JOURNAL OF BACTERIOLOGY, Dec. 2006, p. 8441–8451 Vol. 188, No. 24
0021-9193/06/$08.00+0 doi:10.1128/JB.01188-06

Overlapping and Specialized Functions of the Molybdenum-Dependent Regulators MopA and MopB in *Rhodobacter capsulatus*[▽]

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The phototrophic purple bacterium *Rhodobacter capsulatus* encodes two similar but functionally not identical molybdenum-dependent regulator proteins (MopA and MopB), which are known to replace each other in repression of the *modABC* genes (coding for an ABC-type high-affinity Mo transport system) and *anfA* (coding for the transcriptional activator of Fe-nitrogenase genes). We identified further Mo-regulated (*mor*) genes coding for a putative ABC-type transport system of unknown function (MorABC) and a putative Mo-binding protein (Mop). The genes coding for MopA and the ModABC transporter form part of a single transcriptional unit, *mopA-modABCD*, as shown by reverse transcriptase PCR. Immediately upstream of *mopA* and transcribed in the opposite direction is *mopB*. The genes coding for the putative MorABC transporter belong to two divergently transcribed operons, *morAB* and *morC*. Expression studies based on *lacZ* reporter gene fusions in mutant strains defective for either MopA, MopB, or both revealed that the regulators substitute for each other in Mo-dependent repression of *morAB* and *morC*. Specific Mo-dependent activation of the *mop* gene by MopA, but not MopB, was found to control the putative Mo-binding protein. Both MopA and MopB are thought to bind to conserved DNA sequences with dyad symmetry in the promoter regions of all target genes. The positions of these so-called Mo boxes relative to the transcription start sites (as determined by primer extension analyses) differed between Mo-repressed genes and the Mo-activated *mop* gene. DNA mobility shift assays showed that MopA and MopB require molybdenum to bind to their target sites with high affinity.

Molybdenum serves as a cofactor for many redox enzymes catalyzing basic reactions in the nitrogen, sulfur, and carbon cycles. There are two distinct types of molybdoenzymes. Mo-nitrogenase, which catalyzes the reduction of N_2 to ammonia, has a unique molybdenum-iron-sulfur cofactor called FeMo-co. All other molybdoenzymes, such as nitrate reductase, dimethyl sulfoxide (DMSO) reductase, and xanthine dehydrogenase, contain a cofactor (called Mo-co) in which a mononuclear Mo atom is coordinated to the sulfur atoms of a pterin.

Many bacteria actively take up molybdate by use of a high-affinity ABC-type transport system comprising three proteins (25). ModA, the molybdate-binding protein, is localized in the periplasm in gram-negative bacteria or attached to the outer side of the cytoplasmic membrane in gram-positive bacteria. ModB is the membrane integral channel protein, and ModC is the cytoplasmic ATPase. In *Escherichia coli*, the *modABC* genes constitute a single operon, whose expression requires Mo starvation (8). When the intracellular Mo content is high, a molybdate-dependent regulator, ModE, binds to the *modABC* promoter and represses transcription of the Mo transport operon. ModE consists of two functionally distinct domains, an N-terminal DNA-binding domain and a C-terminal molybdate-binding domain (9). In the presence of molybdenum, ModE binds to a region of the *modABC* promoter with a dyad symmetric element (the so-called Mo box) that overlaps the transcription start (3).

ModABC-like Mo transport systems and ModE-like regulators are widespread in bacteria. In addition to *E. coli*, these proteins have been characterized in greater detail for *Anabaena variabilis* (30), *Azotobacter vinelandii* (20), *Bradyrhizobium japonicum* (6), *Rhodobacter capsulatus* (29), and *Staphylococcus carnosus* (21).

Molybdate binding involves a conserved domain of about 70 amino acids, the Mop domain (for a review, see reference 22). Mop domains occur in three classes of cytoplasmic proteins with distinct functions. Molbindins (Mop proteins), which are implicated in Mo homeostasis within the cell, consist solely of Mop domains present either as single Mop domains or tandem Mop repeats. The Mo-dependent regulatory protein ModE contains a C-terminal tandem Mop repeat, and a single Mop domain occurs in the C-terminal domain of ModC.

While *E. coli* contains only a single copy of *modE*, the phototrophic purple bacterium *Rhodobacter capsulatus* codes for two ModE-like regulator proteins (MopA and MopB) (29). In addition, *R. capsulatus* can synthesize a Mo-dependent nitrogenase (Mo-nitrogenase) and an alternative iron-only nitrogenase (Fe-nitrogenase), the latter being expressed only in the absence of molybdenum (for a review, see reference 17). MopA and MopB replace each other in Mo repression of *anfA* (which codes for the transcriptional activator of Fe-nitrogenase genes) and the *mopA-modABCD* genes (13).

In the present study, we compared expression of known Mo-controlled genes (*anfA* and *mopA-modABCD*) and newly identified Mo-regulated genes (*morABC*, coding for a putative ABC-type transporter, and *mop*, coding for a putative Mo homeostasis protein) in *R. capsulatus* by genetic means. Mo-dependent transcription control depends on direct interaction of MopA and MopB with their respective target promoters as shown by DNA mobility shift assays. While MopA and MopB

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▽ Published ahead of print on 6 October 2006.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli		
DH5α	Host for plasmid amplification	10
BL21(DE3)	Host for expression of $MopA_{His}$ and $MopB_{His}$	Novagen, Darmstadt, Germany
S17-1	RP4-2 (Tc::Mu) (Km::Tn*7*) integrated in the chromosome	27
R. capsulatus		
B10S	Spontaneous Sm^r mutant of *R. capsulatus* B10	12
KS94A	*anfA*::[Sp] insertion mutant of B10S	29
R423AI	*mopA*::[Gm>] insertion mutant of B10S	13
R423BI	*mopB*::[Gm>] insertion mutant of B10S	13
R423CI	Δ(*mopA mopB*)::[Gm] deletion mutant of B10S	13
R438II	*mopA*::[<Gm] polar insertion mutant of B10S	29
Plasmids		
pAW12	pBluescript KS derivative carrying Δ*morABC*::[Km]	This study
pBluescript KS	High-copy expression vector; Ap	Stratagene, Amsterdam, The Netherlands
pBSL86	Km cassette flanked by polylinker; Ap	1
pET22b(+)	High-copy His tag expression vector; Ap	Novagen, Darmstadt, Germany
pJW32	pET22b(+) derivative carrying $mopA_{his}$	This study
pJW33	pET22b(+) derivative carrying $mopB_{his}$	This study
pJW42	pUC18 derivative carrying a *mop* promoter fragment	This study
pJW45	pUC18 derivative carrying the *morA-morC* intergenic region	This study
pJW59	pML5 derivative carrying a *mop-lacZ* transcriptional fusion	This study
pKS131A	pPHU236 derivative carrying an *anfA-lacZ* translational fusion	13
pML5	Mobilizable *lac* fusion broad-host-range vector; Tc	14
pMOT15	pML5 derivative carrying a *morA-lacZ* transcriptional fusion	This study
pMOT16	pML5 derivative carrying a *morC-lacZ* transcriptional fusion	This study
pPHU236	Mobilizable *lac* fusion broad-host-range vector; Tc	11
pSL21I	pML5 derivative carrying a *modA-lacZ* transcriptional fusion	This study
pSL21II	pML5 derivative carrying a *mopB-lacZ* transcriptional fusion	This study
pWKR459	*mob* Tc Km	7

[a] Ap, ampicillin; Gm, gentamicin; Km, kanamycin; Tc, tetracycline; Sm, streptomycin; Sp, spectinomycin.

replaced each other in repressing transcription of *anfA*, *mopA-modABCD*, *morAB*, and *morC*, only MopA was required for activation of *mop* gene expression.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Methods for conjugational plasmid transfer between *E. coli* S17-1 and *R. capsulatus*, the selection of mutants, rich medium (PY), molybdenum-free minimal medium (AK-NL), growth conditions, and antibiotic concentrations were as previously described (see reference 26 and references therein).

