The *modE* gene product mediates molybdenum-dependent expression of genes for the high-affinity molybdate transporter and *modG* in *Azotobacter vinelandii*

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The *Azotobacter vinelandii* *mod* locus, which is involved in high-affinity molybdate transport and the early events in Mo metabolism, consists of two divergently transcribed operons, *modG* and *modEABC*. *modA*, *modB* and *modC* encode the components of the high-affinity molybdate transporter, and *modG* encodes a Mo-binding protein. High concentrations of Mo repressed transcription of both operons. The *modEABC* operon was also repressed by tungstate and to a lesser extent by vanadate. *modE*, the first gene in the *modEABC* operon, controlled the Mo-dependent transcription of both operons. It was not involved in the metal regulation of alternative nitrogenase gene expression. Although a *modE* mutant constitutively expressed genes encoding the molybdate transporter, it had a reduced rate of Mo accumulation.

Keywords: molybdenum, molybdate, regulation and transport, vanadium, nitrogenase

INTRODUCTION

Mo is required for the synthesis of the molybdopterin-based cofactors (Mo-co) of about 20 redox enzymes, and the Fe₇S₈Mo cofactor (FeMoco) of Mo nitrogenase (Dean *et al.*, 1993; Kim & Rees, 1992; Stiefel, 1993). Mo is present at low concentrations and neutral pH in the environment as the oxyanion molybdate, MoO₄²⁻. Bacteria employ a high-affinity ATP-binding cassette (ABC) transporter to efficiently scavenge traces of molybdate from the environment (Fleishmann *et al.*, 1995; Maupin-Furlow *et al.*, 1995; Mouncey *et al.*, 1995; Rech *et al.*, 1995; Wang *et al.*, 1993). In *Azotobacter vinelandii*, a distinct low-affinity transporter has been shown to be responsible for molybdate acquisition when the concentration of molybdate is high (> 10 µM) (Mouncey *et al.*, 1995; Premakumar *et al.*, 1996). *Escherichia coli* grown in low sulfate concentrations also transports molybdate by the sulfate transporter, though it does so much less efficiently than with the high-affinity molybdate transporter (Rosentel *et al.*, 1995).

There are only a few well-documented cases of transcriptional regulation by Mo among the genes known to be

involved in Mo transport and metabolism. The first concerns genes for molybdate transport. The high-affinity molybdate transporter is encoded by three genes, *modA*, *modB* and *modC*, which are located in a single operon. Transcription of *E. coli modA* and *modC*, which respectively encode the periplasmic molybdate-binding protein and the inner-membrane ATP-binding protein of the transporter, is repressed by high concentrations of molybdate (Miller *et al.*, 1987; Rech *et al.*, 1995; Surin *et al.*, 1985). Low-affinity molybdate transport in *A. vinelandii* is repressed in a mutant strain that expresses the high-affinity system constitutively, and it consequently accumulates Mo by this system, even when the concentration of Mo is high (Mouncey *et al.*, 1995). Secondly, Mo transcriptionally regulates the reciprocal expression of the Mo and alternative nitrogenases in *A. vinelandii* (Jacobitz & Bishop, 1992; Jacobson *et al.*, 1986; Luque & Pau, 1991; Walmsley *et al.*, 1994). In addition to these cases, Mo has also been implicated in the regulation of *E. coli narG* and *frdA* expression through the involvement of the *narX* gene product (Stewart, 1993). A putative regulatory motif has been identified in the promoter of the *E. coli modABCD* operon and the regulatory system responsible for the control of Mo transport is currently being elucidated (Maupin-Furlow *et al.*, 1995; Rech *et al.*, 1995). *modR* (also called *modE*) has recently been shown to be involved in the transcriptional regulation of the *E. coli modABCD* operon (Walkenhorst *et al.*, 1995).

