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Involvement of NtcB, a LysR Family Transcription Factor, in Nitrite Activation of the Nitrate Assimilation Operon in the Cyanobacterium *Synechococcus* sp. Strain PCC 7942

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Nitrite, either exogenously supplied or endogenously generated by nitrate reduction, activates transcription of the nitrate assimilation operon (*nirA-nrtABCD-narB*) in *Synechococcus* sp. strain PCC 7942 cells treated with L-methionine-DL-sulfoximine (an inhibitor of glutamine synthetase), in which there is no negative feedback resulting from fixation of the ammonium generated by nitrite reduction (Kikuchi et al., J. Bacteriol. 178:5822–5825, 1996). Other transcription units related to nitrogen assimilation, i.e., the *nirB-ntcB* operon, *glnA*, and *ntcA*, were not activated by nitrite. Nitrite did not activate *nirA* operon transcription in a mutant with a deletion of *ntcB*, an ammonium-repressible gene encoding a LysR-type DNA-binding protein. Introduction of plasmid-borne *ntcB* into the *ntcB* deletion mutant restored the response of the cells to nitrite, indicating that NtcB activates the *nirA* operon in response to nitrite. Supplementation of nitrite or nitrate to nitrogen-starved cultures of the wild-type strain, but not of the *ntcB* deletion mutant, caused activation of the *nirA* operon without L-methionine-DL-sulfoximine treatment of the cells. The results suggested that the positive-regulation mechanism of *nirA* operon transcription plays a role in rapid adaptation of nitrogen-starved cells to changing availability of nitrate and nitrite.

Transcription of the nitrate assimilation operon, *nirA-nrtABCD-narB*, of the cyanobacterium *Synechococcus* sp. strain PCC 7942 is repressed by ammonium (20) and activated by nitrate or nitrite in the medium (11). Ammonium represses transcription through its fixation into Gln, but Gln is not the direct regulator of transcription (20). We have proposed that cyanate, a metabolite of Gln via carbamoylphosphate, acts as the metabolic signal for the ammonium-promoted repression of the *nirA* operon (21). Depletion of ammonium from the medium or inhibition of ammonium fixation with L-methionine-DL-sulfoximine (MSX) derepresses the operon and induces its transcription, showing no requirement for nitrate or nitrite (20). Under the derepressing conditions, however, nitrate and nitrite further activate transcription (11). The positive effect of nitrate and nitrite is manifest in MSX-treated cells, in which there is no negative feedback by the ammonium generated internally by reduction of nitrate and nitrite (11). In the absence of MSX, the negative regulation by internally generated ammonium overrides the positive regulation, and the effects of nitrate and nitrite are marginal (11). Nitrite is the actual activator of transcription and nitrate must be reduced to nitrite to activate the operon (11).

In the genomic DNA region upstream of the *nirA* operon are the *nirB* and *ntcB* genes, which are required for maximum nitrate assimilation and which form an operon oriented divergently from *nirA* (19). Since the predicted NtcB protein belongs to the LysR family of transcription factors, many members of which activate a divergently transcribed operon located upstream (7, 17), we once suspected that *ntcB* might be involved in regulation of the *nirA* operon. However, the levels of the *nirA* operon transcript in the wild-type strain and an *ntcB* deletion mutant, measured in the absence of MSX, were not greatly different, and we concluded that *ntcB* is unlikely to regulate the *nirA* operon (19). In the present study, we reinvestigated the relationship between NtcB and *nirA* operon transcription in MSX-treated cells and found that NtcB is required for the nitrite-responsive positive regulation of the *nirA* operon. In the cells subjected to prolonged starvation of nitrogen, the NtcB-dependent, nitrite-responsive activation of the *nirA* operon without MSX treatment was obvious. The physiological significance of the dual regulation of the *nirA* operon, i.e., derepression by nitrogen depletion and activation by nitrite, in adaptation to changing availability of nitrogen in the natural environment is discussed.

