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Nitrate Assimilation Gene Cluster from the Heterocyst-Forming Cyanobacterium *Anabaena* sp. Strain PCC 7120

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A region of the genome of the filamentous, nitrogen-fixing, heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120 that contains a cluster of genes involved in nitrate assimilation has been identified. The genes *nir*, encoding nitrite reductase, and *nrtABC*, encoding elements of a nitrate permease, have been cloned. Insertion of a gene cassette into the *nir-nrtA* region impaired expression of *narB*, the nitrate reductase structural gene which together with *nrtD* is found downstream from *nrtC* in the gene cluster. This indicates that the *nir-nrtABCD-narB* genes are cotranscribed, thus constituting an operon. Expression of the *nir* operon in strain PCC 7120 is subjected to ammonium-promoted repression and takes place from an NtcA-activated promoter located 460 bp upstream from the start of the *nir* gene. In the absence of ammonium, cellular levels of the products of the *nir* operon are higher in the presence of nitrate than in the absence of combined nitrogen.

Cyanobacteria are organisms belonging to the domain *Bacteria* (31) that are considered the inventors of oxygenic photosynthesis and the phylogenetic ancestors of chloroplasts (9). Nowadays, cyanobacteria are phototrophs that preferentially use inorganic carbon and nitrogen for growth. Nitrate and ammonium are excellent sources of nitrogen for cyanobacteria in general, and many cyanobacterial strains are also able to perform nitrogen fixation and thus to grow at the expense of atmospheric nitrogen (14). Many filamentous, nitrogen-fixing cyanobacteria develop, under conditions of aerobiosis and depletion of combined nitrogen, specialized cells called heterocysts where the machinery of nitrogen fixation is confined and can operate efficiently (47).

The assimilation of nitrate takes place through three successive steps (14). First, nitrate enters the cyanobacterial cell mainly by means of an active transport system. Then, intracellular nitrate is reduced to ammonium through two sequential reactions catalyzed by nitrate reductase and nitrite reductase, respectively. Last, ammonium resulting from nitrate assimilation, as is also the case for that taken up from the external medium or resulting from nitrogen fixation, is incorporated into carbon skeletons mainly via the glutamine synthetase/glutamate synthase cycle to generate all nitrogen-containing metabolites of the cell.

In the unicellular, non-nitrogen-fixing cyanobacterium *Synechococcus* sp. strain PCC 7942, three genes, namely, *narA*, *narB*, and *narC*, that are essential for the reduction of nitrate to nitrite have been cloned (22, 23). Also, a cluster of genes that contains the genes *nir* (encoding nitrite reductase), *nrt ABCD* (encoding four components of a multicomponent transport system for nitrate and nitrite), and *narB* (encoding nitrate reductase) has been characterized (24–26, 32, 37, 39). It has been shown that this cluster of genes behaves as an operon (24, 27, 39). In the unicellular, non-nitrogen-fixing cyanobacterium *Synechocystis* sp. strain PCC 6803, the *nirA* gene is found ca. 400 kb apart from the *nrtABCD* genes (21). Information about some nitrate assimilation genes in three filamentous, non-heterocyst-forming cyanobacteria is also available. A cluster of genes containing only *nirA* and *nrtABC* has been characterized in *Phormidium laminosum* (30). In *Plectonema boryanum*, *nirA* and *nrtA* are clustered together and might be part of an operon including some other genes as well (40). The *narB* gene from *Oscillatoria chalybea* has been isolated and sequenced (41), but no information on any other nitrate assimilation gene of this cyanobacterium is available. On the other hand, no data on nitrate assimilation genes in any heterocyst-forming cyanobacterium have been reported to date.

In all cyanobacteria tested to date, nitrate assimilation is regulated through repression provoked by the presence of ammonium in the external medium that modulates synthesis of nitrate reductase, nitrite reductase, and the nitrate/nitrite transport system, as well as of glutamine synthetase (14). In *Synechococcus* sp. strain PCC 7942, *Phormidium laminosum*, and *Plectonema boryanum*, expression of the *nir* operon has been shown to be subjected to negative control by ammonium (27, 30, 40). The *Synechococcus ntcA* gene that encodes a global transcriptional regulator of nitrogen assimilation genes that are subjected to ammonium-promoted repression has been identified (42, 43). The NtcA protein is required for expression of the *nir* operon and of the *glnA* gene encoding glutamine synthetase (27). The *ntcA* gene has been shown to be widespread among cyanobacteria (16). The *ntcA* gene from the filamentous, heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120 has been cloned and sequenced (16, 44), and the NtcA protein has been shown to be essential for growth at the expense of both nitrate and dinitrogen (17, 45). While the presence of neither nitrate nor nitrite is required as an inducer for the expression of nitrate reductase and nitrite reductase in non-nitrogen-fixing cyanobacteria, high levels of activity of those enzymes in the nitrogen fixers are attained only when either nitrate or nitrite is available in the external medium (14).

This report deals with the cloning, from the genome of *Anabaena* sp. strain PCC 7120, of a cluster of genes involved in nitrate assimilation, the determination of its genetic structure, and the study of the expression of genes in the cluster as well as its regulation by the nitrogen regimen imposed on the cells.

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MATERIALS AND METHODS

Strains and growth conditions. *Anabaena*/*Nostoc* sp. (hereafter referred to as *Anabaena* sp.) strain PCC 7120 (36) and its heterocyst-defective derivative strain

TABLE 1. Plasmids and cyanobacterial strains used in this work

Strain(s) or plasmid(s)	Origin and relevant characteristics	Source or reference(s)
PCC 7120	Wild-type *Anabaena*/*Nostoc* strain	35, 36
EF116	Derivative of *Anabaena* sp. strain PCC 7120 unable to fix nitrogen under aerobic conditions	46
CSE1	Sm^r Sp^r derivative of strain EF116 unable to assimilate nitrate; C.S3 gene cassette substituting a part of the *nir-nrtA* gene region (see Fig. 1)	This work
CSE2	*ntcA* mutant derivative of *Anabaena* sp. strain PCC 7120	17
CSE9, CSE10	Nm^r derivatives of strain PCC 7120; C.K1 gene cassette inserted in both orientations into the *nrtA* gene (see Fig. 1)	This work
CSE16	Sm^r Sp^r derivative of strain PCC 7120; C.S3 gene cassette inserted into *orf136* (see Fig. 1)	This work
CSE17	Sm^r Sp^r derivative of strain PCC 7120; C.S3 gene cassette inserted into *orf398* (see Fig. 1)	This work
CSE18	Sm^r Sp^r derivative of strain EF116 unable to assimilate nitrate; C.S3 gene cassette inserted into the *Cla*I site upstream of the *nir* gene (see Fig. 1)	This work
pBluescript SK(+)	Cloning vector	Stratagene
pCSE2	*Xba*I DNA fragment of about 5.75 kb, carrying part of the nitrate assimilation gene cluster from strain PCC 7120, cloned in pBluescript SK(+) (see Fig. 1A)	This work
pCSE5, pCSE21	*Hin*dIII DNA fragment of about 8 kb, carrying part of the nitrate assimilation gene cluster from strain PCC 7120 (see Fig. 1A), cloned in pBluescript SK(+) or in pRL500, respectively	This work
pCSE21aB	*Hin*dIII fragment from pCSE21 in which the 2.35-kb *Cla*I fragment comprising *nir* and part of the *nrtA* gene has been substituted by gene cassette C.S3 (see Fig. 1A), cloned in pRL278; used to generate mutant strain CSE1	This work
pCSE26	Religated longest fragment of *Eco*RV-digested pCSE21	This work
pCSE44B, pCSE45B	*Hin*dIII fragment from pCSE5 bearing gene cassette C.K1 inserted into the *Eco*RV site of the *nrtA* gene in the same orientation as this gene (pCSE44B) or in the opposite orientation (pCSE45B) (see Fig. 1A), cloned in pRL277; used to generate mutant strains CSE9 and CSE10, respectively	This work
pCSE78B	*Hin*dIII fragment from pCSE26, bearing gene cassette C.S3 inserted into the *Bgl*I site of *orf136* (see Fig. 1A), cloned in pRL278; used to generate mutant strain CSE16	This work
pCSE81B	*Hin*dIII fragment from pCSE26, bearing gene cassette C.S3 inserted into the *Pvu*II site of *orf398* (see Fig. 1A), cloned in pRL278; used to generate mutant strain CSE17	This work
pCSE88B	*Hin*dIII fragment from pCSE26, bearing gene cassette C.S3 inserted into the *Cla*I site upstream of the *nir* gene (see Fig. 1A), cloned in pRL278; used to generate mutant strain CSE18	This work
pRL277	Cloning vector containing the *sacB* gene from *Bacillus subtilis* and determinants for Sm^r and Sp^r	4
pRL278	Cloning vector containing the *sacB* gene from *Bacillus subtilis* and determinants for Km^r and Nm^r	4
pRL443	Km^s derivative of conjugative plasmid RP4	12
pRL500	Positive selection vector	11
pRL528	Plasmid bearing genes encoding methylases for *Ava*I and *Ava*II sites	12
pRL591-W45	Plasmid bearing a gene encoding a methylase for *Ava*III sites	10

