

Physiological and Genetic Analyses Leading to Identification of a Biochemical Role for the *moeA* (Molybdate Metabolism) Gene Product in *Escherichia coli*†

ADNAN HASONA, RAMESH M. RAY,‡ AND K. T. SHANMUGAM*

Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611

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A unique class of chlorate-resistant mutants of *Escherichia coli* which produced formate hydrogenlyase and nitrate reductase activities only when grown in medium with limiting amounts of sulfur compounds was isolated. These mutants failed to produce the two molybdoenzyme activities when cultured in rich medium or glucose-minimal medium. The mutations in these mutants were localized in the *moeA* gene. Mutant strains with polar mutations in *moeA* which are also *moeB* did not produce active molybdoenzymes in any of the media tested. *moeA* mutants with a second mutation in either *cysDNCJI* or *cysH* gene lost the ability to produce active molybdoenzyme even when grown in medium limiting in sulfur compounds. The CysDNCJIH proteins along with CysG catalyze the conversion of sulfate to sulfide. Addition of sulfide to the growth medium of *moeA cys* double mutants suppressed the MoeA⁻ phenotype. These results suggest that in the absence of MoeA protein, the sulfide produced by the sulfate activation/reduction pathway combines with molybdate in the production of activated molybdenum. Since hydrogen sulfide is known to interact with molybdate in the production of thiomolybdate, it is possible that the MoeA-catalyzed activated molybdenum is a form of thiomolybdenum species which is used in the synthesis of molybdenum cofactor from Mo-free molybdopterin.

Molybdoenzymes play essential metabolic roles in most organisms from bacteria to plants and animals (34). All molybdoenzymes other than dinitrogenase contain molybdenum cofactor, which consists of a unique molybdopterin (MPT) complexed with molybdenum (1, 12, 23, 31, 34). In *Escherichia coli*, the biologically active form of the cofactor in molybdoenzymes is MPT guanine dinucleotide (MGD) (5, 22, 23). Synthesis of this cofactor in an active form requires transport of molybdate into the cell, activation of molybdate, synthesis of the MPT moiety, and incorporation of molybdate into MPT. Although molybdate transport and the various steps in the organic part of MGD biosynthesis are well characterized (17, 24, 33; see references 10, 22, and 23 for reviews), very little is known about the activation and incorporation of molybdenum into the cofactor (22).

Mutants which are defective in molybdate metabolism can be isolated as chlorate-resistant mutants (8, 9). A large fraction of these mutants are pleiotropic for all molybdoenzyme activities in the cell, and these comprise the three genetic loci involved in MGD synthesis, *moa*, *mob*, and *moeB* (see references 10, 22, 29, and 31 for reviews). The *mod* gene products comprise the molybdate transport system through which molybdate is transported into the cell and the Mod⁻ phenotype can be suppressed by increasing molybdate concentration in the medium. The *mog* mutants which produced formate hydrogenlyase (FHL) activity containing the molybdoenzyme formate dehydrogenase-H (FDH-H) but not nitrate reductase activity was proposed to be defective in molybdochelatase (13,

32). This molybdochelatase is apparently required for production of active nitrate reductase and not for FDH-H.

The *moe* operon codes for two proteins, and only the physiological role of the second gene product, MoeB protein, is known. The MoeB protein activates MPT synthase, which catalyzes the conversion of MPT precursor (precursor Z) to MPT by introducing the needed sulfur to which Mo is coordinated in the molybdenum cofactor (20, 22). The MoeB protein, MPT synthase sulfurylase, is the known S donor in the activation of MPT synthase. The physiological role of MoeA protein coded by the first gene in the two member *moe* operon is not known. Mutants which are defective in *moeA* (*chlE* [29]) produced about 6% of the wild-type levels of MPT (12), although no molybdoenzyme activity was found in these *moeA* mutants. Since the MoeB protein acts as an S donor in MPT synthesis, it is possible that the first gene product, MoeA protein, also has a similar role in linking S metabolism and Mo metabolism in the cell.

During our analysis of molybdate transport-defective mutants, we identified a subgroup of chlorate-resistant mutants with a unique phenotype. Mutations in this class of mutants were mapped in the *moeA* gene at 18.6 min on the *E. coli* chromosome (3, 18). The MoeA⁻ phenotype was suppressed when the growth medium was supplemented with sulfide. In this report, we present the physiological and genetic characteristics of *E. coli moeA* mutants and propose a role for the MoeA protein in the activation of molybdenum by sulfurylation.

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MATERIALS AND METHODS

Bacterial strains. Bacterial strains, which are derivatives of *E. coli* K-12, and plasmids used in this study are presented in Table 1.

Media and growth conditions. L broth (LB) was used as rich medium for growth of organisms under aerobic conditions, and LB with glucose (0.3%; LBG) was used as rich medium for anaerobic growth. LBG was supplemented with

* Corresponding author. Mailing address: Department of Microbiology and Cell Science, Box 110700, University of Florida, Gainesville, FL 32611. Phone: (352) 392-2490. Fax: (352) 392-5922. E-mail: shan@micro.ifas.ufl.edu.

† Florida Agricultural Experiment Station Journal Series no. R06089.

