Nitrite-Responsive Activation of the Nitrate Assimilation Operon in Cyanobacteria Plays an Essential Role in Up-Regulation of Nitrate Assimilation Activities under Nitrate-Limited Growth Conditions

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NtcB of the cyanobacterium Synechococcus elongatus strain PCC 7942 is a LysR family protein that enhances expression of the nitrate assimilation operon (nirA operon) in response to the presence of nitrite, an intermediate of assimilatory nitrate reduction. Inactivation of ntcB in this cyanobacterium specifically abolishes the nitrite responsiveness of nirA operon expression, but under nitrate-replete conditions (wherein negative feedback by intracellularly generated ammonium prevails over the positive effect of nitrite) activity levels of the nitrate assimilation enzymes are marginally higher in the wild-type cells than in the mutant cells, raising the issue of whether the nitrite-promoted regulation has physiological importance. On the other hand, the strains carrying ntcB expressed much higher nitrate assimilation enzyme activities under nitrate-limited growth conditions than under nitrate-replete conditions whereas the ntcB-deficient strains showed levels of the enzyme activities lower than those seen under the nitrate-replete conditions. Although the ntcB mutant maintained a constant cell population in a nitrate-limited chemostat when grown as a single culture, it was diluted at a rate expected for nondividing cells when mixed with the wild-type cells and subjected to nitrate limitation in the chemostat culture system. These results demonstrated that the nitrite-promoted activation of the nitrate assimilation operon is essential for up-regulation of the nitrate assimilation activities under the conditions of nitrate limitation and for competitive utilization of nitrate.

In cyanobacteria, expression of the genes involved in nitrate assimilation, i.e., the nrt genes for the nitrate/nitrite transporter (NRT), narB for nitrate reductase (NR), and nirA for nitrite reductase (NiR), is repressed by the presence of ammonium (3, 4, 15, 17, 20, 21). Ammonium must be fixed via the glutamine synthetase-glutamate synthase cycle to exert its negative effects on transcription (3, 8, 21). Derepression of transcription by removal of ammonium from medium (or by inhibition of ammonium assimilation) results in induction of the nitrate assimilation genes, showing no requirements for nitrate or nitrite (3, 4, 8, 21). This is a part of the global nitrogen control in cyanobacteria, which involves NtcA, a Crp family protein, as the transcriptional activator (7). Tanigawa et al. recently showed by in vitro experiments that 2-oxoglutarate (2-OG), which serves as the acceptor of the newly fixed nitrogen in the glutamine synthetase-glutamate synthase cycle, activates transcription from NtcA-dependent promoters in a concentration-dependent manner (22). It has also been shown that the intracellular 2-OG concentration is low in the presence of ammonium and is increased by nitrogen deprivation (16). Taken together, these findings suggest that 2-OG acts as the coinducer of transcription of the NtcA-dependent genes, conferring ammonium sensitivity to their expression in vivo.

Besides the NtcA-dependent induction involving 2-OG as the coinducer, expression of the nitrate assimilation genes is subjected to up-regulation by nitrite, the intermediate of the nitrate assimilation pathway (2, 8, 12). Studies using Synechococcus elongatus strain PCC 7942 showed that the nitrite-promoted regulation is specific to the nitrate assimilation operon (nirA-nrtABCD-narB [designated nirA operon]) and is mediated by NtcB, a LysR family protein (1). NtcB requires the activity of NtcA to exert its positive effect on transcription (12), indicating that it is unable to promote transcription by itself. Since the activity of NtcA in transcriptional activation is down-regulated by assimilation of the ammonium generated intracellularly by nitrate reduction (21), the positive effect of nitrite is marginal in cells supplied with sufficient amounts of nitrate (8); the effect of nitrite is hence prominent in cells treated with either L-methionine-DL-sulfoximine (MSX [an inhibitor of glutamine synthetase]) or 6-diazo-5-oxo-L-norleucine (DON [an inhibitor of glutamate synthase]) to prevent ammonium assimilation (2, 8, 12). In accordance with these observations, an ntcB-deficient S. elongatus mutant (NIC1) which is defective specifically in the nitrite responsiveness of nirA operon transcription (1), showed only a small decrease in the activity levels of the nitrate assimilation enzymes during steady-state growth in nitrate-sufficient medium (19). These results raise the issue of whether or not the nitrite stimulation of the nitrate assimilation genes is of physiological importance. In the present study, we used NRT-less mutants and chemostat cultures of the cyanobacterium to investigate the physiological role of the nitrite responsiveness under nitrate-limited growth conditions. By examining the effects of inactivation of the trans-acting factor (NtcB) and modification of the cis-acting element, it was shown that the nitrite-responsiveness of nirA operon transcription is essential for high-level expression of the nitrate assimilation enzymes during growth with limiting supply of nitrate.