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The products of two genes in the *A. vinelandii* *mod* locus, *modE* and *modG*, contain a tandem repeat of a sequence homologous to the molybdopterin-binding proteins, Mop, of *Clostridium pasteurianum* (Mouncey *et al.*, 1995). Whereas the Mop-like tandem repeat constitutes the whole of ModG, it is restricted to the C-terminal half of ModE. *modE* is the first gene in the *modEABC* operon which is transcribed divergently from *modG*. Mutational analysis of *modE* and *modG* indicated that their gene products are not involved in molybdate transport, but they may have a role in the homeostasis and early metabolism of Mo (Mouncey *et al.*, 1995). Genes homologous to *A. vinelandii* *modE* are present in *E. coli* (accession nos U07867 and U27192), *Haemophilus influenzae* (amino acid position numbers 491878–4919447, TIGR database; Fleishmann *et al.*, 1995) and *Rhodobacter capsulatus* (Wang *et al.*, 1993). In *E. coli* and *H. influenzae*, a single *modE*-like gene is transcribed divergently from the *modABCD* operon, while in *R. capsulatus* there are two copies of *modE*-like genes that are called *mopA* and *mopB*. *mopA* is cotranscribed with *modABCD*, like *modE* in *A. vinelandii*. *mopB* is divergently transcribed from the *R. capsulatus* *mopAmodABCD* operon. The alternative nitrogenase of *R. capsulatus* is not repressed by Mo in a double *mopA mopB* mutant as it is in the wild-type strain. This suggests that both *mopA* and *mopB* are involved in Mo-dependent regulation of the alternative nitrogenase (Wang *et al.*, 1993). In this paper, we describe the metal-dependent regulation of *A. vinelandii* *modEABC* and *modG* operons, and show that the regulation of both is mediated by the product of *modE*. However, the Mo-dependent expression of alternative nitrogenase structural genes was independent of the *modE* control, and the Mo accumulation of a *modE* mutant was not that predicted if the product of *modE* was solely involved in the transcriptional regulation of Mo transport.

METHODS

Bacterial strains and growth conditions. *E. coli* strains were grown at 37 °C in Luria Bertani (LB) medium (Miller, 1972) and *A. vinelandii* was grown at 30 °C in modified Burks medium (Pau *et al.*, 1989). The latter was made Mo-free by treatment with 8-hydroxyquinoline followed by continuous liquid-liquid extraction with 1,2-dichloroethane as described previously (Mouncey *et al.*, 1995). Analytical grade chemicals, and plastic or glassware soaked for >16 h in a surface-active cleaning agent (2.5 %, v/v, Decon 90; Decon Laboratories), were used to reduce contamination by transition metals. Highly purified sodium molybdate, vanadium pentoxide and sodium tungstate were added to the media as required (Puratronic grade, <10 p.p.m. of total metal impurities; Johnson Matthey Chemicals). Sodium nitrate (10 mM) or ammonium acetate (20 mM) were added as necessary. Antibiotics were added at the following final concentrations: carbenicillin, 200 µg ml⁻¹ (*E. coli*); kanamycin (Km), 2 µg ml⁻¹ (*A. vinelandii*) and 25 µg ml⁻¹ (*E. coli*); spectinomycin, 2 µg ml⁻¹ (*A. vinelandii*) and 25 µg ml⁻¹ (*E. coli*). Strains used are shown in Table 1.

DNA manipulations and transformation. Standard procedures were used, as described in Sambrook *et al.* (1987). Chromosomal mutants in *mod* genes were constructed by the introduction of either the promoterless *lacZ*-Km-resistance cassette from pKOK6.1 or the spectinomycin-resistance cassette (Ω) from

pHP45 into *mod* genes cloned in a plasmid vector, followed by the recombination of the mutated gene into the chromosome of the wild-type strain UW136 (Mouncey *et al.*, 1995). pKOK6.1 has stop codons in all three reading frames upstream of the *lacZ* gene. *A. vinelandii* cells were made competent by growth on solid medium lacking Mo and V, and transformed by the method of Page & von Tigerstrom (1979). The *vnfH-lacZ modE- Ω* and *anfH-lacZ modE- Ω* double mutants were constructed by transforming the *vnfH-lacZ* and *anfH-lacZ* mutants MV107 and MV143 (Walmsley *et al.*, 1994) with plasmid pLAM86441, which contains *modE- Ω* (Mouncey *et al.*, 1995). *modA-KOK anfH-lacZ*, *modB-KOK anfH-lacZ* and *modC-KOK anfH-lacZ* double mutants were constructed by transforming strain MV143 with the plasmids pNM144, pNM206 and pNM238, which respectively contain *modA*, *modB* and *modC*, with the cassette from pKOK6.1 inserted in the orientation where the *lacZ* gene is not transcribed (Mouncey *et al.*, 1995).

