

## The *narA* Locus of *Synechococcus* sp. Strain PCC 7942 Consists of a Cluster of Molybdopterin Biosynthesis Genes

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**The *narA* locus required for nitrate reduction in *Synechococcus* sp. strain PCC 7942 is shown to consist of a cluster of genes, namely, *moaA*, *moaC*, *moaD*, *moaE*, and *moaA*, involved in molybdenum cofactor biosynthesis. The product of the *moaC* gene of strain PCC 7942 shows homology in its N-terminal half to MoaC from *Escherichia coli* and in its C-terminal half to MoaB or Mog. Overexpression of the *Synechococcus moaC* gene in *E. coli* resulted in the synthesis of a polypeptide of 36 kDa, a size that would conform to a protein resembling a fusion of the MoaC and MoaB or Mog polypeptides of *E. coli*. Insertional inactivation of the *moaA*, *moaC*, *moaE*, and *moaA* genes showed that the *moaA*-*moaA* gene cluster is required for growth on nitrate and expression of nitrate reductase activity in strain PCC 7942. The *moaCDEA* genes constitute an operon which is transcribed divergently from the *moaA* gene. Expression of the *moaA* gene and the *moa* operon was little affected by the nitrogen source present in the culture medium.**

Nitrate is probably the most abundant source of combined nitrogen for cyanobacterial nutrition, its assimilation being a process closely linked to photosynthesis (9). Nitrate is transported into the cyanobacterial cell by a multicomponent transport system of the ABC (ATP-binding cassette) type (34). Once inside the cell, nitrate is reduced to ammonium by two sequential reactions catalyzed by nitrate reductase and nitrite reductase, respectively. Ammonium is incorporated into carbon skeletons mainly via the glutamine synthetase/glutamate synthase cycle (9).

In *Synechococcus* sp. strain PCC 7942, the *nir* gene encoding nitrite reductase (22), the *nrtABCD* genes encoding the components of the nitrate transport system (34), and the *narB* gene encoding nitrate reductase (1, 45) are clustered together and constitute an operon (24, 51). Two other loci, *narA* and *narC*, involved in nitrate reduction in *Synechococcus* sp. strain PCC 7942 have been identified and cloned by means of complementation of nitrate reductase-deficient, Tn901-induced mutants with a gene library from strain PCC 7942 (20, 21). These loci are not clustered together in the *Synechococcus* genome. With regard to regulation, ammonium acts as a nutritional repressor of the nitrate assimilation system (9). The NtcA protein found in cyanobacteria (9, 10, 52) acts as a transcriptional activator that controls the expression of cyanobacterial genes subjected to repression by ammonium such as the *nir* operon (23).

Nitrate reductases from cyanobacteria are monomeric molybdoenzymes of about 75 kDa that use reduced ferredoxin as a physiological electron donor (9). Molybdoenzymes other than nitrogenase catalyze either oxidative hydroxylations or reductive dehydroxylations, and its molybdenum center is constituted by a molybdenum-pterin cofactor in the form of molybdopterin (MPT), molybdopterin guanine dinucleotide (MGD), molybdopterin cytosine dinucleotide, or others (40). In *Escherichia coli*, the pathway for molybdenum cofactor biosynthesis is the subject of intense research. The genes responsible for the transport of molybdate (*modABC*) (29), for MPT

biosynthesis (*moaABCDE* and *moaAB*) (33, 36, 44), and for assembly of molybdenum into MPT (*mog*) (18) have been identified and sequenced, as is also the case for *mobA*, which is involved in the addition of GMP to MPT during the synthesis of the MGD form of the molybdenum cofactor (16, 17, 35).

In cyanobacteria, information about molybdenum cofactors is scarce. Some molybdenum cofactor, partially bound to a carrier protein that would stabilize the cofactor, has been reported to be present in the soluble fraction of *Nostoc muscorum* (3). In *Anabaena variabilis*, the existence of common and specific genes for the synthesis of the iron-molybdenum (of nitrogenase) and molybdenum cofactors has been inferred (28); in *Anabaena* sp. strain PCC 7120, inactivation of a *moaA* gene leads to loss of nitrate reductase activity (41), while the *moaB*-like *hesA* gene found downstream of the *nifHDK* operon seems to be necessary for attaining full nitrogenase activity (5). Recently, the entire genome of *Synechocystis* sp. strain PCC 6803 has been sequenced (19), and a cluster of open reading frames (ORFs) showing similarity to the *moaA*, *moaA*, *moaC*, and *moaE* genes of *E. coli* has been found close to the *nir* gene. Although not identified by the authors, ORF *ssr1527*, also found in this gene cluster, would encode a putative MoaD homolog (see below).

In this report, we describe a genetic analysis of the *Synechococcus* sp. strain PCC 7942 *narA* locus and show that it consists of five genes whose products are essential for nitrate reduction and would be involved in the biosynthesis of molybdopterin.

