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Molybdenum-Sensitive Transcriptional Regulation of the *chlD* Locus of *Escherichia coli*

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The *chlD* gene in *Escherichia coli* is required for the incorporation and utilization of molybdenum when the cells are grown with low concentrations of molybdate. We constructed *chlD-lac* operon fusions and measured expression of the fusion, Mo cofactor, and nitrate reductase activities under a variety of growth conditions. The *chlD-lac* fusion was highly expressed when cells were grown with less than 10 nm molybdate. Increasing concentrations of molybdate caused loss of activity, with less than 5% of the activity remaining at 500 nM molybdate; when tungstate replaced molybdate, it had an identical affect on *chlD* expression. Expression of *chlD-lac* was increased in cells grown with nitrate. Strains with *chlD-lac* plus an additional mutation in a *chl* or *nar* gene were constructed to test whether the regulation of *chlD-lac* required the concerted action of gene products involved with Mo cofactor or nitrate reductase synthesis. Mutations in *narL* prevented the increase in activity in response to nitrate; mutations in *chlB*, *narC*, or *narI* resulted in partial constitutive expression of the *chlD-lac* fusion: the fusion was regulated by molybdate, but it no longer required the presence of nitrate for maximal activity. Mutations in *chlA*, *chlE*, or *chlG* which affect Mo cofactor metabolism, did not affect the expression of *chlD-lac*.

Molybdenum is an essential trace element for virtually all bacteria, plants, and animals. The concentration of soluble molybdenum in most environments is very low (25), and organisms have developed specific, high-affinity systems for processing it. Although the requirement for molybdenum is well established, little is known about how cells acquire and process it. However, all molybdoenzymes, with the exception of nitrogenase, incorporate molybdenum into a pterin-containing Mo cofactor (14), and thus the understanding of molybdenum metabolism in one organism should have far-reaching implications for understanding Mo cofactor metabolism in other biological systems.

In *Escherichia coli* the *chlD* gene is required for cells to produce active molybdoenzymes when grown in media with low concentrations of molybdenum (11), and mutations in this gene result in the loss of activity of all molybdoenzymes. Growth of the cells in media with 1 mM molybdate circumvents the requirement for the *chlD* product and restores the activity of the molybdoenzymes, even in cells in which the entire *chlD* gene is deleted (11). The presence of the *chlD* gene product affects the accumulation of nitrate reductase subunits (8, 30) as well as the amount of active Mo cofactor (21).

Other *chl* gene products are also involved in the synthesis and utilization of the Mo cofactor. Mutations in the *chlA* and *chlE* genes result in no measurable Mo cofactor (21). The *chlB* gene product facilitates the assembly of active nitrate reductase but is not incorporated into the membrane-bound form of this enzyme (26). The *chlB* gene product may perform a similar function for the other cellular molybdoenzymes, since mutations in this gene result in the loss of activity of all molybdoenzymes. The *chlD* and *chlG* gene products are required for the utilization of molybdenum (5, 21, 32). Thus, the activity of *chlD* is interrelated with the activities of the other *chl* genes, as well as with the structural genes for nitrate reductase.

Nitrate reductase, which allows the cell to use nitrate as a terminal electron acceptor for anaerobic electron transport, is induced by anerobic growth and by nitrate in a two-step induction pattern (27) mediated by two separate regulatory signals: the *fnr* gene mediates induction by anaerobiosis (4, 31), and the *narL* gene mediates the second, nitrate-specific step of induction (31). The *narCHI* operon codes for the A and B subunits of the nitrate reductase and the associated cytochrome (7, 18, 32).

We are interested in the factors which affect the expression of the *chlD* gene and, ultimately, with the ways in which this gene affects cellular molybdenum levels and Mo cofactor metabolism. We constructed strains carrying operon fusions of the *lac* genes to the *chlD* promoter by using the specialized Mu dl(Ap *lac*) bacteriophage developed by Casadaban and Cohen (3). The activity of β-galactosidase in these *chlD-lac* fusion strains most likely reflects transcription from the *chlD* promoter. We found that the *chlD-lac* fusion strains are highly regulated by the amount of molybdate and nitrate in the growth media. Also, the *chlB*, *narC*, *narI*, and *narL* genes affected the expression of the *chlD* gene. These results show that molybdenum utilization, Mo cofactor metabolism, and nitrate reductase induction are linked.

(A preliminary account of these results has appeared previously [J. B. Miller and N. K. Amy, Fed. Proc. 43:1720, 1984].)

MATERIALS AND METHODS

Strains and growth conditions. The *E. coli* K-12 and bacteriophage strains used in this study are listed in Table 1. For strain construction and storage, bacteria were grown in Luria broth (20). Unstabilized Mu d(Ap *lac*) insertion strains were grown at 30°C; stabilized fusions were grown at 37°C. We took great care to reduce the molybdenum contamination in the medium. All reagents were the purest obtainable

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TABLE 1. *E. coli* K-12 and bacteriophage strains

