FEBS Letters 354 (1994) 89–92

FEBS 14707

DNA binding activity of NtrC from *Rhizobium* grown on different nitrogen sources

Eduardo J. Patriarca, Anna Riccio, Sergio Colonna-Romano, Roberto Defez, Maurizio Iaccarino*

International Institute of Genetics and Biophysics, CNR, Via Marconi 10, 80125 Naples, Italy

Received 13 September 1994; revised version received 27 September 1994

Abstract The DNA-binding activity of the NtrC protein can be demonstrated in gel retardation assays with concentrated protein extracts of *Rhizobium etli*. Using extracts from either the wild type or a *ntrC* mutant strain and an antiserum raised against the NtrC protein, we demonstrate specific binding of NtrC to the upstream regulatory region of the *glnII* gene, where two putative NtrC-binding sites are present. KNO_3-grown bacteria contain less NtrC protein and more NtrC-binding activity than NH_4Cl-grown bacteria, thus showing that with this protocol it is possible to detect changes in NtrC-binding activity. The advantages of this assay system in comparison with that using pure proteins is discussed.

Key words: Two-component system; Nitrogen regulation; Gel retardation

1. Introduction

The response to nitrogen starvation is the most extensively investigated signal transduction pathway of prokaryotes. The two terminal components of this pathway are the protein kinase NtrB and the transcriptional regulatory protein NtrC, encoded by the *ntrB* and *ntrC* genes, respectively [1–2]. NtrC acts either to activate or repress transcription of its target genes through the binding to specific DNA sequences [3]. The transcriptional activity of NtrC is modulated by phosphorylation/dephosphorylation in a reaction catalyzed by NtrB and the regulatory protein P_{II} (encoded by the *glnB* gene). P_{II} in turn appears to be regulated by the uridyl transfer/uridyl removing enzyme (encoded by the *glnD* gene), which senses the intracellular glutamine to α-ketoglutarate ratio [4–5]. In enteric bacteria, under nitrogen-limiting conditions, the active (phosphorylated) form of NtrC enhances transcription at its own promoter and as a consequence NtrC concentration increases 14-fold. Therefore, a switch from nitrogen-excess to nitrogen-limiting conditions causes an increase both in NtrC activity (namely phosphorylation) and concentration [6–7].

It was previously shown that in *Rhizobium leguminosarum* biovar *phaseoli* (recently reclassified as *Rhizobium etli* [8]) the transcription of the *glnII* gene, coding for the glutamine synthetase II enzyme, is regulated in response to nitrogen availability [9]. The *glnII* gene is transcribed from a single −24/−12 (σ^{54}-dependent) nitrogen-regulated promoter [10], which is induced 8- to 10-fold when the wild type strain of *R. etli* is grown on KNO_3 as compared to NH_4Cl as a sole nitrogen source. A promoter deletion analysis shows that a *cis*-acting sequence (between positions −316 and −219 with respect to the transcription initiation site) is essential to promote *glnII* transcription. Furthermore, the absence of promoter activity in a *ntrC* mutant strain indicates that this *cis*-acting sequence functions through the binding of either NtrC or another *trans*-acting factor(s), absent or inactive in the $ntrC^-$ strain.

In *R. etli* the expression of the *ntrB* and *ntrC* genes is 2-to-3-fold higher when bacteria are grown in NH_4Cl as compared to growth in KNO_3 and is negatively autoregulated by NtrC [11]. The switch from nitrogen-excess to nitrogen-limiting conditions causes an increase in transcription of *glnII*, a putative target of NtrC action, while NtrC concentration slowly decreases to a 2-to-3-fold lower level.

We demonstrate here that NtrC is the activator of *glnII* transcription since it specifically binds to the *glnII* promoter regulatory region. By using a method previously described [12] for the preparation of yeast DNA-binding proteins we show that NtrC-binding activity is higher in protein extracts prepared from KNO_3-grown bacteria as compared to that from NH_4Cl-grown bacteria, thus suggesting that with this method it is possible to detect changes in NtrC-binding activity in the presence of other factor(s) that might influence it.

