Molybdate and Regulation of *mod* (Molybdate Transport), *fdhF*, and *hyc* (Formate Hydrogenlyase) Operons in *Escherichia coli*†

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Escherichia coli mutants with defined mutations in specific *mod* genes that affect molybdate transport were isolated and analyzed for the effects of particular mutations on the regulation of the *mod* operon as well as the *fdhF* and *hyc* operons which code for the components of the formate hydrogenlyase (FHL) complex. $\Phi(hyc'-lacZ^+)$ *mod* double mutants produced β -galactosidase activity only when they were cultured in medium supplemented with molybdate. This requirement was specific for molybdate and was independent of the *moa*, *mob*, and *moe* gene products needed for molybdopterin guanine dinucleotide (MGD) synthesis, as well as Mog protein. The concentration of molybdate required for FHL production by *mod* mutants was dependent on medium composition. In low-sulfur medium, the amount of molybdate needed by *mod* mutants for the production of half-maximal FHL activity was increased approximately 20 times by the addition of 40 mM of sulfate. *mod* mutants growing in low-sulfur medium transported molybdate through the sulfate transport system, as seen by the requirement of the *cysA* gene product for this transport. In wild-type *E. coli*, the *mod* operon is expressed at very low levels, and a *mod*⁺ merodiploid *E. coli* carrying a *modA-lacZ* fusion produced less than 20 units of β -galactosidase activity. This level was increased by over 175 times by a mutation in the *modA*, *modB*, or *modC* gene. The addition of molybdate to the growth medium of a *mod* mutant lowered $\Phi(modA'-lacZ^+)$ expression. Repression of the *mod* operon was sensitive to molybdate but was insensitive to mutations in the MGD synthetic pathway. These physiological and genetic experiments show that molybdate can be transported by one of the following three anion transport systems in *E. coli*: the native system, the sulfate transport system (*cysTWA* gene products), and an undefined transporter. Upon entering the cytoplasm, molybdate branches out to *mod* regulation, *fdhF* and *hyc* activation, and metabolic conversion, leading to MGD synthesis and active molybdoenzyme synthesis.

Synthesis of active molybdoenzymes by *Escherichia coli* is dependent on the various gene products required for the transport of molybdate into cells (5, 9, 12, 25, 29, 39, 40, 43), metabolic conversion of molybdate to an appropriate form, and incorporation into the pterin component of molybdopterin guanine dinucleotide (MGD) during maturation of the apoprotein to active enzyme (6, 8, 34, 46; see references 13, 38, and 46 for reviews). Several mutant strains of *E. coli* which are defective in one or more of these steps have been isolated and described. Mutants defective in the first step of this process, molybdate transport, were isolated as chlorate-resistant derivatives (*chlD* [renamed as *mod*]) (41) whose phenotype can be suppressed by supplementation of the growth medium with molybdate (5, 9, 12, 13, 29, 40, 43, 45). The rates of molybdate transport and internal molybdate concentrations of some of these mutants were determined (5, 12, 40). Recent studies have established that the *mod* operon contains four genes, of which three represent the various components of the periplasmic binding protein-dependent transport system for molybdate (29). Transcription analysis of a *mol-lac* fusion mutant strain with defects in both molybdate transport and utilization re-

vealed that the operon is transcribed to high levels under molybdate starvation conditions but becomes repressed by the addition of molybdate to the growth medium (31). However, the genetic location of the *lac* fusion in this mutant strain is not known (29). By using another *mol-lac* fusion mutant (*molR-lac*), the sulfate transport system and a nonspecific anion transport system were implicated in molybdate transport (25). The nature of this mutation is unknown, but this mutant allowed the complete *mod* operon to be cloned (29).

In this communication, a role for sulfate transport system in molybdate metabolism is demonstrated by using defined *mod* mutants. These mutants are also used to elucidate regulation of the *mod* operon (molybdate transport) and the molybdate dependence of the *fdhF* and *hyc* operons (coding for the components of the formate hydrogenlyase [FHL] complex) in *E. coli*.

MATERIALS AND METHODS

Bacterial strains. The bacterial and phage strains used in this study are listed in Table 1. Plasmids pSE1004, which carries DNA of the entire native *mod* operon, and pSE1009, carrying *modABC* genes, were described previously (25, 29). Strain SE2011 was previously designated an *fhlB* mutant (28). The *lac* fusion DNA from this strain was cloned and sequenced to identify the location of the *lac* fusion junction in the *E. coli* chromosome. On the basis of these experiments, the *lac* fusion in strain SE2011 is in the *hycD* gene, which codes for a 33-kDa membrane protein (3).

