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Nitrogen Deprivation of *Anabaena* sp. Strain PCC 7120 Elicits Rapid Activation of a Gene Cluster That Is Essential for Uptake and Utilization of Nitrate

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A transposon bearing *luxAB*, encoding luciferase, as a reporter of transcription was used to identify genes that are activated rapidly upon deprivation of *Anabaena* sp. strain PCC 7120 of fixed nitrogen. The three transposon-marked loci that were identified as responding most rapidly and strongly are closely linked and situated within *nirA* and *nrtC* and between *nrtD* and *narB*, genes whose products are responsible for uptake and reduction of NO_2^- and NO_3^-. A strain bearing a transcriptional fusion of *narB* to *luxAB* was constructed. Luminescence catalyzed by LuxAB was used to report on the expression of the interrupted genes. Whether these genes are regulated only coordinately is discussed.

Cyanobacteria are ecologically and geographically diverse photosynthetic prokaryotes that are principal components of the food chain in many aquatic ecosystems. Their similarities to chloroplasts of eukaryotic plants, including their use of H_2O as a photosynthetic electron donor and similarities identified by sequencing, have led to the conclusion that cyanobacteria were the evolutionary predecessors of chloroplasts (10, 18). Nitrate, often the most abundant source of fixed nitrogen available for assimilation into microorganisms and plants, is reduced to nitrite, which in plants is further reduced to ammonium within chloroplasts (16). The uptake and utilization of nitrate by the nondiazotrophic, unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942 have been well studied (16, 37). Nitrate and its catabolite, nitrite, are actively transported into cells by a membrane transporter complex that is encoded by the gene cluster *nrtABCD* (28, 30, 38, 39). This cluster is flanked at its 5′ end by the gene *nirA*, which encodes a ferredoxin-dependent nitrite reductase (29, 44), and at its 3′ end by the gene *narB*, which encodes a ferredoxin-dependent nitrate reductase (3, 41). The *nirA-nrtABCD-narB* sequence is transcribed as a single operon, whose expression is regulated by the availability of nitrogen (38, 44). Luque et al. (28) interpreted the observation that insertional mutants of *nirA* showed basal levels of nitrate reductase activity as indicating that *narB* can be driven to a limited extent by one or more constitutive promoters present within this cluster.

Even cyanobacteria that can fix dinitrogen use fixed nitrogen preferentially, perhaps because of the metabolic expense of maintaining a microaerobic environment for nitrogenase (13, 17). *Anabaena* sp. strain PCC 7120, a filamentous cyanobacterium that can fix N_2 in differentiated cells called heterocysts, has become a model organism for studies of prokaryotic cellular differentiation and pattern formation (8, 50). We have used derivatives of transposon Tn*5* that contain *luxAB* as a transcriptional reporter to identify genetic loci that are transcriptionally activated in response to nitrogen deprivation (49). Three of the mutants isolated bear a transposon within the *nirA-nrtABCD-narB* gene cluster that is required for nitrate uptake and utilization. We have studied certain aspects of the transcriptional regulation of this cluster of genes.

MATERIALS AND METHODS

Anabaena sp. strain PCC 7120 and derivatives of it were grown under the conditions described by Hu et al. (23). Mutants were grown in the presence of neomycin sulfate (Sigma Chemical Co., St. Louis, Mo.) at 20 μg ml^{-1} in liquid medium supplemented with nitrate or nitrite or at 10 μg ml^{-1} in the absence of those nitrogen sources and at 200 μg ml^{-1} in agar-solidified medium without supplemental nitrogen or at 400 μg ml^{-1} in such medium with supplemental nitrogen. Plasmid pRL1472a, which provided an endogenous source of aldehyde as substrate for measurements of luciferase activity in the presence of different nitrogen sources and for photon-counting microscopy (14), was selected with 5 μg of erythromycin ml^{-1}. Fixed nitrogen was added, where specified, as 2 mM ammonium chloride plus 5 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer, pH 7.5, or as equimolar concentrations of the Na^+ and K^+ salts of nitrate (unless otherwise noted, totaling 5 mM in liquid or 10 mM in agar) or nitrite (totaling 2.5 mM in liquid or 5 mM in agar). Petri dishes with NH_4^+-containing agar were prepared within 2 days of use. Cells in NH_4^+-containing medium were sedimented and resuspended in fresh medium no less frequently than once every 2 days, and filters bearing cells atop NH_4^+-containing agar were equally frequently transferred to fresh petri dishes. L-Methionine-DL-sulfoximine (MSX) was used at 5 μM. Nitrate reductase was measured by the method of Herrero et al. (20, 22).

DNA was manipulated by standard procedures (31). Suicide plasmid pRL796 was constructed for site-directed interruption and transcriptional reporting of the *narB* gene. To make this plasmid, the 0.9-kb *Hpa*I-*Mun*I internal fragment of *narB* (see Fig. 1 and 2) was fused at its *Mun*I end to an *Eco*RI-*Sst*I fragment from the polylinker of pUC19 (52) and the resulting fragment was cloned between the *Sma*I and *Sst*I sites of pRL561 (12). Plasmids were transferred to *Anabaena* cells by triparental mating (11). Transposon Tn*5*-765 (in plasmid pRL765; GenBank accession number U55819) was constructed by addition of a *Bam*HI fragment from pRL488 (12) containing *Vibrio fischeri luxAB* to the *Bam*HI site of transposon Tn*5*-764 in plasmid pRL764SX. That plasmid differs from pRL1058 (49) in the following two ways: (i) deletion of a *Sma*I fragment within the transposon eliminates the bleomycin resistance and streptomycin resistance determinants, reduces the length of the transposon, and leaves, in addition to a unique *Xba*I site, an adjacent unique *Bam*HI site for the insertion of reporter genes; and (ii) elimination of a *Sma*I site from a polylinker in the *oriT* region of the plasmid permits facile replacement of the kanamycin resistance determinant between *Xmn*I and *Sma*I sites with an alternative selective marker. Mutants generated upon transfer of Tn*5*-765 to *Anabaena* sp. strain PCC 7120 were screened (49) for rapid activation of luminescence in response to nitrogen stepdown. Samples for pulsed-field gel electrophoresis were prepared and gels were run as described by Kuritz et al. (24).

