The molybdenum cofactor biosynthesis protein MobA from *Rhodobacter capsulatus* is required for the activity of molybdenum enzymes containing MGD, but not for xanthine dehydrogenase harboring the MPT cofactor

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Abstract

The requirement of MobA for molybdoenzymes with different molybdenum cofactors was analyzed in *Rhodobacter capsulatus*. MobA is essential for DMSO reductase and nitrate reductase activity, both enzymes containing the molybdopterin guanine dinucleotide cofactor (MGD), but not for active xanthine dehydrogenase, harboring the molybdopterin cofactor. In contrast to the *mob* locus of *Escherichia coli* and *R. sphaeroides*, the *mobB* gene is not located downstream of *mobA* in *R. capsulatus*. The *mobA* gene is expressed constitutively at low levels and no increase in *mobA* expression could be observed even under conditions of high MGD demand.

Keywords: Molybdoenzyme; Molybdenum cofactor biosynthesis; Molybdopterin guanine dinucleotide synthase; *mob* locus; *Rhodobacter capsulatus*

1. Introduction

Molybdoenzymes from all organisms with the exception of dinitrogenase, contain a common cofactor named the molybdenum cofactor (Moco). The common component of all forms of Moco is molybdopterin (MPT), a pterin with a unique dithiolene group, to which molybdenum is complexed [19]. In most bacteria, including *Escherichia coli*, molybdopterin is further modified by covalent attachment of GMP to the terminal phosphate group of molybdopterin via a pyrophosphate link, to form the molybdopterin guanine dinucleotide cofactor (MGD). Alternatively, other nucleotides like CMP, AMP or HMP can be attached to molybdopterin in prokaryotes [2]. *E. coli* mutants defective in the *mob* locus are able to synthesize molybdopterin, but lack the ability to form molybdopterin guanine dinucleotide [17], suggesting...
that the mob locus is required for the addition of a GMP moiety to molybdopterin to produce MGD. The mob locus in E. coli contains two genes, mobA and mobB, which are expressed constitutively at low levels [9]. The product of the mobA gene, protein FA, was characterized to be the molybdopterin guanine dinucleotide synthase [16]. The mobB gene product functions as a nucleotide binding protein with high affinity for GTP [5]. In contrast to E. coli, in which all molybdoenzymes contain MGD, the phototrophic purple bacterium Rhodobacter capsulatus was shown to possess different kinds of molybdoenzymes: dinitrogenase, containing the unique iron molybdenum cofactor (FeMoco), DMSO reductase containing the bis-molybdopterin guanine dinucleotide cofactor [23] and xanthine dehydrogenase containing the molybdopterin cofactor (MPT) as found in all eukaryotic molybdoenzymes [13]. Therefore, R. capsulatus offers a possibility of analyzing the function and the regulation of the mob locus, when molybdoenzymes with different molybdenum cofactors are synthesized.

This work describes the identification, cloning and DNA sequence analysis of the mob locus in R. capsulatus which, in contrast to other bacteria, contains no mobB gene downstream of mobA. The influence of mutations in mobA on the activity of different molybdoenzymes was analyzed. In addition, the expression of the mobA gene was studied under different growth conditions and in different Moco biosynthesis mutants.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Methods for conjugal plasmid transfer between E. coli and R. capsulatus and the selection of mutants, anaerobic growth conditions, growth media and antibiotic concentrations were as previously described ([11,13] and references therein).

| Table 1 |
|------------------|------------------|
| **Bacterial strains and plasmids used in this study** |
| **Strain or plasmid** | **Genotype and/or characteristics** | **Reference or source** |
| E. coli | | |
| S17-1 | RP4-2 (Tc::Mu) (Km::Tn7) integrated into the chromosome | [25] |
| JM83 | Host for pUC plasmids | [27] |
| TP1000 | MC4100, (mobAB) | [17] |
| R. capsulatus | | |
| KS36 | ΔnifHDK::Spc, derivative of B10S | [28] |
| SL113III | mobA::Gm, insertion mutant of KS36 | This work |
| R507 | moaA::Gm, deletion mutant of KS36 | This work |
| IJS8 | moaA::Gm, insertion mutant of KS36 | McEwan (Brisbane) |
| R423CII | Δ(mobAB)::Gm, deletion mutant of KS36 | [28] |
| **Plasmids** | | |
| pUC8 | Ap’, Lac+ | [27] |
| pWKR102A | Cm’, Gm’, mob | [4] |
| pML5B+ | Broad host range lacZ-fusion vector | [12] |
| pFR400 | R. sphaeroides napABC genes cloned into pHU231 | [20] |
| pSL106 | 6-kb EcoRI fragment carrying mobA of R. capsulatus cloned into pUC8 | This work |
| pSL113III | 3-kb PstI-EcoRI fragment of pSL106 cloned into the PstI site of pSUP202, carrying a gentamicin resistance cassette cloned into the BglII site within mobA | This work |
| pSL150 | mobA-lacZ fusion vector; 533-bp EcoRV-HindIII fragment from the 5’ end of mobA cloned into pML5B+ | This work |
| pSL151 | mobA-lacZ fusion vector; 11.4-kb BamHI fragment from pSL150 cloned into pWKR102A | This work |
2.2. DNA biochemistry

DNA isolation, restriction enzyme analysis, agarose gel electrophoresis and cloning procedures were performed using standard methods [22]. Restriction endonucleases, T4 DNA ligase, and Klenow polymerase were purchased from Pharmacia and used as recommended by the supplier.