### MATERIALS AND METHODS

**Organisms, growth conditions, and plasmids.** *Synechococcus* sp. strain PCC 7942 was routinely grown photoautotrophically under white light with shaking (90 rpm) at 30°C in the BG11 medium (17.6 mM NaNO<sub>3</sub> as the nitrogen source) described previously (43). When ammonium was used as the nitrogen source, nitrate was omitted and 4 mM NH<sub>4</sub>Cl and 8 mM N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES)-NaOH buffer (pH 7.5) were supplied, rendering medium BG11<sub>0</sub>NH<sub>4</sub><sup>+</sup>. For growth on plates, the medium was solidified with separately autoclaved 1% agar (Difco Laboratories). The plates were incubated at 30°C in the light. *Synechococcus* strains as well as plasmids used in this work are listed in Table 1. Mutant strain FM6 was grown in BG11<sub>0</sub>NH<sub>4</sub><sup>+</sup> medium, and mutant strains CSLM26, CSLM27, CSLM35, and CSLM40 were grown in BG11<sub>0</sub>NH<sub>4</sub><sup>+</sup> medium supplemented with 10 µg of kanamycin/ml. Mutant strains CSLM32 and CSLM34 were grown in BG11<sub>0</sub>NH<sub>4</sub><sup>+</sup> medium supplemented with 2 µg of streptomycin and 2 µg of spectinomycin/ml. Mutant

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TABLE 1. Cyanobacterial strains and plasmids used in this work

Strain or plasmid	Origin and relevant characteristics	Reference or source
<b>Strains</b>		
PCC 7942	Wild-type <i>Synechococcus</i> strain	42
CSLM26	Km <sup>r</sup> derivative of strain PCC 7942; <i>moaA::lacZ</i> -C.K3 gene fusion	This work
CSLM27	Km <sup>r</sup> derivative of strain PCC 7942; <i>moaA::lacZ</i> -C.K3 gene fusion	This work
CSLM32	Sm <sup>r</sup> Sp <sup>r</sup> derivative of strain PCC 7942; gene cassette C.S3 inserted into the <i>moaA</i> gene	This work
CSLM34	Sm <sup>r</sup> Sp <sup>r</sup> derivative of strain PCC 7942; gene cassette C.S3 inserted into the <i>moaC</i> gene	This work
CSLM35	Km <sup>r</sup> derivative of strain PCC 7942; <i>moaC::lacZ</i> -C.K3 gene fusion	This work
CSLM37	Km <sup>r</sup> Sm <sup>r</sup> Sp <sup>r</sup> derivative of strain CSLM26; double mutant (gene cassette C.S3 inserted into the <i>moaC</i> gene and <i>moaA::lacZ</i> -C.K3 gene fusion)	This work
CSLM40	Km <sup>r</sup> derivative of strain PCC 7942; <i>nir::lacZ</i> -C.K3 gene fusion	This work
FM6	Derivative of strain PCC 7942; transposon Tn901 inserted into the <i>narA</i> locus; transformable to the wild-type phenotype by plasmid pNR12	26
<b>Plasmids</b>		
pBluescript SK(+)	Cloning vector	Stratagene
pCSLM6	4,758-bp <i>XhoI</i> DNA fragment from pNR1211 cloned into the <i>XhoI</i> site of pBluescript SK(+)	This work
pCSLM8	<i>Synechococcus</i> sp. strain PCC 7942 <i>nir</i> gene cloned into the <i>NcoI</i> site of expression vector pTrc99A	46
pCSLM26	Derivative of pCSLM6; 335-bp <i>NheI</i> DNA fragment internal to the <i>moaA</i> gene in pCSLM6 replaced by the <i>lacZ</i> -C.K3 transcriptional reporter cassette from pPE20 (see below); used to generate mutant strain CSLM26	This work
pCSLM27	Derivative of pCSLM6; <i>lacZ</i> -C.K3 transcriptional reporter cassette from pPE20 inserted into the <i>StuI</i> site of the <i>moaA</i> gene; used to generate mutant strain CSLM27	This work
pCSLM32	Derivative of pCSLM6; gene cassette C.S3 inserted into the <i>StuI</i> site of the <i>moaA</i> gene; used to generate mutant strain CSLM32	This work
pCSLM34	Derivative of pCSLM6; gene cassette C.S3 from pRL463 (see below) inserted into the <i>HpaI</i> site of the <i>moaC</i> gene; used to generate mutant strain CSLM34	This work
pCSLM35	Derivative of pCSLM6; transcriptional reporter cassette <i>lacZ</i> -C.K3 from pPE20 inserted into the <i>HpaI</i> site of the <i>moaC</i> gene; used to generate mutant strain CSLM35	This work
pCSLM40	Derivative of pCSLM8; a 99-bp <i>NaeI</i> DNA fragment internal to the <i>nir</i> gene in pCSLM8 substituted by the <i>lacZ</i> -C.K3 transcriptional reporter cassette from pPE20; used to generate mutant strain CSLM40	This work
pCSLM43	<i>Synechococcus</i> sp. strain PCC 7942 <i>moaC</i> gene cloned into the <i>BamHI</i> site of the expression vector pGEX-4T-2 (see below)	This work
pGEX-4T-2	Expression vector for the production of GST-fused proteins	Pharmacia
pNR12	Cosmid containing 19-kb <i>SalI</i> DNA fragment from the genome of strain PCC 7942 that includes the <i>narA</i> locus	20
pNR1211	<i>XhoI</i> DNA fragment of 4,758 bp from pNR12 including the <i>narA</i> locus cloned into the <i>XhoI</i> site of pACYC177	20
pPE20	Promoterless <i>lacZ</i> gene followed by gene cassette C.K3 (transcriptional reporter cassette) cloned into the <i>BamHI</i> site of pUC18/19	48
pRL463	Plasmid pUC18/19 containing the C.S3 gene cassette cloned into the <i>BamHI</i> site of the LEHE1 polylinker	8
pTrc99A	Expression vector	Pharmacia

strain CSLM37 was grown in BG11<sub>0</sub>NH<sub>4</sub><sup>+</sup> medium supplemented with 2 µg of streptomycin/ml, 2 µg of spectinomycin/ml and 10 µg of kanamycin/ml.

