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Involvement of *chlA*, *E*, *M*, and *N* Loci in *Escherichia coli* Molybdopterin Biosynthesis

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All molybdenum enzymes except nitrogenase contain a common molybdenum cofactor, whose organic moiety is a novel pterin called molybdopterin (MPT). To assist in elucidating the biosynthetic pathway of MPT, two MPT-deficient mutants of *Escherichia coli* K-12 were isolated. They lacked activities of the molybdenum enzymes nitrate reductase and formate dehydrogenase, did not reconstitute apo nitrate reductase from a *Neurospora crassa nit-1* strain, and did not yield form A, a derivative of MPT. By P1 mapping, these two mutations mapped to *chlA* and *chlE*, loci previously postulated but never definitely shown to be involved in MPT biosynthesis. The two new mutations are in different genetic complementation groups from previously isolated *chlA* and *chlE* mutations and have been designated as *chlM* and *chlN* (closely linked to *chlA* and *chlE*, respectively). The reported presence of Mo cofactor activity in the *chlA1* strain is shown to be due to in vitro synthesis of MPT through complementation between a trypsin-sensitive macromolecule from the *chlA1* strain and a low-molecular-weight compound from the *nit-1* strain.

All molybdenum enzymes except nitrogenase contain a common cofactor, which is a complex of a novel 6-alkyl pterin with the Mo atom (27, 29, 30). This pterin, which has been named molybdopterin (MPT), is widely distributed in many organisms and is required for several essential metabolic pathways (22, 25, 27, 37, 46, 55, 56, 66). The Mo enzymes of wild-type *Escherichia coli* include respiratory nitrate reductase (22, 25), formate dehydrogenase (25, 37), tertiary amine *N*-oxide reductase (59), and biotin-D-sulfoxide reductase (13). Mutants defective in nitrate reductase can be selected by anaerobic growth on chlorate, which is reduced to the highly toxic chlorite ion by nitrate reductase and is thus lethal to wild-type cells (54). Some of the mutants with these mutations, formerly *chlC*, now *narG*, *H*, *I*, *K*, and *L*, are defective in the nitrate reductase protein or its regulatory genes (59). Several other mutant classes, *chlA*, *B*, *D*, *E*, and *G*, lack all four known Mo enzymes (5, 25, 59). The *chlD* and *chlG* mutants are restored to a wild-type phenotype by growth on very high Mo, suggesting that their defect is in Mo transport or processing rather than in MPT synthesis (13, 25, 52, 59). Strains with mutations in *chlB* are postulated to lack a factor necessary for insertion of Mo cofactor into protein (16, 41), and those with mutations in *chlA* and *chlE* are thought to lack the Mo cofactor (5, 25, 52, 59).

To date, however, no *E. coli* mutant has been shown unequivocally to lack MPT. The same is true of all putative Mpt^- mutants in numerous other organisms (8, 9, 11, 14, 43, 53, 58, 61, 68), with the sole exception of the *Neurospora crassa nit-1* mutant (33). We have applied both a refined *nit-1* reconstitution assay procedure and a direct physicochemical assay for MPT to the analysis of potential Mpt^- mutants in *E. coli*, and we report definitive characterization of true Mpt^- mutants in *E. coli*.

(A preliminary report of this investigation has been presented [Johnson and Rajagopalan, Fed. Proc. 42:1895, 1983].)

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MATERIALS AND METHODS

Nomenclature. The following phenotype designations are used: Chl^r, chlorate resistant anaerobically; Fdh, formate dehydrogenase phenotype; Nar, nitrate reductase phenotype; and Mpt, MPT phenotype. Following established nomenclature (34), the addition of a superscript + or − denotes the phenotype of anaerobic growth on the following media: LN, lactate-nitrate medium; PN, proteose peptone-nitrate medium; GF, glycerol-fumarate medium.

The traditional term "Mo cofactor" is used here to refer to the complete, biologically active cofactor, containing both the pterin and the Mo atom, whereas the name MPT denotes the cofactor's metal-free pterin moiety, which is biologically active when supplied with exogenous Mo as molybdate. Since MPT is also a component of Mo cofactor, when characterizing bacterial strains the term MPT will refer to the total population of MPT-containing molecules, whether or not they are coordinated to Mo.

Media and chemicals. Trypticase soy broth (TSY) and Trypticase were from BBL Microbiology Systems; tryptone, yeast extract, and agar were from Difco Laboratories. M9 minimal medium (48) and medium E (65) were as described previously. Carbon sources with these minimal media were 0.2%, added after autoclaving. When needed, amino acids and vitamins were added as described previously (48). Trypticase (1.7%) was chosen as a supplement to M9 in experiments with rich media. When added, tetracycline was 20 μg/ml, ampicillin was 100 μg/ml, and neomycin was 100 μg/ml. For plates, 1.5 to 2.0% agar was added to liquid media. Molybdenum and selenium were added as Na_2MoO_4 and Na_2SeO_3, respectively, at a level of 1 μM each unless otherwise specified.

Chlorate medium contained TSY, molybdate, selenate, and 20 mM $NaClO_3$; the last was autoclaved separately. EMB-galactose medium was as described previously (48). LN medium was modified from its original formula (64) and contained M9 salts, molybdate, selenate, 0.1 M KNO_3, 0.04% tryptone, and 0.4% DL-lactate (sodium salt, adjusted to pH 7.0); the last was autoclaved separately. PN medium was as described previously (59), with the addition of

TABLE 1. Bacterial strains[a]

