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Identification of a New Gene, *molR*, Essential for Utilization of Molybdate by *Escherichia coli*†

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A mutation in a new gene, *molR*, prevented the synthesis in *Escherichia coli* of molybdoenzymes, including the two formate dehydrogenase isoenzymes, nitrate reductase and trimethylamine-*N*-oxide reductase. This phenotype was suppressed by supplementing the media with molybdate. Thus, the *molR* mutant was phenotypically similar to previously described *chlD* mutants, thought to be defective in molybdate transport. The *molR* gene is located at 65.3 min in the *E. coli* chromosome, in contrast to the *chlD* gene, which maps at 17 min and thus can be readily distinguished. The *molR* gene is also cotransducible with a hitherto unidentified gene essential for the production of 2-oxoglutarate from isocitrate, designated *icdB* (located at 66 min). The *molR* mutant strain SE1100 also failed to produce the hydrogenase component of formate hydrogenlyase (HYD3) in molybdate-unsupplemented media. The amount of molybdate required by strain SE1100 for the production of parental levels of formate hydrogenlyase activity was dependent on the growth medium. In Luria-Bertani medium, this value was about 100 μM, and in glucose-minimal medium, 1.0 μM was sufficient. In low-sulfur medium, this value decreased to about 50 nM. The addition of sulfate or selenite increased the amount of molybdate needed for the production of formate hydrogenlyase activity. These data suggest that in the absence of the high-affinity molybdate transport system, *E. coli* utilizes sulfate and selenite transport systems for transporting molybdate, preferring sulfate transport over the selenite transport system.

Several proteins in *Escherichia coli* contain molybdate in the form of molybdopterin (20, 22, 39). The two formate dehydrogenase (FDH-H and FDH-N) isoenzymes, nitrate reductase and trimethylamine-*N*-oxide reductase, produced only under anaerobic growth conditions, are molybdoproteins and play critical roles in the anaerobic metabolism of *E. coli*. Products of several genes are needed for effective transport and biochemical conversion of molybdate to molybdopterins and incorporation of molybdopterin into the appropriate site in the apoprotein (6, 14, 37–40). Among the several genes, *chlD* and *chlG* are unique, because the effects of a mutation in either gene can be readily suppressed by an increase in the molybdate concentration in the medium (38, 40). The actual physiological roles of the *chlD* and *chlG* products are not known, but the *chlD* gene product is conceived to be a component of the molybdate transport system in the cell (19, 37).

The *chlD* mutant strain lacks the ability to produce hydrogen gas as a fermentation end product in the absence of externally added high concentrations of molybdate. This is due to the absence of FDH-H, a component of the formate hydrogenlyase (FHL) complex which contains electron transport protein(s) and a specific hydrogenase isoenzyme (FHL-hydrogenase; HYD3) besides the FDH-H (15, 16, 32, 35, 36). Supplementation of the growth medium with 1 mM molybdate leads to the synthesis of active FHL by this mutant. Mutations in *chlG* allow H_2 production (40). Recently, Schlensog et al. have reported that the transcription of *fdhF* and *hyd-17* genes which code for FDH-H and HYD3, respectively, requires molybdate (36). During the course of our studies on the hydrogen metabolism of *E. coli*, we isolated mutant strain SE1100, which lacked FHL activity. The mutational effect in this strain can be readily alleviated by increasing the molybdate concentration in the medium. Although the overall phenotypic characteristics of mutant strain SE1100 (*molR*) were similar to those of previously described *chlD* mutants (39), the *molR* mutant can be distinguished genetically. In this paper, the physiological, biochemical, and genetic characteristics of *molR* mutant strain SE1100 are presented.

MATERIALS AND METHODS

Bacterial strains. Bacterial and bacteriophage strains used in this study are listed in Table 1.

Media and growth conditions. Luria-Bertani (LB) medium was used as the rich medium without or with glucose (0.3%; LBG) supplementation. The glucose-minimal medium composition is as follows: Na_2HPO_4, 6.25 g; KH_2PO_4, 0.75 g; NaCl, 2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 1 g; $FeSO_4 \cdot 7H_2O$, 10 mg; $Na_2MoO_4 \cdot 2H_2O$, 10 mg; Na_2SeO_3, 0.1623 mg; glucose, 3 g; and deionized water, 1 liter. The glucose concentration of the medium was increased to 10 g/liter for anaerobic growth of *E. coli*. A low-sulfur medium which still supported good anaerobic growth contained (in 1 liter of deionized water) Trypticase Peptone (BBL Microbiology Systems), 1.0 g; yeast extract (BBL), 0.5 g; NaCl, 1.0 g; phosphate or HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.0), 0.1 M; and glucose, 10 g. Bacterial cultures were grown under aerobic or anaerobic conditions, as described previously (21, 33, 34).

Genetic experiments. Hfr-mediated conjugation, complementation analysis with *E. coli* F′ elements, and transduction experiments with bacteriophage P1 were performed as described by Miller (27). Transposon Tn*5* mutagenesis of plasmids pSE1004 and pSE1009 was as described previously (34). The physical location of Tn*5* in the *molR* gene was identified as described previously (9, 34).

Construction of Hfr (PO-*molR*). In order to construct a

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TABLE 1. Bacterial and phage strains used in this study