Media and growth conditions. L broth (LB) supplemented with glucose (0.3%; LBG) as needed was used as the rich medium. As needed, sodium molybdate and sodium formate were added at the indicated concentrations. The compositions of glucose-minimal medium and low-sulfur medium (LSM) were described previously (25). Bacterial cultures were grown under anaerobic conditions as previously described (24, 25, 28). Whenever aerobic cultures were needed, cells were grown in 125-ml flasks with 10 ml of medium. Fresh medium was inoculated with

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TABLE 1. Bacterial strains and phages used in this study

Strain or phage	Genotype	Source and/or reference
Strains		
BW545	$\Delta(lacU)169 rpsL$	Laboratory collection
MC4100	<i>araD139</i> $\Delta(argF-lacU)205 rpsL150 relA1 flbB5301 deoC1 ptsF25$	CGSC 6152
JBM239-7	MC4100 $\Phi(chlD-lac)2002 [\Phi(mol'-'lacZ^+)2002]$	N. K. Amy (31)
JRG94	<i>leuB6 gal-31 chlD4 (mod-4) bioA2 rpsL129 thi-1</i>	CGSC 4458
RK4353	MC4100 <i>gyrA219 non-9</i>	V. Stewart
RK5202	RK4353 <i>chlD202::Mu cts (modC202)</i>	V. Stewart
VJS720	<i>chlD247::Tn10 (modB247)</i>	V. Stewart
VJS1779	RK4353 <i>moe-251::Tn10d(Tc)</i>	V. Stewart
VJS1780	RK4353 <i>mob-252::Tn10d(Tc)</i>	V. Stewart
VJS1782	RK4353 <i>moa-254::Tn10d(Tc)</i>	V. Stewart
VJS1784	RK4353 <i>mog-256::Tn10d(Tc)</i>	V. Stewart
ECL594	<i>sdh⁺ $\Phi(sdh-lac)$ arcB1 zgi::Tn10</i>	E. C. C. Lin (17)
ECL618	<i>arcA2 zji::Tn10</i>	E. C. C. Lin (18)
JM2314	<i>araD139</i> $\Delta(argF-lac)169 flbB5301 \Delta(his-gnd)296 cysA97 relA1 fda-2(Ts)$ <i>galP79 rpsL150 deoC1</i>	CGSC 6810
CB64	<i>trp-75 cysB93 tfj-8</i>	CGSC 5783
M3s	MC4100 $\Phi(hyc-'lacZ^+)$	A. Bock (35)
M9s	MC4100 $\Phi(fdhF-'lacZ^+)$	A. Bock (35)
GW5310	BW545 $\Phi(hyc-'lacZ^+)$	G. Walker (48)
SE2011	BW545 $\Phi(hycD-'lacZ^+)$	Laboratory collection
SE1188	<i>gal trpA9761 iclR trpR rpsL fnr zcj-5::Tn10</i>	Laboratory collection
SE1328	M3s <i>modB::Tn5</i>	P1 transduction (M3s $\times$ SE1325 [25])
SE1334	M9s <i>modB::Tn5</i>	P1 transduction (M9s $\times$ SE1325)
SE1473	GW5310 <i>modB::Tn5</i>	P1 transduction (GW5310 $\times$ SE1325)
SE1704	<i>modB::Tn5 zgg-3::Tn10</i>	Laboratory collection
SE2010	MC4100 $\Phi(modA-'lacZ^+)101$	This study
SE2069	BW545 $\Phi(modA-'lacZ^+)102$	This study
SE1581	BW545 <i>mol</i>	This study
SE1586	BW545 <i>mol</i>	This study
SE1587	BW545 <i>mol</i>	This study
SE1592	BW545 <i>modA115</i>	This study
SE1595	BW545 <i>modC118</i>	This study
SE1597	BW545 <i>modC120</i>	This study
SE1602	BW545 <i>modB125</i>	This study
SE1605	BW545 <i>modB128</i>	This study
SE1714	SE2011 <i>modB247::Tn10</i>	P1 transduction (SE2011 $\times$ VJS720)
SE1736	BW545 $\Phi(hycD-lacZ)$	P1 transduction (BW545 $\times$ SE2011)
SE1797	SE2069 <i>arcB1 zgi::Tn10</i>	P1 transduction (SE2069 $\times$ ECL594)
SE1804	SE2069 <i>arcA2 zji::Tn10</i>	P1 transduction (SE2069 $\times$ ECL618)
SE1808	SE2069 <i>fnr zcj::Tn10</i>	P1 transduction (SE2069 $\times$ SE1188)
SE1849	SE2069 <i>moa::Tn10</i>	P1 transduction (SE2069 $\times$ VJS1782)
SE1850	SE2069 <i>mob::Tn10</i>	P1 transduction (SE2069 $\times$ VJS1780)
SE1851	SE2069 <i>moe::Tn10</i>	P1 transduction (SE2069 $\times$ VJS1779)
SE1852	SE2069 <i>mog::Tn10</i>	P1 transduction (SE2069 $\times$ VJS1784)
SE1853	JM2314 <i>modB::Tn5</i>	P1 transduction (JM2314 $\times$ SE1704)
SE1854	CB64 <i>modB::Tn5</i>	P1 transduction (CB64 $\times$ SE1704)
SE1855	SE1736 <i>moa::Tn10</i>	P1 transduction (SE1736 $\times$ VJS1782)
SE1856	SE1736 <i>mob::Tn10</i>	P1 transduction (SE1736 $\times$ VJS1780)
SE1857	SE1736 <i>moe::Tn10</i>	P1 transduction (SE1736 $\times$ VJS1779)
SE1858	SE1736 <i>mog::Tn10</i>	P1 transduction (SE1736 $\times$ VJS1784)
Phages		
P1	Tn9 Cm ^r <i>clr-100</i>	Laboratory collection
λ RZ5	λ 'bla' <i>'lacZ lacY⁺</i>	R. P. Gunsalus
λ SE1	λ $\Phi(modA-'lacZ^+) lacY^+ bla^+$	This study
λ placMu53	<i>imm' trp' lacZ⁺ lacY⁺ lacA' 'uvrD' xho::kan Mu[cIts62 ner⁺A⁺S]</i>	Laboratory collection (4)
λ pMu507	c1857 Sam7 Mu[cIts62 ner ⁺ A ⁺ B ⁺]	Laboratory collection (4)