For  $\beta$ -galactosidase assays, *Synechococcus* strains were grown in 70-ml glass tubes containing 35 ml of the medium indicated in each experiment, bubbled with air-CO<sub>2</sub> (98:2) at 30°C in the light. At the mid-exponential growth phase (cultures with about 3 to 5 µg of chlorophyll/ml), samples containing an amount of cells corresponding to about 2 µg of chlorophyll were withdrawn for determination of protein content and  $\beta$ -galactosidase activity.

For nitrate reductase assays and for growth rate determinations, *Synechococcus* strains were grown in 70-ml glass tubes containing 35 ml of BG11<sub>0</sub>NH<sub>4</sub><sup>+</sup> medium without antibiotics; after extensive washing, the cells were transferred to and incubated in the medium in each experiment bubbled with air-CO<sub>2</sub> (98:2) at 30°C in the light.

For isolation of DNA and RNA, *Synechococcus* strains were grown in 240-ml glass flasks containing 150 ml of BG11 medium bubbled with air at 30°C in the light. Cultures with a cell density corresponding to 3 to 5 µg chlorophyll/ml were used.

*E. coli* DH5 $\alpha$ , GM48, BL21, and HB101 were grown in Luria-Bertani medium at 37°C with shaking (200 rpm). For growth of *E. coli* on plates, medium solidified with 1.5% agar was used. Antibiotics were used at standard concentrations (2).

Growth rates were estimated from the increase of protein concentration in the cultures. The growth rate constant corresponds to  $\ln 2/t_d$ , where  $t_d$  represents the doubling time.

**Generation of mutant strains.** Insertions of either *HincII*-ended gene cassette C.S3 from plasmid pRL463 (8) or *SmaI*-ended gene cassette *lacZ*-C.K3 from plasmid pPE20 (48) at the *StuI* restriction site, which is internal to the *Synechococcus moaA* gene, of plasmid pCSLM6 were made to render plasmids pCSLM32 (*moaA::C.S3*) and pCSLM27 (*moaA::lacZ*-C.K3), respectively. Similar insertions

were made into the *moaC* gene, disrupting it at the *HpaI* restriction site of pCSLM6 to render plasmids pCSLM34 (*moaC::C.S3*) and pCSLM35 (*moaC::lacZ*-C.K3). The *moaA* gene was also mutated by substitution of a 335-bp, *NheI* DNA fragment at the 3' terminus of the gene, after digestion of pCSLM6 with *NheI* and treatment with the Klenow enzyme (2), by *SmaI*-ended gene cassette *lacZ*-C.K3, rendering plasmid pCSLM26. The *nir* gene was mutated by substitution of a 99-bp, *NaeI* DNA fragment by *SmaI*-ended gene cassette *lacZ*-C.K3, rendering plasmid pCSLM40 (Table 1). Restriction analysis of plasmids pCSLM26, pCSLM27, pCSLM35, and pCSLM40 confirmed that the antibiotic resistance genes present in the inserted gene cassettes were, in every case, in the same orientation as the *Synechococcus* genes disrupted by the gene cassette.

Plasmids pCSLM26, pCSLM27, pCSLM32, pCSLM34, pCSLM35, and pCSLM40 were transferred to *Synechococcus* sp. strain PCC 7942 by means of transformation (12) for generation of mutant strains CSLM26, CSLM27, CSLM32, CSLM34, CSLM35, and CSLM40, respectively. For generation of mutant strain CSLM37, pCSLM34 was transferred to strain CSLM26 (Table 1). After transformation, cells were spread onto nitrocellulose filters (Nuclepore; REC85) set successively atop BG11<sub>0</sub>NH<sub>4</sub><sup>+</sup> solid medium (incubated for 48 h) and BG11<sub>0</sub>NH<sub>4</sub><sup>+</sup> with the appropriate antibiotics (incubated for 3 weeks). Individual colonies were selected and, after recloning, maintained in BG11<sub>0</sub>NH<sub>4</sub><sup>+</sup> solid medium with antibiotics.

**DNA manipulations.** PCR using EcoTaq DNA polymerase (EcoGen S.R.L.), plasmid constructions, DNA electrophoresis, isolation of DNA fragments from agarose gels, ligation, and transformation of *E. coli* were carried out by standard methods (2). Restriction endonucleases were used according to the manufacturer's recommendations or by standard methods (2). Sequencing was performed in double-stranded DNA by the chain termination method with a T7 Sequencing kit (Pharmacia LKB) and [<sup>35</sup>S]deoxyadenosine 5'-( $\alpha$ -thio)triphosphate (1,000 to

1,500 Ci/mmol). Both strands of the DNA were sequenced. Computer searching for homologies was made by using the FASTA and TFASTA algorithms included in the Genetics Computer Group package (7). Isolation of DNA from *Synechococcus* strains was performed essentially as described by Cai and Wolk (6). For Southern blots, restriction endonuclease-digested DNA or PCR products were subjected to electrophoresis in agarose gels and transferred to Genescreen Plus membranes (Dupont) as instructed by the manufacturer. Probes were labeled with [ $\alpha$ - $^{32}$ P]dCTP (3,000 Ci/mmol). Prehybridization and hybridization were performed essentially as described by Frías et al. (10) in a solution containing 5× SSPE (0.8 M NaCl, 10 mM sodium phosphate, 1 mM EDTA [pH 7.4]), 5× Denhardt's solution (47), 0.5% (wt/vol) sodium dodecyl sulfate (SDS), and 100  $\mu$ g of nonhomologous DNA/ml, under high-stringency conditions at 65°C and under low-stringency conditions at 55°C.

