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Molybdenum Cofactor (Chlorate-Resistant) Mutants of *Klebsiella pneumoniae* M5al Can Use Hypoxanthine as the Sole Nitrogen Source

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Selection for chlorate resistance yields *mol* (formerly *chl*) mutants with defects in molybdenum cofactor synthesis. Complementation and genetic mapping analyses indicated that the *Klebsiella pneumoniae mol* genes are functionally homologous to those of *Escherichia coli* and occupy analogous genetic map positions. Hypoxanthine utilization in other organisms requires molybdenum cofactor as a component of xanthine dehydrogenase, and thus most chlorate-resistant mutants cannot use hypoxanthine as a sole source of nitrogen. Surprisingly, the *K. pneumoniae mol* mutants and the mol^+ parent grew equally well with hypoxanthine as the sole nitrogen source, suggesting that *K. pneumoniae* has a molybdenum cofactor-independent pathway for hypoxanthine utilization.

Nitrate (NO_3^-) serves two roles in bacterial metabolism; it can be the sole source of nitrogen for assimilation, and it can be an electron acceptor for anaerobic respiration. *Klebsiella* spp. will both assimilate and respire nitrate. By contrast, *Escherichia coli* K-12 and *Salmonella typhimurium* LT2 do not assimilate nitrate during aerobic growth, although both respire nitrate during anaerobic growth. Previous studies have established that nitrate assimilation and respiration are distinct processes in bacteria such as *Klebsiella* spp. (1, 4) and *Pseudomonas aeruginosa* (10). Molybdenum cofactor is a component of both respiratory and assimilatory nitrate reductase (25, 35). Chlorate (ClO_3^-) is reduced in vivo by nitrate reductase and other molybdoenzymes to yield a toxic product. Thus, mutants deficient in molybdenum cofactor biosynthesis can be selected on the basis of chlorate resistance. It is established that virtually all *E. coli* chlorate-resistant (*mol* [formerly *chl*]) mutants have alterations in molybdenum cofactor biosynthesis or assembly (25, 35).

Genetic analysis of *E. coli* mutants has identified five *mol* loci: *moa* (formerly *chlA*) and *moe* (formerly *chlE*), whose products are involved in synthesis of molybdopterin (24); *mod* (formerly *chlD*), which encodes a molybdate uptake system (11, 28); *mob* (formerly *chlB*), whose product is required for synthesis of molybdopterin guanine dinucleotide (13); and *mog* (formerly *chlG*), whose role is unknown (25, 35). The *moa*, *mod*, and *moe* loci each contain at least two genes (11, 14, 22).

Molybdenum cofactor is required for the activities of several other enzymes, including formate dehydrogenase in bacteria and xanthine dehydrogenase in fungi and other eukaryotes (6, 21). In the ascomycete *Aspergillus nidulans*, molybdenum cofactor (*cnx*) mutants fail to grow with hypoxanthine as the sole nitrogen source, and ability to use hypoxanthine is used as one diagnostic test to determine whether a nitrate-nonassimilating strain has a mutation in molybdenum cofactor synthesis or in a nitrate reductase structural or regulatory gene (6, 10). *E. coli* K-12 does not use xanthine as a sole nitrogen source (33).

Early genetic analyses of nitrate metabolism in *K. aerogenes* S45 employed Chl^r *mol* mutants (39). Subsequently, we have isolated *K. pneumoniae* M5al mutants defective in nitrate assimilation (4) and respiration (5) by screening procedures rather than by direct selection for chlorate resistance. All of these mutants were fully Chl^s, as expected, because only mutations in *mol* genes confer the Chl^r phenotype (35). Unexpectedly, we found that all of the *K. pneumoniae mol* mutants grew well with hypoxanthine as the sole nitrogen source. Thus, we wished to genetically characterize a representative collection of *K. pneumoniae mol* mutants to determine whether the genetics of molybdenum cofactor biosynthesis in this organism is similar to that in *E. coli*.

Nomenclature. Chl designates resistance (Chl^r) or sensitivity (Chl^s) to chlorate. Nas designates the ability (Nas^+) or inability (Nas^-) to use nitrate as a sole nitrogen source. Nar designates the ability (Nar^+) or inability (Nar^-) to use nitrate as an electron acceptor for anaerobic respiration. The Nas^+ and Nar^+ phenotypes require expression of assimilatory or respiratory nitrate reductase and molybdenum cofactor; Chl^r strains are Nas^- and Nar^-, but Nas^- and Nar^- strains are not necessarily Chl^r.