Strain	Genotype	Source
MC4100	F Δ*lacU169 araD139 rpsL thi*	M. Casadaban (3)
MAL103	F⁻ Mu cts d1(Ap *lac*) Mu cts Δ(*proAB lacIPOZYA*)*XIII strA*	M. Casadaban (3)
RK4353	MC4100 but *gyrA non*	V. Stewart (32)
RK4353 derivatives		
RK4920	*zcg-622*::Tn*10* (Tn*10* near *narG*)	V. Stewart
RK4921	*zbh-623*::Tn*10* (Tn*10* between *chlD* and *chlA*)	V. Stewart
RK4922	*zbi-624*::Tn*10* (Tn*10* near *chlE*)	V. Stewart
RK4923	*zaa-625*::Tn*10* (Tn*10* near *chlG*)	V. Stewart
RK4940	*zif-629*::Tn*10* (Tn*10* near *chlB*)	V. Stewart
RK5200	*chlA200*::Mu cts	V. Stewart
RK5201	*chlE201*::Mu cts	V. Stewart
RK5202	*chlD202*::Mu cts	V. Stewart
RK5206	*chlG206*::Mu cts	V. Stewart
RK5208	*chlB207*::Mu cts	V. Stewart
RK5267	*narI204*::Tn*10*	V. Stewart
RK5272	*narG209*::Tn*10* (now designated *narC*)	V. Stewart
RK5278	*narL215*::Tn*10*	V. Stewart
MC4100 derivatives		
JBM235	*chlD2001*::Mu d1	This study
JBM239	*chlD2002*::Mu d1	This study
JBM239-7	As JBM239 but λp_L(209) (Mu)Φ(*chlD-lac*)	This study
NKA2	*chlD2003*::Mu d1	This study
NKA16	*chlD2004*::Mu d1	This study
NKA17	*chlD2005*::Mu d1	This study
NKA20	*chlD2006*::Mu d1	This study
NKA21	*chlD2007*::Mu d1	This study
JBM239 derivatives		
JBM400	*chlG206*::Mu cts *zaa-625*::Tn*10*	This study
JBM401	*chlA206*::Mu cts *zbh-623*::Tn*10*	This study
JBM402	*chlE201*::Mu cts *zbi-624*::Tn*10*	This study
JBM403	*chlB207*::Mu cts *zif-629*::Tn*10*	This study
JBM404	*narL215*::Tn*10*	This study
JBM405	*narC209*::Tn*10*	This study
JBM406	*narI204*::Tn*10*	This study
Phage strains		
P1 vir_a		A. J. Clark
λp_L(209)	*lacYZO* ΔW*209-trp'AB'*::(+Mu)	V. Stewart

commercially. The glassware was soaked in Radiacwash (Atomic Products Corp., New York, N.Y.) to remove residual molybdenum. For enzyme or cofactor assays, bacteria were grown in modified M9 medium containing 0.5 mM monobasic potassium phosphate, 0.2% glucose, and thiamine (10 μg/ml). The total phosphate in the medium was lowered to 10 μg/ml to reduce the molybdenum contamination from the phosphate stocks. The medium was further depleted of molybdenum by chromatography on immobilized ethylenediamine (17). In addition, some batches of media were treated with *Azotobacter vinelandii* UW cells to remove contaminating molybdenum (12). Similar results were obtained by both methods.

When indicated, the media were supplemented with one or more of the following: ampicillin (25 μg/ml), tetracycline (15 μg/ml), potassium chlorate (20 mM), sodium molybdate, sodium tungstate, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (40 μg/ml), or sodium nitrate (20 mM).

Anaerobic cultures were grown in anaerobic jars equipped with palladium catalyst and gas-generating envelopes (GasPak; Becton Dickinson and Co., Paramus, N.J.). Bacteria were harvested at an optical density of 0.3 to 0.6 at 590 nm.

Isolation and characterization of *chl*::Mu d(Ap *lac*) fusions. Cultures of MC4100 were infected with serial dilutions of a Mu d1 lysate prepared by heat induction of strain MAL103 as described by Casadaban and Cohen (3). Lysogens were obtained by selecting for cells that were ampicillin resistant, Lac⁺, and chlorate resistant under anaerobic conditions. Chlorate resistance selects for cells with mutations in *chlA*, *chlB*, *chlD*, *chlE*, and *chlG*. Mutations in these genes result in the loss of the activities of all the molybdoenzymes, and the cells can grow anaerobically on chlorate, which is lethal to wild-type cells. Cells with a Mu d(Ap *lac*) fusion with the *chlD* gene were identified by a combination of phenotypic characterization and genetic mapping. The cells were grown in media with and without supplementation with 100 μM Na_2MoO_4 or 20 mM $NaNO_3$ and assayed for molybdenum cofactor and nitrate reductase activities. Cells with a typical *chlD* phenotype had very low nitrate reductase and molybdenum cofactor activities when the cells were grown without added molybdate, and these activities were restored to near wild-type values when the cells were grown in the presence of 100 μM molybdate. The strains were mapped by bacteriophage P1-mediated transduction by using strains with Tn*10* insertions linked to the known *chl* genes. All our *chlD* mutants showed 65 to 85% linkage with *zbh-623*::Tn*10* and less than 5% linkage with *zbi-624*::Tn*10*, as described previously (32), showing that the Mu d1 was inserted in the *chlD* region. We determined that the fusion strains carried a single Mu lysogen by heat treatment which resulted in the simultaneous loss of the Mu characteristics of Lac⁺ and temperature sensitivity. We verified that none of the strains had acquired an additional *fnr* mutation since they grew in minimal medium supplemented with 0.2% glycerol and 20 mM sodium fumarate.