Strain	Genotype	Source or reference
Escherichia coli		
AT2446	Hfr *thi-1 metC69 relA1 spoT1*	CGSC[a] 4504
AT2699	F⁻ *hisG1 thyA3 metC69 argG6 lacY1 gal-6 malA1 rpsL tsx-1* λ^r λ^-	CGSC 4524
BW545	Δ(*lacU*)*169 rpsL*	G. Walker (45)
CSH26	F⁻ *ara* Δ(*lac-pro*) *thi*	Laboratory collection
Hfr3000	Hfr *thi-1 relA1 spoT1* λ^- *supQ80*	CGSC 259
LE392	F⁻ *supF supE hsdR galK trpR metB lacY tonA*	J. Beckwith
MBM7014	F⁻ *araC*(Am) *araD* Δ(*argF-lacU*)*169 trp*(Am) *malB*(Am) *rpsL relA thi supF*	G. Weinstock
MC4100	F⁻ *araD139* Δ(*argF-lacU*)*205 rpsL150 relA1 flbB5301 deoC1 ptsF25*	CGSC 6152
P3478	F⁻ *thyA36 polA1 deoC2* IN(*rrnD-rrnE*)*1*	CGSC 4303
SE29	*thi-1 leu-6 suc-10 bioA2? galT27 rpsL129 chlC3 hup*	(21)
SE1001	*cysC43 thr-1 leu-6 thi-1 lacY1 galK2 ara14 xyl-5 mtl-1 proA2 his-4 argE3 rpsL31 tsx-33 supE44*	Laboratory collection
SE1100	BW545 Φ(*molR*::*lacZ*⁺)*1*	This study
SE1119	Hfr3000 Φ(*molR*::*lacZ*⁺)*1*	P1 transduction (Hfr3000 × SE1100)
SE1130	SE1100 *molR*⁺ *zgg-3*::Tn*10*	This study
SE1157	MC4100 *icdB zgg-3*::Tn*10*	This study
SE1158	MC4100 *icdB zgg-3*::Tn*10*	This study
SE1187	SE1100 λ P1(209)	This study
SE1319	P3478 *molR2*::Tn*5*	This study
SE1325	SE1001 *molR2*::Tn*5*	P1 transduction (SE1001 × SE1319)
SE1433	SE1100 *icdB zgg-3*::Tn*10*	This study
SE1438	CSH26 Φ(*molR*::*lacZ*⁺)*1*	P1 transduction (CSH26 × SE1100)
SE1443	SE-1438(F'ts114 *lac*⁺ *zzf*::Tn*10*)	This study
SE1449	SE-1100 *hup zgf-1*::Tn*10*	This study
SE1455	SE-1100 *hup zgf-1*::Tn*10*	This study
SE1459	SE-1443 Hfr (PO-*molR*)	This study
Salmonella typhimurium		
TT627	*strA1 pyrC7*(F'ts114 *zzf*::Tn*10 lac*⁺)	J. Roth (7)
Phages		
P1	Tn*9* Cm^r *clr-100*	L. Csonka
λ NK421	*b*221 *rex*::Tn*5* *c*I857 *O*am23 *P*am80	J. Beckwith
λ NK561	*b*221 *c*I::Tn*10* *O*am29 *P*am80	N. Kleckner (13)
λ p1(209)	(*b-xis*)[+'Mu)::(*trp'BA'lac'OZY*)]	G. Walker
λ p*lac*Mu53	*imm 'trp'lacZ*⁺*lacY*⁺*lacA' 'uvrD' xho*::*kan* Mu[*c*Its62 *ner*⁺*A*⁺*S*]	G. Weinstock (5)
λ pMu507	*c*I857 *S*am7 Mu[*c*Its62 *ner*⁺*A*⁺*B*⁺]	G. Weinstock (5)

[a] CGSC, *E. coli* Genetic Stock Center.

donor Hfr strain in which the point of origin for DNA transfer is in the *molR* gene, a temperature-sensitive episome, F'ts114 *lac*⁺ *zzf*::Tn*10* (7), was introduced by conjugation from donor strain TT627 into strain SE1438, which carries the Φ(*molR-lacZ*⁺)*1* mutation in the chromosome. Exconjugants carrying the F' element were selected at 30°C on lactose-minimal medium containing tetracycline and streptomycin. One of the exconjugants (Tc^r Sm^s Lac⁺; strain SE1443) was incubated at 42°C, and those strains in which the episome has recombined into the chromosome by using the *lac* homology were selected as colonies on lactose-minimal medium containing tetracycline (strain SE1459).

Random mutagenesis with transposon Tn*10*. To facilitate strain constructions, random transpositions of Tn*10* into the chromosome of *E. coli* MC4100 were obtained by using λ-NK561 by the method of Foster et al. (13), with some modifications. Strain MC4100 grown to mid-log phase (5×10^8 CFU/ml) in LB medium containing 0.4% maltose was harvested by centrifugation at room temperature and suspended in 10 mM $MgSO_4$. Phage λ-NK561 was added to the cell suspension at a multiplicity of 1.0 and allowed to adsorb for 30 min at room temperature. Cells were sedimented by centrifugation, and the resulting cell pellet was suspended in 1.0 ml of LB medium. The infected cell suspension was plated on LB plates containing tetracycline, and the plates were incubated at 37°C. About 5,000 tetracycline-resistant colonies were pooled. Phage P1 grown in this pool of cells was used for further experiments.

Isolation of mutations in genes near the *molR* gene. Two different types of mutations which cotransduce in bacteriophage P1-mediated transduction experiments were selected. The first type was selected to isolate Tn*10* insertions in a gene near the *molR* gene and use the Tc^r phenotype of the mutant for mapping purposes. The second type was an auxotrophic mutation(s) in the region, isolated by the localized mutagenesis procedure.

For isolation of Tn*10* mutants, phage P1 grown in the MC4100-Tn*10* pool was used to transduce strain SE1100 to tetracycline resistance. The Tc^r transductants were tested for Km^s [loss of Φ(*molR*::*lacZ*⁺)1; Km^r phenotype]. Three Tc^r Km^s mutants (from a total of 233 Tc^r transductants) were found to produce FHL activity. These three mutants had no other identifiable phenotype besides that of tetracycline resistance. One of these three strains, SE1130, was selected and designated *zgg-3*::Tn*10*.

Localized mutagenesis. Mutations in genes near the *molR* gene were introduced by using the procedure of Hong and Ames (18) as modified by Murgola and Yanofsky (28). Bacteriophage P1 grown in strain SE1130 was mutagenized with hydroxylamine and used to transduce strain MC4100.

