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cis-Acting Sequences Required for NtcB-Dependent, Nitrite-Responsive Positive Regulation of the Nitrate Assimilation Operon in the Cyanobacterium *Synechococcus* sp. Strain PCC 7942

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There are three binding sites for NtcA (*nir*I, *nir*II, and *nir*III), the global nitrogen regulator of cyanobacteria, in the DNA region between the two divergently transcribed operons (*nirA* and *nirB* operons) involved in nitrate assimilation in *Synechococcus* sp. strain PCC 7942. Using the *luxAB* reporter system, we showed that *nir*I and *nir*III, which are located 23 bp upstream from the −10 promoter element of *nirA* and *nirB*, respectively, are required for induction by nitrogen depletion of the *nirA* and *nirB* operons, respectively. The induction of *nirA* operon transcription was a prerequisite for the nitrite-responsive positive regulation of the transcription by NtcB, a LysR-type protein. The NtcA-binding site *nir*II, located in the middle of the *nirA-nirB* intergenic region, and a potential binding site for a LysR-type protein ($TGCAN_5TGCA$; designated L1), located between *nir*I and *nir*II, were required for the nitrite-responsive, NtcB-dependent enhancement of *nirA* operon transcription. Although the requirement for the L1 site was consistent with the involvement of the LysR family protein NtcB in transcriptional regulation, NtcB did not bind to the *nirA* regulatory region in vitro in the presence of nitrite and NtcA, suggesting the involvement of some additional factor(s) in the regulation. An L1-like inverted repeat with the consensus sequence $TGCN_7GCA$ was conserved in the *nirA* promoter region of cyanobacteria, being centered at position −23 with respect to the NtcA-binding site corresponding to *nir*I, which suggested the common occurrence of nitrite-responsive regulation of the nitrate assimilation operon among cyanobacteria.

Nitrate, a major source of nitrogen for cyanobacteria in their natural environment, is transported into the cells by an active transport system (NRT) and reduced to ammonium by the sequential action of nitrate reductase (NR) and nitrite reductase (NiR). The resulting ammonium is fixed into amide group of glutamine by glutamine synthetase, and the amide nitrogen is subsequently transferred to other compounds by various glutamine amidotransferases to form various nitrogenous compounds. In the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942, the genes encoding NRT (*nrtABCD*) (26, 27, 30), NR (*narB*) (4, 18), and NiR (*nirA*) (21, 38) form an operon, *nirA-nrtABCD-narB* (*nirA* operon) (38), the transcription of which is repressed by ammonium. Transcription of the *nirA* operon is induced by removal of ammonium from the medium or by inhibition of ammonium assimilation with L-methionine-DL-sulfoximine (MSX; an inhibitor of glutamine synthetase) or 6-diazo-5-oxo-L-norleucine (DON; an inhibitor of glutamine amidotransferases) (38). Upstream of the *nirA* operon are the two genes required for maximum nitrate assimilation (*nirB* and *ntcB*), which form another ammonium-repressible operon (*nirB* operon) transcribed divergently from the *nirA* operon (36). Under inducing conditions, nitrite, either added exogenously or generated internally from nitrate, further activates transcription of the *nirA* operon but not that of the *nirB* operon (2, 17). Since nitrite is reduced by NiR and the resulting ammonium negatively regulates *nirA* operon transcription, the nitrite-promoted positive regulation of the *nirA* operon is clearly discernible only when ammonium fixation is inhibited (17) or when cells are exposed to prolonged stress of nitrogen deficiency (2).

The *nirA* and *nirB* operons and other ammonium-repressible transcription units require the Crp-type DNA-binding protein NtcA (40) for their expression. In the promoter region, the NtcA-dependent transcription units have the consensus sequence $GTAN_8TACN_{22}TAN_3T$, in which $GTAN_8TAC$ is the NtcA-binding site and TAN_3T is presumably the −10 promoter element (20). There are three NtcA-binding sites, designated *nir*I, *nir*II, and *nir*III (20), in the 286-bp region between *nirA* and *nirB* (36). Two of these, *nir*I and *nir*III, are thought to constitute the promoter of the *nirA* (20) and *nirB* (36) operons, respectively, but the role of the other NtcA-binding site (*nir*II), which is located in the middle of the *nirA-nirB* intergenic region, remains unclear. The nitrite-promoted positive regulation of *nirA* operon transcription, on the other hand, requires a LysR-type DNA-binding protein encoded by the *ntcB* gene (2), but the *cis*-acting sequence involved is yet to be identified. The binding sites of the transcriptional regulators of LysR family generally have an inverted repeat structure built around the motif $TN_{11}A$ (LysR motif [11]). There are two such inverted repeats in the *nirA-nirB* intergenic region, both of which are located between *nir*I and *nir*II (designated L1 and L2 [Fig. 1]). In this study, we used *luxAB* transcriptional fusions to monitor the activities of fragments of the *nirA* and *nirB* regulatory regions under various nitrogen conditions. One of the potential binding sites for a LysR-type protein (L1), having the sequence $TGCAN_5TGCA$, and the NtcA-binding site *nir*II are shown to be involved in the nitrite-responsive regulation of the *nirA* operon. We show that NtcB and nitrite do not promote transcription of the *nirA* operon but enhance transcription once it is induced by the action of NtcA. The physiological significance of the nitrite-responsive enhancement of *nirA* operon transcription during nitrate-limited growth is discussed.