In-frame N-terminal *modE* deletion mutation. Two DNA fragments with complementary 18 bp 5' overhangs on either side of the sequence encoding the N-terminal domain of ModE were first synthesized by PCR using Vent polymerase (New England Biolabs). For each reaction, 2.5 pmol primers and 100 ng template were used. The DNA was denatured for 1 min at 95 °C, the primers were annealed at 54 °C for 1 min, and the products were extended over 2 min at 72 °C. The 644 bp fragment containing DNA that encodes the promoter region and start codon of *modE* was obtained using primers 94018 (5'-TGAGCGGATAACAATTTTCACACAGGAAACAGCTATGACCATG-3') and 94110 (5'-GTTGCGCGCGCTGGTCTTCATCGTCTGGTAGCCTGC-3') and pNM631 as a template. pNM631 contains *modE* and the sequence up to the *BclI* site in the 5' end of *modA*. The 1636 bp fragment with a sequence that encodes the C-terminal end of *modE* was obtained using primers 94108 (5'-AAGACCAGCGCGCGCAACACAGTTTCGCCGGCATCG-3') and 94017 (5'-GTTTTCCAGTCACGACGTTGTAAAACGACGGCCAG-3') and pMac75 as a template. The 18 bp 5' overhangs are underlined above. The plasmids used are described by Mouncey *et al.* (1995). The two PCR products were purified using Wizard PCR purification columns (Promega), annealed, and a single cycle of a PCR reaction was performed in the absence of primers. Primers 94018 and 94017 were then added and the PCR reaction was allowed to proceed for a further 19 cycles to give a 2162 bp product which contained the in-frame N-terminal deletion. The reaction was performed as above, except that the annealing temperature was 40 °C. The product was digested with *PstI* and cloned into *PstI*-digested pTZ18 to give plasmid pNM796. The sequence and in-frame deletion of the cloned DNA was confirmed using the chain-termination DNA sequencing method with [³⁵S]dATP α S, 7-deaza-dGTP and a modified T7 polymerase (Sequenase, series 2; Amersham). DNA from plasmid pNM796 was then separately mixed with the DNA from plasmid pNM239, which contains the KOK cassette inserted into *modC* in the Lac⁺ orientation. The DNA mixture was then used to transform *A. vinelandii* strain RP20, which possesses a *modE:: Ω* insertion mutation. Transformants were selected on the basis of their Km resistance and checked for the spectinomycin-sensitive phenotype. The *modE:: Ω* mutation in the parent strain was replaced by the region of plasmid pNM796 which contains the *modE* N-terminal deletion and its flanking DNA, and the KOK cassette was inserted into *modC*. The resulting strain, RP55, lacks the sequence encoding the N-terminal domain of *modE*.

Enzyme assay. β -Galactosidase activity of whole cells permeabilized with chloroform and SDS was determined by the hydrolysis of ONPG at 30 °C as previously described by Miller