‡ Present address: Department of Pharmacology and Biophysics, University of Tennessee School of Medicine, Memphis, TN 38163.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source or reference
Strains		
BW545	$\Delta(lacU)169 rpsL$	Laboratory collection
VJS1779	RK4353 <i>moe-251::Tn10d(Tc)</i>	V. Stewart
VJS1783	RK4353 <i>moe-255::Tn10d(Tc)</i>	V. Stewart
C26	<i>gal-26 chlE5 (moeA5) supE42</i>	CGSC 4459
JT1	<i>cysN-Km</i>	CGSC 7057
JM73	<i>cysJ90</i>	CGSC 5748
SE1581	BW545 <i>moeA101</i>	This study
SE1588	BW545 <i>moeA103</i>	This study
SE1905	SE1588 <i>zhh::Tn10</i>	This study
SE1595	BW545 <i>modC118</i>	25
SE2147	AH69 <i>zhh::Tn10</i>	This study
AH1	SE1588 <i>cysN-Km</i>	SE1588 \times P1 (JT1)
AH8	JM73 <i>moeA103 zhh::Tn10</i>	JM73 \times P1 (SE1905)
AH10	JT1 <i>moeA103 zhh::Tn10</i>	JT1 \times P1 (SE1905)
AH30	<i>moeB101-Km</i>	This study
AH69	<i>moeA113 $\Delta zhiK$-Km</i>	This study
AH131	BW545 <i>cysN-Km</i>	BW545 \times P1 (JT1)
AH134	AH131 <i>moeA113</i>	AH131 \times P1 (SE2147)
Plasmids		
pACYC184	Tc ^r Cm ^r cloning vector	6
pBR322	Am ^r Tc ^r cloning vector	4
pMAK705	<i>rep(Ts)</i> Cm ^r	11
pUC19	Am ^r <i>lacZ'</i> cloning vector	35
pUC4K	Km ^r Am ^r	Pharmacia
pFGH1	pACYC184 based, <i>moeA</i> ⁺ <i>B</i> ⁺	Laboratory collection
pAH1	pUC19 based, <i>moeA</i> ⁺ <i>B</i> ⁺	This study
pAH2	pUC19 based, <i>moeA</i> ⁺ <i>B</i> ⁺	This study
pAH3-1	pUC19 based, (<i>moeB</i> -Km) Am ^r	This study
pAH6	pUC19 based, Δ <i>moeA</i> <i>moeB</i> ⁺	This study
pAH11	pMAK705 based, (<i>moeB</i> -Km) <i>rep(Ts)</i> Cm ^r	This study
pAH20	pUC19 based, <i>moeA</i> ⁺ Δ <i>moeB</i>	This study
pAH43	pUC19 based, <i>moeA113</i> <i>moeB</i> ⁺	This study
pAH44	pAH43 <i>zhiK</i> -Km	This study
pAH55	pMAK705 based, <i>moeA113</i> <i>moeB</i> ⁺ <i>zhiK</i> -Km	This study

formate (15 mM; LBGf), sodium molybdate (1 mM; LBG-Mo), or sodium nitrate (20 mM; LBGn), as needed. The composition of glucose-minimal medium was reported previously (16). Limiting-sulfur medium (LSM) (25) had the following composition: Na₂HPO₄, 14.89 g; KH₂PO₄, 6.09 g; NaCl, 0.5 g; NH₄Cl, 1.0 g; MgCl₂, 0.2 g; Trypticase Peptone (BBL), 1.0 g; yeast extract (BBL), 0.5 g; glucose, 10 g; and deionized water, 1 liter. In all experiments described in this study, both the glucose-minimal medium and LSM were supplemented with 0.1 mM molybdate. Bacterial cultures were grown under anaerobic conditions as previously described (25, 27).

Genetic and molecular biological experiments were performed as described previously (17, 25). DNA was sequenced by using custom primers based on DNA sequence, using the Sanger dideoxy procedure (26) and Sequenase 2.0 (Amersham, Arlington Heights, Ill.). DNA sequence was manipulated with the Genetics Computer Group software (7) or GenePro (Riverside Scientific, Seattle, Wash.).

Isolation of chlorate-resistant mutants. Chlorate-resistant mutants of strain BW545 were isolated as described previously (17). A culture in the mid-exponential phase of growth was serially diluted and spread on L agar supplemented with potassium chlorate (2 mg ml⁻¹). These plates were incubated under anaerobic conditions at 37°C for 3 days. Small chlorate-resistant colonies were transferred to L agar by replica plating and incubated aerobically at 37°C for 16 h. Chlorate-resistant mutants were tested for the ability to produce dihydrogen when cultured in LBG-Mo and LSM-Mo. Chlorate-resistant mutants which were FHL positive only when grown in LSM with molybdate were used in this study.

Enzyme assay. FHL and nitrate reductase activities of cultures in the late exponential phase of growth were determined by procedures described previously (16, 25). All FHL assays were carried out with whole cells instead of crude extracts to avoid oxygen inactivation of the enzyme. Sodium nitrate (20 mM) was included in all media used for culturing cells for determination of nitrate reductase activity.

Construction of *moe* plasmids. A 4.4-kb *SphI* fragment which contained the entire *moeAB* operon including the upstream regulatory region was removed from a larger plasmid, pFGH1, and cloned into plasmid vector pUC19 at the

SphI site (plasmid pAH1 [Fig. 1]). DNA coding for the *moeAB* operon without the upstream regulatory sequences was removed from plasmid pFGH1 as a 2.3-kb *AvaI-SphI* fragment and cloned into plasmid vector pUC19 at the *AvaI-SphI* sites (plasmid pAH2 [Fig. 1]). Plasmid pAH6, which carries a deletion of *moeA* DNA and is still *MoeB*⁺, was constructed by removing a *BssHII-MluI* fragment within the *moeA* gene in plasmid pAH2 and self-ligating the large DNA fragment (Fig. 1). Plasmid pAH20 lacks the 574-bp *BstEII-SphI* fragment in plasmid pAH2 (Fig. 1) and produced only *MoeA*⁺ protein.