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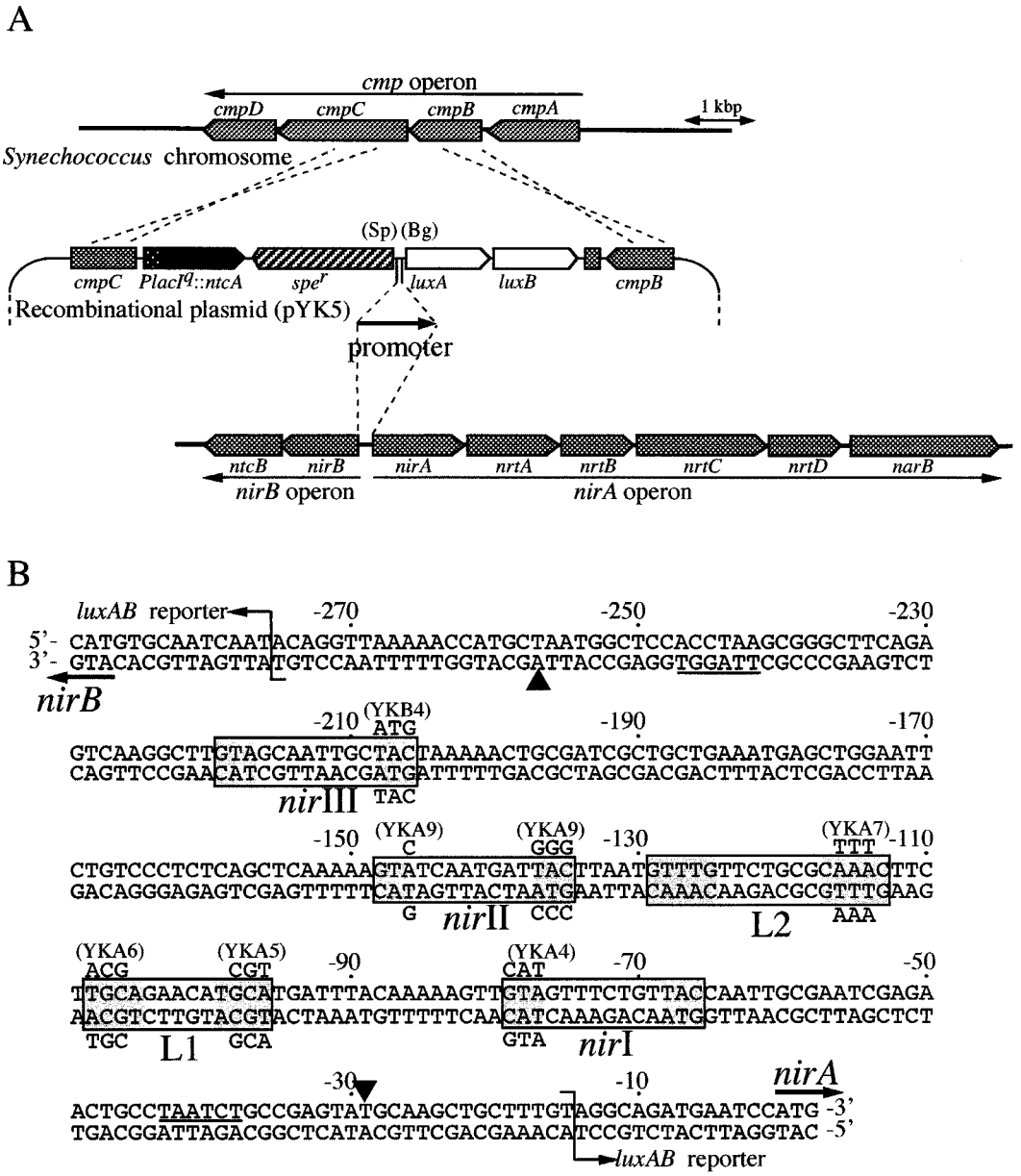

FIG. 1. (A) Schematic representation of the insertion of transcriptional *luxAB* fusions into the *cmp* locus of the *Synechococcus* chromosome. The recombinational plasmid pYK5, which cannot replicate in the cyanobacterium, contains a promoterless *luxAB* gene cluster, a spectinomycin resistance cassette (*spe*^{r}), and a *PlacI*^{q}::*ntcA* transcriptional fusion, which are flanked by *Synechococcus cmpB* and *cmpC* genes. Various fragments of the *nirA-nirB* intergenic region were cloned between the *Spe*I (Sp) and *Bgl*II (Bg) sites preceding the *luxAB* gene cluster. Transformation of *Synechococcus* with the plasmid resulted in spectinomycin-resistant strains in which homologous recombination had transferred the reporter construct to the cyanobacterial chromosome. (B) The *nirA-nirB* intergenic region of *Synechococcus* sp. strain PCC 7942, showing the nucleotide sequences of both DNA strands. Numbers above the sequences indicate positions of the nucleotides with respect to the translation start site of *nirA*. The transcription start sites of the *nirA* and *nirB* operons are indicated by filled triangles. The putative −10 elements of the promoters of the *nirA* and *nirB* operons are underlined. The three NtcA-binding sites (*nir*I, *nir*II, and *nir*III) and the two potential binding sites for LysR-type DNA-binding proteins (L1 and L2) are boxed. The letters above and below the sequences represent the base replacements created in strains YKA4, YKA5, YKA6, YKA7, YKA9, and YKB4. Sites of the *luxAB* fusion are also indicated.

MATERIALS AND METHODS

Strains and growth conditions. A derivative of *Synechococcus* sp. strain PCC 7942, which is cured of the resident small plasmid pUH24 (strain R2-SPc [19]; hereafter designated simply strain PCC 7942), is the parental strain of all of the cyanobacterial strains used in this study. A deletion mutant of the *nrtABCD* genes (NA3 [23]), lacking specifically the high-affinity nitrate-nitrite transporter, was the genetic background into which reporter gene fusions were introduced by transformation. An *ntcA* deletion mutant (NIC2) was constructed from strain PCC 7942 by deleting the *ntcA* coding region and 108 and 18 bp, respectively, of its 5′ and 3′ flanking sequences from the genome by the marker exchange-eviction mutagenesis method (32), using a 3.8-kbp *nptI-sacB* cartridge excised from pRL250 (6) as the selection marker. All *Synechococcus* strains were grown photoautotrophically under continuous illumination provided by fluorescent lamps (70 microeinsteins m^{-2} s^{-1}) at 30°C. The basal medium used was a nitrogen-free medium obtained by modification of BG11 medium (35) as previously described (39). Ammonium-containing medium and nitrate-containing medium were prepared by addition of 3.75 mM $(NH_4)_2SO_4$ and 60 mM KNO_3, respectively, to the basal medium. Both media were buffered with 20 mM HEPES-KOH (pH 8.2). When appropriate, spectinomycin was added to the media at 1 μg/ml. The cultures were aerated with 2% (vol/vol) CO_2 in air. *Escherichia coli* DH5α and NM522, used as hosts for plasmid constructions and protein expression, respectively, were grown on Luria-Bertani medium supplemented with ampicillin (50 μg/ml) and/or spectinomycin (15 μg/ml) when appropriate.

Transcription of the *nirA* operon and the reporter gene fusions was induced by

treatment of ammonium-grown cyanobacterial cells with MSX or DON or by transfer of the ammonium-grown cells to ammonium-free media. MSX and DON were added to cyanobacterial cultures in the mid-logarithmic phase of growth with or without simultaneous addition of $NaNO_2$. The final concentrations of MSX, DON, and $NaNO_2$ were 0.1, 0.15, and 5 mM, respectively. 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 by resuspension and recentrifugation, and inoculated into the basal medium and the nitrate-containing medium.

