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Promoters Controlling Expression of the Alternative Nitrogenase and the Molybdenum Uptake System in *Rhodobacter capsulatus* Are Activated by NtrC, Independent of σ^{54}, and Repressed by Molybdenum

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The alternative nitrogenase of *Rhodobacter capsulatus* is expressed only under conditions of nitrogen and molybdenum depletion. The analysis of *anfA-lacZ* fusions demonstrated that this dual control occurred at the level of transcription of *anfA*, which encodes a transcriptional activator specific for the alternative nitrogenase. The *anfA* promoter was found to be activated under nitrogen-limiting conditions by NtrC in a σ^{54}-independent manner. In addition, *anfA* transcription was repressed by traces of molybdenum. This molybdenum-dependent repression of *anfA* was released in *R. capsulatus* mutants carrying either lesions in the high-affinity molybdenum uptake system (*modABCD*) or a double deletion of *mopA* and *mopB*, two genes encoding molybdenum-pterin-binding proteins. The expression of the molybdenum transport system itself was shown to be negatively regulated by molybdenum and, unexpectedly, to be also regulated by NtrC. This finding is in line with the presence of two tandemly arranged DNA motifs located in front of the *R. capsulatus mopA-modABCD* operon, which are homologous to *R. capsulatus* NtrC binding sites. Mapping of the transcriptional initiation sites of *mopA* and *anfA* revealed promoter sequences exhibiting significant homology to each other but no homology to known prokaryotic promoters. In addition, a conserved DNA sequence of dyad symmetry overlapping the transcriptional initiation sites of *mopA* and *anfA* was found. Deletions within this element resulted in molybdenum-independent expression of *anfA*, indicating that this DNA sequence may be the target of MopA/MopB-mediated repression.

Molybdenum is an essential trace element required for the activities of several enzymes. These molybdenum enzymes contain either molybdopterin cofactors or the iron-molybdenum cofactor which is present only in nitrogenase. In addition to the conventional molybdenum nitrogenase, the phototrophic purple bacterium *Rhodobacter capsulatus* harbors an alternative nitrogenase, which is devoid of heterometals (24, 39, 40). The alternative nitrogenase of *R. capsulatus* is repressed by traces of molybdenum (39). Repression of alternative nitrogenases by molybdenum was also found for *Azotobacter vinelandii*, an obligate aerobic soil bacterium which is able to fix nitrogen via the molybdenum, the vanadium, or the iron-only nitrogenase (for a review, see reference 2). The molybdenum repression of the alternative nitrogenase in *R. capsulatus* is released in mutants unable to import molybdate by a high-affinity molybdenum uptake system (*modABCD*) or in a mutant strain devoid of *mopA* and *mopB*, which code for molybdenum-pterin-binding proteins (46). As found for most members of the superfamily of ABC transporters (11), the high-affinity molybdate transport system of *R. capsulatus* consists of a periplasmic substrate-binding protein (ModA), a transmembrane protein forming the entry pathway (ModB), an ATP-binding protein which couples ATP hydrolysis to translocation of the substrate into the cytoplasm (ModC), and a fourth protein (ModD) of unknown function (46). The corresponding genes are organized in one transcriptional unit together with *mopA*, forming the *mopA-modABCD* operon. The *mopB* gene is located immediately upstream of *mopA* and is divergently transcribed to the *mopA-modABCD* operon (Fig. 1B). A similar organization of the *mod/mop* gene region, including two variants of genes encoding molybdenum-pterin-binding proteins (*modE* and *modG*), was found in *A. vinelandii* (31). In contrast, only single copies of genes coding for molybdenum-pterin-binding proteins have been identified in *Escherichia coli* (45) and *Haemophilus influenzae* (5).

The expression of both nitrogenase systems in *R. capsulatus* is controlled by the concentration of fixed nitrogen. Since *R. capsulatus* contains genes homologous to *ntrC* (*nifR1*), *ntrB* (*nifR2*), *ntrA* (*nifR4*), and *glnB* (*nifR5*), regulatory mechanisms similar to the Ntr system of enteric bacteria have been proposed (12, 21, 24). The Ntr system of enteric bacteria comprises a complex signal-transducing cascade responding to the intracellular concentrations of fixed nitrogen (for a review, see reference 27). Under conditions of nitrogen depletion, the phosphorylated form of NtrC activates, in concert with σ^{54}, an alternative sigma factor of RNA polymerase encoded by *rpoN*, a variety of different genes involved in nitrogen metabolism. In contrast to NtrC from enteric bacteria, *R. capsulatus* NtrC (NifR1) is not responsible for the regulation of genes involved in general nitrogen metabolism (17, 22). Instead, only *glnB*, *nifA1*, and *nifA2* were identified to be targets of NtrC regulation. Furthermore, the activity of each of these promoters is independent of σ^{54}, and mapping of the transcription initiation sites revealed promoter elements which might be recognized by an RNA polymerase containing an as yet unidentified sigma factor (6–8, 32).

In this report, we present evidence that the promoters of *anfA*, encoding a transcriptional activator specific for the alternative nitrogenase system, and of the *mopA-modABCD*

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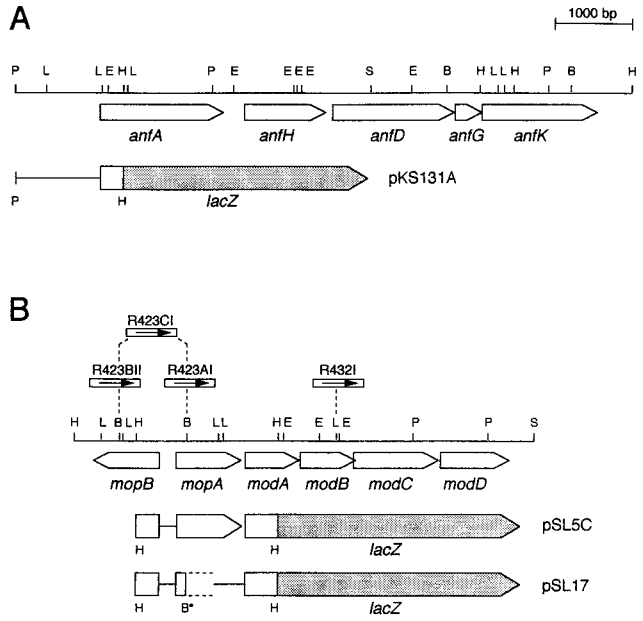