The strains containing the *chlD-lac* fusions were stabilized by using the phage λ-p_L(209) as described by Komeda and Ino (16). The desired recombinants were selected as temperature resistant, lambda immune, ampicillin sensitive, chlorate resistant, and Lac⁺. In all cases, the lambda recombinants displayed the same phenotype for Mo cofactor, nitrate reductase, and β-galactosidase activity as the original Mu-containing strains. We verified that expression of the *lac* genes in the fusion strain JBM239-7 was dependent upon the *chlD* gene sequence by the method described by Silhavy et al. (28).

Isolation of *chlD-lac* strains carrying additional mutations. To construct strains with the *chlD-lac* fusion plus an additional *chl* mutation, we first used P1-mediated transduction to move a Tn*10* insertion near the *chl* locus of interest into a strain carrying a *chl*::Mu cts insertion in that locus. For example, we moved the Tn*10* from RK4923 into RK5206. Tetracycline-resistant recombinants which retained the molybdenum cofactor and nitrate reductase phenotype appropriate to the *chl* locus of interest were retained. The *chl*::Mu cts insertion and nearby Tn*10* insertion were then transferred into the *chlD-lac* fusion strain JBM239 by P1-mediated transduction. Isolates from this transduction which were tetracycline resistant and Lac⁺ were assayed for molybdenum cofactor and nitrate reductase activities. Mutants carrying both the *chlD-lac* fusion and an additional *chl*::Mu cts insertion were identified from these activities. Similarly, to construct strains with the *chlD-lac* plus an additional *nar* mutation, *nar*::Tn*10* insertions were placed into JBM239 by P1-mediated transduction followed by selection of tetracy-

cline-resistant transductants and the lack of nitrate reductase activity. As a final check of the construction of the double mutants, the original phenotype of JBM239 was restored when P1-mediated transduction was used to replace the addition *chl* or *nar* mutations in the double mutants with wild-type alleles.

Assays. Molybdenum cofactor activity in *E. coli* extracts was measured by the ability of the extracts to restore activity to the cofactor-deficient nitrate reductase in extracts of the *nit-1* mutant of *Neurospora crassa* as described previously (2). Mo cofactor activity is expressed as units of NADPH-nitrate reductase reconstituted during the 10-min complementation; 1 U of nitrate reductase activity produces 1 nmol of nitrite per min. The *E. coli* nitrate reductase activity was measured by methyl viologen-linked reduction of nitrate to nitrite (19) with lysed protoplasts or in cells permeabilized by chloroform and sodium dodecyl sulfate as described in the β-galactosidase assay. Specific activity was expressed as nanomoles of nitrite formed per minute per milligram of protein. The activity of β-galactosidase was assayed by the rate of hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as described by Miller (22). Activity was expressed as nanomoles of *o*-nitrophenol produced per minute per milligram of protein. The concentrations of molybdate and tungstate used in our growth media did not interfere with this assay.

Sources of chemicals. Ultrex-brand monobasic potassium phosphate was from J. T. Baker Chemical Co., Phillipsburg, N.J., ammonium nitrate was from Fisher Scientific Co., Pittsburgh, Pa., and Gold Label sodium chloride, potassium chloride, and sodium sulfate were from Aldrich Chemical Co., Inc., Milwaukee, Wis. Agar and dehydrated culture media were purchased from Difco Laboratories, Detroit, Mich. All other chemicals were reagent grade from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Characterization of the *chlD-lac* strains. We constructed *lac* fusions with the *chlD* gene in *E. coli* and measured the expression of β-galactosidase activity, which reflects the activity of the *chlD* promoter. We obtained seven strains which mapped in the *chlD* region and had molybdenum cofactor and nitrate reductase activities typical of *chlD* mutants (Table 2). When these cells were grown in media with less than 10 μM molybdate, the Mo cofactor and nitrate reductase activities were much reduced compared with those of the wild type; growth in media with 100 μM molybdate restored Mo cofactor and nitrate reductase activities to wild-type levels. The β-galactosidase activity in these strains was strongly regulated by the concentration of molybdate in the media of anaerobically grown cells. Since strain JBM239 had high β-galactosidase and nitrate reductase activities, we used it for the further studies; most of our experiments were also performed with strain JBM235, which gave similar results (data not shown).

TABLE 2. Properties of *E. coli* strains[a]

Strain	β-Galactosidase sp act with:		Nitrate reductase sp act with:		Molybdenum cofactor[b] sp act with:	
	NO_3^-[c]	NO_3^- + MoO_4^{2-}[c]	NO_3^-	NO_3^- + MoO_4^{2-}	No ions	MoO_4^{2-}
MC4100	0	0	56	220	18	30
RK5202	0	0	1	140	3.6	34
JBM235	25	2	11	95	2.8	32
JBM239	529	28	2	261	4.8	29
NKA2	619	30	3	312	7.0	16
NKA16	255	48	1	200	3.2	11
NKA17	216	24	2	275	3.6	10
NKA20	39	13	2	239		14
NKA21	88	52	3	80	2.8	8

[a] Cells were grown anaerobically overnight in minimal medium with or without 20 mM $NaNO_3$ or 0.1 mM Na_2MoO_4.
[b] Molybdenum cofactor was assayed with 10 mM Na_2MoO_4 in the assay mixture.
[c] Addition to growth media.