Strain	Relevant Genotype[b]	Origin, reference
AB1885	*galK2 uvrB5 fnr*[c] λ⁻	G. Sancar (24)
CY346	(MuNeo[r])[d]	J. Cronan (12)
H712	*trp-45* λ⁻	CGSC[e] (50)
JC241	F128::Mu 18A-1/*recA*[d]	J. Cronan (12)
JC10240	Hfr PO45 *srlA300*::Tn*10* *recA56*	W. Brill (42)
JRG94	F⁻ *bioA2 gal-31 chlD4*	CGSC (64; C122 in that reference)
JRG97	F⁻[f] *gal-26 chlE5* λ⁻	CGSC (64; C26 in that reference)
KL715	F147 (*chlD*⁺ *gal*⁺ *chlA*⁺ *chlE*⁺ *pyrD*⁺)/*pyrD34* *recA1 thyA33* λ⁻	CGSC (39)
LCB382[g]	F⁻ *thr-1 leuB6 argH purE43* *gal-6 rpsL9 chlA1* χ[r] λ⁻ *fnr*[c]	CGSC (54)
MG1655	F⁻ λ⁻	CGSC (18)
MJ1	F⁺	R. Greene
MJ7	MJ1 F⁻ *rpsL*	Spontaneous Str[r] MJ1; acridine orange → F⁻
MJ17	MG1655 *rpsL*	Spontaneous Str[r] MG1655
MJ122	JRG94 *gal*⁺ *chlD*⁺ λ⁻	P1 MJ7 × JRG94 → Gal⁺
MJ345	LCB382 *gal*⁺	P1 MJ17 × LCB382 → Gal⁺
MJ392	JRG97 *gal*⁺	P1 MJ17 × JRG97 → Gal⁺
MJ397	MJ7 *gal*	Spontaneous dGal[r] MJ7[h]
MJ402	MJ122 *bio*⁺ *chlM*	P1 N23 × MJ122 → Bio⁺
MJ411	MJ122 *bio*⁺ *chlN*	P1 N51 × MJ122 → Bio⁺
MJ421	MJ7 *chlA1*	P1 MJ345 × MJ397 → Gal⁺
MJ429	MJ421 *gal*	Spontaneous dGal[r] MJ421
MJ431	MJ7 *chlM*	P1 MJ402 × MJ397 → Gal⁺
MJ439	MJ431 *gal*	Spontaneous dGal[r] MJ431
MJ441	MJ7 *chlN*	P1 MJ411 × MJ397 → Gal⁺
MJ453	MJ7 *chlE5*	P1 MJ392 × MJ397 → Gal⁺
MJ591	MJ421 (Mu 18A-1) (Mu Neo[r])	CY346 lysate × MJ421 → Neo[r]; × JC241 → Amp[r] Neo[r]; retested for Nar⁻
MJ592	MJ431 (Mu 18A-1) (Mu Neo[r])	As MJ591 but using MJ431, not MJ421
MJ593	MJ441 (Mu 18A-1) (Mu Neo[r])	As MJ591 but using MJ441, not MJ421
MJ594	MJ453 (Mu 18A-1) (Mu Neo[r])	As MJ591 but using MJ453, not MJ421
MJ600	MJ7 *bioA2 chlE5*	P1 MJ122 × spontaneous dGal[r] MJ453 → Gal⁺
MJ603	MJ7 *bioA2 chlN*	P1 MJ122 × spontaneous dGal[r] MJ441 → Gal⁺
N23	MJ7 *chlM*	Nar⁻ Mpt⁻ mutant of MJ7
N51	MJ7 *chlN*	Nar⁻ Mpt⁻ mutant of MJ7
RE103	F⁻ cmlA1	CGSC (57)

molybdate and selenate. GF medium (35) was modified to consist of medium E salts, 0.02 M potassium fumarate, and 0.4% (vol/vol) glycerol; the last two were autoclaved separately.

Chicken intestinal alkaline phosphatase (1.3 U/mg) was from Worthington Diagnostics. All inorganic chemicals were of reagent grade. Periodic acid was prepared by adjusting 50 mM sodium metaperiodate to pH 3.0 with 1 N HCl. Florisil and high-performance liquid chromatography (HPLC)-grade methanol were from Fisher Scientific Co. Deionized water was prepared with a Continental Systems deionizer and was used for all chemical solutions and media. Other biochemicals were from Sigma Chemical Co.

Fungal, bacterial, and phage strains. *N. crassa nit-1* (allele 34547) spores were stored in desiccated silica at 22°C. All bacterial strains used in the present work are listed in Table 1; all are *E. coli* K-12 derivatives. Strains LCB382, JRG94, and JRG97 were obtained from the same *E. coli* Genetic Stock Center stocks used by Amy (3). Phage P1.L4 (10) and $\lambda b_2 c$ were obtained from R. Greene. Phage f1 was obtained from R. Webster.

Genetic methods. Mutagenesis was with ethyl methanesulfonate as previously described (51). Spontaneous *gal* mutants were selected by resistance to 2-deoxygalactose as described (2), except that incubation was done aerobically without chlorate. Episomes were cured with acridine orange (48). The presence of F was tested by spot tests with the male-specific phage f1, and the presence of λ prophage was tested by cross-streaking with $\lambda b_2 c$ (48). Reversion to *chl*⁺ and *fnr*⁺ was tested by centrifuging a 5-ml aerobic culture in TSY, suspending it in 0.5 ml of saline, and plating samples. Cell counts were determined in triplicate as described previously (48).

P1 transduction was performed as described by Miller (48) with phage lysates prepared by confluent lysis. Mini-mu transduction was performed with lysates prepared by heat induction of lysogens (12). Rapid Hfr mating was performed as described previously (38) with the Hfr kit supplied by B. Bachmann, selecting for *chl*⁺ on LN anaerobically. Other Hfr matings and F′ crosses for strain construction were performed in liquid suspension (48).

Scoring of phenotypes. Selection for *chl*⁺ was on LN or PN anaerobically. Selection for *gal*⁺ was on M9 or medium E-galactose; scoring for *gal* as an unselected marker was on EMB-galactose. Chl[r] was scored on chlorate medium anaerobically. Chl[r] cannot be selected for in P1 transduction because of the high spontaneous mutation rate to *chl*, 10^{-5} to 10^{-6} (1). Cml[r] was scored as described previously (57), and auxotrophic markers were scored on appropriately supplemented M9 or medium E-glucose lacking the specific nutrient being tested. The presence of *fnr*⁺ was scored by

[a] All strains are derivatives of *E. coli* K-12.

[b] λ⁺ is considered wild type for *E. coli* K-12; only λ⁻ is noted. The λ genotypes listed for MJ7, MJ122, MJ397, MJ421, MJ431, MJ441, MJ453, N23, and N51 were verified by cross-streaking with $\lambda b_2 c$.

[c] When a P1 lysate of LCB382 was used to transduce H712 to Trp⁺, 2.5% of 358 transductants were PN⁻ (*fnr*). When a P1 lysate of H712 was used to transduce AB1885 to PN⁺ (*fnr*⁺), 2.0% of 100 transductants were Trp⁺. These values are similar to those previously reported for *fnr-trp* contransduction (34).

[d] Mu 18A-1 is Mu *cts62* Δ*B-R* Amp[r] (mini-Mu); Mu Neo[r] = Mu *cts62 kil* Neo[r] (helper Mu).

[e] CGSC, *E. coli* Genetic Stock Center, Yale University.

[f] Although originally reported to be F⁺, our purified stocks are F⁻.

[g] LCB382 has also been referred to as 382 (3, 47) and JP382 (60); LCB382 is the most recent CGSC strain designation.

[h] dGal, 2-Deoxygalactose.

anaerobic growth on PN for chl^+ strains and by anaerobic growth on GF for *chl* strains (34, 59).

To screen colonies for nitrate reductase and to score *chl* as an unselected marker, a dye overlay assay for formate-dependent nitrate reductase was used, modified from a previously published method (17), with 8-anilino-1-naphthalene sulfonic acid as the dye-coupling agent. Wild-type Fdh^+ Nar^+ (chl^+ fnr^+) colonies give a well-defined dark purple spot, somewhat larger than the colony itself. Strains containing *chlA*, *B*, *E*, or *G* are completely colorless; *chlC* and *chlD* and *fnr* give a light purple color which is clearly distinct both from the other *chl* mutants and from the wild type, with the *fnr* mutants having more color than *chlC* or *chlD* mutants. By this technique, a plate with up to 400 small colonies or a grid of 100 large colonies could easily be scored.