Table 1. Bacterial strains used

Bacterial strain	Genotype	Reference
<i>Azotobacter vinelandii</i>		
UW136	<i>rif^r</i>	Bishop & Brill (1977)
MV107	<i>vnfH7::Tn5-B21</i>	Walmsley <i>et al.</i> (1994)
MV143	<i>rif^r anfH1::Tn5-B21</i>	Walmsley <i>et al.</i> (1994)
RP2	<i>modE::'lacZ-Km(Lac⁺)</i> , $\phi(modE-'lacZ-Km)$ (Hyb) from pNM149	Mouncey <i>et al.</i> (1995)
RP4	<i>modA::'lacZ-Km(Lac⁺)</i> , $\phi(modA-'lacZ-Km)$ (Hyb) from pNM145	Mouncey <i>et al.</i> (1995)
RP6	<i>modB::'lacZ-Km(Lac⁺)</i> , $\phi(modB-'lacZ-Km)$ (Hyb) from pNM207	Mouncey <i>et al.</i> (1995)
RP8	<i>modC::'lacZ-Km(Lac⁺)</i> , $\phi(modC-'lacZ-Km)$ (Hyb) from pNM239	Mouncey <i>et al.</i> (1995)
RP10	<i>modA::'lacZ-Km(Lac⁺)</i> , $\phi(modA-'lacZ-Km)$ (Hyb) from pNM145, <i>modE::Ω</i> from pLAM86441	This work
RP12	<i>modG::'lacZ-Km(Lac⁺)</i> , $\phi(modG-'lacZ-Km)$ (Hyb) from pLAM86682	Mouncey <i>et al.</i> (1995)
RP14	<i>modG::'lacZ-Km(Lac⁺)</i> , $\phi(modG-'lacZ-Km)$ (Hyb) from pLAM86682, <i>modE::Ω</i> from pLAM86441	Mouncey <i>et al.</i> (1995)
RP20	<i>modE::Ω</i> from pLAM86441	Mouncey <i>et al.</i> (1995)
RP21	<i>vnfH7::Tn5-B21</i> , <i>modE::Ω</i> from pLAM86441	This work
RP23	<i>anfH1::Tn5-B21</i> , <i>modE::Ω</i> from pLAM86441	This work
RP36	<i>anfH1::Tn5-B21</i> , <i>modA::'lacZ-Km (Lac⁻)</i> , $\phi(modA-'lacZ-Km)$ (Hyb) from pNM144	This work
RP37	<i>anfH1::Tn5-B21</i> , <i>modB::'lacZ-Km (Lac⁻)</i> , $\phi(modB-'lacZ-Km)$ (Hyb) from pNM206	This work
RP38	<i>anfH1::Tn5-B21</i> , <i>modC::'lacZ-Km (Lac⁻)</i> , $\phi(modC-'lacZ-Km)$ (Hyb) from pNM238	This work
RP55	<i>modE</i> N-terminal deletion from pNM796, <i>modC::'lacZ-Km (Lac⁺)</i> , $\phi(modC-'lacZ-Km)$ (Hyb) from pNM239	This work
RP61	<i>modG::Km-'lacZ (Lac⁻)</i> , $\phi(modG-Km-'lacZ)$ (Hyb) from pLAM86681, <i>modE::Ω</i> from pLAM86441, <i>vnfH7::Tn5-B21</i>	This work
RP62	<i>modG::'lacZ-Km (Lac⁻)</i> , $\phi(modG-'lacZ-Km)$ (Hyb) from pLAM86681, <i>modE::Ω</i> from pLAM86441, <i>anfH1::Tn5-B21</i>	This work
<i>Escherichia coli</i>		
71-18	$\Delta(lac-proAB)$ <i>thi supE</i> (F' <i>proAB lacI^a</i> ZAM15)	Yanisch-Perron <i>et al.</i> (1985)

(1972). Each assay was performed in triplicate and the results are the means of assays from three separate cultures. The maximum standard deviation was 12% of any mean value.

Western blot analysis. After overnight growth in medium containing different concentrations of Mo, *A. vinelandii* cells were harvested by centrifugation and resuspended to give equal cell densities ($OD_{600} = 1$). A 100 μ l quantity of the resuspended cells was mixed with 100 μ l 2 $\times$ SDS sample buffer, boiled, and then centrifuged to remove cell debris. Samples (10 μ l) of the extracted proteins were separated by SDS-PAGE on 12.5% (w/v) polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore) by semi-dry electrotransfer in 10 mM CAPS and 10% (v/v) methanol. The periplasmic molybdate-binding protein (ModA) was detected immunochemically with the enhanced chemiluminescence Western blotting system (ECL; Amersham) using polyclonal rabbit antiserum against purified *A. vinelandii* molybdate-binding protein (1:2000 dilution) and a goat anti-rabbit horseradish-peroxidase-labelled second antibody (1:10000 dilution) (Mouncey *et al.*, 1995). The density of the luminescence signals was quantified from the digitized film image using the NIH Image 1.55 program. There was a linear relationship between density and protein concentration from 0 to 125 ng purified molybdate-binding protein.

⁹⁹Mo accumulation. ⁹⁹Mo was recovered from spent ⁹⁹Mo/^{99m}Tc radionuclide generators by elution with disodium

hydrogen phosphate (Baldock *et al.*, 1994). ⁹⁹Mo was added to 2.5 ml cells ($OD_{600} = 1$) in modified Burks medium at 30 °C. At timed intervals, 500 μ l aliquots of cells were removed, filtered with 2.5 mm diameter, 0.45 μ m pore-size, HAWP-type membrane filters (Millipore) on a multiple filter vacuum manifold, and washed with 10 ml 150 mM NaCl and 30 mM potassium phosphate, pH 7.2. The filters were dried and radioactivity was measured in a liquid scintillation counter. The rates of ⁹⁹Mo accumulation were linear over 20 min.

