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The *patB* Gene Product, Required for Growth of the Cyanobacterium *Anabaena* sp. Strain PCC 7120 under Nitrogen-Limiting Conditions, Contains Ferredoxin and Helix-Turn-Helix Domains

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A mutant of *Anabaena* sp. strain PCC 7120, called PAT-2, that grows poorly under nitrogen-fixing conditions, has been isolated. The heterocysts of the mutant strain develop much more slowly than those of the wild type and are spaced more closely in an older culture of the mutant than in the wild type. The wild-type gene that complements the mutation in PAT-2, called *patB*, was isolated and characterized. The predicted 529-amino-acid PatB protein contains a region very similar to the Fe_4S_4 bacterial-type ferredoxins near its N terminus and a helix-turn-helix motif near its C terminus. This pattern of domains resembles those of transcriptional regulators in both prokaryotes and eukaryotes. The mutation in strain PAT-2 is the deletion of G at nucleotide 1342 in the *patB* gene, resulting in the loss of a 62-amino-acid fragment from the C terminus of the PatB protein, including the helix-turn-helix motif.

Cyanobacteria, a diverse group of prokaryotes, are capable of oxygen-evolving photosynthesis, similar to green plants. Some cyanobacteria also carry out nitrogen fixation. Different strategies are employed by nitrogen-fixing cyanobacteria to solve the problem of fixing nitrogen while simultaneously producing oxygen during photosynthesis. Certain filamentous cyanobacteria, such as *Anabaena* sp. strain PCC 7120, differentiate specialized nitrogen-fixing cells, called heterocysts, at regular intervals along the filaments to confine these two biochemically incompatible processes to two physically separated compartments (12). The heterocysts provide the anaerobic environment that is required by the nitrogen fixation machinery, which includes the nitrogenase complex and its electron transport system. The multilayered heterocyst envelope, containing an inner glycolipid and an outer polysaccharide layer, serves as a physical diffusion barrier to external oxygen (34). The internal membranes of the heterocysts undergo extensive reorganization while the O_2-producing photosystem II is eliminated and the ATP-generating photosystem I is retained (31). Channels connecting the heterocysts and neighboring vegetative cells are constructed to facilitate material exchanges between the two cell types. The fixed nitrogen in the heterocyst is exported to the neighboring vegetative cells in the form of glutamine (27, 32). In return, the vegetative cells continue photosynthesis and provide the heterocysts with carbohydrates that provide reductant and a source of energy for nitrogen fixation.

Gene expression and protein synthesis in heterocysts and vegetative cells are programmed differently. Early studies by Fleming and Haselkorn (13) revealed that some proteins are synthesized in both cell types, but other proteins are synthesized exclusively in one cell type. Furthermore, among the heterocyst-specific proteins, some appear early in the proheterocysts and may disappear from mature heterocysts. Other proteins are synthesized late in mature heterocysts. These results indicate that gene expression during heterocyst development is highly regulated spatially and temporally. Enhanced transcription of the *hetR* gene, which is known to be required for heterocyst development, begins no later than 3 h after the cells are deprived of combined nitrogen (7). The expression of *hetR* occurs only in the cells that are going to differentiate into heterocysts, as seen by chemiluminescence in cells containing a *lux* gene fused to the upstream region of the *hetR* gene (40).

In the laboratory, *Anabaena* sp. strain PCC 7120 differentiates heterocysts both terminally and internally along the filaments at intervals of about 10 cells, although the intervals are slightly longer in older cultures. Division of vegetative cells along the axis of the filaments lengthens the interval between heterocysts, but the one-dimensional spacing pattern is maintained by the formation of new heterocysts midway between two existing heterocysts (36, 41). The placement of heterocysts can be altered by physical breakage of the filaments, by modulation of the light intensity, or by treatment with 7-azatryptophan (28) or rifampin (41). Apparently, many, if not all, of the cells in a filament have the potential to develop into heterocysts. The mechanism that allows only cells at certain positions in the filament to differentiate into new heterocysts must involve intercellular communication. It has been suggested that the establishment and maintenance of the heterocyst spacing pattern depend on the interaction between diffusible substances that originate in the heterocyst and move along the filament with other factors, presumably proteins, that both interact with the diffusible substances and control, directly or indirectly, gene expression (18, 38, 39).

Mutations affecting the heterocyst pattern have been induced in several *Anabaena* strains, using UV or chemical mutagenesis (8, 37) or transposon mutagenesis (11). In one case, the mutation produced an extreme phenotype: no heterocysts formed at all (8). The wild-type gene complementing this mutation, called *hetR*, was cloned and sequenced (7). The sequence of HetR did not reveal any of the motifs characteristic of transcription-regulating proteins.