Changes of transcriptional activity upon transfer of cells between different media were measured by quantitating the luminescence of homogeneous spots of cells (7, 49). The results presented are averages from three or four independent experiments. The luciferase activities of individual filaments were recorded as described by Elhai and Wolk (12).

Nucleotide sequence accession numbers. The nucleotide sequences of *nirA* and *narB* were obtained by automated sequencing (Applied Biosystems, Inc., Foster City, Calif.) of overlapping fragments from both strands of DNA of

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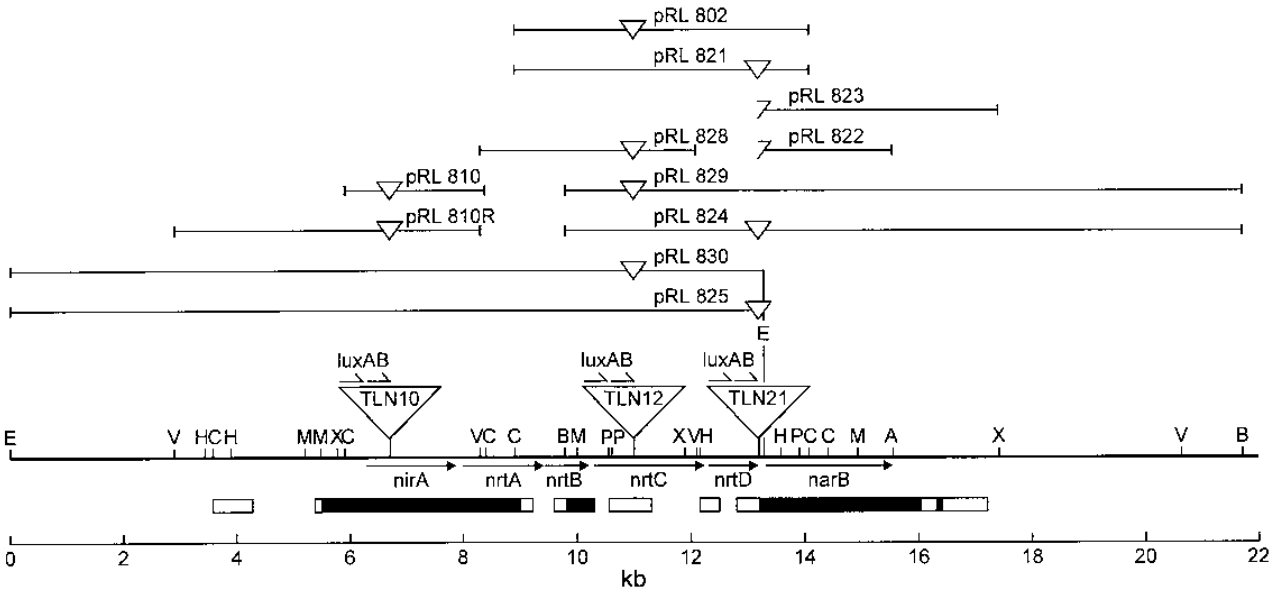

FIG. 1. Maps of the chromosomal region (thick line) bearing insertions of Tn*5*-765 in strains TLN10, TLN12, and TLN21 and of plasmids derived from that region. All sites for *Bsp*1407I (B), *Cla*I (C), *Eco*RI (E), *Eco*RV (V), *Hin*dIII (H), *Hpa*I (P), *Mun*I (M), and *Xba*I (X) are shown for the region from 2.9 to 17.4 kb. Plasmid pRL822 was recovered with *Ase*I (A); plasmids pRL824 and pRL829 were recovered with *Bsp*1407I; plasmids pRL802, pRL810, and pRL821 were recovered with *Cla*I; plasmids pRL825 and pRL830 were recovered with *Eco*RI; plasmids pRL810R and pRL828 were recovered with *Eco*RV; and plasmid pRL823 was recovered with *Xba*I. Each site of insertion of Tn*5*-765 is represented by a triangle; the L end of the transposon that bears *luxAB* as reporter is to the left in each case. Regions sequenced on only one strand are shown as open boxes, and regions with complete, overlapping sequencing on both strands are shown as solid boxes. *nirA* and *narB* genes encode nitrite reductase and nitrate reductase, respectively. *nrtA*, *nrtB*, *nrtC*, and *nrtD* are genes that encode nitrate- and nitrite-permease proteins identified and here positioned on the basis of similarities of deduced partial amino acid sequences with corresponding genes from *Synechococcus* sp. strain PCC 7942 (38, 39).

transposon-bearing plasmids (see Fig. 1) and have been submitted to GenBank under accession numbers U61496 and L49163, respectively.

RESULTS

Forty-six independent colonies derived from transposition of Tn*5*-765 appeared initially to show increased luminescence when transferred on a filter from solid medium containing NH_4^+ to the same medium lacking a source of fixed nitrogen. Of these, seven gave rise to strains (TLN9, -10, -12, -14, -17, -21, and -38) that consistently showed rapid induction of luminescence. TLN17, which showed much lower luminescence upon induction than did the others, was not further studied. Upon transfer from NH_4^+ to N_2 (see below), TLN9 showed 2.7-, 5.5-, 8.0-, 8.7-, and 9.4-fold induction in 1, 2, 3, 4.5, and 6 h; TLN14 showed 8.8-, 17.8-, 24.8-, 28.3-, and 27.8-fold induction during the same intervals; and TLN38 showed 5.9-, 9.3-, 10.3-, 7.0-, and 5.2-fold induction during the same intervals (the results for TLN9, TLN14, and TLN38 are in each case averages from four experiments). The transposon was excised from TLN14 with *Cla*I and *Eco*RV separately. The resulting plasmids (pRL826 and pRL827, respectively) were sequenced outwards from the transposon on one strand, and an open reading frame was identified. Upon BLAST searching (1), it was found that the intercepted genetic sequence appeared to encode a protein which, over a stretch of 120 amino acids, showed approximately 41% identity (58% similarity) to the *Escherichia coli* regulatory protein KdpE (48) (see Fig. 3e). The chromosomal insertion in TLN14 was localized tentatively by pulsed-field gel electrophoresis. Upon cleavage with *Bln*I (an isoschizomer of *Avr*II) plus *Sph*I, band AvrA was replaced with two bands, one about 20 kb larger than AvrH and one of about 254 kb; the latter was labeled upon hybridization with a fragment from the right end of the transposon. After digestion with *Pst*I and hybridization, a band of approximately equal size was labeled and a new band of ca. 170 kb was observed. Tn*5*-765 lacks sites for *Sal*I; hybridization to SalA was observed.

