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Nitrate Reductase Activity and Heterocyst Suppression on Nitrate in *Anabaena* sp. Strain PCC 7120 Require *moeA*

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Mutants of *Anabaena* sp. strain PCC 7120 that form heterocysts when grown on nitrate-containing media were isolated following nitrosoguanidine mutagenesis. Six independent mutants were isolated, and the characterization of one mutant, strain AMC260, which forms 6 to 8% heterocysts in the presence of nitrate, is presented. A 1.8-kb chromosomal fragment that complemented the AMC260 mutant was sequenced, and a 1.2-kb open reading frame, named *moeA*, was identified. The deduced amino acid sequence of the predicted *Anabaena* sp. strain PCC 7120 MoeA polypeptide shows 37% identity to MoeA from *Escherichia coli*, which is required for the synthesis of molybdopterin cofactor. Molybdopterin is required by various molybdoenzymes, such as nitrate reductase. Interruption of the *moeA* gene in *Anabaena* sp. strain PCC 7120 resulted in a strain, AMC364, that showed a phenotype similar to that of AMC260. We show that AMC260 and AMC364 lack methyl viologen-supported nitrate reductase activity. We conclude that the inability of the *moeA* mutants to metabolize nitrate results in heterocyst formation on nitrate-containing media. Northern (RNA) analysis detected a 1.5-kb *moeA* transcript in wild-type cells grown in the presence or absence of a combined nitrogen source.

One of the primary goals in developmental biology is to understand how one type of cell differentiates into another more specialized type of cell (20, 35). Some developmental processes are influenced by environmental cues, and one of the simplest systems in which this has been investigated is the induction of spore formation in *Bacillus subtilis* by nutrient limitation (13). Cyanobacterial heterocyst development provides another example of cellular differentiation regulated by the environment: heterocyst formation is repressed by the presence of combined nitrogen (39).

Anabaena sp. strain PCC 7120 is a filamentous cyanobacterium that is capable of both oxygenic photosynthesis and aerobic nitrogen fixation. Vegetative cells are the site for photosynthesis, while nitrogen fixation occurs in terminally differentiated cells called heterocysts. Filaments of *Anabaena* sp. strain PCC 7120 grown in the presence of combined nitrogen, such as $NaNO_3$, contain only vegetative cells. Once the source of combined nitrogen in the medium becomes limiting, approximately 1 in every 10 vegetative cells in the filament differentiates into a heterocyst. The two cell types are interdependent: the vegetative cells depend on heterocysts for fixed nitrogen, and heterocysts depend on vegetative cells for the photosynthate as the source of the reductant. Several structural, biochemical, and genetic changes take place during the differentiation of heterocysts. These include synthesis of a multilayered cell envelope, formation of narrow channels between the two cell types, reorganization of internal membranes that results in the loss of photosystem II and accessory pigments, rearrangement of DNA elements from within nitrogen fixation genes and hydrogen uptake genes, and expression of genes specifically required for nitrogen fixation (8, 39).

Several genes that are involved in the regulation of heterocyst differentiation in *Anabaena* sp. strain PCC 7120 have been identified. *hetR* is an autoregulated gene necessary for activation of other genes required for differentiation of heterocysts. Inactivation of *hetR* blocks differentiation of heterocysts, and strains containing extra copies of *hetR* form heterocysts even in media containing a source of combined nitrogen (5, 6). Mutations in *hetP* also block heterocyst differentiation, and strains containing extra copies of *hetP* form multiple contiguous heterocysts in media lacking combined nitrogen (14). Another regulatory gene, *ntcA*, which is required for turning on genes involved in nitrogen metabolism, also affects heterocyst differentiation (15, 37).

In our efforts to identify genes that affect heterocyst development in *Anabaena* sp. strain PCC 7120, nitrosoguanidine-mutagenized cells were screened for mutants that formed heterocysts on nitrate-containing medium. One mutant, AMC260, formed 6 to 8% heterocysts when grown in a medium containing nitrate, a phenotype that we will refer to as heterocyst constitutive (Het^c). We show that the Het^c phenotype of AMC260 is due to the inability of the cells to reduce nitrate. This article reports the identification of the *moeA* gene, which is involved in molybdopterin biosynthesis.

MATERIALS AND METHODS

Strains and growth conditions. *Escherichia coli* strains were grown as previously described (16). For selective growth, the media were supplemented with 100 μg of ampicillin, 50 μg of kanamycin, 17.5 μg of chloramphenicol, and 50 μg of spectinomycin per ml. *Anabaena* sp. strain PCC 7120 and derived strains were grown on BG-11 or BG-11_0 medium essentially as previously described (16, 37) except as noted. For *Anabaena* sp. strain PCC 7120 cultures, the following antibiotics were used at the indicated final concentrations: neomycin, 25 μg/ml, and spectinomycin and streptomycin, 2 μg/ml each for solid medium and 1 μg/ml each for liquid medium.

Nitrosoguanidine mutagenesis. Twenty milliliters of *Anabaena* sp. strain 7120 cells from a mid-log-phase culture were collected after centrifugation at 2,700 × *g* for 5 min. The cells were washed with 10 mM citrate buffer, pH 6, and then resuspended in 18 ml of the same buffer. Two milliliters of 1-mg/ml *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine was added to the resuspended cells. After 90 min at room temperature, the cells were collected by centrifugation and washed twice with 20 ml of BG-11 medium. The cells were then resuspended in 100 ml of BG-11 medium and allowed to recover for 4 to 6 days at a light intensity of 80 to 100 microeinsteins $m^{-2}\ s^{-1}$ at 30°C until the culture turned green. Filaments were fragmented by sonication to lengths of two to three cells each and spread onto BG-11 plates to obtain approximately 500 colonies per plate. Colonies were screened individually at a magnification of ×400 with a light microscope for filaments forming heterocysts constitutively on the nitrate-containing medium. Photomicrographs were taken with an Olympus BX50 microscope with Nomarski (differential interference contrast) optics on Kodak Ektachrome 160T color slide film.

