

ModE-Dependent Molybdate Regulation of the Molybdenum Cofactor Operon *moa* in *Escherichia coli*

LISA A. ANDERSON,¹ ELIZABETH McNAIRN,¹ TORBEN LEUBKE,¹ RICHARD N. PAU,^{2†}
AND DAVID H. BOXER^{1*}

Department of Biochemistry, University of Dundee, Dundee DD1 5EH, Scotland,¹ and Nitrogen Fixation Laboratory, John Innes Centre, Norwich NR4 7UH,² United Kingdom

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The expression of the *moa* locus, which encodes enzymes required for molybdopterin biosynthesis, is enhanced under anaerobiosis but repressed when the bacterium is able to synthesize active molybdenum cofactor. In addition, *moa* expression exhibits a strong requirement for molybdate. The molybdate enhancement of *moa* transcription is fully dependent upon the molybdate-binding protein, ModE, which also mediates molybdate repression of the *mod* operon encoding the high-affinity molybdate uptake system. Due to the repression of *moa* in molybdenum cofactor-sufficient strains, the positive molybdate regulation of *moa* is revealed only in strains unable to make the active cofactor. Transcription of *moa* is controlled at two sigma-70-type promoters immediately upstream of the *moaA* gene. Deletion mutations covering the region upstream of *moaA* have allowed each of the promoters to be studied in isolation. The distal promoter is the site of the anaerobic enhancement which is Fnr-dependent. The molybdate induction of *moa* is exerted at the proximal promoter. Molybdate-ModE binds adjacent to the -35 region of this promoter, acting as a direct positive regulator of *moa*. The molybdenum cofactor repression also appears to act at the proximal transcriptional start site, but the mechanism remains to be established. Tungstate in the growth medium affects *moa* expression in two ways. Firstly, it can act as a functional molybdate analogue for the ModE-mediated regulation. Secondly, tungstate brings about the loss of the molybdenum cofactor repression of *moa*. It is proposed that the tungsten derivative of the molybdenum cofactor, which is known to be formed under such conditions, is ineffective in bringing about repression of *moa*. The complex control of *moa* is discussed in relation to the synthesis of molybdoenzymes in the bacterium.

Molybdenum is an essential trace element required for the activity of several enzymes in the form of a molybdenum cofactor which is found in bacteria, plants, and animals. All oxo-molybdoenzymes described to date contain a molybdenum cofactor which consists in its most simple form of a pterin, molybdopterin, complexed to molybdenum. Molybdopterin has a terminally phosphorylated, four-carbon alkyl side chain with a dithiolene group, the two sulfur atoms of which are ligands to the molybdenum (28). Crystallographic structures of several molybdoenzymes are now available (4, 18, 19, 33).

In *Escherichia coli* the *mo*-loci are responsible for molybdenum transport and the synthesis of the molybdenum cofactor, and all of these loci have been characterized (12, 13, 15, 26, 29, 30). The *moa* and *moe* loci are required for molybdopterin biosynthesis. The first three genes (*moaABC*) of the five genes at the *moa* locus (30) are required for the first committed step of molybdopterin biosynthesis, namely the formation of precursor Z from a guanosine nucleotide or closely related compound (39).

The *modABCD* operon of *E. coli* encodes the high-affinity molybdate uptake system of the bacterium. Strains defective at this locus are pleiotropically unable to synthesize active molybdoenzymes, such as nitrate reductase. The introduction of high levels of molybdate into the growth medium, however, restores molybdoenzyme activities to *mod* strains, indicating that molybdate can enter the bacterium by another route un-

der such conditions (6, 27, 31). Transcription of *modABCD* is repressed under conditions of high molybdate availability (23, 29, 31). The repression is mediated by the ModE protein, which is encoded by a second operon expressed divergently from *modABCD* (7, 20, 25, 38).

In vitro studies have demonstrated that dimeric ModE directly binds two molybdate ions with a K_d of about 0.8 μ M and that the molybdate-ModE complex binds to *mod* DNA at a site which spans the *modA* transcription start site. This is consistent with its role as a transcriptional repressor of *modA* (2, 8, 22). The ModE protein has a wider role in mediating molybdate regulation of other operons which include *dmsA*, the structural operon for the molybdoenzyme dimethyl sulfoxide reductase (21), *hyc*, the hydrogenase 3 structural operon (34), and *narG*, which encodes the molybdoenzyme nitrate reductase A (34). Hall and colleagues recently reported the crystallographic structure of ModE, which appears to possess distinct DNA- and molybdate-binding domains (9).

Genetic analysis of the regulation of *moa* has revealed that its expression is enhanced under anaerobic growth conditions but is repressed in strains able to synthesize active molybdenum cofactor (3). McNicholas et al. (22) reported that molybdate-ModE is able to bind at the *moa* promoter region and that *moa* expression displayed a twofold ModE dependency. However, a dependency of *moa* expression on molybdate has not been demonstrated. We report here that internal molybdate availability exerts a major influence on *moa* expression via a mechanism in which ModE acts directly as a positive regulator at one of the two *moa* promoters. This effect is distinct from and in addition to the anaerobic molybdenum-cofactor regulation of *moa*. This work exploits our previous finding that ModE is able to bind tungstate, a molybdate analogue, in a manner that is indistinguishable from that of molybdate (2).

* Corresponding author. Mailing address: Department of Biochemistry, MSI/WTB Complex, University of Dundee, Dundee DD1 5EH, Scotland, United Kingdom. Phone: 44 (0) 1382 345561. Fax: 44 (0) 1382 201063. E-mail: d.h.boxer@dundee.ac.uk.

† Present address: Department of Biochemistry and Molecular Biology, University of Melbourne, Parkville, Victoria 3010, Australia.

