JOURNAL OF BACTERIOLOGY, Sept. 2003, p. 5037–5044 Vol. 185, No. 17
0021-9193/03/$08.00+0 DOI: 10.1128/JB.185.17.5037–5044.2003

Open Reading Frame *all0601* from *Anabaena* sp. Strain PCC 7120 Represents a Novel Gene, *cnaT*, Required for Expression of the Nitrate Assimilation *nir* Operon

José E. Frías, Antonia Herrero, and Enrique Flores*

Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas-Universidad de Sevilla, Centro de Investigaciones Científicas Isla de la Cartuja, E-41092, Seville, Spain

Received 10 March 2003/Accepted 27 May 2003

Expression of the nitrate assimilation *nir* operon in the filamentous, heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120 requires the action of both the global nitrogen control transcription factor NtcA and the pathway-specific transcriptional regulator NtcB. In the genome of this cyanobacterium, the *ntcB* gene is found in a cluster of genes located in the complementary strand, upstream from the *nir* operon. Just downstream of *ntcB*, there is an open reading frame, *all0601* (previously designated *orf356* and now designated the *cnaT* gene), that putatively encodes a protein similar to proteins with glycosyl transferase activity and that is also present clustered together with *ntcB* homologues or nitrate assimilation structural genes in other cyanobacterial genomes. An insertional mutant of *cnaT* was generated and found to be unable to assimilate nitrate, although it could use ammonium or dinitrogen as a source of nitrogen for growth. In the mutant, under derepression conditions, *nir* operon mRNA (as determined by RNA-DNA hybridization and primer extension analysis) and enzymes of the nitrate reduction system (i.e., nitrate reductase and nitrite reductase) were expressed at low or undetectable levels. Inactivation of *cnaT* did not impair expression of *ntcB*, and expression of *cnaT* itself was constitutive and regulated by neither NtcA nor NtcB. Regulation of expression of the *nir* operon in *Anabaena* sp. strain PCC 7120 by CnaT and the previously described regulatory elements, NtcA and NtcB, is discussed.

Cyanobacteria are phototrophs that carry out oxygenic photosynthesis and likely represent the phylogenetic ancestors of eukaryotic algae and higher-plant chloroplasts (18). Nitrate and ammonium are excellent sources of nitrogen for cyanobacteria in general, and many strains are also able to use urea and/or fix atmospheric nitrogen (11). In the absence of combined nitrogen, some filamentous cyanobacteria, such as *Anabaena* sp. strain PCC 7120, differentiate heterocysts, which are specialized cells in which the nitrogen fixation machinery is confined under aerobic conditions (47).

In cyanobacteria, the assimilation of nitrate involves its incorporation into the cell through an active transport system, followed by intracellular two-step reduction to ammonium sequentially catalyzed by ferredoxin-nitrate reductase and ferredoxin-nitrite reductase (11). Genes encoding nitrite reductase (*nir*), an ABC-type nitrate/nitrite uptake permease (*nrtABCD*), and nitrate reductase (*narB*) are clustered together and constitute the *nir* operon (*nir-nrtABCD-narB*) in the genomes of *Synechococcus* sp. strain PCC 7942 (25, 27, 32, 39, 41) and *Anabaena* sp. strain PCC 7120 (6, 14). In addition, several genes involved in molybdenum cofactor (molybdopterin guanine dinucleotide [38]) biosynthesis whose mutation prevents expression of nitrate reductase activity have been characterized in *Synechococcus* sp. strain PCC 7942 (36, 37).

In all cyanobacteria tested to date, expression of the *nir* operon is subject to negative control by ammonium (22). The *ntcA* gene encodes a transcription factor belonging to the CAP family that is required for expression of genes that are subject to repression by ammonium and is widespread among cyanobacteria (16, 26). In *Anabaena* sp., the NtcA protein is essential for growth at the expense of nitrate or dinitrogen (13, 45) and regulates the expression of genes that encode proteins involved in nitrate assimilation, like the *nir* operon, or in dinitrogen fixation (22). The NtcA protein has recently been shown to respond in vitro to 2-oxoglutarate (42, 43), a putative C-to-N ratio signal in cyanobacterial cells (12, 31).

In addition to NtcA, a LysR-type transcriptional regulator, NtcB, is involved in regulation of *nir* operon expression (1, 2, 15, 28, 40). In *Anabaena* sp. strain PCC 7120, the NtcB protein is required for growth at the expense of nitrate and, together with NtcA, for activation of transcription of the *nir* operon in response to withdrawal of ammonium (15). In the genomes of both *Synechococcus* sp. strain PCC 7942 (40) and *Anabaena* sp. strain PCC 7120 (15), the *ntcB* gene is located in the opposite DNA strand, close to (and upstream from) the *nir* operon. Although in most cyanobacteria expression of the *nir* operon takes place in the absence of combined nitrogen (6), higher levels of the *nir* operon mRNA are usually found in cells incubated in the presence of nitrate (14, 24, 26). A role for NtcB in mediating nitrate-nitrite induction of the *nir* operon has been suggested (1, 28, 40), but it has also been shown that NtcB is required in some cyanobacteria for high-level expression of the *nir* operon in the absence of nitrate (2, 15).

In this study, we characterized a gene, *cnaT* (open reading frame [ORF] *all0601*), from *Anabaena* sp. strain PCC 7120 that

* Corresponding author. Mailing address: Instituto de Bioquímica Vegetal y Fotosíntesis, CSIC-Universidad de Sevilla, Avda. Américo Vespucio s/n, E-41092, Seville, Spain. Phone: 34 954 489523. Fax: 34 954 460065. E-mail: flores@cica.es.

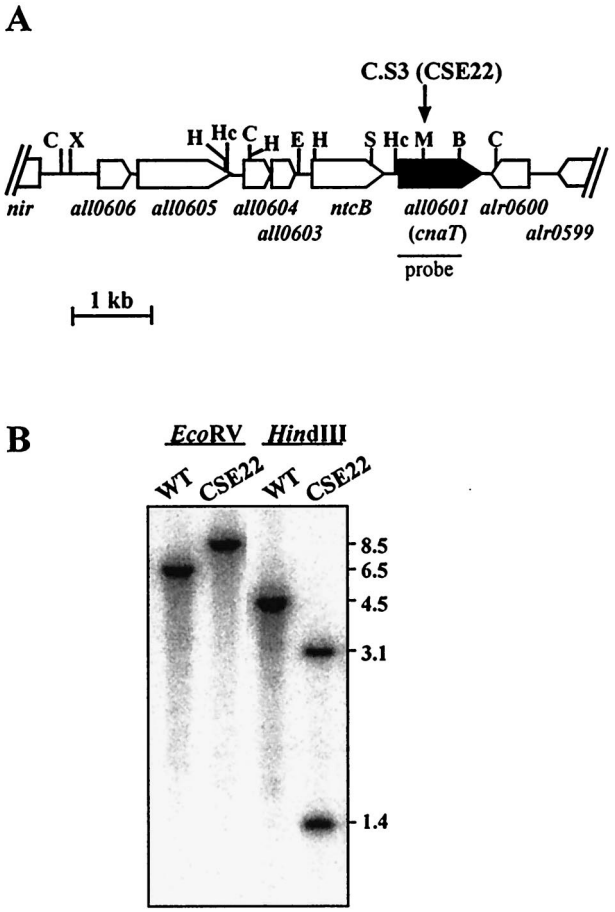

FIG. 1. (A) Genomic region of *Anabaena* sp. strain PCC 7120 bearing *cnaT* (ORF *all0601*). Genes and ORFs are indicated by large arrows, which also show the directions of transcription. The location of the probe used in the experiment whose results are shown in panel B and the restriction site into which gene cassette C.S3 was inserted to generate mutant strain CSE22 are also indicated. Abbreviations for restriction endonuclease sites: B, *Bst*XI; C, *Cla*I; E, *Eco*RV; Hc, *Hinc*II; H, *Hin*dIII; M, *Msc*I; S, *Spe*I; X, *Xba*I. (B) Southern blot analysis of the structure of the *all0601* region in mutant strain CSE22. Total DNA from wild-type strain PCC 7120 (WT) or strain CSE22 was digested with *Eco*RV or *Hin*dIII and hybridized to the probe shown in panel A (808-bp *Hinc*II/*Bst*XI DNA fragment containing most of the *all0601* ORF). Sizes (in kilobases) are indicated on the right.