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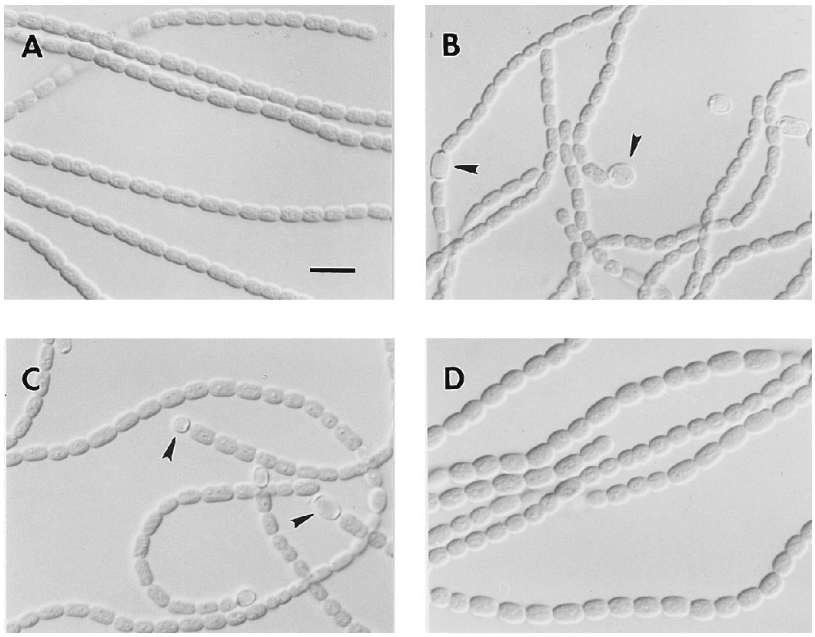

FIG. 1. Light micrographs of *Anabaena* sp. strain PCC 7120 wild-type and *moeA* mutants. (A) Wild-type *Anabaena* sp. strain PCC 7120; (B) *moeA* mutant strain AMC260; (C) reconstructed *moeA* mutant strain AMC364; (D) complemented strain AMC363. All cultures were grown to early stationary phase on BG-11 medium, which contains $NaNO_3$. Arrowheads indicate heterocysts. Bar, 10 μm.

Cosmid shuttle vector library. An *Anabaena* sp. strain PCC 7120 cosmid library containing 20- to 40-kb fragments of vegetative-cell DNA was made in the conjugal shuttle vector pDUCA7M. pDUCA7M is a modified version of pDUCA7 (7) that lacks the *Sal*I site between *oriT* and *oriV*. Total genomic DNA from *Anabaena* sp. strain PCC 7120 was partially digested with *Cpf*I, and the fraction containing 20- to 40-kb fragments was isolated from a preparative agarose gel and dephosphorylated. Complementary pDUCA7M "arms" were generated by digestion with two separate sets of restriction endonucleases. One set was *Bam*HI and *Sal*I, and the other was *Bam*HI and *Kpn*I. After digestion, the pDUCA7M vector arm fragments containing the *cos* site were purified by gel electrophoresis. The *Anabaena* sp. strain PCC 7120 *Cpf*I insert fragments were ligated with both pDUCA7M vector arms in a molar ratio of 1:1:1 and packaged in vitro into bacteriophage lambda (26). Packaged phage particles were then used to infect *E. coli* AM830, which is strain DH5αMCR containing the helper plasmid pRL528 (12). Infected cells were plated on medium containing kanamycin and chloramphenicol. Approximately 1,200 primary clones were inoculated into individual wells of 96-well plates.

The cosmid library was transferred into strain AMC260 by triparental mating (12). Exconjugant colonies were selected with neomycin, and complementation of the Het^c phenotype was determined by light microscopy.

Inactivation of *moeA* in *Anabaena* sp. strain PCC 7120. A 315-bp *Msp*I fragment internal to the *moeA* gene was cloned into the vector pAM580 to create pAM1546. pAM580 is a Sp^r and Sm^r shuttle vector that cannot replicate in *Anabaena* sp. strain PCC 7120 (9). pAM1546 was transferred into *Anabaena* sp. strain PCC 7120 via conjugation, and exconjugants were selected on BG-11 plates containing 2 μg each of spectinomycin and streptomycin per ml. One exconjugant, AMC364, was analyzed further for interruption of the *moeA* gene; insertion of pAM1546 into the *moeA* locus was confirmed by Southern blot analysis.

DNA manipulations. DNA manipulations and recombinant-DNA techniques were performed as previously described (9). *Anabaena* sp. strain PCC 7120 vegetative-cell DNA was isolated as described previously (16). Restriction endonucleases and other DNA-modifying enzymes were used according to the manufacturer's recommendations or standard protocols. For Southern analysis, DNA was transferred from agarose gels to Magna Charge membranes (Micron Separations Inc.) with 50 mM NaOH–1 M NaCl. DNA probes were labeled with a random-primer kit (Boehringer Mannheim), and Southern hybridizations were performed as described previously (16).