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However, when extra copies of the wild-type *hetR* gene were introduced into wild-type cells, the heterocyst frequency increased because of the induction of heterocysts in strings of two, three, or more cells (7). Recently, we described a mutant of *Anabaena* sp. strain PCC 7120 that differentiates heterocysts mostly at the ends of filaments (23). This mutant, PAT-1, grows poorly under nitrogen-fixing conditions. The wild-type gene that complements the mutation in PAT-1, called *patA*, was cloned and sequenced. The C-terminal region of the predicted PatA protein is very similar to that of CheY and other response regulators involved in bacterial signal transduction. This result suggests that a similar signal transduction circuit may be involved in the establishment of the heterocyst pattern. The *patA* mutation suppresses the multiheterocyst phenotype produced by extra copies of the wild-type *hetR* gene, suggesting that PatA and HetR interact in some way to control the heterocyst pattern.

Here we describe another mutant of *Anabaena* sp. strain PCC 7120, called PAT-2, that displays an elevated frequency of heterocysts in older cultures. The sequence of the gene, *patB*, that complements the mutation in PAT-2 contains a region similar to the Fe_4S_4 bacterial-type ferredoxins in an N-terminal domain and a helix-turn-helix (HTH) motif in a C-terminal domain.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* MC1061 and DH5α were used as the hosts for plasmid preparation and for conjugal transfer of DNA to *Anabaena* sp. strain PCC 7120 (25). For selective growth, Luria broth was supplemented with the following antibiotics and concentrations: 100 μg of ampicillin per ml, 50 μg of kanamycin per ml, 10 μg of chloramphenicol per ml, and 20 μg of spectinomycin per ml. *Anabaena* sp. strain PCC 7120 and its mutants were grown in BG-11 medium (29). For plate culture, BG-11 liquid medium was supplemented with 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH 8.0), 1 mM NaS_2O_3, and 1.5% agar (purified; BBL). For selective growth, 30 μg of neomycin per ml and 10 μg of chloramphenicol per ml (final concentrations) were added. A combination of spectinomycin and streptomycin was used at a final concentration of 2 μg/ml (each). BG-11 N^+ medium contained either 17 mM $NaNO_3$ or 5 mM $(NH_4)_2SO_4$; N^- medium lacked both.

Mutagenesis and isolation of mutants. Tn*5* mutagenesis was attempted as described previously (5). *E. coli* MC1061 was successively transformed with plasmids pRK24, pRL528, and pBR322::Tn*5*. Equal volumes of *E. coli* and *Anabaena* sp. strain PCC 7120 cell suspensions were mixed and spread on a nitrocellulose membrane placed on BG-11 agar plates, grown for 3 days, at which time the membranes were transferred to new BG-11 plates containing neomycin (30 μg/ml). When well-separated green colonies appeared (about 2 weeks), the membranes were transferred to BG-11 N^- plates. Colonies that turned yellow around the edges on these plates were selected and maintained on BG-11 plates.

Complementation of mutant PAT-2. Complementation with a cosmid bank containing fragments of wild-type DNA was conducted essentially as described previously (8). A similar procedure was used for subsequent confirmatory complementation. In this case, the *E. coli* donor carried plasmids pRK24 and pRL528 and the complementing plasmid. After 3 days of incubation on a BG-11 agar plate, the membrane that carried the conjugal mixture of *E. coli* and *Anabaena* cells was transferred to a BG11 N^- plate containing 30 μg of neomycin per ml. Successful complementation gives rise to a green lawn, whereas the mutant exhibits a yellow-green color.

DNA subcloning. The DNA of a cosmid that complemented mutant PAT-2 was partially digested with *Sau*3AI and fractionated by agarose gel electrophoresis. Fragments of 1 to 2, 2 to 4, and 4 to 14 kb were isolated with a GeneClean kit (Bio 101) and separately ligated into the *Bam*HI site of pCCB110 (a derivative of shuttle vector pRL25c with the polylinker of pIC20R inserted into the *Eco*RI site [3]). The plasmid banks were subsequently conjugated into *Anabaena* mutant PAT-2 for the complementation test.

DNA sequence analysis. Plasmid pJHL59c3 complements mutant PAT-2 and contains a 6-kb DNA fragment inserted in vector pCCB110. Nested deletions were constructed with an Erase-a-Base kit from Promega Biotec. The complete sequence of one strand of a 3.5-kb fragment was determined by using the sequencing kit from U.S. Biochemical Corp. The sequence of the other strand was determined by utilizing synthetic primers complementary to the sequenced strand. Sequences were analyzed by using the University of Wisconsin Genetic Computer Group's software package and programs written by William Buikema and Conrad Halling for the Apple Macintosh.

Nucleotide sequence accession number. The DNA sequence of the *patB* gene has been submitted to the GenBank database under accession number L06674.

RESULTS

Isolation of mutants with altered heterocyst spacing patterns. We tried to introduce the Tn*5* transposon carried on pBR322 into wild-type *Anabaena* sp. strain PCC 7120 cells by the conjugation system developed by Elhai and Wolk (10, 42). Exconjugants that were resistant to neomycin (30 μg/ml) were screened for their ability to grow on N^- plates. Colonies that gradually turned yellow around the edges, an expected Fix^- phenotype, were selected and maintained on N^+ plates. These Fix^- mutants were examined with a microscope. Of 89 mutants examined, 2 exhibited altered heterocyst spacing patterns. The first mutant, PAT-1, forms only terminal heterocysts (23), while the second, PAT-2, eventually forms many more heterocysts than the wild type. This report describes the gene that complements mutant PAT-2.