2. Materials and methods

2.1. Bacterial strains and media

R. etli wild type strain CE3 and strain CFN2012, a *ntrC*::Tn*5* derivative of CE3 [9], were grown at 30°C in minimal medium (RMM; [13]) with NH_4Cl or KNO_3 as sole nitrogen source, each at a concentration of 1 $g \cdot l^{-1}$. When NH_4Cl was used, 100 mM 3-(*N*-morpholino)propanesulfonic acid pH 7.2 was also added [14]. Antibiotics used were ($\mu g \cdot ml^{-1}$): nalidixic acid (20); kanamycin (30).

2.2. Preparation of protein extracts

Extracts were prepared using minor modifications of a protocol previously described [12]. The bacterial pellet from a 200 ml logarithmic phase *R. etli* culture (OD_{590} of 0.5 to 0.6) was resuspended with 10 ml of cold extraction buffer (200 mM Tris-HCl pH 8, 400 mM $(NH_4)_2SO_4$, 1 mM EDTA, 10% glycerol, 1 mM PMSF and 7 mM β-mercaptoethanol). After centrifugation for 5 min at 5,000 rpm, the pellets were resuspended in 2 ml of extraction buffer and disrupted by vortexing for 5 min in a cold room after addition of an equal volume of glass beads. Samples were incubated for 15 min on ice and the supernatant was transferred to a microfuge tube. After centrifugation for 15 min at 10,000 rpm the supernatant (about 1.5 ml) was treated with solid $(NH_4)_2SO_4$ to a final concentration of 40% (w/v) and left on a rotating wheel for 15 min in a cold room. After centrifugation for 15 min at 10,000 rpm the pellet was resuspended to a final concentration of 5 to 10 mg/ml in 20 mM HEPES pH 8, 5 mM EDTA, 20% glycerol, 1 mM PMSF and 7 mM β-mercaptoethanol and aliquots were frozen in ethanol-dry ice and stored at −80°C. Protein concentration was determined by the Bio-Rad assay using bovine serum albumin as a standard.

*Corresponding author. Fax: (39) (81) 725 7319.

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(ethanesulfonic acid); EDTA, (ethylenedinitrilo)tetraacetic acid.

SSDI 0014-5793(94)01094-3

2.3. Preparation of DNA probes

A DNA fragment from plasmid pAR27 carrying the *glnII* upstream region [10] was cleaved with *Pvu*II and *Ava*II (probe 'a') or *Ava*II and *Hin*dIII (probe 'b'). DNA fragments were separated by agarose gel electrophoresis and eluted; their 3'- or 5'-ends were labeled by using [α-^{32}P]dCTP, [α-^{32}P]dATP and the Klenow fragment. End-labeled DNA fragments were separated from the unincorporated nucleotides by agarose gel electrophoresis, electroeluted and concentrated by ethanol precipitation.

2.4. Binding reaction and mobility shift assay

The incubation mixture for DNA–protein binding followed, with minor modifications, that previously described [15] and contained, in 20 μl total volume, 20 mM HEPES, pH 7.6, 2 mM EDTA, 0.1 M NaCl, 0.1 mM PMSF, 0.7 mM β-mercaptoethanol, 10% glycerol, 2 μg of bovin serum albumin, 0.25 to 5 μg of concentrated protein extract, 1–2 ng of the appropriate end-labeled DNA fragment and 0.5 μg (unless otherwise indicated) of poly(dI-dC). The binding reaction was allowed to proceed for 10 min at room temperature. Samples were loaded immediately on non-denaturating 5% polyacrylamide gels (acrylamide-bisacrylamide ratio 29:1) in 45 mM Tris-borate pH 8.4, 1 mM EDTA, 10% glycerol. After electrophoresis (5 mA for about 12 h at room temperature), gels were fixed in 10% acetic acid, dried on a filter paper and autoradiographed.