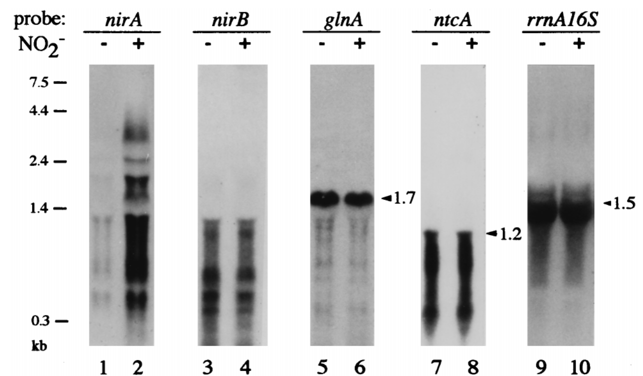

FIG. 1. Northern analysis of RNA from *Synechococcus* sp. strain PCC 7942 showing the effects of nitrite on the transcription of *nirA* (lanes 1 and 2), *nirB* (lanes 3 and 4), *glnA* (lanes 5 and 6), and *ntcA* (lanes 7 and 8) in MSX-treated cells. The 16S rRNA band, detected with the *rrnA* 16S rRNA gene as a probe, is also shown (lanes 9 and 10). Cells were grown with ammonium, the culture was separated into two portions, and total RNA was extracted from the cells 80 min after addition of MSX alone (lanes 1, 3, 5, 7, and 9) and MSX plus nitrite (lanes 2, 4, 6, 8, and 10). Molecular sizes (kilobases) are indicated.

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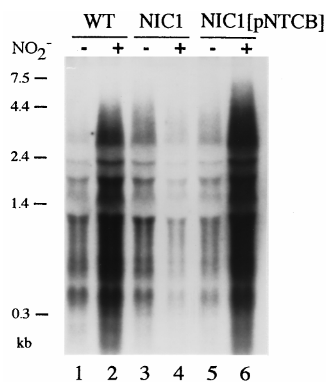


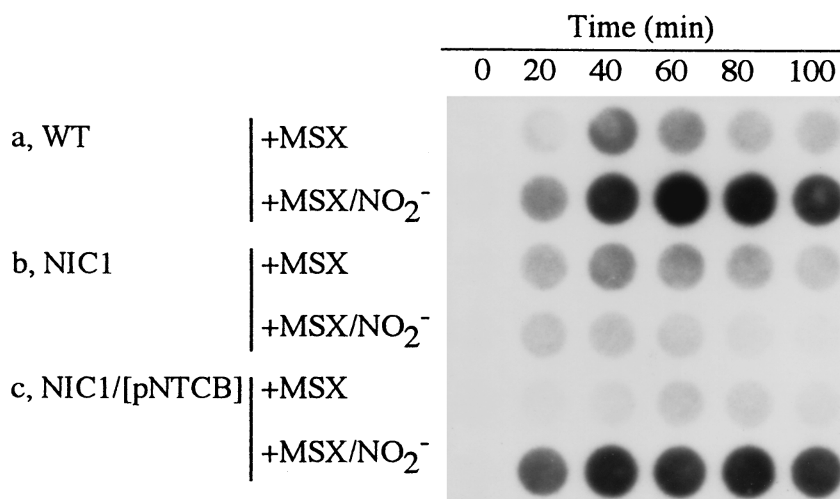
FIG. 2. Northern analysis of RNA from the wild-type PCC 7942 strain (WT; lanes 1 and 2), the *ntcB*-deficient mutant (NIC1; lanes 3 and 4), and the NIC1 mutant transformed with a plasmid carrying *ntcB* (NIC1[pNTCB]; lanes 5 and 6), showing the dependence on *ntcB* of the nitrite-promoted activation of *nirA* operon transcription. Cells were grown with ammonium, the culture was separated into two portions, and total RNA was extracted from the cells 80 min after addition of MSX alone (lanes 1, 3, and 5) and MSX plus nitrite (lanes 2, 4, and 6).

MATERIALS AND METHODS

Strains and growth conditions. A derivative of *Synechococcus* sp. strain PCC 7942 which is cured of the resident small plasmid pUH24 (R2-SPc [12]; hereafter designated simply as strain PCC 7942) and an *ntcB*-deficient mutant strain (Δ*ntcB*::*kan*; hereafter designated as NIC1) derived therefrom (19) were grown photoautotrophically at 30°C under CO_2-sufficient conditions as described previously (21). The basal medium used was a nitrogen-free medium obtained by modification of BG11 medium (18) as previously described (21). Ammonium-containing medium was prepared by addition of 3.75 mM $(NH_4)_2SO_4$ to the basal medium. Both media were buffered with 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)–KOH (pH 8.2). When appropriate, kanamycin, spectinomycin, and streptomycin were added to the media at 10, 2, and 2 μg/ml, respectively.