Construction of *moeA* deletion strain. Strain AH69, which carries an internal deletion of *moeA* and a kanamycin resistance (Km^r) gene cartridge which did not affect production of *MoeB*⁺ activity (*moeA113*), was constructed by first deleting the *EcoRV-MluI* fragment within the *moeA* gene in plasmid pAH1 (Fig. 1). The *MluI* site was modified with DNA polymerase I (Klenow fragment) before self-ligation in the construction of plasmid pAH43. A 661-bp *BssHII* fragment located 260 bp upstream of the *moeAB* operon (Fig. 1) was removed from plasmid pAH43 for insertion of Km^r gene from plasmid pUC4K (Pharmacia Biotech, Piscataway, N.J.). After the ends of the large fragment were filled in, a 1.3-kb *HincII* fragment containing Km^r gene was ligated to produce plasmid pAH44. The entire *SphI* fragment containing *moe* operon and the upstream Km^r gene (*zhiK*-Km) was removed from plasmid pAH44 and ligated into the *SphI* site in plasmid pMAK705 (11) (yielding plasmid pAH55) for mobilization into the *E. coli* chromosome. The *moeA113* allele from plasmid pAH55 was recombinated into the chromosome of strain BW545, creating strain AH69. Strain AH69 is *MoeA*⁻ and *MoeB*⁺.

Deletion of *moeA* DNA in strain AH69 was confirmed by PCR amplification of chromosomal DNA from strain AH69 and its parent, strain BW545. Two primers flanking the *moeA* gene (primer 1, ATATGGCATGTAAAGGCAGG; primer 2, CCTGATCGCTGAGTTCGCC) were synthesized (Genosys, Woodlands, Tex.) and used to amplify the *moeA* DNA. An expected PCR product of 1.3 kb was detected when the chromosomal DNA from strain BW545 served as the template. A fragment of this size was absent when the chromosomal DNA from *moeA* deletion strain AH69 was used as the template. Instead of an expected PCR product of 0.6 kb, DNA corresponding to 0.4 kb was seen after

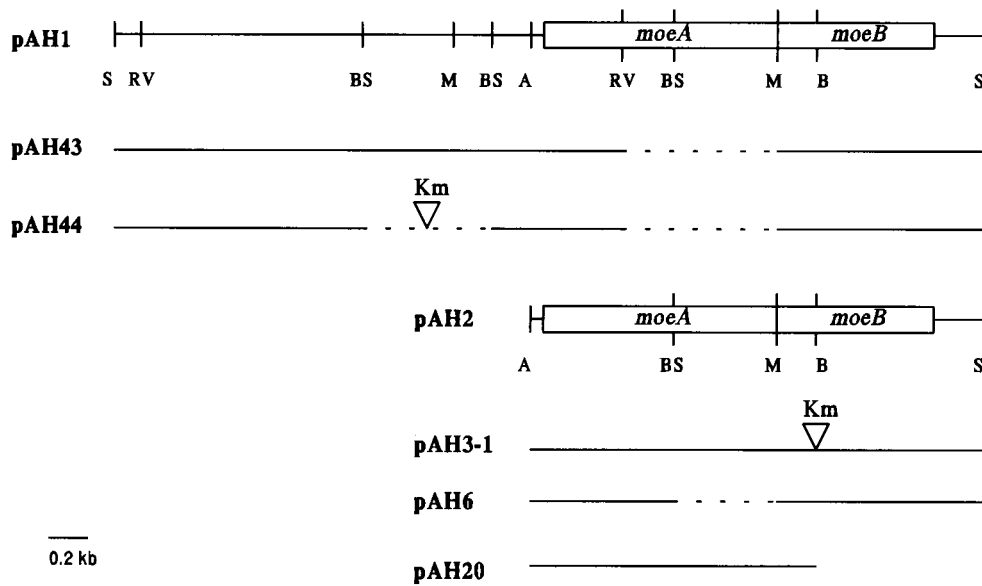


FIG. 1. Restriction maps of the *moe* plasmids used in this study. Solid lines represent *E. coli* chromosomal DNA present in the plasmid; dashed lines indicate regions of chromosomal DNA that were deleted in the construction of the plasmid. Vector DNA is not presented. Each inverted triangle shows the position of insertion of a *Km*^r gene. A, *Ava*I; B, *Bst*EII; BS, *Bss*HIII; M, *Mlu*I; RV, *Eco*RV; S, *Sph*I.

agarose gel electrophoresis. The *moeA113* and *zbiK*-*Km* mutations in strain AH69 cotransduced (phage P1) with each other at a frequency close to 100% and with *gal* and *mod* genes at about 20%. The *moeA113* mutation was also complemented by plasmid pAH20 (see Results). These results are in agreement with the fact that strain AH69 carries a deletion in the *moeA* gene.

Construction of *moeB*-*Km* strain. To construct a *moeB*-*Km* strain, a *Hinc*II fragment containing the *Km*^r gene from plasmid pUC4K was ligated into the *Bst*EII site in the *moeB* gene in plasmid pAH2, which had been previously modified by DNA polymerase I (Klenow fragment), yielding plasmid pAH3-1. A *Sph*I-*Sac*I (from vector DNA) fragment containing the entire chromosomal DNA insert from plasmid pAH3-1 was removed and ligated into the *Sph*I-*Sac*I sites of plasmid pMAK705 (11), resulting in plasmid pAH11. Strain BW545 was transformed with plasmid pAH11, and the *moeB*-*Km* mutation was transferred to the chromosomal DNA by homologous recombination as previously described (11), generating strain AH30. The *moeB*-*Km* mutation cotransduced (phage P1) with *gal* and *mod* mutations at a frequency of 20%, and the mutation was complemented by plasmid pAH6, containing only the *moeB*⁺ gene. The observed phenotype of strain AH30, genetic characteristics, and complementation profile are in agreement with the reported phenotype of the *moeB* mutation (20, 22).