3. Results

3.1. Band shift assays

By means of gel retardation assays the interaction of *trans*-acting factors with different ^{32}P-labeled DNA fragments from the *glnII* upstream region used as probes was investigated (Fig. 1A). Probe 'a' (179 bp DNA fragment), in which no putative NtrC binding sites are observed, spans the nucleotides from position −494 to −316 with respect to the transcription initiation site. Deletion of this DNA region does not affect *glnII* promoter activity [10]. Probe 'b' (98 bp DNA fragment), spans the sequence from position −316 to −219, which is essential for *glnII* promoter activity [10]. Sequence analysis of this DNA region reveals the presence of two putative NtrC-binding sites between positions −260 and −296, with a center-to-center distance of 21 bp and therefore located on the same face of the DNA helix. The probes were incubated with concentrated protein extracts prepared from strains CE3 (wild type) or CFN2012 (*ntrC*$^-$) (Fig. 1B). No retardation band is observed when probe 'a' is incubated with extracts (1 μg) prepared from either the wild type or the *ntrC*$^-$ strain (Fig. 1B, lanes b and c). The protein extract (1 μg) from the wild type strain incubated with probe 'b' cause the appearance of retardation complexes (indicated as b_I, b_{II} and b_{III}). No significative retardation of this probe is observed with extracts (1 μg) prepared from the *ntrC*$^-$ strain (Fig. 1B, lane f). When the amount of non specific competitor poly(dI-dC) is increased from 0.5 to 1 μg, complex b_I is almost absent whereas complexes b_{II} and b_{III} are unaffected (Fig. 1C, lanes a, b and c). At a concentration of 2 μg of poly(dI-dC), complex b_I is competed out, whereas complexes b_{II} and b_{III} are unaffected (data not shown).

3.2. The NtrC protein causes formation of specific complexes

Protein extracts from the wild type strain (1 μg) were incubated with different dilutions of a polyclonal antiserum raised against the *R. etli* NtrC protein [11] before addition of the DNA fragment carrying the *glnII* promoter regulatory region (probe 'b' in Fig. 1). The results (Fig. 2A) clearly show that the two slowest-migrating complexes (indicated as b_{II} and b_{III}) indeed contain the NtrC protein, since new slow-migrating complexes