PCR and DNA sequencing. The 1.8-kb insert from pAM1550 was subcloned from the pDUCA7M vector as a *Sma*I-*Nhe*I fragment into pBluescript SK(+) to form pAM1551. Double-stranded sequencing of the entire insert was done by the Advanced DNA Technologies Laboratory, Department of Biology, Texas A&M University. Sequence data were analyzed by the sequence analysis software package of the Genetics Computer Group (Madison, Wis.) and the BLAST program (1) accessed through the National Center for Biotechnology Information GenBank electronic mail server.

Identification of the mutation in AMC260 was done by direct sequencing of PCR fragments generated from approximately 5 ng of genomic DNA. PCR was carried out in a MiniCycler (MJ Research, Cambridge, Mass.) with *Taq* DNA polymerase. For amplification of the *moeA* gene from the AMC260 chromosome, the primers 5′-CCAACTGCAACAAGTAG-3′ and 5′-GAGTCTGTT TATGTCG-3′ were used; the reaction consisted of 40 cycles of 94°C for 1 min, 38°C for 1 min, and 72°C for 2 min. The amplified fragment was sequenced with a SequiTherm cycle sequencing kit (Epicentre Technologies, Madison, Wis.) and [α-^{32}P]dATP (DuPont/NEN Research Products). The base change identified at position 936 was confirmed by sequencing the other strand from a PCR fragment produced in an independent amplification reaction.

Northern (RNA) analysis. RNA from vegetative cells of *Anabaena* sp. strain PCC 7120 was prepared from 100-ml cultures by an acidic hot phenol method (29). RNA from differentiating filaments was prepared from frozen samples of a large-scale heterocyst induction of wild-type *Anabaena* sp. strain PCC 7120 as previously described (37). Thirty-microgram RNA samples were transferred from a formaldehyde-agarose gel to a Magna Charge membrane and the Northern blot was hybridized as previously described (16). A strand-specific probe was generated by following the random-primer labeling protocol except that a single specific primer was used.

Primer extension analysis. The 5′ termini of *moeA* transcripts were identified by primer extension analysis with the primers AMO-176 (5′-GCTGCCCATA AATCGAC-3′) and AMO-177 (5′-GCTGTATCCCTGACTGA-3′). The primers were labeled at their 5′ ends with [γ-^{32}P]ATP and T4 polynucleotide kinase, and primer extension analysis was done as described previously (33). Forty micrograms of RNA isolated from vegetative cells was used. DNA sequencing ladders were generated with the respective primers. Minor primer extension products greater and smaller than the major band seen with AMO-176 (see Fig. 6) were absent when AMO-177 was used.

Determination of nitrate reductase activity. Nitrate reductase activity was measured in wild-type *Anabaena* sp. strain PCC 7120, AMC260, AMC364, and AMC363 with a methyl viologen-based assay (17).

Growth rate determination. Cultures were grown on a rotating platform at 30°C and a light intensity of 80 to 100 microeinsteins m^{-2} s^{-1}. Growth was monitored spectrophotometrically as optical density at 750 nm. For BG-11 medium, 3-ml samples were removed from 100-ml cultures at 24-h intervals for optical density measurements. To avoid pipetting errors caused by filament clumping, individual 2-ml $BG\text{-}11_0$ medium cultures were grown in test tubes (12 by 100 mm). For each measurement, a tube was removed from the incubator and the filaments were dispersed by brief treatment in a sonic water bath. Plotted values are the means of three separate samples. Antibiotics were not added to media during growth rate experiments.

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited in GenBank under the accession number U34309.