Plasmids used for integration of *luxAB* transcriptional fusions into the *Synechococcus* genome. Fusions of the *nirA* and *nirB* regulatory sequences and a promoterless *luxAB* gene cluster were constructed on plasmids and transferred into the *cmp* locus of the *Synechococcus* genome (25) through homologous recombination between the transforming plasmid DNA and the recipient cyanobacterial chromosome. The plasmids used as the donors of the *luxAB* transcriptional fusions were derivatives of pYK5 (Fig. 1A), which is based on pUC119 and does not replicate in *Synechococcus*. For construction of pYK5, a 1.8-kbp *Bgl*II-*Sph*I fragment of *Synechococcus* DNA carrying the *cmpB-cmpC* region (25) was cloned into pUC119 to form pG23. A 2.1-kbp DNA fragment carrying a promoterless *luxAB* gene cluster of *Vibrio harveyi* (from nucleotide −12 with respect to the *luxA* initiation codon to nucleotide +66 with respect to the *luxB* termination codon [8] and [15]) was amplified by PCR as a *Bgl*II-*Nhe*I fragment. A *PlacI*^{q}::*ntcA* transcriptional fusion, in which *ntcA* coding region is fused to the *lacI*^{q} promoter (nucleotides −247 to −1 with respect to the *lacI*^{q} initiation codon), was prepared by overlap extension PCR (14), using plasmid pTrc99A (3) and *Synechococcus* chromosomal DNA as templates. A spectinomycin resistance cassette flanked by strong transcriptional terminators (31) was excised from plasmid pRL463 (9) by digestion with *Sal*I. The *PlacI*^{q}::*ntcA* transcriptional fusion, the promoterless *luxAB* gene cluster, and the spectinomycin resistance cassette were sequentially cloned into pT7Blue T-Vector (Novagen) to form a 5.0-kbp insert flanked by *Nhe*I recognition sites, which was subsequently excised from the plasmid and ligated into the *Xba*I site in the *cmpC* fragment in pG23 to form pYK5. The interrupted *cmpBC* sequence in pYK5 allows homologous recombination between the plasmid and the cyanobacterial chromosome, and the spectinomycin resistance gene confers a selection marker for transformants. The *PlaqI*^{q}::*ntcA* transcriptional fusion was introduced to allow constitutive expression of NtcA. When transferred from pYK5 to the genome of the *ntcA* deletion mutant NIC2, the *PlaqI*^{q}::*ntcA* fusion complemented the defect of the mutant in nitrate assimilation, verifying functional expression of NtcA. The *nirA* and *nirB* regulatory sequences were cloned between the unique *Spe*I and *Bgl*II recognition sites of pYK5, which are located 48 and 12 bases, respectively, upstream of the *luxA* start codon (Fig. 1A).

For preparation of the *nirA* and *nirB* regulatory sequences to be inserted into pYK5, a 261-bp segment of the 286-bp *nirA-nirB* intergenic region, carrying nucleotides −275 to −15 with respect to the translation start site of *nirA* (i.e., nucleotides −272 to −12 with respect to the translation start site of *nirB* [Fig. 1B]), was amplified by PCR and cloned into pT7Blue T-Vector in two orientations. Deletions and subcloning from the cloned DNA segments, using the internally located *Eco*RI and *Eco*T22I restriction sites and the other restriction sites in the polycloning site of the vector, yielded various fragments of the *nirA-nirB* intergenic region carrying one or two of the three NtcA-binding sites (see Fig. 3), which were flanked by *Spe*I and *Bam*HI recognition sequences originating from the pT7Blue vector. The *nirA* and *nirB* regulatory sequences with base substitutions were generated by overlap extension PCR using oligonucleotide primers carrying mismatches with the wild-type sequence (13) and cloned into pT7Blue T-Vector. After confirmation of the nucleotide sequence, the cloned regulatory regions were excised from the plasmids with *Spe*I and *Bam*HI and cloned between the *Spe*I and *Bgl*II sites of pYK5 (Fig. 1A). The resulting plasmids were used to transfer the *luxAB* transcriptional fusions into the chromosome of the NA3 mutant to yield strains YKA1-YKA9 and YKB1-YKB4. pYK5 was used to transform NA3 to yield strain YKC, which was used as a promoterless control for the *luxAB* reporter.

Expression of plasmid-borne *ntcA* and *ntcB* in *Synechococcus*. A PCR-amplified *ntcA* gene, in which an *Nco*I recognition site had been created at the translation start site, was cloned into pT7Blue T-Vector. After verification of nucleotide sequence, the *ntcA* gene was excised from the plasmid with *Nco*I and *Xba*I and cloned between the *Nco*I and *Xba*I sites of the shuttle expression vector pSE2 (2). The resulting plasmid (pNTCA), encoding a modified NtcA protein in which the second amino acid residue had been changed from Leu to Val, was used for expression of *ntcA* in the *ntcA* deletion mutant NIC2. Another pSE2 derivative carrying *ntcB*, pNTCB (2), was used for NtcA-independent expression of *ntcB* in NIC2. Complementation of the defects of the mutants of *ntcA* and *ntcB* with pNTCA (see Results) and pNTCB (2), respectively, in the absence of isopropyl-β-D-thiogalactopyranoside (IPTG) indicated IPTG-independent expression of the genes from the plasmids.

Preparation of NtcA and NtcB proteins and gel shift assay. The PCR-amplified *ntcA* (see above) and *ntcB* (2) genes cloned into pT7Blue T-Vector were excised from the plasmids with *Nco*I and *Xba*I and, after blunting of the termini, cloned individually into the *Sal*I site in the polylinker of the expression vector pMAL-c2 (33). The resulting plasmids, designated pMNTCA and pMNTCB, carried chimeric genes encoding translational fusions of the maltose-binding protein with NtcA and NtcB, respectively. Cells of *E. coli* NM522 transformants carrying pMAL-c2, pMNTCA, and pMNTCB were grown in Luria-Bertani medium. Expression of the chimeric genes was induced by 1 mM IPTG, and the recombinant proteins were purified on amylose resin (33). The purified fusion proteins were cleaved with factor Xa and used for gel retardation assays.

The DNA fragment used as the probe, carrying nucleotides −173 to −15 with respect to the *nirA* start codon, was labeled at both termini with ^{32}P, using the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP. Gel retardation assays were performed essentially as described by Buratowski and Chodosh (5), with supplementation of 5 mM nitrite to the buffers used for the binding reactions, preparation of the gels, and electrophoresis. Gels containing 4% polyacrylamide were used for separation of the free probe and the protein-DNA complexes. Gels were dried, and the signals were detected with a Bio-image analyzer (Fuji Photo Film).

Isolation and analysis of DNA and RNA. Chromosomal DNA was extracted and purified from the cells of the wild-type and the reporter strains of *Synechococcus* sp. strain PCC 7942 as described by Williams (41). Manipulations and analyses of DNA were performed according to standard protocols (34). Total RNA was extracted and purified from *Synechococcus* cells by the method of Aiba et al. (1). For Northern hybridization analysis, RNA samples (10 μg per lane) were denatured by treatment with formaldehyde, fractionated by electrophoresis in 1.2% agarose gels that contained formaldehyde, transferred to positively charged nylon membranes (Hybond N+; Amersham), and hybridized with the following ^{32}P-labeled double-stranded DNA probes: a 640-bp *Nco*I-*Ava*I fragment of *nirA* (38) and a 1.3-kbp PCR-amplified fragment of *ndhB* (24) from *Synechococcus* sp. strain PCC 7942.