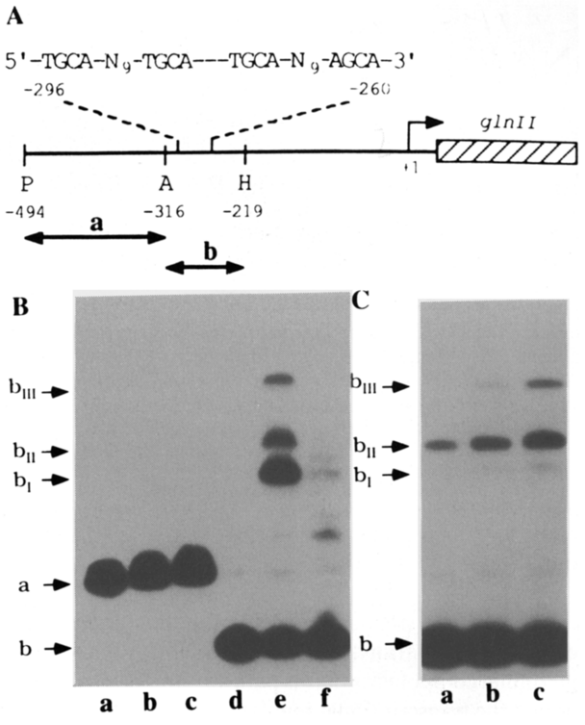

Fig. 1. (A) Physical map of the *glnII* (EMBL/Genbank: X67296) upstream region. Restriction enzymes symbols: P (*Pvu*II), A (*Ava*II), H (*Hin*dIII). Numbers indicate nucleotide position with respect to the transcription initiation site (+1). The sequence of putative NtrC-binding sites are shown. Double arrows indicate the DNA fragments used as probes (a and b) in gel retardation experiments. (B) Complex formation between DNA fragments carrying the *glnII* promoter region and protein extracts (1 μg) from *R. etli* CE3 (wild type) and CFN2012 (*ntrC*$^-$) strains grown in minimal medium with KNO_3 (1 g · l^{-1}) as a sole nitrogen source. Sources of extracts: none (lanes a and d), from strain CE3 (lanes b and e) and from strain CFN2012 (lanes c, f). (C) Effect of poly(dI-dC) (1 μg as compared. to 0.5 μg in samples of Fig. 1B) on complex formation with probe 'b'. Additions to the DNA: 0.25 μg of protein extract prepared from strain CE3 grown with KNO_3 (1 g · l^{-1}) as a nitrogen source (lane a), 0.5 μg (lane b) and 1 μg (lane c).

(supershifts indicated as b', b'' and b''') are observed in the presence of anti-NtrC antibodies. Moreover, these complexes become more pronounced when the antiserum concentration is increased (from 8,000- to 1,000-fold diluted), while the amounts of b_{II} and b_{III} decrease concomitantly (Fig. 2A, lanes f to c). No supershift is observed when the preimmune serum was added (40-fold diluted) to the binding mixture (Fig. 2A, lane g). The antiserum against NtrC even at the highest concentration used (40-fold diluted, Fig. 2B lane c) has no influence on the fastest complex b_I indicating that this complex does not contain NtrC. The supershift of complexes b_{II} and b_{III} is also obtained when the antiserum is added to the mixture after the binding reaction (data not shown), thus indicating that the anti-NtrC antibodies do not prevent the NtrC-DNA complex formation and react with the NtrC protein bound to DNA. This result is not surprising because the antiserum was raised against the central domain of *R. etli* NtrC protein that does not contain the DNA binding domain [11].

3.3. Effect of nitrogen source on the ability to form DNA–protein complexes

When the DNA fragment corresponding to the *glnII* pro-

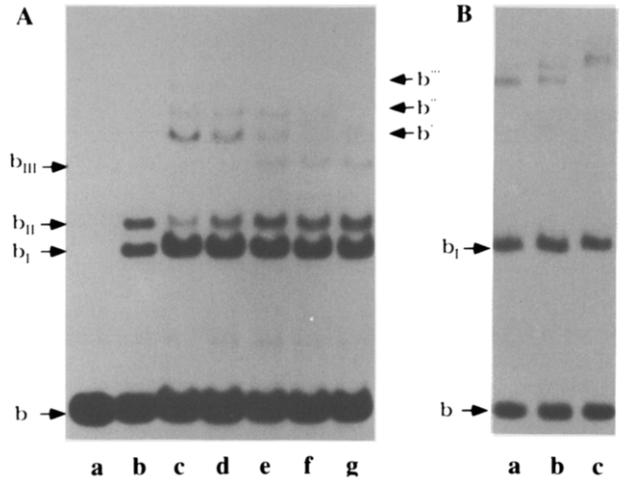

Fig. 2. (A) Effect of an anti-NtrC serum on complex formation with the *glnII* promoter regulatory region (probe 'b' in Fig. 1). Additions to DNA: none (lane a), 1 μg of protein extract prepared. from strain CE3 grown with KNO_3 ($1 \cdot g\ l^{-1}$) as a nitrogen source (lanes b to g), 1 ml of antiserum diluted 1,000-fold (lane c), 2,000-fold (lane d), 4,000-fold (lane e), and 8,000-fold (lane f), 1 μl of preimmune serum 40-fold diluted (lane g). (B) As in part A but with 1 μl of anti-NtrC serum diluted 400-fold (lane a), 200-fold (lane b), 40-fold (lane c).

moter regulatory region (Fig. 1A, probe 'b') is incubated with increasing amounts (0.25 to 5 μg) of extracts from wild type cells grown on KNO_3, the NtrC-DNA complexes (b_{II} and b_{III}) are more evident (Fig. 3A, lanes b to e) than when incubated with extracts from NH_4Cl-grown cells (lanes g to j). Moreover, when the amount of protein extracts from KNO_3-grown cells is increased from 2.5 μg to 5.0 μg (Fig. 3B lanes d and e), complex b_{III} becomes more pronounced, while complex b_{II} decreases concomitantly.