For some of the experiments where *chl* was the unselected marker in a two-point cross, a rapid mapping technique was developed. The original transduction plate, containing Mo and Se but lacking nitrate, e.g., for gal^+ colonies, was overlaid with formate-nitrate-phosphate as described above, except that 30 min was allowed before the color reagents were overlaid. This gave results identical to those obtained when colonies were purified first and then placed on a grid and scored for *chl* on TSY plates.

Growth conditions and preparations of extracts. *N. crassa nit-1* cells were grown, induced for apo-nitrate reductase, harvested, and stored as described previously (4). Extracts were prepared just before use with a Duall glass grinder, with 1 g of mycelia per 2 ml of *nit-1* grinding buffer (0.10 M potassium phosphate, 5 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1% ethanol) (23). Dithiothreitol and phenylmethylsulfonyl fluoride (in ethanol) were always added just before grinding because of their instability in aqueous solution.

E. coli cells were harvested at the late log or early stationary phase by centrifugation at 12,000 × *g* for 10 min. For *nit-1* reconstitution and dephospho-form A assays, the pellet was suspended in freshly mixed 10 mM sodium ascorbate–5 mM EDTA, pH 7.4 (bacterial resuspension buffer), in a ratio of approximately 5 ml/g of wet weight, and stored at −20°C until use. Extracts were prepared with a French pressure cell as described previously (4).

For nitrate reductase and formate dehydrogenase assays, the cell pellet was washed once with 1 volume of cold 50 mM potassium phosphate (pH 7.4), equal to the original culture volume, and centrifuged. The pellet was taken up in 10 mM potassium phosphate (pH 7.4), at 5 ml/g of wet weight, and stored at −20°C until assayed.

***E. coli* enzyme assays.** All assays were performed at room temperature (21 to 23°C) with whole cell suspensions. Nitrate reductase and 2,6-dichlorophenolindophenol–phenazine methosulfate-linked formate dehydrogenase were assayed as described previously (3), except that benzyl viologen was substituted for methyl viologen in the nitrate reductase assay because of its greater permeability in whole cells (32), and the nitrite detection reagents described elsewhere (33) were used. Nitrate reductase based on nitrate-dependent oxidation of reduced benzyl viologen was assayed as described previously (36). All assays were linear with respect to time and amount of cells added. Enzyme activities were calculated by using extinction coefficients at 600 nm of 21,000 for 2,6-dichlorophenolindophenol and 7,400 for reduced beneyl viologen (36), and assuming a two-electron reduction of nitrate and 2,6-dichlorophenolindopherol, two-electron oxidation of formate, and one-electron oxidation of reduced benzyl vilogen. Absorbances and rates were determined on Perkin-Elmer 575, Varian Cary 219, or Shimadzu UV-240 spectrophotometers.

MPT assay by *nit-1* reconstitution. The standard method for reconstitution of *nit-1* apo-nitrate reductase, originally described by Nason and co-workers (27, 49), involves mixing the cofactor source with a fresly prepared *nit-1* crude extract, incubating at room temperature for 10 to 60 min, and then assaying samples for NADPH-dependent nitrate reductase. This method is adequate as a qualitative comparison among samples tested at the same time under identical conditions. However, it is not quantitative, mainly because the *nit-1* apo-nitrate reductase is very labile and is gradually inactivated by incubation at room temperature, so that wide variability is the rule rather than the exception in studies with this assay (20, 27, 67).

Quantitative reconstitution of excess *nit-1* apo-nitrate reductase with all the MPT added to a reconstitution mixture is possible if the reconstitution is carried out at 4°C for 24 h (19, 20). Reconstitution with various amounts of the MPT source gives a typical titration curve, with a slope of nitrate reductase activity per microgram of sample. This can be transformed to MPT concentration by using the turnover number for nitrate reductase. We have used 20,000 per Mo per min as the turnover number for nitrate reductase (20, 23, 26).

The protein-free MPT-Mo complex is extremely labile, so that high (10 mM) exogenous Mo (as molybdate) must be present during *nit-1* reconstitution for maximal activity (20, 67). The reconstitution of *nit-1* apo-nitrate reductase is thus an assay for MPT, rather than for the intact Mo cofactor. The reconstitution mixtures used here contain 10 mM Mo.

For *nit-1* reconstitution, a mixture of 20 μl of *nit-1* extract, 20 μl of various dilutions of bacterial extract, and 10 μl of 50 mM Na_2MoO_4 was incubated at 4°C for 20 to 40 h (20). After reconstitution, the entire reconstitution mixture was assayed by adding to it 0.45 ml of NADPH-nitrate reductase assay solution (4), incubating at 22°C for a given time, adding 1.0 ml nitrite detection reagents (33), letting color develop for 20 min, and centrifuging, and then reading absorbance at 540 nm. NADPH inhibits the nitrite detection reaction, but is also consumed during the assay, resulting in nonlinear assays if this effect is not corrected for (20, 33). The NADPH-regenerating system of isocitrate and isocitrate dehydrogenase (20) was added to the assays to provide linear reaction rates.

To prepare G-25 excluded and included fractions of *nit-1* and bacterial extracts, Pharmacia PD-10 Sephadex G-25 columns were equilibrated and eluted with the same buffer used to prepare the bacterial extract. To obtain good separation between the excluded and included fractions, 2.0 ml of bacterial extract was applied to the column, 1.0 ml of eluate was discarded, 2.5 ml was collected as the excluded volume, 2.0 ml was discarded, and 4.0 ml was collected as the included volume.

MPT assay by analysis of dephospho-form A. A previously described method was adapted for converting molybdopterin in cell extracts to its oxidized, fluorescent degradation product, dephospho-form A (30). All samples were shielded from light because of the light sensitivity of 6-substituted pterins (7). To 1 ml of cell extract (in bacterial resuspension buffer) was added 0.1 ml of 1 N HCl and 1 ml of 1% I_2–2% KI. The suspension was placed in a boiling water bath for 20 min total; after the first 5 min, an additional 0.5 ml of I_2-KI was added. The sample was then cooled to room temperature, and the residual iodine was reduced by the addition of

1 ml of fresh 1% sodium ascorbate. The pH was adjusted to 8.5 with 2 M ammonium hydroxide, and the sample was centrifuged for 10 min at 17,000 × *g*. To the supernatant was added 20 μl of 1.0 M magnesium chloride and 100 μl of alkaline phosphatase (2.0 mg/ml in 0.10 ammonium bicarbonate). The sample was incubated overnight at room temperature and then analyzed by HPLC.

In the case of samples preincubated for 40 h at 4°C to test for in vitro complementation, further treatment was necessary to obtain adequate resolution of dephospho-form A during HPLC. To a 0.5-ml sample was added 0.5 ml of 2 M ammonium hydroxide; the entire 1 ml was applied to a 0.5- by 0.5-cm column of QAE-Sephadex (fine; acetate form). The column was washed with 5 ml of water and 0.5 ml of 0.01 N acetic acid. A broad fluorescent peak was then eluted with 2.5 ml of 0.01 N acetic acid; this peak was used for HPLC analysis. All samples were stored at −20°C until analysis by HPLC.