Because of the much greater induction of luciferase activity in TLN10, TLN12, and TLN21 upon transfer of those strains from NH_4^+ to N_2 (see below) and the clustering of the corresponding sites of transposon insertion (see below), the remainder of our experimentation was restricted to those three strains and to a fourth, related mutant. The chromosomal insertion in TLN10 was localized by pulsed-field gel electrophoresis as follows: upon cleavage with a combination of *Bln*I plus *Sph*I, the AvrE band was cut to yield fragments of ca. 358 and 146 kb, with the latter labeled in a Southern blot upon hybridization with a DNA fragment from the right end of Tn*5*-765. Fragment SalD was labeled. Although electrophoresis should have resolved any fragment of 800 kb or less, the *Pst*I digest resembled that of DNA from wild-type *Anabaena* sp. strain PCC 7120. Digestion of DNA from TLN21 with *Bln*I plus *Sph*I gave rise to new bands of ca. 365 and 141 kb.

Tn*5*-765 was excised from strain TLN10 with *Cla*I and *Eco*RV; from strain TLN12 with those two enzymes, *Eco*RI, and *Bsp*1407I; and from TLN21 with *Bsp*1407I, *Cla*I, *Eco*RI, and (excluding the left end of the transposon) *Ase*I and *Xba*I. Restriction mapping indicated that the resulting plasmids overlapped extensively (Fig. 1). Sequencing of portions of the recovered plasmids (open and closed boxes in Fig. 1) and comparison with the amino acid sequences of the proteins encoded by the *nirA-nrtABCD-narB* region of the chromosome of *Synechococcus* sp. strain PCC 7942 (3, 29, 36, 39, 44) (Fig. 2, 3a through d, 4, and 5) suggested that (i) a sequence of genes that corresponds closely to the series in *Synechococcus* sp. strain PCC 7942 is present in *Anabaena* sp. strain PCC 7120 and (ii) the transposons in TLN10, TLN12, and TLN21 had inserted within *nirA*, within *nrtC*, and between *nrtD* and *narB*, respectively. Figure 4 also compares the predicted sequence of the