EF116 (which is unable to fix dinitrogen under aerobic conditions) (46) were routinely grown photoautotrophically at 30°C under white light (25 to 30 W/m^2) with shaking for liquid cultures. Media used for growth were BG11 ($NaNO_3$ as the nitrogen source) (36), $BG11_0$ (BG11 medium lacking nitrate), and $BG11_0NH_4^+$ [BG11 medium lacking nitrate and supplemented with 2.5 mM NH_4Cl and 5 mM *N*-tris (hydroxymethyl)methyl-2-aminoethane sulfonic 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 (SM), 2 or 5 μg/ml; spectinomycin (SP), 2 or 5 μg/ml; and neomycin (NM), 30 μg/ml. In liquid cultures, antibiotic concentrations used were as follows: SM, 2 μg/ml; SP, 2 μg/ml; and NM, 5 μg/ml. Strains CSE1, CSE2, CSE16, CSE17, and CSE18 were routinely grown in $BG11_0NH_4^+$ medium supplemented with SM and SP, and strains CSE9 and CSE10 were routinely grown in $BG11_0$ medium supplemented with NM. For derepression experiments, cells grown in $BG11_0NH_4^+$ medium were washed twice with $BG11_0$ medium, resuspended in the media indicated in each experiment, and bubbled with air at 30°C in the light.

Escherichia coli DH5α, HB101, and ED8654 were grown in Luria-Bertani medium at 37°C. For growth on plates, medium solidified with 1.5% agar was used. When necessary, antibiotics were added at standard concentrations (2).

Growth rates were estimated from the increase of protein concentration in the cultures. Protein concentration was determined by a modified Lowry procedure (28) in 0.2-ml aliquots periodically withdrawn from the cultures. The growth rate constant (μ) corresponds to $\ln 2/t_d$, where t_d represents the doubling time.

Generation of mutant strains. In vitro-generated constructs bearing a gene cassette inserted into the nitrate assimilation gene cluster of *Anabaena* sp. strain PCC 7120 were cloned in *sacB*-bearing vectors (the *sacB* gene determines sensitivity to sucrose and can be counterselected for in *Anabaena* sp., allowing positive selection for double recombinants) (5). The resulting plasmids were transferred by means of conjugation (12) to *Anabaena* sp. strain PCC 7120 or to strain EF116 to generate strains bearing mutations in the nitrate assimilation genomic region. For generation of strains CSE1, CSE9, CSE10, CSE16, CSE17, and CSE18, *E. coli* HB101 containing plasmid pCSE21aB, pCSE44B, pCSE45B, pCSE78B, pCSE81B, or pCSE88B, respectively (see Table 1), and helper plasmids pRL528 (12) and pRL591-W45 (10) was mixed with *E. coli* ED8654 carrying the conjugative plasmid pRL443 and thereafter with strain PCC 7120 or strain EF116. The resulting cell suspension was spread onto nitrocellulose filters (Nucleopore; REC-85) set successively atop $BG11_0NH_4^+$ solid medium supplemented with 5% Luria-Bertani medium (incubated for 24 h), $BG11_0NH_4^+$ solid medium (incubated for 24 h), and $BG11_0NH_4^+$ solid medium supplemented with SM and SP or with NM (depending on the transferred gene cassette: cassette C.S3 [11] encodes resistance to SM [Sm^r] and SP [Sp^r] and cassette C.K1 [11] encodes resistance to NM [Nm^r] and kanamycin [Km^r]) and incubated until colonies showed up. Some antibiotic-resistant, exconjugant clones were grown in liquid medium supplemented with the same antibiotics. Liquid cultures were sonicated in a cleaning bath to give a mean filament length of three to four cells per filament and plated on solid $BG11_0NH_4^+$, antibiotic-containing medium supplemented with 5% sucrose. Double recombinants were identified as clones resistant to the antibiotic for which resistance was encoded in the inserted gene cassette, resistant to sucrose, and sensitive to the antibiotic for which a resistance determinant was present in the vector portion of the transferred plasmid and by Southern or PCR analysis.

DNA isolation, Southern blot analysis, PCR analysis, and DNA sequencing. Isolation of DNA from *Anabaena* sp. was performed as previously described (5). For Southern blots, restriction endonuclease-digested DNA was subjected to electrophoresis in agarose gels and transferred to GeneScreen Plus membranes (Dupont) following the instructions of the manufacturer. Labelling of probes with ^{32}P and hybridization under low- and high-stringency conditions (used for heterologous and homologous hybridizations, respectively) were performed as described previously (16). Colony blotting and hybridization for subgenomic library screening were performed as described elsewhere (38). PCR amplification was carried out in a 50-μl reaction volume containing ca. 2 ng of genomic DNA from the cyanobacterial strain to be checked, a 0.2 mM concentration of each deoxynucleoside triphosphate, 50 pmol each of oligonucleotides *nir*-2 and *nir*-14 (see below), and 5 U of *Taq* polymerase and buffer (from Boehringer). The program used for amplification was denaturation for 2 min at 95°C, annealing for