encodes a new protein involved in nitrate assimilation through its influence on expression of the *nir* operon at the transcriptional level.

MATERIALS AND METHODS

Strains and growth conditions. *Anabaena* (*Nostoc*) sp. (referred to in this paper as *Anabaena* sp.) strain PCC 7120 (35) and its heterocyst-defective derivative strain EF116 (which is unable to fix dinitrogen under aerobic conditions) (46) were routinely grown photoautotrophically at 30°C under white light (about 25 microeinsteins $\cdot s^{-1} \cdot m^{-2}$) with shaking for liquid cultures. The media used for growth were BG11 medium (with $NaNO_3$ as the nitrogen source) (34), $BG11_0$ medium (BG11 medium without nitrate), and $BG11_0NH_4^+$ medium [$BG11_0$ medium supplemented with 2 mM NH_4Cl and 4 mM *N*-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES)–NaOH buffer (pH 7.5)]. For growth on plates, medium solidified with separately autoclaved 1% agar (Difco) was used. When appropriate, antibiotics were added to plates at the following final concentrations: streptomycin, 5 μg/ml; spectinomycin, 5 μg/ml; erythromycin, 5 μg/ml; and neomycin, 30 μg/ml. In liquid cultures, the following antibiotic concentrations were used: streptomycin, 2 μg/ml; spectinomycin, 2 μg/ml; and erythromycin, 5 μg/ml. Strains CSE2 (13), CSE19 (15), CSE22, and CSE22F were routinely grown in $BG11_0NH_4^+$ medium supplemented with streptomycin and spectinomycin. CSE221 and CSE222 were routinely grown in $BG11_0NH_4^+$ medium supplemented with streptomycin, spectinomycin, and erythromycin. For derepression experiments, cells grown in $BG11_0NH_4^+$ medium were washed three times with $BG11_0$ medium, resuspended in the media indicated below, and bubbled with a mixture of air and CO_2 (1% [vol/vol] CO_2) at 30°C in the light (75 to 100 microeinsteins $\cdot s^{-1} \cdot m^{-2}$). All media used for derepression experiments were supplemented with 12 mM $NaHCO_3$.

Escherichia coli DH5α, HB101, XL1-Blue, and ED8654 were grown in Luria-Bertani medium as described previously (3).

Plasmids. Plasmid pCSE95 bears a 3.25-kb *Cla*I DNA fragment from *Anabaena* sp. strain PCC 7120, which contains ORF *all0601* (previously designated *orf356* [15] and now designated the *cnaT* gene). Plasmid pCSE109 was constructed by inserting *Hinc*II-ended gene cassette C.S3 (9), excised from pRL463 (plasmid pUC18-19/L.HEH1/C.S3) (J. Elhai and C. P. Wolk, unpublished data; nomenclature of Elhai and Wolk [9]), into the *Msc*I site of *cnaT* carried on pCSE95. The C.S3 cassette has transcription terminators at both ends (33). The Klenow fragment-filled 5.25-kb *Cla*I insert from pCSE109 (*cnaT*::C.S3) was cloned into one of the two *Eco*RV sites of the *sacB*-carrying, mobilizable vector pRL278 (4), partially digested with *Eco*RV, to generate plasmid pCSE109B. Plasmid pCSE117 was generated by inserting a 2.4-kb *Sal*I/*Pst*I DNA fragment conferring resistance to chloramphenicol and erythromycin from pRL271 (4) into *Sal*I/*Pst*I-digested pBR322. Plasmid pCSE118 was constructed by inserting a 1.5-kb *Spe*I/*Pst*I DNA fragment containing *cnaT* and the 3′ end of the *ntcB* gene from pCSE95 into *Spe*I/*Pst*I-digested pCSE117.

Generation of mutant strains. Plasmids with mutant versions of *Anabaena* ORFs were transferred to the cyanobacterial parental strain by conjugation (10). For generation of the *cnaT* mutant, a *sacB*-mediated method for positive selection of double recombinants in *Anabaena* sp. was used (5). For generation of strains CSE22, CSE221, and CSE222, *E. coli* HB101 containing plasmid pCSE109B and helper plasmids pRL528 (10) and pRL591-W45 (8) (for CSE22) or pCSE118 and helper plasmid pRL623 (8) (for CSE221 and CSE222) was mixed with *E. coli* ED8654 carrying the conjugative plasmid pRL443. Next, each cell suspension was mixed with strain PCC 7120 (for CSE22) or CSE22 (for CSE221 and CSE222). After this, the resulting cell suspensions were spread onto nitrocellulose filters (REC-85; Nuclepore). The filters were set successively on top of solid medium ($BG11_0NH_4^+$ medium for CSE22 and $BG11_0NH_4^+$ medium containing streptomycin and spectinomycin for CSE221 and CSE222) supplemented with 5% Luria-Bertani medium and incubated for 24 h, on top of the same medium that was not supplemented and incubated for 24 h, and on top of the same medium supplemented with streptomycin and spectinomycin (for CSE22) or with erythromycin (for CSE221 and CSE222) until colonies appeared. In the case of generation of mutant strain CSE22, once the colonies appeared, the filter was set on top of $BG11_0NH_4^+$ solid medium containing streptomycin, spectinomycin, and 5% sucrose. All the sucrose-resistant colonies obtained were checked for sensitivity to neomycin (resistance to neomycin is encoded in the vector portion of plasmid pRL278). Strain CSE22F was generated like strain CSE22, except that strain EF116 was used as the parental strain. Double recombinants were identified by the sucrose-resistant Sm^r Sp^r Nm^s phenotype. In all cases the genomic structure of the resultant *Anabaena* sp. mutant strain was checked by Southern analysis.

DNA isolation, Southern blot analysis, and DNA sequencing. Isolation of DNA from *Anabaena* sp. was performed as previously described (5). For Southern blotting, restriction endonuclease-digested DNA was subjected to electrophoresis in agarose gels and transferred to Hybond-N^+ membranes by following the instructions of the manufacturer. Labeling of probes with ^{32}P and hybridization were performed as described previously (16). Manual sequencing was carried out by the dideoxy chain termination method with a T7 sequencing kit (Amersham Pharmacia Biotech) and [α-^{35}S]thio-dATP.

