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Molybdopterin guanine dinucleotide cofactor in *Synechococcus* sp. nitrate reductase: identification of *mobA* and isolation of a putative *moeB* gene

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Abstract The *narC* locus required for assimilatory nitrate reduction in the cyanobacterium *Synechococcus* sp. strain PCC 7942 was found to carry a *mobA* gene for molybdopterin guanine dinucleotide biosynthesis. Insertional inactivation of this gene blocked production of nitrate reductase in *Synechococcus* cells. We have previously described *Synechococcus* genes encoding homologues to molybdopterin biosynthesis proteins including MoaA, MoaC/MoaB, MoaD, MoaE, and MoeA, but not to MoeB. A cyanobacterial gene putatively encoding a protein composed of an amino-terminal domain of 260 amino acids homologous to *Escherichia coli* MoeB and of a carboxy-terminal extension of 130 amino acids was identified. *Synechococcus* mutants bearing only inactive versions of this putative *moeB* gene could not be isolated suggesting that it has function(s) additional to molybdopterin biosynthesis.

Key words: Nitrate reductase; Molybdenum cofactor; *mobA*; *moeB*; *Synechococcus* sp.

1. Introduction

Nitrate reductases are molybdoenzymes that catalyze the two-electron reduction of nitrate to nitrite. In bacteria, three distinct groups of nitrate reductases are found: assimilatory, respiratory, and periplasmic nitrate reductases [1–3]. All bacterial nitrate reductases are homologous proteins that also share sequence similarity with some other proteins like formate dehydrogenase which are involved in anaerobic respiration [4]. All of these proteins carry molybdenum in a molybdenum cofactor, some of them in the form of molybdopterin guanine dinucleotide (MGD) and others in the form of other dinucleotides or of a molybdopterin (MPT)-Mo complex [5]. Biosynthesis of MPT in *Escherichia coli* requires the products of the genes of the *moaABCDE* operon and of *moeB* [6]. The MoaA, MoaB, and MoaC proteins catalyze the synthesis of a molecule known as 'precursor Z'; an enzyme, MPT synthase, composed of MoaD and MoaE then adds dithiolene sulfurs to precursor Z to give MPT. MPT synthase sulfurylase, the MoeB protein, restores sulfur to the MoaD subunit of MPT synthase, an essential activity of the MPT biosynthetic process [6]. Conversion of MPT into the Mo-MGD complex requires the products of the *mobA* and *moeA* genes, and appears to be facilitated by the *mog* gene product (a putative molybdenum chelatase [7]). While MoeA appears to participate in activation and incorporation of molybdenum [8], MobA adds GMP (from GTP) to form the dinucleotide [6,9].

Nitrate is widely used as a nitrogen source for the growth of cyanobacteria [10]. In the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942, three genetic loci, *narA*, *narB*, and *narC* [11,12], have been identified as essential for the reduction of nitrate to nitrite, the first intracellular step in assimilatory nitrate reduction [10]. The *narB* locus has been identified as the nitrate reductase structural gene, *narB*, which is clustered together with genes for nitrite reductase and the nitrate/nitrite permease [13–16]. The *narA* locus contains genes encoding homologues to MoeA and the various Moa proteins mentioned above [17]. The *narC* locus, on the other hand, had not been characterized until now. In this work, we describe the identification of the gene required for nitrate reduction present in the *narC* locus, *mobA*. We also describe our attempts to identify a *Synechococcus moeB* gene, the only other gene strictly required for MGD biosynthesis which is not present in the above mentioned *nar* loci.

2. Materials and methods

2.1. Cyanobacterial strains and growth conditions

Synechococcus sp. strain PCC 7942 was grown photoautotrophically under white light with shaking (90 rpm) at 30°C in BG11 medium (17.6 mM $NaNO_3$ as the nitrogen source [18]) or $BG11_0NH_4^+$ medium (nitrate omitted and 4 mM NH_4Cl and 8 mM *N*-Tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES)-NaOH buffer (pH 7.5) added). For growth on plates, the medium was solidified with separately autoclaved 1% agar (Difco). Strain FM1 was grown in $BG11_0NH_4^+$ medium and strains CSLM71a, CSLM71b, CSLM91a, and CSLM91b were grown in $BG11_0NH_4^+$ medium supplemented with 10 to 25 μg of kanamycin (Km)/ml.