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and for competitive utilization of nitrate under the nitrate-limited conditions.

### MATERIALS AND METHODS

**Strains and general growth conditions.** A derivative of *S. elongatus* strain PCC 7942, which is cured of the resident small plasmid pUH24 (R2-Spe [9]; hereafter designated simply as strain PCC 7942), is the parental strain of all of the cyanobacterial strains used in this study. NIC1 is an *ntcB*-deficient mutant strain (*ΔntcB::kan*) previously described (1). NA3, an NRT-less mutant (*ΔnirA*) constructed by deleting the *nir* genes from the *nir* operon (14), was the genetic background of the *P. iridoidgenes* strain YKA1, YKA2, and YKA5 (12), and *nirA* was fused individually to the *luxAB* gene in plasmid pYK5 (12), and the resulting *P. iridoidgenes*:luxAB fusions were transferred to the chromosome of the wild-type strain (YKA1a), the NA3 mutant lacking NRT (YKA1, YKA2, and YKA5), and the NA4 mutant lacking NRT and NtcB (YKA2b). The three NtcA-binding sites *nirI, nirII, and nirIII* are indicated by open, dotted, and closed boxes, respectively. The *cis*-acting sequence required for the nitrate responsiveness of *P. iridoidgenes* (L1) is indicated by filled circles. X indicates site-specific nucleotide changes in the L1 site. The bioluminescence data are the means of five measurements from cells incubated in ammonium-containing medium (open bars) and nitrate (60 mM)-containing medium (filled bars).

**RESULTS**

**Effects of endogenously generated nitrite on expression of *P. iridoidgenes*:luxAB fusions in NRT-less strains grown with nitrate.** As previously observed, the reporter strain YKA1 (carrying a transcriptional fusion of the *nirA* operon promoter and *luxAB* in an NRT-deficient background) showed high-level expression of *luxAB* when grown with nitrate (60 mM) (12). The level of *luxAB* expression in YKA1 was much higher than that in YKA2 and YKA5, and the NA4 mutant lacking NRT and *ntcB* (YKA2b). The three *ntcA*-binding sites *nirI, nirII, and nirIII* are indicated by open, dotted, and closed boxes, respectively. The *cis*-acting sequence required for the nitrate responsiveness of *P. iridoidgenes* (L1) is indicated by filled circles. X indicates site-specific nucleotide changes in the L1 site. The bioluminescence data are the means of five measurements from cells incubated in ammonium-containing medium (open bars) and nitrate (60 mM)-containing medium (filled bars).