Although PAT-2 was originally picked from neomycin-resistant colonies, it subsequently proved to be neomycin sensitive. The PAT-2 strain does not contain Tn*5* or the *npt* gene on the basis of Southern blotting (data not shown). The mutation in PAT-2 is a frameshift (see below) not directly related to the attempted transposon mutagenesis. (See the Discussion for an explanation of the impermanent Neo^r phenotype).

Strain PAT-2 grows as well as the wild type in medium containing combined nitrogen. Under these conditions, the two strains are morphologically identical. In the absence of fixed nitrogen, mutant PAT-2 grows poorly. The filaments fragment in shaken N^- liquid medium to the point that filaments longer than 15 cells are rarely seen in a 5-day-old culture. Because this fragmentation in liquid cultures of PAT-2 made it impossible to determine the spacing of heterocysts, we examined the filaments of cells grown on plates. On N^- plates, the heterocysts of PAT-2 filaments were spaced at intervals of three to eight cells. We have

observed different phenotypes under different growth conditions. When PAT-2 was grown in N^- liquid medium without or with gentle shaking (150 rpm), heterocyst formation was delayed by ~21 h. Wild-type cells formed proheterocysts 15 h following the removal of combined nitrogen, and these proheterocysts matured at 21 h. A large proportion of the vegetative cells seemed to be dividing at 21 h, as evidenced by septum initiation. In contrast, PAT-2 heterocysts did not appear until 42 h after induction. Most PAT-2 vegetative cells were round, with no sign of cell division at 21 h or later. When the mutant strain was grown with vigorous stirring and bubbling with a mixture of air and 2% CO_2, the timing of heterocyst differentiation was the same as that of the wild type and the nitrogenase activity was comparable to that of the wild type, as determined by whole-cell acetylene reduction assay under aerobic or anaerobic (helium as top gas) conditions. The growth rate, however, was much lower than that of the wild type. These results suggest that the defect in the PAT-2 strain is in the vegetative cells, possibly involving their ability to fix CO_2 under nitrogen-fixing conditions.

The growth rates of the mutant and wild type were determined by measuring chlorophyll, extracted from whole cells with 100% methanol (10). Curiously, after the methanol extraction, the pelleted mutant cells were colorless, whereas the pelleted wild-type cells were blue. The blue color is indicative of the phycobiliproteins, revealed after the chlorophyll has been removed. Although the PAT-2 mutant does not appear to be deficient in phycobiliproteins on the basis of the color of the cells growing in liquid, this result suggests that the bilins in the mutant are more labile than in the wild type.

Complementation of the mutation. The PAT-2 strain was complemented by conjugation with a cosmid library of wild-type DNA fragments, selecting for rapid growth on medium lacking combined nitrogen (8). Thirty cosmids isolated from complemented colonies contained a common 10.5-kb fragment. One of these cosmids was partially digested with *Sau*3AI, and the resultant fragments of 1 to 2, 2 to 4, and 4 to 14 kb were subcloned into shuttle vector pCCB110. These three fragment libraries were used to complement strain PAT-2. Two weeks after the exconjugants were transferred to N^- plates containing neomycin (30 μg/ml), well-separated large green colonies were seen in a yellow-green background on the conjugation plates of the 4- to 14-kb fragment bank. Tiny green colonies were seen on the conjugation plates of the shorter-fragment banks. This result could be explained if the large green colonies arose from complementation, while the tiny green colonies resulted from homologous recombination between the wild-type sequence carried on the plasmid and the mutated sequence in the chromosomal DNA. This interpretation was confirmed by subsequent complementation tests and plasmid sequencing. Plasmids were isolated from four colonies of each complementation category and retested for complementation of the PAT-2 strain. All four isolates of the 4- to 14-kb category complemented PAT-2 again, but the four isolates of the shorter-fragment categories failed to complement or to rescue the mutation by recombination. In fact, the sequences of the latter four plasmids were converted to the mutant sequences, probably by marker exchange (see below).