Construction of *R. capsulatus morABC* mutant strain AW12. A 3.5-kb DNA fragment carrying the *R. capsulatus mor* gene region (see Fig. 2) was PCR amplified using primer pair PAW3-U/PAW3-L (Table 2). A 2.5-kb BamHI-XhoI fragment from the PCR amplification product was cloned into vector plasmid pBluescript KS. Subsequently, a 1.5-kb SmaI fragment encompassing the entire *morA* gene, the *morA-morC* intergenic region, and large parts of *morB* and *morC* was replaced by a 1.2-kb SmaI kanamycin cartridge from pBSL86. Finally, insertion of a 8.8-kb XhoI fragment (containing a tetracycline resistance gene and the *mob* locus of PR4) from pWKR459 led to the mobilizable hybrid plasmid pAW12. Conjugational transfer of pAW12 from *E. coli* S17-1 into *R. capsulatus* and selection for marker rescue were carried out as described earlier (12, 29).

β-Galactosidase assays. *R. capsulatus* strains carrying reporter fusions between Mo-regulated genes and the promoterless *E. coli lacZ* gene, namely, *anfA-lacZ*, *mopA-modA-lacZ*, *morA-lacZ*, *morC-lacZ*, and *mop-lacZ* (Table 1), were grown in Mo-free AK-NL minimal medium containing either 9.5 mM serine (nitrogen-limiting conditions) or 20 mM ammonium (nitrogen-sufficient conditions). When required, 10 μM Na_2MoO_4 was added. Following growth to late exponential phase, β-galactosidase activities were determined by the sodium dodecyl sulfate-chloroform method (19).

RNA isolation, transcriptional analysis by RT-PCR, and primer extension. *R. capsulatus* wild-type cultures were grown in Mo-free AK-NL minimal medium containing 9.5 mM serine as the sole nitrogen source. When required, 10 μM Na_2MoO_4 was added. Total RNA was isolated using the Micro-to-Midi total RNA purification system according to the instructions of the manufacturer (Invitrogen, Karlsruhe, Germany). Specific transcripts were analyzed with the ThermoScript reverse transcriptase PCR (RT-PCR) system (Invitrogen). To analyze transcription of the *mopA-modABCD*, *morAB*, *morC*, and *mop* operons, the primers shown in Table 2 were used for cDNA synthesis and/or second-strand synthesis and subsequent PCR amplification steps. Primer extension was carried out as described previously (4) using synthetic oligonucleotide primers (Table 2) to map the transcription start sites of *morC* and *mop*, respectively.

Overexpression of His-tagged *R. capsulatus* MopA and MopB proteins in *E. coli*. The *mopA* and *mopB* coding regions were PCR amplified with primer pairs PJW27-U/PJW27-L and PJW28-U/PJW28-L (encompassing recognition sites for NdeI and XhoI, respectively) (Table 2), using *R. capsulatus* chromosomal DNA as a template. Subsequently, the 0.8-kb NdeI-XhoI fragments with either *mopA* or *mopB* were cloned into expression vector pET22b(+), resulting in hybrid plasmids pJW32 ($mopA_{his}$) and pJW33 ($mopB_{his}$), respectively. The plasmids were transformed into *E. coli* strain BL21(DE3), which served as a host for overexpression of the tagged *R. capsulatus* MopA and MopB proteins ($MopA_{His}$ and $MopB_{His}$). Purification of the recombinant proteins was carried out as described previously (23).

DNA mobility shift assays. DNA fragments encompassing Mo-regulated promoters were obtained by PCR amplification with appropriate primer pairs (Table 2), using chromosomal DNA as a template. Amplification products were purified using a NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany) prior to ^{32}P labeling of 5′ ends with T4 polynucleotide kinase (Fermentas, St. Leon-Rot, Germany). Different amounts (up to 150 pmol) of either $MopA_{His}$ or $MopB_{His}$ in buffer B (40 mM NaH_2PO_4 [pH 8.0], 500 mM NaCl) in a total volume of 16 μl were preincubated at room temperature. When required, 250 nmol Na_2MO_4 was added at the beginning of the preincubation phase. After 10 min, a mixture consisting of 1 μl ^{32}P-labeled DNA (5 fmol/μl), 1 μl poly(dI-dC) (1 μg/μl), and 2 μl binding buffer (25 mM HEPES [pH 8.0], 50 mM K-glutamate, 50 mM $MgSO_4$, 1 mM dithiothreitol, 0.1 mM EDTA, 0.05% Igepal CA-630) was added to the protein samples. After incubation at 30°C for 20 min, samples were