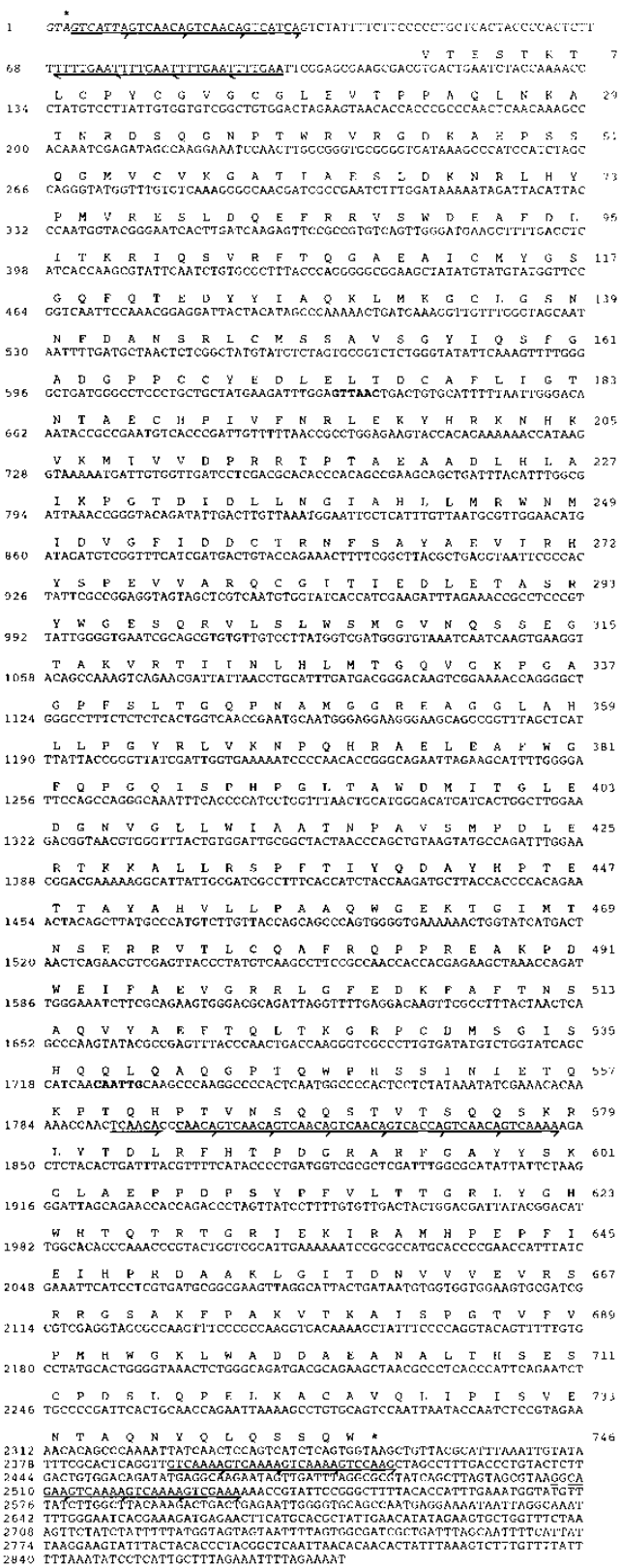

FIG. 2. Nucleotide sequence of *narB* and contiguous DNA and amino acid sequence of NarB by translation. The first 9 bp shown constitute the sequence repeated upon insertion of transposon Tn*5*-765 in mutant TLN21 and include the trinucleotide TAG that by comparison of the product of translation of the approximate 5′ sequence (determined by sequencing of a single DNA strand) with the sequence of NrtD of *Synechococcus* sp. strain PCC 7942 (Fig. 3d) represents the termination (*) codon of PCC 7120 *nrtD*. The *Hpa*I site (GTTAAC) and *Mun*I site (CAATTG) used for insertional mutagenesis are in bold. The following five series of direct repeats are shown (doubly underlined except for singly underlined variant nucleotides): four repeats of GTCAACA, four repeats of TTTTGAA, eight more repeats of GTCAACA, and two sets of four repeats of GTCAAAA.

NirA protein from *Anabaena* sp. strain PCC 7120 with all or portions of the sequences of nitrite reductases from certain other organisms and with portions of the predicted sequence of a bacterial sulfite reductase. Figure 2 presents the complete sequence of *narB*; as indicated in that figure legend, the non-anucleotide sequence duplicated upon insertion of the transposon in TLN21 appears to contain the stop codon of *nrtD*. The predicted amino acid sequence of NarB from PCC 7120 is compared with corresponding sequences from *Synechococcus* sp. strain PCC 7942 and from other sources in Fig. 5. Because the *nirA-nrtABCD* series of genes from PCC 7120 had already been cloned and were being characterized (15, 16), we sequenced the entirety of only *nirA* and *narB*.

Mutant TLN10 did not grow consistently and was at best sickly on either NO_2^- or NO_3^-. Mutant TLN12 grew healthily on NO_2^- and NO_3^- and showed a heterocyst frequency approximating that of the N_2-grown wild-type strain in the presence of up to 80 mM NO_3^-. Mutant TLN21 grew healthily in the presence of 5 mM NO_3^-, forming no heterocysts in older cultures and only occasional heterocysts, e.g., one or two per filament of at least 50 cells, in young, dilute cultures and thus evidently expressing limited nitrate reductase activity. Single-crossover recombination of pRL796 with the chromosome of PCC 7120 produced strain SR796, which lacked an intact copy of *narB*, as established by Southern hybridization (data not presented). SR796 grew healthily on NO_2^- and NO_3^-; heterocysts did not form on NO_2^- but formed abundantly on NO_3^-. Whereas the means of several measurements of nitrate reductase activity of permeabilized cells of wild-type *Anabaena* sp. strain PCC 7120 grown on NO_3^- and N_2 were 0.74 ± 0.24 and 0.55 ± 0.04 nmol of NO_2^- produced μg of chlorophyll a^{-1} min^{-1}, respectively, the corresponding mean activity of SR796 grown in the presence or absence of nitrate and presence or absence of 10 μg of neomycin sulfate ml^{-1} was maximally about 1% as great and probably not significantly different from zero. When grown on NH_4^+ as the sole nitrogen source and at low cell density (Table 1), TLN10, TLN21, and SR796 (all bearing pRL1472a) showed low levels of luminescence (Table 1). The values increased greatly during growth on N_2 and to a lesser extent in the presence of NO_2^- or NO_3^- (Table 1). The activity of LuxAB in vegetative cells of N_2-grown TLN10 and much more faintly in N_2-grown SR796 was visualizable by photon-counting microscopy; varied spatial gradients of luminescence were observed along the filaments (Fig. 6).

Mutants TLN10, TLN12, TLN21, and SR796 responded to transfer from NH_4^+ to N_2 with increased expression of *luxAB* within 0.5 h (Fig. 7a and b). The expression of *luxAB* increased the most rapidly initially (with some variation at 1 h; compare Fig. 7a and b) and over the 6-h duration of the experiments monotonically in TLN10, the expression of *luxAB* by TLN12 and TLN21 increased only a bit more slowly initially but peaked and decreased after 2 to 3 h, and the expression of *luxAB* by SR796 increased the most slowly and then also decreased. Blockage of assimilation of NH_4^+ by addition of MSX in the continued presence of NH_4^+ led to qualitatively similar but quantitatively less pronounced activation of *luxAB* expression (Fig. 7d). The expression of *luxAB* by TLN10, TLN21, and SR796 decreased with modestly differing time courses upon