Identification of the *patB* gene. Nested deletions were constructed starting with the complementing plasmid pJHL59c3, which contained 6 kb of *Anabaena* sp. strain PCC 7120 DNA (Fig. 1). Five plasmids containing progressively deleted DNA fragments were used to complement the PAT-2 strain. One clone, pJHL59c3-10, that carries a 3.5-kb fragment of *Anabaena* DNA, complemented the mutation. Another clone, pJHL59c3-9, that contained a fragment only 300 bp shorter than that in pJHL59c3-10, did not. Therefore, the complete sequence of one strand of the 3.5-kb fragment in pJHL59c3-10 was determined. This fragment contains two complete open reading frames (ORFs) of 1.6 and 0.7 kb and a partial ORF (Fig. 1). In order to determine whether the 1.6-kb ORF alone or both ORFs are required to complement the mutation in PAT-2, serial deletions were made from the other end of the 6-kb fragment in pJHL59c3 and used in subsequent complementation tests. Plasmids containing the intact 1.6-kb ORF alone complemented the mutation, but plasmids containing an incomplete ORF failed to do so (Fig. 1A). Thus, the 1.6-kb ORF, named *patB*, is the gene whose function is impaired in the mutant strain PAT-2. The nucleotide sequence of the noncoding strand of *patB* was determined by using the deletions created for complementation. Gaps in the sequence were filled by using synthetic primers complementary to the coding strand. Comparison to the sequences in GenBank using FASTA did not yield any sequences similar to ORF2 or ORF3.

Sequence of the *patB* gene. Figure 2 presents the nucleotide sequence of the *patB* gene and the predicted amino acid sequence. The *patB* gene encodes a protein of 529 amino acids with 11% acidic residues and 14% basic residues. Analysis of the derived protein sequence by the PLOT.A/HYD program (26) using the parameter set developed by Kyte and Doolittle (21) revealed that the overall hydrophobicity alternates between hydrophobic and hydrophilic regions, except for a 25-amino-acid segment between residues 148 and 173 which lies on the hydrophobic side (data not shown). This region is also more rigid than the rest of the sequence, as revealed by analysis of the same sequence by PLOT.A/KAS (a flexibility predictor) (26). If the PatB protein associates or interacts with a membrane, this region may play a role in such function.

Structural features of the PatB protein. The predicted amino acid sequence of PatB was compared with the PROSITE database (2) of protein patterns by using the MacPattern program (15). This search identified a pattern characteristic of Fe_4S_4 bacterial-type ferredoxins near the N terminus of the PatB protein. Eight cysteine residues that serve as ligands to the Fe atoms are conserved in all ferredoxins, including the PatB protein (Fig. 3). Ferredoxins are low-molecular-weight proteins that function as electron donors in a number of oxidation-reduction processes. Even if the PatB protein were involved in a similar type of oxidation-reduction reaction, it seems unlikely that this is its sole function, because the ferredoxin-like element in PatB accounts for only one-ninth of the protein and deletions near the C-terminal end of the protein rendered it unable to complement the mutation in PAT-2 cells (Fig. 1).

Further analysis of the DNA sequence of the *patB* gene with the DNAlysis program (6) revealed a HTH motif, which is one of the structural elements involved in protein-DNA interaction (30), near the C terminus of the PatB protein (Fig. 4). This HTH motif and its neighboring sequences are similar to those of several small proteins involved in transcriptional regulation, such as the immunity control ORF3 polypeptide in *Bacillus subtilis* phage φ105 (19) and the repressor protein (DicA) of the division inhibition gene *dicB* in *E. coli* (4) (Fig. 4).

Overall the structure of PatB is similar to a group of transcription regulators that include FixK in *Bradyrhizobium japonicum* (1), FNR in *E. coli* (17, 33), and *lin-11*-encoded