RNA isolation and analysis. RNA from *Anabaena* sp. strain PCC 7120 was prepared as described previously (17, 19). The resulting RNA preparations were treated with RNase-free DNase I to eliminate contaminating DNA. For Northern blot analysis, RNA (approximately 20 to 30 μg) was subjected to electrophoresis in denaturing formaldehyde gels, transferred to Hybond-N^+ membranes, and subjected to hybridization at 65°C as described previously (7). Labeling of probes with ^{32}P was performed as described previously (16). Primer extension experiments were performed as described elsewhere (3) by using 20 to 25 μg of RNA and oligonucleotide nir-1 (14), orf356-1 (5′CAG CAG ATC CCT GAA TAC AAT ACT C3′; complementary to nucleotides 27 to 3 of *Anabaena cnaT*), or orf356-4 (5′ CTT TCT CCC GTA TGG TTC CCA C3′; complemen-

TABLE 1. Nitrate reductase and nitrite reductase activities of *Anabaena* sp. strain PCC 7120 and mutant strains CSE22 (*cnaT*), CSE221, and CSE222[a]

Strain	Nitrate reductase activity (mU · mg of protein^{-1})			Nitrite reductase activity (mU · mg of protein^{-1})		
	Nitrate	Dinitrogen	Ammonium	Nitrate	Dinitrogen	Ammonium
PCC7120	69.2 ± 22.5	15.6 ± 6.38	11.3 ± 4.0	15.3 ± 4.6	ND	ND
CSE22	6.0 ± 5.8	0.1 ± 0.1	0.1 ± 0.1	ND	ND	ND
CSE221	86.9 ± 21.8	13.3 ± 2.4	9.4 ± 1.2	13.6 ± 1.8	ND	ND
CSE222	80.3 ± 9.5	16.1 ± 1.3	12.5 ± 4.4	12.7 ± 2.7	ND	ND

[a] Ammonium-grown cells were washed, resuspended in media with different sources of nitrogen, and incubated as indicated in Materials and Methods for derepression experiments. Enzymatic activities were determined after 4 h under derepression conditions. The values are the means ± standard deviations of the results of three independent experiments. ND, not detectable.

tary to nucleotides 62 to 41 of *Anabaena cnaT*) as the primer. Results were visualized and quantified with a Cyclone storage phosphor system and Opti-Quant image analysis software (Packard).

Enzyme activities. Nitrate reductase (20) and nitrite reductase (21) activities were measured with dithionite-reduced methyl viologen as the reductant in cells made permeable with mixed alkyltrimethylammonium bromide. The cells added to the enzymatic assay mixtures for nitrate reductase and nitrite reductase contained 5 and 25 μg of chlorophyll *a*, respectively. One unit of activity corresponded to 1 μmol of nitrite produced per min (nitrate reductase) or 1 μmol of nitrite removed per min (nitrite reductase).

RESULTS

***cnaT*.** ORF *all0601*, previously designated *orf356* (15) and now designated the *cnaT* gene (the *cna* refers to cyanobacterial nitrate assimilation and the *T* refers to the fact that it is required to attain high transcript levels [see below]), is located just downstream of the *ntcB* gene in the genome of *Anabaena* sp. strain PCC 7120 (15, 23). The *cnaT* gene should encode a 356-amino-acid polypeptide that shows overall homology to proteins with glycosyl transferase activity and, in particular, to anthranilate phosphoribosyltransferase, the *trpD* gene product of the tryptophan biosynthesis pathway. However, *Anabaena* sp. strain PCC 7120 contains two other ORFs that are more likely to represent true *trpD* genes, *alr1153* and *alr0409*, the latter of which is located in a putative tryptophan biosynthesis gene cluster (23). Thus, for instance, whereas the *cnaT* product exhibits 26% identity to the *Rhodobacter* TrpD protein, the *alr1153* and *alr0409* products exhibit 39 and 41% identity, respectively, to this TrpD protein.

ORFs of unknown function homologous to *cnaT* are found in other cyanobacterial genomes, and in every case the *cnaT* homologue is located adjacent to *ntcB* or other nitrate assimilation genes. Thus, gene 59 (contig 434) of *Nostoc punctiforme* (encoding a product with 80% identity to CnaT at the amino acid level) is just downstream of *ntcB*, *sll1634* of *Synechocystis* sp. strain PCC 6803 (encoding a product with 54.9% identity to CnaT) is just upstream of the nitrate/nitrite permease and nitrate reductase genes, *tll1358* of *Thermosynechococcus elongatus* strain BP-1 (encoding a product with 53.7% identity to CnaT) is just downstream of *ntcB*, and gene 1086 of *Synechococcus* sp. strain WH8102 (encoding a product with 43% identity to CnaT) is located close to a cluster of putative nitrate transport genes.

In bacteria, genes with related functions are frequently clustered together and sometimes constitute operons. Because of the proximity of *cnaT* to *ntcB* or to nitrate assimilation structural genes, we examined the effect of inactivation of *cnaT* in *Anabaena* sp. strain PCC 7120.

Isolation and characterization of a *cnaT* mutant. An inactivated version of *cnaT* was constructed by inserting in vitro gene cassette C.S3 into ORF *all0601* and transferring the inactivated version to *Anabaena* sp. strain PCC 7120 by conjugation. Selection for double recombination was applied, which led to allele substitution (Fig. 1) (see Materials and Methods for details). Figure 1B shows that no wild-type copies of *cnaT* could be detected in one of the selected clones, which was designated CSE22. In this clone, the wild-type version of the

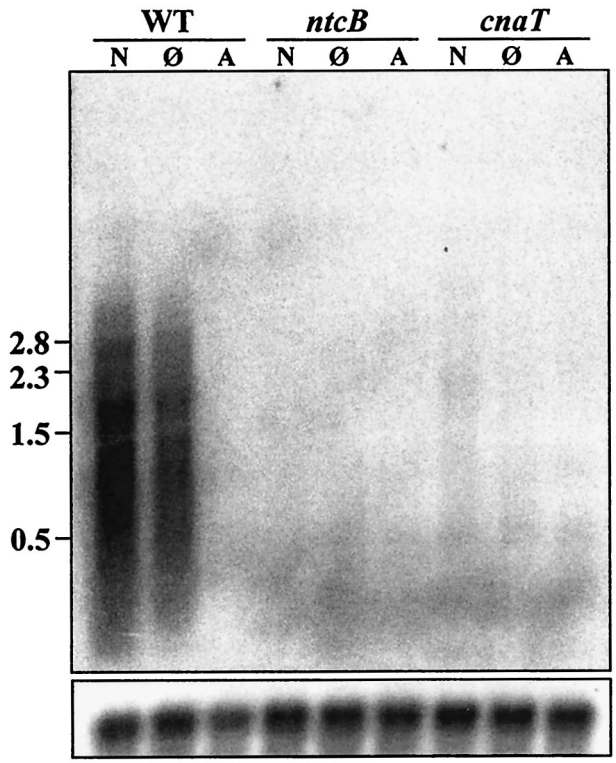

FIG. 2. Northern blot analysis of expression of the *nir* gene in wild-type strain PCC 7120 (WT) and mutant strains CSE19 (*ntcB*) and CSE22 (*cnaT*). Hybridization assays were carried out by using RNA isolated from cells grown with ammonium and incubated for 4 h in medium containing nitrate (lanes N), medium containing no combined nitrogen (lanes Ø), or medium containing ammonium (lanes A). As a hybridization probe, an *Xba*I-*Eco*RV DNA fragment from nucleotide 86 to nucleotide 2,479 with respect to the *nir* operon *tsp* was used. Hybridization to *rnpB* (44) served as a loading and transfer control (lower panel). The positions of some size markers (in kilobases) are indicated on the left.