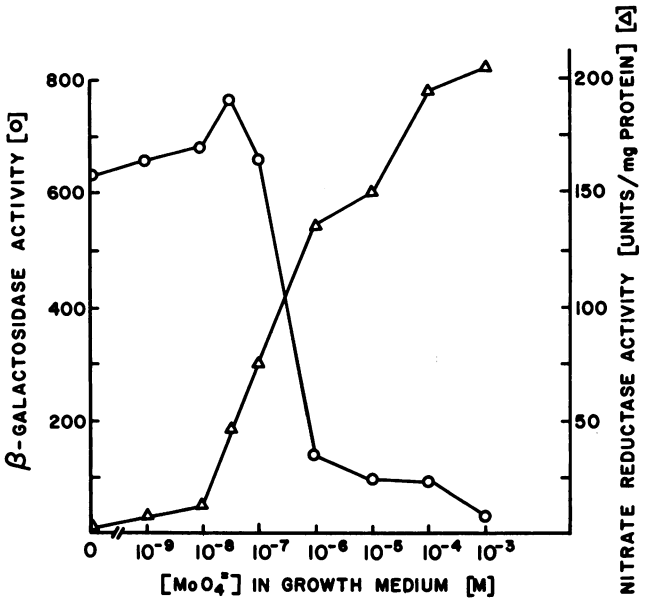

FIG. 1. β-Galactosidase (O) and nitrate reductase (Δ) activities in strain JBM239-7 grown anaerobically in minimal medium with 20 mM $NaNO_3$ and the indicated concentrations of Na_2MoO_4 added.

The β-galactosidase and nitrate reductase activities in JBM239-7 cells in response to increasing concentrations of molybdate in the growth media were measured (Fig. 1). The β-galactosidase activity was high when the cells were grown anaerobically with nitrate in media with less than 100 nM molybdate; when the cells were grown in media with additional molybdate, activity decreased precipitously to less than 5% of maximal activity. Nitrate reductase activity increased with increasing amounts of molybdate in the growth media. Wild-type cells display maximal nitrate reductase activity when grown with 1 μM molybdate (30).

Effect of nitrate. The presence of nitrate in the growth medium also affected the response of the *chlD-lac* fusion (Fig. 2). When strain JBM239-7 was grown anaerobically in media lacking nitrate, the maximal expression of β-galactosidase activity was much lower than in cells grown in the presence of nitrate. Also, the concentration of molybdate required to effect a 50% loss in activity was decreased in cells grown without nitrate. Thus, expression of the *chlD* gene was increased in cells grown with nitrate, and expression of activity persisted at a higher concentration of molybdate.

Effect of tungsten. Tungstate can serve as a competitive inhibitor of molybdate, and growth of cells in media with tungstate leads to accumulation of inactive molybdoproteins (10, 30). We tested whether the concentration of tungstate in the growth medium affected β-galactosidase expression in

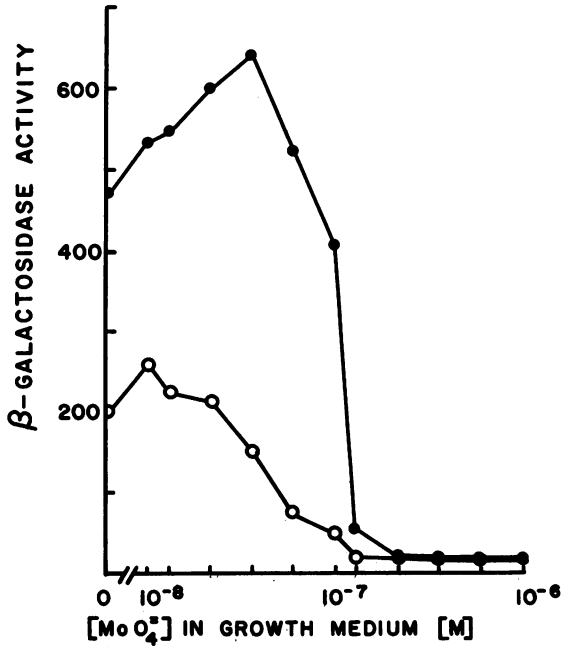

FIG. 2. β-Galactosidase activity in strain JBM239-7 grown anaerobically with (●) and without (○) 20 mM $NaNO_3$ and increasing concentrations of Na_2MoO_4 added to the growth media.

the *chlD-lac* fusion strain. We grew cells anaerobically in media lacking molybdate but with various levels of tungstate (Fig. 3). The β-galactosidase activity was fully expressed in cells grown in media with less than 10 nM tungstate; increasing concentrations of tungstate caused a progressive loss of β-galactosidase activity, with the identical pattern of loss of activity as when cells were grown with molybdate. The addition of tungstate plus nitrate to the growth media also affected the expression of β-galactosidase activity in the same way as seen with molybdate plus nitrate. The addition

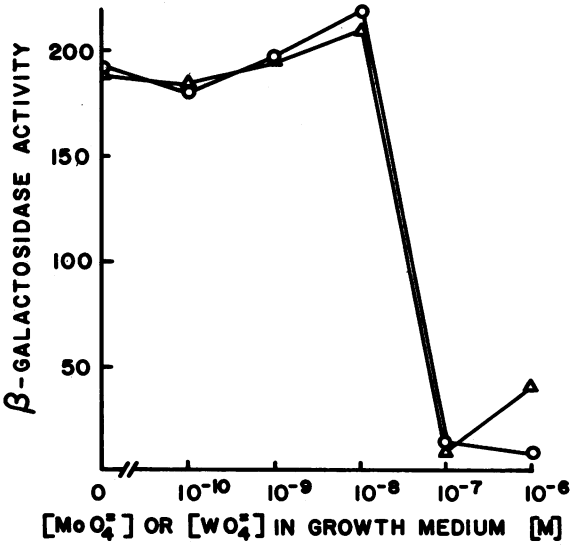

FIG. 3. Effect of the addition of Na_2MoO_4 (○) or Na_2WO_4 (△) to the growth media on β-galactosidase activity in strain JBM239 grown anaerobically.