Historically, five distinct *mol* (*chl*) loci, *chlA*, *chlB*, *chlD*, *chlE*, and *chlG*, have been identified through genetic analysis of *E. coli* mutants. Several of these loci contain more than one gene, and complete characterization of most *chl* loci is in progress. Recently, a new genetic nomenclature has been adopted to designate these genes (29). For example, genes in the historical *chlA* locus are now designated *moaA*, *moaB*, etc. Likewise, the *chlB*, *chlD*, *chlE*, and *chlG* loci are now designated *mob*, *mod*, *moe*, and *mog*, respectively. The designation *mol* is a collective, generic term for the *moa*, *mob*, *mod*, *moe*, and *mog* genes without regard to the specific locus or function.

Media and culture conditions. Liquid cultures for nitrate reductase enzyme assays were grown in Yoch and Pengra nitrogen-free medium (NF medium), which was prepared as described previously (30), except that the final concentration

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of $FeSO_4$ was 0.1 mM. To avoid iron precipitation, neutralized EDTA was mixed with the $FeSO_4$ solution, and the mixture was aerated vigorously overnight (16). Nitrogen sources were added as indicated. Aerated cultures were grown in 20 ml of medium contained in 1-liter flasks, with orbital shaking at 400 rpm. Anaerobic cultures were grown in tubes sealed with rubber stobbers. The tubes were completely filled by injecting culture medium with a syringe. Liquid cultures for β-galactosidase assays were grown in MOPS (morpholinepropanesulfonic acid) medium as described previously (38). Plates were incubated anaerobically in Brewer jars (3).

Defined media contained 0.2% glucose. Complex and indicator media for routine genetic manipulations were used as described previously (7, 20). MacConkey agar-based indicator media to test for production of formate-nitrate oxidoreductase and glycerol-fumarate oxidoreductase were prepared as previously described (38). Peptone-nitrate agar, for selecting Nas^+ colonies, was prepared as described previously (37). Tests for use of alternate nitrogen sources were performed by streaking colonies on defined ammonium medium and then replica printing colonies to plates prepared with NF medium containing alternate nitrogen sources.

The nitrogen sources $(NH_4)_2SO_4$, $NaNO_3$, adenine, asparagine, histidine, hypoxanthine, and urea were used at final concentrations of 0.1%; $NaNO_2$ was used at a final concentration of 0.03%. The sulfur sources cystine and K_2SO_4 were used at final concentrations of 2 mM. Sodium molybdate (Na_2MoO_4) and sodium tungstate (Na_2WO_4) were added as indicated below.

Chlorate agar contained 0.5% $KClO_3$ except as indicated. Chlorate agar was made with NF medium supplemented with ammonium; with Vogel-Bonner defined medium, which contains ammonium (7); or with Luria-Bertani medium (LB medium), a complex medium (20).

Chlorate sensitivity of *K. pneumoniae* M5al on different media. The wild-type strain *K. pneumoniae* M5al was unable to grow on NF agar supplemented with a nitrogen source and at least 0.1% $KClO_3$. The inhibitory effect of chlorate was exerted irrespective of the nitrogen source used (ammonium, nitrate, adenine, asparagine, histidine, or urea) and was observed even on rich media such as LB. However, chlorate sensitivity was most evident when nitrate was used as the sole nitrogen source; in this case, growth inhibition was observed at 0.01% $KClO_3$. *K. pneumoniae* was sensitive to chlorate both aerobically and anaerobically.

Isolation of *mol* mutants. Spontaneous *mol* mutants of strain M5al were isolated by plating 0.1 ml of stationary LB cultures on NF-ammonium-chlorate or LB-chlorate agar and then incubating them aerobically at 37°C. These mutants were isolated at relatively high frequencies (approximately 10^{-5}).