Materials. Biochemicals were purchased from Sigma Chemical Co. Other organic and inorganic chemicals were from Fisher Scientific and were analytical grade. Restriction endonuclease and DNA-modifying enzymes were purchased from Promega (Madison, Wis.) and New England Biolabs (Beverly, Mass.).

RESULTS AND DISCUSSION

During the course of our studies on molybdate transport, we isolated several spontaneous chlorate-resistant mutants of *E. coli* and separated them into three classes based on the ability to produce FHL activity when grown in different media. Class

I mutants are defective in molybdate transport (*mod*) and produced active molybdoenzymes when grown in medium supplemented with molybdate. Class II mutants are pleiotropic and failed to produce FHL activity in any of the media tested. These mutants are apparently defective in *moa*, *mob*, or *moeB* and were not further characterized. These two classes of mutants were previously described (12, 22, 31).

This selection and screen also yielded a third class of mutants. These mutants are conditional mutants and produced FHL and nitrate reductase activities only when cultured in LSM. Two such mutants, strains SE1581 and SE1588, did not produce FHL activity and produced very low levels of nitrate reductase activity when grown in rich medium (Table 2). Changing the medium from LBG to glucose-minimal medium, even with 0.1 mM molybdate, did not alter the FHL⁻ phenotype of the mutants (SE1581 and SE1588). When cultured in LSM, parent strain (BW545) produced about 30% of the FHL activity obtained with strain BW545 grown in LBG-Mo or glucose-minimal medium with molybdate. However, the nitrate reductase activity of the parent, strain BW545, was not significantly influenced by the growth medium. Both mutant strains, SE1581 and SE1588, produced molybdoenzymes when grown in LSM, but the levels of activity were only about 30 to 40% of that of the parent, strain BW545, grown in the same medium. Production of the two molybdoenzyme activities by

TABLE 2. FHL and nitrate reductase activities of class III chlorate-resistant mutants

Strain	Relevant genotype	Enzyme activity (nmol min ⁻¹ mg of cell protein ⁻¹)						
		FHL				Nitrate reductase		
		LBG	LBG-Mo	Glucose-minimal	LSM	LBGN	LBGN-Mo	LSMN
BW545	Wild type	230	200	210	60	560	650	530
SE1581		<1	<1	<1	20	15	20	225
SE1588		<1	<1	2	20	20	30	200
SE1595	<i>modC</i>	<1	160	ND ^a	40	25	620	370

^a ND, not determined.

TABLE 3. Complementation of the mutation in class III mutants by various plasmids^a

Strain	Relevant genotype	Enzyme activity (nmol min ⁻¹ mg of cell protein ⁻¹)						
		FHL				Nitrate reductase		
		No plasmid	pAH2	pAH20	pAH6	No plasmid	pAH2	pAH20
BW545	Wild type	230	220	220	170	650	730	620
SE1581		<1	160	90	ND ^b	40	500	510
SE1588		<1	180	80	<1	40	610	450
AH30	<i>moeB101</i> -Km	<1	150	<1	135	40	600	40

^a Cultures were grown in LBG medium for FHL activity and LBGN medium for nitrate reductase activity.

^b ND, not determined.

the two mutants (strains SE1581 and SE1588) still required molybdate in LSM (data not presented). Increasing the concentrations of molybdate in the medium to 1 mM or higher did not fully restore the ability of these two strains to produce FHL or nitrate reductase activities in LSM. In contrast, *mod* mutant strain SE1595 responded to molybdate in both LBG and LSM (Table 2).

Since the LSM was previously observed to be limiting in sulfur (16, 25) and strains SE1581 and SE1588 produced FHL and nitrate reductase activities when cultured in LSM, the two mutants were grown in a glucose-minimal medium with 0.1 mM sulfate or with 10 μ g of cystine ml⁻¹ as the sole S source. The glucose-minimal medium with a reduced level of sulfur also failed to support production of FHL activity. When nitrate (30 mM) was also included in these media, growth of strains SE1581 and SE1588 was significantly affected, although the parent, strain BW545, grew to acceptable levels. These results show that the suppression of the FHL⁻ phenotype of strains SE1581 and SE1588 required growth in LSM. It is possible that cystine or sulfate even at 0.1 mM repressed the production of some key component required for molybdoenzyme activity in these mutants. It is possible that depletion of sulfur, which could allow production of this unique component, failed to support production of FHL activity, a membrane-bound complex, because of eventual starvation for sulfur.

Strains SE1581 and SE1588 reverted to the wild-type phenotype at a frequency of about 10⁻⁸, suggesting that the observed phenotype is due to a single mutation. In a typical chlorate-resistant population, about 25% of the cells were class III mutants. Since the phenotype of the class III mutants differs from those of other previously described chlorate-resistant mutants, the mutation in these mutants was localized in the *E. coli* chromosome.

Class III mutants are defective in *moeA*. A recombinant plasmid carrying *E. coli* chromosomal DNA which can complement the mutation in strain SE1588 was obtained after transformation with DNA from an *E. coli* gene bank in plasmid vector pACYC184 and selection using glycerol-nitrate medium without molybdate supplementation. *E. coli* chromosomal DNA present in the plasmid was sequenced, and based on the DNA sequence, the complementing DNA was identified as *moe* DNA. A plasmid containing the *moeAB* DNA (plasmid pAH2) complemented the mutation in all class III mutants. Although the mutation in class III mutants was complemented by *moe*⁺ DNA, the observed phenotype of these mutants is different from the previously reported phenotype of *moeA* and *moeB* mutants (molybdoenzyme defective in all growth media [18, 20, 22]). Because of the unique nature of the phenotype of strain SE1588 (Table 2), the location of the mutation within the *moe* operon was first determined by complementation analysis. For these experiments, two plasmids producing only the MoeA⁺ (plasmid pAH20) or MoeB⁺ (plasmid pAH6)

activity were constructed (Fig. 1). Plasmid pAH6 carries a deletion in *moeA* (*moeA113*) which still allowed production of MoeB⁺ activity.