Strain SE1130 carries the *zgg-3*::Tn*10* mutation, which is cotransducible with the *molR* gene. The tetracycline-resistant transductants were selected, and the auxotrophs in the population were identified by replica plating techniques. Two mutants (strains SE1157 and SE1158) grew in glucose-minimal medium supplemented with 2-oxoglutarate but not with citrate or isocitrate. The defect in these mutant strains is probably in the production of active isocitrate dehydrogenase, although this was not biochemically confirmed. The genotype of these mutants was tentatively designated *icdB* to distinguish it from the previously described *icd* gene which maps at 25 min in the *E. coli* chromosome (2).

Enzyme assays. The preparation of cells for enzyme assays and the conditions used for various enzyme assays were as described previously (21, 33, 34). All enzyme activities were determined with whole cells, to eliminate the problems associated with oxygen inactivation of enzyme activity during the preparation of crude extracts.

The general procedures used for preparation of plasmid DNA, restriction endonuclease digestion, gel electrophoresis, manipulation of DNA, and transformation of plasmid DNA into *E. coli* cells were as described previously (25, 33, 34).

Materials. Biochemicals were purchased from Sigma Chemical Co. Analytical-grade inorganic or organic chemicals were obtained from Fisher Scientific Co. and used without further purification.

RESULTS

Isolation of *molR* mutant. Although later studies identified strain SE1100 as defective in the utilization of molybdate, the initial screen was for mutants lacking FHL activity when grown in LB medium. Since FDH-H is a component of the FHL complex, this enzyme was used as the identifiable marker. For these experiments, strain BW545 was mutagenized with λ-*plac*Mu53 (with λ-pMu507 as a helper), and the resulting Km^r mutants were isolated (5). Mutant strains lacking FDH-H activity were identified by the dye-overlay procedure (24). Four mutant strains which failed to reduce benzyl viologen, using formate as the electron donor, were identified. Among the four mutants, strain SE1100 produced FHL activity when grown in glucose-minimal medium and thus was the mutant of choice in this study.

The FDH-H activity was not detected in strain SE1100, and consequently FHL activity also was not detected when the cells were grown in LB medium. However, low levels of the formate-dependent reduction of phenazine methosulfate, presumably FDH-N activity (associated with nitrate respiration), were detected in the mutant. The levels of hydrogenase (determined as tritium exchange activity) and hydrogen uptake (HUP) activities (using either benzyl viologen or fumarate as the electron acceptor) of the parent and mutant strains were comparable. The addition of glucose (0.3%) or formate (0.1%) to LB medium did not influence the levels of FDH-H and FHL activities of the mutant strain. However, when grown in glucose-minimal medium, strain SE1100 produced both FDH-H and FHL activities, but the levels of the two enzymes in the cell did not reach the levels of the parent strain BW545 grown in the same medium.

E. coli produces three hydrogenase isoenzymes, and transcription of the HYD3 structural gene requires molybdate (3, 4, 35, 36). The presence of hydrogenase in strain SE1100 raised the possibility that this hydrogenase is the isoenzyme involved in the HUP system and that the mutant strain lacks the hydrogenase isoenzyme(s) linked to the FHL. In order to test this possibility, a mutation in the *hup* gene (21), which abolishes the production of HUP hydrogenase activity, was transduced from strain SE29 into strain SE1100. Double mutant (*molR hup*) strain SE1455, cultured in LB-glucose medium, produced very low levels of hydrogenase activity even when assayed by the sensitive tritium exchange method. Loss of this activity correlated with the absence of HUP activity in this double mutant. Strain SE1100 obviously lacks both FDH-H and the FHL-linked hydrogenase (HYD3) activities when cultured in LB medium. Strain SE1100 also produced β-galactosidase activity constitutively under both aerobic and anaerobic conditions.

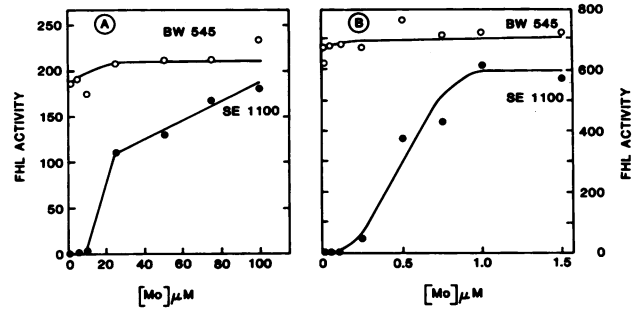

FIG. 1. Effects of addition of molybdate to the growth medium on production of FHL activity by *E. coli* mutant strain SE1100 and its parent strain BW545. (A) LB-glucose medium; (B) glucose-minimal medium. FHL activity is expressed as nanomoles of H_2 produced per minute per milligram of cell protein.

Effects of molybdate. The presence of FHL activity in cells cultured in minimal medium raised the possibility that a medium component present in the minimal medium is essential for the production of active FHL or that a medium component present in the LB medium is detrimental to the synthesis of active FHL. The first possibility was tested by supplementing the LB medium with different compounds present in the minimal medium. These included phosphate, sulfate, NH_4^+, and trace minerals (Fe, Mo, Ni, and Se). Only the trace minerals were found to support the production of FHL activity. Among the different salts present in the mixture, only molybdenum supplementation of the LB medium restored the synthesis of FHL activity. Strain SE1100 produced about 65% of the parent levels of FHL activity in this medium. This phenotypic suppression is unique for molybdate; other compounds, such as selenite, iron, or nickel, had no effect.

The amount of molybdate needed by strain SE1100 to maximally express the FHL activity in LBG medium was about 100 μM (Fig. 1A). The addition of Mo up to 10 μM had no detectable effect on the production of FHL activity. Although the FHL activity of the cells increased with increasing Mo concentration in the growth medium (10 to 100 μM), this increase was moderate between 20 and 100 μM. The concentration of Mo needed for the production of half the maximal activity (110 U) in LBG medium was about 20 μM. The parent strain grown under similar conditions produced more than 80% of the maximum inducible FHL activity in LBG medium which was not supplemented with Mo (Fig. 1A). The addition of Mo did increase the levels of FHL activity, but only slightly (210 U at 20 μM).