HPLC was performed at room temperature with an Alltech C18 reverse-phase column (4.6-mm inner diameter by 25 cm; 10-μm particle size), an LDC Constametric III pump, a Rheodyne 7125 injector with 1.0-ml sample loop, and an LDC FluoroMonitor III. After each sample injection (except for standards), the column was washed with 4.5 mM sulfuric acid in 100% methanol until no more fluorescent material was eluted. Standards were injected several times during the day, and new standard curves were calculated each day.

Dephospho-form A standards were prepared from purified chicken liver sulfite oxidase (28) and quantitated as described previously (29, 30). Fluorescence spectra were obtained on an Aminco-Bowman spectrofluorometer. Protein in extracts was determined by the method of Lowry et al. (40) with crystalline bovine serum albumin as a standard. For whole cells, a modification (21) of the Lowry et al. method was used.

RESULTS

Isolation of MPT-deficient mutants. Out of approximately 15,000 ethyl methanesulfonate-mutagenized colonies derived from MJ7 and grown aerobically (so that anaerobically lethal mutations would be included), 68 were Nar^- in the nitrate reductase overlay assay. After restreaking twice for single colonies, we tested the colonies for MPT by *nit-1* reconstitution. Two isolates, N23 and N51, were Mpt^- in the screening assay. They grew well on minimal and rich media with glucose aerobically and anaerobically and minimal media with succinate aerobically. They did not grow on LN or PN (with or without 1 mM Mo) anaerobically, where nitrate respiration is required owing to lack of fermentable substrate (59, 64), but did grow well aerobically on these media. These mutants were also chlorate resistant (*chl*) anaerobically.

Genetic characterization of N23 and N51. Both N23 and N51 spontaneously reverted to wild type, as measured by colony formation on LN and PN agar anaerobically, at a frequency of 5×10^{-9} and 5×10^{-8}, respectively, a rate consistent with their being single mutations (48). Hfr and F′ mapping suggested that the *chl* mutations of both N23 and N51 were in the *chlD-chlAE* region. P1 transduction analysis showed that the *chl* mutations in N23 and N51 mapped to *chlA* and *chlE*, respectively (5, 59, 64). Two PN^+ revertants each of N23 and N51 were used as P1 donors to transduce MJ122 (*bio chl*$^+$) to Bio^+. Of more than 100 Bio^+ transductants in each cross, all were Nar^+. These results strongly suggested that N23 and N51 each contain only a single mutation affecting MPT biosynthesis.

Two previously characterized pleiotropic *chl* mutants, with *chlA1* and *chlE5* mutations, have been reported to be capable of reconstituting *nit-1* nitrate reductase (3, 47, 60), suggesting that the pleiotropy of those mutants is not due to the absence of Mo cofactor. To characterize the mutations in N23 and N51 more completely, and to compare them with *chlA1* and *chlE5*, we transferred each of the four mutations into the same isogenic background (details not presented). All data reported here, unless otherwise noted, refer to this isogenic set of strains differing only at a single *chl* locus.

Results of complementation analysis by mini-Mu transduction, as described by Cronan (12), demonstrated that each of the *chl* strains tested in this report complemented all of the other strains tested. A definitive proof of complementation was afforded by the instability of the mini-Mu transductants in the absence of selective pressure at 37°C (where the Mu *cts* repressor is partially inactive). For each cross, several PN^+ Amp^r colonies were subcultured aerobically at 37°C, and the colonies of each subculture plate were tested for nitrate reductase by the dye overlay described in Materials and Methods. In each case, 5 to 10% of the colonies had become Nar^- (i.e., *chl*). Because of the evidence that N23 and N51 comprise separate complementation groups in reference to *chlA* and *chlE* mutations, these new mutations have been given the designation of *chlM* and *chlN* respectively.

Molybdenum enzyme expression in *chl* mutants. All strains were grown anaerobically with nitrate to maximally induce nitrate reductase and formate dehydrogenase (25). The *chl* mutants consistently had extremely low Mo enzyme activities and were not affected by high molybdate or other growth variables (Table 2). For Trypticase-grown cells, nitrate reductase was assayed in two different ways to rule out interference from nitrate reductase. Such interference has been reported for a *chlC* mutant of *Salmonella typhimurium*

TABLE 2. Mo enzyme activities[a]

Strain	Enzyme activities in medium plus the following:						
	1 μM Mo		1 mM Mo		1 μM Mo plus 1.7% Trypticase		
	FDH	NaR	FDH	NaR	FDH	NaR	NaR*
chl$^+$	1,144 ± 72	5,680 ± 40	585 ± 35	2,427 ± 228	63.9 ± 7.6	775 ± 0	2,053 ± 22
chlM	0.1 ± 0.3	<0.01	0.2 ± 0.0	<0.01	0.2 ± 0.0	0.13 ± 0.07	2.0 ± 0.1
chlN	0.2 ± 0.2	<0.01	0.1 ± 0.1	0.07 ± 0.08	0.1 ± 0.1	<0.01	<0.1
chlA1	0.0 ± 0.0	<0.01	0.0 ± 0.0	0.06 ± 0.04	0.0 ± 0.0	0.06 ± 0.06	1.0 ± 0.5
chlE5	0.1 ± 0.1	<0.01	0.2 ± 0.1	0.01 ± 0.00	0.2 ± 0.00	<0.01	0.1 ± 0.1

[a] All cultures were grown anaerobically in M9 glucose medium containing 0.10 M KNO_3 plus the indicated addition. Formate dehydrogenase (FDH) and nitrate reductase (NaR) are expressed in nanomoles of formate or nitrate per minute per milligram. NaR activities were assayed by determining nitrite production, except for the values reported in the final column (NaR*) which are the rates of anaerobic, nitrate-dependent, reduced benzyl viologen oxidation. All assays were replicated two or more times. Errors given are the range of two determinations or the standard of greater than two determinations.

TABLE 3. MPT assayed by *nit-1* reconstitution[a]

Strain	*nit-1* fraction	MPT (fmol/mg of bacterial protein) in medium plus the following:		
		1 μM Mo	1 mM Mo	1 μM Mo plus 1.7% Trypticase
chl$^+$	Crude	2.17 ± 0.09 (7)	2.29 ± 0.11 (7)	12.13 ± 0.38 (12) 15.55 ± 0.74 (5) 6.83 ± 0.72 (4)[b]
	G-25 excluded	3.51 ± 0.36 (7)	2.98 ± 0.13 (7)	12.01 ± 0.44 (12) 11.32 ± 0.95 (7) 14.83 ± 0.71 (7) 10.22 ± 0.62 (4)[b]
chlM	Crude	0.000 ± 0.001 (4)	0.002 ± 0.001 (4)	0.007 ± 0.001 (4)
	G-25 excluded	0.003 ± 0.001 (4)	0.001 ± 0.002 (4)	0.007 ± 0.001 (3) <0.000 ± 0.004 (5)
chlA1	Crude	2.19 ± 0.31 (8)	4.78 ± 0.47 (3)	6.34 ± 0.18 (7) 8.38 ± 0.55 (9)
	G-25 excluded	<0.00 ± 0.05 (10)	<0.000 ± 0.008 (7)	<0.000 ± 0.016 (11) 0.52 ± 0.19 (9)
chlN	Crude	0.002 ± 0.002 (4)	<0.000 ± 0.003 (4)	0.002 ± 0.001 (4)
	G-25 excluded	0.003 ± 0.000 (4)	0.002 ± 0.003 (4)	0.001 ± 0.000 (3) 0.004 ± 0.002 (5)
chlE5	Crude	0.013 ± 0.005 (4)	0.039 ± 0.012 (4)	0.725 ± 0.029 (9) 0.069 ± 0.001 (4)[b]
	G-25 excluded	0.014 ± 0.009 (4)	0.060 ± 0.027 (4)	0.334 ± 0.011 (11) 0.081 ± 0.002 (4)[b]

[a] All cultures were grown aerobically in M9-glucose medium plus 1 μM Se and the indicated supplements. Numbers within parentheses are the numbers of data points used for calculations.
[b] Tryptone was substituted for trypticase in growth medium.

