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Positive Regulation by Nitrite of the Nitrate Assimilation Operon in the Cyanobacteria *Synechococcus* sp. Strain PCC 7942 and *Plectonema boryanum*

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In the absence of fixation of ammonium to glutamine, nitrate and nitrite activated transcription of the nitrate assimilation (*nirA-nrtABCD-narB*) operon of *Synechococcus* sp. strain PCC 7942. In a nitrate reductase-deficient mutant, only nitrite activated transcription, indicating that nitrite is the actual activator of the operon. Nitrate and nitrite were also found to activate the transcription of a nitrate assimilation operon in the filamentous nonheterocystous nitrogen-fixing cyanobacterium *Plectonema boryanum*.

Nitrate is a major source of nitrogen for cyanobacteria (11, 12). It is transported into the cells by an active transport system and reduced to ammonium by the sequential action of nitrate reductase (NR) and nitrite reductase (NiR) prior to fixation into the amide nitrogen of glutamine (Gln). As in other microorganisms capable of nitrate assimilation (3, 4, 6, 8, 19, 24), expression of the nitrate assimilation system is inhibited by ammonium (12). In the unicellular non-nitrogen-fixing cyanobacterium *Synechococcus* sp. strain PCC 7942, the genes encoding the nitrate transporter (*nrtABCD*) (25–27), NR (*narB*) (2, 17), and NiR (*nirA*) (20, 32) form the *nirA-nrtABCD-narB* operon (32), and transcription of the operon is repressed by ammonium (32). Ammonium inhibits transcription through its fixation into Gln, but Gln is not the direct regulator of transcription (32). 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 (34).

Transcription of the *nirA* operon of *Synechococcus* sp. strain PCC 7942 is induced simply by removal of ammonium from the medium or by inhibition of ammonium fixation with L-methionine-DL-sulfoximine (MSX), showing no requirement for nitrate (32). Induction of the NiR gene is not dependent on nitrate in a filamentous, nonheterocystous nitrogen-fixing cyanobacterium, *Plectonema boryanum* IAM-M101, either (31). Thus, nitrate has seemingly no specific role in the transcription of the nitrate assimilation genes in the two strains of cyanobacteria. However, during studies of the expression of nitrate assimilation genes in MSX-treated cells, in which there is no negative feedback by the ammonium generated internally by reduction of nitrate and nitrite, we found that nitrate and nitrite do activate the transcription of the nitrate assimilation operons of *Synechococcus* sp. strain PCC 7942 and *P. boryanum*. By use of an NR-deficient mutant of strain PCC 7942 (Δ*narB*::*kan*) (33), the positive effect of nitrate was shown to be due to nitrite generated by reduction of nitrate.

Cells of the cyanobacterial strains were grown photoautotrophically at 30°C under CO_2-sufficient conditions as described previously (34). The basal medium used was a nitrogen-free medium obtained by modification of BG11 medium (30) as previously described (34). Ammonium-containing medium was prepared by adding 3.75 mM $(NH_4)_2SO_4$ to the basal medium. Transcription of the *nirA* operon was induced by treatment of ammonium-grown cells with MSX, an inhibitor of glutamine synthetase, or by transfer of the ammonium-grown cells to ammonium-free media. MSX was added to cyanobacterial cultures in the mid-logarithmic phase of growth with or without simultaneous addition of KNO_3 or $NaNO_2$. For transfer of the cells to ammonium-free media, the ammonium-grown cells were collected by centrifugation at 5,000 × *g* for 5 min at 25°C, washed twice with the basal medium, and inoculated into the basal medium with or without KNO_3. The final concentrations of MSX, KNO_3, and $NaNO_2$ were 0.1, 15, and 5 mM, respectively. Total RNA was extracted and purified from cyanobacterial cells by the method of Aiba et al. (1) and used for Northern (RNA) and dot hybridization analyses. The radioactivity of the RNA dots was quantified with a Bio-image analyzer (Fuji Photo Film).