Measurement of in vivo bioluminescence. For measurement of in vivo luminescence from *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 minute per microgram of Chl. Chl was determined as described by Mackinney (22).

RESULTS

Use of *luxAB* as a reporter of the activity of the promoter of the nitrate assimilation operon. To monitor transcriptional activities and regulation of the *nirA* and *nirB* operon promoters, fusions of various fragments of the *nirA-nirB* intergenic region to promoterless *luxAB* were constructed on plasmid pYK5 and integrated into the *cmpC* gene of the *Synechococcus* chromosome (Fig. 1A). The *cmp* locus was chosen as the target of integration of the promoter-reporter fusions because the *cmpABCD* operon is expressed only under carbon-limited conditions and is not required for normal growth of the cyanobacterium under the high-CO_2 conditions (2%, vol/vol) used in this study (28, 29). Since the steady-state level of *nirA* and *nirB* operon transcription in nitrate-utilizing cells of *Synechococcus* sp. strain PCC 7942 is low due to negative feedback by the ammonium generated internally from nitrate (36, 38, 39), a mutant (NA3) of *Synechococcus* lacking the active nitrate transporter (23) was used as the standard host for *luxAB* fusions, so that a high level of *nirA* operon transcription is maintained in nitrate-containing medium because of the constant stress of nitrogen deficiency. All of the *Synechococcus* reporter strains constructed from NA3 grew normally in ammonium-containing medium; because of nitrogen limitation, growth was slower in nitrate (60 mM)-containing medium, with reduced pigmentation.

Figure 2 shows the time course of bioluminescence from cells of *PnirA*::*luxAB* reporter strain YKA1, carrying nearly the entire *nirA-nirB* intergenic region fused to *luxAB* (Fig. 3), after derepression of *nirA* operon transcription. Luciferase activity was low in ammonium-grown cells and was not affected by transfer of the cells to fresh ammonium-containing medium. Transfer of the cells to nitrogen-free medium induced expression of luciferase, resulting in a 25-fold increase in in vivo luminescence in 24 h. When transferred to nitrate (60 mM)-containing medium, the cells expressed a much higher level of luciferase activity (2×10^7 counts per μg of Chl per min) in 24 h and maintained it during growth with nitrate (Fig. 3). The

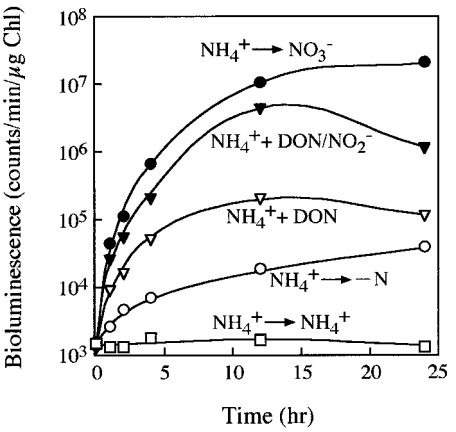

FIG. 2. Effects of various nitrogen conditions on expression of luciferase in *PnirA*::*luxAB* reporter strain YKA1, which carries nearly the entire *nirA-nirB* intergenic region (nucleotides −275 to −15 with respect to the *nirA* translation start site [Fig. 1B and 3]) fused to promoterless *luxAB*. Cells were grown with ammonium, the culture was separated into five portions, and changes in luciferase activity after addition of DON alone (▽) and DON plus nitrite (▼) and after transfer of the cells to nitrogen-free medium (○), nitrate-containing medium (●), and ammonium-containing medium (□) were monitored by measuring in vivo bioluminescence in the presence of *n*-decanal.

YKA1a strain, carrying the same promoter-reporter fusion as YKA1 in a wild-type background, expressed only low luciferase activity (ca. 10^5 counts per μg of Chl per min) when grown in the nitrate-containing medium (not shown), indicating that the constant nitrogen stress in nitrate-grown YKA1 led to the high-level expression of the *luxAB* reporter. Luciferase activity of YKA1 was induced also by addition of DON, an inhibitor of glutamine amidotransferases, to the ammonium-grown cultures (Fig. 2). Nitrite, when added with DON to the ammonium-grown culture, caused a 20-fold increase of luminescence compared to the level obtained by treatment with DON alone. The regulation of luciferase expression, i.e., induction by removal of ammonium or by inhibition of nitrogen assimilation, and activation by nitrite, is essentially the same as that of *nirA* operon transcription, as demonstrated by direct determination of *nirA* mRNA (17, 38). These results verified that the luciferase encoded by *luxAB* can be used as the reporter of the activity of the *nirA* operon promoter. The results also confirmed that the *nirA-nirB* intergenic region contains all of the *cis*-acting elements required for the negative and positive regulation of *nirA* operon expression.

Promoter and other *cis*-acting sequences of *nirA* and *nirB*. Figure 3 summarizes the bioluminescence data from various *PnirA*::*luxAB* and *PnirB*::*luxAB* reporter strains grown continuously in ammonium-containing and nitrate-containing media. When grown with ammonium, luciferase activity in all of the reporter strains was low, being similar to that in ammonium-grown cells of YKC used as the promoterless control for *luxAB* expression. The luciferase activity of nitrate-grown YKC cells was 4.3 times higher than that of the ammonium-grown cells (10^4 counts per μg of Chl per min), presumably because of the lower growth rate and decreased pigmentation of the nitrate-grown cells than of the ammonium-grown cells. Nitrate-grown cells of the *PnirA*::*luxAB* reporter strain YKA3, which carries nucleotides −95 to −15 fused to *luxAB*, showed luciferase activity 110 times higher than that of the ammonium-grown cells, indicating that the 81-bp DNA segment carries the promoter that directs nitrogen-responsive gene expression. YKA2 and YKA1 showed stronger luminescence than YKA3 when grown with nitrate (140 and 200 times that of YKA3, respectively) but not when grown with ammonium (1.1 and 1.2 times that of YKA3, respectively). These results suggested that the region from nucleotides −173 to −96 enhances transcription from the *nirA* operon promoter.

