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ORIGINAL PAPER

# Role of NtcA, a cyanobacterial global nitrogen regulator, in the regulation of sucrose metabolism gene expression in *Anabaena* sp. PCC 7120

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**Abstract** In the heterocyst-forming cyanobacterium *Anabaena* sp. PCC 7120 (also known as *Nostoc* sp. PCC 7120), it has been shown that *spsB* and *susA*, the genes coding for proteins related to sucrose synthesis and cleavage, respectively, exhibit converse expression regarding the nitrogen source. In the nitrogen-fixing filament, *spsB* expression is mostly localized to the heterocysts and *susA* is only expressed in vegetative cells. The aim of this work was to investigate the participation of NtcA, a global nitrogen regulator that operates in cyanobacteria, in the regulation of sucrose metabolism genes in *Anabaena* sp. PCC 7120. The induction of *spsB* expression observed in the filaments upon combined-nitrogen depletion was abolished in an NtcA-deficient mutant. In vitro experiments showed that NtcA binds specifically but with different affinities to two sites in the *spsB* promoter region. When *susA* expression was analyzed after a combined-nitrogen starvation, the levels of mRNA, polypeptide and activity increased in the mutant in comparison with the wild-type strain. Also, NtcA interacted with one site in the promoter region of *susA*. We conclude that sucrose metabolism is coordinated at the transcriptional level with nitrogen metabolism, suggesting a global metabolism regulating role for NtcA.

**Keywords** Cyanobacteria · Sucrose metabolism · Transcriptional regulation · NtcA

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**Abbreviations**

| | |
|---|---|
| EMSA | Electrophoretic mobility shift assay |
| GST | Glutathione *S*-transferase |
| 2-OG | 2-Oxoglutarate |
| Suc | Sucrose |
| Sps | Sucrose-phosphate synthase |
| Sus | Sucrose synthase |

## Introduction

The interconnection between carbon and nitrogen metabolism is crucial to living organisms to survive in changing environments. Microorganisms sense the nutrient supply and adapt their metabolism accordingly, by integrating the information of the different metabolic branches, using multiple regulators (Commichau et al. 2006).

Sucrose (Suc) is the key sugar in plant life. Suc metabolism has been described not only in eukaryotic oxygenic photosynthetic organisms but also in cyanobacteria, a widely distributed group of Gram-negative prokaryotes (Salerno and Curatti 2003). Although the function of Suc in cyanobacteria has been associated with salt stress and with carbon transport in the $N_2$-fixing filaments of heterocyst-forming strains, the significance of Suc metabolism in microorganisms is still not fully understood (Wolk et al. 1994; Salerno and Curatti 2003). The biosynthesis of Suc through the concomitant activities of Suc-phosphate synthase (Sps) and Suc-phosphate phosphatase, its cleavage by Suc synthase (Sus) and hydrolysis to glucose and fructose by invertases have been reported in $N_2$-fixing strains of the *Anabaena* genus. Suc metabolism encoding genes (*spsA*, *spsB*, *sppA*, *susA*, *invA* and *invB*) have been functionally characterized in *Anabaena* sp.

(Porchia and Salerno 1996; Cumino et al. 2001, 2002; Curatti et al. 2000; Vargas et al. 2003). The expression of *susA* decreases after combined-nitrogen starvation and is downregulated in the heterocysts of $N_2$-fixing filaments (Curatti et al. 2002, 2006). Moreover, *susA* overexpression was detrimental for diazotrophic growth (Curatti et al. 2002). In addition, the presence of two Sps proteins (SpsA and SpsB) seems to be important in the $N_2$-fixing filaments, since both *spsA* and *spsB* are upregulated during diazotrophic growth, supporting the role of Suc as carbon carrier molecule along the filaments. Interestingly, only *spsB* is expressed in heterocysts, which led to the suggestion that a Suc cycling may take place in heterocysts during $N_2$ fixation (Cumino et al. 2007).

In cyanobacteria, nitrogen assimilation is tightly regulated in response to the nitrogen and carbon supply. Ammonium is the preferred nitrogen source of combined nitrogen, which is incorporated into carbon skeletons via the glutamine synthetase–glutamate synthase (GS-GOGAT) pathway (Herrero et al. 2001). In the presence of ammonium, genes involved in the utilization of alternative sources of nitrogen are repressed. The metabolite 2-oxoglutarate (2-OG), a substrate of the GS-GOGAT cycle, was shown to act as a signal for the cellular nitrogen supply and is an indicator of the carbon/nitrogen status of the cells (Muro-Pastor et al. 2001; Herrero et al. 2004). A central role in global nitrogen control in cyanobacteria is played by the transcription factor NtcA (encoded by *ntcA*), which belongs to the Crp-FNR family of bacterial transcriptional regulators (Vega-Palas et al. 1992; Korner et al. 2003). NtcA, a dimeric protein composed of identical monomers, activates the promoters of genes subjected to metabolic control by ammonium and its activity has been described as modulated by the redox state of the cell and to respond to 2-oxoglutarate (2-OG) (Jiang et al. 1997; Vázquez-Bermúdez et al. 2002b; Tanigawa et al. 2002; Laurent et al. 2005). The *Anabaena* NtcA monomer structure has been modeled showing that the helix-turn-helix motif of the C-terminal domain is involved in DNA binding (Wisén et al. 2004). The structure of the consensus NtcA-activated promoter comprises a $TAN_3T$ sequence fitting the −10 box of *Escherichia coli* $\sigma^{70}$ consensus promoters and an NtcA-binding site located 20–23 nucleotides upstream from the −10 box. A large number of genes belonging to different cyanobacterial strains have been reported to bear this canonical NtcA-activated promoter (Herrero et al. 2001). The NtcA-core binding site, determined by in vitro selection of DNA sequences, was proposed in early studies as two overlapping palindromic sequences *GT*A($N_8$)T*AC* or T*GT*($N_{10}$)*AC*A, in *Synechococcus elongatus* and *Anabaena* sp. PCC 7120 (also known as *Nostoc* sp. PCC 7120), respectively (Luque et al. 1994; Ramasubramanian et al. 1994). The italicized nucleotides are the most important for NtcA binding and are highly conserved in the binding sites compiled from nine cyanobacterial genomes (Vázquez-Bermúdez et al. 2002a; Su et al. 2005). However, sequences such as *GT*a($N_{7-9}$)t*AC* have also been proposed as NtcA-binding sites (Jiang et al. 1995, 2000; Luque et al. 2001). Interestingly, putative NtcA-binding sequences were recently suggested for the promoter regions of *susA* and *spsB*, key genes of Suc metabolism (Curatti et al. 2006; Cumino et al. 2007).