overnight cultures to a starting density of about 10^7 CFU/ml and allowed to grow in a shaker at 250 rpm at 37°C. When the cell density reached about 10^8 CFU/ml, this culture was used as an inoculum (5% [vol/vol]) for fresh medium. This process was repeated three times before cells were used for enzyme assays.

Genetic experiments. Transduction with P1 and λ phages was performed as described by Miller (32). Phage λ SE1 was constructed by the procedure described by Jones and Gunsalus (20). The methods used for isolation of the *mod* mutants and unique *mol* mutants (strains SE1581, SE1586, and SE1588) used in this study were previously described (29). The mutations in these *mol* mutants were not complemented by *mod* operon DNA (29), although the *mol* mutation mapped at 17 min in the *E. coli* chromosome (1, 11). These three *mol* mutants

(strains SE1581, SE1586, and SE1588) are defective in dihydrogen production when they are grown in LB medium supplemented with 1 mM molybdate. However, these strains produced dihydrogen when they were cultured in LSM containing 0.1 mM molybdate (29). In contrast, *mod* mutants (29) produced dihydrogen in all of the media tested, provided the medium contained molybdate. Plasmid isolation and transformation procedures were those of Maupin and Shanmugam (28).

Enzyme assays. All enzyme assays were performed with whole cells to eliminate problems associated with the oxygen sensitivity of FHL. Cells were grown and harvested for enzyme assays as described previously (24, 25, 28). FHL and nitrate reductase were assayed by procedures described before (25, 28). β -Ga-

TABLE 2. Effects of *mod* mutations on FHL and nitrate reductase activities^a

Strain	Relevant genotype	FHL activity		Nitrate reductase activity	
		– Mo	+ Mo	– Mo	+ Mo
BW545	Wild type	240	230	780	790
MC4100	Wild type	170	210	420	530
SE2010	$\Phi(modA-lacZ)$	<1	190	35	580
SE2069	$\Phi(modA-lacZ)$	<1	160	60	730
SE1595	<i>modC</i>	<1	190	80	1,040
SE1597	<i>modC</i>	<1	200	80	920
SE1605	<i>modB</i>	<1	180	70	750
JBM239-7	$\Phi(mod-lacZ)$	<1	<1	ND ^b	ND

^a Cells were grown in LBG medium with or without sodium molybdate (1 mM). Enzyme activities are expressed in nanomoles minute⁻¹ milligram of cell protein⁻¹.

^b ND, not done.

lactosidase activity in whole cells was determined with chloroform-sodium dodecyl sulfate-permeabilized cells as described by Miller (32). Under the experimental conditions used, the levels of β -galactosidase activity produced by a mutant assayed at different times varied by less than 15%.

FHL activity was also determined qualitatively as the amount of dihydrogen produced by a 16- to 18-h culture. For these experiments, one ml of medium in a tube (12 by 75 mm) was inoculated from an overnight culture (1% [vol/vol]) and the tube was sealed with a serum stopper. The gas phase was replaced with dinitrogen after repeated evacuation and filling. After 16 to 18 h of incubation at 37°C, the amount of dihydrogen present in the gas phase was determined with a gas chromatograph (Varian Associates, Sunnyvale, Calif.) equipped with a molecular sieve (5-Å [0.5-nm]) column and thermal conductivity detector.

Materials. Biochemicals were purchased from Sigma Chemical Co. Other organic and inorganic chemicals were from Fisher Scientific and were analytical grade.

RESULTS

Production of FHL in *mod* mutants. Previous studies with mutants defective in molybdate transport had indicated that the availability of molybdate controlled the transcription of *fdhF* and *hyc* operons coding for the components of the FHL complex as well as the activity of the molybdoenzyme formate dehydrogenase H (FDH-H) in *E. coli* (23, 39). The *mod* operon was described as having four genes (*modABCD*), and mutations in three of them were readily available (29). Since the locations of the mutations in these *mod* mutants are known at the gene level, these mutants were used to reinvestigate the effects of *mod* mutations on FHL activity and *fdhF* and *hyc* transcription. Mutations in any of the first three genes in the *mod* operon (*modABC*) led to defects in FHL and nitrate reductase activities (Table 2). Supplementing the growth medium with molybdate suppressed the mutant phenotype. The defect in FHL is a result of the absence of both FDH-H and hydrogenase 3 (HYD3) activities (data not presented). None of the more than 50 *mod* mutants isolated had a mutation in the *modD* gene, as determined by complementation analysis with *mod*⁺ DNA (29). Deletion strains lacking *modD* also had no detectable phenotype. These results suggest that the ModD protein has no significant role in transporting molybdate across the inner membrane.