Two major non-specific complex are observed: the first complex (b_I) contains a protein(s) which bind(s) more efficiently when extracts are prepared from KNO_3-grown cells as compared to NH_4Cl-grown cells. As shown above, this complex is competed out by high concentrations of poly(dI-dC) and it is not affected by the anti-NtrC serum. However, it is almost absent when extracts are prepared from the *ntrC* mutant strain and is regulated by the nitrogen source. These results suggest that this complex is due to the presence of a DNA-binding protein(s) whose activity or synthesis is regulated by NtrC. In contrast, the binding capacity of the protein responsible for the second non-specific complex, indicated with an asterisk in Fig. 3A, is independent from the nitrogen source or the strain used to obtain the protein extracts. When a high concentration of protein extracts from the *ntrC*$^-$ strain (5 μg) grown on KNO_3 or NH_4Cl (lanes f and k) was used, this complex (asterisk) was predominant. The same protein extracts prepared for gel retardation experiments were dialyzed against 20 mM Tris-HCl buffer pH 8.0 and used to determine the NtrC concentration by immunoblot analysis (not shown). In extracts prepared from the wild type strain the level of NtrC protein is 2-to-3-fold higher when cells are grown on NH_4Cl as compared to KNO_3, confirming previous results obtained with a different extraction procedure [11]. Therefore, extracts from KNO_3-grown cells show more efficient NtrC-binding activity, but lower NtrC concentration than extracts from NH_4Cl-grown cells.

4. Discussion

We report here that the *R. etli* NtrC protein binds, in gel retardation experiments, to the *glnII* promoter regulatory region. In this DNA region two putative NtrC binding sites were identified by sequence analysis (Fig. 1A) and in gel retardation assays two NtrC containing complexes (b_{II} and b_{III}) are observed. These complexes are specific, since they are not competed out by high concentrations of non-specific competitor and contain NtrC, since they are absent using crude extracts prepared from the *ntrC* mutant strain and are specifically supershifted when an anti-NtrC serum is added to the incubation mixture. Moreover, the NtrC binding activity in protein extracts prepared from KNO_3-grown bacteria is more efficient as compared to that found in NH_4Cl-grown bacteria. These results demonstrate that with this protocol it is possible to prepare proteins that in vitro show NtrC-binding activity correlated with the degree of *glnII* transcription. This finding is in agreement with the current model of in vivo NtrC action which postulates that NtrC remains bound to its specific sites and