2.2. Nitrate reductase

For nitrate reductase assays, *Synechococcus* strains were grown in 70-ml glass tubes containing 35 ml of $BG11_0NH_4^+$ medium supplemented with 10 mM $NaHCO_3$ ($BG11_0NH_4^+$'C'), without antibiotics, bubbled with air:CO_2 (98:2) at 30°C in the light. After extensive washing, the cells were transferred to and incubated as above using BG11 medium supplemented with 10 mM $NaHCO_3$ (BG11'C'). Nitrate reductase activity was determined using dithionite-reduced methyl viologen as the reductant in alkyltrimethylammonium bromide-permeabilized *Synechococcus* cells [19]. One unit of enzymatic activity corresponds to the formation of 1 μmol of nitrite per min. Protein quantifications were made by a modified Lowry method [20] using bovine serum albumin as standard. Chlorophyll *a* determinations were made in methanolic extracts [21].

2.3. Nucleic acids isolation and analysis

For isolation of RNA, *Synechococcus* strains were grown in 240-ml glass flasks containing 150 ml of the medium indicated bubbled with air:CO_2 (98:2) at 30°C in the light. Cultures with a cell density corresponding to 3–5 μg chlorophyll/ml were used. Isolation of RNA from *Synechococcus* sp. strain PCC 7942 was performed as described

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[22] with some modifications [23]. Samples were treated with RNase-free DNase I (from bovine pancreas; Boehringer) for elimination of any remaining DNA. For Northern analysis, RNA (approximately 30 μg) was subjected to electrophoresis in denaturing formaldehyde gels, transferred to GeneScreen Plus membranes, and subjected to hybridization following the manufacturer's instructions. Isolation of DNA from *Synechococcus* strains was performed as described [24]. For Southern blots, restriction endonuclease-digested DNA was subjected to electrophoresis in agarose gels and transferred to GeneScreen Plus membranes (Dupont) following the manufacturer's instructions. Prehybridization and hybridization were performed essentially as described [25] under high-stringency conditions at 65°C or under low-stringency conditions at 55°C. Probes were labelled with [α-^{32}P]dCTP (3000 Ci/mmol).

2.4. Plasmid constructions and recombinant DNA and genetic methods

E. coli strains DH5α, GM48, and HB101 were grown in Luria–Bertani (LB) 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 [26]. Restriction of DNA with endonucleases, PCR using Eco*Taq* DNA polymerase (EcoGen S.R.L.) or *Pfu* DNA polymerase (Stratagene), plasmid constructions, DNA electrophoresis, isolation of DNA fragments from agarose gels, ligation, and transformation of *E. coli* were carried out by standard methods [26]. Oligonucleotides used as PCR primers were: ORF131_1, 5′-CTAGATCATCAACTTTCCAATCGC-3′; ORF131_2, 5′-TCAGTCTTGAACGTAGTC-TTGACC-3′; ORF131_3, 5′-GATTGGCAAGCGCTGCAGTTGTCC-3′; mobA4, 5′-GTCAAGACTACGTTCAAGACTGAG-3′; mobA3, 5′-CTAGGACAACTGCAGCGCTTGCCAA-TC-3′; mobA2, 5′-ATGAATTTTGCTGCCTTGATTTTGGC-3′; moeB1, 5′-CTCTATCTGGCTGCAGCGGGGGTAGG-3′; moeB2, 5′-GGCTCCCAAAATGATTTTTAGTGCTTC-3′; moeBsec1, 5′-ATGCTCAACCTCGACACG-3′; and moeBsec4, 5′-CCTAGTAGTTAGTACTGC-3′. Sequencing was performed in double-stranded DNA by the chain termination method with a T7 Sequencing Kit (Pharmacia LKB) and [^{35}S]deoxyadenosine 5′-(α-thio)triphosphate (1000–1500 Ci/mmol). Both strands of the DNA were sequenced.