2.3. Isolation and DNA sequencing of the mob locus in R. capsulatus

A size fractionated EcoRI gene bank was screened using a degenerate oligonucleotide (mobAI: 5’GGCGT(GC)ACC(GC)(CT)(GC)ACCGA(AG)GC-(GC)GGC(AG)A(AG)A(CT)CCTG 3’) synthesized according to conserved amino acid regions in MobA from R. sphaeroides [18], E. coli [1], Haemophilus influenzae [6], Methanococcus jannaschii [3] and Synechocystis sp. strain PCC 6803 [10]. Southern hybridizations were performed according to standard procedures [22]. From Southern hybridizations, positive clones containing a 6-kb EcoRI chromosomal R. capsulatus fragment were identified. To delimit the mob locus on the 6-kb EcoRI fragment, subclones were transformed into the E. coli mob mutant strain TP1000 [17]. Transformed strains were analyzed for nitrate reductase activity using the colony overlay method [8]. DNA sequence analysis was carried out using standard fluorescence labelled primers and appropriate subclones of the 6-kb EcoRI fragment. Sequence data were obtained and processed by using the A.L.F. DNA sequencer (Pharmacia LKB) as instructed by the manufacturer. DNA editing programs were used as described [13].

2.4. Construction of R. capsulatus insertion mutants

For the construction of R. capsulatus mobA interposon mutants, wild-type fragments were cloned by standard methods [22] into mobilizable vector plasmids. The BglII restriction site within the mobA coding region was used to insert the gentamicin resistance gene and the resulting hybrid plasmids pSL113I and pSL113II were mobilized from E. coli S17-1 into R. capsulatus KS36 by filter mating [13]. Mutants were selected for the interposon-encoded resistance, and double cross over events were identified by the loss of the vector-encoded resistance.

2.5. Enzyme assays

Xanthine dehydrogenase activities were assayed as described in [13], with NAD as an electron acceptor. Nitrate reductase and DMSO reductase activities were measured as described previously [7,14], with diithionite-reduced methyl viologen as the electron donor.

2.6. Protein determination

The protein concentration was determined according to Smith et al. [26].

2.7. Construction of mobA-lacZ fusion plasmids

To create transcriptional mobA-lacZ fusions, a 533-bp EcoRV-HindIII fragment (Fig. 1B) carrying the 5′ part of R. capsulatus mobA was cloned into the polylinker of pML5B+ [12] resulting in the replicative reporter plasmid pSL150. To create an integrative mobA-lacZ fusion, an 11.4-kb BamHI fragment from pSL150 containing mobA-lacZ was cloned into pWKR102A [4], resulting in plasmid pSL151.

2.8. β-Galactosidase assays

To determine β-galactosidase activities of R. capsulatus strains carrying the transcriptional mobA-lacZ fusions (pSL150, pSL151), corresponding strains were grown in RCV medium supplemented with tetracycline (0.25 μg ml⁻¹). Ammonium was added at a final concentration of 10 mM, hypoxanthine at 1 mM and DMSO at 30 mM. Following growth in the respective media to late exponential phase, β-galactosidase activities of R. capsulatus strains were determined by the sodium dodecyl sulfate (SDS)-chloroform method [15].

2.9. Nucleotide sequence accession number

The nucleotide sequence of a 795-bp BamHI-EcoRV DNA fragment encompassing the mobA cod-
The region has been submitted to EMBL nucleotide sequence database under accession number AJ131528.

3. Results

3.1. Identification and isolation of the mob locus from R. capsulatus

To identify the mob locus in R. capsulatus, Southern hybridization analysis using a degenerate oligonucleotide (mobAI) synthesized according to conserved regions in MobA amino acid sequences from different organisms was performed as described in Section 2. A 6-kb EcoRI fragment hybridizing to the oligonucleotide was identified in R. capsulatus total DNA and the corresponding clone was isolated from a size-fractionated plasmid gene bank (pSL106, Fig. 1A). Plasmid pSL106 was tested for the ability to complement the mob deficient E. coli strain TP1000 (Section 2). In TP1000 transformants containing pSL106, nitrate reductase was restored (data not shown). To delimit the R. capsulatus mob region on pSL106, subclones were hybridized with the oligonucleotide and tested for their ability to restore nitrate reductase activity in TP1000. The complementation of the E. coli mob mutant strain delimited the R. capsulatus mob region to a 3-kb PstI-EcoRI fragment (Fig. 1A).