In this study, we provide experimental evidence indicating that NtcA is a regulator of Suc metabolism genes acting as an activator of *spsB* and an inhibitor of *susA* expression in *Anabaena* sp. PCC 7120. Suc metabolism is coordinated at the transcriptional level with nitrogen metabolism, which suggests a role of global metabolism regulator for NtcA.

## Materials and methods

### Strains and culture conditions

*Anabaena* (*Nostoc*) sp. PCC 7120 was cultivated in $BG11_0$ medium or $BG11_0NH_4^+$ (Rippka et al. 1979; Curatti et al. 2002), and its derivative strain CSE2, an insertional mutant of the *ntcA* gene (Frías et al. 1994), was grown in the presence of 2 μg $l^{-1}$ spectinomycin and 2 μg $l^{-1}$ streptomycin. To produce the nitrogen step-down, cells grown in $BG11_0NH_4^+$ medium were washed three times with $BG11_0$ and cultivated for 24 h. *Escherichia coli* DH5α and derivative strains were grown in Luria–Bertani medium supplemented with appropriate antibiotics (Sambrook and Russell 2001).

### Protein extraction and immunoblot analysis

Extracts from *Anabaena* cells, protein quantification and immunodetections were performed as previously reported (Cumino et al. 2001; Curatti et al. 2002). Densitometric analyses of signal intensities from blots were quantified using Fotodyne Model Express Zoom Lens System and TotalLab Imagen Analysis Software.

### Enzyme activity assays

SpsB activity was determined on toluene-permeabilized *Anabaena* cells incubated with radiolabelled ADP-[U-$^{14}$C]-Glc and Fru-6P (Cumino et al. 2007). Labeled Suc was separated by chromatography and radioactivity determined in each fraction as previously described (Porchia and Salerno 1996). SusA (the protein product of *susA* expression) activity was measured in the direction of Suc cleavage (Porchia et al. 1999).

## Isolation and manipulation of nucleic acids

DNA experimental procedures were performed according to standard protocols (Sambrook and Russell 2001). Isolation and purification of RNA was carried out as previously described (Cumino et al. 2007).

## Plasmid construction

A DNA fragment of 672 bp corresponding to the NtcA encoding sequence was PCR-amplified with primers 5′-CGGGATCCATGATCGTGACACAAGATAAGG-3′ and 5′-CCCAAGCTTTTAAGTGAACTGTCTGCTGAG-3′, cloned into the pGEMT-Easy vector (Promega) and then introduced into the pGEX4T2 expression vector (Pharmacia Biotech) to produce pGEX4T2::*ntcA*. The 370-bp fragment of the *susA* promoter region was PCR-amplified with primers 5′-GGAATTCTCTCCTATTGTGAGTACT TTTTTAG-3′ and 5′-GGAATTCTCTGACATATTCACC CCTTAAATGA-3′, and cloned into the pGEMT-Easy vector. Automated sequencing was used to verify sequence fidelity of the cloned PCR fragments.

## Real time RT-PCR

Reactions were carried out in a Mastercycler® ep realplex using reagents from Invitrogen. The first reaction mixture (20 µl final volume) contained 0.25 µg reverse specific primer (5′-GACTCACTTTGCGGCTAAACATATC-3′, 5′-GAAGTTTCCGGTTTCTGGCACTTAG-3′ or 5′-GGA TTAGGGATTGGGAAGTTTCTTC-3′ for *spsB*, *susA* or *rnpB*, respectively), 5 µg RNA, 0.5 mM dNTP mix, 1× first strand buffer, 5 mM DTT, 1 µl Rnase OUT™ ribonuclease inhibitor and 0.5 µl SuperScript™ III reverse transcriptase. The RT program was as follows: 50°C for 60 min and 70°C for 15 min. The second step mixture contained 5 µl cDNA, 1× Platinum Taq DNA polymerase buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.5× SYBR® GreenER™, 0.1 µl Platinum Taq DNA polymerase, 1 µM primer mix containing the forward (5′-AACGCA TAGCTTTAATTTCAGTTCA-3′, 5′-GATGCAAGCGAT TTTAGATAGTGAA-3′ or 5′-TAGCTTAACTGATTTG AGGAAAGTC-3′ for *spsB*, *susA* or *rnpB*, respectively) and the reverse (see above) primers. The PCR program was as follows: 95°C for 1 min, 40 cycles at 95°C for 15 s, 55°C for 30 s and at 72°C for 40 s. Product identification was confirmed by a melting curve analysis and visualized on agarose gels. The relative amount of each cDNA was normalized using *rnpB*. Data analyses for a relative quantification of gene expression were performed by the comparative $C_T$ (threshold cycle) method (Livak and Schmittgen 2001).

## Primer extension analysis of 5′ ends

Primer extension experiments were performed from total RNA as previously reported (Curatti et al. 2006; Cumino et al. 2007) using SuperScript II reverse transcriptase (Invitrogen) and specific oligonucleotides for *spsB* and *susA*. Primers were end-labeled with [gamma-$^{32}$P] ATP using T4 polynucleotide kinase (Invitrogen). The cDNA products were purified and resuspended in formamide loading dye and resolved on a 6% polyacrylamide 7 M urea sequencing gel, next to the corresponding sequencing ladder. Nucleotide sequencing was carried out by the dideoxy-chain termination method using a Sequenase Quick-Denature Plasmid Sequencing Kit (USB Corporation). Images of the radioactive gels were obtained by autoradiography.

## Purification of recombinant proteins

GST–NtcA was produced as amino-terminal fusion proteins with glutathione *S*-transferase (GST) and purified by affinity chromatography using glutathione-agarose beads, following the manufacturer's protocol (Sigma). The protein was dialysed to remove the glutathione. NtcA was separated from GST after incubation with 10 units of thrombin (Sigma) per milligram of GST–NtcA. Purified proteins were stored at −20°C in 50% glycerol.