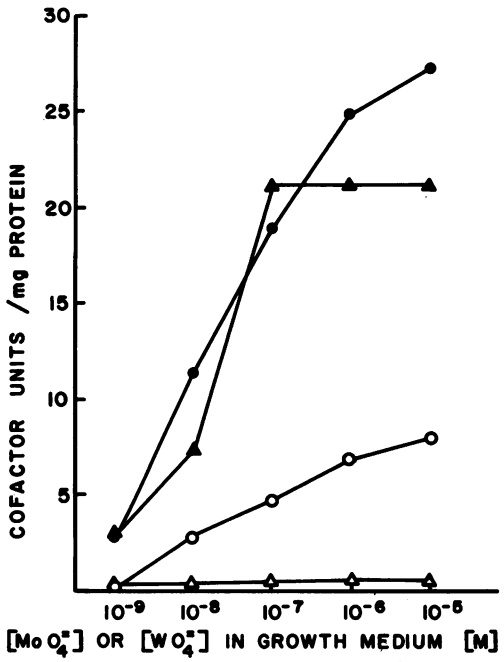

FIG. 4. Effect of the concentration of Na_2MoO_4 (●, ○) or Na_2WO_4 (▲, △) in the growth media on Mo cofactor activity in JBM239. Cells were grown aerobically in minimal medium; lysed protoplasts were prepared and were assayed with (●, ▲) or without (○, △) 10 mM Na_2MoO_4 in the complementation mixture.

of tungstate plus molybdate in the growth medium produced an additive effect (data not shown). The β-galactosidase activity in strain JBM239-7 was not affected by the addition of sodium selenite, sodium selenate, sodium vanadate, zinc sulfate, ferrous sulfate, or chromium potassium sulfate to the growth media. Thus, these data suggest that the *chlD* gene is specifically affected by molybdate as well as by tungstate and that sulfate (which may affect molybdenum transport [33]) did not affect *chlD-lac* expression.

We also measured molybdenum cofactor activity in cells with the *chlD-lac* fusion grown with increasing levels of molybdate or tungstate (Fig. 4). Mo cofactor activity increased when the cells were grown with increasing concentrations of molybdate. This occurred when the cells were assayed either with or without 10 mM molybdate in the complementation mixture. Cofactor assays without molybdate in the complementation mixture measure the amount of Mo-containing cofactor; when molybdate is included in the assay, the total pool of both molybdenum-containing and demolybdo forms of cofactor is detected (2). Increasing the levels of tungstate had a similar effect on Mo cofactor activity. Mo cofactor activity increased when the cells were grown with increasing tungstate and when the cell extract was assayed with molybdate in the complementation mixture. These experiments indicate that the pool of free cofactor was the same when the cells were grown with either molybdate or tungstate and that increased concentrations of either metal caused increased Mo cofactor activity. It is interesting that the concentrations of molybdate and tungstate needed to increase Mo cofactor activity greatly exceeded the amount needed to affect *chlD* gene expression. Thus, molybdate and tungstate have an effect on the synthesis or stability of the Mo cofactor that is different from their effect on *chlD* expression.

Effect of anaerobic and aerobic conditions. Mo cofactor is synthesized constitutively and is present in cells grown both aerobically and anaerobically (2). To determine the extent to which the *chlD* gene was regulated by these conditions in the absence of molybdate, we measured the β-galactosidase activity in strain JBM239-7 cells grown aerobically (371 U), aerobically with nitrate (470 U), anaerobically (384 U), and anaerobically with nitrate (583 U). The presence of molybdate caused the loss of β-galactosidase activity in all cells grown either aerobically or anaerobically.

Regulation of *chlD* expression by the *chl* or *nar* genes. To test whether the regulation of the *chlD-lac* fusion by molybdate required the concerted action of additional gene products involved with Mo cofactor or nitrate reductase synthesis, strains were constructed which carried *chlD-lac* fusion plus an additional mutation in one of the other *chl* or *nar* genes. We then measured β-galactosidase, Mo cofactor, and nitrate reductase activities in these double mutants.

When *chlA* or *chlE* mutations (which cause the loss of all molybdenum cofactor activity) were introduced into strain JBM239, the resulting double mutants (JBM401 and JBM402) completely lacked both molybdenum cofactor and nitrate reductase activities even when the cells were grown in medium with 1 mM molybdate. However, even though Mo cofactor activity was abolished, the response of β-galactosidase activity to the amount of molybdate or nitrate in the growth medium was similar to the response in the parent strain JBM239 (Table 3). When the *chlG* mutation (which reduces the percentage of cofactor that contains molybdenum) was introduced into JBM239, the resulting double mutant (JBM400) had no molybdenum-containing cofactor or nitrate reductase activity during growth in 1 nM molybdate but did have the *chlD* level of demolybdo cofactor. The levels of cofactor and nitrate reductase activity in JBM400 were increased by growth in 100 μM molybdate. The β-galactosidase activity, however, responded to the concentration of molybdate and nitrate in the growth media in the same way as for JBM239, indicating that even though the *chlG* gene affected Mo cofactor levels in this strain, it did not directly affect expression of the *chlD* gene.