When grown with nitrate, the *PnirB*::*luxAB* reporter strain YKB3 exhibited luminescence 38 times stronger than that of the ammonium-grown cells (Fig. 3), showing that the DNA segment from nucleotides −275 to −170 with respect to the *nirA* translation start site (nucleotides −117 to −12 with respect to the *nirB* translation start site), carrying *nir*III and the putative −10 element, can promote nitrogen-responsive gene

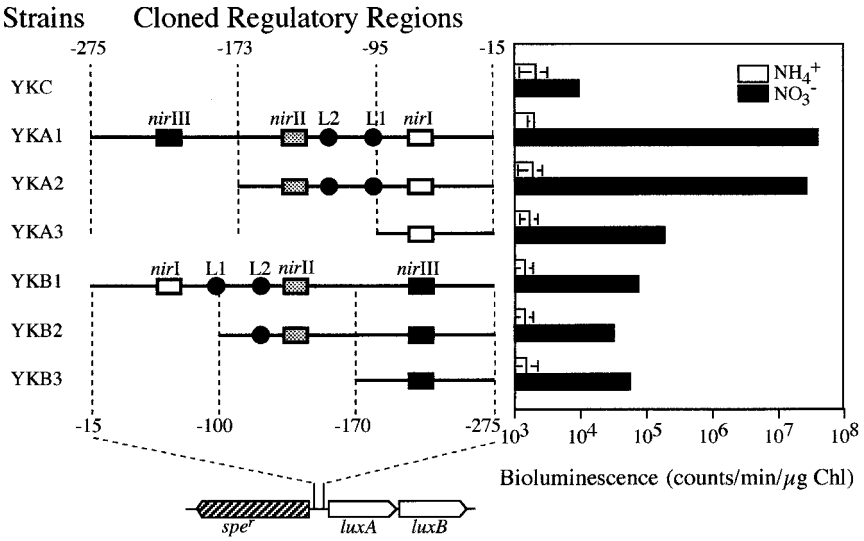

FIG. 3. Effects of upstream regions of *nirA* and *nirB* on *luxAB* expression in ammonium- and nitrate-containing media. Fragments of the *nirA-nirB* regulatory region having the indicated endpoints relative to the translational start site of *nirA* were fused individually to the *luxAB* gene in pYK5 and transferred to the chromosome of *Synechococcus* mutant NA3 to generate *PnirA*::*luxAB* and *PnirB*::*luxAB* reporter strains. The three NtcA-binding sites, *nir*I, *nir*II, and *nir*III, are indicated by open, dotted, and closed boxes, respectively, and the two potential binding sites for LysR-type DNA-binding proteins, L1 and L2, are indicated by filled circles. The bioluminescence data are the means of five measurements, with standard deviations indicated.

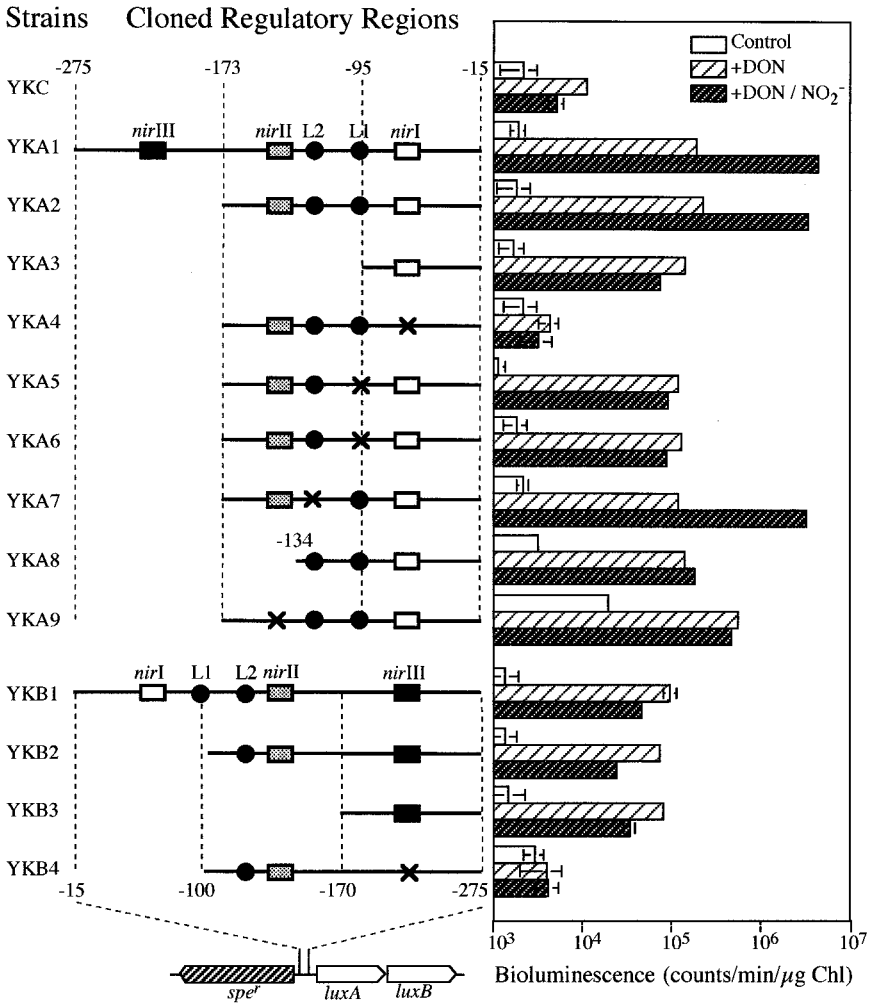

FIG. 4. Effects of DON and nitrite on expression of *luxAB* in various *PnirA*::*luxAB* and *PnirB*::*luxAB* reporter strains. Bioluminescence was measured before (control) and 12 h after addition of DON alone and of DON plus nitrite to ammonium-grown cultures of the *Synechococcus* reporter strains. The three NtcA-binding sites and the two putative binding sites for LysR-type proteins are indicated as in Fig. 3. X indicates the NtcA-binding motifs and the LysR motifs where site-specific nucleotide changes were created. The bioluminescence data are the means of five measurements, with standard deviations indicated.

expression. The DNA region farther upstream, from nucleotides −170 to −15 with respect to the *nirA* start codon, did not affect transcription from the *nirB* operon promoter (Fig. 3, YKB1 and YKB2).

***cis*-acting elements involved in nitrite-responsive regulation of the *nirA* operon.** To further characterize the *cis*-acting element(s) regulating transcription of the *nirA* operon, effects of DON and nitrite on expression of luciferase activity were examined in various *PnirA*::*luxAB* reporter strains (Fig. 4). When nitrogen assimilation was inhibited by DON treatment, YKA1, YKA2, and YKA3 expressed similar levels of luciferase activity, confirming the presence of the nitrogen-responsive promoter in the region from nucleotides −95 to −15 with respect to the *nirA* start codon. Site-specific modification of *nir*I in this region abolished luciferase expression in response to DON (YKA4), confirming that the NtcA-binding site is an essential element of the *nirA* operon promoter.

The presence of nitrite during DON treatment increased the luciferase activity of YKA2 and YKA1 15- and 23-fold, respectively but decreased that of YKA3 by 50% (Fig. 4). In YKA1b and YKA2b, which carry the same promoter-reporter fusions as YKA1 and YKA2, respectively, in an *ntcB* background (Δ*ntcB*::*kan* Δ*nrtABCD*), DON induced luciferase expression but nitrite decreased luciferase activity as in YKA3 (not shown). These results confirmed that the nitrite-promoted increase of luciferase activity in YKA1 and YKA2 represents positive regulation by NtcB and nitrite of the *nirA* operon promoter, as shown previously by the determination of *nirA* mRNA (2).