FIG. 1. Physical maps of the *R. capsulatus anf* and *mod/mop* gene regions. (A) Restriction map of the *anf* gene region containing *anfA* and *anfHDGK*. The *anfHDGK* operon encodes the apoproteins of the alternative nitrogenase, and *anfA* encodes an activator protein specific for the alternative nitrogenase system. Plasmid pKS131A carries a translational *anfA'-'lacZ* fusion. (B) Restriction map and localization of *modABCD*, encoding a high-affinity molybdenum transport system, and *mopA/mopB*, coding for molybdenum-pterin-binding proteins. The localization of interposon insertions in *mopB*, *mopA*, and *modB* strains as well as a *mopA mopB* double-deletion mutant is given above the physical map. The direction of transcription of the gentamicin resistance gene located on the interposons is indicated by arrows in boxes. Interposons are not drawn to scale. Plasmid pSL5C carries a translational *modA'-'lacZ* fusion. Plasmid pSL17 differs from pSL5C by a frameshift mutation within the *mopA* gene introduced by fill-in of the *Bam*HI site (B*). Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; L, *Bcl*I; P, *Pst*I; S, *Sal*I.

operon are activated by NtrC. Therefore, these genes are also members of the *R. capsulatus* Ntr regulon. In contrast to the NtrC-activated promoters of *glnB*, *nifA1*, and *nifA2*, the *anfA* and *mopA* promoters are also negatively controlled by molybdenum, and a region of dyad symmetry overlapping the transcriptional initiation sites may represent the molybdenum-responsive element.

MATERIALS AND METHODS

Bacterial strains 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* and *R. capsulatus* and the selection of mutants, anaerobic growth conditions, and antibiotic concentrations were as previously described (20, 25, 40).

DNA techniques. DNA isolation, restriction enzyme analysis, and cloning procedures were performed according to standard methods (37). Restriction endonucleases, T4 DNA ligase, and Klenow polymerase were purchased from Gibco BRL and used as recommended by the supplier. DNA sequence analysis was carried out with an Auto Read sequencing kit (Pharmacia) according to the protocol devised by Zimmermann et al. (47). Sequence data were obtained and processed by using the A.L.F. DNA sequencer (Pharmacia LKB) as instructed by the manufacturer. PCRs were performed as recommended in the instructions for the GeneAmp kit (Perkin-Elmer Cetus) except that 10% dimethyl sulfoxide was added. A Perkin-Elmer Cetus model 480 DNA Thermal Cycler carried out 30 cycles, each consisting of 60 s at 94°C, 90 s at 50°C, and 60 s at 74°C.

Construction of *lacZ* fusion plasmids. To create a translational *anfA'-'lacZ* fusion, a 1.4-kb *Pst*I-*Hin*dIII fragment (Fig. 1A) carrying the 5′ part of *R. capsulatus anfA* was cloned into the polylinker of pPHU236. This fused codon 86 of *anfA* in the appropriate reading frame to the *lacZ* gene. The resulting plasmid was designated pKS131A.

A translational *anfH'-'lacZ* fusion was constructed by cloning a 615-bp *Eco*RI-*Nru*I fragment from *R. capsulatus* into the polylinker of pPHU235, using a *Sma*I-*Hin*dIII linker. The resulting plasmid, pMKR92, carries an in-frame fusion between *lacZ* and *anfH* at codon 140.

To construct a translational *modA'-'lacZ* fusion, a 1.85-kb *Hin*dIII fragment of *R. capsulatus* (Fig. 1B) was first cloned into pSVB28 to create pWKR471I. Subsequently, the *mopB'*-, *mopA*-, and *modA'*-containing *Hin*dIII fragment could be cloned as an *Eco*RI-*Pst*I fragment into pPHU236. The resulting plasmid, pSL5C, carried *lacZ* fused to codon 155 of *modA*. To construct a *modA'-'lacZ* translational fusion vector devoid of an intact *mopA* gene, plasmid pWKR471I was digested with *Bam*HI, and blunt ends were generated with Klenow polymerase. Religation resulted in a frameshift mutation within the *mopA* gene. Subsequently, the *Eco*RI-*Pst*I fragment was cloned into pPHU236, yielding plasmid pSL17 (Fig. 1B).

Construction of *ntrC* (*nifR1*) and *rpoN* (*nifR4*) interposon mutants. To construct a defined *ntrC* (*nifR1*) interposon mutant, a 2.4-kb *Eco*RI-*Pst*I fragment encompassing *R. capsulatus ntrC* was first cloned into the mobilizable vector plasmid pSUP202 (41). Subsequently, a cassette carrying the kanamycin resistance gene from pHP45Ω-Km (4) was inserted into the *Bcl*I site located within the *ntrC* coding region to yield the hybrid plasmid pPBK2.

To construct a defined *rpoN* (*nifR4*) interposon insertion mutant, a 2.0-kb *Hin*dIII fragment carrying the entire coding region of *R. capsulatus rpoN* was cloned into a mobilizable vector plasmid. A 2.3-kb fragment from plasmid pWKR189 carrying the gentamicin resistance gene was inserted into the unique *Cla*I site within *rpoN* to yield plasmid pKS111.

Plasmids pPBK2 and pKS111 were mobilized from *E. coli* S17-1 into *R. capsulatus* B10S, and double-crossover events were selected as described previously (25). The correct homogenotization of the two interposon insertions was verified in each case by Southern hybridization experiments, and the resulting Nif$^-$ Anf$^-$ phenotype confirmed these mutants.

Site-directed mutagenesis of the *anfA* promoter region. A 25-bp and a 9-bp deletion were introduced into the *anfA* promoter region according to the method described by Deng and Nickoloff (3). The Unique Site Elimination kit (Pharmacia) was used according to the manufacturer's instructions with pKS104II as a template and two mutagenic primers (5′ dCCTTGTGCGGCGCAGCCCGGG AATCACTATATAACGG and 5′ dCCTTGTGCGGCGCAGCCCGGGAGTC GGTCATGTTCGG). Plasmids carrying the corresponding mutations were identified by *Sma*I restriction sites introduced by the primers, and sequence analysis verified the desired mutations. The corresponding *lacZ* fusion plasmids pSL12 and pSL9 were constructed as described for pSL5C.