## Electrophoretic mobility shift assays

DNA target molecules were end-labeled using Klenow (Invitrogen) and an appropriate $^{32}$P-labeled nucleotide (NEN). DNA templates of 30 bp were generated by annealing equal amounts of the two complementary DNA oligomers (5′-GATCCTCTCGTAGTATGTATATAACA ATCG-3′ and 5′-AATTCGATTGTTATATACATACTAC GAGAG-3′, 5′-GATCCTAAATGTAAATGCTGAACAA AAAAG-3′ and 5′-AATTCTTTTTTGTTCAGCATTTAC ATTTAG-3′, 5′-GATCCAGGTTGTTAGCCTACGTTAC CCTAG-3′ and 5′-AATTCTAGGGTAACGTAGGCTAA CAACCTG-3′, 5′-GATCCCTAAGTAAATAAGAACAA GGG-3′and 5′-AATTCCCTTGTTCTTATTTACTTAGG-3′, 5′-GATCCTTCTGTAACAAAGACTACAAAAG-3′ and 5′-AATTCTTTTGTAGTCTTTGTTACAGAAG-3′, and 5′-GATCCAACAGTGAATGCCTATCTACTATA G-3′ and 5′-AATTCTATAGTAGATAGGCATTCACTGT TG-3′, for $spsB_I$, $spsB_{II}$, $susA_I$, $susA_{II}$, $glnA_I$, and negative control *c*-, respectively). Binding reactions contained the following: 200 ng ml$^{-1}$ of poly(dI–dC), 100 mg ml$^{-1}$ BSA, 12% glycerol, 0.5 mM 2-OG, 12 mM Hepes–NaOH, 4 mM Tris–HCl, 700 mM KCl, 1 mM DTT, 1 mM EDTA, 30 mM $MgCl_2$, pH 7.9, 5 pmol of NtcA or 5 pmol of GST (as a control), and 0.05 pmol of DNA template (∼42,000–

65,000 dpm). Control experiments for unspecific binding using oligonucleotides designed on other regions of *spsB* or *susA* promoter were performed in parallel (data not shown). The mixture was incubated at room temperature for 20 min. The effect of 2-OG was assayed at final concentrations ranging from 0 to 3 mM. In competition experiments, 1× is equivalent to 2.5 nM. Samples were electrophoresed onto a 5% nondenaturing polyacrylamide gel with 0.5% TBE. Gels were dried and exposed to films (Kodak MRX). To determine the dissociation constant ($K_D$), the percentage of retarded fragment was plotted against the concentration of NtcA used (0–2 μM) and by fitting to a simplified version of the Hill equation. The $K_D$ value was calculated as the concentration of NtcA effecting retardation of half of the amount of the DNA used (Vázquez-Bermúdez et al. 2002a). Radioactive gels were exposed to a phosphor imager screen and analyzed with a STORM PhosphorImager, using the ImageQuant analysis software (Molecular Dynamics, Sunnyvale, CA).

## Results

### Induction of *spsB* by combined-nitrogen depletion is abolished in an *Anabaena* NtcA-deficient mutant

The *spsB* upregulation in diazotrophically grown cells of *Anabaena* sp. PCC 7120 and the presence of putative NtcA-binding regions (Cumino et al. 2007) led us to investigate the role of NtcA as a transcriptional regulator of Suc synthesis. We compared the expression of *spsB* at different levels in the PCC 7120 strain and in the derivative mutant strain (CSE2) that bears an inactivated version of *ntcA* (Frías et al. 1994). After 24 h of combined-nitrogen step-down, there was only a basal-level of SpsB activity and polypeptides in CSE2 cells (Fig. 1a, b, respectively). A dramatic decrease in *spsB* transcripts was shown by real-time reverse transcriptase-PCR in the CSE2 strain in comparison with an about fivefold increase in PCC 7120 cells after incubation under combined-nitrogen deprivation for 24 h (Fig. 2a).

To test whether the absence of NtcA produces a shift in *tsp* (transcription initiation points) use, we carried out primer extension analysis with RNA isolated from wild-type (7120) or mutant (CSE2) cells grown with ammonium, or with ammonium and then incubated for 24 h under a nitrogen-step down condition. Figure 2b shows the results of a representative primer extension analysis indicating that the levels of $RNA_I$ and $RNA_{II}$ generated from the two *tsps*, previously described (Cumino et al. 2007), increased in the wild-type strain after the nitrogen-

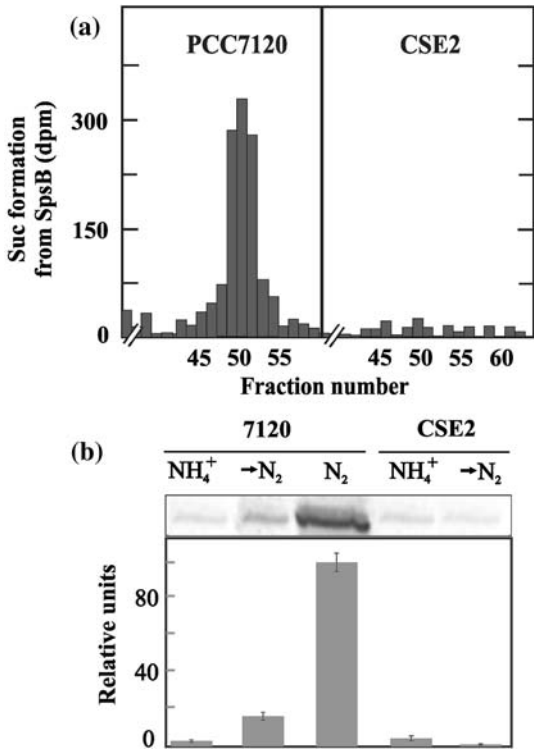


**Fig. 1** Analysis of SpsB activity and polypeptide levels in *Anabaena* sp. PCC 7120 or in the CSE2 mutant strain. **a** SpsB activity measured with ADP-[U-$^{14}$C]-Glc and Fru-6P. **b** Immunoblot analysis of Sps polypeptides in cells grown in ammonium ($NH_4^+$) or in ammonium and then incubated in a medium lacking combined nitrogen for 24 h ($\rightarrow N_2$). The units are relative to the value obtained with the PCC 7120 cells grown in $N_2$ (100%). Data are the mean of three independent experiments

starvation treatment, while in CSE2 cells this effect was not observed.