RESULTS

The *modEABC* and *modG* operons are regulated by Mo, W and V

Strains with chromosomal *lacZ* fusions in *modA*, *modB* and *modC* showed maximal β -galactosidase activity when they were grown in medium that contained less than 10 nM Mo, and the activity was progressively reduced as the concentration of Mo increased above this (Fig. 1). The Mo concentration required for the half-maximal activity was 100 nM, the same as that required for the half-maximal activity of an *E. coli modC-lacZ* fusion strain (Miller *et al.*, 1987). When the *modB* fusion strain was grown in >1 μ M Mo, it showed less than 5% of the maximal β -galactosidase activity (Fig. 1). At between 1

Fig. 1. β -Galactosidase activities of the *A. vinelandii* *modE-lacZ* mutant RP2 (■), *modA-lacZ* mutant RP4 (○), *modB-lacZ* mutant RP6 (●), *modC-lacZ* mutant RP8 (◇), *modG-lacZ* mutant RP12 (▲), *modA-lacZ modE-Ω* double mutant RP10 (□) and *modG-lacZ modE-Ω* double mutant RP14 (◆). The strains were grown overnight in different concentrations of Mo and in the presence of 10 mM NaNO_3 . One hundred per cent activity corresponds to 122 Miller units for *modE-lacZ*, 125 Miller units for *modA-lacZ*, 315 Miller units for *modB-lacZ*, 271 Miller units for *modC-lacZ* and 60 Miller units for *modG-lacZ*.

Fig. 2. Western blot analysis of the periplasmic molybdate-binding protein ModA from the wild-type *A. vinelandii* strain UW136 grown in different concentrations of Mo and different nitrogen sources. Lanes: 1, 31 ng purified periplasmic molybdate-binding protein ModA; 2 and 3, strain UW136 grown in the absence of a source of fixed nitrogen in either the absence of Mo or the presence of 1 mM Mo, respectively; 4 and 5, strain UW136 grown in the presence of 1 mM nitrate in either the absence of Mo or the presence of 1 mM Mo, respectively; 6 and 7, strain UW136 grown in the presence of 20 mM NH_4^+ in either the absence of Mo or the presence of 1 mM Mo, respectively.

and 100 μM Mo, somewhat higher activities were found in the *modA-lacZ* fusion strain, which decreased to approximately 12% of the maximal activity at 1 mM Mo.

Mo-dependent repression of the Mo transporter was also observed at the level of protein synthesis in the wild-type strain by immunochemical detection of ModA, the periplasmic molybdate-binding protein. The amount of ModA in cells grown in 1 mM Mo was 4–8% of that found in cells grown in the absence of Mo (Fig. 2). This

Fig. 3. β -Galactosidase activity of a *modA-lacZ* fusion strain RP4 grown in increasing concentrations of Mo (■), V (○) and W (▲). Cells were grown overnight at 30 °C in the presence of NH_4^+ . One hundred per cent activity corresponds to 98 Miller units in Mo, 100.2 Miller units in V and 94.6 Miller units in W.

was similar to the repression of β -galactosidase activity observed in *modA-* and *modB-lacZ* fusion strains.

The β -galactosidase activity of a *modG-lacZ* fusion strain was also regulated by Mo (Fig. 1) in a similar response to increasing Mo concentration as the *modABC-lacZ* fusion strains, suggesting that the transcription of both operons may be subject to the same Mo-dependent control mechanism.

In *A. vinelandii*, W can replace Mo to produce an inactive nitrogenase, or one with reduced activity which contains both Mo- and W-containing cofactors (Benemann *et al.*, 1973; Hales & Case, 1987). V is incorporated into the cofactor of the *A. vinelandii* V nitrogenase, where it occupies the position equivalent to that occupied by Mo in the Mo nitrogenase cofactor (Eady, 1995). Because Mo, W and V can be incorporated into the cofactors of *A. vinelandii* nitrogenases, it was of interest to determine whether high-affinity Mo transport was regulated by W and V as well as Mo. The β -galactosidase activity of a *modA-lacZ* fusion strain showed that W was as effective as Mo in repressing *modA-lacZ* transcription (Fig. 3). When the *modA-lacZ* fusion strain was grown in the presence of V, β -galactosidase activity decreased to about 50% of its maximum value as the concentration of V increased to 100 μM , which suggests that the Mo sensor may also be able to sense V as effectively as Mo and W up to this concentration. The response of β -galactosidase activity to V concentration above 100 μM differed from that observed with Mo and W, for it increased or remained constant.