Transcription of the *nirA* operon was induced by treatment of ammonium-grown cyanobacterial cells with MSX, an inhibitor of ammonium fixation by glutamine synthetase, or by transfer of the ammonium-grown cells to nitrogen-free medium. MSX was added to cyanobacterial cultures in the mid-logarithmic phase of growth with or without simultaneous addition of $NaNO_2$. The final concentrations of MSX and $NaNO_2$ were 0.1 mM and 1 mM, respectively. For transfer of the cells to nitrogen-free medium, the ammonium-grown cells were collected by centrifugation at 5,000 × *g* for 5 min at 25°C, washed twice with the basal medium by resuspension and recentrifugation, and inoculated into the basal medium.

RNA isolation and analysis. Total RNA was extracted and purified from cyanobacterial cells by the method of Aiba et al. (1). For Northern hybridization analysis, RNA samples (10 μg per lane for detection of *nirA* and *glnA* transcripts and 20 μg per lane for detection of *ntcA* and *nirB* transcripts) were denatured by treatment with formamide, fractionated by electrophoresis on 1.2% agarose gels



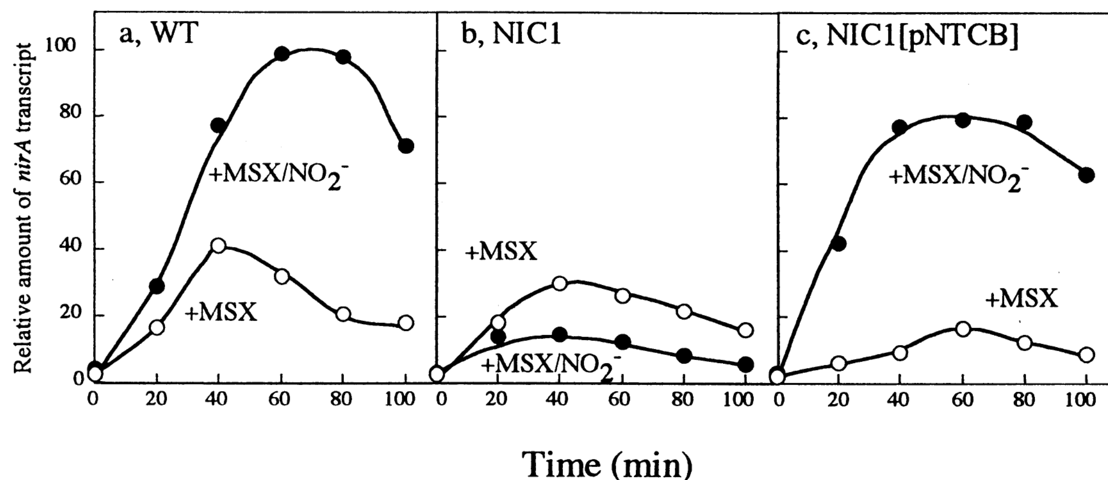


FIG. 3. Changes in *nirA* transcript abundance after addition of MSX alone (+MSX; ○) and MSX plus nitrite (+MSX/NO_2^-; ●) to the ammonium-grown cultures of the wild-type and mutant strains. (A) Autoradiogram on X-ray film showing the results of the dot hybridization analysis with 2.5 μg of RNA per dot. (B) The results of the dot hybridization analysis quantified and plotted relative to the maximum level in the wild-type cells treated with MSX plus nitrite. WT, wild-type strain.

that contained formaldehyde, and transferred to positively charged nylon membranes (Hybond N+; Amersham). For dot hybridization analysis, 1.25-, 2.5-, and 5-μg aliquots of each of the denatured RNA samples were spotted on the nylon membranes with a dot blot apparatus. The blots were allowed to hybridize as described by Church and Gilbert (2) with the following probes: a 640-bp *Nco*I-*Ava*I fragment of *nirA* (20), a 288-bp fragment of *glnA* (3) corresponding to nucleotides −105 to 183 with respect to the translation start site, a 403-bp *Bam*HI-*Hin*dIII fragment of *ntcA* (22), and the entire *nirB* coding region (19). The *glnA*-, and *nirB*-specific probes were prepared by amplification by PCR of the respective sequences, with genomic DNA from *Synechococcus* sp. strain PCC 7942 as the template. The double-stranded DNA probes were labeled with ^{32}P as described by Feinberg and Vogelstein (6). The hybridization signals were detected by autoradiography on X-ray film or by a Bio-image analyzer (Fuji Photo Film). The radioactivity of the RNA dots was quantified with a Bio-image analyzer.