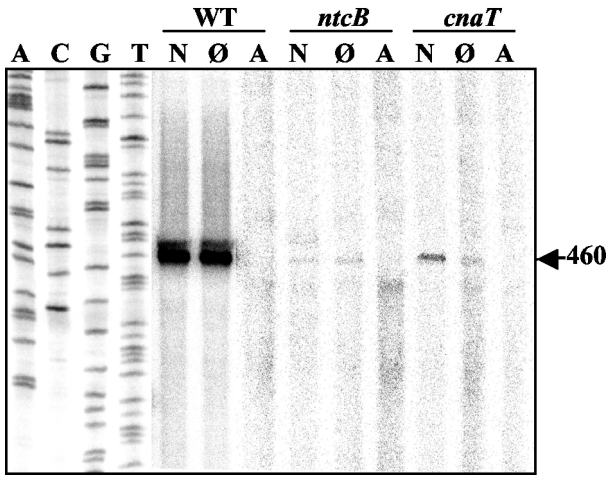

FIG. 3. Primer extension analysis of expression of the *nir* gene in wild-type strain PCC 7120 (WT) and mutant strains CSE19 (*ntcB*) and CSE22 (*cnaT*). Primer extension assays were carried out by using oligonucleotide nir-1 as a primer and RNA isolated from cells grown with ammonium and incubated for 4 h in medium containing nitrate (lanes N), medium containing no combined nitrogen (lanes Ø), or medium containing ammonium (lanes A). The arrowhead indicates the extension product identifying the *nir* operon *tsp*. The sequencing ladders were generated with the same primer used in the primer extension reactions and with plasmid pCSE26 as the template.

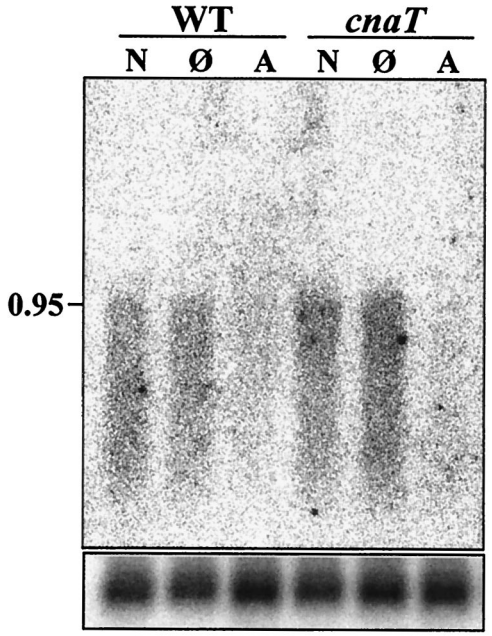

FIG. 4. Expression of the *ntcB* gene in wild-type strain PCC 7120 (WT) and mutant strain CSE22 (*cnaT*) under different nitrogen regimens. RNA isolated from cells grown in medium containing nitrate (lanes N), medium containing dinitrogen (lanes Ø), or medium containing ammonium (lanes A) was used in a Northern blot analysis with a PCR-generated DNA fragment containing most of the *Anabaena ntcB* gene (15) as a probe. Note that CSE22 cells grown in the presence of nitrate use dinitrogen as a nitrogen source. Hybridization to *rnpB* (44) served as a loading and transfer control (lower panel). The size (in kilobases) corresponding to size of the the observed putative transcript is indicated on the left.

cnaT region (6.5-kb *Eco*RV fragment and 4.5-kb *Hin*dIII fragment) had been replaced by the mutated version (8.5-kb *Eco*RV fragment and 3.1- and 1.4-kb *Hin*dIII fragments, respectively, since gene cassette C.S3 has *Hin*dIII sites at both ends).

The ability of mutant strain CSE22 (*cnaT*::C.S3) to grow in liquid medium by using different inorganic sources of nitrogen was examined. As was the case with parental strain PCC 7120, strain CSE22 was able to grow in media containing ammonium or no combined nitrogen. In media containing nitrate, strain CSE22 was able to grow, but it developed heterocysts, in contrast to strain PCC 7120, which developed heterocysts only in the absence of a source of combined nitrogen. The percentage of strain CSE22 cells that were heterocysts in nitrate-containing medium was similar to the percentage of the wild-type strain cells that were heterocysts in the absence of combined nitrogen, about 8%. Because mutants of *Anabaena* sp. strain PCC 7120 impaired in nitrate assimilation develop heterocysts and exhibit nitrogenase activity in the presence of nitrate (29), this observation suggested that strain CSE22 was unable to assimilate nitrate and grew in its presence by fixation of atmospheric nitrogen. The nitrate reductase and nitrite reductase activities in mutant CSE22 were measured and compared to those in parental strain PCC 7120. In contrast to strain PCC 7120, strain CSE22 exhibited only basal or undetectable levels of these enzyme activities under all of the incubation conditions tested (Table 1). However, the levels of nitrate reductase were higher in nitrate-containing medium than in the absence of combined nitrogen.

In order to check whether strain CSE22 really grew at the expenses of atmospheric nitrogen, the same construct used to generate strain CSE22 was transferred by conjugation to heterocyst-defective, Fox$^-$ strain EF116, generating strain CSE22F. Like its parental strain (strain EF116), strain CSE22F was unable to grow in the absence of combined nitrogen. In ammonium-containing media, the growth rate constants for strains EF116 and CSE22F, determined from the increases in the protein contents of the cultures (14), were 0.65 and 0.54 day^{-1}, respectively, and in media containing nitrate as the nitrogen source the growth rate constants were 0.75 and 0.08 day^{-1}, respectively (the values are the means of two independent experiments in which similar results were obtained). These observations confirmed that mutant strain CSE22 was unable to use nitrate as a sole nitrogen source and that in media containing nitrate strain CSE22 grew by fixing atmospheric nitrogen.

To verify that the inability of strain CSE22 to grow with nitrate as the only nitrogen source was a result of inactivation of *cnaT*, we reintroduced a wild-type copy of *cnaT* into strain CSE22. Plasmid pCSE118 (Em^r), containing an intact copy of *cnaT* (see Materials and Methods for details), was transferred by conjugation from *E. coli* to strain CSE22 (Sm^r Sp^r), and Em^r Sm^r Sp^r exconjugants were isolated in the presence of ammonium in the culture medium. The genomic structures of some exconjugants were examined by Southern blot analysis. Integration of pCSE118 upstream and downstream of the gene cassette C.S3 in CSE22 was observed in two of the exconjugants, strains CSE221 and CSE222, respectively (data not shown). Both of these strains had gained the ability to grow in