Bacteriophage Mu dI1734 (MudJ)-induced *mol* mutants were isolated by infecting strain VJSK014 with MudJ as previously described (4). Kanamycin-resistant (Km^r) colonies were then replica printed to various media to search for mutants. The *moa*::MudJ insertion in strain VJSK017 was isolated in a search for nitrate-nonassimilating mutants and has been previously described (4). The *mol*::MudJ insertions in strains VJSK152-157 were isolated by replicating Km^r colonies to Vogel-Bonner defined medium-chlorate-cystine plates, which were incubated aerobically, and those in strains VJSK158-160 were isolated by replicating Km^r colonies to MacConkey-nitrate plates, which were incubated anaerobically. This latter medium differentiates mutants defective in nitrate respiration. All putative *mol*::MudJ insertions were backcrossed to VJSK014 via P1 *kc*-mediated generalized transduction (20) to ensure that they were single insertions in a *mol* locus. All of the *mol* mutants described here were of independent origin.

Complementation analysis of *mol*::MudJ mutants. We used the in vivo cloning method of Groisman and Casadaban (9) to clone mol^+ loci from *E. coli*. Transductions involving pEG5005 were performed essentially as described previously (2, 9). Representative *mol*::Mu *cts* mutants (37) were transduced to Chl^+ by selecting for anaerobic growth on peptone-nitrate-Km plates at 30°C. After preliminary characterization, restriction fragments from representative pEG5005-mol^+ plasmids were subcloned into the general-purpose vector pUC13 or pHG329.

The mol^+ subclones were transformed into *E. coli* *mol*::Mu *cts* strains, including several representative examples of each of the five *mol* loci *moa*, *mob*, *mod*, *moe*, and *mog* (37). Complementation was examined by replica printing colony-purified transformants onto LB-chlorate-glucose plates and onto MacConkey nitrate agar plates. All of these plates were incubated anaerobically. In each case, the subclones complemented only the appropriate *mol*::Mu *cts* lesions. We have not extensively characterized the inserts in these subclones. However, the preliminary restriction maps of the inserts were consistent with the maps described by Reiss et al. (26).

We transformed each mol^+ subclone into each of the *K. pneumoniae mol*::MudJ mutants and then replica plated the transformants to chlorate agar (incubated aerobically), NF agar with nitrate as the sole nitrogen source (incubated aerobically), and MacConkey nitrate agar (incubated anaerobically). For each of the *mol*::MudJ mutants, one (and only one) of the mol^+ plasmids simultaneously restored Chl^s, Nas^+, and Nar^+. These tests revealed that our collection included *moa*::MudJ (two), *mod*::MudJ (five), *moe*::MudJ (one), and *mog*::MudJ (one) mutants (Table 1).

Genetic analysis of *mol*::MudJ mutants. The genetic maps of *E. coli* and *K. pneumoniae* are probably similar, so we reasoned that homologous *mol* genes should have homologous genetic map positions (40). In *E. coli*, *mod* is closely linked to *gal*, *moa* is weakly linked to *gal*, and *moe* is essentially unlinked to *gal*. Thus, we transduced each of our VJSK014 *mol*::MudJ derivatives (Gal^r) into strain VJSK009 (Gal^s), selected for Km^r transductants, and scored inheritance of Gal^r as the nonselected marker. The *mod*::MudJ insertions were approximately 30% linked to *gal*, the *moa*::MudJ insertions were approximately 10% linked to *gal*, and the *moe*::MudJ and *mog*::MudJ insertions were less than 0.5% linked to *gal*. In *E. coli*, *mog* is tightly linked to *thr*. Thus, we also transduced all of the *mol*::MudJ insertions into strain VJSK207 (*thr*::Mu d*tet*). The *mog*::MudJ insertion was approximately 90% linked to *thr*::Mu d*tet*, while the other *mol*::MudJ insertions were less than 2% linked to *thr*::Mu d*tet*. Thus, the two methods employed, plasmid complementation and transductional linkage, yielded identical genetic assignments for the *mol*::MudJ insertions.