Expression of plasmid-encoded NtcB in *Synechococcus*. For expression of cloned genes in strain PCC 7942, the shuttle vector pSE2 was constructed according to the previously described procedure for construction of the shuttle vector pSE1 (16), except that the 2-kbp *Bam*HI fragment of pRL453 (5), carrying the gene that confers resistance to spectinomycin and streptomycin was ligated into the vector in place of the *nptI* gene cassette to provide a selection marker. The pSE2 plasmid replicated in both *Synechococcus* sp. strain PCC 7942 and *Escherichia coli*, conferring spectinomycin and streptomycin resistance to the cells. A PCR-amplified *ntcB* gene, in which a *Bsp*HI recognition site had been created at the translation start site with no amino acid substitutions, was digested with *Bsp*HI and cloned between the *Nco*I and *Xba*I sites of pSE2. The resulting plasmid (pNTCB1) was used for expression of *ntcB* in the *ntcB* deletion mutant.

Other methods. Nitrate reductase (NR) and nitrite reductase (NiR) activities were determined at 30°C by using toluene-permeabilized cells with dithionite-reduced methylviologen as the electron donor (8, 9). Chlorophyll and protein levels were determined according to Mackinney (15) and Lowry et al. (13), respectively.

RESULTS

Effects of nitrite on ammonium-repressible genes related to nitrogen assimilation. In a previous study, we found that nitrite activates transcription of the *nirA* operon in MSX-treated cells of *Synechococcus* sp. strain PCC 7942 (11). Since the *nirA* operon is one of the ammonium-repressible transcription units involved in nitrate assimilation, the effects of nitrite on other ammonium-repressible genes were examined (Fig. 1). Nitrite increased the *nirA* operon transcript level, as previously shown, but did not affect the levels of the transcripts of the *nirB-ntcB* operon (Fig. 1, lanes 3 and 4), *glnA* (lanes 5 and 6), and *ntcA* (lanes 7 and 8). The results showed that nitrite specifically activates the *nirA* operon among the genes involved in assimilation of inorganic nitrogen. The specific activation by nitrite of the *nirA* operon suggested that the molecular mechanism of the nitrite-promoted activation of the operon is distinct from that of the ammonium-promoted repression.

Dependence on *ntcB* of the nitrite-promoted activation of the *nirA* operon. The *ntcB* gene, encoding a LysR-type DNA-binding protein, is a part of the *nirB-ntcB* operon required for maximum nitrate assimilation (19). In an *ntcB* deletion mutant (NIC1) with no apparent defect in *nirB* expression (19), there was no activation by nitrite of the *nirA* operon (Figs. 2 and 3), suggesting involvement of NtcB in the positive regulation of the operon. Introduction into NIC1 of a plasmid-borne *ntcB* gene restored the response of the cells to nitrite (Figs. 2 and 3), indicating that NtcB itself is required for the activation of the *nirA* operon.

Activation by nitrite and NtcB of the *nirA* operon in nitrogen-starved cells. Figure 4 shows the changes in *nirA* transcript abundance caused by nitrogen starvation and subsequent supplementation of nitrite. In both the wild-type strain and the NIC1 mutant, transfer of the cells to nitrogen-free medium induced transcription of the *nirA* operon. In the wild-type cells, the transcripts reached maximum abundance at 40 min after the transfer and then rapidly decreased to a level corresponding to 30 to 40% of the maximum level. In NIC1, the initial rise in the transcript level was similar to that observed in the wild-