Regulation of *fdhF* and *hyc* operons by molybdate. The absence of FHL activities in *modA*, *-B*, or *-C* mutants can be traced to the absence of the molybdoenzyme FDH-H, a component of the FHL complex. Although these strains produced hydrogenase activities, the hydrogenase produced was the HYD2 isoenzyme associated with dihydrogen uptake (30). *mod* mutants lacked the HYD3 isoenzyme associated with the FHL complex (23). Transcription of both *fdhF* and *hyc* operons

was also impaired in *mod* double mutants in the absence of externally added molybdate (Table 3). In these experiments, tungstate can substitute for molybdate in the activation of transcription of *hyc* and *fdhF* operons, although W enzyme is known to be inactive (43). The addition of formate, an effector of FHL synthesis (14), to the medium also enhanced the levels of β -galactosidase activity produced by cultures grown in LBG-Mo medium in a *mod*⁺ background to various levels. For reasons unknown, strain SE1334, an *fdhF-lac modB* double mutant, did not fully derepress the *fdhF* gene in the formate-molybdate medium. Since the medium contained glucose, the intermediate levels of β -galactosidase activity observed in cells cultured in LBG-Mo medium can be ascribed to internally generated formate. These results show that the lack of FHL activity in *mod* mutants is due to the need for a minimum level of molybdate for transcription of the *fdhF* and *hyc* operons and that this effect is independent of formate. Comparable results were also obtained with two other *hyc-lac* mutants (strains M3s and GW5310) and their isogenic *mod* mutants (data not presented). It is interesting that the *hyc* operon does not code for a molybdoprotein (3), and control by molybdate availability could be related to its associated role with FDH-H in the FHL complex.

This regulation of *hyc* transcription is independent of the cell's ability to synthesize MGD, the molybdenum-containing cofactor in FDH-H. Mutants defective in the synthesis of MGD (*moa*, *mob*, and *moe*), as well as a *mog* mutant, are normal in the production of β -galactosidase activity from $\Phi(hycD'-lacZ^+)$ (Table 4). These results also confirm the need for formate in *hyc* operon expression.

In all of these experiments, the levels of FHL in cells were used to conveniently monitor internal levels of molybdate since the transcription of both the *hyc* and *fdhF* operons requires adequate molybdate inside cells and the production of active FDH-H requires MGD. This provided an indirect but qualitative measure of molybdate levels in cells.

Role of sulfate transport system in molybdate transport. To reaffirm the role of the sulfate transport system as an alternate molybdate transport system, specific *mod* mutants were tested for the ability to produce dihydrogen in LSM with and without added molybdate. The results presented in Table 5 show that the amount of molybdate required for production of FHL activity was greatly reduced when cells were grown in LSM (values ranged from 10 to 400 nM, depending on the specific mutation, for supporting half-maximal FHL activity). The optimum amount of molybdate needed for production of maximal activity ranged from 0.26 to 2.0 μ M, depending on the mutant strain. The addition of sulfate, which causes repression of the sulfate transport system (21, 33), increased the molyb-

TABLE 3. Molybdate requirement for transcription of the *fdhF* and *hyc* operons

Strain	Relevant genotype	β -Galactosidase activity ^a			
		No addition	+ Mo	+ Formate	+ Formate and Mo
M9s	$\Phi(fdhF'-lacZ^+)$	230	210	490	600
SE1334	$\Phi(fdhF'-lacZ^+)$	80	210	260	390
	<i>modB::Tn5</i>				
SE2011	$\Phi(hycD'-lacZ^+)$	560	500	1,210	1,400
SE1714	$\Phi(hyc'-lacZ^+)$	100	640	190	1,480
	<i>modB::Tn10</i>				

^a Formate (29.4 mM) and sodium molybdate (1 mM) were added to LBG medium as indicated. Enzyme activities are expressed in nanomoles minute⁻¹ milligram of cell protein⁻¹.

TABLE 4. Effects of mutations in molybdate metabolism on transcription of the *hyc* operon

Strain	Relevant genotype	β -Galactosidase activity ^a	
		LB	LBGF ^b
SE1736	$\Phi(hycD'-lacZ^+)$	320	790
SE1855	$\Phi(hycD'-lacZ^+) moa::Tn10$	340	940
SE1856	$\Phi(hycD'-lacZ^+) mob::Tn10$	400	860
SE1714 ^c	$\Phi(hycD'-lacZ^+) mod::Tn10$	ND ^d	160 (700)
SE1857	$\Phi(hycD'-lacZ^+) moe::Tn10$	320	940
SE1858	$\Phi(hycD'-lacZ^+) mog::Tn10$	320	700

^a Enzyme activities are expressed in nanomoles minute⁻¹ milligram of cell protein⁻¹.