TABLE 1. Bacterial strains, bacteriophage, and plasmids

Strain, bacteriophage, or plasmid ^a	Relevant genotype or characteristics	Source or reference
Strains		
RK4353	F' <i>araD139</i> Δ (<i>argF-lac</i>) <i>U169 deoC1 fbbB5201 gyrA 219 relA1 rpsL150 non-9 ptsF25</i>	37
DB1004	As RK4353; ϕ (<i>moaB::lacZ</i>)4	3
DB1060	As RK4353; [λ p(<i>moaB::lacZ</i>)4]	3
SE1597	Δ (<i>lacU</i>)169 <i>rpsL</i> ; <i>modC118</i>	K. T. Shanmugan
RK4921	As RK4353; <i>zbh-623::Tn10</i> (near <i>mod</i>)	V. Stewart
LA27	As DB1004; <i>modC118 zbh-623::Tn10</i>	This work
LA29	As LA27; <i>modE::Gm</i>	This work
TL28	As DB1060; <i>modC118 zbh-623::Tn10</i>	This work
LA30	As TL28; <i>modE::Gm</i>	This work
MC4100	F' <i>araD139</i> Δ (<i>argF-lac</i>) <i>U169 deoC1 fbbB5201 relA1 rpsL150 ptsF25 rbsR</i>	Lab stocks
RM102	As MC4100; <i>fmr</i> Δ (<i>srl-recA</i>)306::Tn10	A. Bock
MC1000	F' <i>araD139</i> Δ (<i>araABC-leu</i>) 7679 <i>galU galK</i> Δ (<i>lac</i>)X74 <i>rpsL thi</i>	Lab stocks
HORF55	As MC1000; <i>modE::Gm</i>	38
NS3	As MC4100; <i>moeB</i>	Lab stocks
Bacteriophage		
λ p(<i>moa-lacZ</i>)4	λ p(<i>moaB::lacZ</i>)4	This work
Plasmids		
pMLB524	Amp ^r [Lac ⁺]	35
pAA182	Amp ^r <i>lacZ</i>	14
pEM101	1.16-kb <i>Pvu</i> II fragment of <i>moa</i> DNA in pAA182	This work
pEM114*	As pEM101, but 0.74-kb insert	This work
pEM117*	As pEM101, but 0.80-kb insert	This work
pEM118*	As pEM101, but 0.54-kb insert	This work
pEM220†	As pEM101, but 0.37-kb insert	This work

^a *, insert constructed by deleting from the pAA182 multiple cloning site an *Eco*RI site upstream of the insert in plasmid pEM101; †, constructed by deleting from the vector's multiple cloning (*Bam*HI) site downstream of the insert in pEM101.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, and plasmids. All strains (derivatives of *E. coli* strain K-12), bacteriophage, and plasmids used in this study are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) medium at 37°C, but strains carrying a *Muets* prophage were grown at 30°C. Ampicillin was added, where required, to final concentrations of 125 μ g/ml in liquid media and 50 μ g/ml in solid media. Tetracycline and gentamicin were added, where required, to final concentrations of 25 and 10 μ g/ml, respectively. 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) was added to media at 125 μ g/ml when necessary. Isopropyl- β -D-thiogalactopyranoside (IPTG) was used as an inducer for the *lac* operon at a concentration of 0.3 mM. Aerobic growth conditions were achieved by placing 5 to 10 ml of culture in 50-ml conical flasks and incubating with vigorous shaking on an orbital shaker. Anaerobic growth was performed in an anaerobic chamber under an atmosphere containing carbon dioxide and hydrogen (GasPak; BBL Microbiology).

Strain construction. Generalized transduction between *E. coli* strains was carried out using a derivative of bacteriophage P1 essentially as described previously (35). A P1vir lysate was prepared with strain RK4921 (*zbh-623::Tn10* [near *mod*]) and used as the donor in a cross with strain SE1597 (*modC118*). Recombinants selected as Tet^r colonies were screened for loss of molybdenum-dependent formate hydrogenlyase activity by their inability to produce gas during glucose fermentation and for the nitrate reductase-negative phenotype by the nitrite overlay procedure (6). A P1vir lysate prepared with the resultant strain (as SE1597; *zbh-623::Tn10*) was used as the donor in P1-mediated crosses with strains LA27 and TL28 to construct strains LA29 (as LA27; *modE::Gm*) and LA30 (as TL28; *modE::Gm*), respectively. Drug resistance was used for initial selection in all crosses.

Enzymes, reagents, and recombinant DNA techniques. Restriction endonucleases, T4 DNA ligase, Klenow fragment of DNA polymerase I, and nuclease *Bal* 31 were purchased from Northumbria Biologicals. Sequenase was obtained from U.S. Biochemicals Corp. through Cambridge Bioscience (Cambridge, Mass.). Avian myeloblastosis virus reverse transcriptase was purchased from International Biotechnologies Inc. Human placental ribonuclease inhibitor and all radionucleotides were obtained from Amersham PLC. The isolation of plasmid DNA, restriction digestion, ligation, *E. coli* transformation, and agarose gel electrophoresis were essentially as described by Maniatis et al. (17).

The assay for β -galactosidase activity was performed essentially as described by Miller (24). Cells were assayed during early to mid-log phase following permeabilization by the addition of sodium dodecyl sulfate and chloroform. All

assays were performed in triplicate and the mean values were reported in Miller units (24). Individual assays rarely differed from the mean by more than 10%.

Isolation of λ p(*moa-lacZ*) phage and construction of lysogens. Strain DB1004 [ϕ (*moaB::lacZ*)4] was grown to mid-log phase, and the cells were harvested and resuspended in a half-volume of 10 mM magnesium sulfate for lysate preparation. The suspension was exposed (2 min) to short-wave (260-nm) irradiation, diluted 10 times in LB medium, and incubated with shaking at 37°C in foil-covered tubes until lysis occurred (approximately 4 h). Cell debris was removed by centrifugation and the supernatants were used as low-titer lysates for production of Lac⁺ plaques when grown in the presence of X-Gal and IPTG. A high-titer lysate was used to construct *moa::lacZ* reporter lysogens by transduction into appropriate acceptor strains.

To prepare recombinant λ phage DNA, solid polyethylene glycol (PEG 8000) was dissolved at room temperature in a large-scale lysate to a final concentration of 10% (wt/vol). The phage was pelleted and then dispersed in a minimum volume (about 5 ml per liter of lysate) of buffer (50 mM Tris HCl [pH 7.5], 10 mM MgSO₄). Following a phenol-chloroform extraction, the aqueous solution was layered over a glycerol step-gradient (5 to 40% [wt/vol]) and centrifuged at 70,000 \times g for 90 min to pellet the phage. Residual host-derived DNA and RNA were removed using DNase and RNase. The phage coat was liberated by suspension in 0.5% (wt/vol) sodium dodecyl sulfate, 50 mM Tris HCl (pH 7.5), 400 mM EDTA, and 1 mg of proteinase K/ml. DNA was ethanol precipitated and resuspended in a suitable volume of sterile water or 10 mM Tris HCl (pH 8.0), and 1 mM EDTA.

Subcloning the *moa* promoter region and generation of deletion mutations. A plasmid carrying the *moa* promoter region was isolated by shotgun cloning of *Eco*RI-digested phage λ p(*moaB::lacZ*) DNA into the *Eco*RI-digested promoter-cloning vector pMLB524 and by selecting for Lac⁺ transformants of MC4100 (35).