TABLE 2. Primers used for RT-PCR and PCR amplification of selected DNA fragments

Primer	Oligonucleotide sequences (5′→3′)	Relevant characteristics
PAW3-U	ACGGGGAAGCGCGGGGGAAAGAGG	Amplification of *mor* gene region, 3,548 bp
PAW3-L	GCGCGACAGAAAGCCGAACAGC	
PJW1-U	CACCGTTGCACCGCCCACAGT	RT-PCR (*modC-modD*), 694 bp (fragment 1 in Fig. 1)
PJW1-L	TGCCCCCACCGACACCACGATTCT	
PJW2-U	GGTGATCTGCCGCCCCTCCTG	RT-PCR (*modB-modC*), 606 bp (fragment 2 in Fig. 1)
PJW2-L	GGTCGTCGGCTCGGTCATCTATTC	
PJW3-U	CCAGCCCCGCGAAGGTGAAGGA	RT-PCR (*modA-modB*), 688 bp (fragment 3 in Fig. 1)
PJW3-L	TGACAAGGGCGCGGTGCTGAAAAC	
PJW4-U	GACGCATCGGCCGAAAGAAAGAC	RT-PCR (*mopA-modA*), 757 bp (fragment 4 in Fig. 1)
PJW4-L	CGCGCCGGAAAAAGCCCTCAAC	
PJW5-U	GCGCCGTGCCATTGAAA	RT-PCR (*morC-orf1281*), 639 bp (fragment 1 in Fig. 2)
PJW5-L	GGCGCTTGATCCCGACACC	
PJW6-U	ACGGCAAGGCGGGGCGGCAGTAT	RT-PCR (*morA-morB*), 519 bp (fragment 3 in Fig. 2)
PJW6-L	CCAGCACGATCGGCGGAAACACCA	
PJW7-U	GCTTGGCGCGGGGCTCTT	RT-PCR (*morB-orf1277*), 672 bp (fragment 4 in Fig. 2)
PJW7-L	CGGGGCTGACGCAAATCC	
PJW8-U	CGGTCTGGTGCGGATGGGGTCTTC	RT-PCR (*orf413-mop*), 624 bp (fragment 1 in Fig. 3)
PJW8-L	TCGGCGGCGGCTTCGTTGGTGAT	
PJW9-U	CAATATTTGGCGGGCAAGGTCAC	RT-PCR (*mop-orf411*), 559 bp (fragment 3 in Fig. 3)
PJW9-L	GCGCGAAGCAAGGCAGGAGA	
PJW9-U	CAATATTTGGCGGGCAAGGTCAC	RT-PCR (*mop*), 117 bp (fragment 2 in Fig. 3)
PJW8-L	TCGGCGGCGGCTTCGTTGGTGAT	
PJW12-U	GGGCGGCCGTTCCTGTTCCT	*mop* promoter fragment (509 bp) in pJW42
PJW12-L	TCGGCGGCGGCTTCGTTGGTGAT	
PJW18	GAAGGCCCCGTCAGCACCAGAAAT	Primer extension (*morC*)
PJW19	TCGGCGGCGGCTTCGTTGGTGAT	Primer extension (*mop*)
PJW27-U	AACATATGAACGAACAGCCCCTCATCG	Amplification of *mopA* coding region
PJW27-L	TTCTCGAGGGGCATCGCCAGGATGACATG	
PJW28-U	AACATATGACGGACGGTGTGCGCGGGG	Amplification of *mopB* coding region
PJW28-L	TTCTCGAGGGGCAGGGCCAGGATCACATG	
PJW29-U	CCTCGGCGGTCTCGTGGCTTGTCATCA	*morA-morC* intergenic region (1,139 bp) in pJW45
PJW29-L	ACTGCCGCCCCGCCTTGCCGTAAAT	
PJW36-U	CCTCGGCGGTCTCGTGGCTTGTCATC	RT-PCR (*morC*), 363 bp (fragment 2 in Fig. 2)
PJW36-L	CGCGGTCGCTGGGCTTTGTCTTTCA	
PJW49-U	GGCACTGACCGACCTTTTGACC	DNA mobility shift, 222 bp (*mop* promoter)
PJW49-L	AGAATATTGCGTGCGCTGAGTTT	
PJW52-U	ACGGGCAGGCGCGGGGTTCT	DNA mobility shift, 236 bp (*anfA* promoter)
PJW52-L	CGGTAAAGCGTCGGCAGCAGGTTCA	
PJW53-U	GCATCCCAGGCGGTCTTGTAGG	DNA mobility shift, 272 bp (*mopA* promoter)
PJW53-L	ATGAGGCCGCGGGTGATAACG	
PJW54-U	CAGCCCGACATCGAGCGTGAAC	DNA mobility shift, 244 bp (*morC/A* promoter)
PJW54-L	CGGCAGAGGCGGAAAGGAGAAGA	
PJW55-U	ACTGCGCCGCGATCCCCGAGAC	DNA mobility shift, 251 bp (*anfA* internal fragment)
PJW55-L	CGCCGCAATCACCCGCACATCA	

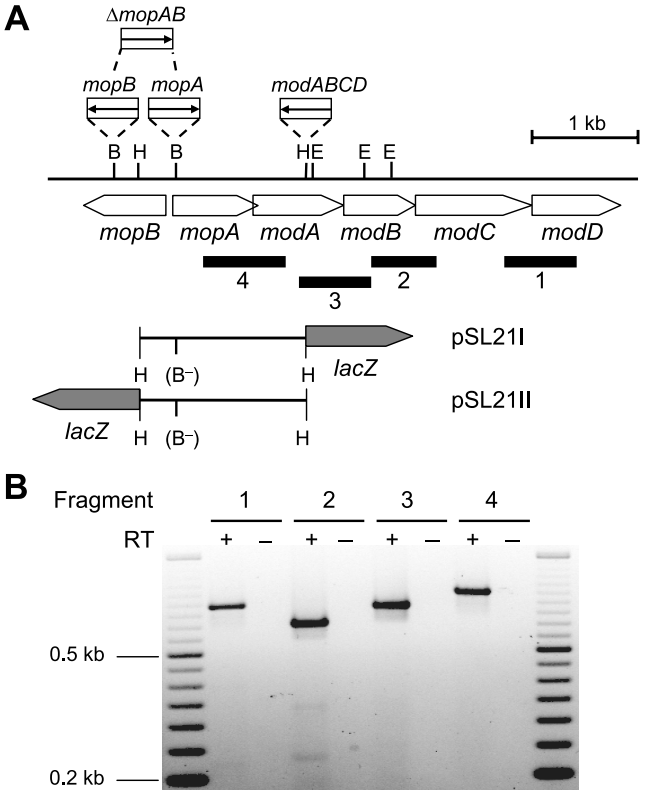

FIG. 1. Transcriptional analysis of the *R. capsulatus mopA-modABCD* gene region. (A) Physical and genetic maps of the *mop-mod* gene region. The physical map is given for BamHI, EcoRI, and HindIII (B, E, and H, respectively). Black bars below the genetic map indicate DNA fragments 1 to 4 emerging from RT-PCR (see Materials and Methods and panel B). The corresponding primer pairs used for RT-PCR are listed in Table 2. Mutant strains defective for either *mopA* (R423AI), *mopB* (R423BI), *mopA* and *mopB* (R423CI), or *modABCD* (R438II) contain gentamicin resistance cassettes, with the directions of transcription of the Gm resistance gene symbolized by arrows. Hybrid plasmids pSL21I and pSL21II, carrying transcriptional *modA-lacZ* and *mopB-lacZ* fusions, respectively, are based on the mobilizable broad-host-range plasmid pML5. In these reporter plasmids, the BamHI sites were destroyed (indicated by B^-) by cutting with BamHI, filling in protruding ends, and blunt-end religation, leading to a frameshift within the *mopA* coding region. Neither the Gm cassette nor the *lacZ* gene is drawn to scale. (B) Transcriptional analysis of the *mopA-modABCD* operon by RT-PCR. Total RNA was isolated from *R. capsulatus* cells grown under Mo-limiting conditions. Either RNA samples were treated with reverse transcriptase to synthesize cDNA (+) or, as a negative control, reverse transcriptase was omitted (−). A 50-bp DNA ladder (Fermentas, St. Leon-Rot, Germany) was used as a length standard.

separated on 6% polyacrylamide gels before ^{32}P-labeled bands were documented using a Hyperscreen X-ray film (Fuji Photo Film Europe, Düsseldorf, Germany).

RESULTS AND DISCUSSION

Genetic organization of selected molybdenum-regulated genes in *R. capsulatus*. Genes coding for two ModE-like Mo-dependent regulators (*mopA* and *mopB*) and a Mo transport system (*modABC*) were previously identified downstream of the structural genes of Mo-nitrogenase, *nifHDK* (29). These genes are organized in two divergently transcribed operons, *mopA-modABCD* and *mopB*. Cotranscription of *mopA-modABCD* was demonstrated by RT-PCR (see Materials and methods) (Fig. 1). Total RNA was isolated from *R. capsulatus* wild-type cells grown in Mo-free minimal medium. After reverse transcription, selected primer pairs (Table 2; Fig. 1) were used to PCR amplify DNA fragments overlapping the gene borders of *mopA-modA*, *modA-modB*, *modB-modC*, and *modC-modD*. The presence of amplification products was completely dependent on the addition of reverse transcriptase to the reaction mixtures, indicating that the RNA was not contaminated with DNA. The presence of PCR products based on all four primer pairs strongly suggested that *mopA-modABCD* comprise a single transcription unit.