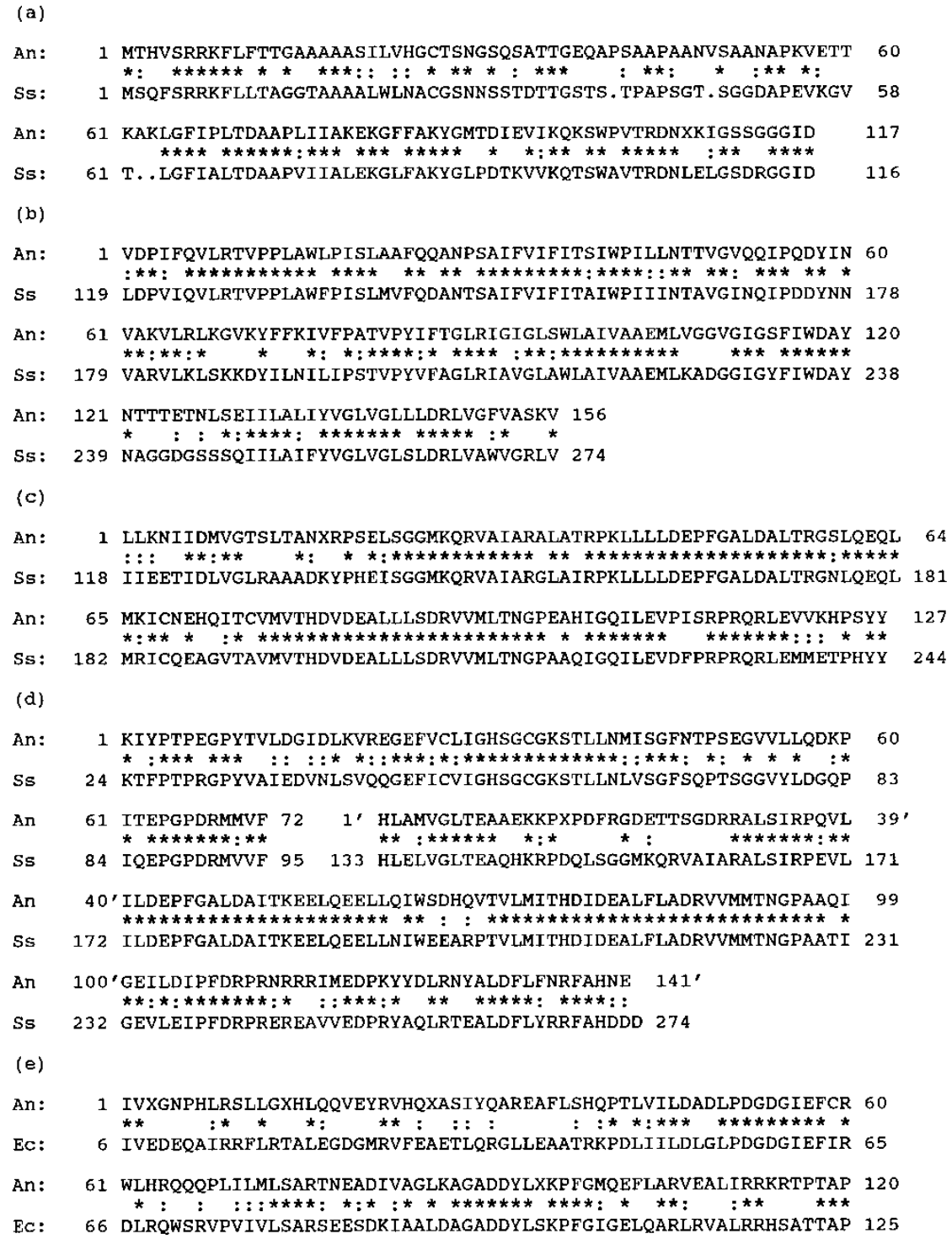

FIG. 3. Partial results of BLAST searches using products of translation of incompletely defined nucleotide sequences from a region 3′ from *nirA* of *Anabaena* sp. strain PCC 7120 (An) compared with the NrtA protein from *Synechococcus* sp. strain PCC 7942 (Ss; 36) (BLAST score, 200; 54% identical [*] and 65% similar [:]) (a), a region between *nirA* and the site of insertion of the transposon in mutant TLN12 compared with the NrtB protein from strain PCC 7942 (39) (BLAST score, 529; 65% identical and 76% similar) (b), a region adjacent to the transposon in mutant TLN12 compared with the NrtC protein from strain PCC 7942 (39) (BLAST score, 465; 73% identical and 82% similar) (c), two regions between the sites of insertion of Tn*5*-765 in mutants TLN12 and TLN21 compared with the NrtD protein from strain PCC 7942 (39) (BLAST score, 502; 67% identical and 81% similar) (d), and a region adjacent to the transposon in mutant TLN14 compared with *E. coli* regulatory protein KdpE (Ec; 48) (BLAST score, 226; 41% identical and 58% similar) (e). Except in panel a, the numbering of the *Anabaena* sequence assigns amino acid position 1 arbitrarily.

FIG. 4. Comparison of the deduced NirA sequence from *Anabaena* sp. strain PCC 7120 (An) with the first 507 amino acids of the sequence of NirA from the filamentous, N_2-fixing but nondifferentiating cyanobacterium *Plectonema boryanum* (Pb; 42) (BLAST score, 1,150); NirA from *Synechococcus* sp. strain PCC 7942 (Ss; 29, 44) (BLAST score 768); all but the first 69 amino acids of the precursor of *Spinacia oleracea* (spinach) NirA (So; 5) (BLAST score, 416); and portions of the (NADPH-dependent) hemoprotein component of *Salmonella typhimurium* sulfite reductase (St; 40) (BLAST score, 99). Amino acids common to all the NirA sequences shown for a particular position are indicated by asterisks; those also common to *S. typhimurium* sulfite reductase are underlined. The cysteines thought to be involved in binding of the Fe_4S_4 and siroheme prosthetic groups (40) are in bold print, and their asterisks are highlighted. Dots indicate gaps introduced to enhance the alignment.

transfer of those strains from N_2 to NH_4^+ (Fig. 7c) (see Discussion). TLN21 showed an extensive increase in *luxAB* expression upon transfer from NH_4^+ to either NO_2^- or NO_3^- (Fig. 8a) but little or no increase when NH_4^+ was supplemented with but not replaced by NO_2^- or NO_3^- (Fig. 8b).

DISCUSSION

A transposon bearing *luxAB*, encoding luciferase, as a reporter of transcription was used to identify genes activated rapidly upon deprivation of *Anabaena* sp. strain PCC 7120 of fixed nitrogen. The three loci (in strains TLN10, TLN12, and TLN21) that were identified as responding most rapidly and strongly proved to be structurally closely linked and situated within a series of genes whose products are responsible for assimilation of NO_2^- and NO_3^-. The (virtual) absence of nitrate reductase activity in cells of SR796 and the continued presence of heterocysts in that strain in the presence of NO_3^- but not NO_2^- substantiate the idea, based on sequence similarity (Fig. 5), that NarB is the nitrate reductase of *Anabaena* sp. strain PCC 7120. The nitrate reductase activity of TLN21 may be driven by a weak promoter situated in IS*50*R of Tn*5*-765. The poor and inconsistent growth of TLN10 in the presence of NO_2^- and NO_3^- is interpretable as being due to the