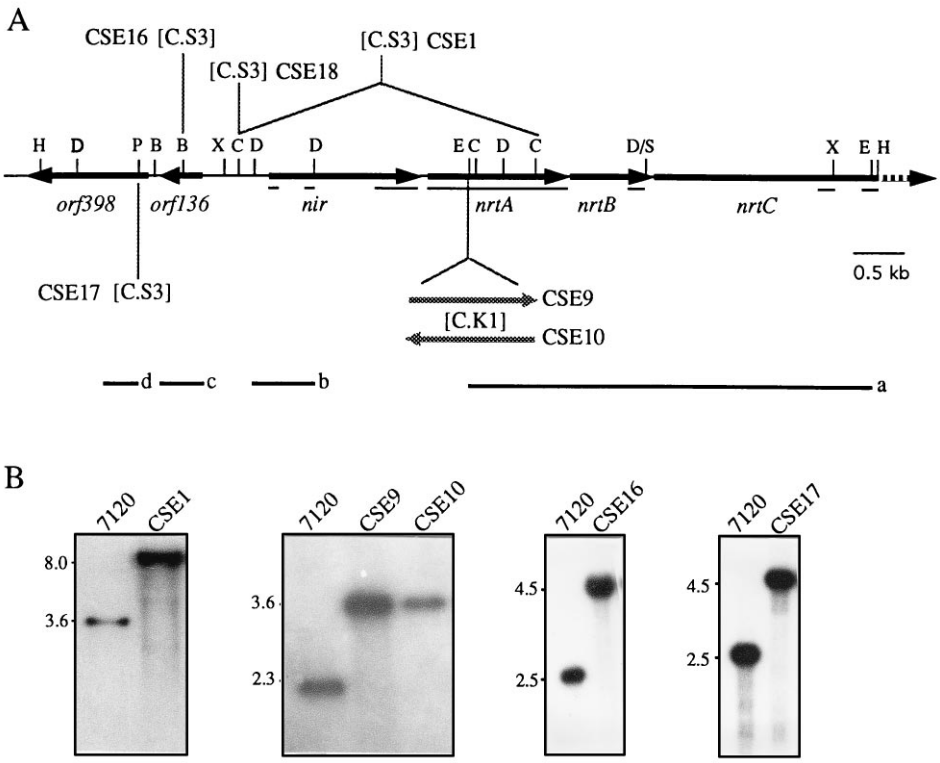

FIG. 1. Genomic region of *Anabaena* sp. strain PCC 7120 that contains a cluster of genes involved in nitrate assimilation. (A) Genes identified by hybridization with *Synechococcus* probes and/or by sequencing are indicated by thick arrows that also indicate the direction of transcription. Thin lines underneath indicate the extent of some sequenced DNA fragments. Additionally, the *nir* upstream region from the start of the *nir* gene to the end of *orf398* was completely sequenced. Mutant strains generated in this work, locations of the corresponding mutations, and the name of the inserted gene cassette are also presented. Thick lines followed by lowercase letters indicate the DNA fragments used as probes in Southern blot analyses shown in panel B. B, *Bgl*I; C, *Cla*I; D, *Dra*I; E, *Eco*RV; H, *Hin*dIII; P, *Pvu*II; S, *Sca*I; X, *Xba*I. (B) Southern blot analyses for identification of fully segregated mutant clones. Total DNA from the indicated strains was digested (for Southern blots shown from left to right) with endonuclease *Eco*RV, *Cla*I, *Cla*I, or *Cla*I and hybridized, respectively, to probes a to d shown in panel A. Sizes are shown on the left in kilobases.

1 min at 50°C, and polymerization for 3 min at 72°C. Products of amplification were visualized by electrophoresis in agarose gels stained with ethidium bromide. DNA fragments used for sequencing were generated by subcloning with restriction enzymes. Sequencing was carried out by the dideoxy chain termination method with a T7 sequencing kit and [α-^{35}S]thio-dATP. For the *nrtA* gene and *orf136* both strands were sequenced.

RNA isolation and analysis. RNA from *Anabaena* sp. was prepared as described previously (18) by using 150 ml of cultures containing ca. 4 to 5 μg of chlorophyll/ml. The resulting RNA preparations were treated with RNase-free DNase I to eliminate contaminating DNA. For Northern blot analysis, RNA (approximately 25 μg) was subjected to electrophoresis in denaturing formaldehyde gels, transferred to GeneScreen Plus membranes, and subjected to hybridization following the manufacturer's instructions. When oligonucleotide *nir*-3 (5′GCGCAGCCTTCCCGCAGGG3′, complementary to nucleotides −288 to −306 with respect to the start of the *nir* gene) was used as a probe, it was labelled in its 5′ end with polynucleotide kinase from Boehringer and [γ-^{32}P]ATP (5,000 Ci/mmol), and hybridization was performed at 42°C as described elsewhere (2). Primer extension experiments were performed as described elsewhere (2), using 50 μg of RNA and, as a primer, oligonucleotide *nir*-1 (5′CGCCGCCGATGTT TCACTGAAGG3′, complementary to nucleotides −344 to −366 with respect to the *nir* gene), *nir*-2 (5′GAGGCTGGCTTTGGGGG3′, complementary to nucleotides 36 to 20 of the *nir* gene), *nir*-3 (see above), *nir*-12 (5′GCTATCCAACC CAAACCTACCC3′, complementary to nucleotides 47 to 26 of *orf136*), or *nir*-14 (5′AGTCTAGCCTTGTTTTACCAAC3′, complementary to nucleotides −34 to −55 with respect to the start of *orf136*). For hybridization of blots of restricted plasmid DNA (2 pmol, resolved by electrophoresis in agarose gels) with total cyanobacterial RNA, 15 μg of RNA was partially hydrolyzed by incubation in 125 mM NaOH for 40 min at 0 to 4°C and labelled with T4 polynucleotide kinase (from Boehringer) and [γ-^{32}P]ATP (4,500 Ci/mmol) (1). Hybridization was performed as described previously (13) at 65°C in a solution containing the following: Tris-HCl (pH 8), 50 mM; bovine serum albumin, 0.2%; Ficoll, 0.2%; sodium pyrophosphate, 0.1%; sodium dodecyl sulfate, 1%; NaCl, 1 M; and yeast tRNA (Boehringer), 100 μg/ml. Results were quantified with the InstantImager scanner for beta particles (Packard).

Enzyme activities. Nitrate reductase (20) and nitrite reductase (19) were measured with dithionite-reduced methyl viologen as the reductant in cells made permeable with mixed alkyltrimethylammonium bromide (MTA-5 from Sigma). The amount of cells added to an enzymatic assay for nitrate reductase and nitrite reductase contained 5 and 40 μg of chlorophyll, respectively. Activity units correspond to micromoles of nitrite produced (nitrate reductase) or removed (nitrite reductase) per minute.

Nitrate uptake. Nitrate uptake assays were performed according to the method of Flores et al. (15). Ammonium-grown cells (4 to 5 μg of chlorophyll/ml) were derepressed by incubation for 5 h in BG11 medium (see above). Cells were then harvested by filtration, washed with 10 mM Tricine-NaOH buffer (pH 8.1), resuspended in the same buffer to 10 μg of chlorophyll/ml, and placed in air-opened conical flasks with shaking under illumination. Uptake assays were started by addition of $NaNO_3$ (0.1 or 17.6 mM, final concentration) after a 10-min preincubation in the presence (for uptake of 17.6 mM nitrate) or in the absence (for uptake of 0.1 mM nitrate) of 1 mM L-methionine–DL-sulfoximine. Nitrate disappearance or ammonium release was determined by estimating the concentration of the corresponding ion in the medium in aliquots of the cell suspensions after removal of the cells by filtration through Millipore HA 0.45-μm-pore-size filters. Nitrate concentration was estimated through its absorption at 210 nm in acid solution (7), and ammonium was measured with glutamate dehydrogenase (3).

Nucleotide sequence accession numbers. The nucleotide sequences of *nrtA* (1,918 bp) and the *nir* upstream region (2,536 bp) reported in this paper will appear in the EMBL/GeneBank/DDBJ nucleotide sequence data libraries under accession numbers X99709 and X99708, respectively.