Genetic analysis of spontaneous *mol* mutants. We used transduction to rapidly identify the affected *mol* locus in spontaneous *mol* mutants. Bacteriophage P1 lysates prepared on four of the spontaneous *mol* mutants were used to infect one representative each of *moa*::MudJ, *mod*::MudJ, *moe*::MudJ, and *mog*::MudJ, and the transduction mixtures were plated on NF-nitrate plates to select Nas^+ (Chl^s) transductants. Three of the mutants transduced each of the recipients, except for the *moa*::MudJ strain, so these strains (KS10, KS11, and KS12) presumably carry lesions in the

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TABLE 1. Strains and plasmids

Strain or plasmid	Description	Reference or source
K. pneumoniae strain		
M5al	Prototroph	23[a]
KS10	*moa-102*	This study
KS11	*moa-103*	This study
KS12	*moa-104*	This study
KS13	*moe-105*	This study
VJSK009	*hsdR1* Gal^s	4
VJSK014	*Δlac-2001 hsdR1* Gal^r Tn7	4
VJSK017	As VJSK014 but *moa-106*::MudJ (Lac$^-$)	This study
VJSK152	As VJSK014 but *mod-107*::MudJ (Lac$^+$)	This study
VJSK154	As VJSK014 but *moa-108*::MudJ (Lac$^-$)	This study
VJSK155	As VJSK014 but *mod-109*::MudJ (Lac$^-$)	This study
VJSK156	As VJSK014 but *mod-110*::MudJ (Lac$^-$)	This study
VJSK157	As VJSK014 but *mog-111*::MudJ (Lac$^+$)	This study
VJSK158	As VJSK014 but *mod-112*::MudJ (Lac$^+$)	This study
VJSK159	As VJSK014 but *mod-113*::MudJ (Lac$^+$)	This study
VJSK160	As VJSK014 but *moe-114*::MudJ (Lac$^-$)	This study
VJSK207	As VJSK009 but *thr*::Mu d*tet*	Laboratory collection
Plasmids		
pEG5005	Ap^r Km^r; Mud5005 cloning vector	9
pHG329	Ap^r; *lacZ* α polylinker	32
pUC13	Ap^r; *lacZ* α polylinker	41
pVJS501	*E. coli moa*$^+$; ~9-kb *Pst*I-*Bgl*II insert in pHG329	This study
pVJS502	*E. coli mob*$^+$; ~7-kb *Bam*HI insert in pUC13	This study
pVJS503	*E. coli mod*$^+$; ~10-kb *Pst*I insert in pUC13	This study
pVJS504	*E. coli moe*$^+$; ~4-kb *Sph*I insert in pHG329	This study
pVJS505	*E. coli mog*$^+$; ~7-kb *Eco*RI insert in pUC13	This study

[a] Courtesy of R. A. Dixon (University of Sussex, Brighton, United Kingdom).

moa locus. The other mutant tested (KS13) transduced each of the recipients, except for the *moe*::MudJ strain, so this mutant presumably carries a lesion in the *moe* locus. Control experiments in which *mol*::MudJ mutants were crossed with each other established the utility of this experiment for the characterization of unknown *mol* mutants.

Summary of genetic analyses. Our combined complementation, genetic, and phenotypic analyses indicate that the *K. pneumoniae mol* loci are homologous to those of *E. coli* in both function and genetic map position. Earlier work with *K. aerogenes* S45 led to a similar conclusion (40). Thus, there seems to be nothing unusual about selection for Chl^r in *K. pneumoniae*. Our relatively small collection of *mol* mutants was biased toward *moa* and *mod* mutants. In *E. coli*, *moa* mutants predominate, while *moe* and *mod* mutants are also quite frequent (37). Mutations in *mob* and *mog* are much less frequently recovered in *E. coli*, and indeed our small collection contained no examples of *mob* and only one of *mog*.

We believe that the relatively high proportion of *mod*::MudJ mutants recovered was due to the fact that we included cystine in the media used to isolate *mol*::MudJ mutants. *mod* mutants are phenotypically leaky, in that added molybdate can restore nearly wild-type molybdoenzyme function. Sulfur sources repress the sulfate transport system, which allows some molybdate entry into *mod* strains, and therefore makes for a more stringent Chl^r phenotype (15).

Although the collection of *mol* mutants that we have genetically analyzed is small, our characterization of several dozen independent spontaneous *mol* mutants revealed none with phenotypes other than those reported here (17).

Phenotypic characterization of *mol* mutants. All of the *mol* mutants had inhibited anaerobic growth on 0.5% chlorate, a concentration that did not cause inhibition of aerobic growth. Anaerobic growth inhibition of *mol* mutants caused by chlorate was merely bacteriostatic, as the *mol* mutants subsequently grew well upon transfer to aerobic conditions.