^b Glucose and formate were added to LB at final concentrations of 0.3% and 15 mM, respectively.

^c Medium contained LB and formate (15 mM). Value in parentheses was obtained with cells grown in LB-formate medium supplemented with sodium molybdate (1 mM).

^d ND, not done.

date requirement for half-maximal activity in all of the strains tested by 18-fold or higher. The amount of molybdate needed for maximal activity in the presence of sulfate was also increased by about 5- to 10-fold. Among these strains and for reasons unknown, *modB* and *modC* mutants required higher concentrations of molybdate than did *modA* mutants. The mutation in strain JBM239-7 apparently lies outside the *mod* operon, as seen by the inability of plasmid pSE1004 to complement the mutation in this strain (29). The amount of molybdate needed by this strain for FHL production was the lowest (Table 5).

If molybdate is transported by the sulfate transport system in the absence of a functional molybdate transport system, then a *cysA* mutation which eliminates the transport of sulfate into the cell (33, 42) should severely affect molybdate entry even in LSM. Such is the case, as seen from the results presented in Table 6. Strain JM2314, defective in sulfate transport because of a *cysA* mutation, produced FHL activity in all of the media tested without additional molybdate supplementation because of its *mod*⁺ genotype. Although strain SE1325, a *modB::Tn5* mutant, produced FHL activity when it was grown in LSM, strain SE1853, a *cysA modB* double mutant, lacked FHL activity when it was grown in LSM. Increasing the molybdate concentration in LSM to 50 μ M led to the production of FHL activity in both strains.

The *cysB* gene product is an activator of the operons coding for the various enzymes in cysteine biosynthesis, including the sulfate transport system (*cysPTWAM*) (16, 33, 42). Some *cysB* mutants are known to derepress various *cys* operon functions (15, 21). When *modB::Tn5* was transduced into the *cysB93* background, the production of FHL activity became independent of medium composition (Table 6), suggesting that this *cysB93* allele derepresses the sulfate transport system. These results support the conclusion that molybdate is transported by the sulfate transport system in *E. coli*. It is not known whether the sulfate transport system is used for molybdate transport only in the absence of the molybdate-specific transport system or if both systems are capable of transporting molybdate in wild-type cells under certain conditions, as in the case of cells cultured in LSM.

The ability of higher molybdate concentrations to support the production of FHL by *mod* mutants grown in LB indicates that another, probably nonspecific anion transport system is also involved in molybdate transport.

Regulation of the *mod* operon. Strain SE2069, a $\Phi(modA'-$

TABLE 5. Amounts of molybdate needed for expression of FHL activity by *mol* mutants in LSM^a

Strain	Mo concn for half-maximal activity		Optimum [Mo]	
	- SO ₄ ⁻²	+ SO ₄ ⁻²	- SO ₄ ⁻²	+ SO ₄ ⁻²
BW545	ND ^b	ND	<12 nM	<12 nM
SE2010	0.016	0.43	0.26	3.0
SE2069	0.100	1.95	1.00	5.0
SE1595	0.370	7.00	1.80	ND
SE1597	0.230	6.50	1.20	ND
SE1605	0.200	5.00	1.90	ND
JBM239-7	0.010	0.24	0.13	0.5

^a Cells were grown in LSM with different concentrations of molybdate. The concentrations of molybdate required for production of half-maximal FHL activity and the minimum amounts of Mo needed for production of maximum activity were determined. Data are micromolar Mo concentrations, unless otherwise indicated. The sodium sulfate concentration in the growth medium was 40 mM.

^b ND, not done.

lacZ⁺) mutant (29), produced high levels of β -galactosidase activity when it was cultured in LBG medium (Table 7), which will not support the production of FHL activity in *mod* mutants. The addition of molybdate to LBG medium lowered the production of *mod*-dependent β -galactosidase activity. Culturing strain SE2069 in LSM (without externally added molybdate) increased the derepressed levels of β -galactosidase activity by about twofold to about 3,700 units (data not presented). Complementation of the *mod* mutation in strain SE2069 by plasmids pSE1004 and pSE1009 lowered the production of β -galactosidase activity even in the absence of added molybdate. Similar results were also obtained with strain SE2010, another $\Phi(mod'-lacZ^+)$ mutant (data not presented). These results suggest that the *mod* operon is expressed at very low levels in *mod*⁺ strains.