![FIG. 1. Effects of the presence of NRT, modification of the *cis*-acting element required for the response to nitrite, and inactivation of *ntcB* on expression of *P. iridoidgenes*:luxAB transcriptional fusions in ammonium- and nitrate (60 mM)-containing media. Fragments of the *nirA*-*ntcB* regulatory region having the indicated endpoints relative to the promoters in question were cloned into the expression vector pGEM-T Easy (Promega) and subjected to continuous cultivation, serial dilutions of the effluent were plated on solid ammonium-containing medium with and without kanamycin to determine the wild-type and the mutant cell population numbers; the total cell population was obtained by counting colonies on the medium containing no kanamycin, and the mutant cell population was obtained by counting colonies on the kanamycin-containing medium. The population of the wild-type cells was obtained by subtracting the mutant cell population from the total cell population. Measurement of in vivo bioluminescence. For measurement of in vivo luminescence from the *Synechococcus* cells carrying *luxAB* transcriptional fusions, 1 ml of cell culture containing 0.001 to 0.5 μg of chlorophyll (Chl) was transferred to a test tube and mixed with 20 μl of 0.1% n-decanal emulsion. Bioluminescence of the cell suspension was measured with a luminometer (ARGUS-50; Hamamatsu Photonics) immediately after the addition of n-decanal. Intensity of bioluminescence was expressed in counts of photons per hour per microgram of Chl. Other methods. NR and NIR activities were determined at 30°C using toluene-permeabilized cells with dithionite-reduced methylviologen as the electron donor (5, 6). The rate of nitrate uptake by the cells was determined at low external nitrate concentrations (<100 μM) by monitoring the decrease in nitrate concentration in medium as previously described (14) and was regarded as the activity of the nitrate transporter (NRT). Nitrate and nitrite levels were determined with a flow-injection analyzer (NOX-1000; Tokyo Chemical Industry Co., Ltd.). Chl levels were determined as described by Mackinney (11).
lacking the nirIII site for binding of NtcA, expressed luxAB to a level comparable to that in YKA1 as previously shown (12), the YKA5 strain, differing from YKA2 in carrying base substitutions in the cis-acting element (L1) required for the nitrite enhancement of PnirA::luxAB expression (12), expressed luciferase activity to a level slightly higher than that in YKA1a (Fig. 1). By comparing the PnirA::luxAB expression levels in cells treated with DON (an inhibitor of ammonium assimilation) alone to the results seen with DON plus nitrite, Maeda et al. previously showed that modification of the L1 site specifically abolishes the nitrite responsiveness and does not affect the basal-level expression from the nirA operon promoter, as is induced by NtcA (12). The present results therefore suggested that the positive regulation mechanism involving nitrite plays an essential role in high-level expression of PnirA::luxAB under the nitrate-limited growth conditions.

Maeda et al. previously showed that ntcB inactivation in *S. elongatus* strain PCC 7942 results in specific loss of the nitrite responsiveness, as does the modification of L1 (12, 13). Since the nucleotide sequence of L1 conforms to the structure of the binding sites for LysR-type proteins, it is likely that L1 constitutes the binding site for NtcB (12). In accordance with this assumption, the level of luxAB expression in the YKA2b mutant, an ntcB-deficient derivative of YKA2, was similar to that in YKA5 under the nitrate-limited growth conditions (Fig. 1) as well as in the presence of DON (12).

Effects of ntcB mutation on activity levels of nitrate assimilation enzymes in NRT-less strains. To examine the effects of the nitrite-responsive enhancement of nirA operon transcription on the activity levels of NR and NiR under nitrate-limited conditions, cells of the wild-type strain, the NRT-less mutant NA3, and the ntcB-deficient derivative of NA3 (NA4) were grown in nitrate-containing medium in batch cultures and their NR and NiR activities were compared (Table 1). While the cultures of the wild-type strain were blue-green, those of NA3 and NA4 looked yellowish green due to reduced level of phycocyanin, indicating that the mutants were under stress of nitrogen deficiency. As expected from the higher levels of *PnirA::luxAB* expression in the NRT-less YKA1 strain compared to the results seen with the YKA1a strain having active NRT (see above), the NA3 cells expressed higher NR and NiR activities than the wild-type cells. Presumably due to limited supply of the cofactors required for assembly of NR and NiR holoenzymes, however, the differences in the enzyme activities between NA3 and the wild-type strain were (two to three times) smaller than those observed between the luciferase activities of YKA1 and YKA1a (Fig. 1). The NR and NiR activities in the NA4 mutant were only 35 and 20% of the corresponding activities in NA3, respectively, and were even lower than those in the wild-type, nitrate-replete cells (Table 1). These results demonstrated the importance of the NtcB-dependent, nitrite-responsive enhancement of nirA operon transcription in the high-level expression of NR and NiR activities during growth with a limiting supply of nitrate.