Plasmid pCS16 carries a 871-bp *Bam*HI DNA fragment from pNR1934 [12] cloned in the *Bam*HI site of vector pIC-20H. Plasmid pCS13 carries a 503-bp *Bam*HI-*Sal*I DNA fragment from pNR1934 [12] cloned in the *Bam*HI-*Sal*I sites of vector pIC-20H. Plasmids pCSLM71a and pCSLM71b carry the insert of pCS16 with gene cassette C.K1 [27] substituting for the 231-bp *Cla*I-*Bam*HI DNA fragment internal to *mobA*, along the insert of pCS13. Plasmid pCSLM62 carries a 507-bp DNA fragment, cloned in vector pGEM-T (Promega), generated by PCR using as primers oligonucleotides moeB1 and moeB2 and as template genomic DNA from *Synechocystis* sp. strain PCC 6803. Plasmids pCSLM91a and pCSLM91b carry an *orf392*-bearing DNA fragment of 1184 bp, generated by PCR using *Pfu* polymerase and oligonucleotides moeBsec1 and moeBsec4 as primers, in which a *Hin*cII-ended C.K1 gene cassette was inserted into the *Eco*RV site internal to *orf392*. Plasmids were transferred to *Synechococcus* sp. strain PCC 7942 by means of transformation [28]. Cells were spread onto nitrocellulose filters (Nucleopore; REC85) set successively atop $BG11_0NH_4^+$ solid medium (incubated for 48 h) and $BG11_0NH_4^+$ with Km at 10–25 μg/ml (incubated for 3 weeks). Individual colonies were selected and, after recloning, maintained in $BG11_0NH_4^+$ solid medium with Km.

2.5. Nucleotide sequence accession numbers

Nucleotide sequences have been deposited in the EMBL/GenBank/DDBJ nucleotide sequence data libraries under accession numbers Y13330 for the *mobA* region and Y16560 for the putative *moeB* gene.

3. Results

3.1. Identification of mobA

Synechococcus sp. strain FM1 is a Tn*901*-induced mutant that lacks nitrate reductase activity and can be rescued by plasmid pNR1934, defining it as a *narC* locus mutant [29]. Plasmid pNR1934 carries a 6.1-kb *Sal*I DNA fragment from *Synechococcus* sp. strain PCC 7942 [12]. Restriction endonuclease mapping coupled to Southern blot analysis indicated that a 0.9-kb *Bam*HI DNA fragment, which can be detected in strain PCC 7942 DNA using as a probe the insert of pNR1934, was missing from strain FM1 DNA which instead showed a much larger hybridization band (Fig. 1). The nucleotide sequence of a fragment of 1553 bp containing the 0.9-kb *Bam*HI fragment was determined and found to bear two complete ORFs and the 3′ end of another one (Fig. 2). The incompletely sequenced ORF encodes a polypeptide that shows homology to the *lap* gene, encoding leucine aminopeptidase, from *Arabidopsis thaliana* (64% identity in the carboxy-terminal 125 amino acids [30]). The ORF immediately downstream of *lap* would encode a 194-amino acid polypeptide with 28% identity to the MobA proteins of both *E. coli* [31] and *Bacillus subtilis* [32]. Finally, downstream of the putative *mobA* gene, in the opposite strand, an ORF of 131 codons (*orf131*) is found whose product would show homology only to that of an ORF of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 [33], *slr0651* (54% identity in a 127-amino acid overlap).

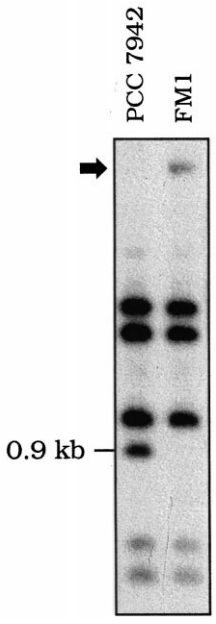

Fig. 1. Localization of Tn*901* in the *narC* locus of mutant strain FM1. Genomic DNA from the indicated strain was simultaneously digested with *Bam*HI, *Bgl*II and *Xho*I and subjected to Southern blot analysis using the 6.1-kb *Sal*I insert of plasmid pNR1934 as a probe. The arrow points to the DNA fragment that includes Tn*901* in mutant strain FM1.