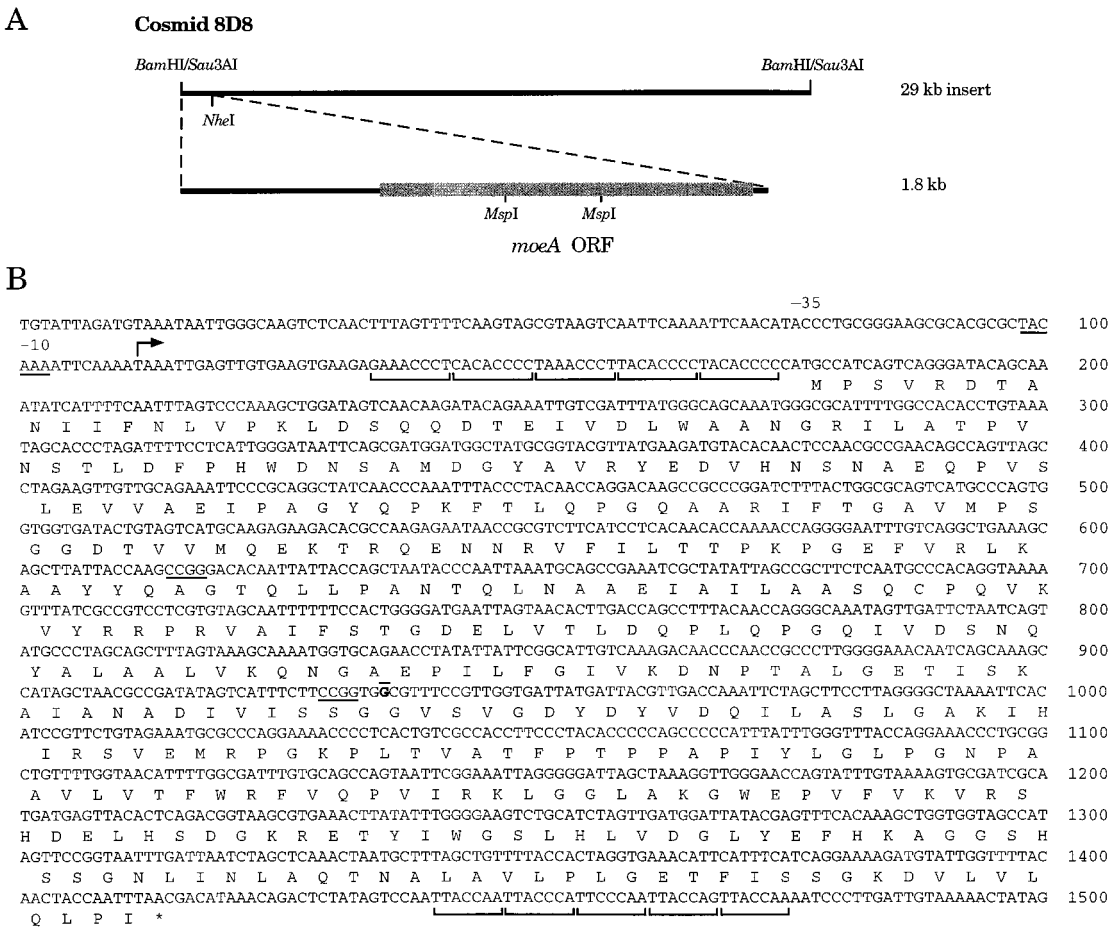

FIG. 2. Cosmid clone 8D8 and nucleotide sequence of *moeA*. (A) Location of *moeA* on cosmid 8D8. The *Msp*I sites indicate the internal fragment of *moeA* used for insertional mutagenesis. (B) Nucleotide sequence and predicted amino acid sequence of *moeA*. The putative transcription start site is indicated by an arrow, and regions corresponding to −10 and −35 are indicated. *Msp*I sites used for insertional inactivation of *moeA* are underlined. The guanosine residue at position 936, which was replaced by adenine in mutant AMC260, is shown in boldface and overlined. Five 8-bp repeats with a consensus sequence of TACACCCC are marked by half brackets upstream of the start codon, and five 7-bp repeats with a consensus sequence of TTACCAA are indicated downstream of the stop codon. Asterisk, stop codon.

RESULTS AND DISCUSSION

Complementation of the Het^c mutant AMC260. *Anabaena* sp. strain PCC 7120 was chemically mutagenized with nitrosoguanidine, and treated cells were plated on BG-11 medium, which contains $NaNO_3$ as the source of combined nitrogen. Surviving colonies were individually screened under a light microscope for filaments that formed heterocysts. Six Het^c mutants were isolated. The percentage of heterocysts in the different mutant strains was between 4 and 20%. The presence of ammonia in the growth medium repressed the Het^c phenotype in all the mutants. One mutant, AMC260, formed 6 to 8% heterocysts in BG-11 medium (Fig. 1B) and 8 to 10% heterocysts in BG-11_0 medium, which lacks a source of combined nitrogen.

The mutation in the AMC260 strain was complemented with a cosmid library containing 20- to 40-kb fragments of *Cpf*I-digested *Anabaena* sp. strain PCC 7120 chromosomal DNA cloned into shuttle vector pDUCA7M. One cosmid clone, 8D8, complemented the mutation in AMC260 and allowed suppression of heterocysts on BG-11 medium. The 8D8 cosmid insert was mapped to approximately 6.2 Mb (24) on the *Anabaena* sp. strain PCC 7120 chromosome by hybridization to Southern blots of pulsed-field gels. The 8D8 probe hybridized to *Sal*I fragment I, *Pst*I fragment F, and the *Bln*I (*Avr*II) fragment F doublet (F2 by deduction; data not shown). It has previously been shown that mutants affected in nitrate uptake and metabolism have a Het^c phenotype on nitrate-containing media (2, 27). Since 8D8 mapped to a locus distant from other known genes involved in nitrogen metabolism, including *narA*, *narC*, and *ntcA* (3, 38), we were encouraged to pursue this new locus.

In order to narrow down the region necessary for complementation of the Het^c phenotype of AMC260, cosmid 8D8 was partially digested with *Cla*I and religated. Subclones containing different *Cla*I fragments were transferred into strain AMC260 via conjugation. A cosmid subclone that had lost all internal *Cla*I fragments still complemented the AMC260 mutant. Further subcloning by removal of an *Nhe*I fragment resulted in a clone, pAM1550, containing only 1.8 kb of *Anabaena* sp. strain PCC 7120 DNA; strain AMC363, which is AMC260 containing pAM1550, showed complementation of the Het^c phenotype (Fig. 1D). Sequencing of the 1.8-kb fragment identified a 1.2-kb open reading frame that encodes a 413-amino-acid polypeptide with a predicted molecular weight of 44,534 and a pI of 6.1 (Fig. 2). Comparison of the predicted amino acid sequence with database sequences revealed 56% similarity and

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7120  ......MPSVRDTANIIFNLVPKLDSQQDTEIVDLWAANGRILATPVNST   44
E.c.  MEFTTGLMSLDTALNEMLSRVTPLTAQETLPLVQCF...GRILASDVVSP   47

7120  LDFPHWDNSAMDGYAVRYEDVHNSNAEQPVSLEVVAEIPAGYQPKFTLQP   94
E.c.  LDVPGFDNSAMDGYAVRLADI...ASGQPLPVAGKSFAGQPYHGEW..PA   92

7120  GQAARIFTGAVMPSGGDTVVMQEKTRQENNRVFILTTPKPGEFVRLKAAY  144
E.c.  GTCIRIMTGAPVPEGCEAVVMQEQTEQMDNGVRFTAEVRSGQNIRRRGED  142