(6). The assays differed somewhat, but did not significantly change the vast differences in nitrate reductase activities between *chl*$^+$ and *chl* strains.

MPT assay by *nit-1* reconstitution. The most common assay for MPT is reconstitution of apo-nitrate reductase from the *N. crassa nit-1* mutant (27, 49). The mutants isolated by us, *chlM* and *chlN*, lacked any detectable MPT, whereas the *chlA1* and *chlE5* mutants previously studied in nonisogenic backgrounds did appear to have MPT when used to reconstitute crude *nit-1* extracts (Table 3). Whereas *chlA1* was comparable to *chl*$^+$ in its ability to reconstitute activity in crude *nit-1* extracts, *chlE5* had only 6% of the *chl*$^+$ activity (Table 3). The levels of apparent MPT were higher in cells grown on rich, as compared to minimal, medium for *chl*$^+$, *chlA1*, and *chlE5*. The increase for *chl*$^+$ is similar to that previously reported (3).

In vitro complementation during *nit-1* reconstitution. Reconstitution of *nit-1* has been extensively used to characterize possible Mpt$^-$ mutants in many species (3, 11, 44, 45, 47, 68). However, even with the modification used here to obtain quantitative results, the potential for in vitro formation of active cofactor is present when crude extracts of *nit-1* and any other mutant are mixed. Either extract might supply a precursor of MPT which an enzyme in the other extract could convert to MPT, giving a false-positive result. When gel-filtered excluded fractions of *nit-1* extracts were used as the source of apo-nitrate reductase, the wild type, *chlM*, and *chlN* were virtually unaffected, whereas a moderate decrease in activity was seen for *chlE5* (Table 3). For *chlA1*, however, there was a dramatic decrease in apparent MPT when excluded *nit-1* was used, suggesting that *chlA1* is in fact an MPT$^-$ mutant and that its reconstitution of crude *nit-1* is not due to the presence of MPT in *chlA1*, but rather to in vitro formation of MPT by a process requiring a low-molecular-weight compound from *nit-1* and some factor from *chlA1*. A similar effect for *chl*$^+$ suggests that the *chl*$^+$ extract also contains the complementation factor found in *chlA1*.

When *chlA1* extract was gel filtered on Sephadex G-25, all of its complementation factor activity was in the excluded volume, required crude *nit-1* for detection, and was totally abolished by trypsin treatment. For *chl*$^+$ and *chlE5* extracts, most of the apparent MPT activity was also in the excluded fraction, but was detectable with gel-filtered *nit-1* as well as crude *nit-1*. A Mo cofactor-containing macromolecular fraction without known enzymatic function has been reported previously and defined functionally as a carrier protein (4). Neither pure MPT nor carrier protein-bound MPT is affected by trypsin (4, 19, 33).

Dephospho-form A assay for MPT. MPT itself is extremely unstable in the presence of oxygen (19). Treatment of Mo enzymes with acidic I_2 and KI at 100°C causes the elimination of the vicinal sulfhydryls and the oxidation of the pterin nucleus, yielding form A, a stable, fluorescent, biologically inactive pterin with the 6-alkyl substituent, $-C{\equiv}C-CHOHCH_2OPO_3^{2-}$ (Fig. 1) (29). The yield of form A from pure Mo enzymes is about 50% of their MPT content. Form A is highly fluorescent, and its dephospho form can be readily characterized by reverse-phase HPLC (29, 30, 33). The form A assay, although much more difficult to perform,

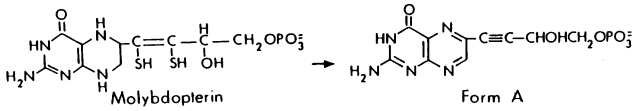

FIG. 1. Postulated structure of MPT and known structure of form A, an oxidized derivative of MPT.

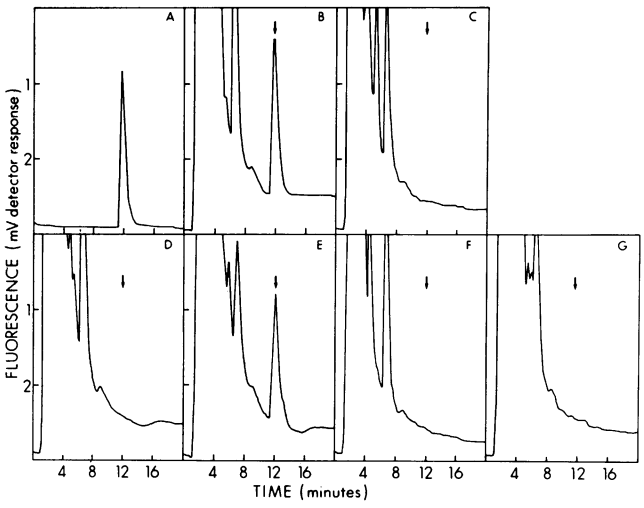

FIG. 2. HPLC of bacterial dephospho-form A preparations. The C18 column was run in 10% methanol at 2.00 ml/min. The fluorescence detector was set to the 1 scale. For panel A, 15 μl of dephospho-form A standard, containing 1.98 pmol total, was injected. For panels B through G, bacterial extracts were treated as described in Materials and Methods to give dephospho-form A preparations, diluted 3.1- to 3.3-fold from original extracts; 25 μl of each were then injected as follows: B, *chl*$^+$; C, *chlM*; D, *chlA1*; E, *chlA1*. Extracts were made 571 nM in sulfite oxidase monomer before treatment for dephospho-form A (F, *chlN*; G, *chlE5*). The arrows indicate the elution position of dephospho-form A standard when it was mixed with the bacterial dephospho-form A preparations before injection (data not shown). Cells were grown aerobically in M9 glucose plus 1 μM Mo, 1 μM Se, and 1.7% Trypticase.