Strain SE1100 lacked FHL activity when grown in glucose-minimal medium which was not supplemented with Mo (Fig. 1B). The cells produced FHL activity in response to increasing Mo concentration in the medium, and maximum levels of FHL activity were reached at about 1 μM, com-

TABLE 2. Effects of selenite on Mo-dependent hydrogenase, FDH-H, and FHL activities in *molR* mutant strain SE1100[a]

Strain	Relevant genotype	Additions to medium	Activities of:			
			FHL	FDH-H	HYD	HUP (BV)
BW545	Wild type	None	313	528	688	1,724
		Mo	149	ND	ND	1,957
		Mo and Se	334	ND	ND	2,028
SE1100	*molR*	None	UD	UD	762	1,492
		Mo	219	247	974	1,905
		Mo and Se	UD	UD	865	2,606
SE29	*hup*	None	337	335	552	59
		Mo	229	787	433	46
		Mo and Se	469	239	623	137
SE1455	*molR hup*	None	UD	UD	192	60
		Mo	335	382	780	187
		Mo and Se	ND	UD	610	380

[a] Activities of enzymes are expressed as nanomoles per minute per milligram of cell protein, except for hydrogenase, which is expressed as nanomoles of tritiated water produced per hour per milligram of cell protein. HUP activity was determined by using benzyl viologen (BV) as an electron acceptor. Whole cells grown in LB-glucose medium were used in the assay. Sodium molybdate and sodium selenite were added to the medium at final concentrations of 25 μM and 1 mM, respectively. ND, Not done; UD, undetectable.

pared with the requirement of about 100 μM in LBG medium (Fig. 1A). The amount of Mo needed for 50% of FHL activity was about 0.5 μM. It is also interesting to note that the FHL activity produced by both the parent and the mutant strains was about three times higher in glucose-minimal medium than in LBG medium. The difference could not be accounted for by the presence of formate in the glucose-minimal medium, since strain SE1100 grown in LB-glucose medium still produced lower levels of FHL activity, although formate was produced by the cultures as a fermentation product. The pH values of the cultures at the time of harvest were comparable in both media (about 5.5). These results suggest that there is a component(s) in the LB medium which suppresses the production of FHL activity; this component(s) is presented in a later section.

In the absence of molybdate, strain SE1100 did not produce either the 80-kilodalton selenopeptide associated with FDH-H or the 110-kilodalton selenopeptide, a component of FDH-N determined after labeling the selenocysteine in the proteins with $^{75}SeO_3^{2-}$ and subjecting them to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8). The addition of Mo to the growth medium led to the synthesis of both proteins. The selenopeptides associated with both FDH isoenzymes could be readily detected in parent strain BW545 in LB medium which was not supplemented with Mo.

Selenite effect. During the course of these studies, it was observed that when both Mo and Se were present in the medium at high concentrations, the levels of FHL activity produced by the mutant strain were lower than when Mo was the lone supplement. The addition of Mo alone (25 μM) to LBG medium restored the ability to produce FHL activity to *molR* mutant strain SE1100 and *molR hupA* double mutant strain SE1455, although not to the parental levels (Table 2). Both FDH-H and HYD3 (HYD activity produced by strain SE1455) activities were also detectable in cells grown with Mo. The addition of 1 mM selenite to this Mo-supplemented medium completely abolished the Mo-dependent production of FDH-H and FHL activities. Selenite had no detectable effect on the production of HYD3 by strain SE1455. Although the hydrogenase activity was unaltered and stayed at a higher level in the double mutant, H_2-dependent benzyl viologen reduction increased only to 380 U from 187 U with Mo alone. This value is still less than 20% of the HUP activity observed in the Hup^+ strain SE1100. These results show that selenite has a detrimental effect on FDH-H production and not on FHL-HYD (HYD3) production and that this effect can be seen only in the *molR* genetic background.

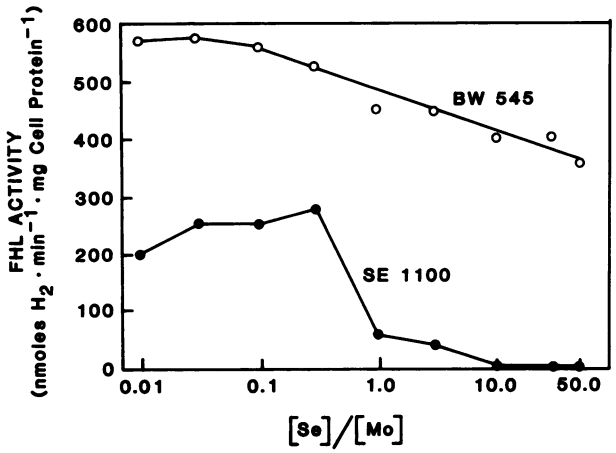

FIG. 2. Effects of increasing selenite concentration on FHL activity of *E. coli* SE1100 and BW545. Cells grown in LB-glucose medium with 25 μM molybdate and various concentrations of selenite were assayed for FHL activity.

The effect of selenite on the Mo-mediated production of FHL activity in strain SE1100 is dependent on the ratio of the two compounds (Fig. 2). A gradual increase in FHL activity in the mutant strain was observed, until a maximum of about 290 U at a ratio of 0.5 (Se/Mo ratio) was reached. At a ratio of 1.0, the amount of FHL produced by the cell is about 20% of the maximum value. FHL activity was not detected in the mutant beyond the ratio of 10.0. Parent strain BW545 showed very little change in the amount of FHL activity present in the cell up to a Se/Mo ratio of 0.1 in the medium. Increasing the selenite concentration beyond this level led to a gradual decline in the amount of FHL activity. High levels of FHL activity can be detected even at a ratio of 50 (400 U to an initial value of 580 U).

In these experiments, the concentration of Mo was kept at 25 μM, although the optimal Mo concentration needed in the medium for the mutant strain was found to be about 100 μM (Fig. 1). This suboptimal concentration of Mo was used in these experiments in order to avoid the toxic effects of high Se concentrations on cell growth, especially at high Se/Mo ratios. The use of LBG medium for these experiments was necessitated by the inability of *E. coli* to grow in glucose-minimal medium supplemented with high concentrations of selenite.