Further subcloning of the *moa* promoter region gave rise to pEM101, which contained a 1.16-kb fragment of *moa* DNA in the *Sma*I site of pAA182, a transcriptional reporter plasmid carrying a promoterless *lacZ* gene. Deletion mutations in the *moa* promoter region were isolated as described below.

For 5' deletions, plasmid pEM101 was digested with *Eco*RI and treated with nuclease *Bal* 31, and overhangs were filled in with Klenow fragment. Following digestion with *Bam*HI, the blunt-end *Bam*HI fragments were ligated to *Sma*I-*Bam*HI-cleaved pAA182. The plasmids were characterized by agarose gel electrophoresis following *Eco*RI-*Bam*HI digestion or by direct DNA sequence determination.

For 3' deletions, plasmid pEM101 was digested with *Bam*HI and treated with nuclease *Bal* 31, and overhangs were filled in with Klenow fragment and reli-

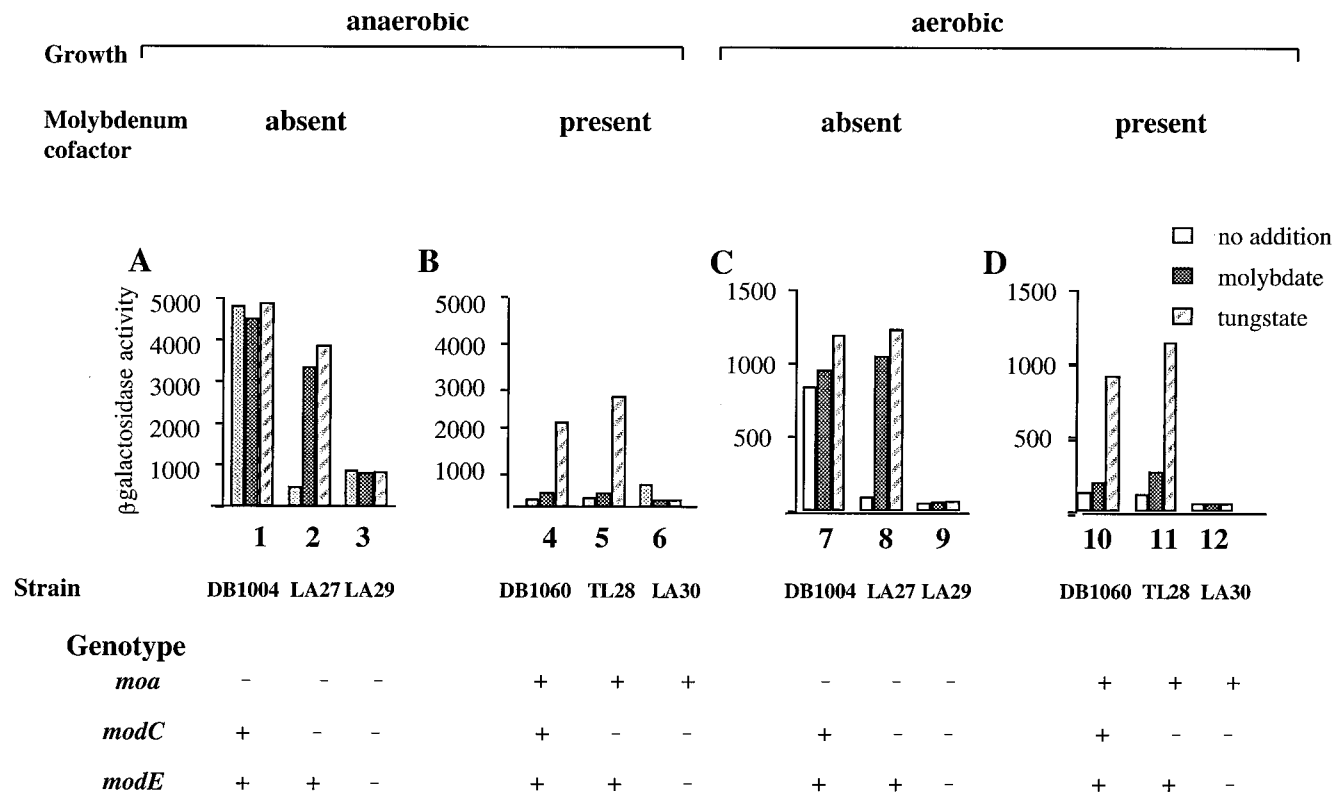


FIG. 1. Expression of *moa*: ModE-dependent molybdate and tungstate regulation. All growths were performed at 37°C in LB medium. Additions to the growth media were sodium molybdate and sodium tungstate to a final concentration of 10 mM, as indicated. β -Galactosidase activity is expressed in Miller units (24). (A) Strains defective in molybdenum cofactor (*moa*), under anaerobic growth. (B) Strains which were molybdenum cofactor sufficient, under anaerobic growth. (C) Strains defective in molybdenum cofactor (*moa*), under aerobic growth. (D) Strains which were molybdenum cofactor sufficient, under aerobic growth. The strains used were as follows: 1 and 7, DB1004 [$\phi(moaB::lacZ)4$]; 2 and 8, LA27 (as DB1004; *modC118*); 3 and 9, LA29 (as LA27; *modE::Gm*); 4 and 10, DB1060 [$\lambda\phi(moaB::lacZ)4$]; 5 and 11, TL28 (as DB1060; *modC118*); 6 and 12, LA30 (as TL28; *modE::Gm*).

gated. The deleted plasmids were characterized by sequencing through the insert DNA region.

The extent of *moa* DNA remaining in the deletion constructs pEM114 and pEM117 was determined by DNA sequencing using the oligonucleotide primer L9 (5'-GCCCTGGTGGCAATCT-3'), which hybridizes to *moa* DNA between nucleotides +87 and +72, located between S1 and the translational start of *moaA*. The extent of the DNA insert remaining in plasmid pEM118 could not be determined in this manner, presumably due to loss of the primer binding site. The size of this insert was estimated from its electrophoretic mobility. The deletion end point in plasmid pEM220 was determined by DNA sequencing using an oligonucleotide primer which hybridizes to a region upstream of the distal transcriptional start site.

Double-stranded DNA sequencing reactions were performed as described previously (32).

The insert in plasmid pEM101 comprises 1,164 bp of chromosomal DNA, of which 587 nucleotides lie upstream of the translational start of *moaA*.