When the *chlB* mutation (which affects the assembly of nitrate reductase [26]) was introduced into JBM239, the resulting double mutant (JBM403) retained the *chlD* level of Mo cofactor but lacked all nitrate reductase activity. The β-galactosidase activity in this strain remained strongly regulated by molybdate (Fig. 5), but the *chlB* mutation affected the regulation of the *chlD* locus in that there was no decrease in β-galactosidase activity when nitrate was omitted from the growth medium. Also, the amount of molybdate required during growth to repress β-galactosidase activity was similar whether or not nitrate was present in the growth medium. The *chlB* mutation caused regulation of the *chlD-lac* fusion in cells grown both with and without nitrate to appear the same as that seen in the parent strain (JBM239) when nitrate was present in the growth medium.

TABLE 3. β-Galactosidase activity in a *chlD-lac* fusion strain and its *chlG*, *chlA*, and *chlE* derivatives

Strain	Class	Sp act with following additions to growth media[a]:			
		None	NO_3^-	MoO_4^{2-}	MoO_4^{2-} + NO_3^-
JBM239	*chlD-lac*	360	488	20	20
JBM400	*chlD-lac chlG*	216	387	10	12
JBM401	*chlD-lac chlA*	254	452	16	19
JBM402	*chlD-lac chlE*	220	394	17	19

[a] Cells were grown anaerobically with or without 20 mM $NaNO_3$ or 0.1 mM Na_2MoO_4.

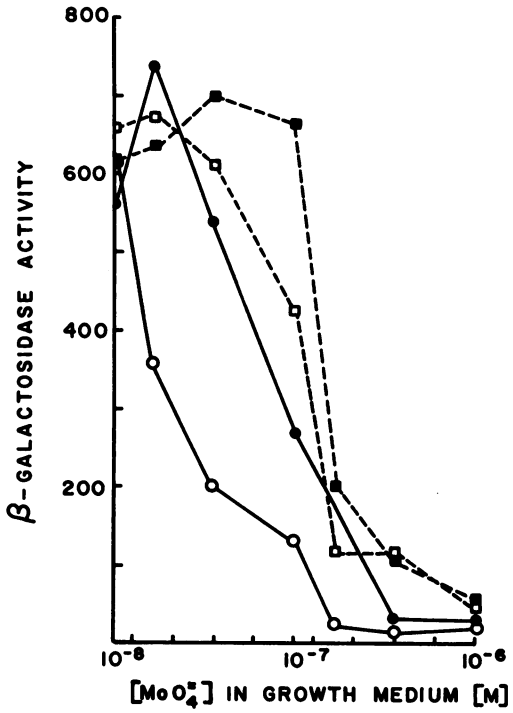

FIG. 5. Effect of added Na_2MoO_4 and $NaNO_3$ in the growth media on β-galactosidase activity in strain JBM239 (●, ○) and strain JBM403 (■, □), which has a mutation in the *chlB* gene in addition to the *chlD-lac* fusion. Cells were grown with (●, ■) or without (○, □) 20 mM $NaNO_3$.

When a mutation in *narC* (which causes the loss of the A subunit of nitrate reductase) was introduced into JBM239, the resulting strain (JBM405) had no detectable nitrate reductase activity. The β-galactosidase activity was affected (Fig. 6A) in that there was no difference in the maximal expression of β-galactosidase activity with or without nitrate, and the concentration of molybdate required for repression of β-galactosidase activity remained the same for cells grown anaerobically with and without nitrate.

When the *narL* mutation (which causes the loss of a nitrate-sensitive positive regulator for nitrate reductase expression [31]) was introduced into JBM239, the resulting double mutant (JBM404) had low levels of nitrate reductase activity which were not inducible by nitrate. The β-galactosidase activity (Fig. 6A) was low and did not increase when the cells were grown with nitrate, and thus this profile appeared the same as obtained for the parent strain when grown without nitrate.

When a mutation in the *narI* gene, which causes the loss of the cytochrome associated with nitrate reductase, was introduced into the *chlD-lac* strain, the profile of β-galactosidase activity in response to molybdenum and nitrate in the growth media was similar to that seen in the parent strain (JBM239) when grown with nitrate.

Thus, mutations that affect the induction (*narL*), the synthesis (*narC*, *narI*), or the assembly (*chlB*) of nitrate reductase also affected the regulation of the *chlD* locus, whereas mutations that affect Mo cofactor, *chlA*, *chlE*, and *chlG*, did not alter the regulation of *chlD*.