RESULTS AND DISCUSSION

Cloning of a cluster of nitrate assimilation genes. A region from the genome of *Anabaena* sp. strain PCC 7120 that contains a cluster of genes involved in nitrate assimilation has been cloned. To clone it, a 1.5-kb *Sac*I DNA fragment containing part of the *nir* operon from the unicellular, non-nitrogen-fixing cyanobacterium *Synechococcus* sp. strain PCC 7942 (24) was used as a probe in heterologous hybridization with Southern

TABLE 2. Growth rates and nitrate reductase and nitrite reductase activities of *Anabaena* sp. strain PCC 7120, strain EF116, and some derivative mutants in culture media with different sources of nitrogen

Strain	μ (day^{-1})[a] in:			Nitrate reductase[b] (mU/mg of protein) in:			Nitrite reductase[b] (mU/mg of protein) in:		
	NO_3^-	N_2	NH_4^+	NO_3^-	N_2	NH_4^+	NO_3^-	N_2	NH_4^+
nir-nrtA mutants									
EF116[c]	0.73	ND	0.66	72.29	5.07	3.37	30.62	3.43	0
CSE1	<0.01	ND	0.58	0	0	0	0	0	0
nrtA mutants									
PCC 7120[c]	0.84	0.65	0.88	84.7	14.4	12.6	25.0	10.6	2.55
CSE9	0.52	0.48	0.67	82.2	11.4	12.9	26.9	9.1	0.82
CSE10	0.53	0.57	0.60	102.7	11.8	4.5	37.0	19.9	1.57
Mutants of *orf136* and *orf398*									
PCC 7120[c]	ND	ND	ND	58.9	22.0	3.0	8.5	2.7	0.1
CSE16	ND	ND	ND	52.8	27.8	1.6	9.1	3.9	0.1
CSE17	ND	ND	ND	69.1	38.3	1.9	4.1	2.7	0
nir leader region mutants									
EF116[c]	ND	ND	ND	61.8	15.7	2.4	9.2	5.4	0.2
CSE18	ND	ND	ND	0.7	0.6	0.3	0.2	0	0

[a] Ammonium-grown cells of the indicated strain were used to inoculate (at 0.2 μg of chlorophyll/ml) Erlenmeyer flasks containing 50 ml of medium with the indicated source of nitrogen. Cultures were incubated under standard conditions (see Materials and Methods). To estimate the growth rate, protein content was measured in aliquots picked up periodically from each culture. ND, not determined.

[b] Ammonium-grown cells of the indicated strain were washed, resuspended in medium with the indicated source of nitrogen, and incubated as indicated in Materials and Methods for derepression experiments. Enzymatic activities were determined after 5 h in derepression conditions.

[c] Parental.

blots of total DNA from strain PCC 7120. Hybridization was ascribed to an *Xba*I fragment of ca. 5.75 kb from the *Anabaena* genome. A subgenomic gene library from strain PCC 7120 was constructed by cloning *Xba*I restriction fragments of ca. 5 to 6 kb in cloning vector pBluescript SK(+). From this subgenomic library, clones exhibiting hybridization to the 1.5-kb *Sac*I DNA fragment from the strain PCC 7942 *nir* operon were identified, and the plasmid contained in a positive clone was named pCSE2 (Fig. 1A and Table 1) and selected for further studies. To try to extend the cloned region, the *Sca*I-*Xba*I fragment of ca. 1.8 kb from the insert of pCSE2 was used as a probe in homologous hybridization with Southern blots of *Hin*dIII-restricted total DNA from strain PCC 7120. A ca. 8-kb *Hin*dIII-hybridizing fragment was identified and cloned in pBluescript SK(+), producing plasmid pCSE5 (Fig. 1A and Table 1).

By means of heterologous hybridization using restricted plasmid pCSE2 and, as probes, internal fragments from the *nir* and *nrtA* genes from *Synechococcus* sp. strain PCC 7942 (not shown) and by partial sequencing (Fig. 1A), the presence in the cloned genomic region of *Anabaena* sp. strain PCC 7120 of genes corresponding to *nir*, *nrtA*, *nrtB*, and *nrtC* of *Synechococcus* sp., as well as the relative locations of those genes, was determined. Partial DNA sequence analysis indicates that the *Anabaena nir-nrtABC* genes encode proteins that share considerable sequence identity with those from *Synechococcus*. The identity ranged from 40 to 70% (amino acid sequence comparisons were made by using the program GAP contained in the Genetics Computer Group package [8]) within the regions sequenced, which included the entire *nrtA* gene, almost 600 bp of the *nir* gene, about 150 bp of the *nrtB* gene, and about 360 bp of the *nrtC* gene. Recently, Y. Cai and C. P. Wolk (6) have found that *nrtD* and *narB* are also located in this gene cluster, downstream from *nrtC*.

Sequencing of the *Anabaena* genomic DNA upstream of the *nir* gene, cloned in pCSE5, revealed the presence of two open reading frames that would encode polypeptides of 136 (*orf136*) and 398 (*orf398*) amino acids (Fig. 1A and Table 1) (the last 85 nucleotides of *orf398* are not included in pCSE5 but were sequenced from an overlapping clone [not shown]; *orf398* starts with a GTG codon). The putative *orf136* product shows homology to iron-sulfur proteins involved in electron transport (e.g., 30% identity to the cytochrome $b_{6\text{-}f}$ complex iron-sulfur subunit precursor from *Synechocystis* sp. strain PCC 6803). The putative product of *orf398* shows homology to proteins of the CpcE/RpcE/PecE family (e.g., 24% identity to CpcE from *Synechococcus* sp. strain PCC 7002) that comprises enzymes involved in the synthesis of bilins or in the chromophorilation of biliproteins.

Mutants of the *nir* and *nrtA* genes. To test the involvement of the cloned gene cluster in the assimilation of nitrate in *Anabaena* sp. strain PCC 7120, various strains bearing mutations in the above-described gene cluster were generated. Gene cassette C.S3 encoding Sm^r and Sp^r (11) was inserted between the two external *Cla*I sites of the insert of pCSE5, leading to substitution, by gene cassette C.S3, of two *Cla*I DNA fragments containing the entire *nir* gene and part of the *nrtA* gene (see Fig. 1A and Materials and Methods for details). The

TABLE 3. Nitrate uptake in *Anabaena* sp. strain PCC 7120 and *nrtA* mutants[a]

Strain	Nitrate uptake at nitrate concn of:	
	17.6 mM[b]	0.1 mM[c]
PCC 7120	203.6	544.4
CSE9	175.5	49.2
CSE10	84.0	9.5

[a] Ammonium-grown cells of the indicated strain were washed, resuspended in BG11 medium (17.6 mM $NaNO_3$), and incubated for 5 h as indicated in Materials and Methods for derepression experiments. Cells were then harvested and used for nitrate uptake assays (see Materials and Methods). Note that the rates of nitrate uptake in the presence of 17.6 and 0.1 mM $NaNO_3$ should not be compared with each other, since both uptake assays were carried out under different experimental conditions (see Materials and Methods).

[b] Data are expressed as nanomoles of NH_4^+ (produced) per minute per milligram of chlorophyll of cells treated with L-methionine–DL-sulfoximine.

[c] Data are expressed as nanomoles of NO_3^- (disappeared) per minute per milligram of chlorophyll.

