig. 3). The guanine residues of the TGT motifs of both NifA-binding sites were protected from DMS-dependent methylation by the N-truncated NifA protein, similar to that observed in vivo with the *K. pneumoniae* NifA protein [5]. This result showed that the N-truncated NifA protein binds to both UAS sites present in the *nifB* promoter region, indicating that the N-truncated form of *H. seropedicae* NifA is capable of recognizing and binding the NifA-binding sites in vitro.

The DNA-protein interaction experiments as well as the protein purification were performed under air, with no precautions to exclude oxygen. Since in vivo experiments have shown that the *H. seropedicae* NifA protein is oxygen-sensitive ([23], Table 1), the observed DNA-binding might have been due to a transcriptionally inactive protein or that oxygen damage was partial and only a functionally active population of N-truncated NifA bound to the *nifB* promoter. DNA-binding of a functionally inactive protein would be consistent with the hypothesis that the C-terminal domain binds DNA and the oxygen-sensitivity is associated with the cysteine motif located in the central domain and ID linker and may not interfere with the DNA-binding activity. Attempts to visualize in vitro open complex formation [27] of the *nifB* promoter catalyzed by the purified N-truncated NifA were unsuccessful (data not shown), which suggests that this protein was transcriptionally inactive, although, capable of binding DNA. Another hypothesis is that the NifA protein requires another protein or co-factor for oxygen-sensibility in vivo, suggesting that the protein may be oxygen-sensitive in vivo but not in vitro at least for DNA-binding. If so, this factor is present in *E. coli*. We have also observed that the DNA-binding activity was lost when the protein was stored in liquid nitrogen (not shown) suggesting that the DNA-binding activity is unstable to freezing. Attempts to purify the His-Tag-N-truncated NifA protein under anaerobic conditions have been unsuccessful to date. The oxygen effect on the protein needs further investigation.

Recently, Passaglia and co-workers [28] published a report that crude extracts of *A. brasilense* cells, expressing a GST-NifA fusion protein, was capable of binding to the *A. brasilense nifH* promoter in the presence of oxygen. However, no transcription activation or DNA-binding was reported for the purified protein.

This report is the first to describe an in vitro analysis of a purified oxygen-sensitive NifA protein. Our results showed DNA-binding activity of the expressed protein as well as the functional significance of the *nifB* promoter NifA-binding regions. They also confirm that the central and C-terminal domains of NifA are involved in the oxygen sensitivity and the ammonia-sensing function is related to the N-terminal domain.

Acknowledgements: We thank Ms. Roseli Prado for the technical assistance and Mr Valter A. de Baura for synthesizing the oligonucleotide primers used in this work. This research was supported by FINEP, PRONEX, CNPq and FUNPAR.

References

[1] Baldani, J.I., Baldani, V.L.D., Seldin, L. and Dobereiner, J. (1986) Int. J. Syst. Bacteriol. 36, 86–93.
[2] Young, J.P.W. (1992) Biological Nitrogen Fixation. in: Phylogenetic Classification of Nitrogen Nitrogen-fixing Organism (Stacey, G., Burris, R.H., Evans, H.J., Eds.), pp. 43-86, Chapman and Hall, New York.
[3] Souza, E.M., Funayama, S., Rigo, L.U., Yates, M.G. and Pedrosa, F.O. (1991) J. Gen. Microbiol. 137, 1511–1522.
[4] Machado, I.M.P., Yates, M.G., Machado, H.B., Souza, E.M. and Pedrosa, F.O. (1995) Braz. J. Med. Biol. Res. 29, 1599–1602.
[5] Rego, F.G.M., Pedrosa, F.O., Yates, M.G., Chubatsu, L.S., Steffens, M.B., Rigo, L.U. and Souza, E.M. (submitted).
[6] Fischer, H.M. (1994) Microbiol. Rev. 58, 352–386.
[7] Szeto, W.W., Zimmerman, J.L., Sundaresan, V. and Ausubel, F.M. (1984) Cell 36, 1035–1043.
[8] Gubler, M. and Hennecke, H. (1988) J. Bacteriol. 170, 1205–1214.
[9] Benelli, E.M., Souza, E.M., Yates, M.G., Rigo, L.U., Buck, M., Moore, M., Harper, A. and Pedrosa, F.O. (1998) in: Highlights on Nitrogen Fixation Research (Martinez and Hernández, Eds.) (in press).
[10] Lee, H.S., Berger, D.K. and Kustu, S. (1993) Proc. Natl. Acad. Sci. USA 90, 2266–2270.
[11] Berger, D.K., Narberhaus, F. and Kustu, S. (1994) Proc. Natl. Acad. Sci. USA 91, 103–107.
[12] Berger, D.K., Narberhaus, F., Lee, H. and Kustu, S. (1995) J. Bacteriol. 177, 191–199.
[13] Austin, S., Buck, M., Cannon, W., Eydmann, T. and Dixon, R. (1994) J. Bacteriol. 176, 3460–3465.
[14] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[15] Laemmli, U.K. (1970) Nature 277, 680–685.
[16] Miller, J.H. (1990) Experiments in molecular genetics, pp. 325-355, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
[17] Rickwood, D. and Hames, B.D. (1990) Gel Electrophoresis of Nucleic Acids: A Practical Approach, 2nd edn., IRL Press.
[18] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, 2nd edn, Cold Spring Harbor Laboratory Press, New York.
[19] Morret, E. and Buck, M. (1988) Proc. Natl. Acad. Sci. USA 85, 9401–9405.
[20] Minchin, S.D., Austin, S. and Dixon, R.A. (1988) Mol. Microbiol. 2, 433–442.
[21] Tuli, R. and Merrick, M.J. (1988) J. Gen. Microbiol. 134, 425–432.
[22] Cannon, F.C., Dixon, R.A. and Postgate, J.R. (1976) J. Gen. Microbiol. 93, 11–25.
[23] Souza, E.M., Pedrosa, F.O., Drummond, M., Rigo, L.U. and Yates, M.G. (1999) J. Bacteriol. 181, 681–684.
[24] Drummond, M.H., Contreras, A. and Mitchenall, L.A. (1990) Mol. Microbiol. 4, 29–37.
[25] Dixon, R. (1998) Arch. Microbiol. 169, 371–380.
[26] Arsene, F., Kaminski, P.A. and Elmerich, C. (1996) J. Bacteriol. 178, 4830–4838.
[27] Oguiza, J.A. and Buck, M. (1997) Mol. Microbiol. 26, 655–664.
[28] Passaglia, L.M.P., Van Soom, C., Schrank, A. and Schrank, I.S. (1998) Braz. J. Med. Biol. Res. 31, 1363–1374.