In the presence of plasmid pAH2 (*moeAB*), strains SE1581 and SE1588 regained the ability to produce FHL and nitrate reductase activities when cultured in LBG medium (Table 3). Plasmid pAH20, which contains only an intact *moeA*⁺ gene, also complemented the mutation in strains SE1581 and SE1588 but not in *moeB* mutant strain AH30. However, the level of FHL activity produced by strains SE1581(pAH20) and SE1588(pAH20) was lower than the amount produced by the parent strain with or without the plasmid (less than 40%) as well as the amount produced by the mutant strains with plasmid pAH2. Similar results were also obtained with other *moeA* mutants (data not presented). This lower level of FHL activity produced by strains SE1581(pAH20) and SE1588(pAH20) is apparently due to overproduction of MoeA protein from the plasmid without a corresponding increase in MoeB. The reason for this is unclear. Plasmid pAH6, which contains only *moeB*⁺ DNA, complemented the *moeB101* mutation in strain AH30 and not the mutation in strain SE1588. The levels of nitrate reductase activity in the plasmid-containing mutant strains SE1581 and SE1588 were about 70 to 85% of the parent levels and were not significantly altered by the *moe* genotype of the plasmid. These results suggest that the mutation in the class III chlorate-resistant mutants, such as SE1581 and SE1588, is located in the *moeA* gene. The *moeA* mutation in these strains was also cotransducible by phage P1 with the *mod* operon as well as with the *gal* operon. The cotransduction frequency between the *moeA* mutation and *gal* was about 20%. This finding is in agreement with the known locations of these two operons on the *E. coli* chromosome map (17 min for the *gal-mod* region and 18.6 min for the *moe* operon [3]).

Based on these results, *moeA* mutants can be defined as having a molybdoenzyme activity-negative phenotype when grown in rich medium and a molybdoenzyme-positive phenotype when cultured in medium low in sulfur compounds or in chemically defined medium.

Mutant strains which are defective in *moeA* have been previously described, and these strains were reported to be defective in molybdoenzyme activity even when grown in minimal medium (32). Mutations in three of these mutants (C26, VJS1779, and VJS1783) were mapped by using plasmids pAH2, pAH6, and pAH20, carrying different regions of the *moe* operon (data not presented). All three mutants produced FHL activity when plasmid pAH2 (*moeA*⁺B⁺) was present in the cell. Plasmid pAH20 carrying only the *moeA*⁺ DNA failed to complement the *moe* mutations in these mutants. All three mutant strains with plasmid pAH6 (Δ *moeA* *moeB*⁺) produced FHL activity only when grown in LSM, indicating that the mutation in each of these mutants is located in the *moeA* gene with a polar effect on *moeB* gene expression. In the presence of

TABLE 4. Effect of *cys* mutation on production of FHL and nitrate reductase activities by *moeA* mutant strain SE1588

Strain	Relevant genotype	Enzyme activity (nmol min ⁻¹ mg of cell protein ⁻¹)			
		FHL		Nitrate reductase	
		LBG	LSM	LBGN	LSMN
BW545	Wild type	240	65	570	530
SE1588	<i>moeA103</i>	<1	35	50	320
AH69	<i>moeA113</i>	<1	25	40	300
AH131	<i>cysN</i> -Km	230	45	630	570
AH1	<i>cysN</i> -Km <i>moeA103</i>	<1	<1	35	55
AH134	<i>moeA113 cysN</i> -Km	<1	<1	50	40
JM73	<i>cysJ</i>	130	25	720	450
AH8	<i>cysJ moeA103</i>	<1	<1	50	60

plasmid pAH6, these three mutants produced MoeB protein from the plasmid DNA, and the defect in *moeA* was suppressed by growth in LSM. Similar results were obtained when FHL was replaced by nitrate reductase as the assay system (data not shown).

To confirm that the observed phenotype of strains SE1581 and SE1588 is due to a mutation in the *moeA* gene, a Δ *moeA moeB*⁺ mutant (*moeA113*; strain AH69) was constructed. The *moeA113* mutation was also complemented by plasmid pAH20 and not by plasmid pAH6 (data not shown). The phenotype of strain AH69 was similar to that of strain SE1588 (Table 4).

Role of *cys* gene products in suppression of *moeA* mutation. Our previous studies show that the *mod* mutants, which are defective in molybdate accumulation, are capable of utilizing the sulfate transport system coded by the *cysUWA* (previously *cysTWA* [3, 15, 25]) genes for molybdate transport. The uptake of molybdate via the sulfate transporter required cultivation of *mod* mutants in LSM to activate the production of sulfate transport components. Addition of cystine to LSM prevented production of molybdoenzyme activity since cystine is known to significantly reduce the level of the sulfate transporter (15, 16). Suppression of the *moeA* mutation by growth in LSM suggests that the *cys* gene products are also responsible for molybdoenzyme production in *moeA* mutants grown in LSM. The *moeA* mutants were found to be molybdate transport competent (data not presented). Double mutants with a *moeA* mutation and a second mutation in either *cysU*, *cysW*, or *cysA* still produced FHL and nitrate reductase activities when cultured in LSM, suggesting that the suppression of the *moeA* phenotype in LSM is not due to increased molybdate transport through the sulfate transport system. Thus, the *cys* gene products needed for suppression of the MoeA⁻ phenotype must be in the cysteine biosynthetic pathway after the sulfate transport process.