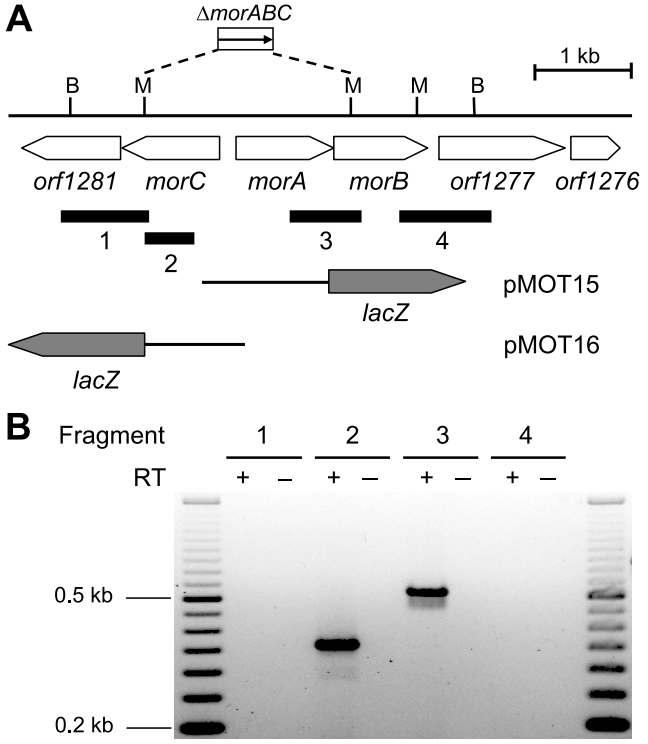

FIG. 2. Transcriptional analysis of the *R. capsulatus mor* gene region. (A) Physical and genetic maps of the *mor* gene region. The physical map is given for BamHI and SmaI (B and M, respectively). Black bars below the genetic map indicate DNA fragments 1 to 4 emerging from RT-PCR (see Materials and Methods and panel B). The corresponding primer pairs used for RT-PCR are listed in Table 2. The *morABC* deletion mutant AW12 contains a kanamycin resistance cassette (not drawn to scale). Hybrid plasmids pMOT15 and pMOT16, carrying transcriptional *morA-lacZ* or *morC-lacZ* fusions, respectively, are based on the mobilizable broad-host-range plasmid pML5. (B) Transcriptional analysis of the *morAB* and *morC* operons by RT-PCR. Total RNA was isolated from *R. capsulatus* cells grown under Mo-limiting conditions. Either RNA samples were treated with reverse transcriptase to synthesize cDNA (+) or, as a negative control, reverse transcriptase was omitted (−). A 50-bp DNA ladder (Fermentas, St. Leon-Rot, Germany) was used as a length standard.

Using the ModABC proteins as query to screen the *R. capsulatus* genome database (www.ergo-light.com), we identified a related ABC-type transport system encoded by open reading frames Rc1279, Rc2331, and Rc1280. Since this study revealed that expression of these genes is repressed by molybdenum (see below), we propose new designations, namely, *morA*, *morB*, and *morC* (for *Mo-r*egulated genes) (Fig. 2). In addition,

TABLE 3. Expression of Mo-controlled *lacZ* reporter fusions in *R. capsulatus* wild-type and mutant strains

Strain(plasmid)	Genetic background	Reporter fusion	β-Galactosidase activity[a]			
			+Mo/−N	−Mo/−N	+Mo/+N	−Mo/+N
B10S(pKS131A)	Wild type	*anfA-lacZ*	1 ± 1	63 ± 10	1 ± 1	0 ± 0
R423AI(pKS131A)	*mopA*	*anfA-lacZ*	1 ± 1	73 ± 11	1 ± 1	0 ± 0
R423BI(pKS131A)	*mopB*	*anfA-lacZ*	0 ± 0	64 ± 10	0 ± 0	0 ± 0
R423CI(pKS131A)	Δ(*mopAB*)	*anfA-lacZ*	72 ± 18	67 ± 9	2 ± 2	1 ± 1
B10S(pSL21I)	Wild type	*modA-lacZ*	11 ± 1	70 ± 7	6 ± 2	4 ± 3
R423AI(pSL21I)	*mopA*	*modA-lacZ*	7 ± 9	88 ± 5	7 ± 2	3 ± 2
R423BI(pSL21I)	*mopB*	*modA-lacZ*	6 ± 5	78 ± 11	13 ± 6	6 ± 0
R423CI(pSL21I)	Δ(*mopAB*)	*modA-lacZ*	87 ± 9	89 ± 13	0 ± 0	2 ± 2
B10S(pSL21II)	Wild type	*mopB-lacZ*	21 ± 3	22 ± 2	18 ± 2	18 ± 1
R423AI(pSL21II)	*mopA*	*mopB-lacZ*	18 ± 3	18 ± 4	18 ± 4	23 ± 7
R423BI(pSL21II)	*mopB*	*mopB-lacZ*	16 ± 1	22 ± 1	16 ± 2	18 ± 4
R423CI(pSL21II)	Δ(*mopAB*)	*mopB-lacZ*	17 ± 4	20 ± 4	21 ± 2	20 ± 2
B10S(pMOT15)	Wild type	*morA-lacZ*	43 ± 3	369 ± 28	37 ± 3	117 ± 3
R423AI(pMOT15)	*mopA*	*morA-lacZ*	51 ± 4	441 ± 52	48 ± 3	67 ± 27
R423BI(pMOT15)	*mopB*	*morA-lacZ*	120 ± 19	408 ± 44	70 ± 11	171 ± 7
R423CI(pMOT15)	Δ(*mopAB*)	*morA-lacZ*	452 ± 66	435 ± 63	175 ± 19	202 ± 21
B10S(pMOT16)	Wild type	*morC-lacZ*	438 ± 27	733 ± 76	148 ± 14	387 ± 39
R423AI(pMOT16)	*mopA*	*morC-lacZ*	411 ± 48	791 ± 84	147 ± 13	368 ± 10
R423BI(pMOT16)	*mopB*	*morC-lacZ*	454 ± 32	785 ± 61	196 ± 18	456 ± 34
R423CI(pMOT16)	Δ(*mopAB*)	*morC-lacZ*	891 ± 63	862 ± 56	388 ± 36	494 ± 41
B10S(pJW59)	Wild type	*mop-lacZ*	551 ± 51	40 ± 15	316 ± 40	64 ± 5
R423AI(pJW59)	*mopA*	*mop-lacZ*	24 ± 3	25 ± 20	41 ± 5	43 ± 3
R423BI(pJW59)	*mopB*	*mop-lacZ*	382 ± 18	18 ± 4	333 ± 22	22 ± 2
R423CI(pJW59)	Δ(*mopAB*)	*mop-lacZ*	44 ± 13	36 ± 11	57 ± 17	43 ± 5

[a] *R. capsulatus* strains were grown under phototrophic conditions in AK-NL minimal medium either without addition of Mo (−Mo) or in the presence of 10 μM Na_2MoO_4 (+Mo). N-sufficient or N-limiting conditions were achieved by addition of either 15 mM $(NH_4)_2SO_4$ (+N) or 9.5 mM serine (−N), respectively. β-Galactosidase activity is given in Miller units (19). Results represent the means and standard deviations of three independent measurements.

a *mop*-like gene (Rc412) coding for a small protein similar to the Mop domain of ModC was identified 2.3 kb downstream of *nifB1* coding for a protein involved in biosynthesis of the cofactor of Mo-nitrogenase, FeMo-co. As was the case for *anfA* and the *mopA-modABCD* operon (13), putative Mo boxes (binding sites for MopA and/or MopB) were identified in the intergenic region between *morAB* and *morC* as well as in the *mop* promoter region (Fig. 5A), giving a first hint for Mo regulation of both the putative transporter MorABC and the putative Mo homeostasis protein Mop.