A 5-bp deletion was created by introducing an *Xba*I site into the *anfA* promoter region by fusion of two PCR products. Two oligonucleotides (5′ dAAT CACGTCTAGACGGAGTCGGTCATGTTCGG and 5′ dCTCCGTTATATCT AGATTCCATATA) and either the M13 reverse or universal primer were used to amplify the corresponding fragments, using plasmid pMKR95 as a template. The resulting PCR products were purified (QIAquick PCR Purification kit; Qiagen), digested with *Xba*I-*Hin*dIII and *Xba*I-*Pst*I, respectively, and cloned into pUC8 digested with *Pst*I-*Hin*dIII. After confirmation of the DNA sequence of the resulting *Pst*I-*Hin*dIII fragment, this fragment was cloned into pPHU236, resulting in the *lacZ* fusion plasmid pMKR118. Filling in the *Xba*I site of pMKR118 by Klenow polymerase resulted in the *lacZ* fusion plasmid pMKR118-X, which contains a 1-bp deletion.

β-Galactosidase assays. To determine the β-galactosidase activities of *R. capsulatus* strains carrying *lacZ* fusions on broad-host-range vector plasmids, strains were grown in molybdenum-free medium (38) supplemented with tetracycline (0.25 μg/ml). For growth under nitrogenase-derepressing conditions, serine was added to a final concentration of 10 mM. Nitrogenase-repressing conditions were achieved by addition of 10 mM NH_4Cl. To determine promoter activities under Mo-repressing conditions, sodium molybdate was added to a final concentration of 10 μM. 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 described previously (12, 29).

RNA isolation and primer extension analysis. RNA was prepared from *R. capsulatus* cells harboring either plasmid pKS131A (*anfA'-'lacZ*) or plasmid pSL5C (*modA'-'lacZ*). One hundred-milliliter cultures were grown in medium treated with activated carbon to remove traces of molybdenum (38). To derepress the alternative nitrogenase, serine was used as the nitrogen source, and the cells were grown under anaerobic photosynthetic conditions, harvested in exponential growth phase, chilled with crushed wet ice, and centrifuged at 4°C. Cell lysis was achieved by repeated passages through a French Press cell, and RNA was isolated as described by Rather and Moran (34). The primer extension procedure was performed with SuperScript reverse transcriptase (Gibco BRL), using the following oligonucleotides: 5′ dGCCCAAGCTCGAGTGCCTCGA, corresponding to codons 7 to 13 of the *anfA* gene, and 5′ dCCCGCGCGTTGCAG GCTCAG, corresponding to codons 10 to 16 of the *mopA* gene. Conditions for primer extension were as described by Vögtli and Hütter (44). T4 polynucleotide kinase (Pharmacia) was used to label oligonucleotides with [γ-^{32}P]ATP (Amersham). Analysis of primer extension products was performed by separation on 8% polyacrylamide gels next to sequencing ladders generated with the same oligonucleotides.

Nucleotide sequence accession number. The nucleotide sequence of a 1,966-bp DNA fragment encompassing the *anfA* coding region, which directly abuts the

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or characteristics	Reference or source
Bacterial strains		
E. coli		
DH5α	Host for pUC and pPHU plasmids	10
S17-1	RP4-2 (Tc::Mu) (Km::Tn*7*) integrated into the chromosome	41
R. capsulatus		
B10S	Spontaneous Sm^r mutant of *R. capsulatus* B10	20
PBK2	*ntrC*::Km insertion mutant of B10S	This work
KS111	*rpoN*::Gm insertion mutant of B10S	This work
R423AI	*mopA*::Gm insertion mutant of B10S	This work
R423BII	*mopB*::Gm insertion mutant of B10S	This work
R423CI	Δ(*mopA mopB*)::Gm deletion mutant of B10S	This work
R432I	*modB*::Gm insertion mutant of B10S	This work
Plasmids		
pBluescriptKS+	Ap^r, *lacZ*α	Stratagene
pSVB28,30	Ap^r, *lacZ*α	1
pUC8,9	Ap^r, *lacZ*α	43
pHP45Ω-Km	Ap^r, Km^r	4
pSUP202	Ap^r, Tc^r, Cm^r, *mob*	41
pPHU235,236	Broad-host-range *lacZ* translational fusion vectors, Tc^r	12
pWKR189	Ap^r, Gm^r	30
pWKR423AI	pUC9 (Tc^r, *mob*) carrying *R. capsulatus mopA*::Gm	46
pWKR423BII	pUC9 (Tc^r, *mob*) carrying *R. capsulatus mopB*::Gm	46
pWKR423CI	pUC9 (Tc^r, *mob*) carrying *R. capsulatus* Δ(*mopA mopB*)::Gm	46
pWKR432I	pUC9 (Tc^r, *mob*) carrying *R. capsulatus modB*::Gm	46
pWKR471I	1.9-kb *Hin*dIII fragment of *R. capsulatus* carrying *mopB′*, *mopA*, and *modA′* cloned into pSVB28	This work
pKS104II	2.5-kb *Pst*I fragment of *R. capsulatus* carrying *anfA′* cloned into pSVB30	This work
pKS131A	Broad-host-range plasmid carrying an *anfA′-′lacZ* translational fusion	This work
pMKR92	Broad-host-range plasmid carrying an *anfH′-′lacZ* translational fusion	This work
pSL5C	Broad-host-range plasmid carrying a *modA′-′lacZ* translational fusion	This work
pPBK2	Mobilizable plasmid carrying *ntrC*::Km	This work
pKS111	Mobilizable plasmid carrying *rpoN*::Gm	This work
pSL17	pSL5C carrying a frameshift mutation in *mopA*	This work
pSL12	pKS131A derivative carrying a 25-bp deletion in the *anfA* promoter region	This work
pSL9	pKS131A derivative carrying a 9-bp deletion in the *anfA* promoter region	This work
pMKR118	pKS131A derivative carrying a 5-bp deletion in the *anfA* promoter region	This work
pMKR118-X	pKS131A derivative carrying a 1-bp deletion in the *anfA* promoter region	This work
pMKR95	1.4-kb *Pst*I-*Hin*dIII fragment of *R. capsulatus* carrying *anfA′* cloned into pBluescriptKS+	This work

sequence of the *anfHDGK* operon published previously (40), will appear in the EMBL nucleotide sequence database under accession number X75972.