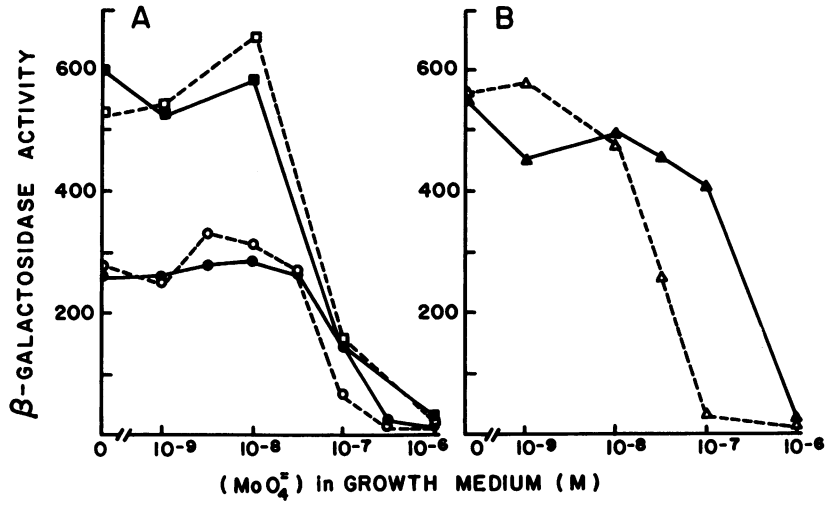

FIG. 6. Effect of Na_2MoO_4 and $NaNO_3$ in the growth media on β-galactosidase activity in strains with the *chlD-lac* fusion plus an additional mutation in a *nar* gene. (A) Strains JBM405 (with *narC*) (□) and JBM406 (with *narL*) (○). (B) Strain JBM404 (with *narI*) (△). Cells were grown anaerobically without (-----) or with (——) 20 mM $NaNO_3$.

DISCUSSION

The synthesis and assembly of nitrate reductase require molybdenum plus the concerted actions of the *fnr* gene, a positive regulator for expression of anaerobic enzymes (4, 31); the *nar* genes, which are structural genes plus regulatory elements for nitrate reductase (7, 18, 32); and the *chl* genes, which are involved with the synthesis and activation of the Mo cofactor (1, 21). Molybdenum is required for the activity of a number of these gene products. The presence of molybdate or tungstate is essential for the accumulation of the subunits of nitrate reductase (8, 30); for the accumulation of active Mo cofactor in both wild-type and *chlD* cells (1, 21); for the activity of the PA protein, which is the *chlA* gene product (9); and also for the increase in expression of the *chlI* gene in response to nitrate (23).

Our experiments with the *chlD-lac* operon fusions show that the *chlD* gene is expressed when cells are grown in media with low concentrations of molybdenum. This presumably allows cells to generate adequate molybdenum for Mo cofactor to supply the needs of the cells. When cells are grown with high concentrations of molybdate, the synthesis of the *chlD* gene product is greatly reduced, and alternate processes, perhaps nonenzymatic, are sufficient to supply molybdenum for incorporation into the cofactor. Expression of *chlD* can occur both aerobically and anaerobically, with or without nitrate.

The presence of tungstate in the growth media affected the expression of *chlD* in a way identical to that seen with molybdate. A large excess of tungstate can compete with molybdate, and growth of cells in media with tungstate has been used to produce a complete molybdenum deficiency because, even when very pure chemicals are used in the growth media, the cells scavenge traces of molybdenum and produce some molybdoenzymes. However, very different physiological responses are evident in cells grown in molybdenum-free media with or without the addition of tungsten (1, 8, 10, 23, 29). Our results indicate that tungstate may produce two effects: (i) it may reduce the expression of *chlD*, and (ii) it may stabilize the Mo cofactor or other gene products. Thus, the states of molybdenum deficiency produced by growing cells with or without tungstate are certainly not equivalent.

Strains with the *chlD-lac* fusion combined with an additional *chl* or *nar* mutation show that the regulation of *chlD* by molybdenum is not mediated by any of the *chl* or *nar* genes we tested. The presence of high concentrations of molybdate always resulted in greatly reduced gene expression, even in the absence of the *chl* or *nar* gene products.

Expression of *chlD* is stimulated two- to threefold in response to nitrate under anaerobic conditions. Growth of the cells in media with nitrate and low concentrations of molybdate resulted in increased expression of the fusion and allowed the fusion to be expressed at higher concentrations of molybdate. This regulation by nitrate appears to be mediated by the *narL* product, since cells with the *chlD-lac* fusion plus a *narL* mutation do not show increased expression in response to nitrate. It is advantageous for the cell to have *narL*-dependent regulation of *chlD* because under anaeroboic conditions with nitrate, both nitrate reductase and formate dehydrogenase are induced, which greatly increases the demand for molybdenum. MacGregor et al. have estimated that 15% of the protein in the cytoplasmic membrane is nitrate reductase (19).

Mutations in *chlB*, *narC*, or *narI* result in partial constitutive expression of the fusion, that is, the fusion is expressed to the same high level in both the presence and absence of nitrate. Thus, the absence of the subunits of nitrate reductase or the *chlB* gene product, the FA protein, results in partial constitutive expression of the fusion in the absence of nitrate.

Figure 7 is a schematic diagram showing the interrelationships among the *chl* and *nar* genes observed in our experiments. The *chlD* gene is expressed constitutively, but the presence of molybdate turns off *chlD* expression. Nitrate induces expression of the *narL* gene product, which in turn stimulates the expression of the *narCHI* operon. The *narL* gene product may also be involved in formation of the apo-nitrate reductase complex with the *narC*, *narI*, and *chlB* gene products. Studies of the reconstitution of nitrate reductase indicate that the *chlB* product may be transiently associated with the nitrate reductase complex but that it is