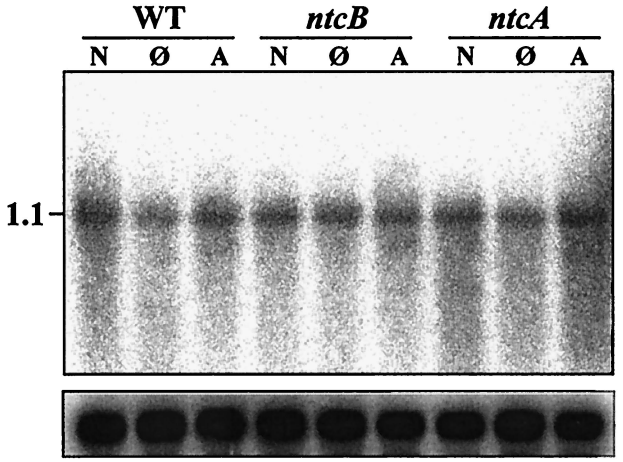

FIG. 5. Expression of *cnaT* in wild-type strain PCC 7120 (WT) and mutant strains CSE19 (*ntcB*) and CSE2 (*ntcA*) under different nitrogen regimens. A Northern blot analysis was performed with total RNA isolated from cells grown with ammonium and incubated for 4 h in medium containing nitrate (lanes N), medium containing no combined nitrogen (lanes Ø), or medium containing ammonium (lanes A). The probe used was the probe shown in Fig. 1A. Hybridization to *rnpB* (44) served as a loading and transfer control (lower panel). The size (in kilobases) corresponding to the size of the observed putative transcript is indicated on the left.

the presence of nitrate without developing heterocysts and had levels of nitrate reductase and nitrite reductase activities similar to those of wild-type strain PCC 7120 (Table 1). We therefore concluded that in *Anabaena* sp. strain PCC 7120 an intact *cnaT* gene is required for assimilation of nitrate and for expression of significant levels of nitrate reductase and nitrite reductase activities. On the other hand, because of the genetic structure of the genomic region in which *cnaT* is located (Fig. 1A) (23), no polar effects of insertion of gene cassette C.S3, which bears transcriptional terminators (33), were expected.

Because of the CnaT homology to tryptophan biosynthesis protein TrpD, it was possible that *cnaT* represented a tryptophan biosynthesis gene specifically required by the cells in nitrate-containing growth medium. We therefore tested whether growth of *Anabaena* sp. strain CSE22F (*cnaT*::C.S3 Fox$^-$) in nitrate-containing medium could be rescued by tryptophan, an amino acid that is expected to be transported by the neutral amino acid permeases of *Anabaena* sp. strain PCC 7120 (30). However, no growth of CSE22F was observed in plates of BG11 medium supplemented with 0.1 or 1 mM tryptophan, whereas growth was observed in $BG11_0NH_4^+$ medium in the presence of the same concentrations of this amino acid.

Strain CSE22F (*cnaT*::C.S3 Fox$^-$) was observed to revert frequently, giving rise to colonies able to grow on BG11 medium. To quantify the reversion frequency, a culture was raised from a CSE22F colony in liquid $BG11_0NH_4^+$ medium supplemented with streptomycin and spectinomycin, the filaments were fragmented by cavitation to obtain short filaments having a mean of five cells per filament, and serial dilutions of the filament suspension were plated on $BG11_0NH_4^+$ and BG11 media supplemented with streptomycin and spectinomycin. The numbers of blue-green colonies that appeared in $BG11_0NH_4^+$ and BG11 media were counted, and in two independent experiments reversion frequencies of 2.5×10^{-6} and 3.2×10^{-6} cell^{-1} were calculated. Fifteen independent revertants were tested and found to be Nas$^+$ Fox$^-$ and, as determined by Southern analysis, to have the mutant *cnaT*::C.S3 region (consistent with their Sm^r Sp^r phenotype) without carrying wild-type chromosome copies (results not shown). Thus, there appeared to be an extragenic suppressor mutation(s) of the *cnaT*::C.S3 mutation, and because of the high frequency of reversion, this suppression phenomenon appeared to result from loss-of-function mutations (i.e., from gene inactivation).

Analysis of *nir* operon expression in strain CSE22. To test whether the phenotype of strain CSE22 (*cnaT*::C.S3) was due to impaired expression of the *nir* operon, Northern blot analysis was carried out with a probe of the *nir* operon covering the *nir* gene and part of the *nrtA* gene. Hybridizations were performed with RNA isolated from cells of wild-type strain PCC 7120 and mutant strain CSE22 incubated under different nitrogen regimens. The *ntcB* mutant strain CSE19 (15) was also included for comparison. As is usual for the *nir* operon transcript (14), only a smear of degraded RNA products was detected (Fig. 2). Whereas in strain PCC 7120 there was a high level of expression of the *nir* operon in media without ammonium (media containing no combined nitrogen or nitrate), a low level of expression was observed for the *cnaT* mutant, which was also the case for the *ntcB* mutant (Fig. 2). Therefore, the requirement for CnaT for growth on nitrate is based on a requirement for this protein for expression of the *nir* operon.

Levels of the 5′ region of the *nir* operon transcript in strain CSE22 (*cnaT*::C.S3) were also analyzed. Primer extension experiments were performed with RNA isolated from cells of wild-type strain PCC 7120 and mutant strains CSE19 and CSE22 incubated under different nitrogen regimens (Fig. 3). Consistent with previously reported data (14), an extension product that ended at nucleotide −460 (with respect to the *nir* gene initiation codon), which was thus identified as a putative transcriptional start point (*tsp*), was obtained. Whereas in strain PCC 7120 a high level of the −460 extension product was observed in media without ammonium (media containing no combined nitrogen or nitrate), in strains CSE19 and CSE22 very low levels of this extension product were observed, although the product was still regulated by ammonium. Additionally, in the *cnaT* mutant, higher levels of this extension product were observed in RNA preparations from cells incubated with nitrate than in RNA preparations from cells incubated without combined nitrogen.

The results described above indicate that the *cnaT* product is required for efficient expression of the *nir* operon. However, no DNA-binding motif could be identified in the CnaT amino acid sequence. Additionally, mobility shift assays were carried out by using a DNA fragment containing the *nir* operon promoter and two different sources of CnaT protein, cell extracts of an CnaT-overproducing *E. coli* strain and purified His_6-CnaT protein, but no retardation was observed in either case under the experimental conditions used (data not shown).

Analysis of *ntcB* expression in strain CSE22. To test whether expression of the positive effector NtcB was affected in mutant strain CSE22 (*cnaT*::C.S3), expression of the *ntcB* gene was studied by Northern blot analysis by using total RNA isolated from cells of wild-type strain PCC 7120 and mutant strain CSE22 grown under various nitrogen regimens (Fig. 4). Similar