***modE* is involved in Mo regulation of the *modEABC* operon and the *modG* gene**

In contrast to the *modA-*, *modB-* and *modC-lacZ* fusion strains described above, a *modE-lacZ* insertion/deletion fusion strain showed no significant change in β -

Fig. 4. β -Galactosidase activities of *vnfH-lacZ* and *anfH-lacZ* fusion strains in the absence and presence of Mo. Cells were derepressed for nitrogenase for 6 h at 30 °C in either the absence (-) or the presence (+) of 100 nM Mo. Strains: MV107, *vnfH-lacZ* (■); RP21, *vnfH-lacZ modE-Ω* (▨); RP61, *vnfH-lacZ modE-Ω modG-KOK* (▩); MV143, *anfH-lacZ* (■); RP23, *anfH-lacZ modE-Ω* (▨); RP62, *anfH-lacZ modE-Ω modG-KOK* (▩). One hundred per cent activity corresponds to 4607 Miller units for *vnfH-lacZ* and 5013 Miller units for *anfH-lacZ*.

galactosidase activity with increasing Mo concentration (Fig. 1). Transcription of a *modA-lacZ* fusion was also constitutive in a strain (strain RP10) with a non-polar insertion/deletion mutation in *modE* (Fig. 1). Further, a non-polar *modE* insertion/deletion mutation relieved the repression of *modG-lacZ* transcription by Mo in strain RP14 (Fig. 1). A mutant (strain RP55) was constructed which contained a *modC-lacZ* fusion as well as an in-frame deletion in *modE*, which removes the sequence encoding the N-terminal domain of ModE. Like the *modE* insertion/deletion mutants, the β -galactosidase activities of this double mutant were constitutive with respect to increasing Mo concentration (data not shown). These results indicate that the *modE* gene product mediates the Mo-dependent repression of both of the divergently transcribed operons in the *A. vinelandii mod* locus.

ModE is not involved in the regulation of alternative nitrogenases by Mo

In *R. capsulatus*, the two ModE-like gene products MopA and MopB regulate the Mo repression of the alternative nitrogenase. Since *A. vinelandii* ModE mediated the Mo-dependent repression of both *modEABC* and *modG*, it was of interest to see whether it also regulated the repression of the alternative nitrogenases. *vnfH* and *anfH* encode the Fe proteins of the V nitrogenase and nitrogenase-3, respectively (Joerger *et al.*, 1989, 1990). Repression is observed with Mo concentrations above 50 nM (Jacobson *et al.*, 1986). Comparison of the β -galactosidase activities of *vnfH-lacZ* and *anfH-lacZ* fusion strains (MV107 and MV143) with the activities of strains which also contained a mutation in *modE* (strains RP21 and RP23) showed that ModE did not mediate the Mo-dependent repression of *vnfH-lacZ* and *anfH-lacZ* (Fig. 4). Repression of *vnfH* and *anfH* by Mo was also observed in strains (RP61 and RP62) with mutations in both *modE* and *modG* (Fig. 4). Further, Mo-dependent repression of *vnfH* and *anfH* was not affected by deletions in *modA*, *modB* or *modC* (strains RP36, RP37 and RP38) (data not shown).

The rates of Mo accumulation in a *modE* mutant

Since ModE mediates the Mo-dependent repression of the molybdate transporter, a non-polar *modE* mutant would be expected to show maximal rates of Mo transport since the high affinity Mo-transport system is being synthesized constitutively. Further, the nitrate reductase activity of the non-polar *modE* mutant is similar to the wild-type strain, except above 100 μ M Mo. The insertion/deletion mutation in *modE* in strain RP20 is non-polar since this strain synthesizes wild-type levels of the periplasmic molybdate-binding protein, the product of *modA* (Mouncey *et al.*, 1995). Nevertheless, at ^{99}Mo concentrations > 10 nM, the rates of ^{99}Mo accumulation in this mutant were significantly lower than in the wild-type strain (Fig. 5a). Since accumulation reflects the rate of influx, efflux, storage and conversion to cofactors, it was also of interest to compare the rate of ^{99}Mo accumulation in cells grown in different nitrogen sources. Mo is utilized for the synthesis of the Mo nitrogenase or nitrate reductase cofactors in the absence of a source of fixed nitrogen or in the presence of nitrate, respectively. In the presence of NH_4^+ , neither cofactor is produced. Mo is then transported into the cell and stored. Although ModA synthesis is not repressed by the presence of NH_4^+ (Fig. 2), the initial rate of ^{99}Mo accumulation was much lower in cells grown in NH_4^+ than in the absence of a source of fixed nitrogen or in the presence of nitrate (Fig. 5b).