MolR is needed for nitrate and trimethylamine-*N*-oxide reductase synthesis. Besides the defect in H_2 production, strain SE1100 also lacked the ability to use NO_3^- as a terminal electron acceptor. When grown in a Mo-deficient, NO_3^--supplemented medium, strain SE1100 lacked NO_3^- reductase activity (Table 3). In the presence of molybdate, the NO_3^- reductase activity of the mutant was comparable to the levels of activity in the parent strain. The production of trimethylamine-*N*-oxide reductase, another molybdoprotein, was also dependent on the presence of molybdate in the

TABLE 3. Levels of Mo-containing enzymes in strains BW545 and SE1100 grown in the presence and absence of Mo and Se[a]

Strain	Additions to medium	Enzyme activities of:	
		Nitrate reductase	TMAO reductase
BW545	None	661	1,396
	Mo	634	1,480
	Mo and Se	661	1,176
SE1100	None	50	23
	Mo	561	876
	Mo and Se	615	524

[a] Units of enzyme activity are expressed as nanomoles per minute per milligram of cell protein. Cells grown in LB medium supplemented with either nitrate or trimethylamine-*N*-oxide (TMAO) were used for the assay. Molybdate and selenite were added as sodium salts at final concentrations of 25 μM and 1 mM, respectively.

medium (Table 3). The synthesis of active NO_3^- reductase and trimethylamine-*N*-oxide reductase was not influenced by the addition of selenite (1 mM) to the molybdate (25 μM)-supplemented medium.

The absence of nitrate reductase activity in strain SE1100 cultured in Mo-deficient medium also manifested itself as a chlorate-resistant phenotype. Strain SE1100 grew normally in LB medium supplemented with chlorate (0.2%) under anaerobic conditions, while the growth of the parent strain BW545 was undetectable in the same medium.

Effects of S compounds. The amount of Mo needed for complete restoration of FHL activity in glucose-minimal medium is less than 1% of the concentration needed for production of maximum levels in LBG medium (Fig. 1). This indicated that the utilization of molybdate in LB medium is inhibited by some medium component. Analysis of different constituents of LB medium indicated that cysteine and cystine inhibited the utilization of Mo for FHL production. In order to evaluate this further, a low-sulfur medium was used.

Glucose-minimal medium with 10 μM SO_4^{2-} supported the anaerobic growth of the organism only at a very low rate. This growth rate was not enhanced by the addition of buffers or trace minerals. In order to overcome this problem, the medium described in Materials and Methods was used as the low-S medium. Both the parent and mutant strains grew well in this medium. The parent strain produced FHL and HUP activities. Strain SE1100 produced low levels of FHL activity, although the HUP activity of the mutant was comparable to the parent values. In this medium, the FHL activity of strain SE1100 was detectable only during the late exponential phase to early stationary phase of growth in media which were not supplemented with molybdate. The FHL activity of the mutant reached about 70 U at a Mo concentration of about 60 nM and stayed constant at this level, which is about 65% of the parent values (Fig. 3). Increasing the concentration of Mo to over 100 μM and decreasing the amount of tryptone and yeast extract in the medium increased the specific activity of FHL in the mutant to values comparable to those obtained with the parent (about 100 U), but the cell yield was lower. However, the amount of Mo needed for the production of 50% of maximal activity under these conditions was found to be less than 10 nM (Fig. 3). The presence of high levels of FHL activity in the parent strain grown without Mo supplementation indicates that the parent strain is capable of utilizing the extremely small amounts of Mo present as a contaminant in the medium components. When the HEPES buffer used in these experiments was replaced

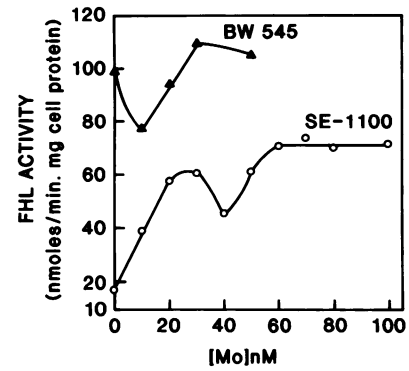

FIG. 3. Effects of increasing molybdate concentration on FHL activity of *E. coli* BW545 and SE1100 grown in low-S medium with HEPES buffer.

with other buffers, like phosphate, MOPS [3-(*N*-morpholino) propanesulfonic acid], TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid)], PIPES [piperazine-*N*,*N*'-bis (2-ethanesulfonic acid)], MES [2-(*N*-morpholino)ethanesulfonic acid], or Tricine [*N*-tris(hydroxymethyl)methylglycine], no significant difference was observed. In subsequent experiments, phosphate (0.1 M) was used to buffer the medium at a starting pH of 7.0.

Strain SE1100 lacked FHL activity if the Mo-supplemented growth medium also contained cystine. Methionine supplementation (0.5 mg/ml) reduced the FHL activity only partially (33% of the control value). Although the effect of cysteine was similar to that of cystine, cysteine reduced the growth rate and cell yield, probably a consequence of the altered redox potential of the medium. A similar effect was also observed with inorganic S compounds, sulfate, sulfite, and sulfide (5 mM each). Neither sulfate nor cystine influenced the levels of FHL activity in parent strain BW545. The minimum amount of sulfate and cystine required to completely abolish the production of FHL activity was found to be 2.0 mM and 7 μg/ml (29 μM), respectively, in the low-S medium with 50 nM molybdate (Fig. 4). Increasing the concentration of molybdate in the SO_4^{2-}-supplemented medium reversed the inhibitory effect of sulfate. In the presence of sulfate (5 mM), the amount of molybdate needed for the production of 50% of the maximal activity was increased from less than 10 nM to about 200 nM.