### Binding of NtcA to the *spsB* promoter region

A detailed sequence inspection of the *spsB* promoter region suggested the presence of two putative NtcA-binding sites (Fig. 2c), one of them ($spsB_{II}$) was not described previously (Cumino et al. 2007). To further characterize the role of NtcA on *spsB* transcription, we performed electrophoretic mobility shift assay (EMSA) using a 477-bp DNA fragment corresponding to the *spsB* promoter region that contained the nitrogen-regulated *tsps*. Two retarded bands were identified suggesting that NtcA may be interacting with two different sites (data not shown). Accordingly, annealed oligonucleotides containing those putative binding sites were retarded by NtcA in preliminary gel shift analyses. Increasing concentrations of 2-OG was also evaluated, which resulted in an enhancement of the NtcA binding to the DNA fragments comprising either $spsB_I$ or $spsB_{II}$ (not shown and Fig. 3a). The relative dissociation constants ($K_D$) of the NtcA binding to $spsB_I$ and $spsB_{II}$

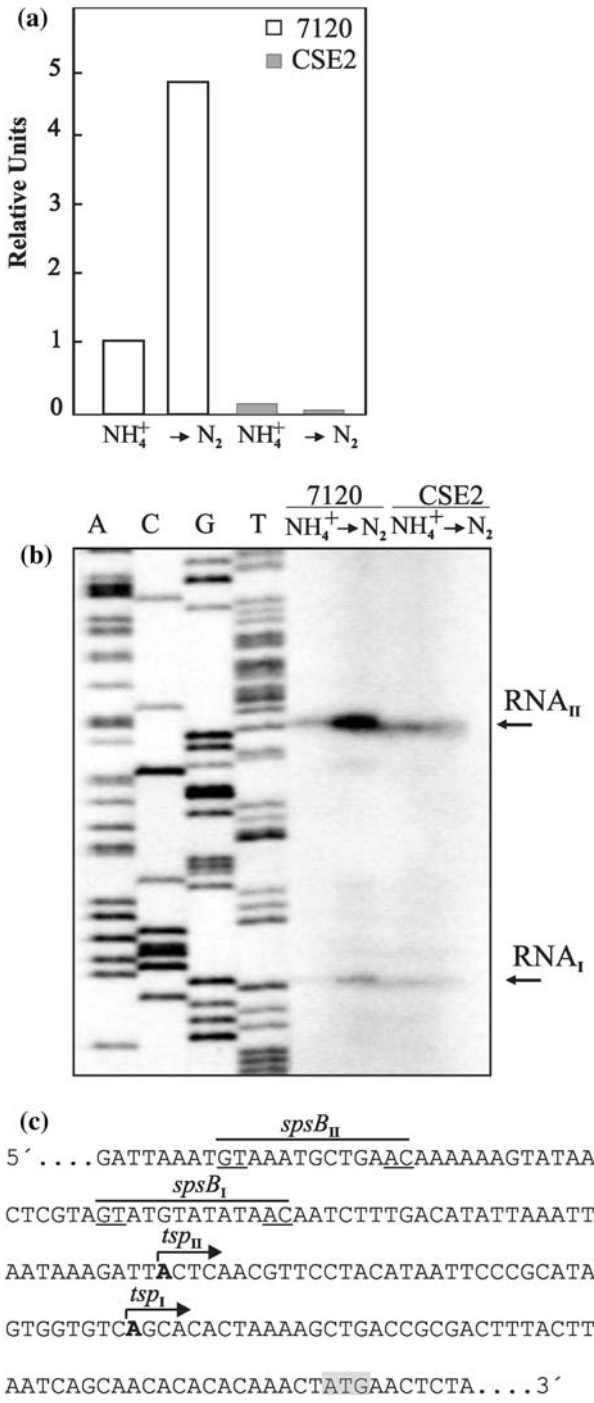


**Fig. 2** Effect of NtcA on the *spsB* transcription level in *Anabaena* sp. PCC 7120 and in the CSE2 mutant strain. **a** Quantitative real-time PCR. The units are relative to the value obtained with the PCC 7120 cells grown in $NH_4^+$, taken arbitrarily as equal to 1. **b** Primer extension analysis. *Arrowheads* indicate two putative *tsp* positions from which $RNA_I$ and $RNA_{II}$ were generated. **c** Nucleotide sequence covering the two putative *tsp* positions on the *spsB* promoter region. The potential sites for NtcA binding are named $spsB_I$ and $spsB_{II}$, and the conserved nucleotides in the consensus signature are *underlined*. *Arrows* indicate the *tsp* positions and in *bold* are the +1 points. The translation start codon is shadowed in *gray*

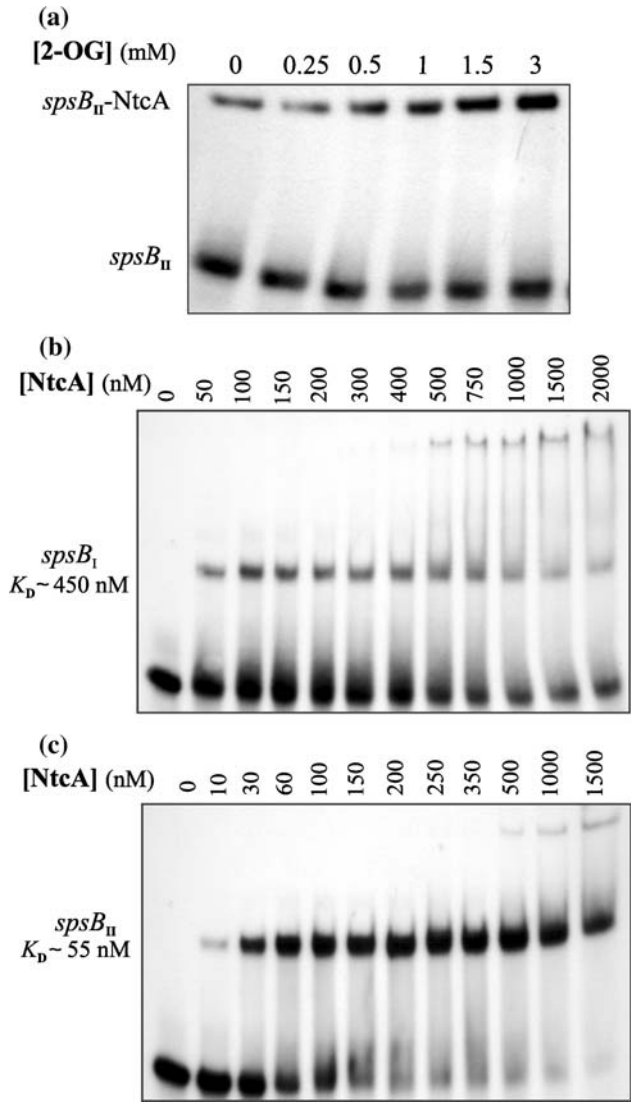


**Fig. 3** NtcA binding to the *spsB* promoter. **a** Effect of increasing 2-OG concentration on NtcA binding to the fragment $spsB_{II}$. **b** $K_D$ of NtcA to oligonucleotides containing $spsB_I$. **c** $K_D$ of NtcA to oligonucleotides containing $spsB_{II}$. Data are the mean of three independent experiments

[about 450 (±52) and 55 (±7) nM, respectively] were calculated from EMSA, where increasing amounts of NtcA were incubated with a fixed concentration of DNA (Fig. 3b, c).