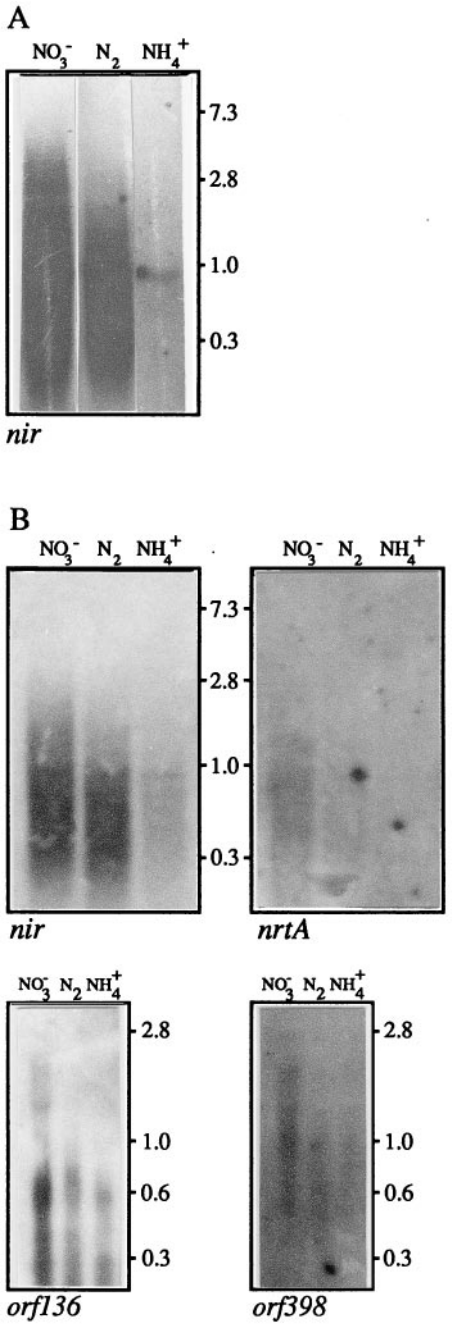

FIG. 2. Expression in *Anabaena* sp. strain PCC 7120, under different nitrogen regimens, of various genes of the nitrate assimilation gene cluster. (A) RNA isolated from cells grown on dinitrogen (N_2), nitrate (NO_3^-), or ammonium (NH_4^+) was used in Northern blot analysis using as a probe a ca. 0.75-kb *Dra*I restriction fragment containing the N-terminal portion and 126 bp upstream of the *nir* gene (see fragment b in Fig. 1). (B) Northern blot analysis of total RNA isolated from cells grown on ammonium (NH_4^+) or grown on ammonium and incubated for 3 h in medium containing nitrate (NO_3^-) or no combined nitrogen (N_2). The probes used were the same ca. 0.75-kb *Dra*I fragment used in panel A (*nir*), a 642-bp *Cla*I restriction fragment internal to the *nrtA* gene (Fig. 1), a PCR-generated DNA fragment comprising nucleotides −46 to +444 with respect to the start of *orf136* and a PCR-generated DNA fragment comprising nucleotides +61 to +454 with respect to the start of *orf398* (fragments c and d in Fig. 1). Sizes are given on the side in kilobases.

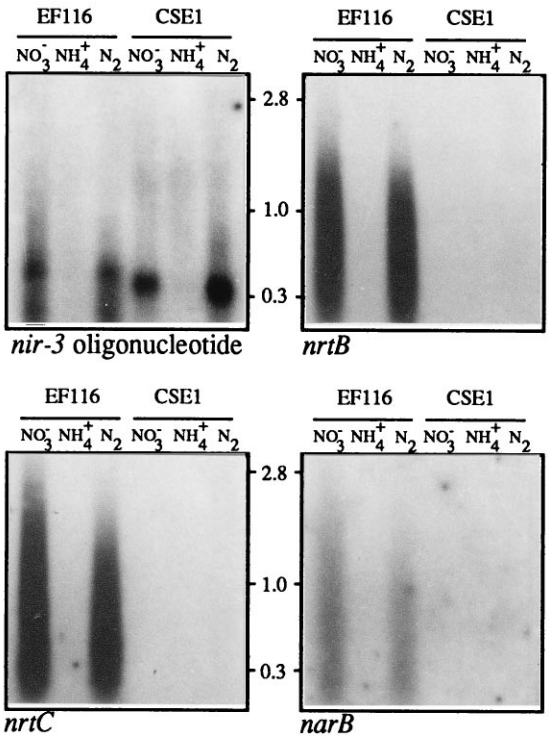

FIG. 3. Expression in strains EF116 and CSE1, under different nitrogen regimens, of different parts of the nitrate assimilation gene cluster. RNA isolated from cells grown on ammonium (NH_4^+) or grown on ammonium and incubated for 3 h in medium containing nitrate (NO_3^-) or no combined nitrogen (N_2) was used in Northern blot analysis. The probes used were oligonucleotide *nir*-3, complementary to nucleotides −288 to −306 with respect to the start of the *nir* gene (see Materials and Methods); a ca. 0.4-kb *Bst*NI restriction fragment internal to the *nrtB* gene; a ca. 0.35-kb *Xba*I-*Hin*dIII restriction fragment internal to the *nrtC* gene (Fig. 1); and a 1,564-bp *Eco*RI-*Acc*I restriction fragment, excised from plasmid pRL822RM2, containing most of the *narB* gene and 18 bp upstream of it (6). Sizes are given on the side in kilobases.

C.S3-bearing insert was transferred to plasmid vector pRL278 (4) that encodes Nm^r and Km^r and contains the *sacB* gene that can be counterselected for in *Anabaena* sp., and the resulting plasmid (pCSE21aB) was transferred to strain EF116 by means of conjugation selecting for Sm^r and Sp^r encoded in the C.S3 gene cassette. Four clones that exhibited Sm^r, Sp^r, resistance to sucrose, and sensitivity to NM (Nm^s) were selected. Southern analysis was performed to test for the presence of wild-type and mutant chromosomes in those clones. Results presented in Fig. 1B show that no wild-type chromosomes could be detected in one of the selected clones (named CSE1) and that in this clone the wild-type version of the *nir-nrtA* region (3.6-kb *Eco*RV fragment) had been substituted by the mutated version (7.6-kb *Eco*RV fragment).

The ability of mutant strain CSE1 to grow using different sources of inorganic nitrogen was tested. As shown in Table 2, while strain EF116 exhibited similar growth rates with either nitrate or ammonium, strain CSE1 proved able to use ammonium but not nitrate as a nitrogen source. Nitrate reductase and nitrite reductase activities were measured in strains EF116 and CSE1 (Table 2). Whereas, as shown previously (29), strain EF116 exhibited high levels of activity of both nitrate reductase and nitrite reductase in medium containing nitrate as the sole nitrogen source and low activity levels in both ammonium-containing medium and medium lacking combined nitrogen, strain CSE1 lacked nitrate reductase and nitrite reductase ac-