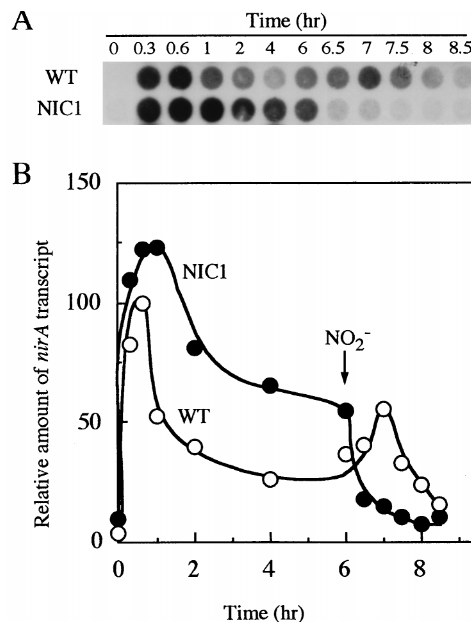


FIG. 4. Effects of nitrogen starvation and subsequent supplementation of nitrite on *nirA* transcript abundance in the cells of the wild-type strain (WT) and NIC1. Cells were transferred from ammonium-containing medium to nitrogen-free medium at time zero, and $NaNO_2$ was added to the cultures to a final concentration of 1 mM after a 6-h incubation under nitrogen starvation conditions (indicated by an arrow). (A) Autoradiogram on X-ray film showing the results of the dot hybridization analysis with 1.25 μg of RNA per dot. (B) The results of the dot hybridization analysis quantified and plotted relative to the maximum level in the wild-type cells. A representative of three sets of essentially the same results, obtained with three independent sets of cultures, is shown.

type strain, but the subsequent decrease in the transcript level was slower. Consequently, the transcript level in the mutant was higher than that in the wild type after reaching the maximum level. Addition of nitrite to the cells after 6 h of nitrogen starvation caused a transient increase in the amount of *nirA* operon transcript in the wild-type strain, whereas in the mutant strain, nitrite caused a rapid decrease in the transcript level. Regulation of the *nirA* operon was thus altered in the *ntcB* mutant in the absence of nitrite as well as in its presence. The results also showed that NtcB and nitrite can activate *nirA* operon transcription in the nitrogen-starved cells without MSX treatment.

Activation of *nirA* operon transcription in nitrogen-starved cells was caused also by nitrate, a more commonly available nitrogen source in the natural environment, and the increase in the transcript level was accompanied by increases in NR and NiR activities (Fig. 5). Similar to nitrite, nitrate caused neither an increase in the level of *nirA* operon transcript nor increases in NR and NiR activities in the *ntcB* deletion mutant (Fig. 5).

DISCUSSION

By using MSX-treated cells, in which there is no negative regulation of the *nirA* operon, nitrite was shown to stimulate *nirA* operon transcription in the wild-type strain but not in the *ntcB* mutant (Figs. 2 and 3). Introduction of plasmid-borne *ntcB* into the *ntcB* deletion mutant restored the response of the cells to nitrite stimulation (Figs. 2 and 3). These findings indicate that *ntcB* is responsible for the nitrite-promoted activation of the *nirA* operon. NtcB thus activates a divergently transcribed operon located upstream, as many other LysR-type