RESULTS

Transcriptional regulation of genes encoding the alternative nitrogenase in *R. capsulatus*. The alternative nitrogenase of *R. capsulatus* is repressed by traces of molybdenum in the growth medium (39, 46). To determine at which level the molybdenum repression occurs, translational *lacZ* fusions to *anfH*, encoding the nitrogenase reductase of the alternative nitrogenase, and to *anfA*, which codes for a transcriptional activator protein specific for the alternative nitrogenase, were constructed. The *R. capsulatus* AnfA protein showed a domain structure similar to those of the transcriptional activator proteins AnfA and VnfA of *A. vinelandii* (15). The predicted amino acid sequence of *R. capsulatus* AnfA exhibited the highest homology to *A. vinelandii* AnfA (65%) and less homology to the transcriptional activator of the vanadium nitrogenase VnfA (56%). In contrast, the homology between *R. capsulatus* AnfA and NifA, the activator protein of the molybdenum nitrogenase system (25), is restricted to the central domain, and the overall homology is only 25%. Interposon mutagenesis of *R. capsulatus anfA* demonstrated that expression of the structural genes of the alternative nitrogenase (*anfH′-′lacZ*) strictly depends on this transcriptional activator (data not shown). As shown in Table 2, the *anfA′-′lacZ* fusion located on plasmid pKS131A (Fig. 1A) is expressed only under nitrogen and molybdenum depletion. In the presence of 10 μM molybdate or in the presence of ammonia, no expression was observed. A similar regulation pattern was found for the *anfH′-′lacZ* fusion (data not shown). Since AnfA is a transcriptional activator specific for *anfH* expression, the molybdenum regulation of the alternative nitrogenase occurred primarily at the level of *anfA* expression. To exclude the possibility that expression of *anfA* is affected only at the translational level and not at the transcriptional level, an *anfA* transcriptional fusion was analyzed. It could be demonstrated that this *anfA-lacZ* fusion was also expressed only in the absence of ammonia and molybdenum (data not shown), indicating that the *anfA* promoter itself is under dual negative control.

In *R. capsulatus*, expression of the conventional molybdenum nitrogenase is controlled by a regulatory cascade resembling the Ntr system of other diazotrophs. To test whether the ammonia regulation of *anfA* occurred via this Ntr-like system, the *anfA′-′lacZ* fusion plasmid pKS131A was introduced into *R. capsulatus* strains carrying lesions in *ntrC* (*nifR1*) or *rpoN* (*nifR4*, encoding σ^{54}). As shown in Table 2, the expression of *anfA* strictly depends on the transcriptional activator protein NtrC but does not require σ^{54}.

Effects of *mod* and *mop* mutations on the expression of *anfA*. Previously, two classes of *R. capsulatus* mutants which are able to express the alternative nitrogenase even in the presence of

TABLE 2. Expression of *anfA'-'lacZ* and *modA'-'lacZ* translational fusions in the *R. capsulatus* wild-type strain and different mutant strains

Strain	Relevant genotype	Fusion	β-Galactosidase activity[a]			
			−N −Mo	−N +Mo	+N −Mo	+N +Mo
B10S(pKS131A)	Wild type	*anfA'-'lacZ*	120 ± 26	2 ± 1	1 ± 1	1 ± 1
PBK2(pKS131A)	*ntrC*	*anfA'-'lacZ*	6 ± 1	1 ± 1	1 ± 1	1 ± 1
KS111(pKS131A)	*rpoN*	*anfA'-'lacZ*	115 ± 20	6 ± 1	1 ± 1	1 ± 1
R432I(pKS131A)	*modB*	*anfA'-'lacZ*	120 ± 15	51 ± 8	ND	ND
R423AI(pKS131A)	*mopA*	*anfA'-'lacZ*	123 ± 10	3 ± 1	ND	ND
R423BII(pKS131A)	*mopB*	*anfA'-'lacZ*	120 ± 6	3 ± 1	ND	ND
R423CI(pKS131A)	Δ(*mopAB*)	*anfA'-'lacZ*	121 ± 12	89 ± 11	ND	ND
B10S(pSL17)	Wild type	*modA'-'lacZ*	298 ± 16	2 ± 1	1 ± 1	1 ± 1
PBK2(pSL17)	*ntrC*	*modA'-'lacZ*	1 ± 1	1 ± 1	1 ± 1	1 ± 1
KS111(pSL17)	*rpoN*	*modA'-'lacZ*	91 ± 12	3 ± 1	1 ± 1	1 ± 1
R432I(pSL17)	*modB*	*modA'-'lacZ*	247 ± 31	176 ± 8	ND	ND
R423AI(pSL17)	*mopA*	*modA'-'lacZ*	271 ± 6	6 ± 2	ND	ND
R423BII(pSL17)	*mopB*	*modA'-'lacZ*	280 ± 19	4 ± 2	ND	ND
R423CI(pSL17)	Δ(*mopAB*)	*modA'-'lacZ*	239 ± 30	263 ± 45	ND	ND

[a] Determined by the SDS-chloroform method (12, 29) and expressed in Miller units. Mean values and standard deviations were calculated from at least three independent measurements. *R. capsulatus* cultures were grown photoheterotrophically in molybdenum-free medium (38) under nitrogenase-derepressing conditions (−N) with serine as the nitrogen source or under repressing conditions (+N) in the presence of ammonia. Sodium molybdate was added (+Mo) to a final concentration of 10 μM. ND, not determined.

high concentrations of molybdenum were identified: (i) mutants in *modABC*, encoding components of a high-affinity molybdate uptake system, and (ii) *mopA mopB* double mutants (46). To analyze the effects of *mod* and *mop* mutations on the expression of *anfA*, the *anfA'-'lacZ* fusion plasmid pKS131A was introduced into the corresponding mutant strains (Fig. 1B). The molybdenum repression of *anfA* is released in a *modB* mutant, which is unable to express the transmembrane protein of the transport complex (Table 2). In addition, no molybdenum repression of *anfA* could be observed in the *mopA mopB* double mutant (Fig. 1B), whereas single mutations in either *mopA* or *mopB* had no effect.