**RNA isolation and RT-PCR analysis.** Isolation of RNA from *Synechococcus* sp. strain PCC 7942 was performed as described by Mohamed and Jansson (32), with the modifications described in Luque et al. (23). Samples were treated with RNase-free DNase I (from bovine pancreas; Boehringer) for elimination of any remaining DNA. For retrotranscription-PCR (RT-PCR) experiments, 4  $\mu$ g of strain PCC 7942 total RNA was mixed with 20 pmol of the oligonucleotide 5'-ATTGACCTTGAGGATCGGTAAGCG-3' (complementary to nucleotides 479 to 456 with respect to the translation start of the *moaA* gene) in the presence of 50 mM Tris-HCl, 8 mM MgCl<sub>2</sub>, 30 mM KCl, and 1 mM dithiothreitol, pH 8.5 (AMV [avian myeloblastosis virus] buffer), heated for 2 min at 90°C, and immediately cooled down to 55°C. Then 1 mM each deoxynucleoside triphosphate, 20 U of RNA Guard (Pharmacia), and 50 U of AMV reverse transcriptase (Boehringer) were added, and the extension reaction was developed for 1 h at 54°C in a volume of 20  $\mu$ l. To control for the presence of contaminating DNA, samples containing 4  $\mu$ g of the RNA preparation, 20 pmol of the same oligonucleotide, and 1  $\mu$ g of RNase A (DNase free; Boehringer) were incubated, in a 20- $\mu$ l reaction volume, at 37°C for 1 h. PCR was carried out with 35  $\mu$ l of retrotranscription mixture (diluted 10-fold with 10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) or RNase-treated sample (see above) as the template, and oligonucleotides 5'-ATTGACCTTGAGGATCGGTAAGCG-3' (complementary to nucleotides 479 to 456 with respect to the *moaA* translation start; same as above) and 5'-GGTCTATCAGCGCGTTACTCAAGG-3' (complementary to nucleotides 74 to 97 with respect to the *moaC* translation start) as primers. Control samples containing the same oligonucleotides and strain PCC 7942 genomic DNA as the template were run in parallel. PCR consisted of 35 cycles of template denaturation at 95°C for 1 min, annealing with the oligonucleotides for 1 min at 69°C, and DNA extension at 72°C for 2 min. One half of each sample was resolved by electrophoresis in 1% agarose gels and transferred to membranes for Southern blot analysis. A 0.53-kb, *Pvu*II DNA fragment from plasmid pNR1211 (Table 1), internal to the *moa* operon (see Fig. 2), was used as the probe.

**Expression of the *Synechococcus moaC* gene in *E. coli*.** A 1,030-bp DNA fragment, containing the *moaC* gene and part of the *moaD* gene, from an unmethylated pCSLM6 plasmid restricted with *Cla*I and *Sac*I and treated with Klenow enzyme was isolated and cloned into the *Bam*HI site of plasmid pGEX-4T-2 (Pharmacia), made blunt ended with Klenow enzyme, to render plasmid pCSLM43. *E. coli* BL21 carrying plasmid pCSLM43 was used for overproduction of the glutathione *S*-transferase (GST)-MoaC fusion protein after induction with 1 mM isopropyl- $\beta$ -D-thiogalactoside (IPTG). Preparation of cell extracts from BL21(pCSLM43), purification of GST-MoaC protein by using bulk glutathione-Sepharose 4B in batch, and thrombin cleavage of fusion proteins were carried out as recommended by the manufacturer. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) as described by Schleif and Wensink (49), using a 12% acrylamide running gel with an upper 4% acrylamide stacking gel.

**Enzyme assays and analytical procedures.** Nitrate reductase activity was determined by using dithionite-reduced methyl viologen as the reductant in alkyltrimethylammonium bromide-permeabilized *Synechococcus* cells (14).  $\beta$ -Galactosidase activity was determined as described by Schaefer and Golden (48) by colorimetric assay with *o*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) (31). One unit of enzymatic activity corresponds to the formation of 1  $\mu$ mol of product (nitrite or *o*-nitrophenol) per min. Protein quantifications were made by a modified Lowry method (27), using bovine serum albumin as the standard. Chlorophyll *a* determinations were made in methanolic extracts as described by MacKinney (25).

**Nucleotide sequence accession number.** The nucleotide sequence of the *moaE* and *moa* genes reported in this paper will appear in the EMBL/GenBank/DBJ nucleotide sequence data libraries under accession no. X99625.

## RESULTS

**Identification of the *narA* locus.** FM6 is a Tn901-induced mutant derived from *Synechococcus* sp. strain PCC 7942 that is impaired in nitrate reductase activity. This mutant is readily transformable to the wild-type phenotype by the 4.7-kb, *Xho*I DNA fragment from strain PCC 7942 that is cloned in plasmid pNR1211. This genetic locus has been named *narA* (20). For localization and identification of the nitrate reduction-related

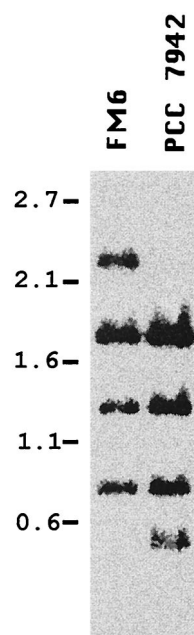


FIG. 1. Localization of Tn901 in the *narA* locus of mutant strain FM6. Genomic DNA from the indicated strain was simultaneously digested with *Xho*I, *Bgl*II, and *Pvu*II and subjected to Southern blot analysis using the 4.7-kb *Xho*I insert of plasmid pNR1211 as a probe. The positions and sizes (in kilobases) of some standards are indicated to the left.

gene(s) corresponding to the *narA* locus, genomic DNA from mutant strain FM6 was restricted with the endonucleases *Xho*I, *Bgl*II, and *Pvu*II and subjected to Southern blot analysis using as a probe the 4.7-kb, strain PCC 7942 DNA fragment of pNR1211. Compared to strain PCC 7942 DNA, mutant FM6 DNA showed a clear change in the hybridization pattern indicative of a Tn901 insertion into a 0.53-kb, *Pvu*II DNA fragment (Fig. 1). Sequencing of this *Pvu*II fragment revealed the existence of two ORFs. The putative product of one of them showed homology to the large subunit of the MPT-converting factor, the MoaE polypeptide of *E. coli*.