3.2. Nucleotide sequence analysis and interposon mutagenesis of the R. capsulatus mob region

The exact location of the R. capsulatus mob locus within the 3-kb PstI-EcoRI fragment was determined by partial DNA sequence analysis (Fig. 1B). Complete sequence analysis of a 795-bp BamHI-EcoRV fragment revealed the presence of an open reading frame (mobA, Fig. 1B) encoding a protein of 191 amino acids with a deduced molecular mass of 19814 Da. R. capsulatus MobA is 47% identical to MobA from R. sphaeroides [18] and shows high similarities to MobA proteins from other organisms.
Downstream of mobA, the 3’ end of another ORF (moaC) transcribed in the opposite direction was identified (Fig. 1B). The deduced amino acid sequence of the C-terminus of the corresponding protein shares a high degree of similarity to MoaC of E. coli [21] and other organisms (data not shown). The stop codons of both genes overlap each other. It is worth noting that the mob locus maps approximately 3 kb downstream of the structural gene for DMSO reductase (dorA, [24]) in the R. capsulatus chromosome (Fig. 1A). No coding region for mobB could be identified within the 3-kb EcoRI-PstI fragment.

To inactivate the mobA gene in R. capsulatus, defined insertion mutations were constructed. For this purpose an interposon encoding gentamicin resistance was inserted into the BglII site within the mobA gene (Fig. 1B). The resulting plasmids, designated pSL113I and pSL113II, no longer restored nitrate reductase activity in E. coli mutant strain TP1000, confirming that the R. capsulatus mobA gene was inactivated by the gentamicin interposon insertion. To analyze the influence of the mobA mutation in R. capsulatus, plasmids pSL113I and pSL113II were introduced into different R. capsulatus mutant strains, as shown below.

3.3. Molybdoenzyme activities in R. capsulatus KS36 and in mobA mutant strains

To study the influence of a mutation in mobA on the activity of enzymes containing different molybdenum cofactors, the activities of xanthine dehydrogenase, periplasmic nitrate reductase and DMSO reductase were analyzed in different R. capsulatus strains (Table 2). To analyze nitrate reductase activity, the corresponding genes for periplasmic nitrate reductase from R. sphaeroides were introduced on a replicative plasmid into R. capsulatus (pFR400 [20]). The activities of the three molybdoenzymes were analyzed in the mobA mutant strains SL113I and SL113II compared to the parental strain KS36 (carrying a nifHDK deletion). As expected, the activity of xanthine dehydrogenase, which was shown to bind the MPT cofactor [13], was not influenced by a mutation in mobA (Table 2). In contrast, nitrate reductase activity and DMSO reductase activity were completely lost in mobA mutant strains (Table 2). The crystal structure of DMSO reductase revealed the presence of a bis-molybdopterin guanine dinucleotide cofactor [23]. Nitrate reductase, for which the crystal structure has not yet been solved, is assumed to contain the same cofactor. In conclusion, MobA from R. capsulatus is absolutely required for enzymes containing the MGD cofactor, but not for xanthine dehydrogenase containing MPT.

3.4. Transcriptional regulation of mobA: expression analysis of mobA-lacZ fusions under different growth conditions and in Moco biosynthesis mutants

As shown in Fig. 1C, two transcriptional mobA-lacZ fusions were constructed by fusing the mobA coding region at the HindIII site to lacZ (Section 2). The reporter plasmid pSL151 is unable to replicate in R. capsulatus and thus has to integrate into the R. capsulatus chromosome by single cross-over.
recombination. This plasmid was used to ensure that no DNA sequences located outside the cloned region influenced the expression of mobA. As shown in Table 3, the expression pattern of the chromosomal mobA-lacZ fusion (pSL151) and the mobA-lacZ fusion located on a replicative plasmid (pSL150), revealed no significant differences under all growth conditions tested. Both reporter plasmids indicate a low constitutive expression of mobA. The three-fold increase of mobA-lacZ expression in strains carrying pSL150 compared to those containing pSL151 can be explained by the copy number of the pSL150 replicon (RSF1010). In the presence of DMSO, large amounts of the MGD containing DMSO reductase are synthesized, whereas hypoxanthine as sole source of nitrogen in the medium ensures maximum levels of the MPT containing xanthine dehydrogenase. However, the relative amount of these two kinds of molybdoenzymes did not influence the expression of mobA. In addition, the expression of mobA was neither affected in strains carrying mutations in the Moco biosynthesis loci moeA, moaA and mobA, nor in a strain defective in the high affinity molybdate uptake system (modABCD−) and in mopAB, encoding proteins involved in gene regulation by molybdenum [28].