The genetic organization of the *mor* and *mop* gene regions was analyzed by RT-PCR essentially as described above for the *mopA-modABCD* operon. These studies revealed that *morAB* formed part of a bicistronic operon, while *morC* formed a monocistronic transcription unit (Fig. 2). While *mod* and *mor* gene expression was repressed by molybdenum, transcription of the *mop* gene was Mo activated (Table 3) (see below). Therefore, in contrast to studies on *mod* and *mor*, for analysis of *mop* gene organization, total RNA from cultures grown in the presence of molybdenum was used. While *mop* expression studies clearly demonstrated the presence of a Mo-activated promoter within the *orf413-mop* intergenic region (Table 3) (see below), RT-PCR studies identified an amplification product overlapping the gene border of *orf413-mop* (Fig. 3). These findings are most likely explained by the presence of two promoters driving expression of the *mop* gene, one immediately upstream of the *mop* coding region and the second either upstream of or within the coding region of *orf413*.

Regulation of *mod*, *mor*, *mop*, and *anf* transcription by molybdenum. To analyze Mo regulation of selected genes, *R. capsulatus* reporter strains carrying fusions between these genes and the promoterless *E. coli lacZ* gene were used (Table 1). In detail, we examined expression of *lac* fusions with *anfA* (pKS131A), *mopA-modA* (pSL21I), *mopB* (pSL21II), *morA* (pMOT15), *morC* (pMOT16), and *mop* (pJW59). The *lac* fusions were introduced into wild-type *R. capsulatus* (B10S) and mutant strains defective for either MopA (R423AI), MopB (R423BI) or both (R423CI). These mutant strains contain a gentamicin resistance (Gm) cassette (Fig. 1A), which drives expression of downstream genes reading in the same direction as the Gm gene (29). Therefore, although *mopA* is the first gene of the *mopA-modABCD* operon, expression of the Mo transport system is not abolished in mutant strains R423AI (*mopA*) and R423CI (*mopA mopB*). In agreement with previous studies (13), transcription of *modA* and *anfA* was not only repressed by molybdenum but also inhibited by ammonium. To analyze whether other Mo-regulated genes were also controlled by the N source, *R. capsulatus* reporter strains were cultivated under four different growth conditions, namely, in the presence or absence of molybdenum in combination with either nitrogen-sufficient conditions (ammonium as the N source) or nitrogen-limiting conditions (serine as the N

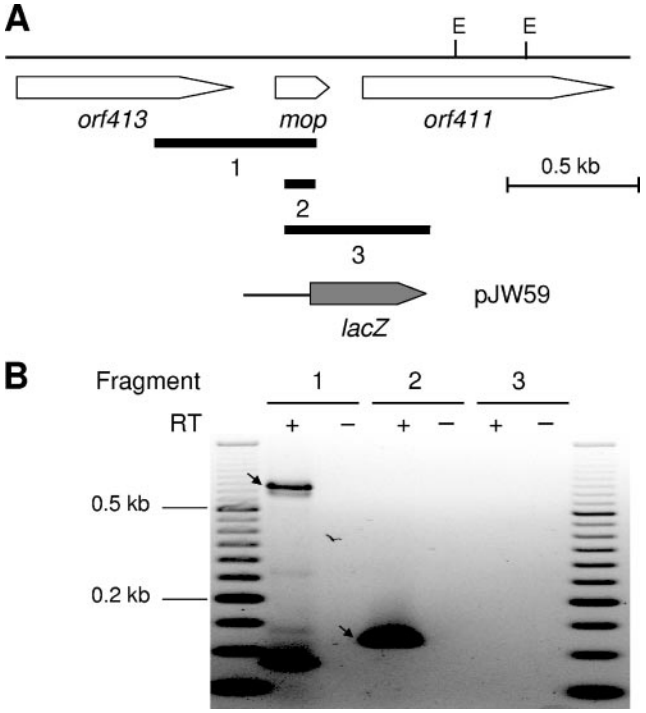

FIG. 3. Transcriptional analysis of the *R. capsulatus mop* gene region. (A) Physical and genetic maps of the *mop* gene region. The physical map is given for EcoRI (E). Black bars below the genetic map indicate DNA fragments 1 to 3 emerging from RT-PCR (see Materials and Methods and panel B). The corresponding primer pairs used for RT-PCR are listed in Table 2. Hybrid plasmid pJW59, carrying a transcriptional *mop-lacZ* fusion, is based on the mobilizable broad-host-range plasmid pML5. (B) Transcriptional analysis of the *mop* gene region by RT-PCR. Total RNA was isolated from *R. capsulatus* cells grown in the presence of 10 μM Na_2MoO_4. Either RNA samples were treated with reverse transcriptase to synthesize cDNA (+) or, as a negative control, reverse transcriptase was omitted (−). Amplification products corresponding to DNA fragments 1 and 2 are marked by arrows. A 50-bp DNA ladder (Fermentas, St. Leon-Rot, Germany) was used as a length standard.

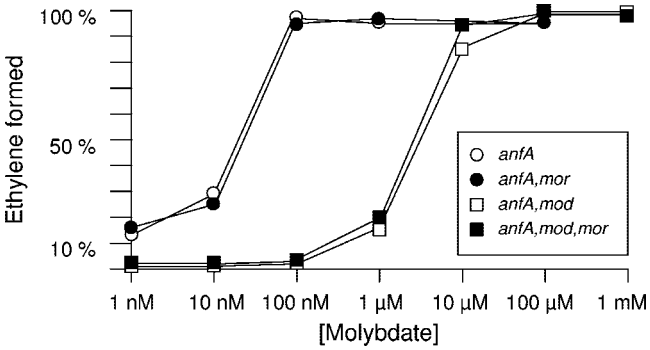

FIG. 4. Effect of increasing molybdate concentrations on the activity of Mo-nitrogenase. *R. capsulatus* strains were grown in AK-NL minimal medium containing the indicated Mo concentrations and 9.5 mM serine as the sole nitrogen source (nitrogenase-derepressing conditions). The activity of Mo-nitrogenase was determined by the reduction of acetylene to ethylene, as assayed by gas chromatography, and is expressed as a percentage of the maximal value obtained in Mo-sufficient medium (100% corresponds to 662 nmol ethylene produced $\times$ h^{-1} $\times$ mg $protein^{-1}$). *R. capsulatus* strains KS94A (*anfA*), KS94A-AW12 (*anfA morABC*), KS94A-R438II (*anfA modABC*), and KS94A-R438II-AW12 (*anfA morABC modABC*) were used.

source), prior to determination of β-galactosidase activities. *R. capsulatus* can efficiently use serine as the sole nitrogen source, but, in contrast to ammonium, serine does not inhibit synthesis of the ModABC transport system or nitrogenase (13).