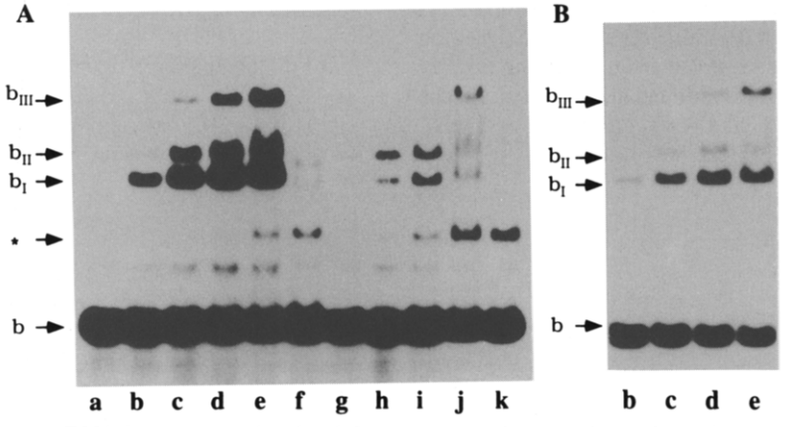

Fig. 3. Complex formation between a DNA fragment carrying the *glnII* promoter regulatory region and protein extracts from *R. etli* CE3 (wild type) and CFN2012 (*ntrC*$^-$) strains grown with $1 \cdot g\ l^{-1}$ of KNO_3 or NH_4Cl as sole nitrogen sources. (A) Probe 'b' (see Fig. 1A) was incubated. with: none (lane a); 0.25 μg of extract from CE3 grown with KNO_3 (lane b), 1.0 μg (lane c), 2.5 μg (lane d) or 5.0 μg (lane e); 5.0 μg of extract from CFN2012 grown with KNO_3 (lane f); 0.25 μg extract from CE3 grown with NH_4Cl (lane g), 1.0 μg (lane h), 2.5 μg (lane i) or 5.0 μg (lane j); 5.0 μg of extract from CFN2012 grown with NH_4Cl (lane k). (B) A shorter exposure time of a portion of the same gel is shown.

contacts the σ^{54}-holoenzyme-promoter complex directly when the DNA between sites and promoter forms a loop. NtrC activates transcription by catalyzing isomerization of closed complexes between RNA polymerase and the promoter to open complexes [16–17].

In enteric bacteria all known NtrC-activatable promoters are preceded by two NtrC binding sites located on the same face of the helix. This is the case of *glnAp2* [18], *nifLA* [19] and *glnHp2* [20] promoters. Since NtrC binds as a dimer to each NtrC binding site, it has been suggested that only the NtrC-phosphate tetramer is capable of activating open complex formation [16]. The presence of two NtrC-binding sites in the *glnII* promoter regulatory region with higher binding affinity in activating conditions supports the notion that the same mechanism of NtrC-dependent transcription activation may be operative also in *R. etli*. If this is the case presumably the faster migrating complex b_{II} contains an NtrC dimer bound to a single site whereas complex b_{III} contains two dimers of NtrC bound to the two sites. This would be a specific advantage of this method since it allows to detect the binding of a single NtrC dimer, which has been shown to be undetectable when purified proteins are used [17].

Phosphorylation of pure NtrC from enteric bacteria induces a strong cooperative binding to two adjacent NtrC binding sites [16]. In our gel retardation experiments (Fig. 3B) it is evident that with increasing amount of protein extracts from KNO_3-grown cells, a shift of complex b_{II} into b_{III} is observed, thus suggesting binding cooperativity.

In the wild type strain of *R. etli* the level of NtrC protein is 2- to 3-fold higher when bacteria are grown on NH_4Cl as compared to KNO_3. Therefore, extracts from KNO_3-grown cells show more efficient binding activity, but lower NtrC concentration than extracts from NH_4Cl-grown cells. This different binding activity of NtrC and the binding cooperativity are probably correlated to differences in the level of NtrC phosphorylation. We obtained preliminary data indicating that *R. etli* NtrC may be phosphorylated either in permeabilized cells [21] with [γ-^{32}P]ATP or in vivo with [^{32}P]orthophosphate and that the level of NtrC phosphorylation is higher in KNO_3-grown cells than in NH_4Cl-grown cells (unpublished results).

The DNA-binding activity of NtrC has been previously demonstrated only with pure components [17,22,23–25]. Moreover, to determine the influence of NtrC-phosphorylation on its binding activity purified NtrB is also required. The method described here may be used to test the NtrC-binding activity using extracts of different strains mutated in components of the signal transduction pathway, like *glnD*, *glnB*, *ntrB* and *ntrC*, without purification of the proteins involved.

Acknowledgements: We thank Maria Ciaramella for useful discussion and Concetta Sole for technical assistance. This study was partially supported by MAF-DPGA, EEC BIOT-0166-C(EDB) and RAISA-CNR subproject 2 (Paper No. 1945).