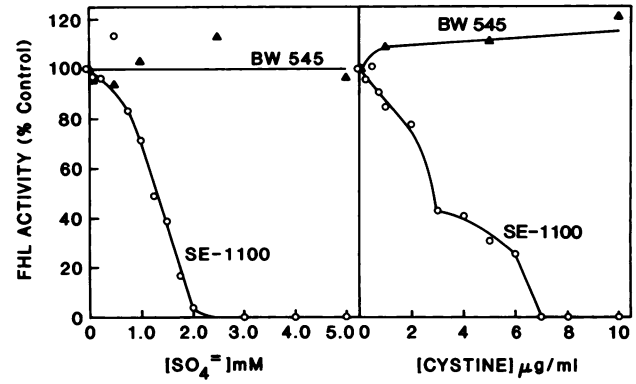

FIG. 4. Effects of increasing concentrations of sodium sulfate (SO_4^{2-}) or cystine on molybdate-dependent production of FHL activity of *E. coli* SE1100 and its parent strain BW545 grown in low-S medium supplemented with molybdate (50 nM).

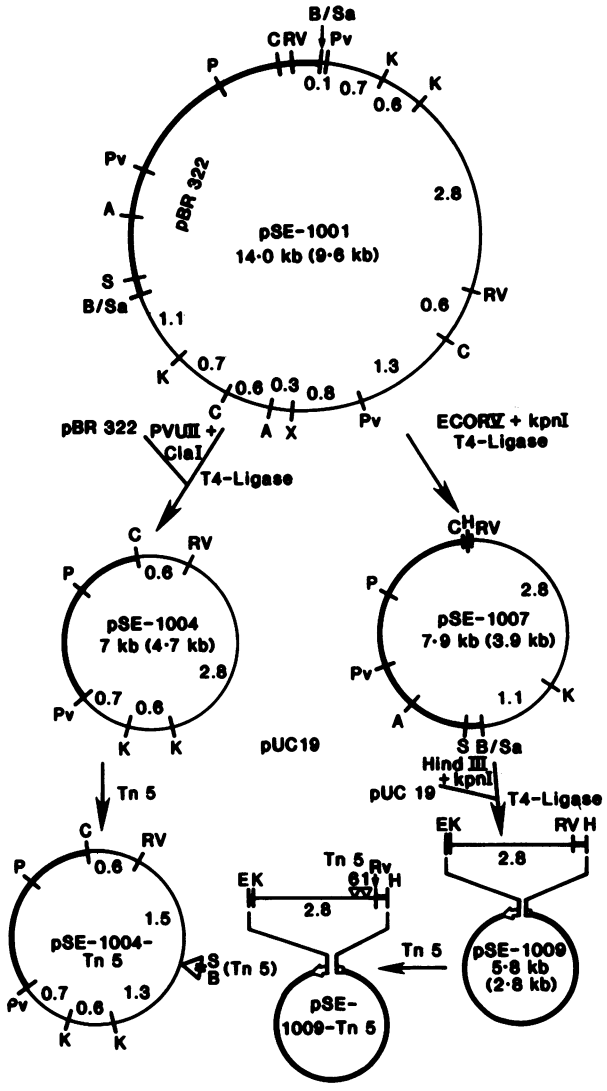

FIG. 5. Plasmid derivatives containing *E. coli* chromosomal *molR* DNA. A, *Ava*I; C, *Cla*I; E, *Eco*RI; RV, *Eco*RV; K, *Kpn*I; B, *Bam*HI; Sa, *Sau*3A; P, *Pst*I; Pv, *Pvu*II; X, *Xho*I; S, *Sal*I; H, *Hin*dIII. The location of the transposon Tn*5* in plasmids pSE1004-Tn*5* and pSE1009-Tn*5* is indicated by a triangle, and the numbers 1 and 6 in plasmid pSE1009-Tn*5* represent two different plasmid isolates. kb, Kilobases.

These results show that in the *molR* genetic background, the amount of molybdate needed for the production of FHL is influenced by the amount of sulfur compounds present in the medium.

Cloning the *molR* gene. Plasmid DNA from an *E. coli* gene bank containing random fragments of genomic DNA (33) was transformed into strain SE1100, and a transformant carrying the *molR* gene in a plasmid was identified as FDH-H positive by using the dye-overlay procedure (24). This plasmid was designated pSE1001 and maintained for further study. Plasmid pSE1001 contained a 9.6-kilobase *E. coli* chromosomal DNA fragment and pBR322 as vector plasmid (Fig. 5). Different segments of the chromosomal DNA were subcloned into vector plasmid pBR322, pUC18, or pUC19. These plasmid derivatives were transformed into strain SE1100, and all these plasmids (Fig. 5) complemented the defect, suggesting that the *molR*$^+$ gene resides within a 2.8-kilobase *Kpn*I-*Eco*RV fragment of *E. coli* chromosomal DNA.

Plasmids pSE1004 and pSE1009 were mutagenized with transposon Tn*5*, and the plasmid derivatives carrying the *molR*::Tn*5* mutation were selected and identified (plasmids pSE1004-Tn*5* and pSE1009-Tn*5*, respectively). The physical location of the transposon in these plasmids was determined, and these results show that the *molR* gene lies close to the *Kpn*I end of the 2.8-kilobase fragment.

Mapping the *molR* gene. Preliminary experiments revealed that the *molR* gene is not located in the region of the *E. coli* chromosome where other *chl* genes are found (17 to 27 min). To facilitate the localization of the *molR* gene in the *E. coli* chromosome, an Hfr-mediated conjugation analysis was carried out. For these experiments, an Hfr strain in which the origin of DNA transfer is located in the *molR* gene was constructed. One such strain, SE1459, was used as the donor, and strain AT2699 (*metC argG*) was used as the recipient. In this conjugation experiment, the donor and recipient cells were separated after 15 min and plated on appropriate media. Results from this experiment showed that the *argG*$^+$ gene was transferred early and that the *metC*$^+$ gene was not transferred during the 15-min conjugation period. The *argG* and *metC* genes map at 69 and 65 min in the *E. coli* chromosome, respectively, and the *molR* gene obviously lies between 65 and 69 min. These results also show that the direction of transcription of the *molR* gene is clockwise and towards the *argG* gene (Fig. 6).