The nitrite activation of luciferase expression in YKA2 but not in YKA3 indicated that the regulatory sequence essential for the nitrite-promoted activation of *nirA* operon transcription is located in the region from bases −173 to −96, which carries the NtcA-binding site *nir*II and the two putative binding sites for LysR-type DNA-binding proteins, L1 and L2 (Fig. 1B). Site-specific modification of either the left half or the right half of the L1 site in YKA2 (Fig. 1B) abolished the nitrite-promoted increase of luciferase expression without affecting the luciferase induction by DON treatment (Fig. 4, YKA5 and YKA6), whereas site-specific modification of L2 in YKA2 (Fig. 1B) had no effect on the regulation of luciferase expression (YKA7). Removal of the NtcA-binding site *nir*II (YKA8) or its site-specific modification (YKA9) also resulted in loss of the response to nitrite. These findings suggested that the NtcA-

binding site *nir*II and one of the potential binding sites for LysR-type proteins, L1, are required for the nitrite-responsive regulation of *nirA* operon transcription.

When treated with DON, the *PnirB*::*luxAB* reporter strains YKB1, YKB2, and YKB3 expressed essentially the same level of luciferase activity (Fig. 4). None of the *PnirB*::*luxAB* reporter strains showed any nitrite-responsive activation of luciferase expression, consistent with our previous conclusion that nitrite does not affect *nirB* operon transcription (2). These findings showed that transcription from the *nirB* operon promoter is regulated simply by repression/derepression and that the region from bases −112 to −17 with respect to the *nirB* start codon carries all of the elements required for regulation. Site-specific modification of *nir*III abolished the luciferase expression in response to DON treatment (YKB4), showing that the NtcA-binding site is essential for the activity of the *nirB* operon promoter.

Dependence on NtcA of the nitrite-responsive, NtcB-dependent activation of *nirA* operon transcription. Although it has a functional *nirB-ntcB* operon, strain YKA4, carrying the *PnirA*::*luxAB* fusion with modified *nir*I, did not express luciferase when treated with DON and nitrite (Fig. 4). This indicated that NtcB and nitrite cannot induce transcription of the reporter genes by themselves even under nitrogen starvation and that induction by NtcA is a prerequisite for enhancement by NtcB of the transcription.

Dependence on NtcA of the action of NtcB was confirmed by determination of the *nirA* mRNA in the *ntcA* deletion mutant NIC2 (Fig. 5). Since induction of the *nirB-ntcB* operon is dependent on NtcA, a plasmid-borne *ntcB* was used for constitutive expression of NtcB in the mutant. In the wild-type strain PCC 7942, MSX treatment of ammonium-grown cells induced *nirA* operon transcription (Fig. 5A, lane 2) and nitrite further enhanced the transcription as previously shown (Fig. 5A, lane 3). The hybridization signal was smeary as previously shown, representing rapid degradation of the primary transcript (38). The level of the transcript of *ndhB*, encoding an NADH dehydrogenase subunit, was not affected by the nitrogen conditions tested (Fig. 5B). In the *ntcA* deletion mutant NIC2, there was no induction of *nirA* operon transcription by treatment of ammonium-grown cells with MSX alone or with MSX plus nitrite (Fig. 5A, lanes 4 to 6). Introduction into NIC2 of a plasmid carrying *ntcA* restored the abilities of the cells to express *nirA* operon upon MSX treatment and to respond to nitrite (Fig. 5A, lanes 7 to 9), whereas introduction of pNTCB, which allows NtcA-independent expression of NtcB (2), restored neither of these abilities (Fig. 5A, lanes 10 to 12). Thus, NtcB and nitrite cannot activate *nirA* operon transcription in the absence of NtcA.

Gel shift assay. To examine whether NtcB binds to the *nirA* operon regulatory region, gel shift assays were performed with NtcB and/or NtcA, using the DNA segment carrying nucleotides −173 to −15 with respect to the *nirA* start codon as the probe. Since translational fusions of NtcB to glutathione *S*-transferase and to a His_6-containing amino acid segment yielded insoluble materials which could not be solubilized, a MalE-NtcB fusion, expressed in *E. coli* as a soluble protein, was purified to near homogeneity (Fig. 6A), cleaved with factor Xa (Fig. 6B), and used for the experiments (Fig. 7). Samples of NtcA were also obtained by cleavage of a MalE-NtcA fusion with factor Xa (Fig. 6). While addition of NtcA to a concentration of 15 nM yielded two clearly retarded bands representing DNA-protein complexes as previously reported by Luque et al. (20) (Fig. 7, lanes 7, 13, and 18), addition of NtcB up to 500 nM did not affect the electrophoretic mobility of the DNA probe (lanes 3 to 6) or of the probe-NtcA complexes (lanes 8 to 11, 14 to 17, and 19 to 22). These results showed that the NtcB protein does not bind to the *nirA* operon regulatory region under the given conditions.

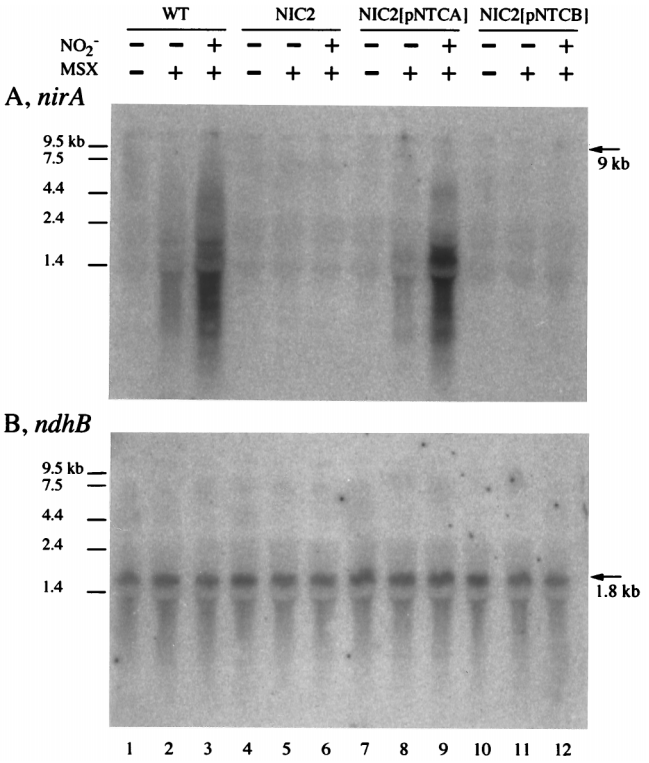

FIG. 5. Northern blot analysis of RNA from wild-type PCC 7942 strain (WT; lanes 1 to 3), the *ntcA* deletion mutant (NIC2; lanes 4 to 6), the NIC2 mutant transformed with a plasmid carrying *Ptrc*::*ntcA* transcriptional fusion (NIC2 [pNTCA]; lanes 7 to 9), and the NIC2 mutant transformed with a plasmid carrying *Ptrc*::*ntcB* transcriptional fusion (NIC2[pNTCB]; lanes 10 to 12), showing the requirement for *ntcA* of the activation of *nirA* operon transcription by NtcB and nitrite. Cells were grown with ammonium, the culture was separated into three portions, and total RNA was extracted from the cells 60 min after addition of nothing (control; lanes 1, 4, 7, and 10), MSX (lanes 2, 5, 8, and 11), and MSX plus nitrite (lanes 3, 6, 9, and 12). The RNA samples (10 μg per lane) were denatured, fractionated by electrophoresis, transferred to positively charged nylon membranes, and hybridized with the probes specific to *nirA* (A) and *ndhB* (B). The arrow in panel A indicates the calculated size of the full-length mRNA of the *nirA* operon, whereas that in panel B shows the position of the *ndhB* transcript.