Transcriptional regulation of the *mopA-modABCD* operon. The high-affinity molybdate uptake system of *E. coli* (26, 45), which corresponds to ModA, ModB, and ModC of *R. capsulatus*, was shown to be negatively regulated by high concentrations of molybdate (28, 35, 36). To analyze the expression of the corresponding *R. capsulatus* genes, a translational *lacZ* fusion to *modA*, which encodes the periplasmic molybdate-binding protein, was constructed. As shown in Fig. 1B, the *mopA* gene is located immediately upstream of *modA* and is part of the *mopA-modABCD* transcriptional unit. Therefore, the *modA'-'lacZ* fusion plasmid pSL5C carries an intact copy of *mopA*. To exclude influences of the plasmid-encoded *mopA* gene product on the expression of *modA'-'lacZ* and to allow the analysis of this *lacZ* fusion plasmid in a *mopA* mutant background, a frameshift mutation within *mopA* was introduced by filling in the *Bam*HI site located in the 5′ end of the *mopA* coding region (Fig. 1B). No differences in the expression of *modA'-'lacZ* could be observed between the resulting *mopA modA'-'lacZ* fusion plasmid pSL17 and the parental plasmid pSL5C (data not shown).

The *modA'-'lacZ* fusion is expressed only in the absence of molybdenum and, surprisingly, only under nitrogen-limiting conditions (Table 2). Therefore, the expression pattern of the *mopA-modABCD* operon is similar to that for the regulation of the *anfA* promoter. As in the case of *anfA*, no differences between translational and transcriptional *modA-lacZ* fusions were observed (data not shown). To determine whether the *mopA* promoter is also dependent on NtrC (NifR1) but independent of σ^{54} (NifR4), the *modA'-'lacZ* fusion plasmid pSL17 was analyzed in the corresponding mutants. As shown in Table 2, *modA* expression was strictly dependent on NtrC but independent of σ^{54}. As demonstrated for the *anfA* promoter, the molybdenum repression of the *mopA-modABCD* promoter is released not only in a *modB* mutant but also in a *mopA mopB* double mutant. In contrast, *mopA* or *mopB* single mutants exhibited no effect on the regulation of both the *mopA* and *anfA* promoters, indicating that the molybdenum-pterin-binding proteins MopA and MopB can substitute for each other in negative regulation of gene expression.

Evaluation of the *anfA* and *mopA-modABCD* promoter sequences: mapping of transcriptional start sites. Since both the *anfA* and *mopA-modABCD* promoters are activated by NtrC in a σ^{54}-independent manner and are both negatively controlled by high concentrations of molybdate, the DNA sequences of these promoter regions were compared (Fig. 2A). Two tandemly arranged sequences resembling the NtrC binding sites mapped in front of *R. capsulatus glnB*, *nifA1*, and *nifA2* (6, 8) were found in front of *anfA* and *mopA*, respectively. As shown in Fig. 3A, these putative NtrC binding sites corresponded to the consensus sequence of NtrC boxes proposed by Foster-Hartnett and Kranz (8), which consists of a $CGCCN_{10}$ (AT rich)-GC motif.

No promoter sequence resembling the σ^{54}-dependent −12/−24 promoter element could be identified in front of *anfA* or *mopA*. This is in line with the finding that expression of these two NtrC-activated promoters is independent of *rpoN* encoding σ^{54} (Table 2). In addition, no sequences corresponding to the canonical −10/−35 promoter of prokaryotes were found in front of *anfA* and *mopA*.

To locate the initiation sites of *anfA* and *mopA* transcription, primer extension experiments were performed. Total RNA was isolated from *R. capsulatus* as described in Materials and Methods, annealed to two oligonucleotide primers complementary to 5′ parts of the *anfA* and *mopA* coding regions, respectively, and extended by reverse transcriptase. The products were analyzed on polyacrylamide gels next to the products of DNA sequencing reactions generated with the same primers. As shown in Fig. 4, a single 5′ mRNA terminus located at a dA residue could be identified for the *anfA* and *mopA-modABCD* transcripts. This residue is located 30 bp upstream of the *anfA* ATG start codon and 38 bp upstream of the *mopA* initiation codon (Fig. 2A). Alignment of the DNA sequences in