To test this possibility, the $\Phi(modA'-lacZ^+)$ mutation from strain SE2069 was transferred to λ (λ SE1) and incorporated into the chromosome (probably at the λ att site). When λ SE1 was present in a *mod*⁺ strain (BW545), the levels of β -galactosidase activity produced by culture were very close to the detection limit (Table 7). However, when λ SE1 was transduced into *mod* mutants, the levels of β -galactosidase activity produced by transductants grown in LBG medium increased to about 2,000 units. The addition of molybdate to the growth medium reduced this level to less than 200 units. In this regard, these transductants were comparable to strain SE2069, a *modA-lac* fusion mutant. Similar results were also obtained when *modA115*, *modB125*, or *modC118* was transduced into strain BW545(λ SE1) (data not presented). These results suggest that molybdate starvation (introduced by a mutation) increases the level of transcription of the *modABCD* operon by over 175 times [about 3,700 units of β -galactosidase activity for

TABLE 6. Effects of *cys* mutations on dihydrogen production by *modB* mutants

Strain	Relevant genotype	FHL activity ^a			
		LBG	LBG-Mo	LSM	LSM-Mo
JM2314	<i>cysA97</i>	+	+	+	+
SE1325	<i>modB::Tn5</i>	-	+	+	+
SE1853	<i>cysA97 modB::Tn5</i>	-	+	-	+
SE1854	<i>cysB93 modB::Tn5</i>	+	+	+	+

^a The molybdate concentrations were 1 mM in LBG and 0.05 mM in LSM. See Materials and Methods for details of FHL assays. +, activity; -, no activity.

strain SE2069 grown in LSM compared with about 20 units for strain BW545(λ SE1) (Table 7)].

After transduction with λ SE1, a class of *mol* mutants with defects in molybdate metabolism (29) [strains SE1581(λ SE1), SE1586(λ SE1), and SE1588(λ SE1)] produced extremely low levels of β -galactosidase activity even when they were cultured in LBG medium (Table 7). The addition of molybdate to LBG medium had no effect on the levels of β -galactosidase activity produced by these strains. Transductants of strain SE1595 (*modC*) carrying λ SE1 that had been isolated from these strains produced high levels of β -galactosidase activity, suggesting that $\Phi(modA'-lacZ^+)$ in λ SE1 had not been altered during initial strain construction. These results suggest that these unique *mol* mutants are capable of transporting molybdate and that the block in molybdate metabolism in these strains is past the branch point of regulation.

Effects of MGD synthesis on regulation of the *mod* operon.

Since the availability of molybdate appeared to regulate transcription of the *mod* operon, the role of MGD synthesis in this regulation was investigated. For these experiments, known mutations in the *moa*, *mob*, and *moe* operons were transduced into strain SE2069 and the levels of β -galactosidase activity produced by these transductants were determined. The results presented in Table 8 show that mutations in the operons coding for the synthesis of MGD had no detectable effect on *mod* operon transcription and regulation. Mutations in the *mog* operon which allow the production of FHL activity but limit the production of nitrate reductase in a molybdate-deficient medium (46) also did not influence *mod* operon transcription (Table 8).

The levels of *mod* operon transcription were enhanced by about twofold when strain SE2069 was cultured under strictly aerobic growth conditions (Table 9). A similar increase was also observed with the *fnr* genetic background under anaerobic growth conditions. Analysis of the *modABCD* operon DNA sequence did not reveal putative FNR protein binding motifs in the sequence, and the twofold increase observed for the *modA-lac fnr* double mutant could be a physiological effect of the *fnr* mutation on molybdate transport. Another global regulator which regulates respiratory systems, Arc (19), did not affect the regulation of *mod* operon transcription under these conditions (Table 9), nor did mutations in the *narL* and *cya* genes (data not presented).

TABLE 7. Effects of *mod* mutations on *mod* operon transcription

Strain	<i>mod</i> genotype(s)	β -Galactosidase activity ^a	
		– Mo	+ Mo
SE2069	$\Phi(modA'-lacZ^+)$	2,400	250
SE2069(pSE1004)	<i>mod(mod⁺)</i>	20	ND
SE2069(pSE1009)	<i>mod(mod⁺)</i>	10	ND
BW545(λ SE1)	<i>mod⁺</i>	20	<10
SE1592(λ SE1)	<i>modA</i>	2,330	165
SE1595(λ SE1)	<i>modC</i>	1,980	150
SE1602(λ SE1)	<i>modB</i>	1,860	60
SE1581(λ SE1)	<i>mol</i>	40	20
SE1586(λ SE1)	<i>mol</i>	70	50
SE1588(λ SE1)	<i>mol</i>	75	90

^a Cultures were grown in LBG medium with and without added sodium molybdate (1 mM). β -Galactosidase activities are expressed in nanomoles minute⁻¹ milligram of cell protein⁻¹. ND, not done.

TABLE 8. Regulation of the *mod* operon

Strain	Relevant genotype	β -Galactosidase activity ^a	
		– Mo	+ Mo
SE2069	$\Phi(modA'-lacZ^+)$	2,400	250
SE1849	$\Phi(modA'-lacZ^+) moa$	2,430	480
SE1850	$\Phi(modA'-lacZ^+) mob$	2,250	360
SE1851	$\Phi(modA'-lacZ^+) moe$	2,650	210
SE1852	$\Phi(modA'-lacZ^+) mog$	2,860	220

^a Cells grown in LBG medium with and without sodium molybdate (1 mM) were assayed as described in Materials and Methods. Enzyme activities are expressed in nanomoles minute⁻¹ milligram of cell protein⁻¹.