The segment of *E. coli* chromosomal DNA between 65 and 69 min is not a region of intense study, and thus very few good genetic markers are available for transductional analysis. The *molR* mutation was also not cotransduced with either *metC* or *argG* genes, although the gene was located in this section by other independent methods (F′ complementation analysis; 23). This necessitated the isolation of other mutations in this region of the chromosome which can be used for mapping the *molR* gene more accurately. Also, the presence of a large amount of extraneous DNA introduced in this region during the isolation of the Φ(*molR*::*lacZ*$^+$) mutation may influence the cotransduction frequencies. In order to overcome this difficulty, the *molR*::Tn*5* mutation from plasmid pSE1004-Tn*5* was transferred to the chromosome of *polA* mutant strain P3478 (*molR2*::Tn*5*) by using the gene replacement technique (17). Several other mutants carrying *icdB* and/or *zgg-3*::Tn*10* mutations which are cotransducible with the *molR* gene were constructed.

Phage P1-mediated transduction analysis. The [Φ(*molR*::*lacZ*$^+$)*1* (Km^r)], *molR2*::Tn*5*, *icdB*, and *zgg-3*::Tn*10* mutations were used in the transduction experiments. Results from these experiments show that the *icdB* and *molR* (*molR2*::Tn*5*) genes were cotransduced at about 13% (Table 4). The *zgg-3*::Tn*10* mutation was cotransduced with the *molR* gene at a frequency of about 43% when the *molR2*::Tn*5* was used as the recipient. When the Φ(*molR*::*lacZ*$^+$)*1* was used as the recipient strain, this value decreased to about 29%. The cotransduction frequency for *icdB* and *zgg-3*::Tn*10* was about 53% (strains SE1100 and SE1157). In another experiment, in which strain AT2699 (*metC argG*) was used as the donor, the cotransduction frequency between the *icdB* gene and *zgg-3*::Tn*10* was also found to be about 58%. In these crosses, none of the *icdB*$^+$ transductants were found to be Met$^-$ or Arg$^-$ when either one of the two *icdB* mutant strains (SE1157 or SE1158) was used as the recipient. In the reverse experiment, *zgg-3*::Tn*10* and *metC*

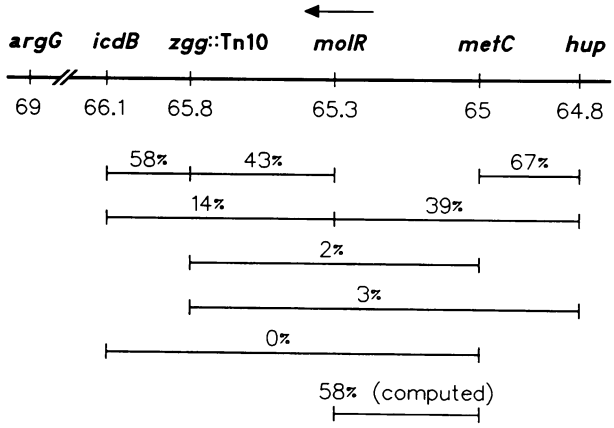

FIG. 6. Genetic map location of *molR*, *icdB*, and *zgg-3*::Tn*10* in the *E. coli* chromosome, based on bacteriophage P1-mediated transduction analysis. The map distances are in minutes. The numbers above the lines represent the cotransduction frequencies. The arrow indicates the direction of transcription of the *molR* gene.

were found to be cotransduced at a very low but detectable frequency (less than 5%).

These results show that the *molR*, *icdB*, and *zgg* (*zgg-3*::Tn*10*) genes are linked to each other. The very low but detectable level of cotransduction between the *zgg* and *metC* genes suggests that these genes are closer to *metC* than to *argG*. It has been reported before that the *hup* genes are near the *metC* gene and that the cotransduction frequency between the *hup* and *metC* genes is about 65% (21). Since repeated attempts failed to transduce the Φ(*molR*::*lacZ*$^+$)*1* mutation into the *metC* strains (AT2446 or AT2699) at high frequency, the cotransduction frequencies between the *hup* gene and *molR* and *zgg-3*::Tn*10* were determined. Results from these experiments showed that the *hup* gene was cotransducible with *molR2*::Tn*5* at a frequency of about 39% and that the cotransduction frequency between the *hup* genes and *zgg-3*::Tn*10* was about 3%.

Although these results indicate that the *hup* gene(s) is located between the *metC* and *molR* genes (on the basis of the cotransduction frequencies of *metC* and *hup* [65%], *hup* and *molR* [40%], and *metC* and *molR* [0%]), the position of the *hup* gene with respect to the *metC* gene was determined directly. For these experiments, an Hfr strain in which the origin of transfer is located in the *hup* gene was constructed by using procedures similar to those used for constructing Hfr-PO-*molR* strains. Conjugation experiments using this strain as the donor and strain AT2699 (*metC argG thyA*) as the recipient revealed that the *hup* gene, although close to the *metC* gene, maps between the *metC* (65 min) and *thyA* (61 min) genes.

These results from the mapping experiments are summarized in Fig. 6. The gene order in this region, among the ones studied, is *hup*, *metC*, *molR*, *zgg-3*::Tn*10*, *icdB*, and *argG*.V

DISCUSSION

The molybdoproteins produced by *E. coli*, FDH-H, FDH-N nitrate reductase, trimethylamine-*N*-oxide reductase, and biotin-sulfoxide reductase contain molybdenum as molybdopterin (20, 39). Several genes whose products are essential for the conversion of molybdate in the medium into molybdopterin have been identified in this organism. These include all the *chl* genes except *chlC*, which codes for the structural gene for nitrate reductase (39). Among the chlorate-resistant mutants, *chlD* mutants are defective in FHL production, and the mutational effect can be reversed by the addition of molybdate to the medium. The *chlD* gene has been cloned, and the DNA sequence has been determined (19). The DNA sequence shares similarities with those of other genes coding for transport components (1), suggesting that the *chlD* gene product is involved in the transport of molybdate into the cell. In support of this putative role, *chlD* mutants accumulated molybdate into the cell at very low levels when the medium concentration was low (37). The *chlD* gene is part of an operon which probably codes for both the periplasmic-binding protein and membrane components needed for the transport of molybdate. In support of this hypothesis, Miller et al. (26) observed, by using Φ(*chlD-lacZ*$^+$) strains, that the transcription of the *chlD* gene is reduced to very low levels when Mo is present in the medium at high concentrations.