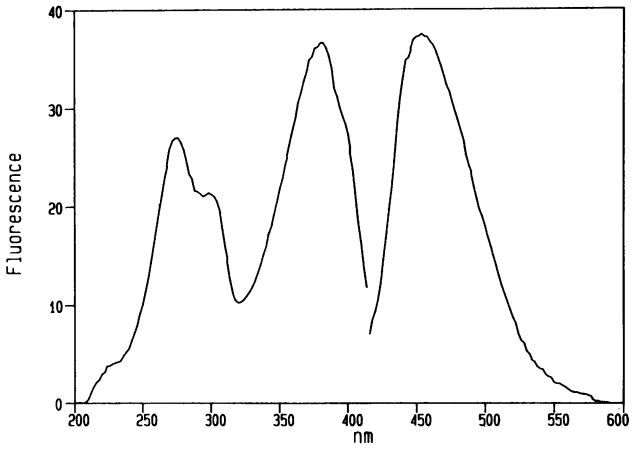

FIG. 3. Fluorescence spectrum of dephospho-form A from *chl*$^+$. Preparative HPLC of 0.40 ml of *chl*$^+$ dephospho-form A preparation (the same preparation analyzed in Fig. 2) was performed with 10% methanol at 2.00 ml/min, collecting 0.5-min fractions. The dephospho-form A peak fraction was diluted 1:1 with 2 M NH_4OH. Uncorrected fluorescence spectra of this peak and the appropriate blank were obtained using the 1 scale, with excitation at 380 nm, emission at 460 nm, damping on (time constant, 2 s), and a scan speed of 30 nm/min. The uncorrected spectra were digitalized at 2-nm intervals, the blank was subtracted from the sample to correct for scattering, and the resultant data were plotted to give the corrected spectrum above.

has several advantages over the *nit-1* reconstitution assay for characterizing potential Mpt$^-$ mutants. The form A assay is specific for the pterin of the Mo cofactor and detects all forms of MPT (19, 29, 67), whereas enzyme-bound forms of MPT require release by denaturing agents to be observed in the *nit-1* assay (19, 20, 27, 47, 49). The form A assay should be unaffected by organic precursors of MPT which might be converted to MPT by *nit-1* extract in vitro, with the exception that a precursor almost identical to MPT might also yield form A or dephospho-form A.

For the form A assay, bacterial extracts were treated as described in Materials and Methods to yield dephospho-form A preparations. The resulting samples were analyzed by C18 reverse-phase HPLC. Dephospho-form A in *chl*$^+$ was easily detectable (Fig. 2B), and its identity was confirmed by its fluorescence spectrum (Fig. 3). The *chlM*, *chlA1*, and *chlN* preparations had no detectable dephospho-form A (Fig. 2C, D, and F). The *chlE5* preparation had a small peak that cochromatographed with authentic dephospho-form A, but its identity could not be established owing to paucity of material.

HPLC of the bacterial dephospho-form A preparations also allows quantitation of MPT. Peak height of standards was consistently proportional to the quantity injected, yielding standard curves with $r^2 > 0.99$. Both form A and *nit-1* reconstitution assays for the same bacterial extracts are presented in Table 4. The results are generally comparable; the higher dephospho-form A values for *chl*$^+$ suggest that the *nit-1* assay did not detect all the MPT-containing species present. The higher MPT values and uncertainties in the dephospho-form A assays for *chlM*, *chlA1*, and *chlN* reflect the lower sensitivity of that assay compared with *nit-1* reconstitution.

Assays for other pterin derivatives of MPT (29) showed that appropriately treated wild-type extracts yielded form A (phospho) and dephospho-form B; these compounds were not detectable in the *chlA1*, *chlM*, and *chlN* extracts tested (M. E. Johnson, Ph.D. thesis, Duke University, Durham, 1985).

In vitro complementation among *chl* mutants. Genetic complementation was observed among all four of the *chl* mutants studied here (Table 3). In vitro complementation among these mutants, if observed, would greatly facilitate the search for intermediates and enzymes involved in the biosynthesis of MPT. In the simplest case of four different structural genes corresponding to the four *chl* mutants

TABLE 4. MPT assayed by dephospho-form A production[a]

Strain	MPT (fmol/μg of bacterial protein)	
	Dephospho-form A assay[b]	G-25-excluded *nit-1* assay
chl$^+$	17.1 ± 1.1 (8)	11.32 ± 0.95 (7)
chlM	0.25 ± 0.20 (4)	<0.000 ± 0.004 (5)
chlA1	0.06 ± 0.03 (3)	<0.000 ± 0.016 (11)
chlN	0.17 ± 0.17 (4)	<0.000 ± 0.002 (5)
chlE5	0.40 ± 0.17 (8)	0.334 ± 0.011 (11)

[a] Each culture was grown aerobically in M9 glucose plus 1.7% trypticase. A portion of each extract was tested for reconstitution of *nit-1* G-25 excluded volume. Another portion was treated to give a dephospho-form A preparation for each extract, and analyzed by HPLC as described in the legend to Fig. 2 and Materials and Methods. Numbers in parentheses are the numbers of data points used for calculating *nit-1* reconstitution as in Table 3 or the numbers of separate HPLC injections of the same dephospho-form A preparation.

[b] The recovery of form A was tested by adding a known amount of pure sulfite oxidase to *chlA1* extract (Fig. 2E). A yield of 0.433 ± 0.010 mol of dephospho-form A per mol of MPT was observed. This figure was used to correct the dephospho-form A HPLC assays to the amount of MPT actually present; the table presents the corrected values. The observed yield was consistent with the reported maximal yeild of 50% (29).

studied here, any combination of two of these *chl* mutants should also give in vitro complementation resulting in MPT synthesis. However, in vitro complementation would not be observed if either of the mutants being tested were altered at a regulatory locus, leading to loss of expression of more than one biosynthetic protein. In vitro complementation might not also be observed if one of the intermediates of biosynthetic enzymes needed for complementation were unstable in the cell-free extract. Tests for in vitro complementation between putative Mpt$^-$ mutants of *Aspergillus nidulans* (15) and *N. crassa* (62) have been uniformly negative; however, exogenous Mo was not added to those complementation mixtures.

To test the mutants studied here, mixtures of all possible binary combinations of *chl* extracts were preincubated 40 h at 4°C to allow new synthesis of MPT to proceed. The mixtures were then assayed by reconstitution of *nit-1* (excluded fraction) or by treatment to yield form A. The same tests were also applied to mixtures of *chlA1* with crude and G-25 fractions of *nit-1*. The most striking in vitro complementation was between *chlA1* and any other *chl* mutant or between *chlA1* and *nit-1* (crude or G-25 included) (Table 5). A smaller, but definite, complementation was observed between *chlM* and *chlN*.

If the *nit-1* assay had been used alone, we could not exclude the possibility that in vitro complementation occurred only between *nit-1* and a *chl* mutant, and not between the two *chl* mutants being tested. In fact, the *nit-1* reconstitution values were higher than the dephospho-form A values for some samples (Table 5), suggesting that subsequent in vitro complementation did occur upon the addition of excluded *nit-1*. However, in vitro complementation significantly above background was also observed with the dephospho-form A assay in the absence of any *nit-1* (Table 5), demonstrating that the complementation between *chlA1* and other *chl* mutants was genuine. The dephospho-form A data in Table 5 also establish that the synthesis of MPT by in vitro complementation does not require added Mo.