Figure 1 shows the Northern hybridization profiles of RNA from *Synechococcus* sp. strain PCC 7942 cells before (lanes 1) and after (lanes 2 to 5) derepression of the *nirA* operon in four different ways. As previously reported (21, 32), the *nirA*-specific probe hybridized with transcripts of a wide range of sizes, representing rapid degradation of a large mRNA (Fig. 1A). The amount of the transcript was negligible in ammonium-grown cells (Fig. 1A, lane 1) and was increased by incubation of the cells in ammonium-free media (Fig. 1A, lanes 2 and 3) or by treatment of the cells with MSX (Fig. 1A, lanes 4 and 5), either in the absence (lanes 2 and 4) or in the presence (lanes 3 and 5) of nitrate. Nitrate was found to increase the level of the *nirA* transcript, particularly in the MSX-treated cells, for which nitrate cannot serve as a nitrogen source (Fig. 1A, lane 5). In accordance with previous observations (34), the level of the *rbcL* transcript was high in the cells that were allowed to assimilate nitrogen (Fig. 1B, lanes 1 and 3) and low in the cells that were deprived of the nitrogen source (Fig. 1B, lane 2) or treated with MSX (Fig. 1B, lanes 4 and 5). The level of the transcript of *ndhB*, encoding an NADH dehydrogenase subunit, was not very much affected by the conditions for derepression of the *nirA* operon (Fig. 1C). These findings showed that nitrate specifically stimulated the accumulation of the *nirA* operon transcript in MSX-treated cells.

The changes in *nirA* transcript abundance after derepression of the *nirA* operon, as quantitated by dot hybridization analysis, are shown in Fig. 2. When transcription was derepressed by

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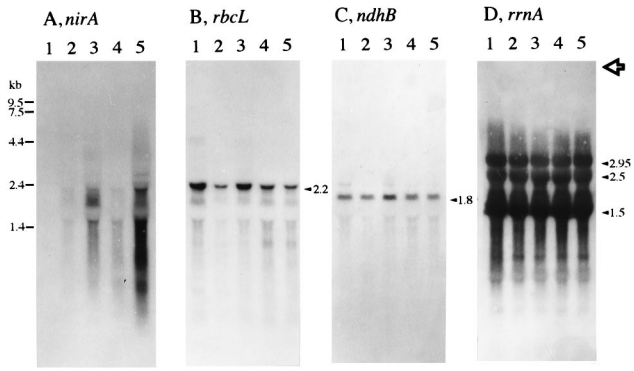

FIG. 1. Northern analysis of RNA from *Synechococcus* sp. strain PCC 7942 cells comparing the effects of four different conditions for derepression of the *nirA* operon on the levels of the transcripts of *nirA* (A), *rbcL* (B), and *ndhB* (C). The rRNA bands, detected with the *rrnA* rRNA genes as a probe, are also shown (D). Cells were grown with ammonium, the culture was separated into four portions, and total RNA was extracted from the cells before (lanes 1) and 80 min after the following treatments: transfer of the cells to the basal medium without (lanes 2) and with (lanes 3) nitrate and addition of MSX without (lanes 4) and with (lanes 5) simultaneous addition of nitrate. RNA (10 μg per lane) was denatured with formamide, separated on a 1.2% agarose–formaldehyde gel, transferred to positively charged nylon membrane (Hybond N+; Amersham), and hybridized as described by Church and Gilbert (5) with the following ^{32}P-labeled double-stranded DNA probes (7): a 640-bp *Nco*I-*Ava*I fragment of *nirA* (32) and a 1.3-kbp PCR-amplified fragment of *ndhB* (23) from *Synechococcus* sp. strain PCC 7942, a 1.5-kbp *Pst*I-*Eco*RI fragment of *rbcL* from *Synechococcus* sp. strain PCC 6301 (29), and a 6.5-kbp *Pst*I fragment of genomic DNA of strain PCC 6301 carrying the rRNA genes (35). The arrow indicates the top of the gel. Arrowheads indicate the positions of the principal hybridizing bands.