Sequencing of the entire *Synechococcus* sp. strain PCC 7942 DNA fragment cloned in pNR1211 was carried out by using several synthetic oligonucleotides as primers and plasmid pNR1211 as the template. Sequence analysis revealed the existence of five ORFs in that fragment (Fig. 2). Putative ribosome binding sites could be found only in front of ORF1, ORF4, and ORF5. No other ORF was found after sequencing 300 bp of the *Synechococcus* DNA adjacent to the *Xho*I site closest to ORF5, using as template cosmid pNR12 (20), whose insert includes that of plasmid pNR1211.

As summarized in Table 2, ORF1 would encode a polypeptide of 403 amino acids that shows homology to MoaA polypeptides from *E. coli* (33) and *Anabaena* sp. strain PCC 7120 (41). ORF2 would encode a 319-amino-acid polypeptide whose N-terminal half shows homology to the MoaC polypeptide of *E. coli* (44) and whose C-terminal half shows homology to MoaB and Mog polypeptides of *E. coli* (it should be noted that MoaB and Mog are themselves homologous to each other) (44, 53). ORF3 would encode a polypeptide of 90 amino acids that in its C-terminal part (amino acids 60 through 90) shows homology to the MoaD polypeptide of *E. coli* (44). The putative product of ORF4 (165 amino acids) shows homology to the *E. coli* MoaE polypeptide (44). ORF5 would encode a polypeptide of 327 amino acids homologous to *E. coli* MoaA (44),



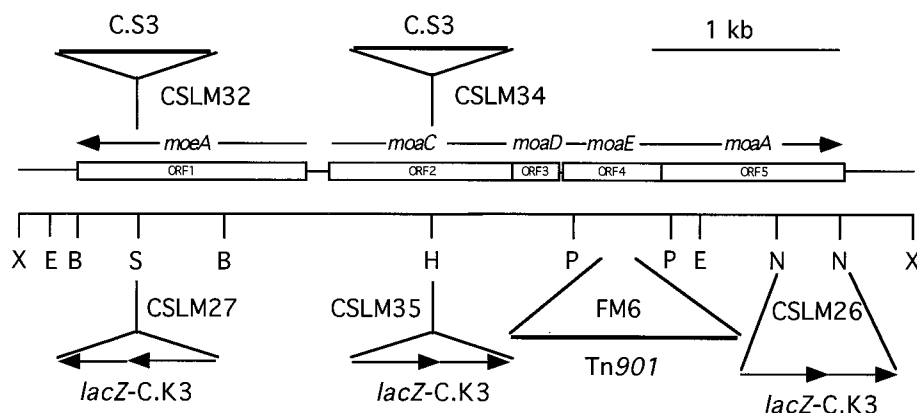


FIG. 2. Structure of a genomic region of *Synechococcus* sp. strain PCC 7942 that contains *moeA* and several *moa* genes. The identities and orientations of gene cassettes inserted at some restriction sites for the generation of cyanobacterial mutants are indicated together with the CSLM denomination of the resulting mutant strain. The location of Tn901 in mutant strain FM6 is also indicated. B, *Bgl*II; E, *Eco*RV; H, *Hpa*I; N, *Nhe*I; P, *Pvu*II; S, *Stu*I; X, *Xho*I.

to *Bacillus subtilis* NarA (11), and to Cnx2 from *Arabidopsis thaliana* (15). All Moa, Moe, and Mog polypeptides of *E. coli* have been shown to be involved in the biosynthesis of Mo-MPT (39). Because of the homologies described above, we propose to name ORF1 as *moeA*, ORF2 as *moaC* (whose product would bear a domain homologous to MoaC and another one homologous to MoaB and Mog from *E. coli*), ORF3 as *moaD*, ORF4 as *moaE*, and ORF5 as *moaA*.

No evidence for the existence in strain PCC 7942 of other genes homologous to those present in the *moaA-moa* cluster here described could be obtained by means of Southern blot analysis under low-stringency conditions using a 3,458-bp, *Eco*RV DNA fragment containing most of the *moa-moe* gene cluster (Fig. 2) as a probe (not shown).

**Overexpression of the *Synechococcus moaC* gene in *E. coli*.** The MoaC polypeptide from strain PCC 7942 was produced in *E. coli* BL21(pCSLM43) cells as a GST-MoaC fusion protein of about 60 kDa. After purification of the GST-MoaC protein and cleavage with thrombin, a 36-kDa MoaC protein was released (Fig. 3). This protein would differ from the native MoaC only in the first two amino acids, which were changed from Met-Ile in the native protein to Gly-Ser in the recombinant protein. The size of MoaC polypeptide derived from the nu-

cleotide sequence of the *Synechococcus moaC* gene would be 33 kDa.