References

[1] Stock, J.B., Ninfa, A.J. and Stock, A.M. (1989) Microbiol. Rev. 53, 450–490.
[2] Parkinson, J.S. and Kofoid, E.C. (1992) Annu. Rev. Genet. 26, 71–112.
[3] Thony, B. and Hennecke, H. (1989) FEMS Microbiol. Rev. 63, 341–358.
[4] Reitzer, L.J. and Magasanik, B. (1987) in: *Escherichia coli* and *Salmonella typhymurium* Cellular and Molecular Biology (Neidhardt, F.C. Ed.) ASM Vol. 1, pp. 302–318.
[5] Merrick, M.J. (1988) In: The Nitrogen and Sulphur Cycle. (Cole J.A. and Ferguson, S. Eds.) Cambridge University Press, pp. 331–361.
[6] Ninfa, A.J. and Magasanik, B. (1986) Proc. Natl. Acad. Sci. USA 83, 5909–5913.
[7] Reitzer, L.J. and Magasanik, B. (1986) Cell 45, 785–792.
[8] Segovia, L., Young, P.W., Martinez-Romero, E. (1993) Int. J. Syst. Bacteriol. 43, 347–377.
[9] Moreno, S., Patriarca, E.J., Chiurazzi, M., Meza, R., Defez, R., Iaccarino, M. and Espin, G. (1992) Res. Microbiol. 143, 161–171.
[10] Patriarca, E.J., Chiurazzi, M., Manco, G., Riccio, A., Lamberti, A., De Paolis, A., Rossi, M., Defez, R. and Iaccarino, M. (1992) Mol. Gen. Genet. 234, 337–345.
[11] Patriarca, E.J., Riccio, A., Taté, R., Colonna-Romano, S., Iaccarino, M. and Defez, R. (1993) Mol. Microbiol. 9, 569–577.
[12] Pfeifer, K., Arcangioli, B. and Guarente, L. (1987) Cell 49, 9–18.
[13] Hooykaas, P.J.J., Clapwijk, P.M., Nuti, M.P., Shilperoort, R.A. and Roersch, A. (1977) J. Gen. Microbiol. 98, 477–484.
[14] Rossi, M., Defez, R., Chiurazzi, M., Lamberti, A., Fuggi, A. and Iaccarino, M. (1989) J. Gen. Microbiol. 135, 629–637.
[15] Jones, R.H., Moreno, S., Nurse, P. and Jones, N. (1988) Cell 53, 659.
[16] Weiss, V., Claverie-Martin, F. and Magasanik, B. (1992) Proc. Natl. Acad. Sci. USA 89, 5088–5092.
[17] Klose, K.E., Weiss, D.S. and Kustu, S. (1993) J. Mol. Biol. 232, 67–78.
[18] Reitzer, L.J. and Magasanik, B. (1985) Proc. Natl. Acad. Sci. USA 82, 1979–1983.
[19] Minchin, S.D., Austin, S. and Dixon, R.A. (1988) Mol. Microbiol. 2, 433–442.
[20] Claverie-Martin, F. and Magasanik, B. (1991) Proc. Natl. Acad. Sci. USA 88, 1631–1635.
[21] Colonna-Romano, S., Patriarca, E.J., Amar, M., Bernard, P., Manco, G., Lamberti, A., Iaccarino, M. and Defez, R. (1993) FEBS Lett. 330, 95–98.
[22] Sasse-Dwight, S. and Gralla, J.D. (1988) Proc. Natl. Acad. Sci. USA 85, 8934–8938.
[23] Hawkes, T., Merrick, M. and Dixon, R. (1985) Mol. Gen. Genet. 201, 492–498.
[24] Ferro-Luzzi, G. and Nikaido, K. (1985) EMBO J. 4, 539–547.
[25] Hirschman, J., Wong, P.K., Sei, K., Keener, J. and Kustu, S. (1985) Proc. Natl. Acad. Sci. USA 82, 7525–7529.