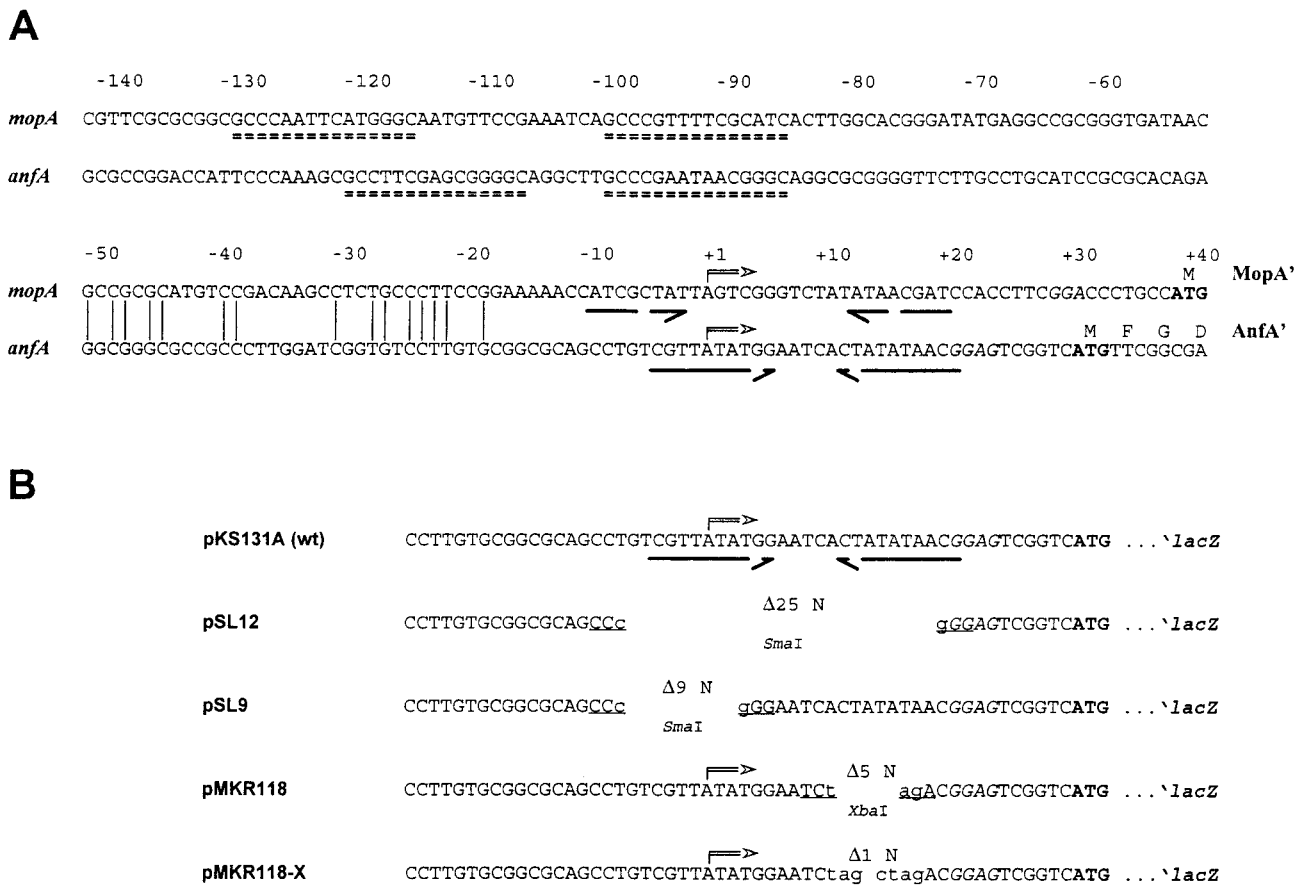

FIG. 2. Nucleotide sequence comparison of the *R. capsulatus anfA* and *mopA* promoter regions and DNA sequence of *anfA* promoter deletion mutants. (A) Alignment of promoter regions of *anfA* and *mopA* according to the transcription initiation sites (marked by arrows at position +1). DNA sequences resembling *R. capsulatus* NtrC binding sites are doubly underlined, and inverted repeats are indicated by broken arrows. Nucleotides identical in both sequences, which may be the recognition sites of RNA polymerase, are connected by vertical lines. The ATG start codons are in boldface, and putative ribosome binding sites are in italics. (B) Comparison of the DNA sequences of *anfA* promoter deletion mutants with the wild-type sequence (wt). Newly introduced restriction sites are underlined, and nucleotides differing from the wild-type sequence are given in lowercase. The plasmid numbers refer to the resulting *anfA'-'lacZ* fusion plasmids.

front of the *anfA* and *mopA* transcription initiation sites revealed considerable nucleotide conservations in the region between positions −20 and −50 (Fig. 2A). However, comparison of these putative *anfA* and *mopA* promoter elements with the NtrC-activated promoters located in front of *glnB*, *nifA1*, and *nifA2* (6–8, 32) revealed no significant homology.

Effects of *cis*-acting mutations on the molybdenum control of the *anfA* promoter. The transcription initiation sites of *R. capsulatus anfA* and *mopA-modABCD* transcripts are located within AT-rich inverted repeats (Fig. 2A). A DNA sequence resembling this element was also found in front of *E. coli modA* (Fig. 3B), which is also negatively regulated by molybdenum (35). As found for *R. capsulatus*, the transcription initiation site in *E. coli* was also localized within this element (35). Comparison of the DNA sequences in front of a number of genes which are likely to be repressed by high molybdenum concentrations in each case revealed the presence of this AT-rich inverted repeat. As shown in Fig. 3B, a high degree of similarity was found between these putative molybdenum response elements located in front of *A. vinelandii modE* and *modG*, homologs to *R. capsulatus mopA* and *mopB* (31), in front of genes encoding transcriptional activator proteins of the alternative nitrogenase systems in *A. vinelandii* (*anfA* and *vnfA* [15]), and in front of *H. influenzae* genes (ORF7 and ORF8; GenBank accession number M94855) coding for a molybdenum-pterin-binding protein and a periplasmic molybdate-binding protein, respectively.

To corroborate the hypothesis that the inverted repeat overlapping the transcription initiation site is responsible for the molybdenum regulation of *R. capsulatus anfA*, four defined deletions were introduced into the *anfA* promoter (Fig. 2B). As shown in Table 3, deletion of the complete element (pSL12) or of the 5′ half of the inverted repeat (pSL9) completely abolished promoter activity. In contrast, deletions within the 3′ part of the putative molybdenum-responsive element resulted in promoters that still exhibited activity under conditions of molybdenum depletion, although the level of expression was twofold less than for the wild-type *anfA'-'lacZ* fusion. However, the deletion of five nucleotides (pMKR118) severely impaired the molybdenum control, whereas the alterations introduced into plasmid pMKR118-X resulting in a single-base-pair deletion totally eliminated molybdenum repression.