### TABLE 1. Enzyme activities involved in nitrate assimilation in the wild-type and mutant strains grown under various nitrogen conditions

<table>
<thead>
<tr>
<th>Culture and strain</th>
<th>Genotypes</th>
<th>Nitrate concn</th>
<th>NRT activity</th>
<th>NR activity</th>
<th>NiR activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Batch cultures</strong></td>
<td></td>
<td></td>
<td>µmol mg of Chl h⁻¹</td>
<td>µmol OD₅₆₂₅ h⁻¹</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>WT</td>
<td>15 mM</td>
<td>25 (3)</td>
<td>185 (13)</td>
<td>90 (3)</td>
</tr>
<tr>
<td>WT</td>
<td>WT</td>
<td>60 mM</td>
<td>—</td>
<td>196 (18)</td>
<td>80 (5)</td>
</tr>
<tr>
<td>NA3</td>
<td>ΔnrtABCD</td>
<td>60 mM</td>
<td>—</td>
<td>361 (63)</td>
<td>243 (50)</td>
</tr>
<tr>
<td>NA4</td>
<td>ΔnrtABCD ΔntcB</td>
<td>60 mM</td>
<td>—</td>
<td>129 (28)</td>
<td>50 (12)</td>
</tr>
<tr>
<td><strong>Continuous cultures</strong></td>
<td></td>
<td></td>
<td>µmol mg of Chl h⁻¹</td>
<td>µmol OD₅₆₂₅ h⁻¹</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>WT</td>
<td>&lt;0.5 µM</td>
<td>85 (2)</td>
<td>855 (68)</td>
<td>778 (31)</td>
</tr>
<tr>
<td>NIC1</td>
<td>ΔntcB</td>
<td>−2 µM</td>
<td>51 (5)</td>
<td>233 (74)</td>
<td>166 (36)</td>
</tr>
</tbody>
</table>

* The values are the averages from three measurements; those in parentheses are standard deviations.

**WT**, wild type.

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**NRT NR NiR**

**NRT NR NiR**

51 (5) 233 (74) 166 (36)

0.08 (0.01) 0.42 (0.13) 0.30 (0.07)

0.21 (0.03) 1.92 (0.14) 1.75 (0.07)

0.08 (0.01) 0.42 (0.13) 0.30 (0.07)
lular Chl content in the former cultures had been decreased due to nitrogen stress. Thus, the high activity levels of the nitrate assimilation enzymes in the chemostat-grown wild-type cultures were to be ascribed partly to the low level of Chl content of the cells. Even when expressed on the basis of turbidity, nevertheless, the enzyme activities in the chemostat-grown wild-type cells were higher than the corresponding activities in the nitrate-replete batch cultures (Table 1). The activity levels in the ntcB mutant were, on the other hand, lower than those in the wild-type batch cultures (Table 1). These results confirmed the results obtained with the NRT-less strains, i.e., that the NtcB-dependent, nitrite-responsive enhancement of nirA operon expression is required for up-regulation of the nitrate assimilation activities in nitrate-limited cells.

**Competition between the wild-type and ntcB mutant strains.** When equal numbers of the wild-type and the mutant cells were mixed and subjected to growth under nitrate-limited conditions in the continuous-culture system, the cell population of the wild-type strain remained constant whereas that of the mutant decreased exponentially, giving a straight line on a logarithmic scale (Fig. 4A, panel b). The slope of the line was $-0.302 \text{ day}^{-1}$ (Fig. 4A, panel b), $-0.297 \text{ day}^{-1}$, and $-0.261 \text{ day}^{-1}$ in three separate experiments. These figures were close to that expected for dilution of nondividing cells at a dilution rate of $0.03 \text{ h}^{-1}$, i.e., $-0.313 \text{ day}^{-1}$. The mutant cells were thus virtually unable to grow in the presence of the wild-type cells under the nitrate-limited conditions. When mixed cultures were subjected to continuous cultivation under nitrate-replete conditions, the proportion of the mutant cells in the total cell population was almost constant (Fig. 4B, panel b). These results demonstrated that the nitrite-responsive, NtcB-dependent enhancement of nirA operon transcription is essential for nitrate assimilation and growth of S. elongatus strain PCC 7942 in competitive environments with a limiting supply of nitrate.