Analysis of transcription initiation sites. Cultures of strain RK4353 carrying plasmid pEM101 were grown to an optical density at 600 nm of 0.6, and total RNA was recovered according to the method of Figueroa et al. (5). The dried RNA was resuspended in 30 μ l of sterile H₂O and hybridized with 0.1 pmol of ³²P-labeled oligonucleotide primer. Primer extension was performed using the method of Inoue and Cech (11). The oligonucleotide primer L9 (see Fig. 2) was used in all the experiments shown. The same oligonucleotide primer was used in DNA sequencing reactions with plasmid pEM101 DNA as the template to produce a reference sequencing ladder, which was electrophoresed adjacent to the transcript-analysis samples in a 6% (wt/vol) denaturing polyacrylamide gel containing 6 M urea.

RESULTS

Molybdate activates *moa* expression under anaerobic growth conditions in a molybdenum cofactor-deficient background. To assess the possible molybdate regulation of *moa*, *moa-lacZ* reporter strains defective in *modC*, which encodes part of the

high-affinity molybdate transporter, were constructed in order to obtain molybdate starvation conditions in normal growth media.

In strains unable to synthesize molybdenum cofactor, reduction of the internal availability of molybdenum (*modC*) resulted in a much-reduced expression of *moa* compared to that found for the parental strain, DB1004 (Fig. 1A). Because strain LA27 (*modC*) is molybdenum cofactor-deficient, the reduced expression cannot arise from the previously described molybdenum cofactor-mediated repression. This suggests that intracellular molybdenum availability acts positively on *moa* via a mechanism that is independent of the molybdenum cofactor.

The internal molybdate deficiency brought about by mutation of *modC* (strain LA27) can be overcome by growth in the presence of excess molybdate. Under such conditions, expression of *moa* returned to levels similar to those found for the parental reporter strain (DB1004). The suppression of the *modC* phenotype with respect to *moa* expression by molybdate addition to the growth medium clearly points to a role for molybdenum in the activation of *moa*.

To establish whether ModE may be responsible for mediating the molybdenum-dependent activation, strain LA29 (*modC*, *modE*) which was additionally defective in *modE* was constructed. Expression of *moa* was much reduced in this strain but the addition of molybdate to the growth medium was without effect (Fig. 1A). The ModE protein is, therefore, required for the molybdenum-dependent activation of *moa*. Since it is established that ModE binds molybdate, it appears

that the internal availability of molybdate, rather than any other form of molybdenum, is the signal for *moa* activation. The ModE-mediated, molybdate-dependent regulation of *moa* can result in a fivefold increase in activation.

Molybdate cannot activate *moa* in a molybdenum cofactor-sufficient background. Baker and Boxer (3) reported that *moa* is repressed in molybdenum cofactor-sufficient strains. As anticipated, in the presence of functional molybdenum cofactor, *moa* was repressed {strain DB1060 [*moa*⁺/ϕ(*moaB-lacZ*)]}, and the addition of molybdate to the growth medium was without effect (Fig. 1B). Strain TL28 [*moa*⁺/ϕ(*moaB-lacZ*), *modC*], in the presence or absence of added molybdate, displayed levels of *moa* expression similar to the repressed levels in strain DB1060. Furthermore, loss of ModE function was without effect, since expression in strain LA30 [*moa*⁺/ϕ(*moaB-lacZ*) *modC modE*] remained low and uninfluenced by the addition of molybdate. The clear ModE-dependent molybdate activation of *moa* observed in molybdenum cofactor-deficient backgrounds is not found in strains possessing functional molybdenum cofactor. It is evident that molybdenum cofactor-dependent repression is dominant over the ModE-requiring molybdate activation. However, high expression of *moa* clearly requires ModE.

Aerobic expression of *moa* displays similar control characteristics to those present during anaerobic growth. The aerobic expression of *moa* was monitored in a manner similar to that described above. The relative patterns of expression were closely similar to those found for anaerobically grown cultures (Fig. 1, compare C and D). In the molybdenum cofactor-deficient strain DB1004, the anaerobic enhancement was four- to fivefold higher, whereas in the molybdenum cofactor-sufficient strain (DB1060), the anaerobic enhancement was much lower. Molybdate availability, therefore, activates *moa* during both anaerobic and aerobic growth in a ModE-dependent manner and is a major positive control on *moa*.

Tungstate activates *moa* expression in a ModE-dependent manner. Tungstate is a close analogue of molybdate in biological systems. Tungstate interacts with isolated ModE indistinguishably from molybdate, and the tungstate-ModE complex also interacts with DNA in a manner similar to that of the molybdate-ModE complex (2). The effects of tungstate on *moa* regulation were investigated.

The addition of tungstate to the growth media of molybdenum cofactor-deficient strains LA27 (*modC*) and LA29 (*modC modE*) influenced *moa* expression in a way similar to that found for molybdate (Fig. 1A and C). The expression of *moa* in strain LA27 was enhanced to a comparable level to that found for the parental strain, DB1004, when grown in tungstate. The addition of tungstate to the growth medium for strain LA29 (*modE*) could not restore the tungstate activation seen in strain LA27. A similar result was also obtained following tungstate addition to the growth medium for strain LA30 (*modC modE*). We conclude that for a molybdenum cofactor-deficient strain, tungstate, like molybdate, is able to activate *moa* expression in a ModE-dependent manner.

In contrast to molybdate, tungstate relieves molybdenum cofactor-dependent repression of *moa*. The low *moa* expression in the (*moa*⁺) merodiploid strain DB1060 can be explained by repression brought about by the molybdenum cofactor sufficiency of the strain. This is revealed by the enhanced expression of *moa* observed when mutations in other (*mo*-) genes required for molybdenum cofactor biosynthesis are introduced into such merodiploids. This repression could be mediated by the cofactor itself, a derivative of the cofactor, or a process which is dependent on the active cofactor (3).

Growth in the presence of tungstate gives rise to a biologically inactive tungsten form of the molybdenum cofactor,

which leads to the formation of inactive forms of molybdoenzymes (16). The expression of *moa* is low in DB1060, a molybdenum cofactor-sufficient strain, but on the addition of tungstate to the growth medium, expression was increased some 10-fold (Fig. 1B and D). This was in contrast to the lack of effect of molybdate addition to this strain. Similarly, the addition of tungstate to the growth medium for strain TL28 (*modC*) gave rise to enhanced *moa* expression; however, molybdate addition was without effect. The presence or absence of functional ModE did not influence this tungstate-specific effect, which was observed only in molybdenum cofactor-sufficient strains.