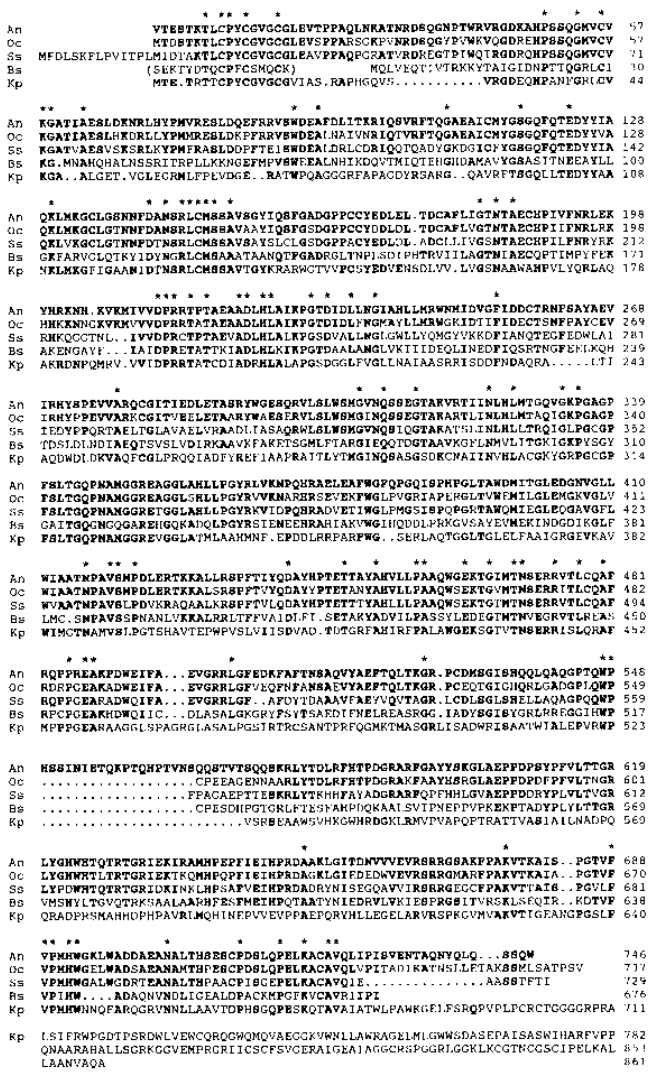

FIG. 5. Comparison of NarB sequences from *Anabaena* sp. strain PCC 7120 (An), *Oscillatoria chalybea* (Oc; 47), *Synechococcus* sp. strain PCC 7942 (Ss; 2), *B. subtilis* (Bs; 33) (in parentheses, a continuation of the translation 5′ from the putative start codon), and *K. pneumoniae* (Kp; 26). Amino acids common to all five sequences are indicated by asterisks. Dots and the space after the parenthesis indicate gaps introduced to enhance alignment. Amino acids in common with the *Anabaena* sp. sequence are shown in bold print.

toxicity of exogenous or NO_3^--derived NO_2^- in the absence of a functional nitrite reductase.

The results of pulsed-field gel electrophoresis indicated that the transposon insertion in strain TLN10 is located ca. 146 kb from an end of AvrE and suggest strongly that it is at ca. 0.71 Mb in the chromosome, oriented clockwise, about 100 kb clockwise from the phycocyanin operon (24). The experimental results are consistent with the interpretation that the transposon in TLN21 maps close to and at a location 3′ from the transposon in TLN10. Finally, the transposon in a fourth rapidly activated locus (in strain TLN14) maps at a distant site, apparently at approximately 5.17 Mb, with the corresponding open reading frame oriented counterclockwise. KdpE, which is similar to the predicted polypeptide product of the open reading frame interrupted in TLN14, is thought to regulate an *E. coli* transport ATPase with high affinity for K^+ that responds to changes in cell turgor (48). The relationship of such a function

TABLE 1. Luminescence of TLN10, TLN21, and SR796 during growth at low cell density with various nitrogen sources[a]

Nitrogen source	Luminescence [10^7 quanta/(μg of chlorophyll *a* · min)][b]		
	TLN10	TLN21	SR796
NH_4^+	63 ± 9 (10)	88 ± 27 (4)	98 ± 18 (4)
NO_2^-		310 ± 50 (2)	356 ± 16 (2)
NO_3^-		758 ± 241 (5)	
N_2	8,992 ± 1,803 (4)	1,234 ± 266 (4)	728 ± 94 (9)

[a] Each strain bore pRL1472a as a source of aldehyde, a substrate of luciferase. Cell density was <2.1 μg of chlorophyll *a* ml^{-1}.

[b] Data are the means ± standard errors of the means of the number of experiments indicated parenthetically.

to nitrogen deprivation is obscure but could involve a structural similarity of NH_4^+, whose uptake might be stimulated, and K^+.

Expression of *luxAB* by transcriptional fusions was used to report on the transcription of genes involved in the assimilation of nitrate. It is notable that there is a decreasing gradient of activation of *luxAB* expression and thus of transcription from TLN10 (in which *luxAB* is fused to *nirA*) to TLN12 (fused to *nrtC*), TLN21 (fused at the 3′ end of *nrtD*), and SR796 (fusion to *narB*) (Fig. 7a and b). This result would be expected if these genes form an operon in PCC 7120. How can such a model account for the subsequent reduction in transcription reported for TLN12, TLN21, and SR796, but not for TLN10? Perhaps an additional regulatory influence affects transcription as monitored in the former three strains. Other results also suggest that this cluster of genes is not regulated only coordinately. First, the ratios of steady-state luminescence in the presence of different nitrogen sources differed strikingly for TLN10, TLN21, and SR796 (Table 1). However, the exceptionally high luminescence of TLN10 grown on N_2 (on average, severalfold higher than that of a strain that bears a P_{rbcLS}-*luxAB* fusion [12]) may itself impose a metabolic burden on cells. If so and if such a burden rendered them more nitrogen deprived than are TLN12, TLN21, and SR796, it would be possible to account for the differences illustrated in Fig. 7b and d and Table 1. Second, averaged from four independent experiments for each of these three strains, the decrease in luminescent intensity attendant upon transfer of SR796 from N_2 to NH_4^+ appeared to lag the corresponding decreases observed for TLN10 and TLN21 (Fig. 7c). Multivariate repeated-measures analysis (27) of the data in Fig. 7c was consistent with the idea (probability of 0.94) that TLN10 and TLN21 were sampled from the same population but gave highly significant values for the hypotheses that SR796 and TLN10 were sampled from the same population (probability of 0.0079) and that SR796 and TLN21 were sampled from the same population (probability of 0.0007). Our data are, therefore, subject to the interpretation that *nirA* and *narB* may be to some extent independently regulated.