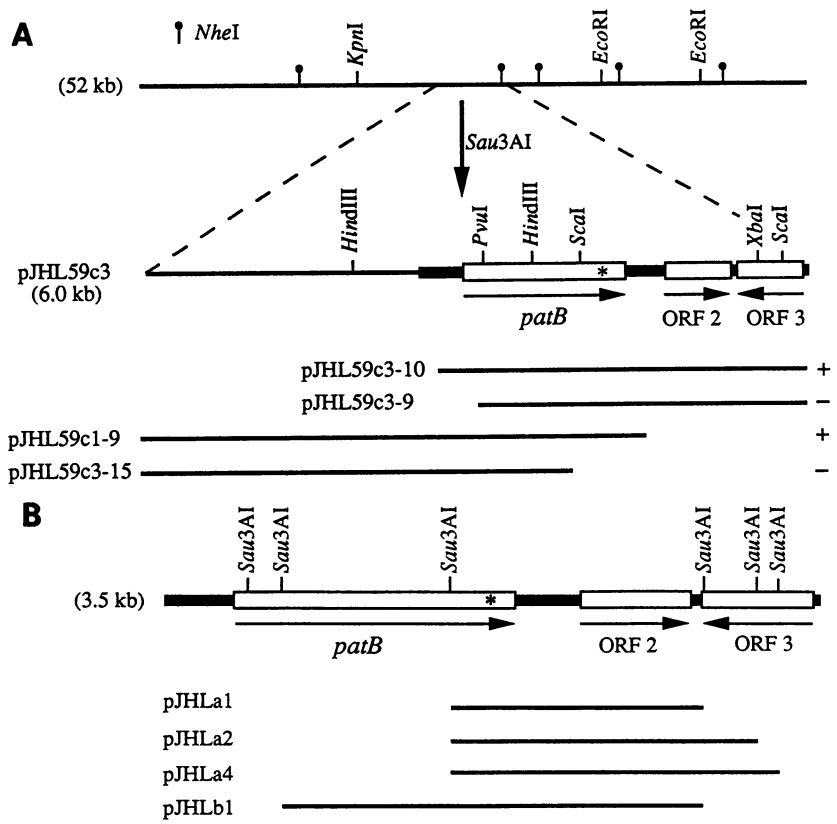

FIG. 1. Physical map of the cosmid containing the *patB* gene, the 3.5-kb fragment that was sequenced, and the deletions used for complementation of the mutation in PAT-2. (A) The upper line represents a 52-kb fragment of *Anabaena* sp. strain PCC 7120 chromosomal DNA contained in two overlapping cosmids. The second line represents the 6-kb DNA fragment carried in plasmid pJHL59c3. The thick section on the right side is the 3.5-kb fragment whose complete sequence was determined. The horizontal arrows indicate the directions of transcription of the *patB* gene and of the ORFs. Whether a plasmid corresponding to the thin horizontal line did (+) or did not (−) complement the mutation in PAT-2 is indicated at the right. The location of the frameshift mutation in PAT-2 is indicated by the asterisk within the *patB* box. (B) Plasmids containing part of the *patB* gene that rescued the mutation in strain PAT-2 by double recombination.

protein in *Caenorhabditis elegans* (22) (see Discussion). All of these proteins contain an iron-binding domain near their N termini and a DNA-binding site near their C termini.

Strain PAT-2 contains a frameshift mutation in *patB*. Since the mutation in strain PAT-2 was not caused by Tn*5* insertion, its nature had to be determined by DNA sequencing. In complementing the mutation, three libraries of *Sau*3AI fragments of wild-type *Anabaena* DNA were constructed as described above. Several plasmids that contained 1- to 2-kb and 2- to 4-kb DNA fragments did not complement the mutation but restored the wild-type phenotype after long incubation, presumably by marker rescue involving homologous recombination between the mutated locus in the chromosome and the wild-type sequence on the plasmids. If this were so, plasmids isolated from the rescued colonies might carry the mutation that was originally in the chromosome of the mutant strain PAT-2. Plasmids from three colonies in the 1- to 2-kb fragment category and one in the 2- to 4-kb fragment category were isolated. None of these plasmids could rescue the marker in the PAT-2 strain in subsequent tests. Both ends of the DNA insert in these four different plasmids were sequenced to determine which segment of the *patB* gene they contained. As shown in Fig. 1B, these plasmids carry a common fragment that contains the 3′ end of the *patB* gene. The sequences of plasmids pJHLa1, pJHLa2, and pJHLa4 were determined past the end of the *patB* gene. A frameshift mutation was found in all three plasmids, caused by the deletion of a single guanine in a run of three at positions 1370 to 1372 in Fig. 2. The new reading frame terminates 60 nucleotides downstream of the shift point. It results in a different amino acid sequence for the last 20 residues of the mutant protein and the deletion of the entire HTH motif.

Northern blot analysis of *patB*. Total RNAs were isolated from wild-type *Anabaena* sp. strain PCC 7120 cells induced for heterocyst formation for 0, 3, 6, 12, and 24 h. These RNAs were probed with the 0.83-kb *Pvu*I-*Sca*I internal fragment of *patB*. As shown in Fig. 5, there was no signal observable in the RNA sample from cells grown under nitrogen-replete conditions (N^+), indicating that the *patB* gene is not expressed during vegetative growth. Four bands of 1.85, 1.4, 1.2, and 0.8 kb hybridized to the probe in the RNA samples from cells grown under nitrogen deprivation conditions for 3 and 6 h, demonstrating that the expression of *patB* is inducible by limitation of combined nitrogen and occurs no later than 3 h after the removal of combined nitrogen. In the RNA samples from cells induced for 12 and 24 h, while the intensities of all four bands increased, an extra band of 3.6 kb appeared in the 12-h sample. The mRNA of 1.85 kb seems to be the message for the *patB* gene, which is 1,590 bp long. The transiently synthesized 3.6-kb message could include the downstream ORF2 or an up-