4. Discussion

The mob locus of E. coli, required for molybdenum guanine dinucleotide synthesis is comprised of two genes, mobA and mobB. The mobA gene encodes protein FA [16], which is essential for the synthesis of the molybdopterin guanine dinucleotide cofactor (MGD), and mobB codes for a GTP binding protein [5]. In contrast, the mob locus of R. capsulatus was shown to contain the mobA gene only. No coding region for mobB could be identified downstream or upstream of mobA, demonstrating that in R. capsulatus, in contrast to R. sphaeroides [18], E. coli [1], H. influenzae [6] and M. jannaschii [3], the mobA and mobB genes are not organized into one transcriptional unit. As found in R. capsulatus, mobB is also not linked to mobA in Synechocystis sp. strain PCC6803 [10]. Whether R. capsulatus lacks the mobB gene or if the mobB gene is located at another position in the chromosome, remains speculative at the moment. However, complementation of an E. coli mobAB double mutant by a plasmid carrying only R. capsulatus mobA proved that even in E. coli, MobBB is not essential for the biosynthesis of the molybdopterin guanine dinucleotide cofactor [5].

R. capsulatus was shown to contain molybdoenzymes with different molybdenum cofactors, like DMSO reductase coordinating the bis-MGD cofactor [23] and xanthine dehydrogenase containing the MPT cofactor [13]. In contrast to E. coli, in which all molybdoenzymes contain MGD, R. capsulatus offers the possibility of conclusively demonstrating that the molybdopterin guanine dinucleotide synthase, MobA, is only essential for MGD biosynthesis and has no further role for MPT biosynthesis. The analysis of defined R. capsulatus mobA mutants revealed

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>lacZ-fusion</th>
<th>+NH₄⁺</th>
<th>+NH₄⁺+DMSO</th>
<th>+Hypoxanthine</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10S</td>
<td>wild-type</td>
<td>pSL151</td>
<td>11 ± 2</td>
<td>11 ± 1</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>R10S</td>
<td>wild-type</td>
<td>pSL150</td>
<td>26 ± 2</td>
<td>27 ± 1</td>
<td>29 ± 4</td>
</tr>
<tr>
<td>KS36</td>
<td>ΔmoeB</td>
<td>pSL150</td>
<td>30 ± 3</td>
<td>34 ± 5</td>
<td>30 ± 1</td>
</tr>
<tr>
<td>SL113I</td>
<td>ΔmoeB, mobA⁻</td>
<td>pSL150</td>
<td>30 ± 3</td>
<td>27 ± 4</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>R507</td>
<td>ΔmoeB, mobA⁻</td>
<td>pSL150</td>
<td>30 ± 5</td>
<td>28 ± 1</td>
<td>NG</td>
</tr>
<tr>
<td>JPS8</td>
<td>ΔmoeB, mobA⁻</td>
<td>pSL150</td>
<td>26 ± 4</td>
<td>31 ± 2</td>
<td>32 ± 3</td>
</tr>
<tr>
<td>R423CII</td>
<td>ΔmoeB, mopABmodABCD⁻</td>
<td>pSL150</td>
<td>29 ± 3</td>
<td>30 ± 4</td>
<td>31 ± 5</td>
</tr>
</tbody>
</table>

[α]β-Galactosidase activity was determined by the SDS-chloroform method [15] and is given in Miller units. Mean values and standard deviations were calculated from at least six independent measurements.

R. capsulatus cultures were grown photoheterotrophically in RCV medium with 10 mM NH₄⁺, 30 mM DMSO or 1 mM hypoxanthine. NG: no growth.
that the activity of MGD containing enzymes like nitrate reductase and DMSO reductase is completely lost, whereas xanthine dehydrogenase activity is not affected in these mutant strains. The ratio of enzymes containing either MGD or MPT cofactors differs in R. capsulatus significantly according to the growth conditions. In the presence of DMSO large amounts of MGD containing DMSO reductase are formed. In contrast, growth with hypoxanthine as the sole source of nitrogen results in increased levels of MPT containing xanthine dehydrogenase. Therefore, expression of mobA could have been the target for regulatory mechanisms ensuring the correct amounts of the respective cofactor. However, analysis of mobA-lacZ fusions revealed no significant difference in the level of expression according to the growth conditions. The expression pattern of R. capsulatus mobA is also not influenced in mutant strains unable to synthesize MGD cofactors (moaA, mobA) or unable to synthesize both MPT and MGD cofactors (moeA). The constitutive expression of R. capsulatus mobA indicates that the regulation of the ratio between MGD and MPT formation, respectively, is not controlled at the transcriptional level of mobA, coding for the molybdopterin guanine dinucleotide synthase in R. capsulatus.

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References