7120  YQAGTQLLPANTQLNAAEIAILAASQCPQVKVYRRPRVAIFSTGDELVTL  194
E.c.  ISAGAVVFPAGTRLTTAELPVIASLGIAEVPVIRKVRVALFSTGDELQLP  192

7120  DQPLQPGQIVDSNQYALAALVKQNGAEPILFGIVKDNPTALGETISKAIA  244
E.c.  GQPLGDGQIYDTNRLAVHLMLEQLGCEVINLGIIRDDPHALRAAFIEADS  242

7120  NADIVISSGGVSVGDYDYVDQILASLGAKIHIRSVEMRPGKPLTVATFPT  294
E.c.  QADVVISSGGVSVGEADYTKTILEELG.EIAFWKLAIKPGKPFAFGKL..  289

7120  PPAPIYLGLPGNPAAVLVTFWRFVQPVIRKLGGLAKGWEPVFVKVRSHDE  344
E.c.  .SNSWFCGLPGNPVSATLTFYQLVQPLLAKLSGNTASGLPARQRVRTASR  338

7120  LHSDGKRETYIWGSLH.LVDGLYEFHKAGGSHSSGNLINLAQTNALAVLP  393
E.c.  LKKTPGRLDFQRGVLQRNADGELEV.TTTGHQGSHIFSSFSLGNCFIVLE  387

7120  LGETFISSGKDVLVLQLPI*                               413
E.c.  RDRGNVEVGEWVEVEPFNALFGGL*                           411
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FIG. 3. Comparison of the predicted MoeA proteins from *Anabaena* sp. strain PCC 7120 and *E. coli* (E.c.). The amino acid sequences of *Anabaena* sp. strain PCC 7120 (413 amino acids) and *E. coli* (411 amino acids) were aligned with the GAP program (default settings) from the Genetics Computer Group software package. The proteins showed 56% similarity and 37% identity over their entire lengths. Vertical lines indicate identical amino acids, and single or double dots indicate evolutionarily conserved amino acid substitutions. Glycine 254 (boldface and underlined) is changed to aspartic acid in the Het^c strain AMC260. Asterisk, stop codon.

37% identity to the 411-amino-acid *E. coli* MoeA protein (Fig. 3).

In *E. coli*, MoeA is necessary for the synthesis of the molybdenum-containing cofactor molybdopterin (23, 30). The *moe* locus contains two translationally coupled genes, *moeA* and *moeB*, both of which are implicated in molybdopterin biosynthesis (30, 40). All molybdoenzymes except nitrogenase share a cofactor, molybdopterin, which is a complex of a novel six-alkyl pterin with a Mo atom (19, 32). Molybdopterin is widely distributed in many organisms and is required for several essential metabolic pathways (18, 21, 22, 25, 31). *E. coli* genes involved in molybdopterin biosynthesis have been identified by screening for mutants that are able to grow in the presence of chlorate. Enzymes containing molybdopterin reduce chlorate to chlorite, which is lethal to cells. Examples of Mo cofactor-requiring enzymes in *E. coli* include nitrate reductase (18, 21), formate dehydrogenase (21, 25), and biotin-D-sulfoxide reductase (11). Chlorate-resistant mutants of cyanobacteria have also been isolated: *Synechococcus* strain PCC 7002 mutants were defective in nitrate reductase activity (36), and *Nostoc muscorum* mutants were defective in nitrate reductase activity and nitrate uptake (2).

The sequences downstream of the *moeA* gene showed the presence of five tandem heptanucleotide repeats that have a consensus sequence of TTACCAA (Fig. 2). Mazel et al. have classified similar short tandemly repeated repetitive sequences, designated STRR sequences, from various cyanobacteria (28). The consensus repeat (opposite strand) found downstream of the *moeA* gene is categorized under the family STRR2 (28). Sequences upstream of the *moeA* open reading frame showed the presence of five tandemly repeated octamer repeats with a consensus sequence of TACACCCC (Fig. 2). This sequence shows some similarity to the STRR1 heptamer (28). It is not known what roles such repeats play, but they have been shown to be present within and between open reading frames in many cyanobacteria (4, 28), and they have been used for taxonomic and ecological studies (28, 34).

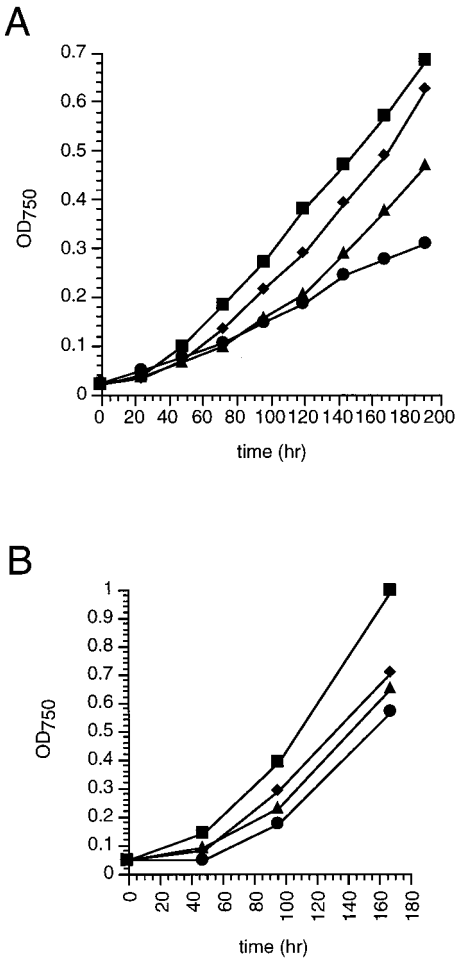

FIG. 4. Growth of wild-type *Anabaena* sp. strain PCC 7120 (■), Het^c mutant strain AMC260 (●), reconstructed mutant AMC364 (▲), and complemented strain AMC363 (◆) on BG-11 medium (A) and on BG-11$_0$ medium (B), which lacks nitrate. OD_{750}, optical density at 750 nm.