**Mutational analysis of the *moeA* and *moa* genes.** Three of the genes found in the insert of pNR1211 were mutated by in vitro gene cassette insertion (Fig. 2 and Table 1) to test their involvement in expression of nitrate reductase activity. *Synechococcus* strains bearing those mutations in the *moeA* or *moa* genes were obtained by genetic transformation of strain PCC 7942 with plasmids bearing the inactivated genes (see Fig. 2 and Materials and Methods for details). Strain CSLM26 bears gene cassette *lacZ-C.K3*, which does not carry transcriptional terminators (30), substituting for the 335-bp *Nhe*I fragment internal to the *moaA* gene; strain CSLM27 bears gene cassette *lacZ-C.K3* inserted into the *Stu*I site of the *moeA* gene; strain CSLM32 bears gene cassette C.S3, which carries transcriptional terminators (38), inserted into the *Stu*I site within the *moeA* gene; strain CSLM34 bears gene cassette C.S3 inserted into the *Hpa*I site within the *moaC* gene; strain CSLM35 bears gene cassette *lacZ-C.K3* inserted at the same *Hpa*I site within the *moaC* gene. We also constructed a double mutant, strain CSLM37, that bears the mutations present in strains CSLM34 and CSLM26. The genetic structure of each of the mutant

TABLE 2. ORFs in the *narA* locus of *Synechococcus* sp. strain PCC 7942

ORF	Proposed gene name	Size (no. of amino acids)	Homologous protein (organism)	% Identity <sup>a</sup>	Overlapping fragment <sup>b</sup>
ORF1	<i>moeA</i>	403	MoeA ( <i>E. coli</i> )	36	1-403
			MoeA ( <i>Anabaena</i> sp.)	32	1-403
ORF2	<i>moaC</i>	319	MoaC ( <i>E. coli</i> )	47	1-145
			MoaB ( <i>E. coli</i> )	27	163-303
			Mog ( <i>E. coli</i> )	28	156-300
ORF3	<i>moaD</i>	90	MoaD ( <i>E. coli</i> )	34	60-90
ORF4	<i>moaE</i>	165	MoaE ( <i>E. coli</i> )	35	10-156
ORF5	<i>moaA</i>	327	MoaA ( <i>E. coli</i> )	46	1-327
			NarA ( <i>B. subtilis</i> )	31	1-327
			Cnx2 ( <i>A. thaliana</i> )	35	1-327

<sup>a</sup> As deduced from the aligned fragment.

<sup>b</sup> Position in the *Synechococcus* polypeptide of the amino acids that could be aligned with the indicated homologous protein (or with a fragment of that protein).

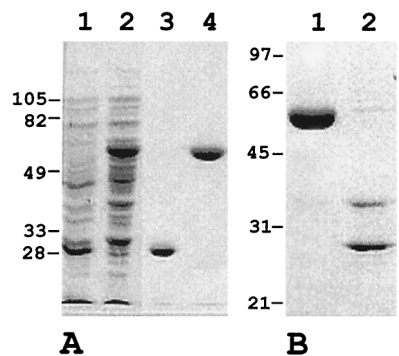


FIG. 3. Expression in *E. coli* and purification of a GST-MoaC fusion protein. (A) SDS-PAGE of a cell extract from strain BL21(pGEX-4T-2) containing GST (lane 1), cell extract from BL21(pCSLM43) containing GST-MoaC (lane 2), purified GST (lane 3), and purified GST-MoaC protein (lane 4). (B) SDS-PAGE of purified GST-MoaC protein (lane 1) and thrombin-treated GST-MoaC protein (lane 2). The arrowhead points to the ca. 36-kDa *Synechococcus* MoaC protein. Positions and sizes (in kilodaltons) of some molecular weight markers are indicated to the left of each panel.

TABLE 3. Growth rates and nitrate reductase activities of mutant strains derived from *Synechococcus* sp. strain PCC 7942

Strain	Relevant genotype	Growth rate (days <sup>-1</sup> ) <sup>a</sup>		Nitrate reductase (mU/mg of protein) <sup>b</sup>
		Ammonium	Nitrate	
PCC 7942	Wild type	2.76	3.07	36.5
CSLM26	<i>moaA::lacZ</i> -C.K3	2.10	<0.01	<0.1
CSLM27	<i>moaA::lacZ</i> -C.K3	1.61	<0.01	<0.1
CSLM32	<i>moaA::C.S3</i>	2.90	<0.01	<0.1
CSLM34	<i>moaC::C.S3</i>	2.06	<0.01	<0.1
CSLM35	<i>moaC::lacZ</i> -C.K3	3.26	<0.01	<0.1
CSLM37	<i>moaC::C.S3</i> , <i>moaA::lacZ</i> -C.K3	1.80	<0.01	<0.1
FM6 <sup>c</sup>	<i>moaE::Tn901</i>	1.80	<0.01	<0.1

<sup>a</sup> Ammonium-grown cells of the wild-type and mutant strains were washed twice with medium lacking combined nitrogen and used to inoculate, at a final concentration of 0.2 µg of chlorophyll/ml, cultures with the indicated nitrogen source. The cultures were bubbled with air-CO<sub>2</sub> (98:2) at 30°C in the light for 24 h and then diluted eightfold with fresh culture medium with the same nitrogen source. To estimate the growth rate, protein content was measured in aliquots sampled periodically from the last set of cultures.

<sup>b</sup> For nitrate reductase activity determinations, ammonium-grown cells of the wild-type and mutant strains were washed twice with medium lacking combined nitrogen and incubated for 6 h in medium containing nitrate as the sole nitrogen source, bubbled with air-CO<sub>2</sub> (98:2) at 30°C in the light. Nitrate reductase activity was then determined in aliquots of each culture.