Double mutants with the *moeA* mutation and a second mu-

tation in any of the genes in the two operons (*cysDNC* and *cysJIH*) coding for proteins in the activation and reduction of sulfate to sulfide (15) failed to produce FHL activity in LSM. Results for two such double mutants involving one *cys* gene from each operon are presented in Table 4. In the construction of the double-mutant strains AH1 and AH10, the *moeA103* and *cysN* mutations were transduced in both directions (Table 1). The *moeA cysN* double mutants lacked FHL and nitrate reductase activities irrespective of the growth medium. Other double mutants with point mutations in *cysD* or *cysC* and *moeA103* had similar phenotypes (data not presented). A double mutant carrying *cysN* mutation and a deletion in the *moeA* gene (*moeA113*) (strain AH134) also failed to produce FHL and nitrate reductase activities (Table 4).

A double mutant with mutations in *cysJ*, a component of the second operon (*cysJIH*) coding for the 3'-phosphoadenosine 5'-phosphosulfate reductase and sulfite reductase (15), and *moeA103* (strain AH8) also lacked FHL and nitrate reductase activities (Table 4). Spontaneous revertants of strain AH8 for Cys⁺ produced both molybdoenzymes when grown in LSM, suggesting that the *cys* and *moeA* mutations are the only mutations causing the observed molybdoenzyme-negative phenotype of strain AH8. Similar results were also obtained with double mutants and Cys⁺ revertants with *cysI* and *cysH* mutations.

The MoeA⁻ phenotype is suppressed by sulfide. Since all of the enzymes in the sulfate activation/reduction pathway are essential for production of FHL and nitrate reductase activities by *moeA* mutants grown in LSM, it is possible that the end product sulfide is necessary for the activation of Mo in the absence of MoeA protein. Apparently, the CysDNCJIH proteins and thus sulfide are not produced in the *moeA* mutants grown in rich medium or glucose-minimal medium due to the presence of S compounds which prevented expression of *cys* gene products (15) to optimum levels for suppression of the MoeA⁻ phenotype.

When wild-type strain BW545 or *cysN* mutant strain AH131 was grown in rich medium or LSM, the amount of nitrate reductase activity produced by the culture was not altered by including sodium sulfide in the growth medium (Table 5). *moeA* mutant strain AH69 produced about 380 U (about 50% of the wild-type level) of nitrate reductase activity when grown in rich medium only when the medium was also supplemented with sodium sulfide. Similarly, the FHL activity of the mutant grown in LBG was also increased from undetectable levels to about 25 U (data not presented). Sulfide also restored the ability of the *moeA cysN* double mutants (strains AH1 and AH134) to produce both molybdoenzyme activities when grown in either rich medium or LSM. Addition of sulfide to glucose-minimal medium failed to suppress the FHL⁻ phenotype of *moeA* mutants, probably due to the presence of sulfate in the medium. When sulfide served as the sole S source,

TABLE 5. Effect of sulfide on nitrate reductase activity produced by *moeA* mutants^a

Strain	Relevant genotype	Nitrate reductase activity (nmol min ⁻¹ mg of cell protein ⁻¹)			
		LBGN	LBGN + S ²⁻	LSMN	LSMN + S ²⁻
BW545	Wild type	670	700	585	530
AH131	<i>cysN</i> -Km	630	725	570	625
AH69	<i>moeA113</i>	40	380	320	300
AH1	<i>moeA103 cysN</i> -Km	55	400	65	360
AH134	<i>moeA113 cysN</i> -Km	50	280	40	275

^a Sodium molybdate concentrations were 1 mM in LBGN and 0.1 mM in LSMN. Sodium sulfide concentrations were 2 mM in LBGN and 0.2 mM in LSMN.

strains SE1581 and SE1588 produced low but detectable levels of FHL activity.

These results suggest that in the absence of MoeA protein in the cell (*moeA* mutant), sulfide, generated by the sulfate activation/reduction pathway, chemically interacts with molybdate in the production of activated molybdenum for incorporation into molybdopterin. Hydrogen sulfide is known to react with molybdate in the production of various thiomolybdate compounds (dithiomolybdate, tetrathiomolybdate, etc.), depending on the ratio of sulfide and molybdate, and this reaction is used in chemical synthesis of thiomolybdate (2). Apparently, cystine is not a sulfur donor in this process since inclusion of cystine at a concentration as high as 200 $\mu\text{g ml}^{-1}$ in LSM or glucose-minimal medium failed to overcome the defect in the *moeA cysN* mutant strain AH1. Inclusion of various thiomolybdates to the culture medium of strain AH1 also did not restore the ability to produce molybdoenzymes, probably due to a lack of thiomolybdate transport. However, thiomolybdate did serve as a source of molybdate for molybdoenzyme synthesis and activity in a *mod* mutant (data not presented).

Putative physiological role of MoeA protein. The results presented above suggest that the MoeA protein catalyzes the formation of activated molybdenum. It is possible that the activated molybdenum is a form of Mo-S complex (thiomolybdate?) and the MoeA protein itself or another protein provides the needed sulfur. This Mo-S complex could serve as the substrate during incorporation of molybdenum into the molybdopterin in the synthesis of molybdenum cofactor. This proposed role of MoeA as a sulfur source for the synthesis of a molybdenum-sulfur complex would be similar to the role of MoeB as a sulfur donor (MPT synthase sulfurylase) in the activation of MPT synthase (20, 22). Thus, the two proteins coded by the *moe* operon would catalyze similar reactions but with differing substrates.