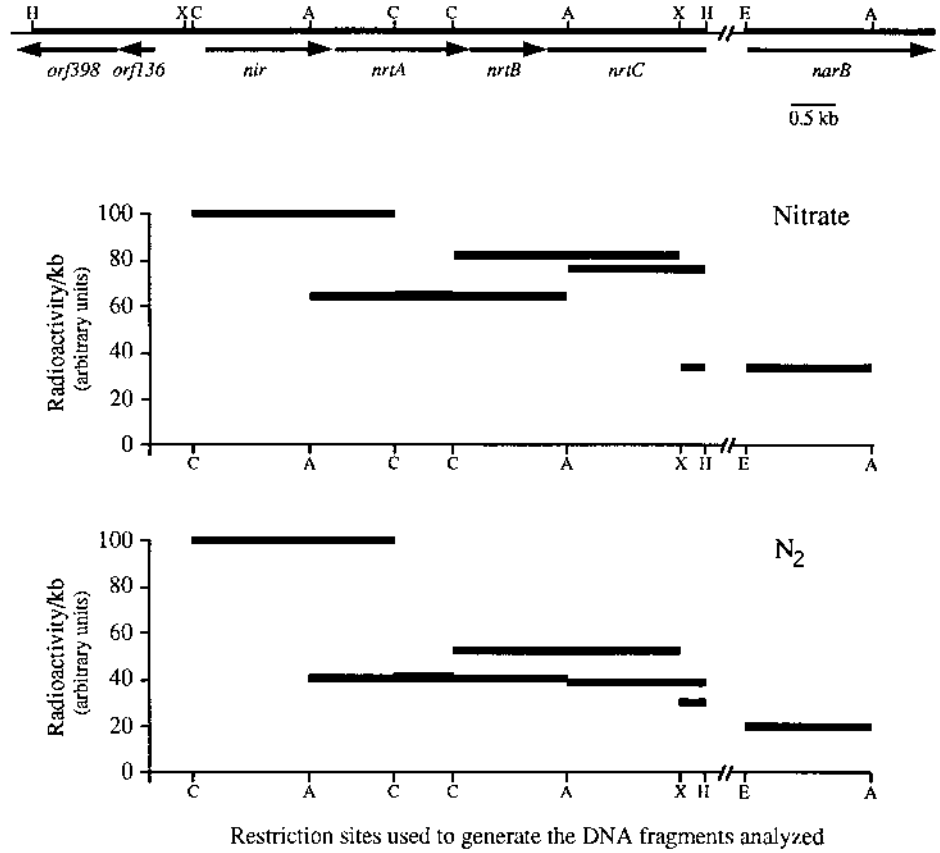

FIG. 4. Levels of expression along the *nir* operon of *Anabaena* sp. strain PCC 7120. The DNA restriction fragments from the *nir* operon represented in the figure by thick segments were excised with the indicated enzymes from plasmid pCSE21 (for fragments of the *nir* or *nrt* gene [see Table 1]) or pRL822RM2 (for the fragment of the *narB* gene [6]) and were subjected to electrophoresis on agarose gels and blotted to filters. Total RNA from ammonium-grown cells incubated for 3 h in medium containing nitrate or no combined nitrogen (N_2) was used as a probe for hybridization (see Materials and Methods for details). The amount of radioactivity in each fragment was normalized to 1 kb (dividing by the number of kilobases of the fragment). The amount of radioactivity associated with each fragment is expressed as a percentage of the radioactivity associated with the fragment showing maximum labelling in each of the two parts of the figure (100% corresponded to different values in the experiment with RNA from nitrate-incubated cells and in that with RNA from no-combined-nitrogen-incubated cells). A, *Acc*I; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; X, *Xba*I.

tivities under any of these growth conditions. These results indicate that in *Anabaena* sp. strain PCC 7120 the function of the *nir* gene is required for the assimilation of nitrate as well as for expression of nitrite reductase (for the role of *nrtA* in nitrate assimilation in strain PCC 7120, see below). The fact that mutagenesis of the *nir* gene with a gene cassette (C.S3) bearing transcriptional terminators (34) resulted in a lack of expression of nitrate reductase points to the nitrate assimilation gene cluster behaving as an operon in this cyanobacterium.

Insertional mutants of the *nrtA* gene were generated from *Anabaena* sp. strain PCC 7120 by means of conjugation of this strain with *E. coli* strains bearing plasmid pCSE44B or pCSE45B (see Table 1). These are Sm^r Sp^r, SacB-encoding plasmids whose vector portion is pRL277 (4) and whose inserts comprise that of pCSE5 modified by insertion of gene cassette C.K1 (which does not include transcriptional terminators), in either of the two orientations, into the *Eco*RV site located inside the *nrtA* gene (see Fig. 1A and Materials and Methods for details). After selection for *Anabaena* strains exhibiting Nm^r (encoded in the C.K1 gene cassette), resistance to sucrose, and Sm^s and Sp^s, two clones that, in Southern analysis, did not show the presence of wild-type chromosomes (2.3-kb *Cla*I fragment) and showed the mutated version (3.6-kb *Cla*I fragment) of the *nrtA* gene were selected (Fig. 1B). These clones were named CSE9 and CSE10, which should bear the Nm^r-encoding gene in the same orientation as the *nrtA* gene and in the opposite orientation, respectively.

Both CSE9 and CSE10 were able to grow in media containing ammonium, nitrate, or no combined nitrogen, although at rates lower than those of parental strain PCC 7120 (Table 2). In contrast to the situation with strain PCC 7120, which develops heterocysts only in the absence of combined nitrogen, both strains CSE9 and CSE10 developed heterocysts in media supplemented with nitrate (1 or 17.6 mM). Nitrate uptake, nitrate reductase, and nitrite reductase activities were determined in mutants CSE9 and CSE10 and compared to those of parental strain PCC 7120. As shown in Table 2, both CSE9 and CSE10 exhibited nitrate reductase and nitrite reductase activities comparable to those of strain PCC 7120. As shown in Table 3, both mutants were drastically impaired in uptake of low (0.1 mM) but not of high (17.6 mM) concentrations of nitrate. These results showing that strains CSE9 and CSE10 were impaired in nitrate uptake at 0.1 mM nitrate concentration indicate that these mutants are affected in the active transport of nitrate and thus that the *nrtA* gene is required for active transport of nitrate in strain PCC 7120. On the other hand, the fact that strains CSE9 and CSE10 showed substantial rates of nitrate

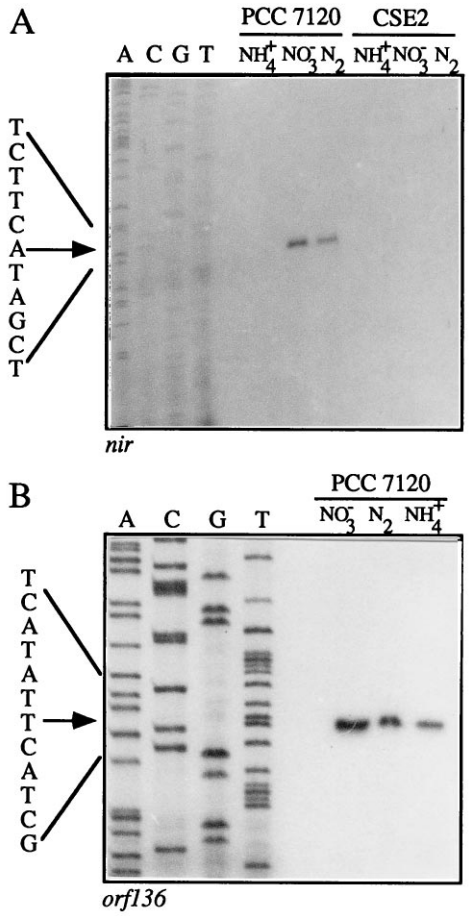

FIG. 5. Presumed transcription start points of *nir* and *orf136*. Primer extension experiments were performed with total RNA from the indicated strain grown on ammonium (NH_4^+) or grown on ammonium and incubated for 3 h in medium containing nitrate (NO_3^-) or no combined nitrogen (N_2). The oligonucleotides used were *nir*-1 (A) and *nir*-14 (B) (see Materials and Methods). Lanes A, C, G, and T contain a dideoxy sequencing ladder of the same DNA region, used as a size marker of the extension products. The nucleotide sequence around the end of each extension product (indicated by a horizontal arrow) is shown at the left.

uptake at high concentrations of nitrate indicates that, in *Anabaena* sp. strain PCC 7120, the *nrtA* gene is dispensable for the uptake of high concentrations of nitrate. As has been suggested for *Synechococcus* sp. strain PCC 7942 (33), nitrate could be taken up substantially in a passive way at those high concentrations of nitrate. Alternatively, a low-affinity nitrate transport system independent of the *nrtA* gene might be expressed in *Anabaena* sp. strain PCC 7120. Anyway, results in Table 3 show that strains CSE9 and CSE10 are to some extent also affected in the uptake of nitrate at 17.6 mM concentration. This, together with the fact that both CSE9 and CSE10 exhibited heterocysts when growing in nitrate-supplemented media, which is indicative of a shortage of nitrogen in the cells, points to a role of the active transport system in the total uptake of nitrate and in the growth at the expense of this nutrient not only at low but also at high concentrations of nitrate.