<sup>c</sup> Although the phenotype of this strain has been previously reported (26), it is included in this experiment for the sake of comparison.

strains in the *moaA-moa* region, as well as the absence of wild-type chromosomes in them, was confirmed by PCR analysis using primers flanking each mutation (not shown). In addition, strain FM6, which bears Tn901 inserted into the 0.53-kb *Pvu*II fragment, was analyzed by PCR using several primers internal to the *moaE* and *moaA* genes. Results obtained (not shown) indicated that Tn901 in strain FM6 is inserted into the *moaE* gene (Fig. 2).

Strains CSLM26, CSLM27, CSLM32, CSLM34, CSLM35, and CSLM37 were unable to grow with nitrate as the sole nitrogen source (Table 3). Moreover, in contrast to strain PCC 7942, none of the mutants exhibited nitrate reductase activity upon incubation in medium containing nitrate as the sole nitrogen source (Table 3).

**Expression of the *moaA* and *moa* genes.** Because we were unable to detect any *moa* transcript in *Synechococcus* sp. strain PCC 7942 by means of Northern analysis, we studied the expression of *moa* genes by subjecting mRNA to RT-PCR (see Materials and Methods for details). For retrotranscription, an oligonucleotide complementary to sequences internal to the *moaA* gene was used as the primer. The resulting cDNA was then amplified by PCR using the same primer used for retrotranscription and another one that should anneal at the beginning of the *moaC* gene. After electrophoresis of the RT-PCR products on an agarose gel, a band of the expected size, 2.1 kb, was observed. This band was verified by Southern blot analysis to hybridize to a probe of the *moaE* gene (Fig. 4, lane 3). Since this RT-PCR product was strictly dependent on the presence of RNA (Fig. 4, lane 2), it cannot be due to amplification of contaminating genomic DNA. These results suggest that the *moaC*, *moaD*, *moaE*, and *moaA* genes of strain PCC 7942 (Fig. 2) are cotranscribed into a single mRNA species. It is worth noting that overlapping of termination and start codons is found between the *moaC* and *moaD* genes, as well as between the *moaE* and *moaA* genes, of strain PCC 7942.

The effect of the presence of ammonium or nitrate in the extracellular medium on the expression of the *moaA* and *moa*

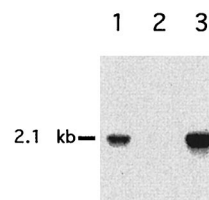


FIG. 4. Southern analysis of RT-PCR products of the *moa* gene cluster of *Synechococcus* sp. strain PCC 7942, using a *moaE* gene probe. The primers used for the RT-PCR corresponded to DNA sequences of the *moaA* and *moaC* genes (see Materials and Methods for primers used). Lane 1, PCR-amplified strain PCC 7942 genomic DNA; lane 2, PCR-amplified, RNase-treated strain PCC 7942 total RNA; lane 3, RT-PCR-amplified RNA. Samples of lanes 2 and 3 were incubated with DNase before the RNase treatment or the retrotranscription reaction. The size of the amplified DNA fragment is indicated to the left.

genes of strain PCC 7942 was studied by measuring β-galactosidase activity in mutant strains bearing gene fusions to the *lacZ*-C.K3 gene cassette. Ammonium-grown cells of mutant strains CSLM26 (*moaA::lacZ*-C.K3), CSLM27 (*moaA::lacZ*-C.K3), CSLM35 (*moaC::lacZ*-C.K3) (Fig. 2), and, as a control, CSLM40 (*nir::lacZ*-C.K3) (Table 1) were incubated for 14 h in media containing either ammonium or nitrate as the sole nitrogen source, and protein and β-galactosidase activities were determined (Table 4). While the activity level of β-galactosidase in CSLM40 (carrying the *nir::lacZ*-C.K3 fusion) was about 4.2-fold higher in nitrate- than in ammonium-incubated cells, only 1.7- to 1.9-fold-higher levels were found in nitrate- than in ammonium-incubated cells of the strains carrying *lacZ* fused to the *moaA* or *moa* genes. Data in Table 4 should be interpreted with caution, however, since β-galactosidase basal levels can be relatively high in *Synechococcus* cells carrying *lacZ*. As an example, β-galactosidase activity in ammonium-grown cells of strain CSLM37, in which *lacZ* is inserted within the *moa* operon about 1.8 kb downstream from the transcriptional terminator present in the C.S3 gene cassette, was about 60 mU/mg of protein. No β-galactosidase activity was detected in wild-type strain PCC 7942.

## DISCUSSION

Some years ago, three genetic loci, *narA*, *narB*, and *narC*, whose mutation leads to impairment of nitrate reductase activity in the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942 were identified and cloned (20, 21). The *narB* locus was later identified as the structural gene for nitrate reductase (1, 45) that is part of an operon of nitrate assimila-

TABLE 4. β-Galactosidase activities of *Synechococcus* sp. mutant strains CSLM40, CSLM26, CSLM27, and CSLM35 incubated with ammonium or nitrate<sup>a</sup>

Strain	Relevant genotype	β-Galactosidase (mU/mg of protein)	
		Ammonium	Nitrate
CSLM40	<i>nir::lacZ</i> -C.K3	113 (18)	475 (90)
CSLM26	<i>moaA::lacZ</i> -C.K3	110 (11)	190 (40)
CSLM27	<i>moaA::lacZ</i> -C.K3	106 (10)	200 (31)
CSLM35	<i>moaC::lacZ</i> -C.K3	116 (22)	216 (19)

<sup>a</sup> Ammonium-grown cells were collected and transferred to media containing the indicated nitrogen source. After 14 h of incubation under culture conditions, total protein and β-galactosidase activity were measured. Data are the medians of four independent experiments (standard deviations are shown into parentheses).

tion genes which includes, in addition to *narB*, the *nir* and *nrtABCD* genes (22, 24, 34). Up to now, nothing was known about the actual function of the genes in the *narA* or *narC* locus.