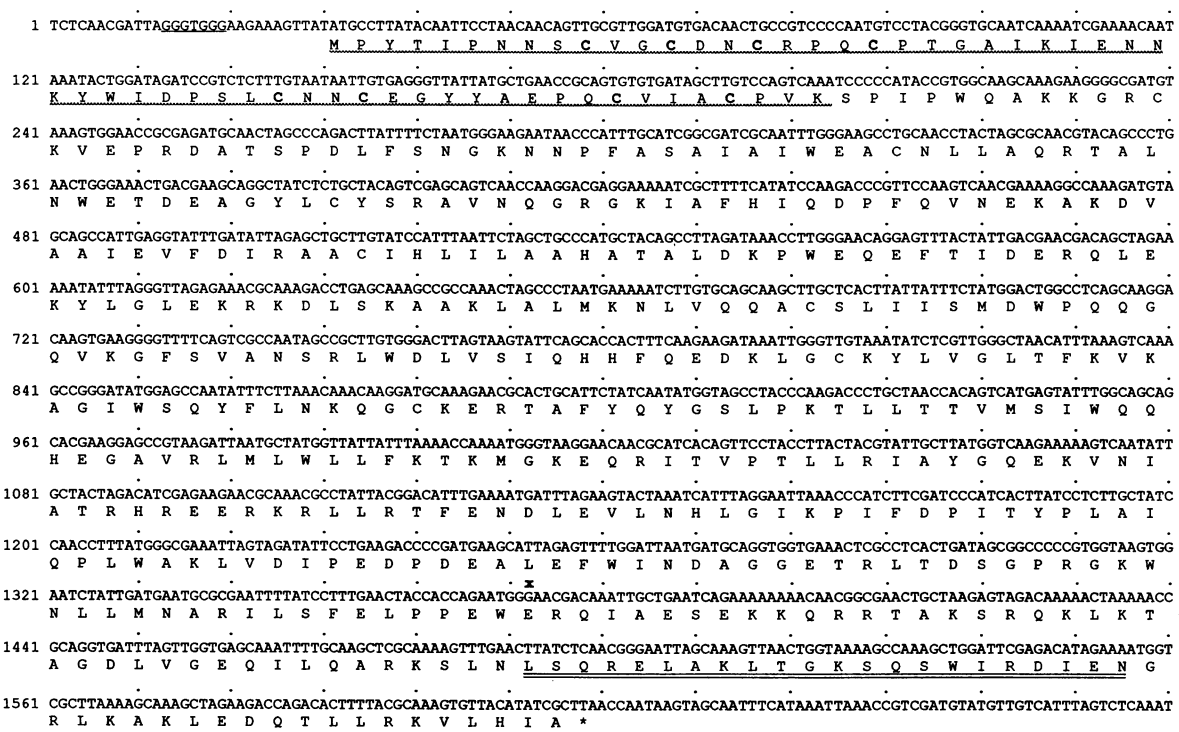

FIG. 2. Nucleotide sequence of the *patB* gene and the derived amino acid sequence of the PatB protein. The underlined sequence is complementary to the 3′ end of 16S rRNA. The wavy underline near the N terminus indicates the ferredoxin-like sequence with the conserved cysteine residues in boldface type. The x indicates the position at which a G is deleted to create a frameshift mutation in the PAT-2 strain. The sequence underlined twice near the C terminus is the HTH motif.

stream gene which is not in the sequenced fragment. Since the remaining three bands are smaller than the size required to encode PatB, they are likely to be degradation products.

Distribution of *patB* in cyanobacteria. The same probe that had been used in the Northern (RNA) analysis was employed again to hybridize to a Southern blot that carried chromosomal DNAs from 24 strains of cyanobacteria, under high-stringency conditions. As shown in Fig. 6, the *patB* gene hybridizes to the DNAs from the filamentous heterocystous nitrogen-fixing cyanobacteria, which includes all of the *Anabaena*, *Nostoc*, and *Calothrix* strains, except for *Anabaena cylindrica* PCC 7122. The *patB* gene does not hybridize with the DNAs from the filamentous nonheterocystous anaerobically nitrogen-fixing cyanobacteria included in the blot, *Phormidium faveolarum* and *Plectonema boryanum*. The lane marked *Oscillatoria tenuis* contains DNA from a strain that clearly contains heterocysts and thus cannot be an *Oscillatoria* strain. The route traveled by that strain between its origin and this laboratory is so complicated that its real name may never be known. The two unicellular non-nitrogen-fixing *Synechocystis* strains do not hybridize either. Strains that are closely related to *Anabaena* sp. strain PCC 7120, such as *Anabaena* sp. strains PCC 7118 and PCC 7119, show strong hybridization and identical or similar restriction patterns, whereas the more distantly related strains show weaker hybridization and different restriction patterns.

DISCUSSION

We have described a mutant of *Anabaena* sp. strain PCC 7120 that grows poorly in nitrogen-free medium. In older cultures of this mutant, the heterocysts are spaced more closely than in wild-type filaments. The mutation in strain PAT-2 is a frameshift caused by the deletion of a G from a string of three G residues near the C-terminal end of the *patB* gene. This mutation appears to be a spontaneous mutation of the type arising from slippage during replication. The mutants probably survived the initial neomycin selection, because a large number of cells was applied to the mating filter,