DISCUSSION

Transcription of the *fdhF* operon, coding for the molybdo-protein FDH-H, and the *hyc* operon, coding for HYD3 and other intermediate electron carriers of the FHL complex in *E. coli*, requires FHLA protein, formate, molybdate, and the σ 54 subunit of RNA polymerase (14, 28, 39). Among these, the requirement of molybdate for *hyc* operon transcription is unique since none of the proteins encoded by the *hyc* operon is known to contain molybdate (3). Results reported above (Table 4) show that molybdate, not molybdopterin or MGD, is the active component of this regulation. The molybdate requirement for transcription of the *hyc* operon also demonstrates that the components of the FHL system are coordinately synthesized to produce the active membrane-bound complex.

Previous studies have established that mutant strains of *E. coli* which exhibit defects in the transport of molybdate also fail to transcribe the *hyc* and *fdhF* operons (23, 27, 39). By using these mutants strains, the genes coding for the transport of molybdate have been cloned and the DNA sequence revealed the presence of four genes (29). Mutant phenotypes, complementation analysis, and homology to other known periplasmic binding protein-dependent transport systems clearly show that three of these four gene products function in the transport of molybdate (ModA, ModB, and ModC proteins [Fig. 1]). Although the role of ModD protein in cells is not known, preliminary experiments suggest that this protein is an outer membrane protein which helps transport molybdate into the periplasm (10).

The sulfate transport system apparently transports molybdate when it is fully derepressed (limiting sulfur conditions) (SBP, CysA, CysT, and CysW proteins [Fig. 1]), and the role of

TABLE 9. Regulation of the *mod* operon by global regulators^a

Conditions and strain	Relevant genotype	β -Galactosidase activity ^b	
		– Mo	+ Mo
Anaerobic			
SE2069	$\Phi(modA'-'lacZ^+)$	1,900	90
SE1797	$\Phi(modA'-'lacZ^+) arcB$	2,000	70
SE1804	$\Phi(modA'-'lacZ^+) arcA$	1,500	40
SE1808	$\Phi(modA'-'lacZ^+) fnr$	3,800	200
Aerobic			
SE2069	$\Phi(modA'-'lacZ^+)$	3,700	160
SE1808	$\Phi(modA'-'lacZ^+) fnr$	4,100	170

^a The culture and assay conditions are described in Materials and Methods.

^b Enzyme activities are expressed in nanomoles minute⁻¹ milligram of cell protein⁻¹.

FIG. 1. Pathways of molybdate transport and metabolism in *E. coli*. This diagram presents some of the known components of molybdate metabolism, leading to the production of FHL activity. ModA, ModB, and ModC are the three molybdate transport components coded by the *modABC* genes (29). SBP is the periplasmic sulfate-binding protein. CysA, CysT, and CysW are the components of the sulfate transporter (42). The third ill-defined transporter (?) is probably a nonspecific anion transport system. Upon entering the cytoplasm, molybdate is channelled to one of three pathways (I, II, or III). See text for details.

each component of the sulfate transport system in molybdate transport needs to be evaluated by direct transport studies. However, the amount of molybdate needed for production of optimum levels of FHL was at least 10 times higher when the sulfate transport system was used by *mod* mutants compared with the amount needed when the native molybdate transport system was used (Table 5). Although physiological and genetic experiments show that sulfate transport proteins function in molybdate transport, *mod*⁺ *cysA* mutants were unable to use the molybdate transport system for transporting sulfate under the growth conditions tested. *cysA* mutants defective in sulfate transport remained Cys⁻ even in the presence of multicopy plasmids containing *mod*⁺ DNA. This may have been due to the very low levels of *mod* operon expression in *mod*⁺ strains (Table 7). When they were cultured in low-molybdate glucose-minimal medium which allows limited derepression of the *mod* operon, *cysA* mutants were phenotypically *cys*⁺ (10). These results show that molybdate and sulfate can be transported by either transport system and that the specific transport system used by anions depends on the regulation and availability of one or the other. Besides the four *mod* gene products, there are other proteins whose requirement for the production of FHL activity (MGD synthesis) can be suppressed by an increase in molybdate concentration, but only in specific media (*mol* mutants [Table 7]). This is in contrast to the *mod* mutants, which respond positively to molybdate in all of the media

tested. Upon entering the cytoplasm, molybdate needs to be reduced to the appropriate valence state for incorporation into molybdopterin, probably in a manner analogous to the reduction of sulfate to sulfide. It is possible that these *mol* mutants are defective in the enzyme(s) responsible for this reduction of molybdate. When various *cys* genes are induced (growth in glucose-minimal medium or LSM), the enzymes responsible for activation and reduction of sulfate (21) may substitute and suppress the mutations in these mutants. The need for higher concentrations of molybdate by these mutants for the production of FHL activity may be explained by the enzyme's interaction with a nonnative substrate (analog?). It is interesting that adenosine 5'-phosphosulfate sulfurylase has been demonstrated to catalyze molybdate-dependent hydrolysis of ATP (47). The reduction of sulfate to sulfide and of molybdate to activated Mo could follow similar steps.