We have mapped the site of insertion of Tn901 in a previously reported *narA* mutant of *Synechococcus* sp. strain PCC 7942, strain FM6 (26), and have determined that the inactivated genomic region carries a cluster of genes whose putative polypeptide products show similarity to genes involved in the biosynthesis of the molybdenum cofactor of nitrate reductase and other molybdoenzymes. The genes identified include homologs of the *moaA*, *moaB* and *mog*, *moaC*, *moaD*, *moaE*, and *moaA* genes from *E. coli* and some other biological sources.

The synthesis of all molybdenum cofactors of molybdoenzymes, except the iron-molybdenum cofactor of nitrogenase, comprises the synthesis of Mo-MPT, which presumably is common to all molybdoenzyme-containing organisms, and, in some cases, the posterior formation of different dinucleotide variants. Synthesis of MPT takes place through the formation of a sulfur-free pterin precursor, termed precursor Z, that is then sulfurylated, leading to MPT and, after incorporation of molybdenum, to the Mo-MPT complex (39). The *moaABC* genes of *E. coli*, which are part of the *moaABCDE* operon, are involved in the biosynthesis of precursor Z. The *moa* operon of *Synechococcus* sp. strain PCC 7942 described in this work contains genes homologous to *moaABC*. While *Synechococcus* MoaA would be similar to other MoaA (or Cnx2) proteins, *Synechococcus* MoaC is unique in that it resembles a fusion protein of MoaC (N-terminal half) and MoaB or Mog (C-terminal half). Sequence similarities do not allow us to conclude whether the C-terminal half of *Synechococcus* MoaC represents a MoaB or a Mog domain. In *Synechocystis* sp. strain PCC 6803, ORFs showing similarity to *moaA* (*shr0901*) and *moaC* (*shr0902*) are also clustered together (19). In this case, *moaC* would be fused to *moaA* (a gene required in a late step of MGD biosynthesis), but the actual assignments of these genes to the corresponding ORFs awaits experimental confirmation. The *moaDE* genes of *E. coli* encode the two subunits of the so-called converting factor or MPT synthase that adds dithiolene sulfurs to precursor Z, thus generating MPT (37). The *Synechococcus* *moa* operon also contains *moaDE* homologs (Table 2 and Fig. 2), as is also the case for the *Synechocystis* genome (19). The putative cyanobacterial MoaD polypeptides are peculiar in that they show appreciable identity to *E. coli* MoaD only in the 30 C-terminal amino acids; notably, however, these include the C-terminal Gly-Gly sequence that is thought to be essential for MoaD function (39). On the other hand, the *moaE* gene, which in *E. coli* encodes MPT synthase sulfurylase that catalyzes the transfer of sulfur to the MoaD subunit of MPT synthase, is not present in the *Synechococcus* *moaA-moa* gene cluster. Finally, the MoaE protein from strain PCC 7942 would be similar to MoaE from *E. coli* that has recently been suggested to be involved in activation of molybdate (13). The fact that *Synechococcus* strains bearing mutations in the *moaA-moa* gene cluster are devoid of nitrate reductase activity indicates that this gene cluster is involved in the synthesis of the Mo cofactor of nitrate reductase. In particular, four of the genes in the cluster (*moaA*, *moaC*, *moaE*, and *moaA*) have been inactivated, the phenotype of the corresponding mutants showing the involvement of these genes in production of an active nitrate reductase. It should be noted that both the *lacZ-C.K3* gene cassette and transposon Tn901 allow transcription of genes located downstream from them in a transcriptional unit (30, 46, 50).

Results of RT-PCR presented in this work show that *Synechococcus* sp. strain PCC 7942 synthesizes mRNA molecules

containing a message for both the *moaC* and *moaA* genes. This finding indicates that the *moa* genes in the identified gene cluster can be expressed as a single mRNA molecule from a promoter located upstream from *moaC*, thus constituting an operon. On the other hand, *moaE*, which is located in the complementary DNA strand, would be expressed independently.

In *Synechococcus* sp. strain PCC 7942, structural genes for nitrate assimilation proteins including nitrite reductase, the components of the nitrate/nitrite transport system, and nitrate reductase, which constitute the *nir* operon, are subjected to repression by ammonium. Results presented here on the expression of the *moaE* gene and the *moa* operon, compared to that of the *nir* operon, using  $\beta$ -galactosidase as a transcriptional reporter indicate that the expression of these Mo cofactor biosynthesis genes is not regulated by the nitrogen source to the same extent as the *nir* operon is. This resembles the situation with the *moa* genes in *E. coli*, whose expression is not affected by the regulatory element NarL, a nitrate-responsive activator of the synthesis of nitrate reductase, or by high levels of nitrate in the growth medium (4). Lack of regulation by the nitrogen source of the *moaE* and *moa* genes in *Synechococcus* sp. strain PCC 7942 would be consistent with a role of these genes in this cyanobacterium, as is also the case for *E. coli*, in the synthesis of the Mo cofactor not only of nitrate reductase but also of some other molybdoenzymes involved in processes other than nitrogen assimilation.

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