DISCUSSION

The results show that *modE* mediates the Mo-dependent regulation of transcription of the two operons in the *mod* locus of *A. vinelandii*. Unlike the case in *E. coli*, where the regulatory gene is encoded in a separate operon, in *A. vinelandii* the regulatory gene is in the same operon as the genes encoding the components of the transporter (Luque *et al.*, 1993; Walkenhorst *et al.*, 1995). *A. vinelandii modE* is presumably autoregulated since the intergenic region between *modE* and *modA* is short and there is no evidence for the presence of a promoter between *modE* and *modA*, or within the 3' end of *modE*.

Fig. 5. (a) Rates of ^{99}Mo accumulation in the wild-type *A. vinelandii* strain UW136 ($\square$) and a *modE* mutant, RP20 ($\triangle$), grown in increasing concentrations of Mo. Cells were grown overnight in the presence of 10 mM NaNO_3 and 100 nM Mo and washed with Mo-free media. The rates of ^{99}Mo accumulation were determined from the amounts accumulated at 0, 5, 11 and 20 min. (b) ^{99}Mo accumulation of *A. vinelandii* wild-type strain UW136 grown in different media with different nitrogen sources. Cells were grown overnight at 30 °C in the presence of 20 mM NH_4Ac ($\circ$) or 10 mM NaNO_3 ($\blacksquare$) or in the absence of a source of fixed nitrogen ($\bullet$). Cells were washed and ^{99}Mo was added at a final concentration of 1.6 μM .

Genes encoding proteins involved in the regulation of transport as well as in the early steps of substrate metabolism are also found in genetic loci that encode the transporters of other oxyanions, such as phosphate, sulfate and arsenate. In the Pst phosphate-transport system of *E. coli*, a regulatory and metabolic role are combined in the product of *phoU*, which has no role in transport (Hryniewicz *et al.*, 1990; Ji & Silver, 1992; Laudenbach & Grossman, 1991; Rosen *et al.*, 1995; Steed & Wanner, 1993; Wanner, 1993). In the sulfate- and arsenate-transport loci, regulatory proteins and sulfate or arsenate reductases are encoded by separate genes (Hryniewicz *et al.*, 1990; Laudenbach & Grossman, 1991; Ji & Silver, 1992; Rosen *et al.*, 1995). ModE is distinctive in that the

two halves of the molecule are very different, as shown by sequence homology, in which the C-terminal half is equivalent to ModG, or a fused dimer of *C. pasteurianum* Mop (Mouncey *et al.*, 1995). This difference receives further support from the predicted secondary structure of ModE, which shows that the N-terminal half consists almost entirely of α -helices, while the C-terminal half consists of β -sheets (data not shown). Since a *modG* mutation does not affect Mo-dependent regulation of the *modEABC* operon, the *modG* protein must have an independent non-regulatory role or have lost its ability to regulate. This suggests that the N-terminal domain of *modE* has a regulatory role. It is not known whether the regulatory function requires interaction between the N- and C-terminal domains, or the N-terminal domain alone.

In *E. coli*, expression of a *modA-lacZ* fusion introduced into the chromosome could only be observed in strains with a mutation in one of the Mo-transporter genes (Rech *et al.*, 1995). This contrasts with the demonstration of ModA synthesis in wild-type *A. vinelandii* cells, though the disparity may be due to the concentration of residual Mo in the *E. coli* culture medium ($<1 \mu\text{M}$), which may have been sufficient to repress transcription of the *mod* operon to low levels unless Mo transport is impaired. The *E. coli* phosphate transporter is involved in the negative control of phosphate-dependent genes (Cox *et al.*, 1989). The observation that Mo represses *modEABC* transcription in *A. vinelandii* strains with an inactivated high-affinity transporter raises the question of whether Mo is sensed intra- or extracellularly, and whether there are components in the regulatory system other than ModE. If the *A. vinelandii* Mo sensor senses intracellular Mo, then it must sense low levels which enter the cell when the high-affinity transporter is inactivated. Our results also show that the repression of the *A. vinelandii* alternative nitrogenase structural gene transcription is independent of *modE* control, and can take place in cells with a non-functional high-affinity Mo transporter. ModE is therefore not a global regulator of Mo-controlled genes in *A. vinelandii*. This was unexpected, since deletion of the two *modE*-like genes (*mopA* and *mopB*) in *R. capsulatus* allowed the alternative nitrogenase to be expressed in the presence of Mo. In *E. coli*, mutations located outside the *mod* locus have been shown to affect Mo-dependent regulation (Hemschemeier *et al.*, 1991; Lee *et al.*, 1990). *A. vinelandii* may likewise possess more than one Mo-dependent regulatory system.