To define more precisely the insertion site of Tn*901* in strain FM1, PCR amplifications were carried out with DNA from strains PCC 7942 and FM1 and pairs of oligonucleotides whose positions are depicted in Fig. 2. Fragments of the expected wild-type sizes were obtained in every case with strain PCC 7942 DNA. With strain FM1 DNA, however, wild-type-size fragments were obtained with oligonucleotide pairs 1+2 and 1+3, but not with 6+4 or 6+5 which gave no detectable PCR product (not shown). This is indicative of the presence of a large DNA insert (i.e. the Tn*901* transposon) within the *mobA* gene. A Km^r-encoding gene cassette, C.K1 [27], was inserted in both orientations into the *mobA* gene (Fig. 2). These constructions were transferred to *Synechococcus* sp. strain PCC 7942 by transformation followed by selection for

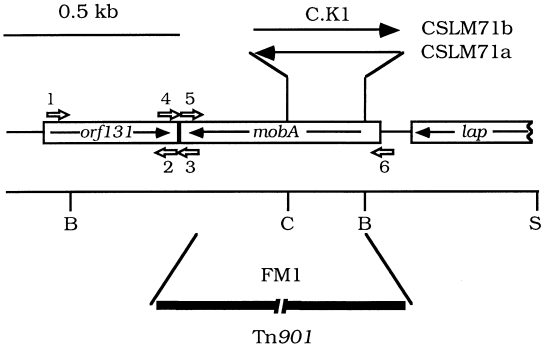

Fig. 2. Structure of a genomic region of *Synechococcus* sp. strain PCC 7942 that contains the *mobA* gene. The orientation of gene cassette C.K1 substituting the 231-bp *Cla*I-*Bam*HI DNA fragment for the generation of cyanobacterial mutants is indicated together with the CSLM denomination of the resulting mutant strain. The location of Tn*901* in mutant strain FM1 is also indicated. For simplicity, the oligonucleotides used as PCR primers to localize the gene disrupted by Tn*901* in strain FM1 are numbered in the figure from 1 to 6: number 1, ORF131_1; number 2, ORF131_2; number 3, ORF131_3; number 4, mobA4; number 5, mobA3; number 6, mobA2 (see Section 2 for details). B, *Bam*HI; C, *Cla*I; S, *Sal*I.

Km^r in $BG11_0NH_4^+$ medium. Clones that, according to PCR and Southern blot analyses, were homozygous for the mutant chromosomes (not shown) were selected for further analysis and named strain CSLM71a (*npt* gene in the same orientation as *mobA*) and strain CSLM71b (*npt* gene in the opposite orientation). None of these strains could be grown with nitrate as the only nitrogen source. Additionally, ammonium-grown cells of these strains incubated for 6 h in BG11'C' (nitrate-containing) medium showed a nitrate reductase activity of <0.1 mU/mg of protein, whereas the activity of strain PCC 7942 cells subjected to the same induction procedure was 104 mU/mg of protein. This corroborates that the mutated ORF is required for production of an active nitrate reductase and, through sequence homology, permits us to conclude that it represents the *Synechococcus mobA* gene.

3.2. Isolation of a putative moeB gene

An essential gene in MPT biosynthesis that has not been found in the characterized *Synechococcus* sp. strain PCC 7942

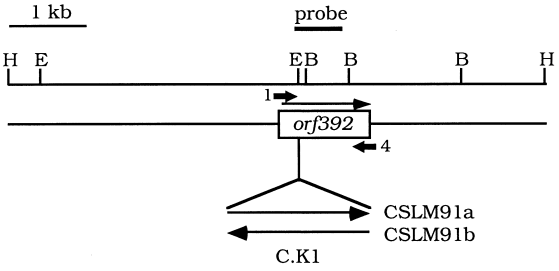

Fig. 3. Restriction map of the insert of plasmid pCSLM58 comprising a genomic region of *Synechococcus* sp. strain PCC 7942 that contains a putative *moeB* gene, and location of the sequences homologous to the *sll1536* probe from *Synechocystis* sp. strain PCC 6803. The oligonucleotides used as PCR primers for the amplification of *moeB* are numbered 1 and 4 (moeBsec1 and moeBsec4 in Section 2, respectively). The orientation of gene cassette C.K1 inserted at the *Eco*RV restriction site internal to *orf392* (putative *moeB* gene) for the generation of cyanobacterial mutants is indicated together with the CSLM denomination of the resulting mutant strain. B, *Bam*HI; E, *Eco*RV; H, *Hin*dIII.