An alternate possibility that the MoeA protein is the S donor for MoeB protein in the activation of MPT synthase small subunit cannot be ruled out. In this role, the MoeA protein would be similar to the NifS protein of *Azotobacter vinelandii*, which apparently provides the needed sulfur for biosynthesis of Fe-Mo cofactor by desulfuration of cysteine (36). It is possible that in the absence of MoeA protein, sulfide serves as a direct source of S for MoeB protein, although at a lower level. However, in other studies of transcription of *hyc-lac* and *nar-lac*, MoeA protein and not MoeB protein was found to be required for optimum expression of the two operons coding for molybdoenzymes, in response to molybdate (unpublished data). Such a requirement for only the MoeA protein is difficult to reconcile with a putative role of MoeA as strictly an S donor for MoeB activity in molybdopterin synthesis. Additionally, the MoeA protein and NifS sequences are not similar. Direct biochemical experiments are needed to establish the role of MoeA protein in molybdenum cofactor biosynthesis and Mo metabolism.

Recently, the crystal structure of FDH-H, a component of the FHL complex, was reported and it was shown that the FDH-H contains two MGD molecules coordinating a single molybdenum through four sulfur atoms (5). A similar structure was identified in the dimethyl sulfoxide reductase from *Rhodobacter capsulatus*, which also contains two MGD molecules (28). The Mo is coordinated in these molybdoenzymes by four S atoms from the two MGD molecules. The MoeA-produced thiomolybdenum species could serve as the donor of Mo in the production of active cofactor by transferring Mo from one Mo-S complex to sulfur in the MPT.

In three different eukaryotes, *Drosophila melanogaster* (14), rat (21), and *Arabidopsis thaliana* (30), the MoeA and Mog

protein homologs constitute a single protein. It is believed that these MoeA-Mog proteins catalyze the same steps in Mo-MPT synthesis in these organisms also. Among the three organisms, only DNA coding for the protein from *Arabidopsis* was isolated by complementation of an *E. coli mog* mutation. However, this *Arabidopsis* cDNA encoding the MoeA-Mog hybrid protein failed to complement the *E. coli moeA* mutation (16a). In *E. coli*, the MoeA and Mog proteins are independent and genetically well separated (*moeA*, 18.7 min; *mog*, 0.2 min [3]). It is possible that the activated molybdenum species produced by the MoeA protein is transferred to the Mog protein as a prelude to incorporation into molybdopterin. Joshi et al. (13) proposed that the Mog protein in *E. coli* is a molybdochelataze which binds molybdenum prior to incorporation into MPT. This suggested role would be in agreement with the proposed model except that the Mo would be a thiomolybdenum species. Alternatively, the possibility that a MoeA-Mog complex catalyzes the synthesis of thiomolybdenum in which the MoeA protein is the sulfur donor and the thiomolybdenum is a protein-bound intermediate cannot be ruled out. However, a soluble or dissociable Mo complex can be formed, as demonstrated by the ability of the *moeA* mutants to produce active molybdoenzymes, such as nitrate reductase, in the presence of molybdate and sulfide in the growth medium.

Conclusion. The results presented above show that in *E. coli*, the MoeA protein is essential for production of active molybdoenzymes and that this requirement for MoeA protein can be suppressed by the enzymes in the sulfate activation/reduction pathway or by supplementation of the medium with sulfide. The MoeA protein apparently catalyzes the production of a Mo-S complex which can be generated at a lower rate by direct interaction between Mo and sulfide. This complex could be a thiomolybdenum species since H_2S and molybdate can yield thiomolybdate. The thiomolybdenum compound is apparently the Mo donor in the production of the Mo cofactor.

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REFERENCES

- Allen, R. M., R. Chatterjee, M. S. Madden, P. W. Ludden, and V. K. Shah. 1994. Biosynthesis of the iron-molybdenum cofactor of nitrogenase. *Crit. Rev. Biotechnol.* **14**:225-249.
- Aymonino, P. J., A. C. Ranade, E. Dieman, and A. Muller. 1969. Study of formation and relative reaction rates of different thioanions of molybdenum and tungsten. *Z. Anorg. Allg. Chem.* **371**:300-305.
- Berlyn, M. K. B., K. B. Low, and K. E. Rudd. 1996. Linkage map of *Escherichia coli* K-12, edition 9, p. 1715-1902. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
- Bolivar, F., R. L. Roriquéz, P. J. Greene, M. C. Betlach, H. L. Heyneker, and H. W. Boyer. 1977. Construction and characterization of new cloning vehicles II. A multipurpose cloning system. *Gene* **2**:95-113.
- Boydington, C. J., V. N. Gladyshev, S. V. Khangulov, T. C. Stadtman, and P. D. Sun. 1997. Crystal structure of formate dehydrogenase H: catalysis involving Mo, molybdopterin, selenocysteine, and an Fe_4S_4 cluster. *Science* **275**:1305-1308.
- Chang, A. C. Y., and S. N. Cohen. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.* **134**:1141-1156.
- Devereux, J., P. Haerberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**:387-395.
- Dubourdieu, M., E. Andrade, and J. Puig. 1976. Molybdenum and chlorate resistant mutants in *Escherichia coli* K-12. *Biochem. Biophys. Res. Commun.* **70**:766-773.
- Glaser, J. H., and J. A. DeMoss. 1972. Comparison of nitrate reductase