MSX, an inhibitor of glutamine synthetase, presumably prevents assimilation not only of exogenous NH_4^+ (when, as in the experiments illustrated in Fig. 7a and b, cells are deprived of that nitrogen source) but also of internally generated NH_4^+, perhaps accounting for the diminished activation observed in the presence of MSX (Fig. 7d). The increase in expression of *luxAB* by TLN21 upon transfer of that strain from NH_4^+ to either NO_2^- or NO_3^- (Fig. 8a) was nearly completely eliminated when NH_4^+ remained present (Fig. 8b), suggesting that the increase is attributable primarily to the lesser effectiveness

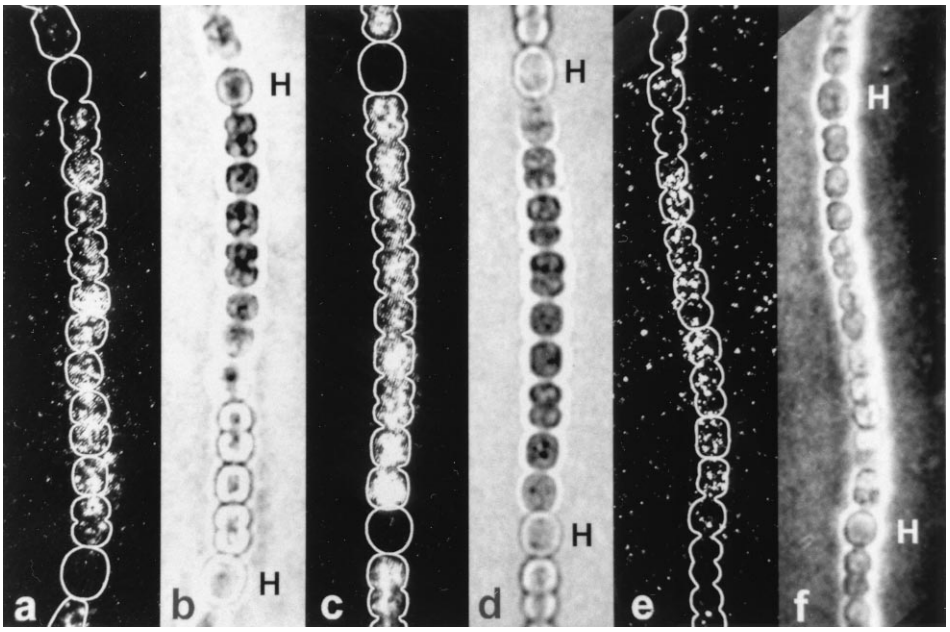

FIG. 6. Representative images of light emission from filaments of TLN10(pRL1472a) (a through d) and SR796(pRL1472a) grown on N_2 (e and f). (b, d, and f) Bright-field video images. H, heterocyst. (a, c, and e) Corresponding images of luminescence recorded during a 20-min exposure, with cell outlines drawn from superimposed bright-field images.

of NO_2^- and NO_3^- as nitrogen sources in strain TLN21 rather than to induction by these two substrates.

Whereas heterocyst differentiation is first evident by transmission light microscopy after ca. 12 h of nitrogen deprivation, the first indication of cells that can be identified as presumptive heterocysts is localized transcriptional activation of *hetR* after 3.5 h of deprivation (7). Our current results show that cells respond physiologically to nitrogen deprivation within 0.5 h and that the initial activation of *nrtC*, *nrtD*, and *narB* moderates, perhaps in response to mobilization of internal nitrogenous reserves, within 2.5 h. Others have observed that the presence of NO_2^- or NO_3^- is essential for all but very low-level activity of nitrite reductase and nitrate reductase in *Anabaena* strains (16, 32, 35; see also references 4, 19, 21, and 43). In contrast, the results presented in Table 1 show that despite abundant availability of cellular nitrogen during growth on N_2, it is under those conditions that the NO_3^--assimilatory genes are most strongly promoted in our cultures of *Anabaena* sp. strain PCC 7120. Moreover, our measurements of nitrate reductase activity confirm that wild-type filaments possess plentiful nitrate reductase activity during growth in the absence of nitrate. Unlike the *nif* genes, whose expression in aerobic cultures of *Anabaena* sp. strain PCC 7120 is restricted to heterocysts (12), the genes for nitrite and nitrate reductase were expressed in vegetative cells of filaments grown on N_2 (Fig. 6).

As shown by Fig. 7d, MSX blocks the repressing effect of NH_4^+ on expression of *luxAB* in all four mutant strains examined. Therefore, repression may be affected by glutamine or, as in *Synechococcus* sp. strain PCC 7942 (44), by a product of its metabolism. Heterocysts transfer fixed nitrogen to contiguous vegetative cells partially at least in the form of glutamine (46). We therefore anticipated that in N_2-grown filaments of TLN10