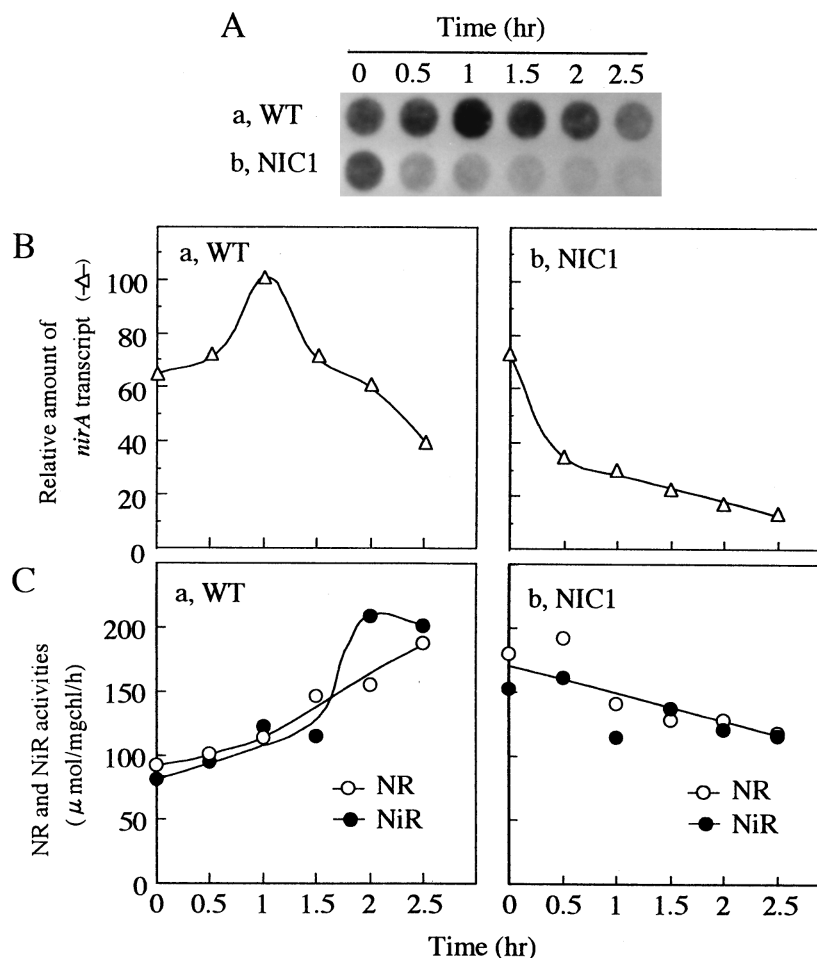


FIG. 5. Changes in *nirA* transcript abundance and NR and NiR activities after addition of nitrate to nitrogen-starved cultures of the wild-type strain (WT) and the NIC1 mutant. Ammonium-grown cells were transferred to nitrogen-free medium, and after a 6-h incubation in the light (time zero), KNO_3 was added to the cell suspensions to a final concentration of 2 mM. (A) Autoradiogram on X-ray film showing the results of the dot hybridization analysis with 1.25 μg of RNA per dot. (B) The results of the dot hybridization analysis quantified and plotted relative to the maximum level in the wild-type cells. (C) NR and NiR activities. One of the two sets of essentially the same results, obtained with two independent sets of cultures, is shown. chl, chlorophyll.

transcriptional regulators do (7, 17), with nitrite presumably being the coinducer.

Due to the negative effect of internally generated ammonium, the positive regulation of *nirA* operon transcription is not obvious after transfer of ammonium-grown cells to nitrate-containing medium (11), and the physiological significance of the regulation under nitrogen-sufficient conditions has remained unclear. The finding of the involvement of NtcB in the regulation provides a clue to the physiological role of the positive-regulation mechanism. The *ntcB* mutant grows slower than the wild-type strain in nitrate-containing medium, with NR and NiR activities being 25 to 35% lower than the wild-type level (19). These observations suggest that the nitrite-promoted, NtcB-dependent activation has a role in optimization of the level of *nirA* operon transcription during continuous growth of the cyanobacterial cells on nitrate. In addition, the present results indicate that the positive-regulation mechanism is required for the substrate-induced activation of the nitrate assimilation operon in nitrogen-starved cells, which leads to an increase in nitrate assimilation activities (Figs. 4 and 5).

Since the *ntcB* mutant shows a sign of nitrogen deficiency not only in nitrate-containing medium but also in ammonium-containing medium, we previously inferred that NtcB, expressed at a low level in ammonium-grown cells, has a role in the optimization of the partitioning of fixed nitrogen among different biosynthetic pathways (19), which inference assumed functioning of NtcB in the absence of nitrite. The higher level of *nirA* operon transcript in the nitrogen-starved cells of the *ntcB* deletion mutant, compared to the wild-type level (Fig. 4), also implies a nitrite-independent function of NtcB; NtcB seems to negatively regulate *nirA* operon transcription in the absence of nitrite. This would be advantageous to the nitrogen-starved cells, since uncontrolled expression of the operon in the absence of the substrate would be wasteful. Combined with the positive-regulation mechanism, the presumed function of NtcB in negative regulation of *nirA* operon transcription would allow efficient utilization of intracellular resources in adaptation to changing availability of nitrogen in the natural environment.

Nitrate is the most common form of combined inorganic nitrogen in the largely nitrogen-deficient natural environment. Since nitrate must be reduced to nitrite to activate transcription of the *nirA* operon (11), expression of the basal activities of transport and reduction of nitrate is a prerequisite for the rapid response of nitrogen-starved cells to external nitrate. While expression of nitrate assimilation genes requires nitrate

in higher plants (10) and nitrate or nitrite in fungi (4), it is induced simply by depletion of the nitrogen source in cyanobacteria (14, 20). The nitrate- and nitrite-independent induction of *nirA* operon transcription seems to have a role in preparing the cells for unpredictable encounters with nitrate in the natural environment.

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