DISCUSSION

Regulation of the alternative nitrogenase in *R. capsulatus*. The negative regulation of the alternative nitrogenase by ammonia and molybdenum was shown to occur at the level of *anfA* transcription. No evidence for further levels of regulation was obtained by the analysis of transcriptional and transla-

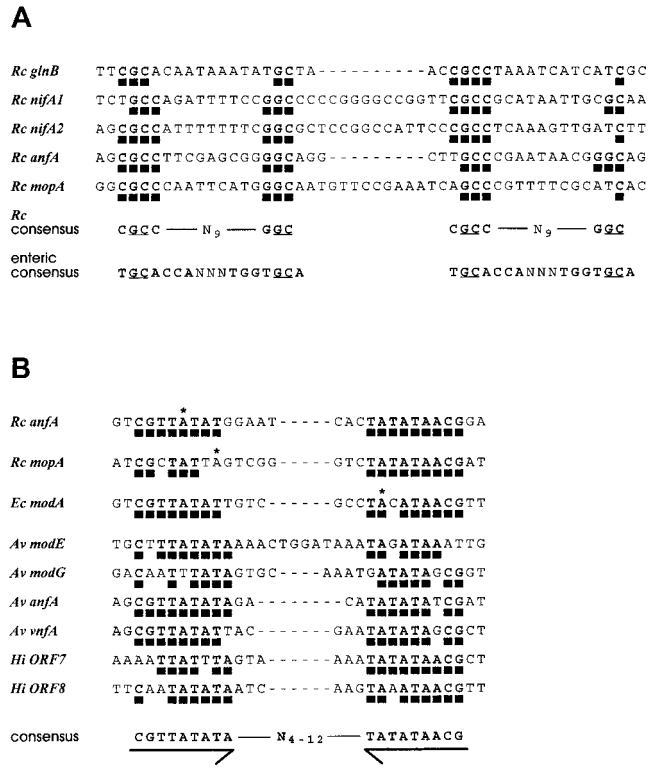

FIG. 3. DNA sequence comparison of the putative NtrC binding sites located in front of *R. capsulatus anfA* and *mopA* with those mapped in front of *glnB*, *nifA1*, and *nifA2*, and comparison of inverted repeat elements located in front of molybdenum-regulated genes from different organisms. (A) DNA sequences of *R. capsulatus* NtrC binding sites aligned for maximum matching. Nucleotides conforming to the consensus sequence are marked by squares. Below this comparison, the consensus sequences of NtrC binding sites from *R. capsulatus* and enteric bacteria are compared; identical nucleotides are underlined. (B) Inverted repeat structures located in front of *R. capsulatus* (*Rc*) *anfA* and *mopA*, *E. coli* (*Ec*) *modA*, *A. vinelandii* (*Av*) *modE*, *modG*, *anfA*, and *vnfA*, and *H. influenzae* (*Hi*) ORF7 and ORF8 are aligned. Nucleotides conforming to the consensus sequence of the inverse repetitive DNA element are marked by squares; transcription initiation sites are marked by asterisks.

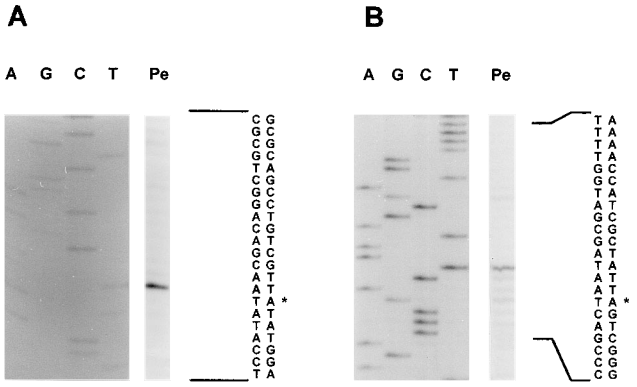

FIG. 4. Locations of the in vivo 5′ terminus of the *R. capsulatus anfA* and *mopA-modABCD* transcripts. Lanes Pe represent primer extension products from mRNA purified from *R. capsulatus* cells grown under nitrogenase-derepressing conditions in molybdenum-free media. Lanes A, G, C, and T show the products of DNA sequencing reactions performed with the primers used for primer extension. The adenine residues corresponding to the 5′ terminus of the *anfA* (A) mRNA or the *mopA-modABCD* transcript (B) are marked by asterisks.

tional *lacZ* fusions to *anfA* and to the structural genes of the alternative nitrogenase *anfHDGK*, which are activated by AnfA in concert with σ^{54}-RNA polymerase. In *A. vinelandii*, however, expression of alternative nitrogenases is also controlled at the level of mRNA translation (14) and at the posttranslational level by modulating AnfA activity (9, 13, 16).

The activity of the *anfA* promoter was shown to be strictly dependent on the transcriptional activator NtrC but independent of σ^{54} (see below). In addition, the *anfA* promoter is negatively regulated by molybdenum. This negative regulation is released not only in *R. capsulatus* mutants carrying lesions in the high-affinity molybdenum transport system, which result in low internal molybdenum concentrations, but also in a *mopA mopB* double mutant. Although the *mopA* gene is located immediately upstream of *modABCD* and forms one transcriptional unit with these genes, the *mopA mopB* double mutant used in this study is able to express the high-affinity molybdenum transport system via a constitutive promoter located on the gentamicin interposon (Fig. 1B). Thus, the *mopA mopB* double mutant is still able to accumulate molybdenum. The release of molybdenum repression of the *anfA* promoter in this double mutant is therefore an indication that MopA and MopB are directly or indirectly involved in transcriptional regulation. The *R. capsulatus* MopA and MopB proteins contain two tandemly arranged domains highly homologous to molybdenum-pterin-binding proteins of *Clostridium pasteurianum*. Therefore, it is assumed that two pterin moieties and two molybdenum atoms can be ligated by these proteins (46). From the deduced amino acid sequences of MopA and MopB, no domains resembling known DNA binding motifs were obvious. Therefore, it is more likely that MopA and MopB themselves do not bind to the *anfA* and *mopA* promoter regions but instead may be the intracellular molybdenum sensor molecules interacting with a putative apo-repressor. The hypothesis that a repressor is responsible for the negative regulation by molybdenum is corroborated by the presence of highly conserved DNA sequences of dyad symmetry overlapping the initiation sites of the *anfA* and *mopA-modABCD* transcripts in *R. capsulatus* and of the *modABCD* transcripts in *E. coli* (35). In addition, deletions within these elements of *R. capsulatus* (this work) and nucleotide exchanges in the corresponding DNA region of *E. coli* (35) resulted in release of molybdenum repression. The hypothesis that MopA and MopB are essential for molybdenum-dependent gene regulation was further tested by analyzing the *anfA′-′lacZ* and *modA′-′lacZ* fusions in the presence of 1 mM molybdate (data not shown). Under these conditions, molybdenum is imported into the cell by the low-affinity transport system. In a *mopA mopB* double mutant, no repression at all could be observed, whereas in a *modB* mutant, both *lacZ* fusions are completely repressed by 1 mM molybdate. These data corroborate the hypothesis that MopA and