TABLE 5. In vitro complementation between *chl* and *nit-1* mutants[a]

Prepn	MPT (fmol/μg of protein in preincubation mixture)	
	G-25-excluded *nit-1* assay	Dephospho-form A assay
chlA1 plus crude *nit-1*	1.23 ± 0.06 (7)	3.67 ± 0.59 (4)
chlA1 plus G-25-excluded *nit-1*	0.00 ± 0.03 (5)	0.24 ± 0.14 (2)
chlA1 plus G-25-included *nit-1*	0.83 ± 0.04 (7)	1.32 ± 0.03 (2)
chlA1 plus *chlM*	4.22 ± 0.04 (6)	2.26 ± 0.26 (4)
chlA1 plus *chlN*	3.41 ± 0.04 (6)	2.09 ± 0.19 (3)
chlA1 plus *chlE5*	3.41 ± 0.03 (6)	1.66 ± 0.24 (6)
chlM plus *chlN*	0.33 ± 0.07 (4)	0.91 ± 0.12 (4)
chlM plus *chlE5*	0.09 ± 0.02 (4)	0.57 ± 0.08 (3)
chlN plus *chlE5*	0.05 ± 0.06 (7)	0.39 ± 0.08 (3)
chlA1 plus buffer	<0.00 ± 0.03 (5)	0.19 ± 0.08 (2)
Crude *nit-1* plus buffer	0.02 ± 0.03 (5)	0.40 ± 0.09 (2)
chlM plus buffer	0.02 ± 0.00 (5)	0.43 ± 0.07 (2)
chlN plus buffer	0.00 ± 0.00 (5)	0.32 ± 0.04 (2)
chlE5 plus buffer	0.12 ± 0.01 (5)	0.60 ± 0.01 (2)

[a] Equal volumes of the indicated extracts were preincubated for 40 h at 4°C (without any added Mo), and then samples were mixed with fresh G-25-excluded *nit-1* plus Mo for regular *nit-1* reconstitution. Another portion of each preincubated mixture was treated to produce dephospho-form A and partially purified by quarternary aminoethyl chromatography before HPLC analysis as described in Materials and Methods. The percent recovery of dephospho-Form A from QAE-Sephadex was 68.5 ± 0.3% with the sample of *chlA1* plus sulfite oxidase previously analyzed for stoichiometry of dephospho-form A production (Fig. 2E). Each bacterial culture was grown in M9 glucose plus 1 μM Mo, 1 μM Se, and 1.7% Trypticase. The *nit-1* extracts and G-25 fractions used for preincubation (not reconstitution) were prepared in bacterial resuspension buffer. Buffer in this table refers to bacterial resuspension buffer. All data are presented as in Table 4, with an additional correction for the partial recovery of dephospho-form A from quarternary aminoethyl chromatography.

DISCUSSION

This report is the first demonstration that there are two biochemically distinguishable complementation groups at both *chlA* (*chlA* and *chlM*) and at *chlE* (*chlE and chlN*), and that all four complementation groups were involved in the biosynthesis of MPT, the organic component of the Mo cofactor. Involvement of *chlA* and *chlE* in cofactor synthesis has been postulated previously on the basis of pleiotropy (25), endogenous production of aponitrate reductase (16, 41, 52, 59), and failure to reconstitute apo-nitrate reductase in *nit-1* (47) and other mutants (60). However, those data did not exclude the presence of tightly bound, nonreconstituting forms of MPT, nor of defects in Mo metabolism, as opposed to pterin (MPT) metabolism. Our application of the dephospho-form A assay in conjunction with *nit-1* reconstitution has established a definite role for *chlA*, *E*, *M*, and *N* in MPT biosynthesis.

Two complementation groups have been demonstrated previously in both *chlA* and *chlE* by abortive transduction (63) and in *chlA* by subcloning as well (60). However, there has been no correlation until now between these complementation groups and their roles in Mo cofactor metabolism. Either *chlM* or *chlN* could be the *E. coli* analog of the *nit-1* mutation. Both are devoid of MPT; they complement each other in vitro to a small extent, but do not significantly complement *nit-1* or *chlE5* in vitro.

The *chlA1* mutant lacks MPT, but complements all of the other *chl* mutants and *nit-1* in vitro. Complementation results from the action of a protein in *chlA1* on a low-molecular-weight compound, possibly an MPT precursor, in *nit-1* (31). The *chlA1* mutant may be distinct from all of the known potential Mpt$^-$ mutants in *A. nidulans* (15) and *N. crassa* (62), because none of those mutants complemented *nit-1* in vitro. None of those complementation tests was performed with added Mo, however, so they are not directly comparable to the experiments reported here.

In an earlier report, it was concluded that the primary defect in the *chlA1* mutant was impairment of Mo insertion into the cofactor (i.e., MPT) (3). That conclusion was based primarily on the finding that *chlA1* extracts required higher levels of Mo (≥40 mM) during *nit-1* reconstitution than did wild-type extracts (10 mM). A similar finding and interpretation have been reported for four allelic *Nicotiana tabacum cnx* mutants (45). We have confirmed that *chlA1* extracts have a higher Mo requirement than *chl*$^+$ during crude *nit-1* reconstitution (31). Our work here, however, establishes that the *chlA1* mutant completely lacks MPT and gives a positive result in the *nit-1* assay only because of in vitro complementation. The primary defect in the *chlA1* mutant is therefore a block in MPT biosynthesis, not a block in Mo insertion.

We do not yet understand the mechanism by which Mo is inserted into MPT during synthesis of Mo cofactor-dependent enzymes or the identity of the Mo complex which is the active species in the process. The high levels of molybdate required during *nit-1* reconstitution by preformed MPT or MPT generated from in vitro complementation are obviously nonphysiologic. It is possible that some factor

facilitating the association of Mo, MPT, and apo-nitrate reductase is inactivated during preparation of *nit-1* extract; when not protein bound, the Mo cofactor is rapidly hydrolyzed to Mo and MPT (67). In any event, our data establish that a high Mo requirement for *nit-1* reconstitution by crude extracts may indicate in vitro complementation rather than the presence of preexisting, Mo-deficient MPT. Experiments purporting to measure Mo-containing cofactor versus demolybdo cofactor (i.e., MPT) in crude extracts by Mo dependence of *nit-1* reconstitution (47) must now be reevaluated in the context of preexisting versus newly generated MPT.