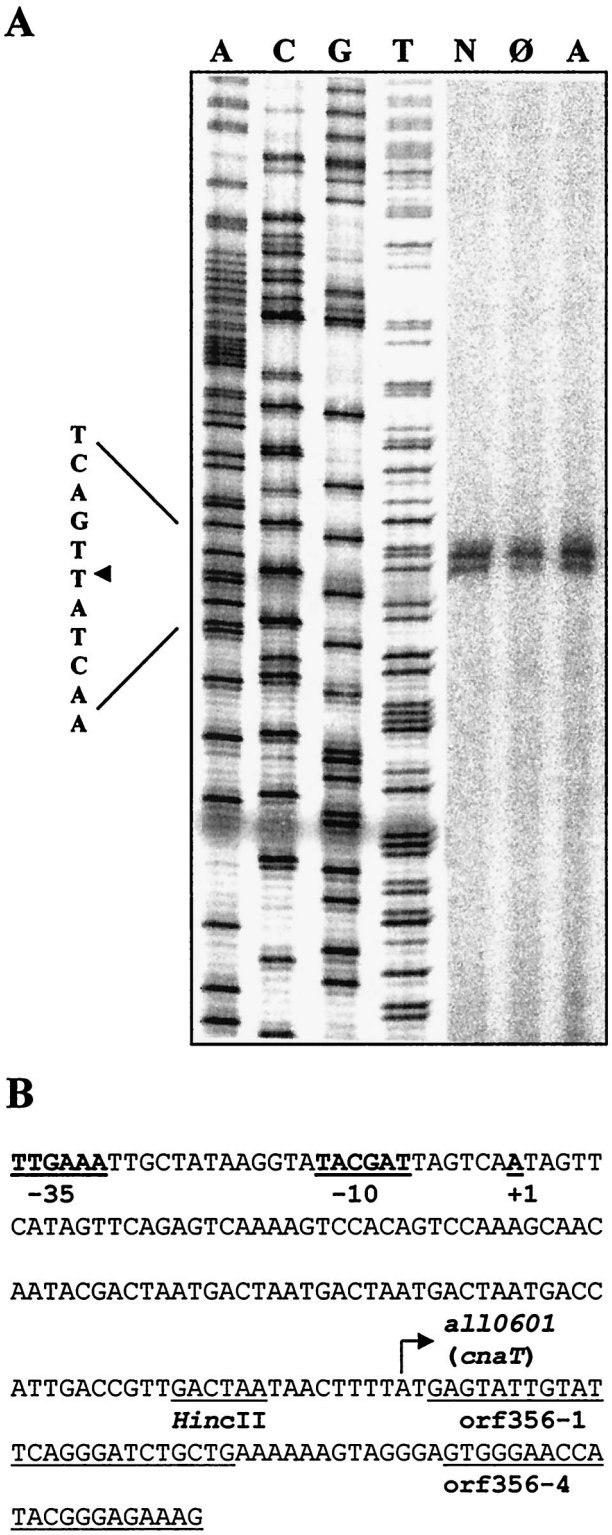

FIG. 6. Identification of the *cnaT* promoter. (A) Primer extension analysis performed with oligonucleotide orf356-1 and total RNA isolated from cells of *Anabaena* sp. strain PCC 7120 grown with ammonium and incubated for 4 h in medium containing nitrate (lanes N), medium containing no combined nitrogen (lanes Ø), or medium containing ammonium (lanes A). The nucleotide sequence around the end of the extension product (indicated by an arrowhead) is shown on the left. The sequencing ladders were generated with the primer used in the primer extension reactions and with pCSE95 as the template. (B) DNA sequence of the 5′ end and sequences upstream of *cnaT*. The locations of oligonucleotides orf356-1 and orf356-4, as well as the locations of the putative *tsp* (+1) and promoter elements (−10 and −35 boxes) of *cnaT*, are indicated. The arrow indicates the start of the *cnaT* ORF.

ammonium-regulated expression of the *ntcB* gene was observed in the two strains, indicating that expression of the *ntcB* gene was not affected in mutant strain CSE22.

Analysis of *cnaT* expression. Expression of *cnaT* was studied by Northern blot analysis by using RNA isolated from cells of wild-type strain PCC 7120 and mutant strains CSE2 (*ntcA*) and CSE19 (*ntcB*) incubated under various nitrogen regimens (Fig. 5). A 1.1-kb transcript whose levels were similar under all the nitrogen conditions tested was detected in the three strains. This observation indicates that expression of *cnaT* is constitutive with respect to the nitrogen source and is not regulated by either NtcA or NtcB. A size of 1.1-kb is consistent with a monocistronic transcript for *cnaT*, which is 1,036 bp long. On the other hand, the *cnaT* probe detected no transcript with RNA isolated from an *Anabaena* strain carrying an insertion of the C.S3 gene cassette into the *Hinc*II restriction site that is located upstream of the *cnaT* initiation codon (Fig. 1 and 6B show the location of the *Hinc*II site) (results not shown). This result confirmed that the transcript observed corresponded to the *cnaT* gene.

The putative *tsp* of *cnaT* was determined by using primer extension analysis. RNA isolated from cells of strain PCC 7120 grown with ammonium and incubated under various nitrogen regimens was used. An extension product, whose 3′ end corresponded to nucleotide −105 relative to the first nucleotide of *cnaT*, was obtained by using two different oligonucleotides, orf356-1 and orf356-4. The results obtained with oligonucleotide orf356-1 are shown in Fig. 6A. Similar levels of the RNA template were present in cells incubated with nitrate, in cells incubated with no combined nitrogen, and in cells incubated with ammonium, which is consistent with the data obtained in Northern blot experiments (Fig. 5). Two sequences, 5′TTGA AA3′ and 5′TACGAT3′, which could conform to the −35 and −10 boxes, respectively, of a σ^{70}-type promoter, were found upstream from this putative *tsp* (Fig. 6B). Nevertheless, it should be noted that only 13 nucleotides separated the two boxes, a distance too short for a typical σ^{70}-type promoter.

DISCUSSION

We identified a novel gene from *Anabaena* sp. strain PCC 7120, *cnaT*, which is required for expression of the *nir* operon. The product of *cnaT* shows significant sequence similarity to putative products of ORFs of unknown function from different cyanobacterial strains (e.g., *Nostoc punctiforme*, *Synechococcus* sp. strain WH8102, *Synechocystis* sp. strain PCC 6803, and *Thermosynechococcus elongatus* strain BP-1). As is the case for *Anabaena* sp. strain PCC 7120, the *cnaT* homologue is located close to genes involved in nitrate assimilation in the other cyanobacterial genomes. The deduced CnaT polypeptide and its cyanobacterial homologues are similar to proteins with glycosyl transferase activity, specifically to anthranilate phospho-

ribosyltransferase, an enzyme that transfers a phosphorylated ribose substrate to anthranilate. Thymidine/pyrimidine-nucleoside phosphorylase is also similar to these proteins.

Mutation of *cnaT* renders *Anabaena* sp. strain PCC 7120 unable to use nitrate as a nitrogen source, as corroborated by the phenotype of heterocyst-defective strain CSE22F. Figure 2 shows that inactivation of *cnaT* impairs accumulation of *nir* operon mRNA under conditions permissive for *nir* operon expression. Impaired accumulation could result from a defect in activation of gene transcription or from decreased stability of the transcripts. Nonetheless, primer extension analysis indicated that inactivation of CnaT has a strong effect on the abundance of the *nir* operon mRNA 5′ end, similar to what happens with inactivation of the transcriptional activators NtcA and NtcB (Fig. 3) (14, 15). This observation suggests that the inability of strain CSE22 (*cnaT*::C.S3) to use nitrate may be due to a defect in activation of transcription of the *nir* operon. However, no DNA-binding motif has been identified in the CnaT sequence, and no indication of binding of the CnaT protein to the *nir* operon promoter has been obtained. Therefore, if CnaT has a role in activation of *nir* operon expression, it may be indirect.