Mouse L929 fibroblastic cells exhibited a phenotype similar to that of the *E. coli mol* mutant strains SE1586 and SE1588 (7). These cells are apparently normal for molybdate transport and molybdopterin precursor synthesis but required millimolar levels of molybdate for the production of active xanthine oxidoreductase. It is possible that these unique *mol* mutants of *E. coli* and L929 fibroblastic cells carry mutations in genes whose functions in cells are similar.

A nonspecific anion transport system which can transport sulfate, selenate, selenite (26), and probably molybdate in *E.*

coli has been described (Fig. 1). This transport system may account for molybdate transport in *mod* mutants cultured in rich medium in which the sulfate transport system is repressed by the presence of sulfur compounds. This third transport system is of low efficiency and requires molybdate at higher concentrations than those for the other two transport systems.

As presented in Fig. 1, molybdate enters cells through the activities of *modABC* gene products. In a *modA*, *-B*, or *-C* mutant, molybdate is transported by one of two alternate molybdate transport systems, the sulfate transport system or the nonspecific anion transport system. In the absence of the sulfate transport system, molybdate is transported via the nonspecific anion transport system. Once molybdate enters cells, regardless of the portal of entry, it branches into the following three separate pathways: (i) regulation of the *mod* operon (pathway I [Fig. 1]); (ii) regulation of the *hyc* and *fdhF* operons (pathway II [Fig. 1]); and (iii) reduction of molybdate for incorporation into the MGD precursor (pathway III [Fig. 1]).

Regulation of the *mod* operon (pathway I [Fig. 1]). A molybdate sensor which also acts as an aporepressor for the *mod* operon binds molybdate and regulates (represses) transcription of the *mod* operon. In a wild-type strain growing in medium without added molybdate, the *mod* operon is normally repressed because of trace amounts of molybdate in the culture medium as a contaminant of medium components (Table 7). Apparently, the Mod proteins in the cell membrane are present at very low levels; consequently, the rate of molybdate transport in a wild-type cell is also very low. In a *mod* mutant, the *mod* operon is fully derepressed in media not supplemented with molybdate while the addition of molybdate to media, regardless of the alternate transport system used by the mutant, represses the *mod* operon. The observed regulation of the *mod* operon by molybdate is similar to the results reported by Amy and her colleagues for JBM239-7 and other *chdD-lac* (*mod*) mutants (31).

The presence of a four-base inverted repeat (TAACGTTA) starting at position +4 in the *modABCD* operon (29) is interesting. This is reminiscent of the 4-base inverted repeats found in the repressor binding regions of the *met* and *trp* operons (AGACGTCT and AGTTAACT, respectively) (22, 36, 37). This 8-base region may serve as the repressor binding region for the *mod* operon in a manner analogous to those in the *met* and *trp* operons.

Besides this specific molybdate-dependent regulation, transcription of the *mod* operon was enhanced by the presence of oxygen (Table 9), probably by the inactivation of FNR protein, which controls the transcription of oxygen-regulated enzyme systems (44) either directly or indirectly. This is in contrast to the reported requirement of anaerobic conditions for expression of the *moa* operon, which codes for the enzymes in the molybdopterin biosynthetic pathway (2). Apparently, the physiological responses of the *mod* and *moa* operons are distinct; thus, they are regulated differently.

Activation of the *hyc* operon (pathway II [Fig. 1]). This is the opposite of repression of the *mod* operon. Whether the *mod* operon repressor also functions in activating the *hyc* operon or another protein plays this role is unknown. Recently, Bock and his associates reported that the *fdhF* and *hyc* operons can be transcribed and translated in an in vitro system with FHLa, IHF, and formate (14). This transcription system does not require the presence of molybdate as an additional effector. Although a role for molybdate in in vivo transcription of the *fdhF* and *hyc* operons can be demonstrated, this need for molybdate was not observed under the conditions used in these experiments in an in vitro system. Molybdate is also not needed for transcription of the *fhla* gene, whose product is the acti-

vator of the *hyc* operon (27). Additional experiments are needed to identify the mechanism by which the molybdate requirement for transcription of the *hyc* operon is transduced.

Molybdate activation and reduction (pathway III [Fig. 1]). Molybdate is converted to an appropriate valence state before its culmination as MGD in the molybdoprotein. Up to a certain point in this pathway, defects can be suppressed by increasing the concentration of molybdate in the medium (*mol* mutants) (29).

In summary, transcriptional analyses of various *mod* and *hyc* operon mutants show that molybdate, not the Mo-molybdopterin component or MGD, is the main effector of control for these operons. Although three independent pathways for molybdate transport in cells exist, the specific pathway of transport for regulation of the *mod* operon depends on the strain genotype and medium composition.

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