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An  PatB  MPYTIPNNSCVGCDNCRPQCPTGAIKI-ENNKYWIDPSLCNNC-EGYYAEPQCVIACPVKSP...
Rm  FER   MAFKIIASQCTQCGACEFECPRGAVNF-KGEKYVIDPTKCNEC-KGGFDTQQCASVCPVSNT...
Rp  FER   -AYKIITSQCTVCGACEFECPNAAIAM-KRGTYVIDAVKCTEC-EGHFDKPQCVAVCPVDNT...
Rc  FER   -AMKIDPELCTSCGDCEPVCPTNAIAP-KKGVYVINADTCTEC-EGEHDLPQCVNAC-MTDN...
Av  FER   MALKIVES-CVNCWACVDVCPSEAISL-AGPHFEISASKCTEC-DGDYAEKQCASICPVEGA...
An  FER   MAYTI-TSQCISCKLCSSVCPTGAIKIAENGQHWIDSELCTNCVDTVYTVPQCKAGCPTCDG...
          .  *    *  *  *   **  *.       * . * .*        **   *
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FIG. 3. Comparison of the amino acid sequences of *Anabaena* PatB (residues 1 to 60) and ferredoxins (FER) from different bacteria. Identical residues are indicated by asterisks, similar residues are shown by dots, and the conserved cysteine residues are shown in boldface type. The analysis used the Clustal computer program with the standard Pam 250 matrix for proteins and a gap penalty of 10. An, *Anabaena* sp. strain PCC 7120; RM, *Rhizobium meliloti*; Rp, *Rhodopseudomonas palustris*; Rc, *Rhodobacter capsulatus*; Av, *Azotobacter vinelandii*.

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An    PatB   ...AGDLVGEQILQARKSLNLSQRELAKLTGKSQSWIRDIENGRLKAKLEDQTLLRKVLHIA
ø105  ORF3   LDGKKLGALIKDKRKEKHLKQTEMAKALGMSRTYLSDIENGRYLPSTKTLSRIAILINL...
              .*. .*. * . **. .*.* *.**  * *.... ******  .. .. . .  ....

An    PatB   ...KTAGDLVGEQILQARKSLNLSQRELAKLTGKSQSWIRDIENGRLKAKLEDQTLLRKVLHIA
Ec    DicA   METKNLTIGERIRYRRKNLKHTQRSLAKALKISHVSVSQWERGDSEPTGKNLFALSKVLQC...
              .*     **.*   **.*. .** ***    *.  ....*.*. ... ..   *.***..
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FIG. 4. Comparison of the primary structure of the putative DNA-binding domain in the *Anabaena* PatB protein (residues 471 to 529) to that in the ORF3 polypeptide of *B. subtilis* phage ϕ105 and that of the *E. coli* DicA protein. Identical residues are indicated by asterisks, similar residues are shown by dots, and the HTH motifs are shown in boldface type. The sequence comparison used the Clustal computer program with the standard Pam 250 matrix for proteins and a gap penalty of 10. The scores for HTH motifs in these different proteins, as determined by the DNAlysis computer program (6), are 6.0, 5.4, and 6.8 standard deviations for PatB, ORF3, and DicA, respectively. A score above 3.0 is significant for an HTH structure (9). An, *Anabaena* sp. strain PCC 7120; Ec, *E. coli*.

providing some protection against the antibiotic and because neomycin, which is light sensitive, decomposes under the experimental conditions. Introduction of foreign DNA into *Anabaena* sp. strain PCC 7120 appears to increase the frequency of spontaneous mutations by 1 to 2 orders of magnitude (3). Although the Tn*5* mutagenesis per se did not work as expected, the transfer of DNA from *E. coli* to *Anabaena* sp. strain PCC 7120 may have contributed to the generation of a larger population of mutants than existed in the untreated culture. The mutant phenotype is due to the *patB* mutation, because it is complemented only by the complete *patB* gene.

The *patB* gene product apparently is not required for growth in the presence of combined nitrogen, since the PAT-2 mutant grows as well as the wild type and there is no detectable expression of the *patB* gene in cells grown under nitrogen-replete conditions. The poor growth of the *patB* mutant under nitrogen-fixing conditions is not caused by a low nitrogen fixation activity, since the nitrogenase activity of the mutant is comparable to that of the wild type. Under the same conditions, the *patA* mutant strain PAT-1 grows faster than the *patB* strain PAT-2, even though the PAT-1 culture contains fewer heterocysts and has lower nitrogenase activity than the PAT-2 strain.

Fleming and Haselkorn (13) demonstrated that a number of classes of proteins are induced when cells are transferred from nitrogen-containing medium to nitrogen-free medium. One group of proteins induced by nitrogen limitation appears in both vegetative cells and cells differentiating into heterocysts. This result suggests that although the vegetative cells look unchanged morphologically, they adopt some new biochemical strategies to cope with a new source of fixed nitrogen.

The structural features of the PatB protein are of interest because PatB contains near its N terminus a region that is similar in sequence to Fe_4S_4-type ferredoxins, including the eight conserved Cys residues that donate ligands to Fe atoms, and at its C terminus PatB contains a DNA-binding HTH motif. Ferredoxins are low-molecular-weight proteins involved in a wide variety of oxidation-reduction processes, such as photosynthesis and nitrogen fixation. Ferredoxins can be reduced at one site in the cell and pass the electrons to other proteins at other sites. It seems unlikely that the PatB protein functions solely as an electron carrier, since the ferredoxin-like part of PatB accounts for only one-ninth of the sequence. Furthermore, the HTH motif, known to bind DNA in many DNA-binding proteins, is essential for the

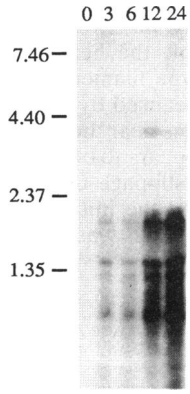

FIG. 5. Northern blot of total RNA (40 μg in each lane) prepared from wild-type *Anabaena* sp. strain PCC 7120 starved for combined nitrogen for 0, 3, 6, 12, and 24 h. The probe was a 0.83-kb *Pvu*I-*Sca*I fragment internal to the *patB* gene. The numbers at the top indicate the length of time (in hours) cells had been induced for heterocyst differentiation. The positions (in kilobases) of molecular size standards are shown to the left.