As mentioned above, activation of expression of the *nir* operon in *Anabaena* sp. strain PCC 7120 requires the functions of both the NtcA and NtcB transcription factors, which exert their effects by binding to the *nir* operon promoter region (15). Thus, mutation of *cnaT* might affect *nir* operon expression via a defect in activation of the *nir* operon promoter by NtcA and/or NtcB. The fact that strain CSE22, unlike *ntcA* mutant strain CSE2 (13), can grow on N_2, a process absolutely dependent on NtcA, indicates that the NtcA protein is functional in strain CSE22. On the other hand, ammonium-regulated expression of *ntcB* is unaffected in mutant strain CSE22 (Fig. 4), indicating that the impaired expression of the *nir* operon in strain CSE22 is not due to impaired expression of the *ntcB* gene.

The molecular mechanism of the effect of CnaT on *nir* operon expression is unknown. However, given the putative glycosyl transferase activity of this protein, it is possible that it acts by modifying another as-yet-unidentified factor of the regulatory system for the *nir* operon promoter. The high reversion frequency observed for the *cnaT*::C.S3 mutant suggests that a negative factor acts downstream of CnaT during regulation of *nir* operon expression, so that a spontaneous mutation of such a factor suppresses the effect of the *cnaT* mutation. It is therefore possible that CnaT acts by modifying a negative factor, which has the effect of liberating the *nir* operon from inhibition of transcription. If this were the case, the function of CnaT would be similar to that of the eukaryotic protein poly(ADP-ribose) polymerase 1, which, in response to DNA damage, promotes base excision repair and modifies, through poly-(ADP-ribosyl)ation, several transcription factors, thereby precluding their binding to DNA (48). Because of the similarity of the phenotypes of the *cnaT* and *ntcB* mutants, the putative negative factor might act by impairing the positive effect of NtcB on *nir* operon transcription. Since, as shown in Fig. 3, inactivation of *cnaT* does not eliminate nitrogen control, it is possible that CnaT represents a sensor of cellular parameters other than nitrogen availability that may be important for modulation of *nir* operon expression. Constitutive expression (at least with regard to the nitrogen source) (Fig. 5) of *cnaT* would ensure the presence of the CnaT protein under different growth conditions, which would be consistent with a sensor role for this protein. Alternatively, CnaT could be required for high-level expression of the *nir* operon under any cellular conditions.

To recapitulate, activation of the *nir* operon promoter in *Anabaena* sp. strain PCC 7120 after withdrawal of ammonium under otherwise permissive growth conditions strictly depends on NtcA (13, 14), which mediates gene activation in response to an increase in the C-to-N ratio of the cells (22, 42, 43). Additionally, NtcB, whose expression is NtcA dependent, is necessary to attain high levels of use of the *nir* operon promoter independent of the presence of nitrate in the culture medium (15). Nonetheless, nitrate has a positive effect on the *nir* operon transcript and on nitrate reductase and nitrite reductase activities that is still observed in the *cnaT* mutant and operates, at least in part, at a posttranscriptional level (14, 15). Our findings indicate that the action of CnaT is also required to attain high levels of expression of the *nir* operon through a mechanism that remains to be established.

ACKNOWLEDGMENTS

We thank A. M. Muro-Pastor and A. Valladares for their generous help during this work. Use of DNA sequences of the DOE-Joint Genome Institute and Kazusa DNA Research Institute databases is acknowledged.

This work was supported by grant BMC2002-03902 from the Ministerio de Ciencia y Tecnología, Spain.

REFERENCES

1. **Aichi, M., and T. Omata.** 1997. Involvement of NtcB, a LysR family transcription factor, in nitrite activation of the nitrate assimilation operon in the cyanobacterium *Synechococcus* sp. strain PCC 7942. J. Bacteriol. **179:**4671–4675.
2. **Aichi, M., N. Takatani, and T. Omata.** 2001. Role of NtcB in activation of nitrate assimilation genes in the cyanobacterium *Synechocystis* sp. strain PCC 6803. J. Bacteriol. **183:**5840–5847.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.)** 2002. Current protocols in molecular biology. Greene Publishing and Wiley-Interscience, New York, N.Y.
4. **Black, T. A., Y. Cai, and C. P. Wolk.** 1993. Spatial expression and autoregulation of *hetR*, a gene involved in control of heterocyst development in *Anabaena.* Mol. Microbiol. **9:**77–84.
5. **Cai, Y., and C. P. Wolk.** 1990. Use of a conditionally lethal gene in *Anabaena* sp. strain PCC 7120 to select for double recombinants and to entrap insertion sequences. J. Bacteriol. **172:**3138–3145.
6. **Cai, Y., and C. P. Wolk.** 1997. Nitrogen deprivation of *Anabaena* sp. strain PCC 7120 elicits rapid activation of a gene cluster that is essential for uptake and utilization of nitrate. J. Bacteriol. **179:**258–266.
7. **Church, G. M., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81:**1991–1995.
8. **Elhai, J., A. Vepritskiy, A. M. Muro-Pastor, E. Flores, and C. P. Wolk.** 1997. Reduction of conjugal transfer efficiency by three restriction activities of *Anabaena* sp. strain PCC 7120. J. Bacteriol. **179:**1998–2005.
9. **Elhai, J., and C. P. Wolk.** 1988. A versatile class of positive-selection vectors based on the nonviability of palindrome-containing plasmids that allows cloning into long polylinkers. Gene **68:**119–138.
10. **Elhai, J., and C. P. Wolk.** 1988. Conjugal transfer of DNA to cyanobacteria. Methods Enzymol. **167:**747–755.
11. **Flores, E., and A. Herrero.** 1994. Assimilatory nitrogen metabolism and its regulation, p. 487–517. *In* D. A. Bryant (ed.), The molecular biology of cyanobacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
12. **Forchhammer, K.** 1999. The P_{II} protein in *Synechococcus* PCC 7942 senses and signals 2-oxoglutarate under ATP-replete conditions, p. 549–553. *In* G. A. Peschek, W. Löffelhardt, and G. Schmetterer (ed.), The phototrophic prokaryotes. Kluwer Academic/Plenum Publishers, New York, N.Y.
13. **Frías, J. E., E. Flores, and A. Herrero.** 1994. Requirement of the regulatory protein NtcA for the expression of nitrogen assimilation and heterocyst development genes in the cyanobacterium *Anabaena* sp. PCC 7120. Mol. Microbiol. **14:**823–832.
14. **Frías, J. E., E. Flores, and A. Herrero.** 1997. Nitrate assimilation gene cluster