Mutants of *orf136* and *orf398*. To generate insertional mutants of *orf136* and *orf398*, plasmids pCSE78B and pCSE81B, respectively, were transferred to *Anabaena* sp. strain PCC 7120 by means of conjugation (see Table 1 and Materials and Methods). Plasmids pCSE78B and pCSE81B were constructed by insertion, in plasmid pCSE26, of gene cassette C.S3 into the *Bgl*I site found inside *orf136* and into the *Pvu*II site located inside *orf398*, respectively (Fig. 1A). After isolation of *Anabaena* strains exhibiting Sm^r and Sp^r (encoded in the C.S3 gene cassette) and resistance to sucrose, one clone mutated in *orf136* (strain CSE16) and one clone mutated in *orf398* (strain CSE17) that in Southern analysis did not show tracks of wild-type chromosomes were selected. In both strain CSE16 and strain CSE17, the 2.5-kb *Cla*I fragment that in the wild-type strain comprises *orf136* and *orf398* was substituted by the C.S3-containing *Cla*I fragment of 4.5 kb (Fig. 1B).

Strains CSE16 and CSE17 were able to grow using nitrate as the nitrogen source (not shown) and exhibited high levels of nitrate reductase and nitrite reductase (although in strain CSE17 nitrite reductase levels seem to be somewhat lower than in the wild-type strain) when incubated in the presence of nitrate, with both activities being repressed by ammonium, as it is the case in the wild-type strain (Table 2). Thus, at least under our laboratory growth conditions, both *orf136* and *orf398* seem to be dispensable for the assimilation of nitrate in *Anabaena* sp. strain PCC 7120.

Analysis of gene expression. Expression of the *Anabaena* sp. strain PCC 7120 gene cluster described above was studied by means of Northern and primer extension experiments using total RNA from cells of parental and mutant strains incubated under various nitrogen regimens. Northern analyses were performed with total RNA isolated from cells of *Anabaena* sp. strain PCC 7120 grown on ammonium, nitrate, or dinitrogen (Fig. 2A) or grown on ammonium and either not further incubated or incubated for 3 h with nitrate or no combined nitrogen (Fig. 2B) and with RNA of strains EF116 and CSE1 grown on ammonium and either not further incubated or incubated for 3 h with nitrate or no combined nitrogen (Fig. 3). The probes used included fragments of the *nir*, *nrtA*, *nrtB*, *nrtC*, and *narB* genes and an oligonucleotide (oligonucleotide *nir*-3 [see Materials and Methods]) complementary to a transcribed (see below) DNA sequence located upstream of the coding region of the *nir* gene. Strong hybridization signals were obtained, with the different probes, with RNA isolated from cells of strain PCC 7120 or EF116 incubated with nitrate or with no combined nitrogen but not with RNA from cells grown on ammonium. When RNA from strain CSE1 was used, a hybridization signal was observed only when RNA isolated from cells incubated with nitrate or no combined nitrogen was probed with the oligonucleotide of the *nir* upstream region.

The facts that similar hybridization signals were observed with RNA from strain PCC 7120 or EF116 by using probes corresponding to the *nir* gene and to genes downstream of it in the cluster and, principally, that insertion of C.S3 in *nir-nrtA* abolished expression of downstream genes (*nrtB*, *nrtC*, and *narB*) strongly suggest that the *nir*, *nrtABCD*, and *narB* genes of *Anabaena* sp. strain PCC 7120 behave as an operon. The RNA representing the message from the whole operon should be longer than 6 kb (the distance from the *tsp* [see below] to the 3′ end of the fragment of the *nrtC* gene contained in plasmid pCSE5 [Fig. 1A]). The fact that the hybridization signal detected was a smear of RNAs from 7.5 to 0.3 kb in size is probably due to low stability of the whole mRNA of the operon, as has been suggested for the *nir* operon mRNA of *Synechococcus* sp. strain PCC 7942 (27, 39).

Northern experiments were also performed with probes of *orf136* and *orf398*. Both probes hybridized to RNAs of similar sizes. This would be consistent with cotranscription of both open reading frames, whose expression seems to take place both in the presence and in the absence of ammonium, being highest in the medium containing nitrate.

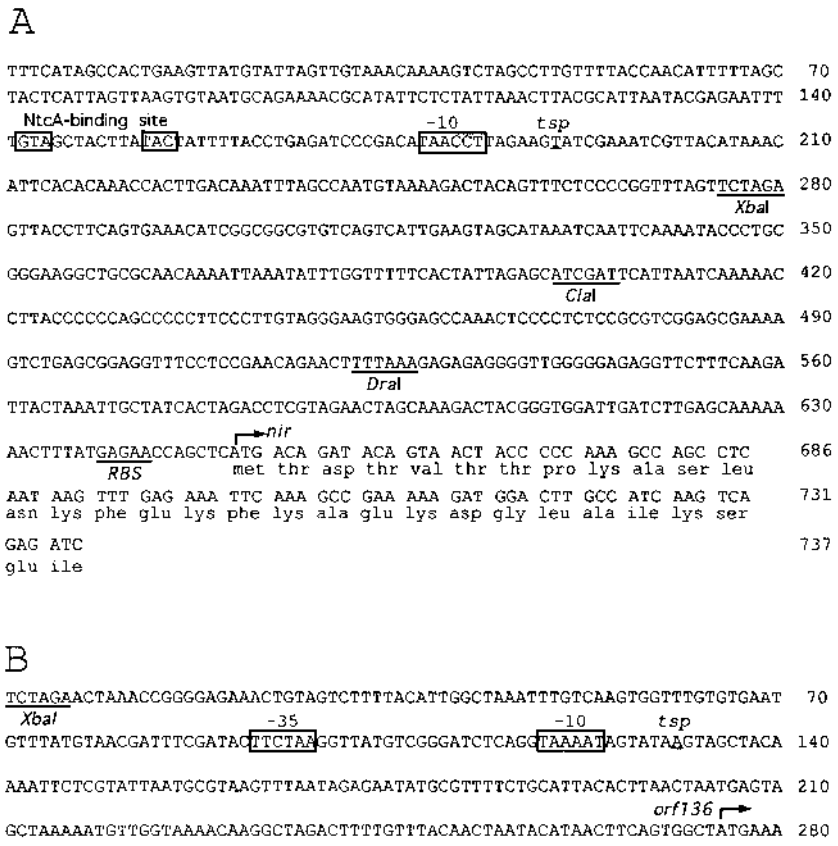

FIG. 6. DNA sequences upstream of the *nir* gene. (A) The first codon of the *nir* gene (whose 31 N-terminal amino acids are shown) is represented by a horizontal arrow. A putative ribosome binding site (*RBS*) is underlined. The position of a presumed transcription initiation site of the *nir* gene (*tsp*, underlined), as well as the putative −10 box and an NtcA-binding site, is also indicated. (B) The start of *orf136* is represented by an horizontal arrow. A presumed transcription initiation site for *orf136* (*tsp*, underlined), as well as putative −10 and −35 promoter sequences, is also indicated. Note that the nucleotide sequence shown in panel B is the antiparallel sequence of the first 280 nucleotides presented in panel A.