## NtcA mutation also affects *susA* expression after a combined-nitrogen starvation

The downregulation of *susA* expression under diazotrophic conditions in *Anabaena* and the similarities between *susA* and other ammonium-activated cyanobacterial genes (Curatti et al. 2002; Curatti et al. 2006) led us to examine also the role of NtcA as a transcriptional regulator. We

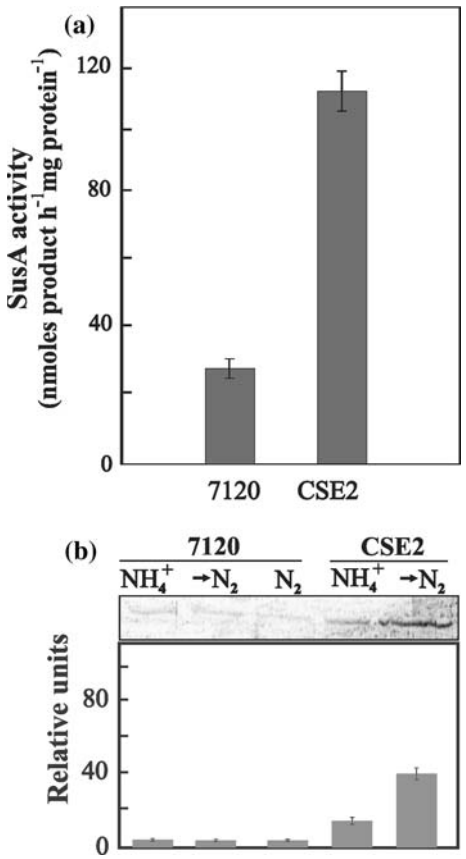


**Fig. 4** Analysis of SusA activity and polypeptide levels in *Anabaena* sp. PCC 7120 or in the CSE2 mutant strain. **a** Sus activity. **b** Immunoblot analysis of SusA polypeptide in cells grown in ammonium ($NH_4^+$) or in ammonium and then incubated in a medium lacking combined nitrogen for 24 h ($\rightarrow N_2$). SusA polypeptide level of the PCC 7120 cells grown in combined nitrogen-free medium ($N_2$) is also shown. Data are the mean of three independent experiments

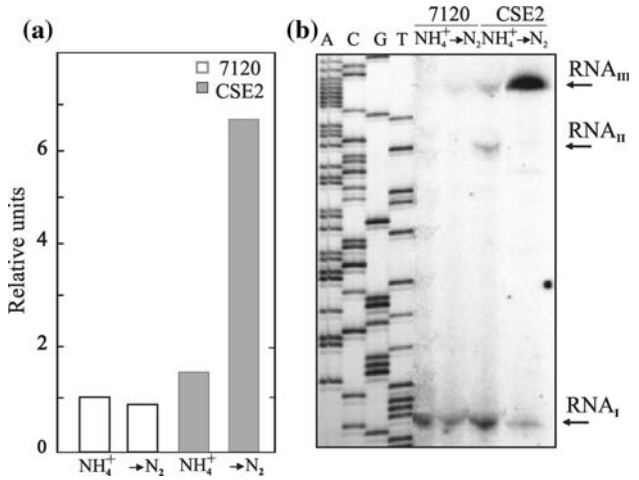


**Fig. 5** Effect of the absence of NtcA in the transcription level of *susA* mRNA measured by quantitative real-time PCR (**a**) or by primer extension analysis (**b**). *Arrowheads* indicate the *tsp* positions. In (**a**), the units are relative to the value obtained for the PCC 7120 cells grown in $NH_4^+$

compared *susA* expression in filaments of the PCC 7120 and CSE2 strains. After 24 h of combined-nitrogen step-down, Sus activity was about 3.5-fold higher in CSE2 cells than in those of PCC 7120, which was in accordance with an increase in the level of SusA polypeptide (Fig. 4). Also, *susA* transcript levels were four times higher in cells of the CSE2 strain than in those of PCC 7120 after 24 h of combined-nitrogen deprivation (Fig. 5a).

To investigate whether *susA* induction and/or derepression in the absence of NtcA took place from already reported (Curatti et al. 2006) or unknown *tsp*s, primer extension assays were conducted with RNA isolated from cells of the two strains grown with ammonium, or after a 24-h-ammonium step-down. It was observed that the level of transcripts generated from the $tsp_{III}$ ($RNA_{III}$) in combined-nitrogen-lacking medium was more abundant in CSE2 than in PCC 7120 cells. Under identical growth condition, $RNA_I$ and $RNA_{II}$ expressions were similar in both strains (Fig. 5b).

The possible involvement of NtcA in the regulation of *susA* expression was investigated in a preliminary in vivo interaction experiment coexpressing *ntcA* under an IPTG-inducible promoter, and *gfp* (GFP-encoding gene) under the control of *Anabaena susA* promoter in *E. coli* cells. A decrease in the fluorescence coming from GFP was determined upon the addition of IPTG (not shown). Although preliminary and performed in a heterologous system, these results suggest that NtcA accumulation in the cells might interfere with transcription from the *susA* promoter.

## Binding of NtcA to the *susA* promoter region

The effect of NtcA on *susA* expression by a direct binding to its promoter region was tested by EMSA using a 370-bp fragment containing the described *tsp*s. The presence of NtcA resulted in the retardation of migration of the DNA fragment in gel electrophoresis (data not shown). To establish whether NtcA binds the potential sites previously proposed (Curatti et al. 2006), we carried out EMSA with a number of annealed oligonucleotides containing the site 1 ($susA_I$) or the site 2 ($susA_{II}$) sequences (Fig. 6a). DNA retardation was only observed for site 1 (data not shown). The $K_D$ of the complex $susA_I$–NtcA was 180 (±16) nM, calculated from EMSA with radiolabeled $susA_I$ and different amounts of NtcA (Fig. 6b).