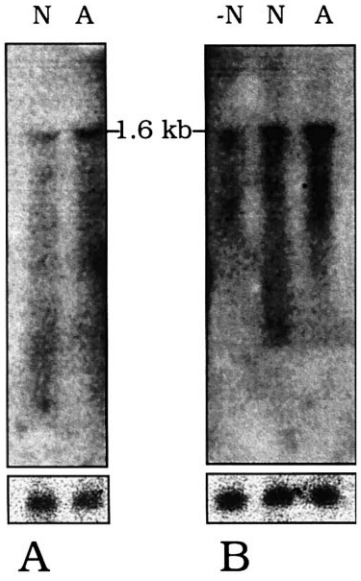

Fig. 4. Transcript levels of the putative *moeB* gene under different nitrogen regimens. A: RNA isolated from *Synechococcus* sp. PCC 7942 cells grown with nitrate (N) or ammonium (A) as the nitrogen source was probed with a 0.5-kb *Bam*HI DNA fragment internal to *orf392* (see Fig. 3). B: RNA isolated from cells grown with ammonium and incubated for 90 min in medium containing ammonium (A), nitrate (N), or no nitrogen source (−N) was probed with the same DNA fragment as above. The lower panels show hybridizations with a probe of the *rpnB* gene [39], carried out as a loading and transfer control.

nar loci is *moeB*. An ORF encoding a putative MoeB homologue, *sll1536*, is present in the chromosome of *Synechocystis* sp. strain PCC 6803 [33]. We sought the isolation of a putative *moeB* gene from strain PCC 7942 by heterologous DNA/DNA hybridization with a probe of *sll1536*. This probe was generated by PCR amplification of strain PCC 6803 DNA and was cloned as the insert of plasmid pCSLM62. A 7.2-kb *Hin*dIII DNA fragment from *Synechococcus* sp. strain PCC 7942 hybridized, under low-stringency conditions, to the *sll1536* probe and was cloned into the *Hin*dIII site of vector pIC-20R giving plasmid pCSLM58. Restriction endonuclease mapping coupled to Southern blot analysis under low-stringency conditions, using as a probe the insert of pCSLM62, localized the putative *sll1536* homologue sequences around a 0.5-kb *Bam*HI DNA fragment within the insert of pCSLM58 (Fig. 3). Sequencing of 1220 bp in this region revealed an ORF (*orf392*) that would encode a 391-amino acid polypeptide with 74% identity to the putative product of *sll1536*.

The putative products of *sll1536* and *orf392* exhibited a higher identity to putative polypeptides from *Mycobacterium tuberculosis* (Rv3116) and *Homo sapiens* (MOCS3) than to MoeB and ThiF of enterobacteria or to HesA of filamentous, nitrogen-fixing cyanobacteria (Table 1). Whereas homology to Rv3116 and MOCS3 extended to the whole putative products of *sll1536* and *orf392*, similarity to MoeB, ThiF or HesA was limited to approximately their first 260 amino acids. The *sll1536* and *orf392* predicted products would therefore be composed of two modules: an amino-terminal domain homologous to MoeB (amino acids 1–260, approximately), and a carboxy-terminal domain (amino acids 261–390, approximately) without similarity to any protein of known function.

Table 1
Polypeptides homologous to the predicted product of strain PCC 7942 *orf392*

Homologous protein (organism)	Size (number of amino acids)	% Identity[a]	Overlapping fragment[b]	Reference
sll1536 product (*Synechocystis* sp.)	392	74	1–391	[33]
Rv3116 (*M. tuberculosis*)	389	51	1–391	[40]
MOCS3 (*H. sapiens*)	406	44	1–391	[41]
Hi1449 (*H. influenzae*)	243	42	8–252	[42]
MoeB (*S. typhimurium*)	249	38	8–258	[43]
MoeB (*E. coli*)	249	39	8–258	[44]
ThiF (*E. coli*)	251	37	11–262	[45]
HesA (*Anabaena* sp.)	266	34	23–290	[46]
HesA (*P. boryanum*)	277	28	8–288	[47]

[a]As deduced from the overlapping fragment.
[b]Position in the *Synechococcus* polypeptide of the amino acids that could be aligned with the indicated homologous protein.