All of the *mol* mutants grew well on plates with hypoxanthine as the sole nitrogen source. The growth rates and yields of the *mol*$^+$ parent (VJSK014) and of representative *moa*::MudJ (VJSK017), *moe*::MudJ (VJSK160), and *mog*::MudJ (VJSK157) strains were indistinguishable in MOPS-glucose medium with hypoxanthine (2.5 mM) as the sole nitrogen source. Each strain doubled in about 100 min and achieved a final growth yield of about 150 Klett units. In contrast, *A. nidulans* molybdenum cofactor mutants (*cnx*) fail to grow with hypoxanthine, as xanthine dehydrogenase is a molybdoenzyme in that organism (6). We previously reported that strain VJSK017 (*moa-106*::MudJ) was unable to grow with hypoxanthine (4). This report was in error. Our laboratory records from 1987 clearly show that this strain grew well with hypoxanthine, and that we were puzzled by this result.

The other phenotypes of the *K. pneumoniae mol* mutants were exactly as expected. All of the *mol* mutants were unable to grow with nitrate as the sole nitrogen source but were fully able to grow with ammonium, nitrite, adenine, asparagine, histidine, and urea. All formed small, dark-red colonies on MacConkey nitrate agar which were similar to those of *E. coli mol* mutants (37), while the parent M5al formed large, salmon-colored colonies indicative of efficient nitrate respiration. All *mol* mutants formed wild-type colonies on MacConkey glycerol-fumarate agar, indicating that none had accumulated secondary mutations in *fnr* (34). All were essentially devoid of gas production, indicating severe defects in formate dehydrogenase activity.

E. coli mod mutants are phenotypically suppressed by including 1 mM molybdate in the culture medium (28).

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Likewise, all of our *K. pneumoniae mod* mutants became Chl[s] Nas[+] Nar[+] when cultured with 1 mM molybdate.

Effects of tungstate on *K. pneumoniae mol* mutant phenotypes. Tungsten is chemically similar to molybdenum, and organisms cultured in the presence of high levels of tungstate incorporate tungsten into molybdoenzymes. Tungsten-substituted enzymes are devoid of enzyme activity (25, 35). Thus, we examined the effects of 1 mM tungstate on the growth of *K. pneumoniae* M5al and its *mol* derivatives. Tungstate had no effect on the growth of *mol*[+] or *mol* strains cultured on NF agar supplemented with ammonium or nitrite. As expected, tungstate abolished growth of *mol*[+] strains on NF-nitrate agar, because it inhibited assimilatory nitrate reductase activity. However, *mol*[+] and *mol* strains all grew vigorously on NF-hypoxanthine agar irrespective of the presence of tungstate. This result further suggests that hypoxanthine utilization in *K. pneumoniae* M5al does not require molybdenum cofactor.

Nitrate reductase activities in *mol* mutants. Nitrate reductase activity was measured as nitrite produced by intact cells in exponentially growing cultures (27). After growth to an A_{560} of 0.5, the cultures (either aerobic or anaerobic) were treated with chloramphenicol (to avoid induction of respiratory nitrate reductase during centrifugation), harvested, washed in saline buffer, and resuspended in one-half of the original volume. The reaction mixture (0.5 M MOPS-KOH, pH 7.0, 0.4 ml; 100 mM KNO_3, 0.2 ml; 1.5 mM methyl viologen, 0.2 ml; 8 mg of $Na_2S_2O_4$ per ml, 0.2 ml; and 0.8 ml of bacterial suspension) was incubated at 37°C for 15 min, vigorously vortexed, and centrifuged. One milliliter of the supernatant was used to measure the nitrite generated during the incubation. Nitrite concentration was estimated by the method of Snell and Snell (31). Protein content was estimated by a modification of the Lowry procedure (18). Specific activity units are reported as micromoles of nitrate $minute^{-1}$ $milligram^{-1}$.

We cultured strain M5al aerobically and anaerobically to determine the activities of assimilatory and respiratory nitrate reductase, respectively. Aerated cultures grown on ammonium had very low levels of activity (0.5 mU/mg), while cultures grown with nitrate as the sole nitrogen source had substantial levels of activity (20 mU/mg). Addition of both ammonium and nitrate resulted in low levels of activity (4 mU/mg). Anaerobic cultures grown on ammonium had relatively low levels of activity (2.6 mU/mg), while nitrate-grown cultures had high levels of activity (approximately 20 mU/mg) irrespective of the addition of ammonium. These results are fully consistent with previous studies which have detected nitrate-inducible, ammonium-repressible assimilatory nitrate reductase and nitrate-inducible, ammonium-insensitive respiratory nitrate reductase in *Klebsiella* spp. (1, 4, 36).