## Relative NtcA-binding affinities for *spsB* and *susA* promoters

Competition experiments were conducted to analyze specificity of the NtcA–DNA complexes and to determine the relative affinity of NtcA for the identified putative binding sites on *spsB* and *susA* promoter regions. EMSAs

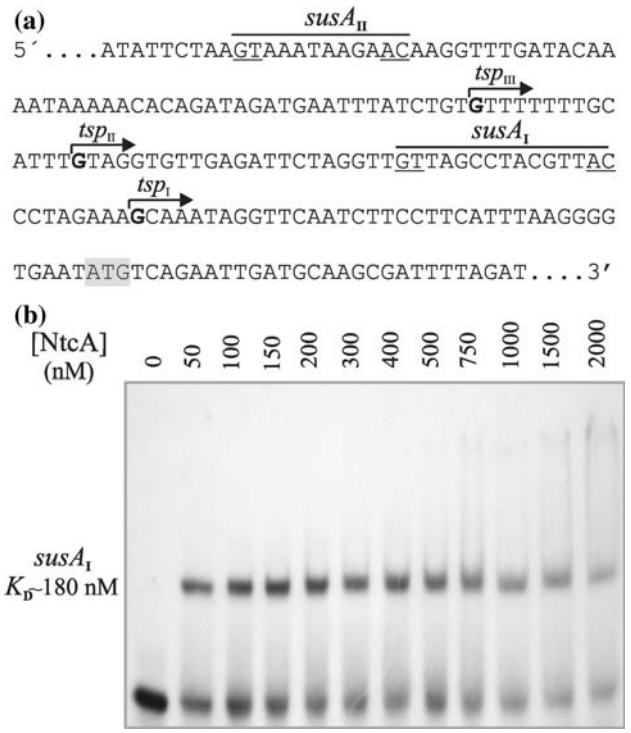


**Fig. 6** Position of putative *tsps* and NtcA binding to *susA* promoter region. **a** Nucleotide sequence covering the three putative *tsp*s on the *susA* promoter region. The two potential sites for NtcA binding are named $susA_I$ and $susA_{II}$, and the conserved nucleotides in the consensus signature are *underlined*. *Arrows* indicate the direction of transcription. In *bold* are the +1 points and shadowed in *gray* is the translation start codon. **b** Determination of $K_D$ of NtcA to $susA_I$. Data are the mean of three independent experiments

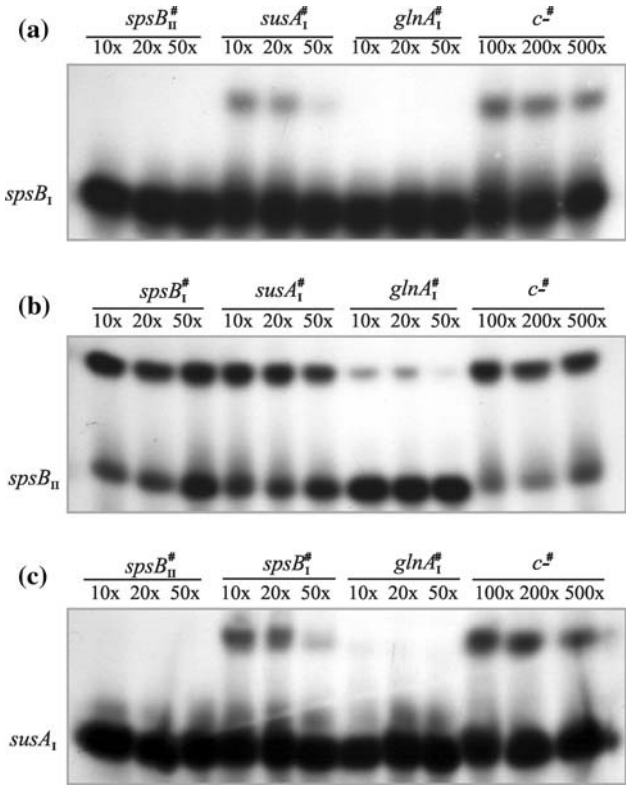


**Fig. 7** Competition experiments with NtcA and *spsB* and *susA* promoter regions. EMSAs were performed with radiolabeled $spsB_I$ (**a**), $spsB_{II}$ (**b**) or $susA_I$ (**c**) oligonucleotides, and 10×-, 20×- or 50×-fold excess of nonradiolabeled $spsB_I^{\#}$, $spsB_{II}^{\#}$, $susA_I^{\#}$, $glnA_I^{\#}$, or with 100×-, 200×- or 500×-fold excess of nonradiolabeled nonspecific competitor fragment ($c\text{-}^{\#}$)

were carried out with NtcA, radiolabeled $spsB_I$ (Fig. 7a), or $spsB_{II}$ (Fig. 7b), or $susA_I$ (Fig. 7c), and different amounts of unlabeled DNA competitors [$spsB_I^{\#}$, $spsB_{II}^{\#}$, $susA_I^{\#}$, $glnA_I^{\#}$ and $c\text{-}^{\#}$ (nonspecific competitor)]. A 50-fold excess of $spsB_{II}^{\#}$ or $glnA_I^{\#}$ fragments significantly interfered with the formation of NtcA–$spsB_I$ or NtcA–$susA_I$ complexes (Fig. 7a, c). In contrast, up to a 50-fold excess of $spsB_I^{\#}$ or $susA_I^{\#}$ fragments did not prevent the formation of the $spsB_{II}$–NtcA complex (Fig. 7b). However, a competition of $spsB_{II}$ with a 50-fold excess of $glnA_I^{\#}$ almost fully eliminated $spsB_{II}$–NtcA interaction (Fig. 7b). A 500-fold excess of $c\text{-}^{\#}$ fragment did not eliminate the binding of NtcA to $spsB_I$, $spsB_{II}$ or $susA_I$ (Fig. 7a–c). Thus, NtcA specifically binds in vitro to the three fragments, with an affinity order of $glnA_I > spsB_{II} > susA_I > spsB_I$. This conclusion was additionally supported by the $K_D$s (Figs. 3b, c, 6b).

## Discussion

Although the knowledge of Suc metabolism in prokaryotic organisms is recent, important achievements have opened new insights into its physiological function in relation with nitrogen metabolism in filamentous $N_2$-fixing cyanobacteria (Salerno and Curatti 2003; Curatti et al. 2006; Cumino et al. 2007).

In this study, we provide evidence of the involvement of NtcA in the transcriptional regulation of Suc metabolism genes. We show that NtcA participates as an activator of *spsB* and as a repressor of *susA* expression by means of different lines of evidence: (1) response of the genes to the presence of ammonium, (2) lack of activation or derepression in a NtcA-deficient genetic background for *spsB* or *susA*, respectively, (3) the presence of DNA sequences similar to characterized NtcA-binding sites in *spsB* and *susA* promoters, and (4) in vitro binding of NtcA to the proposed putative binding sites on *spsB* and *susA* promoters.