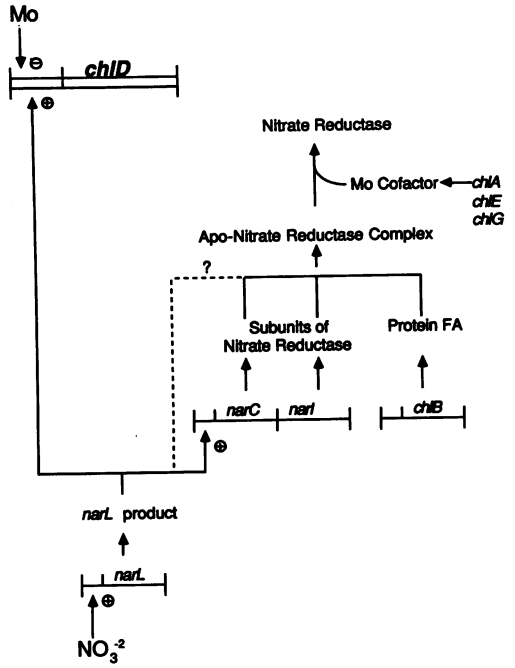

FIG. 7. Model for the regulation of the *chlD* gene. The *chlD* gene is expressed constitutively in the absence of molybdate or nitrate. The presence of molybdate turns off *chlD* expression. Nitrate induces the synthesis of the *narL* gene product which stimulates the expression of the structural genes for nitrate reductase and may also form a complex with the products of the *narC*, *narI*, and *chlB* genes. Excess *narL* can then stimulate the expression of *chlD*.

not associated with active enzyme (26). Excess *narL* gene product that is not bound to this complex can then stimulate the expression of the *chlD* gene in response to nitrate. This model assumes that in the absence of nitrate, the *narL* product is synthesized to a low level and that sufficient complex is present to bind all the *narL* product. When the *chlB*, *narC*, or *narI* genes are not present, the apo-nitrate reductase complex cannot form and, therefore, cannot bind the *narL* product. The *narL* product would then be available to stimulate the expression of *chlD*; this would account for the high level of *chlD* expression in the absence of nitrate seen when the *narC*, *narI*, or *chlB* gene was mutated. This model is highly speculative, but it should stimulate ideas for further experiments.

The regulation of *chlD* can be compared with the regulation of the structural gene for the cytochrome *b* of nitrate reductase. Pascal et al. (23, 24) constructed operon fusions with the *chlI* gene, (which is now designated *narI* [7, 32]) and showed that expression of the fusion was regulated by oxygen and nitrate similarly to the regulation of nitrate reductase activity in wild-type cells, i.e., the fusion was partially expressed in cells grown anaerobically and fully expressed in cells grown anaerobically with nitrate. When a *chlD* mutation was introduced into these strains, there was partial expression with anaerobiosis, but nitrate was no longer sufficient for full expression: the addition of molybdate was also required. Mutations in *chlA*, *chlE*, or *chlB* introduced into the *chlI-lac* fusion strain resulted in partial constitutive expression of activity in the absence of nitrate. Pascal proposed the hypothesis that the "Mo-X cofactor" is a corepressor of the nitrate reductase structural genes. When nitrate is present, the cofactor binds nitrate and, therefore, cannot repress nitrate reductase. According to this model, a defective Mo cofactor is synthesized in *chlA*, *chlE*, or *chlG* mutants and is less effective as a repressor of nitrate reductase, thus explaining the constitutive expression. For the Mo-X cofactor to serve as a corepressor of nitrate reductase, either the *chlD* gene product must be present or the cell must be grown in media with high concentrations of molybdate or tungstate. In contrast, our results indicate that the genes affecting Mo cofactor (*chlA*, *chlE*, and *chlG*) do not affect the expression of *chlD*.

Expression of *chlD* is also required for organisms synthesizing nitrogenase. When plasmids carrying *Klebsiella pneumoniae nif* genes were introduced into wild-type *E. coli* cells, the subunits of nitrogenase were present and the *E. coli* expressed acetylene-reducing activity (15, 29). When the *nif*-containing plasmids were introduced into *E. coli* bearing a *chlD* mutation, nitrogenase activity was absent, but activity could be restored when the cells were derepressed in media with high concentrations of molybdate, indicating that the expression of nitrogenase required a functional *chlD* gene. Dixon et al. (6) constructed *lac* fusions with the *nif* genes of *K. pneumoniae* and found that the concentration of molybdenum did not regulate the expression of any of these genes. Imperial and co-workers (13, 33) have recently isolated Mol^- mutants of *K. pneumoniae* that require high levels of molybdate for nitrogenase and nitrate reductase activity. The mutations in these mutants appear to be equivalent to *chlD* in *E. coli*. In *K. pneumoniae*, cystine suppressed the effects of *mol* mutations on nitrogenase but not on nitrate reductase. However, cystine increased the requirement of molybdate for maximal nitrate reductase activity in both *K. pneumoniae* and *E. coli*. Ugalde et al. (33) postulated that after molybdate enters the cell, it is transformed either by the *mol* (*chlD*) gene product or spontaneously by high intracellular molybdate concentrations into an intermediate (Mo-X) common to both the Mo cofactor and the FeMo cofactor pathways. The Mo-X can then either be incorporated into the pteridine moiety to yield active Mo cofactor for nitrate reductase and other molybdoenzymes or be transformed by the *nifQ* gene for ultimate incorporation into FeMo cofactor.

Regulation of gene expression by metal availability in the *chlD* system reported here joins the well-understood iron utilization system of *E. coli* and the metallothionein genes of mammals as an important and widespread mechanism for cells to deal with fluctuating levels of metals in the environment.

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