Inactivation of the *moeA* gene in *Anabaena* sp. strain PCC 7120. To verify that the Het^c phenotype of the AMC260 strain is due to a mutation in the *moeA* gene, an insertional mutation was created by using an internal fragment of the *moeA* gene. A 315-bp *Msp*I fragment (Fig. 2) was cloned into the mobilizable but nonreplicating vector pAM580 to create pAM1546. Single recombination of pAM1546 with the *Anabaena* sp. strain PCC 7120 chromosome results in two incomplete copies of the *moeA* gene, one lacking the promoter and 5′-end sequences and the other lacking 3′-end sequences. Exconjugant strains resistant to spectinomycin and streptomycin were obtained when plasmid pAM1546 was transferred into *Anabaena* sp. strain PCC 7120 via conjugation. Southern blot analysis of one of the exconjugant strains, AMC364, confirmed the integration of pAM1546, via a single recombinational event, into the *moeA* gene (data not shown). AMC364 formed 8 to 10% heterocysts on nitrate-containing medium (Fig. 1C), values which are similar to those of the original mutant strain AMC260 (Fig. 1B).

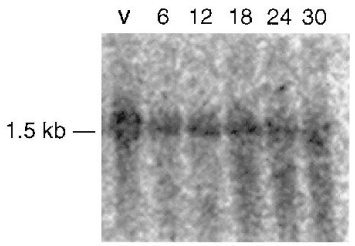

FIG. 5. Northern analysis of *moeA* transcripts from filaments grown in nitrate-containing medium and during induction of heterocyst differentiation. Total RNA was isolated from wild-type *Anabaena* sp. strain PCC 7120 vegetative cells grown in BG-11 medium (lane V) and from filaments collected at the indicated times (in hours) after nitrogen step-down. The position of the 1.5-kb *moeA* transcript is indicated.

The Het^c phenotype of the *moeA* insertional mutant strongly suggests that the phenotype of AMC260 is due to a mutation in the *moeA* gene.

We determined the sequence of *moeA* in AMC260. The *moeA* gene was amplified from chromosomal DNA by PCR. Direct sequencing of the PCR product revealed one base change from G to A at position 936 in the *moeA* sequence (Fig. 2B). This transversion would result in the incorporation of aspartic acid instead of glycine in the predicted MoeA protein sequence. The mutation is in a region of the MoeA protein that is highly conserved between *Anabaena* sp. strain PCC 7120 and *E. coli* (Fig. 3).

Nitrate reductase activity. Since molybdopterin is a component of nitrate reductase, we determined the activity of this enzyme in the *moeA* mutant strains. Nitrate reductase activity was determined for wild-type *Anabaena* sp. strain PCC 7120, Het^c strains AMC260 and AMC364, and the AMC260-complemented strain AMC363. *Anabaena* sp. strain PCC 7120 and AMC363 showed similar levels of nitrate reductase activity, 30 nmol of NO_2^- mg of protein^{-1} min^{-1}, whereas the Het^c mutants AMC260 and AMC364 showed nitrate reductase activity of only 2 nmol of NO_2^- mg of protein^{-1} min^{-1}. The absence of nitrate reductase activity in the Het^c strains is consistent with the inability of these strains to synthesize functional molybdopterin. The presence of the *moeA* gene in *trans* on a shuttle vector in strain AMC363 restores nitrate reductase activity, presumably by restoring molybdopterin synthesis. We conclude that the Het^c phenotype of the *moeA* mutant strains AMC260 and AMC364 is due to the inability of these strains to utilize nitrate as their nitrogen source and that therefore they form heterocysts to utilize atmospheric dinitrogen.

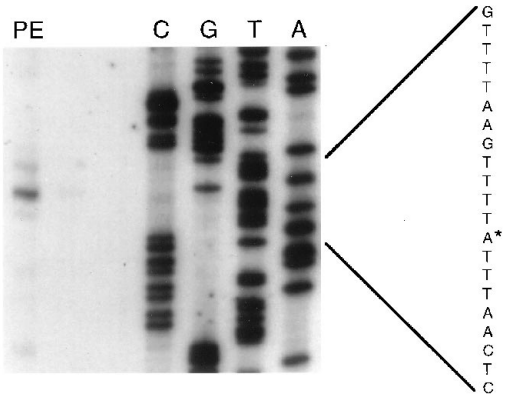

FIG. 6. Primer extension analysis of *moeA* transcripts. Primer AMO-176 was annealed with 40 μg of RNA isolated from vegetative cells grown on BG-11 medium, and the primer extension products (lane PE) were run on a sequencing gel and detected by autoradiography. The sequencing ladders (lanes C, G, T, and A), which represent the template strand, were generated with the primer used for primer extension. The putative transcription start site was mapped to the A residue of the template strand (marked with an asterisk) 64 bp upstream of the ATG start codon.