To test whether *orf392* is involved in the production of nitrate reductase activity, we attempted to construct a mutant with a null mutation in this ORF. The C.K1 gene cassette was inserted in both orientations into the *Eco*RV site of a PCR-generated DNA fragment carrying just *orf392* (see Section 2), giving plasmids pCSLM91a (*npt* gene in the same orientation as *orf392*) and pCSLM91b (*npt* gene in the opposite orientation) (Fig. 3). These plasmids were transferred to *Synechococcus* sp. strain PCC 7942 by transformation followed by selection for Km^r in $BG11_0NH_4^+$ medium, and Km^r transformants were analyzed by PCR. Clones carrying recombinant chromosomes in which the wild-type version of *orf392* had been substituted through a double recombination by the C.K1-inactivated version of the gene were obtained in both cases. No clone homozygous for the mutant chromosomes could however be isolated after prolonged growth in the presence of ammonium and Km. This suggests that *orf392* is essential under the growth conditions tried. Alternatively, the insertion in *orf392* might be lethal because of a polar effect on the expression of another gene. This possibility is unlikely, however, since (i) the *orf392* transcript is not much longer than *orf392* itself (see below), and (ii) the C.K1 gene cassette does not bear any transcription terminator downstream of the *npt* gene [34,35].

Transcript levels of *orf392* were investigated by RNA/DNA hybridization. RNA isolated from strain PCC 7942 cells, grown with ammonium or nitrate as nitrogen source, was probed with the 0.5-kb *Bam*HI DNA fragment internal to *orf392* (see Fig. 3). A transcript of 1.6 kb was observed with both RNA preparations, but it appeared to be somewhat more abundant in ammonium- than in nitrate-grown cells (Fig. 4A). Since *orf392* consists of 1176 bp, the observed transcript is apt to cover the whole gene. The hybridization was also carried out with RNA isolated from cells grown with ammonium, washed, and incubated for 90 min in medium supplemented with ammonium, nitrate or no source of combined nitrogen (−N). A transcript of 1.6 kb was again observed whose abundance with the different nitrogen sources was: ammonium > nitrate > −N, although the differences were not very pronounced (Fig. 4B).

4. Discussion

The MobA protein is required to add a GMP moiety to MPT to give MGD [9]. Therefore, the identification of a *mobA* gene required for the expression of nitrate reductase activity in *Synechococcus* sp. strain PCC 7942 indicates that, in this bacterium, assimilatory nitrate reductase uses a molybdenum cofactor that includes MGD.

A putative *moeB* gene has also been identified in this work, but a strain homozygous for chromosomes carrying an inactivated *moeB* gene could not be isolated. This is consistent with the fact that *moeB* did not appear among the previously recognized *nar* loci that were identified by a nitrate reductase minus phenotype in a collection of transposon-induced mutants [11,12,29]. MoeB is required for MPT biosynthesis and mutants of *moeB* have been isolated in *E. coli* [36]. It seems therefore that the putative MoeB protein of *Synechococcus* sp. strain PCC 7942 identified here would have function(s) additional to, or other than, MPT biosynthesis. In this context, it is of interest that both strain PCC 7942 and strain PCC 6803 putative MoeB proteins have a 130-amino acid carboxy-terminal extension that is not present in *E. coli* MoeB and is only found in putative proteins whose function has not yet been investigated, *M. tuberculosis* Rv3116 and human MOCS3. On the other hand, we have been unable to complement the nitrate reductase minus phenotype of an *E. coli moeB* mutant, strain AH30 [8], using the *Synechococcus orf392* (not shown). The putative *moeB* gene is expressed at levels higher than those of any of the other MGD biosynthesis genes characterized in *Synechococcus* sp. strain PCC 7942. Whereas we have detected, through RNA/DNA hybridization, a transcript for this gene (Fig. 4), we have been unable to observe, using the same experimental approach, the transcripts of *mobA* (this work, not shown), *moeA* or the *moa* operon [17].

Apart from genes encoding a molybdate transport system, a gene related to MGD biosynthesis that has not been characterized in *Synechococcus* sp. strain PCC 7942 is *mobB*, which in *E. coli* appears to be cotranscribed with *mobA* [37]. Because an *E. coli mobB* mutant has a wild-type phenotype [37] and no *mobB* homologue is found in the chromosome of *Synechocystis* sp. strain PCC 6803 which however carries a *mobA* homologue [33], it appears that *mobB* is not essential for MGD biosynthesis. Another gene related to MGD biosynthesis that has been described in *E. coli* is *mog* whose product is homologous to MoaB. MoaC from *Synechococcus* sp. strain PCC 7942 resembles a fusion of the MoaC and MoaB or Mog polypeptides of *E. coli* [17]. However, no ORF that would encode a polypeptide homologous to MoaB/Mog is found in the *Synechocystis* chromosome [33]. This suggests that also MoaB and Mog may not be strictly required for MGD biosynthesis. Consistent with this, no *moaB* mutant has been identified in *E. coli* [38].

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