These observations cannot be explained by the ModE-dependent tungstate enhancement of *moa* demonstrated earlier for the molybdenum cofactor-deficient strains (Fig. 1A and C). This tungstate effect is dependent upon the ability of the strain to synthesize functional molybdenum cofactor under normal growth conditions. The most likely explanation is that the tungstate leads to the formation of a nonfunctional tungsten form of the cofactor, which is unable to effect *moa* repression via the molybdenum cofactor-dependent mechanism.

Identification of two transcriptional start sites at *moa*. Plasmid pEM101 consists of approximately 0.6 kb of DNA upstream of the *moaA* translational start inserted into the multiple cloning site of the transcriptional (LacZ) reporter plasmid vector, pAA182. The *moa* DNA in pEM101 was subcloned from a Lac⁺ bacteriophage λ [ϕ(*moaB-lacZ*)], a transducing phage induced from strain DB1004. That the *moa* promoter region was present in pEM101 was confirmed by the retention of the *moa* expression characteristics by the plasmid (data not shown). This retention also confirmed that *moa* regulation is exerted at the level of transcription.

The insert in plasmid pEM101 comprised 1,164 bp of chromosomal DNA, and part of the DNA sequence which lies upstream of the *moaA* translational start is shown in Fig. 2A. Primer extension analysis revealed two transcriptional start sites (Fig. 2B). The proximal transcriptional start site, S1, lies 130 bp upstream of the translational start of *moaA*; the distal start site, S2, lies a further 87 bp upstream of S1 (Fig. 2A).

Transcripts originated from S2 only when mRNA was isolated from cells grown anaerobically. Transcripts from S1, however, were seen following growth under both aerobic and anaerobic conditions regardless of whether expression from the distal transcriptional start was active.

Analysis of expression from deletion clones. We determined, by using deletion analysis, the expression characteristics of the independent *moa* promoters in order to assess their contribution to the overall control of the locus. Three 5'-deleted plasmids were obtained following *Bal* 31 digestion from the *EcoRI* site in pEM101. These constructs are pEM114 (~0.74 kb insert; carrying only S1), pEM117 (~0.80 kb insert; carrying S1 and S2 with 25 bp upstream of S2), and pEM118 (~0.54 kb; whole promoter region absent). A further deletion, in plasmid pEM220 (insert 0.37 kb), was obtained by *Bal* 31 digestion originating from the *Bam*HI site at the 3' limit of the insert in pEM101. The structures of the deletion clones are summarized in Fig. 3.

Since strains carrying mutations in either *moa* or *moe* cannot synthesize molybdopterin (28), plasmids pEM101, pEM220, pEM117, pEM114, and pEM118 were transformed into the molybdenum cofactor-deficient strain NS3 (*moeB*) in order to assess the capacity of the deletion constructs to respond to *moa* effectors. The results were compared to those for the intact promoter plasmid, pEM101 (Fig. 3).

In the molybdenum cofactor-sufficient background, expression from pEM101 was anaerobically enhanced fivefold. This

A

CACGATCAAGATCAGCCAGCGTACGATTGCGCATATAGTTTCCTGGAGTCAGATTATCCG -241

CGCTACAGTAGCGCAAATTCGCGGGAAACAGCAATTAACTGCCAATATTGAGGATACAA -181

ACTATTTTCTTCTACCTCTAAAGGACGATGCACGCTATGCCTCCCTGATGATGTATATCA -121

pEM117

pEM220

AAGTAAACCGCCATTTTCCTTATTCTGTAGCGAAATAGCACGATCATGACGCTATATA -61

pEM114

S2

CATGATTACATAGCGAAAGTGTGGATGGTAAAAATCTCATTACACGCTAGTATCGGCAT -1

S1

AACCACTAAACACTCTAGCCTCTGCACCTGGGTCAACTGATACGGTGCTTTGGCCGTGAC 60

AATGCTCGTAAAGATTGCCACCAGGGCGAAGGAAGAAATGACTTCGCCTCCCGTATCTGG 120

L9

AAAGGTGTACatggccttcacaactgactgatgcatttgcgcgtaagttttactacttg... 178

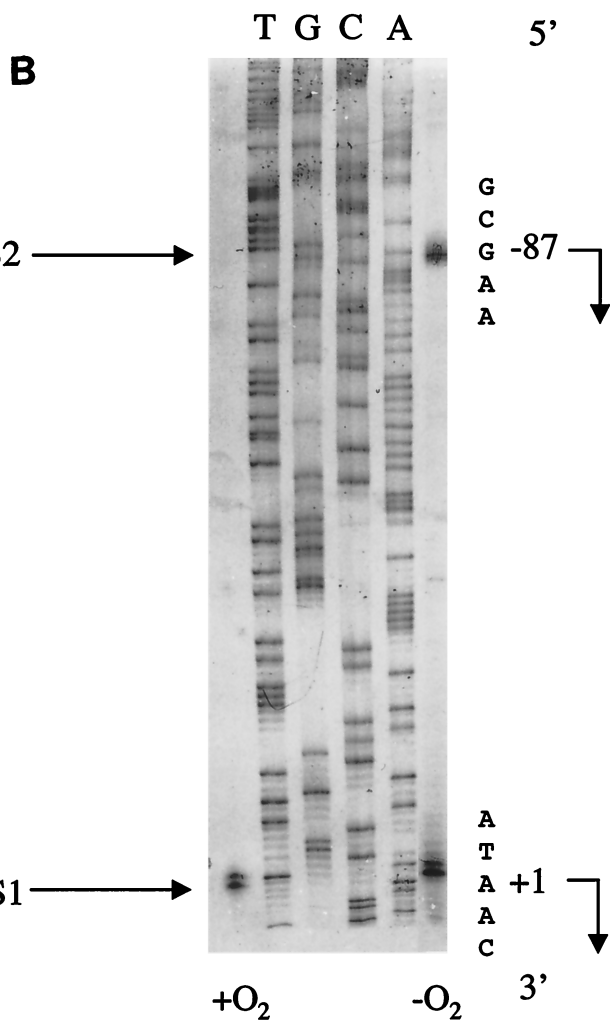


FIG. 2. (A) Nucleotide sequence of the *moa* promoter region. Part of the *moa* upstream sequence is shown. Indicated are the different plasmid-borne 5' (pEM114 and pEM117) and 3' (pEM220) deletion end points. The position of binding of the primer (L9) used in the primer extension reactions is indicated by underlining and the L9 label. Transcriptional start sites, termed S1 (proximal) and S2 (distal), are shown in bold type. The Fnr recognition half-sites are underlined. The start codon of *moaA* is boxed and is in lowercase type, as is the portion of the *moaA* sequence shown. (B) Primer extension analysis of *moa* transcription. RNA was recovered from parental (RK4353) cells carrying plasmid pEM101, grown both aerobically (+O₂) and anaerobically (-O₂) in LB medium. The autoradiograph was reversed prior to photography to show the sequence of the coding strand. The DNA sequence shown was obtained using primer L9 (see Materials and Methods). The nucleotide sequence around each initiation site is shown on the right. The transcript labeled as S1 begins at nucleotide +1, and transcript S2 begins at position -87.

anaerobic expression was still partially repressed, since tungstate addition led to a further threefold enhancement. Such repression was absent from the corresponding *moeB* strain. Expression in this strain was, as anticipated, similar to that of the parental strain when grown with tungstate.