Expression along the *nir* operon of *Anabaena* sp. strain PCC 7120 was studied by means of hybridization of filter-bound DNA from various cloned fragments of the nitrate assimilation gene cluster with ^{32}P-labelled total RNA from wild-type strain PCC 7120 (see Materials and Methods for details). RNA was isolated from ammonium-grown cells that had been incubated for 3 h with nitrate or with no combined nitrogen. In both cases, the strongest hybridization signal was found with the DNA fragment that contained the *nir* gene (Fig. 4) and the weakest hybridization signal was found with the *narB*-containing fragment located at the 3′ end of the operon (about 33% of the 5′-end value with RNA from nitrate-incubated cells and 19% with RNA from no-combined-nitrogen-incubated cells). Intermediate hybridization signals were observed with the DNA fragments covering the three analyzed nitrate transport genes *nrtABC* (65 to 85% of the 5′-end value with RNA from nitrate-incubated cells and 35 to 55% with RNA from no-combined-nitrogen-incubated cells). Thus, the 5′ end of the mRNA of the *nir* operon seems to be more abundant than its 3′ end in cells incubated with either nitrate or no source of combined nitrogen.

Transcription start points. Primer extension experiments were carried out using total RNA from *Anabaena* sp. strain PCC 7120 cells grown on ammonium and either not further incubated or incubated for 3 h in the presence of nitrate or of no combined nitrogen. Oligonucleotides complementary to the 5′ end of the *nir* gene or to sequences upstream of it (see Materials and Methods for details) were used as primers. As shown in Fig. 5A, an extension product whose 3′ end corresponded to nucleotide −460 with respect to the first nucleotide of the *nir* gene was obtained with oligonucleotide *nir*-1 and RNA from cells that had been incubated in medium containing either nitrate or no combined nitrogen but not with RNA from ammonium-grown cells.

As described in the introduction, the NtcA protein is a transcriptional regulator required for the expression of ammonium-repressed genes in cyanobacteria. We have previously shown that in strain PCC 7120 expression of the *nir* gene requires the function of the NtcA protein, since no RNA hybridizing to a probe of the *nir* gene could be detected in a mutant, strain CSE2, that bears a gene cassette inserted into the *ntcA* gene (17). Results shown in Fig. 5A indicate that, in fact, the ammonium-regulated, presumed transcription initiation site of the *nir* operon was not used in the *ntcA* mutant, strain CSE2. The structure of the NtcA-activated promoters has been determined in the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942 (27). They are constituted by a TAN_3T sequence fitting the −10 box of the *E. coli* σ^{70} consensus promoter and an NtcA-binding site including the sequence $GTAN_8TAC$ located ca. 22 nucleotides upstream from the TAN_3T box. As shown in Fig. 6A, the DNA region upstream of the ammonium-regulated, presumed transcription initiation site of the *nir* operon indeed exhibits the structure of an NtcA-activated promoter.

For *orf136*, primer extension experiments using as a primer oligonucleotide *nir*-12 or *nir*-14 (see Materials and Methods) were carried out. An extension product whose 3′ end corresponded to nucleotide −144 with respect to the first nucleotide of *orf136* was obtained when oligonucleotide *nir*-14 was used (Fig. 5B). The template RNA was present in cells incubated with nitrate, no combined nitrogen, or ammonium, although it appeared to be more abundant in cells incubated with nitrate than with no combined nitrogen and in no-combined-nitrogen-incubated than in ammonium-grown cells. Upstream from this presumed transcription initiation site, sequences, 5′TAAAA T3′ and 5′TTCTAA3′, that could conform, respectively, to the −10 and −35 boxes of the *E. coli* σ^{70} consensus promoter are found (Fig. 6B).

To test the existence in the *nir* operon of internal promoters different from the NtcA-regulated promoter above described, one insertional mutant of the leader region of that operon was generated. Plasmid pCSE88B (Table 1) bearing gene cassette C.S3 (which carries transcriptional terminators in both orientations [34]) inserted into the *Cla*I restriction site located in the leader region of the *nir* operon (Fig. 1A) was transferred to strain EF116 by means of conjugation (see Materials and Methods). Strains exhibiting Sm^r, Sp^r (encoded in the C.S3 gene cassette), and resistance to sucrose were selected. The presence in these strains of wild-type copies of the *nir* region, as well as their genetic structure in that region, was tested by PCR amplification (not shown). One clone (strain CSE18) that did not show tracks of wild-type chromosomes and showed evidence of insertion of gene cassette C.S3 in the leader region of the *nir* operon was chosen for further analysis. Strain CSE18 was unable to grow on nitrate (not shown) and exhibited negligible levels of both nitrate reductase and nitrite reductase activities in ammonium-grown as well as in nitrate- or no-combined-nitrogen-incubated cells (Table 2). The results presented are best interpreted in terms of a lack of promoter activity, at least under our experimental conditions, of DNA sequences of the *nir* gene cluster downstream from the *Cla*I restriction site where C.S3 is inserted in strain CSE18.

Concluding remarks. Results presented in this work, together with those of Cai and Wolk (6), demonstrate the presence in the genome of the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120 of a cluster of genes encoding proteins for nitrate assimilation. These genes are *nir* (encoding nitrite reductase), *nrtABCD* (encoding a nitrate permease), and *narB* (encoding the nitrate reductase apoenzyme). This cluster of genes is similar to that found in the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942, but the arrangement of nitrate assimilation genes in some other cyanobacteria is different (see the introduction).

As indicated by the effects, on expression of distal genes, of insertion of gene cassettes in the 5′ region of the cluster, the nitrate assimilation gene cluster of strain PCC 7120 behaves as an operon. The levels of mRNA of the 5′ end of the cluster (the *nir* gene) are higher than those of the 3′ end (the *narB* gene). This could be accounted for by partial premature termination of transcription of the operon or by differential degradation of the two ends of the mRNA. Anyway, the observed lower levels of the 3′ end of the mRNA of the cluster are consistent with the low content of nitrate reductase protein usually found in cyanobacteria.

Expression of the *nir* operon is subjected to ammonium-promoted repression and requires the action of the transcriptional regulator NtcA. The presumed transcription initiation site of the *nir* operon is located 460 bp upstream of the start of *nir*, the first gene in the operon, and is preceded by an NtcA-type promoter. It can be concluded that in *Anabaena* sp. strain PCC 7120 expression of the *nir* operon upon ammonium withdrawal takes place from an NtcA-regulated promoter and thus that the *nir* operon comprising structural genes for nitrate and nitrite assimilation belongs to the NtcA regulon.

We have repeatedly observed that expression of high nitrate reductase and nitrite reductase levels in heterocyst-forming cyanobacteria requires, in addition to an absence of ammonium, the presence of either nitrate or nitrite in the culture medium (14) (Table 2). Levels of the mRNA of the *nir* operon in *Anabaena* sp. strain PCC 7120 also seem to be higher in nitrate-supplemented media than in media lacking a source of combined nitrogen. Utilization of the *nir* operon *tsp* could be stimulated, and/or stability of the *nir* operon mRNA could be enhanced, by nitrate.

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