**DISCUSSION**

Because of the negative effect of assimilation of internally generated ammonium, the positive effect of nitrite on expression of the nirA operon and the nitrate assimilation enzymes is not obvious during growth of S. elongatus strain PCC 7942 in medium containing sufficient amounts of nitrate (1, 8, 19). Under nitrate-limited conditions, by contrast, the nitrite-responsive enhancement of nirA operon leads to a large increase in the activities of the nitrate assimilation enzymes (Table 1). Thus, the nitrite stimulation enables allotment of more nitrogen for synthesis of the nitrate assimilation enzymes in nitrogen-deficient cells than in nitrate-replete cells. Since nitrite is produced from nitrate, the regulatory system provides an effective mechanism to express high activities of the nitrate assimilation enzymes when the substrate is present in the medium and is limiting the growth. In the nitrate-limited cells, ammonium production is presumed to be too slow to cause negative feedback but the same cells are producing required amounts of nitrite sufficient for the enhancement of transcription. It is therefore deduced that the positive regulation system

![FIG. 2. Growth of the wild-type strain (A) and the ntcB mutant NIC1 (B) of S. elongatus strain PCC 7942 in continuous cultures with a limited supply of nitrate. Ammonium-grown cells were collected by centrifugation, suspended in nitrate-containing medium, and inoculated into 1 liter of nitrogen-free medium. Sterile fresh medium containing 1 mM nitrate was continuously pumped into the vessel at a dilution rate of 0.03 h$^{-1}$, and the effluent was collected axenically for measurements of cell density (diamonds) and nitrate concentration (triangles).](http://jb.asm.org/)

![FIG. 3. Appearance of the nitrate-limited chemostat cultures of the wild-type strain (WT) and the ntcB mutant NIC1 of S. elongatus strain PCC 7942.](http://jb.asm.org/)
has very high sensitivity for nitrite. The molecular mechanism of the nitrite sensing is being investigated.

The stimulation by nitrite and NtcB of nitrate assimilation activities is not essential for growth of the cells in a single culture even under nitrate-limited conditions (Fig. 2B). However, it is essential for competitive utilization of limiting amounts of nitrate (Fig. 4A). In a previous study, Aichi and Omata also showed that NtcB is required for induction of the nitrite-responsive positive regulation of the nitrate assimilation operon after replenishment of nitrate to the culture even under nitrate-limited conditions (Fig. 2B). How-

FIG. 4. Competition between the wild-type strain and the ntcB mutant of S. elongatus strain PCC 7942 in mixed continuous cultures with (A) and without (B) limitation of nitrate. Ammonium-grown cells of the two strains were collected by centrifugation and washed with nitrogen-free medium by resuspension and refugation. Equal amounts of the cells of the two strains were inoculated together into 1 liter of nitrogen-free medium (A) and nitrate (15 mM)-containing medium (B), and sterile fresh media containing 1 mM (A) and 15 mM (B) of nitrate was continuously pumped into the vessels at a dilution rate of 0.03 h⁻¹. The effluents were collected axenically for measurements of total cell density (open diamonds) and nitrate concentration (closed triangles) and for counting of the wild-type (closed circles) and the mutant (open circles) cell populations.

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REFERENCES
Cloning and sequencing of the nitrate transport system from the thermo-
phlic, filamentous cyanobacterium Phormidium laminosum: comparative
analysis with the homologous system from Synechococcus sp. PCC 7942. 
Plant Mol. Biol. 28:759–766.

perceive nitrogen status by sensing intracellular 2-oxoglutarate levels. J. Biol.

nitrite permease in the marine cyanobacterium Synechococcus sp. strain PCC 

cation and properties of unicellular blue-green algae (order Chroococcales). 

characterization of two nitrogen-regulated genes of the cyanobacterium Syn-
echococcus sp. strain PCC7942 required for maximum efficiency of nitrogen 

1995. A novel nitrite reductase gene from the cyanobacterium Plectonema 

scriptional regulation of the gene for nitrite reductase from the cyanobac-

Takahashi. 2002. Transcriptional activation of NtcA-dependent promoters 