Growth rate determination. Growth of the original Het^c mutant AMC260 and the reconstructed mutant AMC364 on BG-11 and BG-11$_0$ media was significantly slower than that of wild-type *Anabaena* sp. strain PCC 7120 (Fig. 4). Although the lower growth rate of the mutants on nitrate-containing medium could be due to the presence of heterocysts, which are nondividing cells, their lower growth rate on medium lacking nitrate suggests that they have additional defects. Since molybdopterin is required for enzymes other than nitrate reductase, the *moeA* mutant strains are likely to be deficient in the activity of other molybdoenzymes. This may explain their lower growth rate. Although the complemented strain AMC363 grew at a wild-type rate on BG-11 medium, it grew more slowly than the wild type on BG-11$_0$ medium. The level of expression of *moeA* from the shuttle vector in this strain may not equal wild-type levels on BG-11$_0$ medium.

Transcription of *moeA* during heterocyst induction. The expression of *moeA* in filaments during induction of heterocysts was examined by Northern analysis. A 1.5-kb transcript was detected in RNA from vegetative cells as well as from filaments at all tested time points after induction; there was, however, a gradual decline in message abundance after the nitrogen step-down (Fig. 5). The size of the *moeA* transcript indicates a monocistronic message. The pattern of expression differs from that of heterocyst-specific genes such as *nifH* and from that of genes such as *ntcA* and *glnA*, which are expressed both in the presence of nitrate and in nitrogen-fixing filaments (37). The lower level of *moeA* transcript present in nitrogen-fixing filaments may be related to the lower level of nitrate reductase activity present in filaments grown on dinitrogen (27). A basal level of *moeA* transcription may be required for the synthesis of other molybdopterin-containing molybdoenzymes (32).

Mapping the 5′ termini of *moeA* mRNAs. Two oligonucleotide primers, AMO-176 and AMO-177, were used to prime cDNA synthesis with RNA isolated from vegetative cells grown in the presence of nitrate. Figure 6 shows the result of mapping the 5′ termini of *moeA* transcripts by using the AMO-176 primer. AMO-176 yielded a predominant extension product that maps the *moeA* transcription start site to position 112 (as shown in Fig. 1B), which is 64 nucleotides upstream of the translation start codon. Primer extension with AMO-177 yielded a product that also mapped the start site to position 112 (data not shown). The agreement between these two results indicates that the thymine residue at position 112 is the putative start site of *moeA* transcription. Sequences upstream from this start site show similarity to the *E. coli* σ^{70} −10 consensus hexamer (Fig. 1B). No similarity to the *E. coli* σ^{70} −35 consensus is present. However, most *Anabaena* sp. strain PCC 7120 genes do not show a consensus sequence in the −35 region (10).

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.