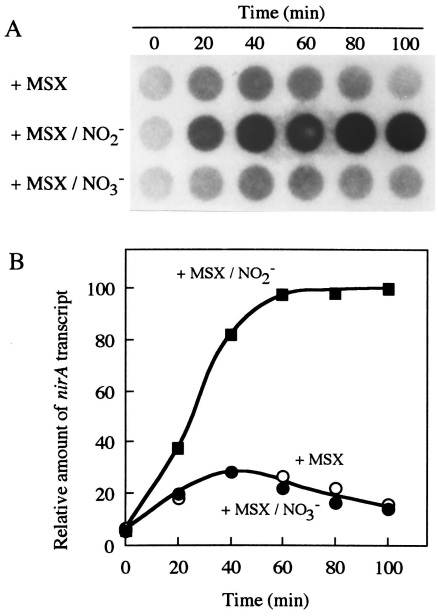

FIG. 3. Changes in the *nirA* transcript abundance in an NR-deficient mutant of *Synechococcus* sp. strain PCC 7942 after addition of MSX alone (+MSX; ○), MSX plus nitrite (+MSX/NO_2^-; ■), and MSX plus nitrate (+MSX/NO_3^-; ●) to the ammonium-grown culture of the mutant. (A) Autoradiogram on X-ray film showing the results of 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 cells treated with MSX plus nitrite.

transfer of ammonium-grown cells to ammonium-free media (Fig. 2A), the level of the transcript reached a maximum at 20 min after the transfer and then gradually decreased, with the transcript level being somewhat higher in the presence of nitrate than in its absence. When transcription was derepressed by treatment of the ammonium-grown cells with MSX (Fig. 2B), there was a lag in the increase of the *nirA* transcript, which was probably due to gradual inhibition of ammonium fixation by MSX. In the cells treated with MSX alone, the level of the

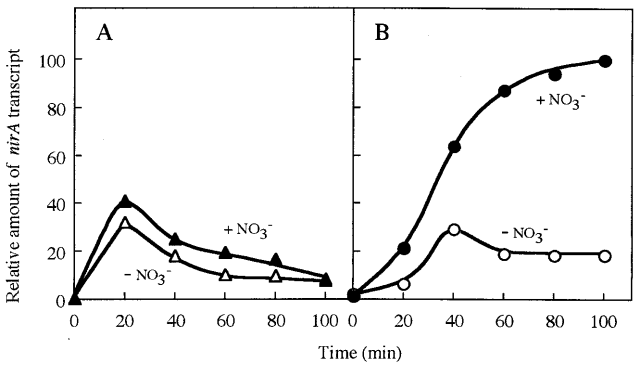

FIG. 2. Changes in the *nirA* transcript abundance in *Synechococcus* sp. strain PCC 7942 cells after derepression of the *nirA* operon in four different ways. The *nirA* operon was derepressed as described in the legend to Fig. 1. (A) Accumulation of the *nirA* transcript upon transfer of ammonium-grown cells to ammonium-free media with (▲) and without (△) nitrate. (B) Accumulation of the *nirA* transcript upon addition of MSX, with (●) and without (○) simultaneous addition of nitrate, to cells growing with ammonium. The amounts of the *nirA* transcript were quantitated by dot hybridization analysis with 2.5 μg of RNA per dot and are shown relative to the maximum level in the cells treated with MSX and nitrate. The *nirA*-specific probe used and the hybridization conditions were the same as those described in the legend to Fig. 1.

nirA transcript reached a maximum at 40 min after the addition of the inhibitor of ammonium fixation and the maximum level was similar to that in the cells transferred to ammonium-free media (Fig. 2B). In the cells treated with MSX and nitrate, on the other hand, the amount of the *nirA* transcript continued to increase for about 80 min and the final transcript level was several times higher than the maximum level reached after addition of MSX alone (Fig. 2B). Addition of rifampin to the cyanobacterial cells that had been treated with MSX caused rapid disappearance of the *nirA* transcript, either in the presence or in the absence of nitrate, with a half-life of approximately 3.5 min (data not shown). The results showed that nitrate did not stabilize the *nirA* transcript but activated *nirA* operon transcription.