- mutants of *Escherichia coli* selected by alternative procedures. *Mol. Gen. Genet.* **116**:1–10.
10. **Grunden, A. M., and K. T. Shanmugam.** 1997. Molybdate transport and regulation in bacteria. *Arch. Microbiol.* **168**:345–354.
 11. **Hamilton, C. M., M. Aldea, B. K. Washburn, P. Babitzke, and S. R. Kushner.** 1989. New method for generating deletions and gene replacements in *Escherichia coli*. *J. Bacteriol.* **171**:4617–4622.
 12. **Hinton, S. M., and D. Dean.** 1990. Biogenesis of molybdenum cofactors. *Crit. Rev. Microbiol.* **17**:169–188.
 13. **Joshi, M. S., J. L. Johnson, and K. V. Rajagopalan.** 1996. Molybdenum cofactor biosynthesis in *Escherichia coli mod* and *mog* mutants. *J. Bacteriol.* **178**:4310–4312.
 14. **Kamdar, K. P., M. Shelton, and V. Finnerty.** 1994. The drosophila molybdenum cofactor gene *cinnamon* is homologous to three *Escherichia coli* cofactor proteins and to the rat protein gephyrin. *Genetics* **137**:791–801.
 15. **Kredich, N. M.** 1996. Biosynthesis of cysteine, p. 514–527. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
 16. **Lee, J. H., J. C. Wendt, and K. T. Shanmugam.** 1990. Identification and characterization of a new gene, *molR*, essential for utilization of molybdate by *Escherichia coli*. *J. Bacteriol.* **172**:2079–2087.
 - 16a. **Mandel, R. R.** Personal communication.
 17. **Maupin-Furlow, J. A., J. K. Rosentel, J. H. Lee, U. Deppenmeier, R. P. Gunsalus, and K. T. Shanmugam.** 1995. Genetic analysis of the *modABCD* operon (molybdate transport) of *Escherichia coli*. *J. Bacteriol.* **177**:4851–4856.
 18. **Nohn, T., Y. Kasai, and T. Saito.** 1988. Cloning and sequencing of the *Escherichia coli chlEN* operon involved in molybdopterin biosynthesis. *J. Bacteriol.* **170**:4097–4102.
 19. **Pateman, J. A., D. J. Cove, B. M. Rever, and D. B. Roberts.** 1964. A common cofactor for nitrate reductase and xanthine dehydrogenase which also regulates the synthesis of nitrate reductase. *Nature* **201**:58.
 20. **Pitterle, D. M., J. L. Johnson, and K. V. Rajagopalan.** 1993. *In vitro* synthesis of molybdopterin from precursor Z using purified converting factor. Role of protein-bound sulfur in formation of the dithiolene. *J. Biol. Chem.* **268**:13506–13509.
 21. **Prior, P., B. Schmidt, G. Grenningloh, I. Pribilla, G. Multhaup, K. Beyreuther, Y. Maulet, P. Werner, D. Langosch, J. Kirsch, and H. Betz.** 1992. Primary structure and alternative splice variants of gephyrin, a putative glycine receptor-tubulin linker protein. *Neuron* **8**:1161–1170.
 22. **Rajagopalan, K. V.** 1996. Biosynthesis of the molybdenum cofactor, p. 674–679. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
 23. **Rajagopalan, K. V., and J. L. Johnson.** 1992. The pterin molybdenum cofactors. *J. Biol. Chem.* **267**:10199–10202.
 24. **Rech, S., C. Wolin, and R. P. Gunsalus.** 1996. Properties of periplasmic ModA molybdate-binding protein of *Escherichia coli*. *J. Biol. Chem.* **271**:2557–2562.
 25. **Rosentel, J. K., F. Healy, J. A. Maupin-Furlow, J. H. Lee, and K. T. Shanmugam.** 1995. Role of molybdate transport system(s) in the regulation of formate hydrogenlyase synthesis in *Escherichia coli*. *J. Bacteriol.* **177**:4857–4864.
 26. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
 27. **Sankar, P., and K. T. Shanmugam.** 1988. Biochemical and genetic analysis of hydrogen metabolism in *Escherichia coli*: the *hydB* gene. *J. Bacteriol.* **170**:5433–5439.
 28. **Schindelin, H., C. Kisker, J. Hilton, K. V. Rajagopalan, and D. C. Rees.** 1996. Crystal structure of DMSO reductase: redox-linked changes in molybdopterin coordination. *Science* **272**:1615–1621.
 29. **Shanmugam, K. T., V. Stewart, R. P. Gunsalus, D. H. Boxer, J. A. Cole, M. Chippaux, J. A. Demoss, G. Giordano, E. C. C. Lin, and K. V. Rajagopalan.** 1992. Proposed nomenclature for the genes involved in molybdenum metabolism in *Escherichia coli* and *Salmonella typhimurium*. *Mol. Microbiol.* **6**:3452–3454.
 30. **Stallmeyer, B., A. Nerlich, J. Schiemann, H. Brinkmann, and R. R. Mandel.** 1995. Molybdenum co-factor biosynthesis: the *Arabidopsis thaliana* cDNA *cnx1* encodes a multifunctional two-domain protein homologous to a mammalian neuroprotein, the insect protein cinnamon and three *Escherichia coli* proteins. *Plant J.* **8**:751–762.
 31. **Stewart, V.** 1988. Nitrate respiration in relation to facultative metabolism in enterobacteria. *Microbiol. Rev.* **52**:190–232.
 32. **Stewart, V., and C. H. MacGregor.** 1982. Nitrate reductase in *Escherichia coli* K-12: involvement of *chlC*, *chlE*, and *chlG* loci. *J. Bacteriol.* **151**:788–799.
 33. **Walkenhorst, H., S. Hemschmeier, and R. Eichenlaub.** 1995. Molecular analysis of the molybdate uptake operon, *modABCD*, of *Escherichia coli* and *modR* a regulatory gene. *Microbiol. Res.* **50**:347–361.
 34. **Wootton, J. C., R. E. Nicolson, J. M. Cock, D. E. Walters, J. F. Burke, W. A. Doyle, and R. C. Cray.** 1991. Enzymes depending on the pterin molybdenum cofactor: sequence families, spectroscopic properties of molybdenum and possible cofactor-binding domains. *Biochim. Biophys. Acta* **1057**:157–185.
 35. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103–119.
 36. **Zheng, L., R. H. White, V. L. Cash, R. F. Jack, and D. R. Dean.** 1993. Cysteine desulfurase activity indicates a role for NifS in metallocluster biosynthesis. *Proc. Natl. Acad. Sci. USA* **90**:2754–2758.