The results of expression studies on Mo-regulated genes (Table 3) may be summarized as follows. (i) In the wild-type background significant expression of both *anfA* and *mopA-modA* occurred only under Mo- and N-limiting conditions (13; this study). (ii) Mo repression was mediated by either MopA or MopB, which are able to replace each other in repression of *mopA-modA* and *anfA*. In other words, MopA autoregulates its own expression. (iii) Like the *anfA* and *mopA-modA* genes, *morA* and *morC* were repressed by MopA or MopB in the presence of Mo. However, Mo repression of *morC* was less pronounced compared to that of *anfA*, *mopA-modA*, and *morA*. Significant expression of MorC in the presence of Mo might suggest that the protein has, in addition to its energizer function of the putative ABC transporter in the absence of Mo, another yet-unknown function. (iv) While *mopA-modA* (like *anfA*) was strongly inhibited by ammonium, the N source had only a minor influence on expression of *morA* and *morC*. (v) In contrast to that of *mopA*, expression of *mopB* was not regulated by molybdenum or ammonium. As a consequence, the cellular MopA/MopB ratio should strongly differ in response to Mo and N availability, if expression data reflect the actual amounts of MopA and MopB protein. (vi) In contrast to the Mod and Mor systems and the Fe-nitrogenase, which were Mo repressed, expression of the putative Mo homeostasis protein Mop was activated by molybdenum. (vii) Interestingly, Mo activation specifically required the MopA protein, whereas MopB had little influence on *mop* transcription. Like *R. capsulatus* MopA, *E. coli* ModE can act as both a repressor and an activator (2). It represses the *modABC* operon and activates transcription of genes involved in Mo-co biosynthesis. It is worth noting, however, that expression of *R. capsulatus* Mo-co biosynthesis genes *moeA* and *moeB* is not Mo regulated (15) (data not shown). (viii) In contrast to that of *mopA-modA* and *anfA*, expression of *mop* was almost unaffected by ammonium. This finding might be explained by a role of the postulated Mo homeostasis protein as an Mo donor not only for Mo-nitrogenase, which is expressed exclusively under nitrogen-limiting conditions, but also for other Mo-containing enzymes such as DMSO reductase and xanthine dehydrogenase (16).

The *morABC* genes are not required for Mo-nitrogenase activity. *R. capsulatus* mutant strains defective for the *modABC* genes are impaired in high-affinity Mo uptake as estimated from the Mo-nitrogenase activity (29). While the parental strain exhibited full Mo-nitrogenase activity at Mo concentrations of as low as 100 nM, a *modABC* mutant strain required at least 100-fold-higher Mo concentrations for maximum Mo-nitrogenase activity (Fig. 4) (29). A second, yet-uncharacterized low-affinity transport system has been discussed as being responsible for Mo uptake at concentrations of above 10 μM (29). Since the *morAB* and *morC* genes were shown to be repressed by molybdenum, we asked whether the putative MorABC transporter was involved in low-affinity Mo uptake. For this purpose, *R. capsulatus* mutant strains defective for

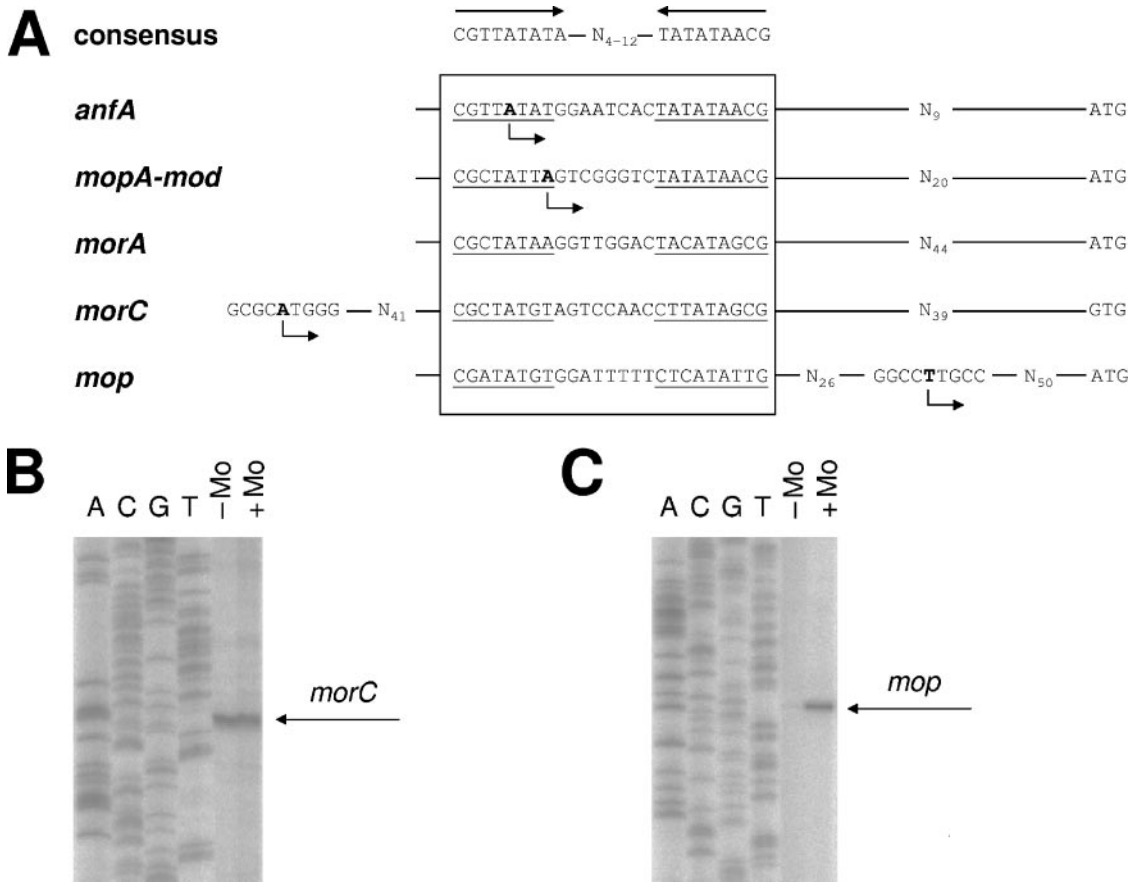

FIG. 5. DNA sequence comparison of Mo-regulated promoters (A) and transcription start site mapping of the *morC* (B) and *mop* (C) genes. DNA sequences of Mo boxes are compared to the consensus as defined by Kutsche et al. (13). The *morA-morC* intergenic region contains a single Mo box, which is thought to control expression of the divergently transcribed *mor* operons (Fig. 2). For clarity, two complementary sequences (*morA* and *morC*) of the same Mo box from this region are shown. The transcription start sites of *anfA* and the *mopA-modABCD* operon were taken from Kutsche et al. (13). To determine the transcription start sites of the other genes, primer extension was carried out with total RNA from *R. capsulatus* cells grown either under Mo-limiting conditions (−Mo) or in the presence of 10 μM Na_2MoO_4 (+Mo). Primers PJW18 and PJW19 (binding to the 5′ regions of *morC* and *mop*, respectively) were used for reverse transcription. The corresponding sequencing reactions (A, C, G, and T) with plasmids pJW45 (*morC*) and pJW42 (*mop*) served as length standards. No transcription start site was mapped for *morA*.

either ModABC (R438II) (Fig. 1), MorABC (AW12) (Fig. 2), or both (R438II-AW12) were assayed for their Mo-nitrogenase activities at different Mo concentrations (Fig. 4). To rule out any interference with Fe-nitrogenase, which does not require molybdenum for activity, Mo-nitrogenase activity was measured in an *anfA* mutant background (KS94A), thus preventing transcription of Fe-nitrogenase genes. Based on Mo-nitrogenase activity, the *morABC* mutant strains were indistinguishable from their parental strains (Fig. 4), strongly suggesting that MorABC is not the previously postulated low-affinity Mo uptake system.