TABLE 3. Expression of *anfA′-′lacZ* translational fusions carrying *cis*-acting promoter mutations in *R. capsulatus* wild type

Strain	Fusion[a]	β-Galactosidase activity[b]		
		−N −Mo	−N +Mo	+N −Mo
B10S(pKS131A)	*anfA′-′lacZ*	120 ± 26	2 ± 1	1 ± 1
B10S(pSL12)	pΔ25-*anfA′-′lacZ*	3 ± 1	3 ± 1	4 ± 1
B10S(pSL9)	pΔ9-*anfA′-′lacZ*	4 ± 1	3 ± 1	3 ± 1
B10S(pMKR118)	pΔ5-*anfA′-′lacZ*	46 ± 3	22 ± 9	3 ± 1
B10S(pMKR118-X)	pΔ1-*anfA′-′lacZ*	48 ± 4	42 ± 6	1 ± 1

[a] DNA sequences of the wild-type *anfA* promoter and the corresponding deletion derivatives are given in Fig. 2B.
[b] Determined as described in footnote *a* of Table 2.

MopB are directly involved in gene regulation. In addition, the expression of fusions carrying *cis*-acting mutations in the 3′ part of the inverted repeat of the *anfA* promoter was also found to be completely independent of molybdenum concentrations even up to 1 mM. The mechanism of Mop-dependent gene regulation by molybdenum seems to be more general, since analogous proteins and promoter elements (Fig. 3B) were also found in *A. vinelandii* (31) and *H. influenzae* (5).

The high-affinity molybdenum transport system is part of the nitrogen fixation regulon in *R. capsulatus*. The *R. capsulatus modABCD* genes, encoding a periplasmic binding protein-dependent molybdenum transport system, are located within a cluster of genes involved in nitrogen fixation (24, 46). The expression of this high-affinity molybdenum transport system of *R. capsulatus* is not only negatively controlled by molybdenum but also dependent on NtrC. This transcriptional activator protein is active only under nitrogen-limiting conditions, and therefore, the high-affinity molybdenum transport system is expressed only under nitrogenase-derepressing conditions. Since there is no evidence for a second high-affinity molybdenum transport system, the *modABCD* gene products have to cover the demand of molybdenum not only for the conventional nitrogenase under nitrogen-limiting conditions but also for molybdopterin-containing enzymes such as dimethyl sulfoxide reductase and xanthine dehydrogenase in the presence of ammonia. It remains speculative whether a low-level expression of *modABCD*, which could not be detected by the methods used in this study and which might be either constitutive or still regulated by molybdenum, is sufficient for supply of these molybdopterin enzymes. However, the high demand of molybdenum needed for nitrogenase activity is fulfilled by increased expression of *mopA-modABCD* under nitrogen-limiting conditions via NtrC activation. This additional regulation of *modABCD* by NtrC was not found in *E. coli*. In this non-nitrogen-fixing enteric bacterium, no effect on the regulation of *modABCD* was observed in strains carrying mutations in genes involved in aerobic and anaerobic control or in nitrate control or in genes necessary for the production of molybdopterin (35, 36). In contrast to *R. capsulatus*, the expression of the high-affinity molybdenum transport system in *E. coli* seems to be negatively regulated only by molybdenum.

NtrC-activated promoters of *R. capsulatus* are independent of σ^{54} and may depend on two different, unidentified sigma factors of RNA polymerase. In *R. capsulatus*, NtrC-dependent activation has been found for promoters in front of *nifA1*, *nifA2*, and *glnB* (6–8, 32) as well as for *anfA* and *mopA-modABCD* (this study). These five promoters were all shown to be independent of σ^{54}, and therefore, *R. capsulatus* NtrC is different from all other NtrC-like activator proteins, which strictly depend on σ^{54} polymerase. Comparison of DNA sequences of these promoters revealed no significant homology either to $-12/-24$ promoters typical for σ^{54} RNA polymerase or to the canonical $-10/-35$ promoter recognized by RNA polymerase containing the major sigma factor (σ^{70} in *E. coli*; σ^{93} in *Rhodobacter sphaeroides* [18]). Instead, conserved nucleotides were identified between positions -7 and -30 in front of *nifA1* and *nifA2* (7, 32), whereas conservations between -20 and -50 were found for the *anfA* and *mopA* promoters. Therefore, it can be speculated that two alternative sigma factors, which clearly differ from σ^{54}, may be responsible for the expression of these NtrC-dependent promoters in *R. capsulatus*. However, since housekeeping promoters of *R. capsulatus* are not yet as clearly defined as those in other prokaryotes, it may also be possible that upon binding of the phosphorylated form of NtrC to tandemly arranged upstream activator sequences, RNA polymerase containing the major sigma factor is able to recognize DNA sequences exhibiting no significant homology to each other and to known promoter sequences.

The Ntr regulon of *R. capsulatus* differs from analogous systems in other bacterial species not only in its mechanism of transcriptional activation by NtrC but also in its target genes. In enteric bacteria, the Ntr system is responsible for the hierarchic control of a large number of genes involved in different aspects of the general nitrogen metabolism. In contrast, *R. capsulatus* NtrC was found to activate only genes directly involved in nitrogen fixation such as *nifA1*/*nifA2* and *anfA*, which code for transcriptional activators of both nitrogenase systems. In addition, the ammonia uptake system (33) and the high-affinity molybdenum transport system are controlled by NtrC. The degradation of proline, which is under control of the Ntr system in enteric bacteria (23, 42), was shown to be independent of NtrC in *R. capsulatus*. Instead, an Lrp-like activator is necessary for the induction of this system (19). Therefore, in the phototrophic purple bacterium *R. capsulatus*, substrate-induced parallel networks may replace the hierarchic Ntr regulatory cascade (for a review, see reference 24).

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