We also assayed nitrate reductase in several of the spontaneous *mol* mutants. None of the mutants had any detectable nitrate reductase activity under any growth conditions.

Regulation of *mod* expression. Expression of genes in the *E. coli mod* locus is repressed by high concentrations of molybdate and is slightly induced by nitrate during anaerobic growth (19). Since three of our *mod*::MudJ strains were Lac[+], and thus presumably carried Φ(*mod-lacZ*) operon fusions, we examined *mod* expression in *K. pneumoniae* as well. Cultures were grown anaerobically in three different media: low-molybdate (0.5 μM) medium, high-molybdate (100 μM) medium, and low-molybdate medium supplemented with nitrate. These media also contained 2 mM sulfate, which represses synthesis of the sulfate transport system. Molybdate can enter cells with low efficiency through the sulfate transport system, so addition of sulfate ensures that *mod* strains are fully limited for molybdate (15).

TABLE 2. Anaerobic expression of Φ(*mod-lacZ*) operon fusions in response to molybdate and nitrate[a]

K. pneumoniae strain	Allele	β-Galactosidase sp act[b] in medium containing:		
		0.5 μM MoO_4^{2-}	100 μM MoO_4^{2-}	0.5 μM MoO_4^{2-} + 40 mM NO_3^-
VJSK152	*mod-107*::MudJ	390	35	390
VJSK158	*mod-112*::MudJ	290	20	290
VJSK159	*mod-113*::MudJ	980	85	1,040

[a] Strains were grown anaerobically in defined media, supplemented as indicated.
[b] Determined as described in Materials and Methods and expressed in arbitrary units.

β-Galactosidase activity was measured in permeabilized cells as described previously (20). Specific activity units ("Miller units") are arbitrary. The results are shown in Table 2. In all three strains, expression of Φ(*mod-lacZ*) was reduced approximately 10-fold by growth in high-molybdate medium. Similar results were observed with *E. coli* Φ(*mod-lacZ*) operon fusions (19). However, in contrast to *E. coli mod*, the *K. pneumoniae* Φ(*mod-lacZ*) fusions were not induced by nitrate (Table 2).

Xanthine utilization by *mol* mutants. Why do *K. pneumoniae mol* mutants retain the ability to use hypoxanthine as a sole nitrogen source? The simplest explanation is that this organism contains a molybdenum-independent pathway for hypoxanthine degradation. Unorthodox results regarding the relationship between the Chl phenotype and hypoxanthine utilization were also obtained in a study of *P. aeruginosa* mutants (8). Recently, it has been shown that *P. aeruginosa* xanthine dehydrogenase contains the Mo-molybdopterin form of the molybdenum cofactor (12), while nitrate reductase presumably contains the Mo-molybdopterin guanine dinucleotide form of the molybdenum cofactor (25). Thus, it is possible that the hypoxanthine-utilizing Chl[r] mutants of *P. aeruginosa* (8) have defects in the *mob* locus, required for synthesis of molybdopterin guanine dinucleotide (19). However, this would not explain hypoxanthine utilization by the *K. pneumoniae moa* and *moe* mutants described here. Thus, the relationship between molybdenum cofactor and hypoxanthine utilization in bacteria deserves further attention.

Summary. The genetic control of molybdenum cofactor synthesis in *K. pneumoniae* appears to be analogous to that in *E. coli*. We identified four distinct loci, *moa*, *mod*, *moe*, and *mog*, which were functionally and genetically homologous to those of *E. coli*. We did not recover any *K. pneumoniae mob* mutants; such mutants represent a minority class of *E. coli mol* mutants. All of the *mol* mutants studied failed to synthesize assimilatory and respiratory nitrate reductases and formate dehydrogenase as revealed by phenotypic tests. However, all were able to use hypoxanthine as the sole nitrogen source, indicating that this organism has a molybdenum-independent pathway for hypoxanthine utilization.

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