Transcription start sites of Mo-regulated genes. Typically, repressor binding sites either overlap or are located downstream of the transcription start site of the respective target genes. This has previously been demonstrated for the Mo boxes implicated in binding of MopA and MopB upstream of *anfA* and the *mopA-modABCD* operon (13). The situation is more complex for the divergently transcribed *morAB* and *morC* operons, which are expected to share a single Mo box located in the intergenic region between the two operons (Fig. 2). The transcription start site of the *morC* gene was determined by primer extension analysis (Fig. 5B). For this purpose, total RNAs isolated from *R. capsulatus* wild-type cultures grown in either the presence or absence of Mo were used as templates for reverse transcription with primer PJW18, complementary to the 5′ ends of *morC* mRNA (see Materials and Methods) (Table 2). Reverse transcripts based on RNA isolated from cultures grown in either the presence or absence of Mo were identical in length and of comparable intensity. This finding was in line with *morC-lacZ* expression studies showing significant expression under both conditions (Table 3). The transcription start site of *morC* mapped upstream of the putative Mo box (Fig. 5A), suggesting that binding of MopA and MopB to this Mo box interferes with transcription. Despite several attempts using three different primers complementary to the 5′ ends of *morA* mRNA, no transcription start site could be determined for *morA*.

In parallel, we determined the transcription start site of the *mop* gene (Fig. 5C). Reverse transcripts based on RNA isolated from cultures grown in either the presence or absence of Mo were identical in length but clearly differed in intensity, which was in line with *mop-lacZ* expression studies showing

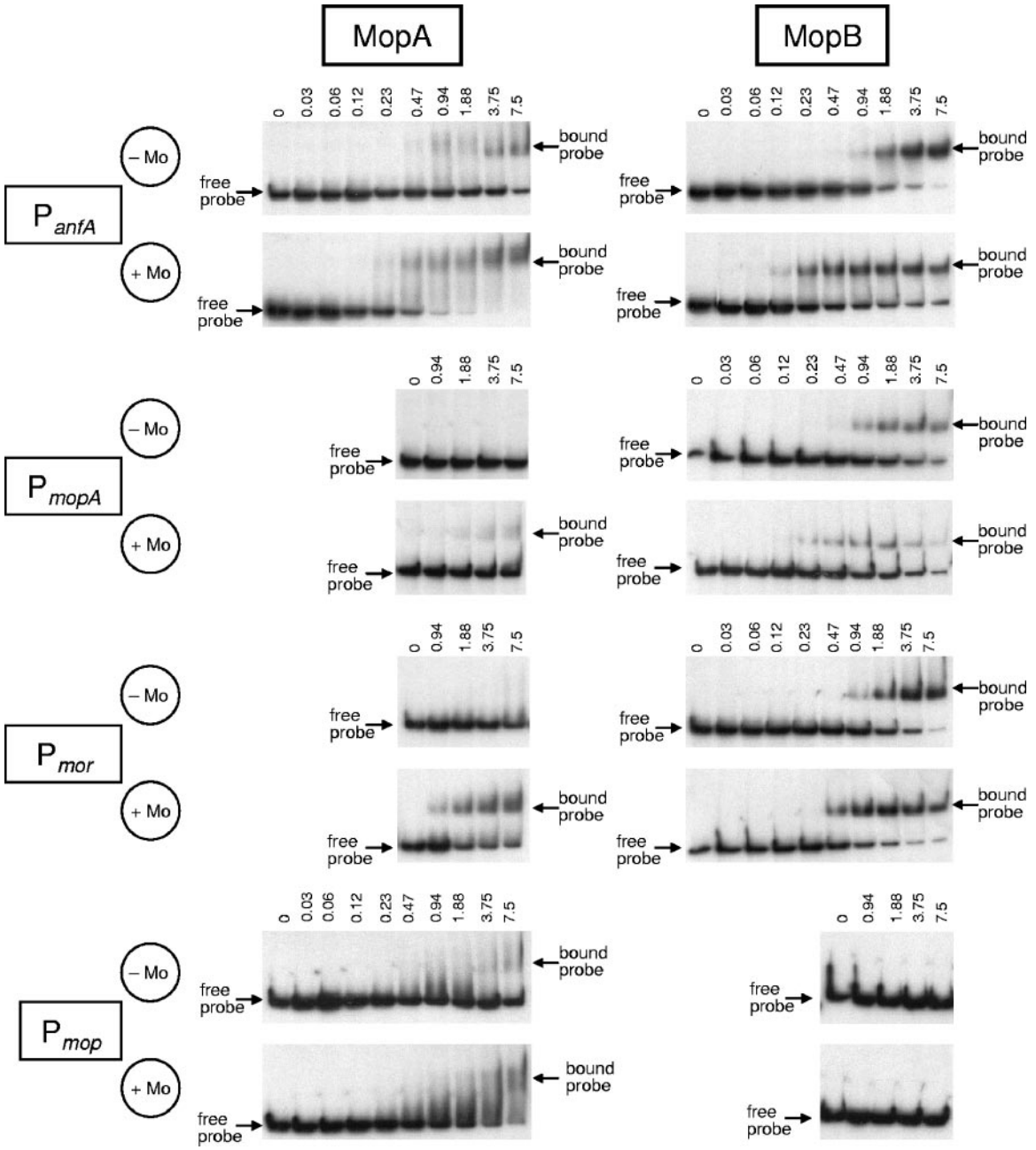

FIG. 6. DNA mobility shift assays with Mo-regulated promoter fragments and purified recombinant MopA and MopB proteins. DNA fragments encompassing the promoters of *anfA* (P_{anfA}), *mopA-modABCD* (P_{mopA}), and *mop* (P_{mop}) and the intergenic region between the divergently transcribed *mor* operons (P_{mor}) were generated by PCR amplification using appropriate primer pairs (Table 2) prior to ^{32}P labeling (see Materials and Methods). Incubation of increasing amounts of MopA and MopB (0, 0.03, 0.06, 0.12, 0.23, 0.47, 0.94, 1.88, 3.75, and 7.5 μM) with labeled DNA fragments was carried out either in the absence (−Mo) or presence (+Mo) of molybdenum. All reactions were performed with 5 fmol ^{32}P-labeled DNA fragment probes.

that maximal *mop* expression occurred in the presence of Mo (Table 3). The *mop* transcription start site was mapped downstream of the putative Mo box (Fig. 5A). As activator binding sites are typically located upstream of the transcription start site, this finding is consistent with MopA-dependent *mop* gene activation.

Binding of MopA and MopB to target promoters. As shown above by expression studies, MopA and MopB regulate transcription of their target genes in response to Mo availability. The presence of conserved Mo boxes in the promoter regions of all target genes suggested that MopA and MopB specifically bind to these promoters. DNA mobility shift assays were carried out to verify this assumption. For this purpose, MopA and MopB were overexpressed and purified as C-terminally His-tagged recombinant proteins from *E. coli* (see Materials and Methods). DNA fragments, ranging from 222 to 272 bp, en-