The general phenotype of the *molR* mutant is indistinguishable from the characteristics of *chlD* mutants. However, the *molR* gene maps at 65.3 min (Fig. 6), while the *chlD* gene is located at 17 min (2, 39) in the *E. coli* chromosome. The genetic map location of the *molR* gene has been identified by several independent methods, alleviating the possibility of fortuitous identification. On the basis of the phenotypic characteristics, the most plausible role for the *molR* gene product is in the transport of molybdate into the cell. If the *chlD* operon is involved in the actual transport of the

TABLE 4. Transductional analysis of *molR*, *hup*, *icdB*, *zgg*-3::Tn*10*, *metC*, and *argG* genes

Recipient (genotype)	Donor (genotype)	Selected phenotype	No. tested	Unselected phenotype (%)
SE1157 (*icdB zgg-3*::Tn*10*)	SE1319 (*molR2*::Tn*5*)	IcdB$^+$	272	Km^r (14)
SE1158 (*icdB zgg-3*::Tn*10*)	SE1319 (*molR2*::Tn*5*)	IcdB$^+$	205	Km^r (13)
SE1100 Φ(*molR-lacZ*$^+$)1	SE1157 (*icdB zgg-3*::Tn*10*)	Tc^r	699	IcdB$^-$ (53) Km^s (29)
SE1325 (*molR2*::Tn*5*)	SE1157 (*icdB zgg-3*::Tn*10*)	Tc^r	380	Km^s (43)
SE1157 (*icdB zgg-3*::Tn*10*)	AT2699 (*metC argG*)	IcdB$^+$	66	Met$^-$ (0) Arg$^-$ (0) Tc^s (58)
SE1158 (*icdB zgg-3*::Tn*10*)	AT2699 (*metC argG*)	IcdB$^+$	87	Met$^-$ (0) Arg$^-$ (0) Tc^s (59)
AT2699 (*metC argG*)	SE1157 (*icdB zgg-3*::Tn*10*)	Tc^r	475	Arg$^+$ (0) Met$^+$ (3)
AT2446 (*metC*)	SE1157 (*icdB zgg-3*::Tn*10*)	Tc^r Met$^+$	601 326	Met$^+$ (1) Tc^r (1)

anion (19, 37), the *molR* gene probably regulates the expression of the *chlD* operon. In agreement with this, other experiments (Lee and Shanmugam, unpublished data) show that the Φ(*chlD-lacZ*$^+$) gene is not expressed in the *molR* genetic background. The transport of molybdate thus requires the MolR protein, which in turn regulates the synthesis of ChlD protein, a putative transport protein. The mechanism by which the MolR protein regulates the *chlD* operon is unknown.

In the absence of either of the two genes in an active form (*molR* and *chlD*), the transport of molybdate in *E. coli* is effected by the sulfate transport system. It is known that molybdate as well as selenate acts as an analog of sulfate in binding to periplasmic proteins and transport and as a substrate for ATP sulfurylase (12, 29, 42, 44). However, in *E. coli*, the amount of sulfate needed for abolishing the FHL production is considerably higher—10,000 times greater than the amount of molybdate (Fig. 4). If sulfate and molybdate compete for the same binding and transport systems, such a large excess would not be needed (42). The possibility that this effect of sulfate is mediated through an unknown intracellular effect (43) can be discounted in these experiments, since even higher concentrations of sulfate did not affect the levels of FHL activity produced by the parent strain BW545.

It is known that in *Salmonella typhimurium*, sulfate represses its own transport system when present at high concentrations in the medium (10, 11, 30). Cysteine and cystine are also potent repressors of the sulfate transport system in *E. coli* (41). The lack of FHL activity in the presence of 50 nM molybdate and 30 μM cystine (Fig. 4B) suggests that the effects of S compounds on FHL activity in strain SE1100 are due to the repression of the sulfate transport system, which, under appropriate conditions, acts as a fortuitous molybdate transport system. The amount of Mo needed for maximal production of FHL activity by strain SE1100 grown in different media (Fig. 1 and 4) may be a consequence of the extent of repression of sulfate transport by S-containing compounds, like sulfate or cystine. In a medium like LB, which is rich in S-containing organic compounds, the only transport system available in the mutant is some other less-efficient system.

The inhibition by selenite, at a Se/Mo ratio of 0.3 to 1.0 (Fig. 2), shows that the two ions compete and that molybdate is not the preferred anion for this transport system. These results also suggest that the transport of molybdate is facilitated by the selenite transport system, which has a low affinity for molybdate. At higher concentrations of selenite, the transport of molybdate is completely inhibited. Another alternative is that selenite at this concentration affects the conversion of Mo to molybdopterins and finally the incorporation of the molybdopterin into apo-FDH, since the FDH-H also contains selenocysteine (8, 31). That this possibility is unlikely is evidenced by the fact that even at a Se/Mo ratio of 50, selenite did not significantly affect the growth or FHL activity of parent strain BW545. However, selenite affected only the levels of FDH, a selenoprotein (Table 2), and not those of nitrate reductase or trimethylamine-*N*-oxide reductase, other molybdoproteins. The likelihood that the presence of high concentrations of selenite in the medium influences the incorporation of molybdopterin into FDH-H only when the internal molybdate pools are low, as in the case of molybdate transport mutants, needs to be investigated.

In summary, *E. coli* has a specific high-affinity molybdate transport system, and the production of this system requires the products of at least two unlinked operons, *chlD* and *molR*. With a mutation in either one of the two operons, molybdate is probably transported by the sulfate transport system. The selenite transport sytem is used in the absence of both the molybdate and sulfate transport systems. Although the results presented in this paper did not include the actual transport of these ions, the study of the levels of FHL activity in strain SE1100 certainly allowed a reasonable deduction of the transport of molybdate by *E. coli*, which needs to be investigated by direct experiments.

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