Plasmid pEM220 obtained from the 3' deletion of pEM101 retains only 8 nucleotides of *moa* DNA downstream of S2. This construct showed little or no promoter activity under aerobic conditions but exhibited a 40-fold enhancement during anaerobic growth. This is consistent with expression from S2 occurring only in anaerobic cultures (Fig. 2B). The addition of tungstate to the anaerobic growth medium was without effect on expression from pEM220, suggesting that expression from S2 is not influenced by molybdate or the molybdenum cofactor. Placing this plasmid into a strain genetically lacking the functional cofactor, however, did lead to a further small increase in anaerobic expression, the significance of which is presently unclear. Plasmid pEM101, however, displayed high levels of anaerobic expression in the *moeB* strain similar to those obtained following the addition of tungstate to the molybdenum cofactor-sufficient strain (RK4353). We conclude that the distal promoter, as indicated from the transcript analysis, is responsible for the anaerobic enhancement of *moa* expression.

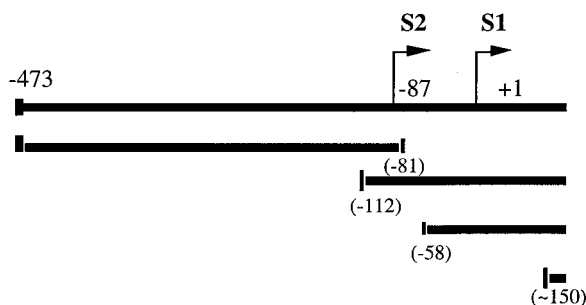


FIG. 3. Properties of promoter-deletion clones. The direction of transcription is indicated by arrows and the position of the first nucleotide in each transcript is given. The DNA present in each of the deleted clones is shown by the broad lines, with the deletion end point given in parentheses. The expression from each of the constructs is given as β -galactosidase activity (Miller units). MoCo⁺ indicates that the strain (RK4353) is able to synthesize functional molybdenum cofactor. MoCo⁻ indicates that the strain (NS3; *moeB*) is unable to synthesize functional molybdenum cofactor. Each plasmid was examined in both a MoCo⁺ strain (RK4353) and a MoCo⁻ strain (NS3; *moeB*). The bacteria were grown in LB medium at 37°C. Growth conditions and additions were as described in Materials and Methods.

The deletion clone pEM114 has lost the distal (S2) start and an additional 28 nucleotides downstream. This plasmid retains the -35 and -10 regions of the proximal promoter. Under all conditions examined, expression from this clone was lower than that for the parental plasmid, pEM101. For this clone in the absence of tungstate, expression was not increased during anaerobic growth. During anaerobic growth the addition of tungstate to the molybdenum cofactor-sufficient parental strain carrying pEM114 raised expression. A similar derepression was also seen when the plasmid was present in the molybdenum cofactor-deficient (*moeB*) strain. Overall, these results suggest that the loss of S2 has two effects. Firstly, the level of expression is lower. Secondly, the anaerobic enhancement is lost. The results for pEM117, which has only 25 bp of DNA upstream of S2, were similar to those found for pEM114.

Transcription-start analysis on pEM114 and pEM117 confirmed that transcription was from S1 in both plasmids. As would be expected from the loss of the -35 region of S2, transcription from S2 in pEM117 could not be detected even following anaerobic growth (data not shown). Expression from these two 5'-deletion clones is much lower than that from the parental plasmid, which may reflect a requirement for sequences 5' to S2 being required for full expression from S1. As anticipated, expression from plasmid pEM118 (both S1 and S2 absent) was negligible under all conditions tested.

In the absence of S2 (pEM117 and pEM114), there is both a tungstate enhancement in the molybdenum cofactor-sufficient parental strain background and a derepression to a similar level in the molybdenum cofactor-deficient strain. We conclude that the promoter associated with S1 is subject to repression in molybdenum cofactor-sufficient strains.

If the two *moa* promoters worked independently in situ, the expression observed when both promoters are functional should equal the sum of their individual expressions. This is not the case. The proximal promoter clearly overlaps part of the distal promoter.

Expression from the distal promoter is insensitive to molybdate. The plasmid pEM101 possesses both promoter regions and retains the overall characteristics of *moa* regulation. Plasmid pEM220 contains only the distal promoter. In order to establish whether the distal promoter is regulated by ModE, the plasmids were each transformed into a *modE* strain (HORF55) and expression was monitored following anaerobic

Plasmid	β -galactosidase activity			
	MoCo ⁺			MoCo ⁻
	Aerobic	Anaerobic	Anaerobic + tungstate	Anaerobic
pEM101	520	2,830	9,410	9,430
pEM220	33	1,350	1,330	2,980
pEM117	44	62	620	680
pEM114	32	42	440	610
pEM118	20	20	20	20

growth (Fig. 4). In the *modE* strain, expression from pEM101 was reduced approximately twofold from the level for the parental strain background. Expression from pEM101 was enhanced some 75% by molybdate addition to the growth medium in the *modE*⁺ strain, but in the *modE*-deficient strain the addition of molybdate was without effect. This is consistent with the results obtained for the chromosomal *moa-lacZ* strains. When only S2 is present (pEM220), neither the presence of molybdate in the growth medium nor the lack of *modE* influences expression. This strongly indicates that the ModE-dependent molybdate control of *moa* is effected through the proximal promoter.