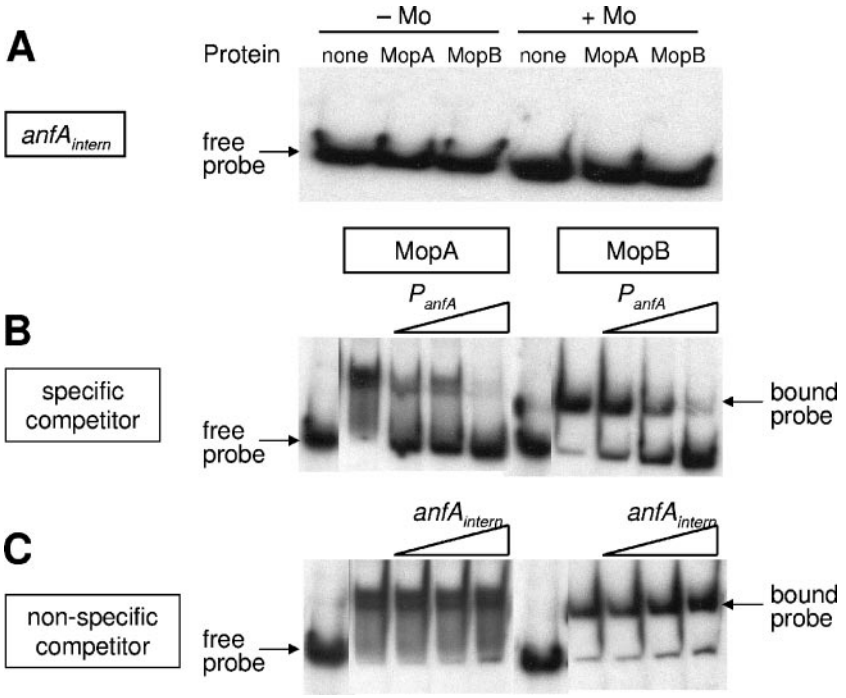

FIG. 7. Specificity controls for binding of MopA and MopB to *anfA* promoter DNA. (A) Use of an internal region of *anfA* ($anfA_{intern}$) as negative control for DNA mobility shift assays. The control DNA fragment ($anfA_{intern}$) (Table 2) was PCR amplified, ^{32}P labeled, and incubated with either 7.5 μM MopA or MopB in the absence (−Mo) or presence (+Mo) of molybdenum. (B) Use of unlabeled *anfA* promoter fragments as specific competitor DNA. MopA or MopB (2.5 μM) and ^{32}P-labeled *anfA* promoter fragments were mixed with a 400-, 800-, or 1,600-fold excess of unlabeled competitor DNA (compared to the labeled probe). (C) Use of $anfA_{intern}$ in competition assays. MopA or MopB (2.5 μM) and ^{32}P-labeled *anfA* promoter fragments were mixed with a 550-, 1,100-, or 2,200-fold excess of unlabeled *anfA* internal fragments (compared to the labeled probe). All reactions were performed with 5 fmol ^{32}P-labeled P_{anfA} probes.

compassing selected promoters were PCR amplified using appropriate primer pairs (Table 2) and radioactively labeled at their 5′ ends (see Materials and Methods). All binding assays were performed in the presence of poly(dI-dC) as competitor DNA.

The results of DNA mobility shift assays with increasing amounts of either $MopA_{His}$ or $MopB_{His}$ are shown in Fig. 6. Both MopA and MopB bound to the *anfA* promoter (P_{anfA}). Binding of both regulators occurred in the absence of Mo but was clearly improved in the presence of Mo. In contrast to binding to the *anfA* promoter, neither MopA nor MopB bound to a control DNA fragment derived from an internal region of *anfA* (Fig. 7A), thus corroborating binding specificity. Binding of MopA and MopB to radioactively labeled *anfA* promoter fragments could be reversed by addition of increasing amounts of nonlabeled *anfA* promoter DNA (Fig. 7B) but not by the internal *anfA* fragment (Fig. 7C). The importance of the Mo box upstream of *anfA* as a *cis*-regulatory element has been demonstrated by analysis of mutant promoters carrying small deletions within this element (13). Taken together, these findings strongly suggest that MopA and MopB control *anfA* expression by binding to this Mo box overlapping the transcription start site (Fig. 5A). Although in vitro binding of MopA and MopB to the *anfA* promoter implies that no additional proteins are required for Mo repression of *anfA*, fine-tuning by other protein factors in vivo cannot be excluded.

Under comparable conditions, binding of MopA to the *mopA-modABCD* promoter (P_{mopA}) and the intergenic region between the divergently transcribed *mor* operons (P_{mor}) was much weaker than binding of MopB, and binding of MopA to P_{mopA} and P_{mor} was not detectable at all in the absence of Mo (Fig. 6). Most interestingly, only MopA (and not MopB) bound to the promoter of the *mop* gene (P_{mop}), coding for a putative Mo homeostasis protein (Fig. 6). Binding of MopA in the absence of Mo was barely detectable. More efficient binding occurred in the presence of Mo. As one would expect for a *cis*-regulatory element serving exclusively as a binding site for MopA, the *mop*-specific Mo box differs at three positions from all the other Mo boxes, which serve as binding sites for both MopA and MopB (Fig. 5A).

Compared to binding to the *anfA*, *mopA*, and *mor* promoters, binding of MopA to the *mop* promoter was fairly weak (even in the presence of Mo). Generally, binding of activator proteins to their target promoters is believed to be much weaker than binding of repressor proteins to operator sequences (5). The suggested role of MopA acting either as a repressor (for *anfA*, *mopA-modABCD*, *morAB*, and *morC* expression) or as an activator (for *mop* regulation) perfectly agrees with this general observation.

Conclusions. MopA and MopB have overlapping functions, as they can substitute for each other in Mo repression of *anfA*, *mopA-modABCD*, *morAB*, and *morC* (13; this study). In addition to its role as a repressor, MopA serves as an activator of transcription of the *mop* gene (this study), which is the first example of a specialized function of MopA in Mo regulation.

It is worth noting that MopB has been described to be essential for activity of DMSO reductase (*dor* encoded) in *R. capsulatus* strain 37b4 (28). A putative Mo box has been identified upstream of the *dorX* gene, consistent with the view that the *dorX* gene is the target for MopB-dependent Mo regulation in strain 37b4 (18). DorX, in turn, has been suggested to activate transcription of an operon whose products are required for Mo-co biosynthesis and, hence, DMSO reductase activity. *R. capsulatus* strain B10S, which was used in this study, also has the capacity to synthesize DMSO reductase. However, in contrast to strain 37b4, strain B10S does not contain a *dorX*-like gene at the equivalent position relative to the other *dor* genes (data not shown) or elsewhere in the chromosome (www.ergo-light.com), suggesting that regulation of DMSO reductase activity differs in the two *R. capsulatus* strains.

The current model for Mo regulation in *R. capsulatus* suggests that the Mo-dependent regulators, MopA and MopB, are involved in regulation of the internal Mo concentration by repressing transcription of the *modABCD* transport operon (13; this study) and thus limiting the amount of the transporter at high Mo concentrations. Mutant strains defective for ModABC express Fe-nitrogenase at Mo concentrations of up to 1 μM, while synthesis of Fe-nitrogenase is repressed at much lower concentrations in the parental strain (29), suggesting that the putative MorABC transporter does not substitute for the ModABC system. Although expression of the *morAB* and *morC* genes is controlled by molybdenum, at present it remains unknown whether the gene products are involved in Mo uptake at all. The presence of the high-affinity Mo transporter ModABC, which provides sufficient Mo for the Mo-nitrogenase at low Mo concentrations, is physiologically favorable, as Mo-nitrogenase is more efficient than Fe-nitrogenase with respect to N_2 reduction rates (24).

MopA-dependent *mop* gene activation occurred only in the presence of Mo, thus ensuring that the putative Mo homeostasis protein is not expressed under Mo-limiting conditions. However, since expression of *mopA* (as part of the *mopA-modABCD* operon) is down-regulated in response to increasing Mo concentrations, MopA-dependent synthesis of the Mop protein is expected to decrease when Mo is abundant.

ACKNOWLEDGMENTS

We thank Thomas Drepper for helpful discussions, Corinna Hasecke and Britta Schubert for analysis of the DMSO reductase gene region from *R. capsulatus* strain B10S, Silke Leimkühler for construction of plasmids pSL21I and pSL21II, and Andrea Kreuz for construction of plasmids pMOT15 and pMOT16.

This work was supported by a financial grant from Deutsche Forschungsgemeinschaft (Ma 1814/3-1).

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