Structures of the *nirA* operon promoters of other cyanobacteria. Figure 8 compares the nucleotide sequences of the *nirA* regulatory regions of four species of cyanobacteria, including *Synechococcus* sp. strain PCC 7942. As previously reported, the *nirA* operons of *Plectonema boryanum* (37) and *Anabaena* sp. strain PCC 7120 (10) have the consensus sequence of the NtcA-dependent, ammonium-repressible promoter, i.e., $GTAN_8TACN_{22}TAN_3T$. The *nirA* gene of *Synechocystis* sp. strain PCC 6803, which was identified by the genome sequencing project (16), also has an NtcA-binding motif and the putative −10 element in its promoter region (Fig. 8). The L1 sequence of *Synechococcus* sp. strain PCC 7942 is centered at nucleotide −23 with respect to the conserved NtcA-binding site (Fig. 8). The other species of cyanobacteria were found to have an L1-like inverted repeat containing a LysR motif ($TN_{11}A$) at the same location as that of L1 with respect to the conserved NtcA-binding site (Fig. 8). On the other hand, the NtcA-binding motif corresponding to *nir*II of *Synechococcus* sp. strain PCC 7942 was not present in the other species of cyanobacteria (not shown).

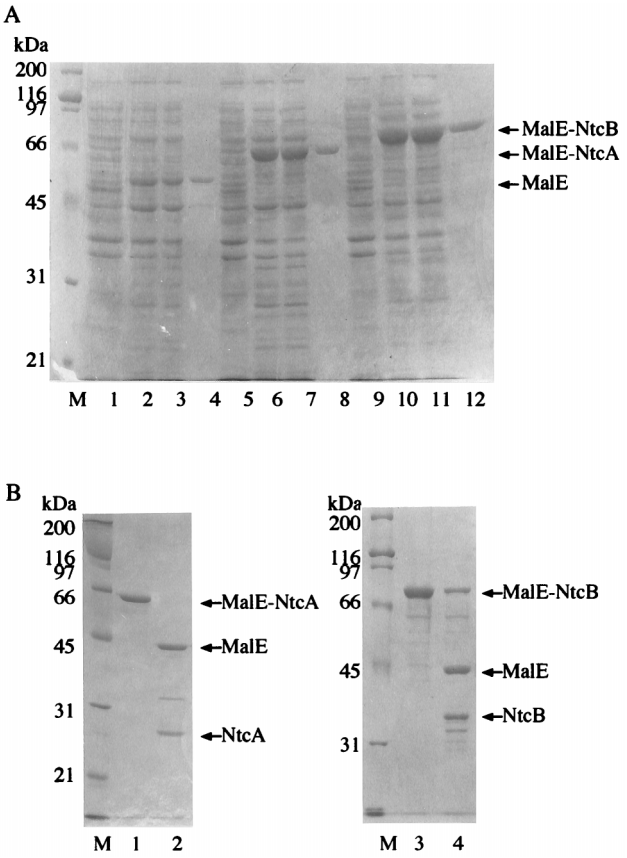

FIG. 6. Preparation of recombinant NtcA and NtcB proteins. (A) Expression in *E. coli* and purification of MalE (lanes 1 to 4) and the MalE-NtcA (lanes 5 to 8) and MalE-NtcB (lanes 9 to 12) fusions. Proteins were separated on a sodium dodecyl sulfate–10% polyacrylamide gel and stained with Coomassie brilliant blue. Lanes 1, 5, and 9, total protein from the *E. coli* expression strains before IPTG treatment; lanes 2, 6, and 10, total protein from the expression strains after a 1-h treatment with IPTG; lanes 3, 7, and 11, soluble fraction from the IPTG-induced expression strains; lanes 4, 8, and 12, proteins purified on amylose resin. (B) Cleavage of the MalE-NtcA (lanes 1 and 2) and MalE-NtcB (lanes 3 and 4) fusions with factor Xa. Protein profiles before (lanes 1 and 3) and after (lanes 2 and 4) treatment with factor Xa were compared. Proteins were separated on sodium dodecyl sulfate–12.5% (lanes 1 and 2) and 10% (lanes 3 and 4) polyacrylamide gels and stained with Coomassie brilliant blue. Lanes M, molecular mass markers (masses are indicated at the left).

DISCUSSION

Transcriptional *luxAB* fusions integrated into the chromosome of *Synechococcus* at the *cmp* locus allowed us to characterize promoters and *cis*-controlling elements regulating expression of *nirA* and *nirB* operons. In accordance with the essential role of NtcA in activation of the cyanobacterial ammonium-repressible transcription units (20), the *PnirA*::*luxAB* and *PnirB*::*luxAB* reporters with modified *nir*I and *nir*III, respectively, did not express luciferase activity when treated with DON (Fig. 4, YKA4 and YKB4), verifying that the conserved NtcA-binding site of ammonium-repressible transcriptional units (20) is an essential promoter element. YKA4 cells treated with DON plus nitrite did not express luciferase activity despite the presence of a functional *ntcB* gene (Fig. 4), indicating that the positive regulation by NtcB and nitrite requires prior induction by NtcA of *nirA* operon transcription. The action of NtcB and nitrite on the *nirA* operon is therefore deduced to be enhancement of the transcription. Site-specific mutagenesis showed that the NtcA-binding site *nir*II, which is located in the middle of the *nirA-nirB* intergenic region, and the putative binding site (L1) for a LysR-type protein, which is located between *nir*I and *nir*II, are required for the nitrite-responsive, NtcB-dependent enhancement of *nirA* operon transcription (Fig. 4). Among the known NtcA-dependent transcription units of *Synechococcus* sp. strain PCC 7942, none but the *nirA* operon has the L1 motif in the regulatory region (7, 12, 36, 40). In accordance with this finding, nitrite has no effect on the transcription of *ntcA*, *glnA*, and the *nirB-ntcB* operon (2). NtcB and nitrite thus seem to specifically activate the *nirA* operon among the transcription units under the global nitrogen control.