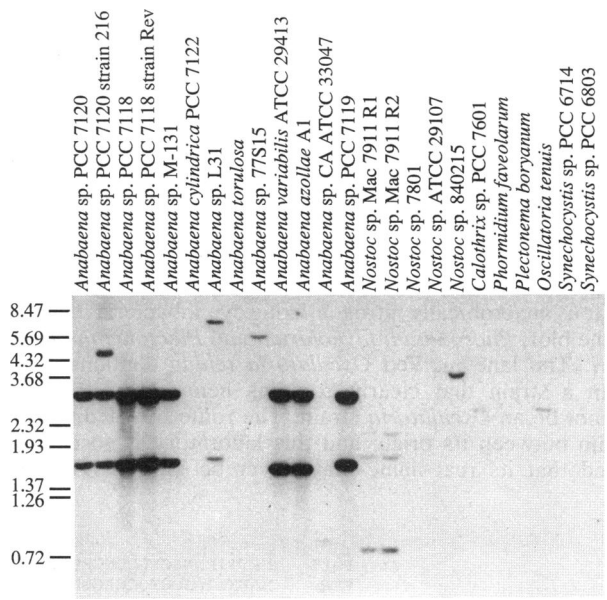

FIG. 6. Hybridization of *patB* to genomic DNA from different cyanobacterial strains. The Southern blot was a generous gift from W. Buikema (7). Each lane contains about 2 μg of genomic DNA digested with *Hin*dIII. The probe was the same one used for the blot in Fig. 5. At the top of the blot are the names of the cyanobacterial strains from which the DNAs were prepared (see text for one strain correction). The positions (in kilobases) of the molecular size markers are shown to the left.

function of PatB. A number of proteins, such as Fnr, FixK, and the product of the *lin-11* gene, have been found to possess a similar primary structure in that they contain an iron-binding site near the N terminus and a DNA-binding site near the C terminus.

The Fnr protein of *E. coli*, required for anaerobic respiration, functions as a transcriptional regulator. Fnr activates the expression of a large number of genes, such as those encoding fumarate reductase, nitrate reductase, and nitrite reductase, under anaerobic conditions. The oxygen-responsive Fnr protein is probably inactive under aerobic conditions and converted to an active form when oxygen is removed. Although the mechanism of this conversion is not completely understood, it has been suggested that cysteine residues in the N-terminal part of Fnr and protein-bound iron are involved (17, 33). Several Fnr-like regulators have also been found in other bacteria. The HlyX protein was identified as a probable regulator of hemolysin synthesis in *Actinobacillus pleuropneumoniae* (24). The Anr protein controls expression of the nitrate reductase and arginine deiminase genes under anaerobic conditions in *Pseudomonas aeruginosa* (16, 43). Additional Fnr-like proteins are rhizobial FixK proteins that are involved in adaptation to symbiotic nitrogen-fixing conditions in nodules.

A cysteine-rich motif, called LIM, that shows similarity to the metal-binding domains of zinc metalloproteins and ferredoxins is present in a number of presumptive eukaryotic transcription factors. The product of the *lin-11* gene in *C. elegans* controls certain asymmetric cell divisions during vulval development (14), the product of the *isl-1* gene in rats is an insulin gene enhancer-binding protein (20) and the product of the *mec-3* gene is required for the differentiation of certain *C. elegans* mechanosensory neurons (35). All three proteins contain two tandem copies of the LIM motif in their N-terminal sequence and also a homeodomain in the C-terminal sequence that has been implicated in DNA binding and in transcriptional regulation. The LIM motif might sense the redox state and affect the regulation of transcription, as suggested by the results of experiments in which the purified LIM region of the *lin-11*-encoded protein was demonstrated to bind zinc and iron and underwent oxidation-reduction by air and dithionite (22).

The HTH motif near the C-terminal end of the *patB* gene identified by computer analysis is homologous to the HTH motif contained in the *B. subtilis* phage φ105 ORF3 polypeptide and in the repressor protein DicA of *E. coli*, both of which are involved in transcriptional regulation (4, 19). Therefore, the PatB protein may function as a transcriptional regulator in response to the redox state or other redox factors in the cell. Alternatively, it may sense the level of iron in the cell and regulate relevant gene expression accordingly. However, the target genes that presumably are regulated by PatB remain to be identified.

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