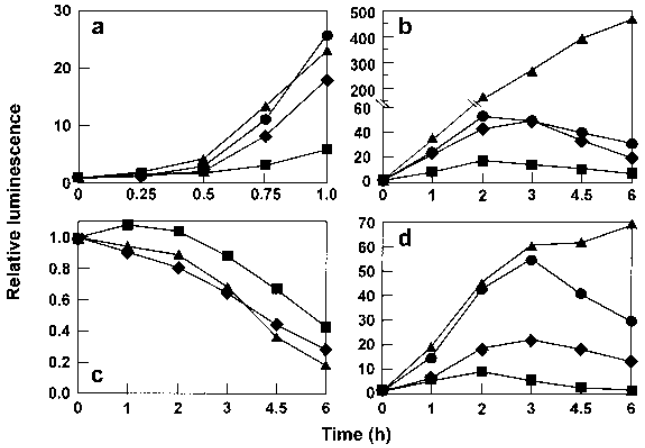

FIG. 7. Response of strains TLN10 (▲), TLN12 (●), TLN21 (◆), and SR796 (■) to transfer between different environmental conditions. (a and b) Transfer from NH_4^+ to N_2. The relative luminescence (the mean ± standard error of the mean of the results from the indicated number of experiments, compared with a value of 1.00 at 0 h) for each of these strains was 1.8 ± 0.4 (n = 3), 1.3 ± 0.2 (n = 4), 1.3 ± 0.1 (n = 4), and 1.7 ± 0.2 (n = 3) at 0.25 h and 4.3 ± 0.2, 3.0 ± 0.5, 2.3 ± 0.3, and 2.0 ± 0.2 at 0.5 h (same number of experiments as before). (c) Transfer from N_2 to NH_4^+. The mean ratio (standard error of the mean/mean) for all data points ≥1 h, averaged over four experiments, was 0.06. (d) Transfer from NH_4^+ to NH_4^+ plus MSX.

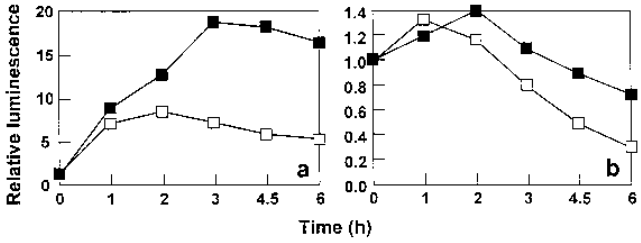

FIG. 8. Response of TLN21 to transfer from NH_4^+ to either NO_2^- (□) or NO_3^- (■) in the absence (a) or continued presence (b) of NH_4^+.

and SR796, there might be lesser expression of *luxAB* near heterocysts than in cells farther from heterocysts and that such a gradient of expression could be used as a bioassay to identify substances that might be responsible for regulation. However, in the nine filaments of TLN10 (examples shown in Fig. 6a through d) and six filaments of SR796 (one shown in Fig. 6e and f) that were imaged, no consistent deficiency of transcription of *nirA* or *narB* was observed in cells contiguous with heterocysts.

Repeats of oligonucleotides, often heptanucleotides, are common features of DNA sequences of *Anabaena* sp. strain PCC 7120 and closely related strains (45); Bauer et al. (6) observed, as we have here, such repeats within the coding sequence of a gene. Andriesse et al. (3) commented that because their sequence of *narB* predicted two possible start codons, it was unclear whether the NarB of *Synechococcus* sp. strain PCC 7942 contains 715 or 729 amino acids. The likelihood of similarities with the other sequences that are now available provides a reason to suggest that it is the second methionine in the open reading frame they reported that is the actual start codon (Fig. 5). No classical example of a ribosome binding site is seen 5′ from the coding region of the PCC 7120 *narB*, but on the basis of the sequence of 16S rRNA from that organism (25), either GGAG or GAAG, which is more likely because it is closer to the GTG start codon, may serve. The predicted mass and pI of the polypeptide portion of *Anabaena* sp. strain PCC 7120 NarB are 83.0 kDa and 7.40, respectively. In the 746 amino acids of PCC 7120 NarB, only 17 cysteines are found; of these (except for a pair, amino acids 167 and 168), only 3 form a spaced cluster, CXXCXXXC (amino acids 9 through 16), which may serve to bind a molybdenum cofactor or nonheme iron of an Fe_xS_x cluster (51). Although the sequence, TXTXCPYCGVGCG, close to the N terminus is highly conserved within cyanobacteria and *Klebsiella pneumoniae*, no corresponding sequence within the predicted NarB of *Bacillus subtilis* was reported (33). However, one codon in advance of their proposed initiation codon is a sequence that translates as TQCPFCSMQC, i.e., TXCPXCXXXC. No plausible initiation codon is present between the previous stop codon and this amino acid sequence; therefore, if this series of amino acids is to be considered part of *B. subtilis* NarB, there would have to be a prior frameshift in the sequence reported. Other regions of high conservation of amino acid sequence among enzymes that contain a pterin molybdenum cofactor correspond to amino acids 142 through 152, 211 through 239, 416 through 422, 441 through 488, and 688 through 693 in the sequence of NarB from *Anabaena* sp. strain PCC 7120. In a structurally related molecule, *E. coli* formate dehydrogenase, C-148 (in the *Anabaena* sequence) is replaced by selenocysteine (51, 53).

Whereas NarB from *Anabaena* sp. strain PCC 7120 shows extensive similarities in amino acid sequence to the nitrate reductases from a diversity of other prokaryotes (Fig. 5), NirA from that same strain shows extensive similarities in amino acid sequence to the nitrite reductases of other cyanobacteria and higher plants (Fig. 4) but very low similarities to the nitrite reductases of heterotrophic bacteria [e.g., a BLAST score of only 52 with the NAD(P)H-dependent nitrite reductase from *B. subtilis* (34)]. It actually shows greater similarities to bacterial sulfite reductases (one example in Fig. 4), which share with nitrite reductases the use of a siroheme cofactor. Higher-plant nitrite reductase is a nucleus-encoded chloroplast enzyme (9). These observations are consistent with two ideas presented by Flores and coworkers (16, 29). First, cyanobacterial *nirA* is evolutionarily related, albeit distantly, to bacterial sulfite reductases. Second, when an endosymbiotic cyanobacterium evolved into a chloroplast, the NirA protein that it encoded remained chloroplast situated, presumably to make use of ferredoxin as a reductant, whereas the gene encoding that protein became relocated to the plant nucleus.

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