In MSX-treated cells, nitrate is reduced via nitrite to ammonium and the resulting ammonium is excreted into the medium (9). Since nitrite was as effective as nitrate in promoting accumulation of the *nirA* transcript (data not shown), we compared the effects of nitrate and nitrite in a mutant of *Synechococcus* sp. strain PCC 7942 defective in NR (33). The presence of nitrite but not nitrate during MSX treatment stimulated accumulation of the *nirA* transcript in the mutant (Fig. 3), demonstrating that nitrate has to be reduced to nitrite to exert its effect on transcription. Exogenously added nitrite did not induce transcription of the *nirA* operon in ammonium-utilizing cells in the absence of MSX, showing that nitrite per se cannot derepress transcription (data not shown).

As in *Synechococcus* sp. strain PCC 7942, *nirA* is the first gene of a large operon in *P. boryanum* and its transcription is induced simply by depletion of ammonium from the medium in the absence of nitrate (31). In accordance with this observation, transcription of *nirA* in *P. boryanum* was induced by addition of MSX alone to the ammonium-grown cultures (Fig. 4), and the maximum transcript level (Fig. 4) was similar to

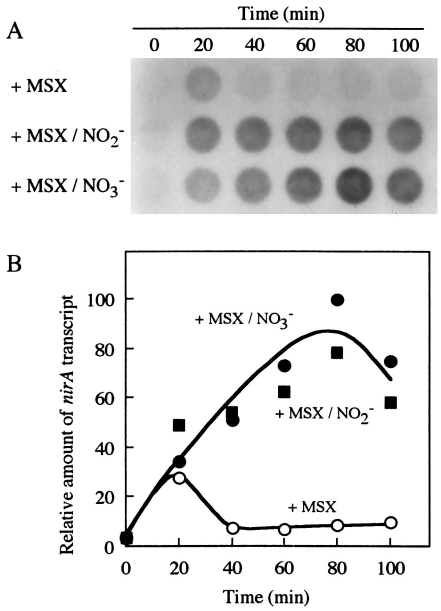

FIG. 4. Changes in the *nirA* transcript abundance in *P. boryanum* caused by addition of MSX alone (+MSX; ○), MSX plus nitrite (+MSX/NO_2^-; ■), and MSX plus nitrate (+MSX/NO_3^-; ●) to the ammonium-grown culture of the cyanobacterium. (A) Autoradiogram on X-ray film showing the results of dot hybridization 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 cells treated with MSX plus nitrate. A 1-kbp *Eco*RI-*Eco*RV fragment of *nirA* from *P. boryanum* (31) was used as a probe.

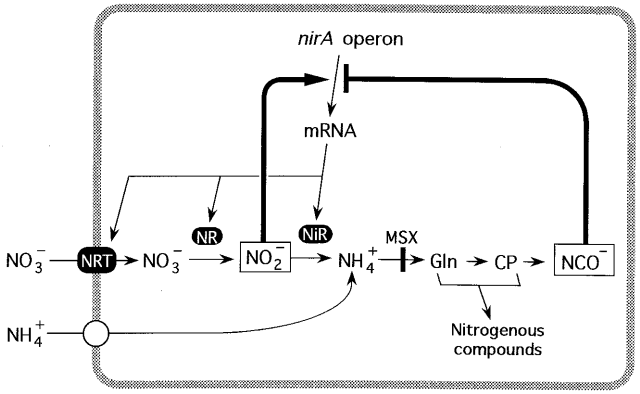

FIG. 5. Scheme for the regulation of the nitrate assimilation operon in *Synechococcus* sp. strain PCC 7942 cells. Nitrite and cyanate act as an activator and a repressor, respectively. CP, carbamoylphosphate; NRT, nitrate transporter encoded by the *nrtABCD* genes. Exogenously added nitrite enters the cells both by passive diffusion and by active transport mediated by NRT (22).