Although two *cis*-acting sequences involved in the nitrite-responsive, NtcB-dependent enhancement of *nirA* operon transcription have been identified, the molecular mechanism of the regulation remains unclear. Dependence on the LysR-type protein NtcB and the requirement for a sequence motif conforming to the structure of the binding sites for LysR-type proteins seemed to suggest that NtcB binds to the L1 site in response to nitrite and thereby enhances transcription. However, NtcB samples obtained by cleavage of a MalE-NtcB fusion did not bind to the L1 site in vitro irrespective of the presence of nitrite and NtcA (Fig. 7), which suggests that one or more additional factors are involved in the regulation. For example, nitrite may modify the DNA-binding activity of NtcB through the action of another protein. It is also possible that NtcB activates transcription of another LysR-type protein, which in turn binds to the L1 motif and regulates *nirA* operon transcription. Further work, including study of the interaction of NtcB and nitrite, is required for elucidation of the molecular mechanism of the regulation.

The use of an NRT-deficient cyanobacterial strain (NA3) as the recipient of the *luxAB* reporter fusions led to expression of high luciferase activity during growth of the reporter strains with nitrate, when the regulatory sequence included the region involved in the nitrite-responsive positive regulation (YKA1 and YKA2 [Fig. 2 and 3]). However, the level of luciferase

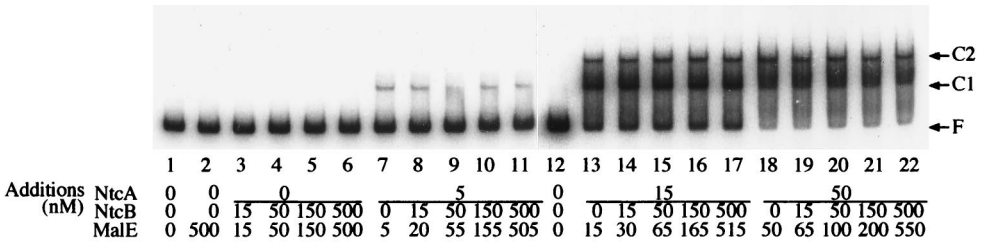

FIG. 7. Mobility shift assays showing retardation in 4% polyacrylamide gel of the ^{32}P-labeled *nirA* promoter segment (nucleotides −173 to −15 with respect to the *nirA* translation start site) by NtcA but not by NtcB. Samples of MalE (Fig. 6A, lane 4) and the MalE-NtcA and MalE-NtcB fusions cleaved with factor Xa (Fig. 6B, lanes 2 and 4) were added to the reaction mixtures to give the indicated concentrations of NtcA, NtcB, and MalE. C1 and C2, DNA-protein complexes; F, free probe.

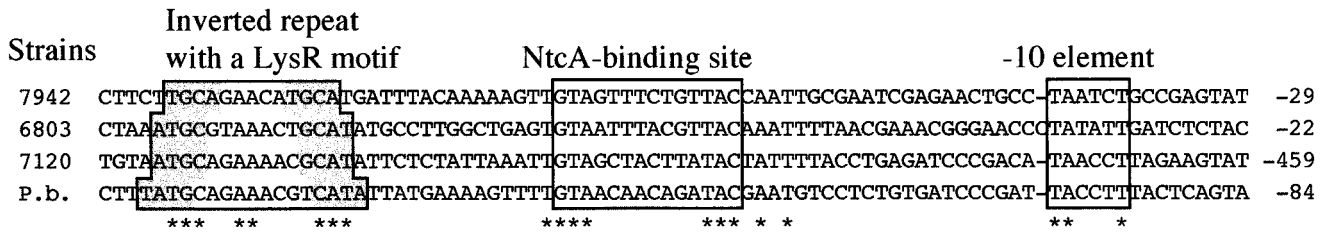

FIG. 8. Alignment of the promoter regions of the *nirA* operons from various species of cyanobacteria. The regions of the putative binding site for a LysR-type DNA-binding protein, the NtcA-binding site, and the −10 sequence are boxed. The nucleotides forming an inverted repeat with a LysR motif ($TN_{11}A$) are shaded. Asterisks indicate the nucleotides conserved in the four promoter sequences. Gaps have been introduced into the sequences to maintain optimal alignment. Numbers at the right indicate positions of the rightward-most bases with respect to the translation start site of *nirA*. Strains: 7942, *Synechococcus* sp. strain PCC 7942; 6803, *Synechocystis* sp. strain PCC 6803; 7120, *Anabaena* sp. strain PCC 7120; P.b., *Plectonema boryanum*.

expression in nitrate-grown YKA3, which lacks the *cis*-acting elements essential for the nitrite enhancement of transcription, was much lower than that in YKA1 and YKA2 (Fig. 3) and was similar to the level obtained simply by derepression of the transcription by DON treatment (Fig. 4). Thus, the high luciferase activity seen in nitrate-grown YKA1 and YKA2 is ascribed to the operation of the nitrite-responsive positive regulation mechanism of *nirA* operon transcription. Due to the limitation of nitrate uptake, the intracellular concentration of nitrite in the reporter strains is believed to be too low to form the level of ammonium that causes repression of the *nirA* operon promoter. The operation of the nitrite-responsive mechanism under these conditions indicates that the nitrite-responding mechanism has a high sensitivity to nitrite. In our previous studies, the positive effect of nitrite on *nirA* operon transcription was clearly discernible after inhibition of ammonium fixation (17) or after exposure of the cells to prolonged nitrogen deficiency (2). The present results, obtained with NRT-deficient strains, predict that the positive regulation mechanism will greatly enhance *nirA* operon transcription even during growth of the wild-type strain, provided that the nitrate levels in the medium are not saturating for uptake and reduction. Since cyanobacterial NRT has an apparent $K_m(NO_3^-)$ of about 1 μM (30), endogenously formed nitrite would enhance expression of the nitrate assimilation operon in the wild-type cells at external nitrate concentrations of 1 μM or lower. Such low concentrations of nitrate are barely maintained during growth of cyanobacterial cells in laboratory cultures but would be common in the natural environment.

We previously showed that nitrite positively regulates *nirA* operon transcription in *P. boryanum* as well as in *Synechococcus* sp. strain PCC 7942 (17). The L1-like inverted repeat of *P. boryanum*, which is present at the same location in the *nirA* regulatory region as in strain PCC 7942 (Fig. 8), is hence likely to be involved in the nitrite stimulation of *nirA* operon transcription in this cyanobacterium. The absence in *P. boryanum* of the NtcA-binding site corresponding to *nir*II indicates that nitrite regulation of the *nirA* operon does not require the additional NtcA-binding site in *P. boryanum*. The *nirA* regulatory regions of *Anabaena* sp. strain PCC 7120 and *Synechocystis* sp. strain PCC 6803 also have no *nir*II NtcA-binding site. However, the occurrence of L1-like motifs in these two strains (Fig. 8) and the identification of a *ntcB* homolog in strain PCC 6803 (16) suggest the common occurrence of nitrite-promoted, NtcB-dependent enhancement of *nirA* operon transcription in cyanobacteria.

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