2. **Bagchi, S. N., and H. N. Singh.** 1984. Genetic control of nitrate reduction in the cyanobacterium *Nostoc muscorum*. Mol. Gen. Genet. **193:**82–84.
3. **Bancroft, I., C. P. Wolk, and E. V. Oren.** 1989. Physical and genetic maps of the genome of the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **171:**5940–5948.
4. **Bauer, C. C., L. Scappino, and R. Haselkorn.** 1993. Growth of the cyanobacterium *Anabaena* on molecular nitrogen: NifJ is required when iron is limited. Proc. Natl. Acad. Sci. USA **90:**8812–8816.
5. **Black, T. A., Y. Cai, and C. P. Wolk.** 1993. Spatial expression and autoregulation of *hetR*, a gene involved in the control of heterocyst development in *Anabaena*. Mol. Microbiol. **9:**77–84.
6. **Buikema, W. J., and R. Haselkorn.** 1991. Characterization of a gene controlling heterocyst differentiation in the cyanobacterium *Anabaena* 7120. Genes Dev. **5:**321–330.
7. **Buikema, W. J., and R. Haselkorn.** 1991. Isolation and complementation of nitrogen fixation mutants of the cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **173:**1879–1885.
8. **Buikema, W. J., and R. Haselkorn.** 1993. Molecular genetics of cyanobacterial development. Annu. Rev. Plant Physiol. Plant Mol. Biol. **44:**33–52.
9. **Carrasco, C. D., K. S. Ramaswamy, T. S. Ramasubramanian, and J. W. Golden.** 1994. *Anabaena xisF* gene encodes a developmentally regulated site-specific recombinase. Genes Dev. **8:**74–83.
10. **Curtis, S. E., and J. A. Martin.** 1994. The transcription apparatus and the regulation of transcription initiation, p. 613–639. *In* D. A. Bryant (ed.), The molecular biology of cyanobacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
11. **Del Campillo-Campbell, A., and A. Campbell.** 1982. Molybdenum cofactor requirement for biotin sulfoxide reduction in *Escherichia coli*. J. Bacteriol. **149:**469–478.
12. **Elhai, J., and C. P. Wolk.** 1988. Conjugal transfer of DNA to cyanobacteria. Methods Enzymol. **167:**747–754.
13. **Errington, J.** 1993. *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. Microbiol. Rev. **57:**1–33.
14. **Fernández-Piñas, F., F. Leganés, and C. P. Wolk.** 1994. A third genetic locus required for the formation of heterocysts in *Anabaena* sp. strain PCC 7120. J. Bacteriol. **176:**5277–5283.
15. **Frías, J. E., E. Flores, and A. Herrero.** 1994. Requirement of the regulatory protein NtcA for the expression of nitrogen assimilation and heterocyst development genes in the cyanobacterium *Anabaena* sp. PCC 7120. Mol. Microbiol. **14:**823–832.
16. **Golden, J. W., L. L. Whorff, and D. R. Wiest.** 1991. Independent regulation of *nifHDK* operon transcription and DNA rearrangement during heterocyst differentiation in the cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **173:**7098–7105.
17. **Herrero, A., E. Flores, and M. G. Guerrero.** 1981. Regulation of nitrate reductase levels in the cyanobacteria *Anacystis nidulans*, *Anabaena* sp. strain 7119, and *Nostoc* sp. strain 6719. J. Bacteriol. **145:**175–180.
18. **Hewitt, E. J., and B. A. Notton.** 1980. Nitrate reductase systems in eukaryotic and prokaryotic organisms, p. 273–326. *In* M. P. Coughlan (ed.), Molybdenum and molybdenum-containing enzymes. Pergamon Press, Inc., Elmsford, N.Y.
19. **Hinton, S. M., and D. Dean.** 1990. Biogenesis of molybdenum cofactors. Crit. Rev. Microbiol. **17:**169–188.
20. **Horvitz, H. R., and I. Herskowitz.** 1992. Mechanisms of asymmetric cell division: two Bs or not to Bs that is the question. Cell **68:**237–255.
21. **Ingledew, W. J., and R. K. Poole.** 1984. The respiratory chains of *Escherichia coli*. Microbiol. Rev. **48:**222–271.
22. **Johnson, J. L.** 1980. The molybdenum cofactor common to nitrate reductase, xanthine dehydrogenase and sulfite oxidase, p. 345–383. *In* M. P. Coughlan (ed.), Molybdenum and molybdenum-containing enzymes. Pergamon Press, Inc., Elmsford, N.Y.
23. **Johnson, M. E., and K. V. Rajagopalan.** 1987. Involvement of *chlA*, *E*, *M*, and *N* loci in *Escherichia coli* molybdopterin biosynthesis. J. Bacteriol. **169:** 117–125.
24. **Kuritz, T., A. Ernst, T. A. Black, and C. P. Wolk.** 1993. High-resolution mapping of genetic loci of *Anabaena* PCC 7120 required for photosynthesis and nitrogen fixation. Mol. Microbiol. **8:**101–110.
25. **Ljungdahl, L. G.** 1980. Formate dehydrogenases: role of molybdenum, tungsten and selenium, p. 463–486. *In* M. P. Coughlan (ed.), Molybdenum and molybdenum-containing enzymes. Pergamon Press, Inc., Elmsford, N.Y.
26. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Martin-Nieto, J., A. Herrero, and E. Flores.** 1991. Control of nitrogenase mRNA levels by products of nitrate assimilation in the cyanobacterium *Anabaena* sp. strain PCC 7120. Plant Physiol. (Bethesda) **97:**825–828.
28. **Mazel, D., J. Houmard, A. M. Castets, and N. Tandeau de Marsac.** 1990. Highly repetitive DNA sequences in cyanobacterial genomes. J. Bacteriol. **172:**2755–2761.
29. **Mohamed, A., and C. Jansson.** 1989. Influence of light on accumulation of photosynthesis-specific transcripts in the cyanobacterium *Synechocystis* 6803. Plant Mol. Biol. **13:**693–700.
30. **Nohno, T., Y. Kasai, and T. Saito.** 1988. Cloning and sequencing of the *Escherichia coli chlEN* operon involved in molybdopterin biosynthesis. J. Bacteriol. **170:**4097–4102.
31. **Rajagopalan, K. V.** 1980. Sulphite oxidase, p. 241–272. *In* M. P. Coughlan (ed.), Molybdenum and molybdenum-containing enzymes. Pergamon Press, Inc., Elmsford, N.Y.
32. **Rajagopalan, K. V., and J. L. Johnson.** 1992. The pterin molybdenum cofactors. J. Biol. Chem. **267:**10199–10202.
33. **Ramasubramanian, T. S., T.-F. Wei, A. K. Oldham, and J. W. Golden.** 1996. Transcription of the *Anabaena* sp. strain PCC 7120 *ntcA* gene: multiple transcripts and NtcA binding. J. Bacteriol. **178:**922–926.
34. **Rouhiainen, L., K. Sivonen, W. J. Buikema, and R. Haselkorn.** 1995. Characterization of toxin-producing cyanobacteria by using an oligonucleotide probe containing a tandemly repeated heptamer. J. Bacteriol. **177:**6021–6026.
35. **Shapiro, L., D. Kaiser, and R. Losick.** 1993. Development and behavior in bacteria. Cell **73:**835–836.
36. **Stevens, S. E., Jr., and R. L. Smith.** 1994. Isolation and characterization of five genotypic mutants of chlorate-resistant cyanobacteria unable to utilize nitrate. Curr. Microbiol. **29:**311–318.
37. **Wei, T.-F., T. S. Ramasubramanian, and J. W. Golden.** 1994. *Anabaena* sp. strain PCC 7120 *ntcA* gene required for growth on nitrate and heterocyst development. J. Bacteriol. **176:**4473–4482.
38. **Wei, T.-F., T. S. Ramasubramanian, F. Pu, and J. W. Golden.** 1993. *Anabaena* sp. strain PCC 7120 *bifA* gene encoding a sequence-specific DNA-binding protein cloned by in vivo transcriptional interference selection. J. Bacteriol. **175:**4025–4035.
39. **Wolk, C. P., A. Ernst, and J. Elhai.** 1994. Heterocyst metabolism and development, p. 769–823. *In* D. A. Bryant (ed.), The molecular biology of cyanobacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
40. **Wuebbens, M. M., and K. V. Rajagopalan.** 1995. Investigation of the early steps of molybdopterin biosynthesis in *Escherichia coli* through the use of *in vivo* labeling studies. J. Biol. Chem. **270:**1082–1087.