from the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **179:**477–486.
15. **Frías, J. E., E. Flores, and A. Herrero.** 2000. Activation of the *Anabaena nir* operon promoter requires both NtcA (CAP family) and NtcB (LysR family) transcription factors. Mol. Microbiol. **38:**613–625.
16. **Frías, J. E., A. Mérida, A. Herrero, J. Martín-Nieto, and E. Flores.** 1993. General distribution of the nitrogen control gene *ntcA* in cyanobacteria. J. Bacteriol. **175:**5710–5713.
17. **García-Domínguez, M., and F. J. Florencio.** 1997. Nitrogen availability and electron transport control the expression of *glnB* gene (encoding PII protein) in the cyanobacterium *Synechocystis* sp. PCC 6803. Plant Mol. Biol. **35:**723–734.
18. **Giovannoni, S. J., S. Turner, G. J. Olsen, S. Barns, D. J. Lane, and N. R. Pace.** 1988. Evolutionary relationships among cyanobacteria and green chloroplasts. J. Bacteriol. **170:**3584–3592.
19. **Golden, S. S., J. Brusslan, and R. Haselkorn.** 1987. Genetic engineering of the cyanobacterial chromosome. Methods Enzymol. **153:**215–231.
20. **Herrero, A., E. Flores, and M. G. Guerrero.** 1985. Regulation of nitrate reductase cellular levels in the cyanobacteria *Anabaena variabilis* and *Synechocystis* sp. FEMS Microbiol. Lett. **26:**21–25.
21. **Herrero, A., and M. G. Guerrero.** 1986. Regulation of nitrite reductase in the cyanobacterium *Anacystis nidulans.* J. Gen. Microbiol. **132:**2463–2468.
22. **Herrero, A., A. M. Muro-Pastor, and E. Flores.** 2001. Nitrogen control in cyanobacteria. J. Bacteriol. **183:**411–425.
23. **Kaneko, T., Y. Nakamura, C. P. Wolk, T. Kuritz, S. Sasamoto, A. Watanabe, M. Iriguchi, A. Ishikawa, K. Kawashima, T. Kimura, Y. Kishida, M. Kohara, M. Matsumoto, A. Matsuno, A. Muraki, N. Nakazaki, S. Shimpo, M. Sugimoto, M. Takazawa, M. Yamada, M. Yasuda, and S. Tabata.** 2001. Complete genomic sequence of the filamentous nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120. DNA Res. **8:**227–253.
24. **Kikuchi, H., M. Aichi, I. Suzuki, and T. Omata.** 1996. Positive regulation by nitrite of the nitrate assimilation operon in the cyanobacteria *Synechococcus* sp. strain PCC 7942 and *Plectonema boryanum.* J. Bacteriol. **178:**5822–5825.
25. **Luque, I., E. Flores, and A. Herrero.** 1994. Nitrate and nitrite transport in the cyanobacterium *Synechococcus* sp. PCC 7942 are mediated by the same permease. Biochim. Biophys. Acta **1184:**296–298.
26. **Luque, I., E. Flores, and A. Herrero.** 1994. Molecular mechanism for the operation of nitrogen control in cyanobacteria. EMBO J. **13:**2862–2869.
27. **Luque, I., A. Herrero, E. Flores, and F. Madueño.** 1992. Clustering of genes involved in nitrate assimilation in the cyanobacterium *Synechococcus.* Mol. Gen. Genet. **232:**7–11.
28. **Maeda, S., Y. Kawaguchi, T. Ohe, and T. Omata.** 1998. *cis*-acting sequences required for NtcB-dependent, nitrite-responsive positive regulation of the nitrate assimilation operon in the cyanobacterium *Synechococcus* sp. strain PCC 7942. J. Bacteriol. **180:**4080–4088.
29. **Martín-Nieto, J., A. Herrero, and E. Flores.** 1991. Control of nitrogenase mRNA levels by products of nitrate assimilation in the cyanobacterium *Anabaena* sp. strain PCC 7120. Plant Physiol. **97:**825–828.
30. **Montesinos, M. L., A. Herrero, and E. Flores.** 1995. Amino acid transport systems required for diazotrophic growth in the cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **177:**3150–3157.
31. **Muro-Pastor, M. I., J. C. Reyes, and F. J. Florencio.** 2001. Cyanobacteria perceive nitrogen status by sensing intracellular 2-oxoglutarate levels. J. Biol. Chem. **276:**38320–38328.
32. **Omata, T., X. Andriesse, and A. Hirano.** 1993. Identification and characterization of a gene cluster involved in nitrate transport in the cyanobacterium *Synechococcus* sp. PCC 7942. Mol. Gen. Genet. **236:**193–202.
33. **Prentki, P., and H. M. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
34. **Rippka, R., J. Deruelles, J. B. Waterbury, M. Herdman, and R. Y. Stanier.** 1979. Generic asssignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. **111:**1–61.
35. **Rippka, R., and M. Herdman.** 1992/93. Pasteur Culture Collection of Cyanobacterial Strains catalogue and taxonomic handbook, vol. I. Institute Pasteur, Paris, France.
36. **Rubio, L. M., E. Flores, and A. Herrero.** 1998. The *narA* locus of *Synechococcus* sp. strain PCC 7942 consists of a cluster of molybdopterin biosynthesis genes. J. Bacteriol. **180:**1200–1206.
37. **Rubio, L. M., E. Flores, and A. Herrero.** 1999. Molybdopterin guanine dinucleotide cofactor in *Synechococcus* sp. nitrate reductase: identification of *mobA* and isolation of a putative *moeB* gene. FEBS Lett. **462:**358–362.
38. **Rubio, L. M., E. Flores, and A. Herrero.** 2002. Purification, cofactor analysis, and site-directed mutagenesis of *Synechococcus* ferredoxin-nitrate reductase. Photosynth. Res. **72:**13–26.
39. **Rubio, L. M., A. Herrero, and E. Flores.** 1996. A cyanobacterial *narB* gene encodes a ferredoxin-dependent nitrate reductase. Plant Mol. Biol. **30:**845–850.
40. **Suzuki, I., N. Horie, T. Sugiyama, and T. Omata.** 1995. Identification and characterization of two nitrogen-regulated genes of the cyanobacterium *Synechococcus* sp. strain PCC 7942 required for maximum efficiency of nitrogen assimilation. J. Bacteriol. **177:**290–296.
41. **Suzuki, I., T. Sugiyama, and T. Omata.** 1993. Primary structure and transcriptional regulation of the gene for nitrite reductase from the cyanobacterium *Synechococcus* PCC 7942. Plant Cell Physiol. **34:**1311–1320.
42. **Tanigawa, R., M. Shirokane, S.-I. Maeda, T. Omata, K. Tanaka, and H. Takahashi.** 2002. Transcriptional activation of NtcA-dependent promoters of *Synechococcus* sp. PCC 7942 by 2-oxoglutarate *in vitro.* Proc. Natl. Acad. Sci. USA **99:**4251–4255.
43. **Vázquez-Bermúdez, M. F., A. Herrero, and E. Flores.** 2002. 2-Oxoglutarate increases the binding affinity of the NtcA (nitrogen control) transcription factor for the *Synechococcus glnA* promoter. FEBS Lett. **512:**71–74.
44. **Vioque, A.** 1997. The RNase P from cyanobacteria: short tandemly repeated repetitive (STRR) sequences are present within the RNase P RNA gene in heterocyst-forming cyanobacteria. Nucleic Acids Res. **25:**3471–3477.
45. **Wei, T.-F., T. S. Ramasubramanian, and J. W. Golden.** 1994. *Anabaena* sp. strain PCC 7120 *ntcA* gene required for growth on nitrate and heterocyst development. J. Bacteriol. **176:**4473–4482.
46. **Wolk, C. P., Y. Cai, L. Cardemil, E. Flores, B. Hohn, M. Murry, G. Schmetterer, B. Schrautemeier, and R. Wilson.** 1988. Isolation and complementation of mutants of *Anabaena* sp. strain PCC 7120 unable to grow aerobically in dinitrogen. J. Bacteriol. **170:**1239–1244.
47. **Wolk, C. P., A. Ernst, and J. Elhai.** 1994. Heterocyst metabolism and development, p. 769–823. *In* D. A. Bryant (ed.), The molecular biology of cyanobacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
48. **Ziegler, M., and S. L. Oei.** 2001. A cellular survival switch: poly(ADP-ribosyl)ation stimulates DNA repair and silences transcription. BioEssays **23:**543–548.