Fnr is required for the anaerobic regulation of *moa* at the distal promoter. The *fnr* gene encodes a positive transcriptional regulator required for the expression of a number of anaerobically expressed genes (36). A putative Fnr recognition sequence (GATGAT-N₄-ATCAAA), centered at -39.5 nucleotides upstream of S2, was found (Fig. 2A). This site differs

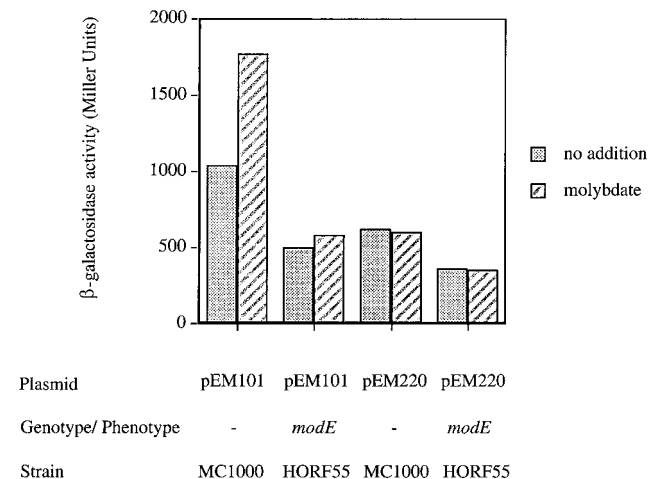
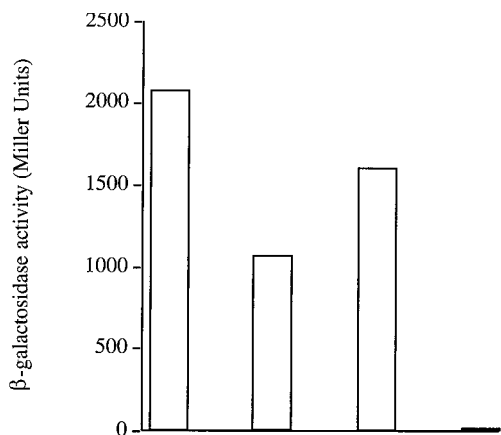


FIG. 4. ModE does not regulate *moa* expression from the distal promoter. The plasmids were present in the strains as indicated. Cells were grown under anaerobic conditions at 37°C in LB medium with 125 μ g of ampicillin/ml. Where indicated, sodium molybdate was added at a final concentration of 10 mM. β -Galactosidase activity is expressed in Miller units (24).



Plasmid	pEM101	pEM220	pEM101	pEM220
Genotype/Phenotype	-	-	<i>fnr</i>	<i>fnr</i>
Strain	MC4100	MC4100	RM102	RM102

FIG. 5. Fnr controls the anaerobic expression of *moa* at the distal promoter. The plasmids were present in the strains as indicated. All growths were performed under anaerobic conditions at 37°C in LB medium with 125 µg of ampicillin/ml. β-Galactosidase activity is expressed in Miller units (24).

from the consensus sequence in only one base. Baker and Boxer previously reported that the *fnr* gene is not required for the anaerobic expression of *moa* (3). In our earlier studies, single-copy *moaB-lacZ* chromosomal translational gene fusions which contained the intact promoter region upstream of the *moa* operon were used. Since the previous results reflected the combined effects of both promoters and the anaerobic enhancement of *moa* can be studied directly using pEM220, we decided to reinvestigate the possible involvement of *fnr* in *moa* regulation.

During anaerobic growth high levels of expression were ob-

served from plasmid pEM101 (both promoters) when present in the parental strain. Plasmid pEM220 (distal promoter only) also displayed a high level of expression, although slightly lower than that for pEM101. When expression from pEM101 was examined in the *fnr* strain, a small reduction to about 70% of the parental strain level was obtained (Fig. 5). This is comparable to the results reported previously by Baker and Boxer (3) for the chromosomal fusions, which were not considered significant at the time. However, when expression of pEM220 was examined in the *fnr* strain, a large reduction (60-fold or more) was observed. It is clear, therefore, that full anaerobic expression of *moa* requires the Fnr protein. This effect is mediated through the distal promoter. The full *fnr* dependency is apparent only when expression from the distal promoter (pEM220) is examined in isolation.

DISCUSSION

Molybdate addition to the growth medium brings about an activation of *moa* expression during both anaerobic and aerobic growth of some four- to fivefold over that found in its absence. Molybdate acts as a major positive regulator of *moa* and its action requires the ModE protein. The molybdate activation of *moa*, however, is revealed only in a molybdenum cofactor-deficient background, since in molybdenum cofactor-sufficient strains, *moa* is effectively repressed. ModE is a major regulator of *moa*, since in *modE* strains *moa* expression levels remain low regardless of growth conditions or molybdate or molybdenum-cofactor availability. A summary of our findings regarding the regulation of *moa* is shown in Fig. 6.

ModE has been best characterized as a transcriptional repressor for the *modABCD* operon (8, 38). Although molybdate-ModE has been shown to mediate enhanced expression of *dmsA* under certain conditions, its mode of action is thought to be indirect, namely that of a transcriptional repressor of a direct repressor of *dmsA* (21). Recently, molybdate-ModE has been proposed as a secondary transcriptional activator of both *hyc* and *narG* (34). In the case of *hyc*, the ModE-molybdate complex is thought to bind about 95 bp upstream of the main formate-dependent positive transcriptional regulator, Fh1A. In