Upon nitrogen deprivation, the presence of NtcA causes the ensuing upregulation of *spsB* expression from the two determined *tsps* (positions –54 and –93) in the PCC 7120 strain. This induction is abolished in the *ntcA* CSE2 mutant, showing a direct or indirect link between *spsB*

expression and NtcA. In contrast, the absence of NtcA gives rise to an increase in *susA* expression, suggesting that NtcA might participate in its regulation as a repressor, as proposed for the *rbcLS* gene (Ramasubramanian et al. 1994; Curatti et al. 2006). Moreover, neither *susA* nor *rbcLS* is expressed in heterocysts where *ntcA* expression also takes place at a high level (Olmedo-Verd et al. 2006). A converse effect of NtcA on the expression of *spsB* and *susA* may be operating in the heterocysts, in agreement with the differential expression of both genes in those cells (Curatti et al. 2006; Cumino et al. 2007).

The in vitro studies show that NtcA specifically interacts with both *spsB* and *susA* up-stream regions. Also, a positive effect of 2-OG, the molecule signaling the carbon/nitrogen balance of the cell, was observed in the NtcA binding to the promoter regions, as it was demonstrated for other NtcA-activated promoters of *Anabaena* sp. PCC 7120 genes (Vázquez-Bermúdez et al. 2002b). The metabolite 2-OG accumulates within nitrogen-starved filaments up to about ~0.1 mM and constitutes the early signal for the initiation of heterocyst differentiation (Laurent et al. 2005).

Depending on the position of its binding site with respect to different promoters, NtcA was reported to act as an activator or a repressor of gene expression in the PCC 7120 strain (Herrero et al. 2001). In the case of *spsB*, two putative NtcA-binding sites ($spsB_{I}$ and $spsB_{II}$, centered at 38.5 and 71.5 nt upstream $tsp_{II}$) were identified in its promoter sequence. While the location of $spsB_{I}$ is consistent with most NtcA-binding sites characterized in NtcA-activated promoters (Luque et al. 1994; Herrero et al. 2001), the highest binding affinity for NtcA was exhibited for the $spsB_{II}$ site. The involvement of two regulator-binding sites is also the case of the *ntcA* promoter from *Anabaena* sp. PCC 7120, which bears NtcA-binding sites at −94.5 and −54.5 nt from one of its *tsp*s (Ramasubramanian et al. 1996). Binding sequences for NtcA have already been found in a similar position in NtcA-activated promoters from other cyanobacteria (Luque et al. 2001). In the case of *susA* promoter, although two putative NtcA-binding sites were proposed previously (Curatti et al. 2006), the in vitro experiments demonstrate that NtcA specifically binds only $susA_{I}$. The location of this NtcA-binding site (43 bp downstream of $tsp_{III}$) is not overlapping the RNA polymerase binding sequence (−35 or −10 boxes) as reported for other NtcA-repressed genes from *Anabaena* sp. PCC 7120 and *Synechocystis* sp. PCC 6803 (Ramasubramanian et al. 1994; Jiang et al. 1995; García-Domínguez et al. 2000). However, a similar arrangement has been described for the *hanA* gene (encoding the histone-like protein, HU) of *Anabaena* sp. PCC 7120 in which an NtcA-binding site is positioned at 68 bp downstream its *tsp* (Khudyakov and Wolk 1996). On the other hand, the molecular mechanism by which NtcA represses transcription from the promoter corresponding to *susA* $RNA_{III}$ is not completely understood. It appears that NtcA binding to $susA_{I}$ alone would be insufficient to explain the *susA* expression differences observed in the CSE2 and PCC 7120 strains when ammonium is not available. Presumably, other unidentified protein factors and/or metabolites that are differentially expressed and/or accumulated in the cells in the presence or absence of ammonium might modulate the NtcA-repressing activity of transcription from the *susA* promoter.

Similar to other NtcA-regulated genes that carry non-consensus NtcA-binding sites in their promoters (Herrero et al. 2001, 2004; Olmedo-Verd et al. 2006; López-Gomollón et al. 2007), the putative sequences of the NtcA-binding sites on *spsB* and *susA* promoter regions did not match exactly the canonical signature sequences. This feature may explain the lower affinity for NtcA obtained for $spsB_{II}$, $susA_{I}$ and $spsB_{I}$ in comparison with that of $glnA_{I}$, since variations in the consensus sequences (either in the triplet sequences or in the spacer length between the GTA and TAC triplets, longer or shorter than eight nucleotides) resulted in a decrease of affinity for NtcA (Jiang et al. 1995, 2000; Luque et al. 2001; Vázquez-Bermúdez et al. 2002a).

Our results strongly suggest that NtcA is required to maintain a high Suc biosynthetic capacity and a low rate of disaccharide cleavage during $N_2$ fixation, supporting a central role for the Suc molecule in the $N_2$-fixing filaments. Thus, it appears that NtcA integrates signals from carbon and nitrogen metabolism, and regulates gene expression accordingly to redirect metabolism as a function of the carbon/nitrogen status of the cell. The demonstration of a direct participation of NtcA in the regulation of the expression of genes for Suc metabolism contributes to highlight the importance of NtcA as a global regulator of metabolism beyond its well-defined function as a global nitrogen regulator.

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## References

Commichau FM, Forchhammer K, Stulke J (2006) Regulatory links between carbon and nitrogen metabolism. Curr Opin Microbiol 9:167–172

Cumino AC, Ekeroth CN, Salerno GL (2001) Sucrose-phosphate phosphatase from *Anabaena* sp. strain PCC 7120: isolation of the protein and gene revealed significant structural differences from the higher-plant enzyme. Planta 214:250–256

Cumino A, Curatti L, Giarrocco L, Salerno GL (2002) Sucrose metabolism: *Anabaena* sucrose-phosphate synthase and sucrose-phosphate phosphatase define minimal functional domains shuffled during evolution. FEBS Lett 517:19–23

Cumino AC, Marcozzi C, Barreiro R, Salerno GL (2007) Carbon cycling in *Anabaena* sp. PCC 7120. Sucrose synthesis in the heterocysts and possible role in nitrogen fixation. Plant Physiol 143:1385–1397

Curatti L, Porchia AC, Herrera-Estrella L, Salerno GL (2000) A prokaryotic sucrose synthase gene (*susA*) isolated from a filamentous nitrogen-fixing cyanobacterium encodes a protein similar to those of plant. Planta 211:727–735

Curatti L, Flores E, Salerno GL (2002) Sucrose is involved in the diazotrophic metabolism of the heterocyst-forming cyanobacterium *Anabaena* sp. FEBS Lett 513:175–178

Curatti L, Giarrocco L, Salerno GL (2006) Sucrose synthase and RuBisCo expression is similarly regulated by the nitrogen source in the nitrogen-fixing cyanobacterium *Anabaena* sp. Planta 5:891–900

Frías JE, Flores E, Herrero A (1994) Requirement of the regulatory protein NtcA for the expression of nitrogen assimilation and heterocyst development genes in the cyanobacterium *Anabaena* sp. PCC 7120. Mol Microbiol 14:823–832