that observed after transfer of the ammonium-grown cells to nitrogen-free medium (data not shown). As for the wild-type PCC 7942 strain, the presence of either nitrate or nitrite in the medium during the MSX treatment stimulated accumulation of the *nirA* transcript (Fig. 4).

Nitrate induces transcription of nitrate assimilation genes in higher plants (6), fungi (6, 24), and bacteria (4, 10, 28), and nitrite has been shown to be an alternative inducer in the fungus *Aspergillus nidulans* (13, 36) and the bacterium *Klebsiella pneumoniae* (4, 10). The present results revealed that nitrate and nitrite activate the nitrate assimilation genes in the cyanobacteria *Synechococcus* sp. strain PCC 7942 and *P. boryanum* as well (Fig. 1 to 4). Furthermore, our results clearly demonstrate that nitrate must be reduced to nitrite to regulate transcription in strain PCC 7942 (Fig. 3). Since nitrate and nitrite both activate *nirA* transcription in *P. boryanum*, it is likely that the pathway to activation is the same in both organisms.

We recently proposed that cyanate, arising from decomposition of carbamoylphosphate, acts as a negative regulator of the *nirA* operon of *Synechococcus* sp. strain PCC 7942 (34). Figure 5 shows a scheme for the regulation of the nitrate assimilation operon, including both negative regulation by cyanate and positive regulation by nitrite. The pronounced effect of external nitrate on *nirA* operon transcription in the presence of MSX (Fig. 2B) but not in its absence (Fig. 2A) can be explained in terms of feedback regulation by cyanate of the transcription of the operon (Fig. 5); in the absence of MSX, ammonium generated by reduction of nitrate is fixed to Gln to negatively regulate transcription of the *nirA* operon through formation of cyanate. Fixation of ammonium also promotes negative regulation of nitrate transport into the cells (18), which would lower the intracellular nitrate and nitrite concentrations. The combined effects of ammonium-promoted repression and inducer exclusion would mask the positive regulation of *nirA* operon transcription. In the MSX-treated cells, on the other hand, the effect of positive regulation was apparent probably because ammonium generated from nitrate is not fixed to Gln and has no negative effect on transcription of the *nirA* operon or on transport of nitrate. Although its effect is apparently marginal in the absence of MSX, the positive regulation mechanism of the *nirA* operon transcription probably contributes to finely tuned regulation of the nitrate assimilation genes in response to changing availability of inorganic nitrogen sources in the natural environment.

Since expression of NR (14) and NiR (16) activities does not strictly require the presence of nitrate in *Synechococcus* sp. strain PCC 6301 (formerly *Anacystis nidulans*), a close relative of strain PCC 7942, nitrate and nitrite probably have no major role in the posttranscriptional control of enzyme synthesis in these strains. In *P. boryanum*, transcription of *nirA* does not require the presence of nitrate but expression of NiR activity requires nitrate (31), indicating posttranscriptional involvement of nitrate or nitrite in NiR biosynthesis. In the filamentous nitrogen-fixing cyanobacteria which develop heterocysts for dinitrogen fixation in the absence of combined nitrogen, expression of NR activity requires not only the absence of ammonium but also the presence of nitrate (14, 15), but it is unknown which step(s) of NR expression depends on nitrate. Determination of the role(s) of nitrate in the heterocystous strains would be necessary for a comprehensive understanding of the positive regulation of the expression of the nitrate assimilation system in cyanobacteria.

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