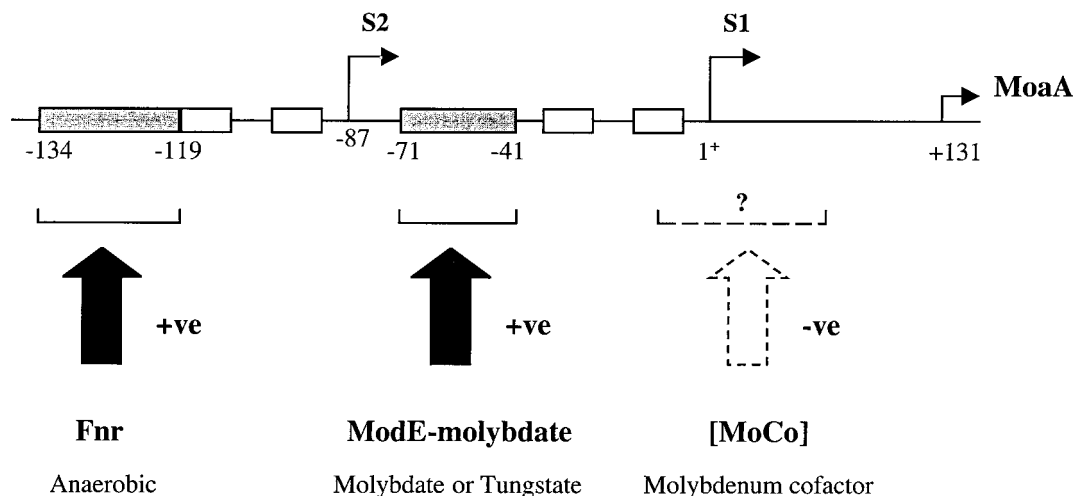


FIG. 6. Regulation of *moa* expression. The diagram is a schematic representation of the DNA sequence upstream of *moaA* indicating the two transcriptional start sites, S1 and S2, along with their $\sigma^{70} - 10$ and -35 regions, which are shown as open boxes. Numbering of the DNA sequence is with reference to the S1 transcription start site as +1. Anaerobic regulation is effected by the positive global regulator Fnr, the putative binding site for which is shown as the filled box (-134 to -119) upstream of S2. The positive regulation by molybdate is effected by the ModE-molybdate complex, which acts at the filled boxed region (-71 to -41) upstream of S1. Tungstate is also capable of effecting this regulation. Molybdenum cofactor (MoCo)-mediated negative regulation appears to be exerted at the S1 transcription start site. The mechanism for this regulation is presently unknown. Tungstate in the growth medium is able to alleviate the molybdenum cofactor repression.

the regulation of *narG*, ModE-molybdate does not bind directly to the *narG* promoter region but binds to the *narK-narXL* intragenic region. This suggests that its effect on *narG* is mediated via the *narXL* operon (34). McNicholas et al. (22), by employing DNaseI-protection experiments, defined the ModE binding site at *moa* and we have confirmed this by gel shift analysis (data not shown). The ModE-molybdate binding site at *moa* is centered at -57.5 with respect to the proximal *moa* transcription start site and, therefore, is adjacent to the -35 region of this promoter. The magnitude of the ModE-dependent molybdate activation of *moa*, the direct binding of ModE to the proximal *moa* promoter region, and the persistent low levels of *moa* expression in *modE* strains point to molybdate-ModE being a major direct, positive transcriptional regulator of this operon. Previously, McNicholas et al. (22) reported, from analysis of a molybdenum cofactor-sufficient strain, a twofold, ModE-dependent but molybdate-independent enhancement of *moa* expression.

The molybdate regulation of both the *hyc* and *narG* operons also displays a ModE-independent, MoeA-dependent component (10). There is no evidence for such regulation of *moa*, since all the molybdate effects appear to be totally dependent upon ModE.

Enhanced expression of *moa* under anaerobic growth conditions has been reported previously (3). This effect, like the molybdate effect, is only fully apparent in strains that lack functional molybdenum cofactor. Only a modest twofold enhancement of *moa* to a very low level can be seen in molybdenum cofactor-sufficient strains. In molybdenum cofactor-deficient strains also lacking ModE activity, anaerobiosis elevates *moa* expression about eightfold over the aerobic levels but only to a relatively modest level (~ 800 Miller units). However, anaerobic growth of such strains with a functional ModE results in a further fourfold increase in *moa* expression (Fig. 1). Under aerobic growth conditions, the ModE-dependent molybdate activation effects a 10-fold or so enhancement to an intermediate (1,100 units) level of expression. ModE-molybdate, therefore, is required for full *moa* expression.

The molybdate- and anaerobic-dependent enhancements of *moa* appear independent and distinct. This interpretation is consistent with our finding of two promoters at *moa*. Analysis of expression from the individual *moa* promoters clearly showed that the global anaerobic transcriptional regulator, Fnr, acts positively at the highly active, distal *moa* promoter. Full expression of *moa*, during anaerobic growth with high molybdate availability, requires the action of the two positive regulators, ModE and Fnr. ModE and Fnr mediate their effects specifically and distinctly at the proximal and distal promoters, respectively. The promoter subclones containing only the proximal promoter generally displayed poor expression. Given the close proximity of the transcriptional start sites, it is likely that the proximal promoter region overlaps the distal transcription start. Further work is required to illuminate how expression from each of the two promoters is related to that of the other.

The molybdate and anaerobic control of *moa* described above is apparent only in strains that are molybdenum cofactor-deficient. That molybdenum cofactor sufficiency leads to tight *moa* repression was identified by Baker and Boxer (3). It is abundantly clear from the present work that the molybdenum cofactor-dependent repression of *moa* is dominant over both the molybdate and anaerobic effects. The mechanism of this repression has not been established but it appears from the analysis of the promoter subclones that the repression is mediated at the proximal promoter region. Experiments designed

to isolate a molybdenum cofactor-dependent repressor are currently under way.

A large enhancement of *moa* transcription is observed when tungstate is present in the growth medium. ModE binds tungstate in a virtually identical manner to molybdate, and ModE-tungstate can bind to the *modA* promoter (2). In strains that are deficient in molybdenum cofactor it is clear that tungstate can also bring about a ModE-dependent activation of *moa*. Under these conditions, tungstate and molybdate are equivalent with respect to their positive effect on *moa* expression.

In strains genetically able to synthesize molybdenum cofactor, the effect of tungstate in the growth medium is more complicated. Under these conditions tungstate leads to a full derepression of *moa* via a mechanism that is independent of ModE. This demonstrates a second ModE-independent tungstate effect on *moa* which is dependent upon the capacity of the bacterium to synthesize active molybdenum cofactor in the absence of tungstate. It is known that tungsten can substitute for molybdenum in the molybdenum cofactor to form a biologically inactive derivative (16). We propose that the tungsten cofactor derivative cannot mediate *moa* repression. This gives a plausible explanation for this tungstate effect on *moa*. Either the tungsten-cofactor derivative is unable to bind to the putative molybdenum cofactor-repressor, or the tungsten cofactor-repressor complex cannot productively bind to the proximal transcriptional start region.

The *moa* operon encodes the enzymes required for the first dedicated step of molybdopterin synthesis. It is appropriate, therefore, that this locus should be subject to transcriptional control, and the expression of *moa* is indeed highly regulated. The regulation is generally consistent with what is known concerning molybdoenzyme biosynthesis in *E. coli*. The positive molybdate control should ensure that molybdopterin biosynthesis is down regulated under conditions of limited molybdate availability. When there is sufficient molybdenum cofactor, further molybdopterin synthesis would not be required. Almost all of the *E. coli* molybdoenzymes are present predominantly during anaerobic growth. The anaerobic molybdoenzymes nitrate reductases and DMSO reductase under fully induced conditions are of high abundance in the bacterium. This points to a relatively high requirement for the molybdenum cofactor during anaerobiosis. The anaerobic regulation of *moa*, therefore, acts to ensure appropriate molybdopterin availability for the synthesis of these metabolically important enzymes.

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