García-Domínguez M, Reyes JC, Florencio FJ (2000) NtcA represses transcription of *gifA* and *gifB*, genes that encode inhibitors of glutamine synthetase type I from *Synechocystis* sp. PCC 6803. Mol Microbiol 35:1192–1201

Herrero A, Muro-Pastor AM, Flores E (2001) Nitrogen control in cyanobacteria. J Bacteriol 183:411–425

Herrero A, Muro-Pastor AM, Valladares A, Flores E (2004) Cellular differentiation and the NtcA transcription factor in filamentous cyanobacteria. FEMS Microbiol Rev 28:469–487

Jiang F, Hellman U, Sroga GE, Bergman B, Mannervik B (1995) Cloning, sequencing, and regulation of the glutathione reductase gene from the cyanobacterium *Anabaena* PCC 7120. J Biol Chem 270:22882–22889

Jiang F, Mannervik B, Bergman B (1997) Evidence for redox regulation of the transcription factor NtcA, acting both as an activator and a repressor, in the cyanobacterium *Anabaena* PCC 7120. Biochem J 327:513–517

Jiang F, Wisén S, Widersten M, Bergman B, Mannervik B (2000) Examination of the transcription factor NtcA-binding motif by in vitro selection of DNA sequences from a random library. J Mol Biol 301:783–793

Khudyakov I, Wolk CP (1996) Evidence that the *hanA* gene coding for HU protein is essential for heterocyst differentiation and cyanophage A-4(L) sensitivity of, *Anabaena* sp. strain PCC 7120. J Bacteriol 178:3572–3577

Korner H, Sofia HJ, Zumft WG (2003) Phylogeny of the bacterial superfamily of Crp-Fnr transcription regulators: exploiting the metabolic spectrum of controlling alternative gene programs. FEMS Microbiol Rev 27:559–592

Laurent S, Chen H, Bedu S, Ziarelli F, Peng L, Zhang CC (2005) Nonmetabolizable analogue of 2-OG elicits heterocyst differentiation under repressive conditions in *Anabaena* sp. PCC 7120. Proc Natl Acad Sci USA 102:9907–9912

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\triangle\triangle C}{}_{T}$ method. Methods 25:402–408

López-Gomollón S, Hernandez JA, Wolk CP, Peleato ML, Fillat MF (2007) Expression of *furA* is modulated by NtcA and strongly enhanced in heterocysts of *Anabaena* sp. PCC 7120. Microbiology 153:42–50

Luque I, Flores E, Herrero A (1994) Molecular mechanism for the operation of nitrogen control in cyanobacteria. EMBO J 13:2862–2869

Luque I, Zabulon G, Contreras A, Houmard J (2001) Convergence of two global transcriptional regulators on nitrogen induction of the stress acclimation gene *nblA* in the cyanobacterium *Synechococcus* sp. PCC 7942. Mol Microbiol 1:937–947

Muro-Pastor MI, Reyes JC, Florencio FJ (2001) Cyanobacteria perceive nitrogen status by sensing intracellular 2-oxoglutarate levels. J Biol Chem 276:38320–38328

Olmedo-Verd E, Muro-Pastor AM, Flores E, Herrero A (2006) Localized induction of the *ntcA* regulatory gene in developing heterocysts of *Anabaena* sp. strain PCC 7120. J Bacteriol 188:6694–6699

Porchia AC, Salerno GL (1996) Sucrose biosynthesis in a prokaryotic organism: presence of two sucrose-phosphate synthases in Anabaena with remarkable differences compared with the plant enzymes. Proc Natl Acad Sci USA 93:13600–13604

Porchia AC, Curatti L, Salerno GL (1999) Sucrose metabolism in cyanobacteria: sucrose synthase from Anabaena sp PCC 7119 is remarkably different from the plant enzymes with respect to substrate affinity and amino-terminal sequence. Planta 210:34–40

Ramasubramanian TS, Wei TF, Golden JW (1994) Two *Anabaena* sp. strain PCC 7120 DNA-binding factors interact with vegetative cell- and heterocyst-specific genes. J Bacteriol 176:1214–1223

Ramasubramanian TS, Wei TF, Oldham AK, Golden JW (1996) Transcription of the *Anabaena* sp. strain PCC 7120 *ntcA* gene: multiple transcripts and NtcA binding. J Bacteriol 178:922–926

Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY (1979) Generic assignments, strain stories and properties of pure cultures of cyanobacteria. J Gen Microbiol 111:1–61

Salerno GL, Curatti L (2003) Origin of sucrose metabolism in higher plants: when, how and why? Trends Plant Sci 8:63–69

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Su Z, Olman V, Mao F, Xu Y (2005) Comparative genomics analysis of NtcA regulons in cyanobacteria: regulation of nitrogen assimilation and its coupling to photosynthesis. Nucleic Acids Res 33:5156–5171

Tanigawa R, Shirokane M, Maeda Si S, Omata T, Tanaka K, Takahashi H (2002) Transcriptional activation of the NtcA-dependent promoters of *Synechococcus* sp. PCC 7942 by 2-oxoglutarate in vitro. Proc Natl Acad Sci USA 99:4251–4255

Vargas W, Cumino A, Salerno GL (2003) Cyanobacterial alkaline/neutral invertases. Origin of sucrose hydrolysis in the plant cytosol? Planta 216:951–960

Vázquez-Bermúdez MF, Flores E, Herrero A (2002a) Analysis of the binding sites for the nitrogen-control transcription factor NtcA in the promoters of *Synechococcus* nitrogen-regulated genes. Biochim Biophys Acta 1578:95–98

Vázquez-Bermúdez MF, Herrero A, Flores E (2002b) 2-Oxoglutarate increases the binding affinity of the NtcA (nitrogen control) transcription factor for the *Synechococcus* sp. strain PCC 7942. FEMS Microbiol Lett 221:155–159

Vega-Palas MA, Flores E, Herrero A (1992) NtcA, a global nitrogen regulator from the cyanobacterium *Synechococcus* that belongs to the Crp family of transcriptional regulators. Mol Microbiol 6:1853–1859

Wisén S, Bergman B, Mannervik B (2004) Mutagenesis of the cysteine residues in the transcription factor NtcA from *Anabaena* PCC 7120 and its effects on DNA binding in vitro. Biopchim Biophys Acta 1679:156–163

Wolk CP, Ernst A, Elhai J (1994) Heterocyst metabolism and development. In: Bryant DA (ed) The molecular biology of cyanobacteria. Kluwer, Dordrecht, pp 769–823