Demonstration of *A. vinelandii* ModA synthesis in the presence of NH_4^+ is in accord with early observations which showed that Mo uptake in *A. vinelandii* was not repressed when the cells were grown in media containing a source of fixed nitrogen, as it was in *Klebsiella pneumoniae* and *C. pasteurianum* (Elliott & Mortenson, 1975; Pienkos & Brill, 1981). W represses Mo transport in *C. pasteurianum* and *Azospirillum brasilense* and *modC* expression in *E. coli* (Elliott & Mortenson, 1975; Chauret *et al.*, 1992; Miller *et al.*, 1987). In *Azotobacter chroococcum*, a species closely related to *A. vinelandii*, the progressive increase in V nitrogenase activity with rising V concentration in the nanomolar range is indicative of high-

affinity V transport (Robson *et al.*, 1986). However, unlike tungstate and molybdate, vanadate does not compete for radioactive molybdate bound to the periplasmic molybdate-binding protein of *A. vinelandii*, which suggests that the high-affinity molybdate-uptake system is not used to transport V into the cell (Mouncey *et al.*, 1995). This may reflect the different species of V and Mo oxyanions in aqueous solution at low concentrations and approximately neutral pH (Willsky, 1990). Mo is present as MoO_4^{2-} and V as a mixture of H_2VO_4^- and HVO_4^{2-} , protonated forms of VO_4^{3-} with greater similarity to phosphate than to molybdate. In *Neurospora crassa*, vanadate uptake occurs via a phosphate-transport system (Bowman, 1983). The demonstration that transcription of the *mod* operon is repressed by V up to a concentration of 100 nM suggests that the metal sensor responds to similar species of Mo and V up to this concentration of V. The lack of repression at higher V concentrations may reflect concentration-dependent changes in the species of vanadate which the sensor recognizes. Monomeric species of vanadate decrease with increasing V concentration (Willsky, 1990).

Measurement of ^{99}Mo accumulation does not distinguish between processes which affect the rate of molybdate transport or subsequent steps in Mo metabolism. The molybdate transporter is fully expressed in non-polar *modE* mutants, which show wild-type nitrate reductase activity, except at high Mo concentration (Mouncey *et al.*, 1995). It was therefore surprising to find that the rates of ^{99}Mo accumulation of cells in the presence of >10 nM molybdate were significantly reduced in a *modE* mutant (RP20) compared to the wild-type strain, though they were sufficient for nitrate reductase cofactor biosynthesis. One explanation is that the rate of Mo accumulation reflects the level of intracellular Mo. Such a situation may be seen in wild-type cells grown in the presence of different nitrogen sources. Since the expression of the molybdate transporter was found to be comparable in all cases, the lower rate of accumulation in the presence of NH_4^+ probably reflects the absence of cofactor biosynthesis, and only represents Mo transported into the cells and bound to 'storage' proteins. The absence of lags in the rates indicates that the differential rates do not depend on *de novo* protein synthesis. The lower initial rate of ^{99}Mo accumulation in the non-polar *modE* mutant may likewise be due to the absence of an intracellular sink or pool for Mo. The similarity of the C-terminal half of ModE with a tandem repeat of *C. pasteurianum* Mop, and ModG, suggests that ModE may be a bifunctional protein. In addition to functioning as a negative regulator, it may have a role as an intracellular Mo-binding protein. Alternatively, it is possible that ModE regulates other unknown genes that are involved in Mo transport or accumulation.

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NOTE ADDED IN PROOF

Recently, molybdate-dependent binding of the product of *E. coli modE* to the promoter of *modABCD* has been observed (Grunden *et al.*, 1996, *J Bacteriol* **178**, 735–744).

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