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LITERATURE CITED

1. **Adhya, S., P. Cleary, and A. Campbell.** 1968. A deletion analysis of prophage lambda and adjacent genetic regions. Proc. Natl. Acad. Sci. USA **61**:956–962.
2. **Alper, M. D., and B. N. Ames.** 1975. Positive selection of mutants with deletions of the *gal-chl* region of the *Salmonella* chromosome as a screening procedure for mutagens that cause deletions. J. Bacteriol. **121**:259–266.
3. **Amy, N. K.** 1981. Identification of the molybdenum cofactor in chlorate-resistant mutants of *Escherichia coli*. J. Bacteriol. **148**:274–282.
4. **Amy, N. K., and K. V. Rajagopalan.** 1979. Characterization of molybdenum cofactor from *Escherichia coli*. J. Bacteriol. **140**:114–124.
5. **Bachmann, B. J.** 1983. Linkage map of *Escherichia coli* K-12, edition 7. Microbiol. Rev. **47**:180–230.
6. **Barrett, E. L., and D. L. Riggs.** 1982. Evidence for a second nitrate reductase activity that is distinct from the respiratory enzyme in *Salmonella typhimurium*. J. Bacteriol. **150**:563–571.
7. **Blakley, R. L.** 1969. The biochemistry of folic acid and related pteridines, p. 58–105. North-Holland Publishing Co., Amsterdam.
8. **Bright, S. W. J., P. B. Norbury, J. Franklin, D. W. Kirk, and J. L. Wray.** 1983. A conditional-lethal *cnx*-type nitrate reductase-deficient barley mutant. Mol. Gen. Genet. **189**: 240–244.
9. **Buchanan, R. J., and J. L. Wray.** 1982. Isolation of molybdenum cofactor defective cell lines of *Nicotiana tabacum*. Mol. Gen. Genet. **188**:228–234.
10. **Caro, L., and C. M. Berg.** 1971. P1 transduction. Methods Enzymol. **21**:444–458.
11. **Claassen, V. P., L. F. Oltmann, S. Bus, J. v.'t Riet, and A. H. Stouthamer.** 1981. The influence of growth conditions on the synthesis of molybdenum cofactor in *Proteus mirabilis*. Arch. Microbiol. **130**:44–49.
12. **Cronan, J. E., Jr.** 1984. Mini-Mu-duction as a test for genetic complementation in *Escherichia coli*. J. Bacteriol. **158**:357–361.
13. **del Campillo-Campbell, A., and A. Campbell.** 1982. Molybdenum cofactor requirement for biotin sulfoxide reduction in *Escherichia coli*. J. Bacteriol. **149**:469–478.
14. **Fankhauser, H., F. Bucher, and P. J. King.** 1984. Isolation of biochemical mutants using haploid mesophyll protoplasts of *Hyoscyamus muticus*. IV. Biochemical characterization of nitrate non-utilizing clones. Planta **160**:415–421.
15. **Garrett, R. H., and D. J. Cove.** 1976. Formation of NADPH-nitrate reductase activity in vitro from *Aspergillus nidulans niaD* and *cnx* mutants. Mol. Gen. Genet. **149**:179–186.
16. **Giordano, G., L. Grillett, J. Pommier, C. Terriere, B. A. Haddock, and E. Azoulay.** 1980. Precursor forms of the subunits of nitrate reductase in *chlA* and *chlB* mutants of *Escherichia coli* K12. Eur. J. Biochem. **105**:297–306.
17. **Glaser, J. H., and J. A. DeMoss.** 1971. Phenotypic restoration by molybdate of nitrate reductase activity in *chlD* mutants of *Escherichia coli*. J. Bacteriol. **108**:854–860.
18. **Guyer, M. S., R. R. Reed, J. A. Steitz, and K. B. Low.** 1980. Identification of a sex-factor-affinity site in *E. coli* as bp. Cold Spring Harbor Symp. Quant. Biol. **45**:135–140.
19. **Hageman, R. V., and K. V. Rajagopalan.** 1985. Characterization of molybdopterin, the organic portion of the molybdenum cofactor, p. 133–141. *In* P. W. Ludden and J. E. Burris (ed.), Nitrogen fixation and CO_2 metabolism. Elsevier Science Publishing Co., New York.
20. **Hawkes, T. R., and R. C. Bray.** 1984. Quantitative transfer of the molybdenum cofactor from xanthine oxidase and from sulphite oxidase to the deficient enzymes of the *nit-1* mutant of *Neurospora crassa* to yield active nitrate reductase. Biochem. J. **219**:481–493.
21. **Herbert, D., P. J. Phipps, and R. E. Strange.** 1971. Chemical analysis of microbial cells. Methods Microbiol. **5B**:210–314.
22. **Hewitt, E. J., and B. A. Notton.** 1980. Nitrate reductase systems in eukaryotic and prokaryotic organisms, p. 273–326. *In* M. P. Coughlan (ed.), Molybdenum and molybdenum-containing enzymes. Pergamon Press, Inc., Elmsford, N.Y.
23. **Horner, R. D.** 1983. Purification and comparison of *nit-1* and wild-type NADPH:nitrate reductases of *Neurospora crassa*. Biochim. Biophys. Acta **744**:7–15.
24. **Howard-Flanders, P., R. P. Boyce, and L. Theriot.** 1966. Three loci in Escherichia coli K-12 that control the excision of pyrimidine dimers and certain other mutagen products from DNA. Genetics **53**:1119–1136.
25. **Ingledew, W. J., and R. K. Poole.** 1984. The respiratory chains of *Escherichia coli*. Microbiol. Rev. **48**:222–271.
26. **Jacob, G. S., and W. H. Orme-Johnson.** 1980. Prosthetic groups and mechanism of action of nitrate reductase from *Neurospora Crassa* [sic], p. 327–344. *In* M. P. Coughlan (ed.), Molybdenum and molybdenum-containing enzymes. Pergamon Press, Inc., Elmsford, N.Y.
27. **Johnson, J. L.** 1980. The molybdenum cofactor common to nitrate reductase, xanthine dehydrogenase and sulphite oxidase, p. 345–383. *In* M. P. Coughlan (ed.), Molybdenum and molybdenum-containing enzymes. Pergamon Press, Inc., Elmsford, N.Y.
28. **Johnson, J. L., B. E. Hainline, and K. V. Rajagopalan.** 1980. Characterization of the molybdenum cofactor of sulfite oxidase, xanthine oxidase, and nitrate reductase: identification of a pteridine as a structural component. J. Biol. Chem. **255**:1783–1786.
29. **Johnson, J. L., B. E. Hainline, K. V. Rajagopalan, and B. H. Arison.** 1984. The pterin component of the molybdenum cofactor: structural characterization of two fluorescent derivatives. J. Biol. Chem. **259**:5414–5422.
30. **Johnson, J. L., and K. V. Rajagopalan.** 1982. Structural and metabolic relationship between the molybdenum cofactor and urothione. Proc. Natl. Acad. Sci. USA **79**:6856–6860.
31. **Johnson, M. E., and K. V. Rajagopalan.** 1986. In vitro system for molybdopterin biosynthesis. J. Bacteriol. **169**:110–116.
32. **Jones, R. W., and P. B. Garland.** 1977. Sites and specificity of the reaction of bipyridylium compounds with anaerobic respiratory enzymes of Escherichia coli. Biochem. J. **164**:199–211.
33. **Kramer, S., R. V. Hageman, and K. V. Rajagopalan.** 1984. *In vitro* reconstitution of nitrate reductase activity of the *Neurospora crassa* mutant nit-1: specific incorporation of molybdopterin. Arch. Biochem. Biophys. **233**:821–829.
34. **Lambden, P. R., and J. R. Guest.** 1976. Mutants of *Escherichia coli* K12 unable to use fumarate as an anaerobic electron acceptor. J. Gen. Microbiol. **97**:145–160.
35. **Lambden, P. R., and J. R. Guest.** 1976. A novel method for isolating chlorate-resistant mutants of *Escherichia coli* K12 by anaerobic selection on a lactate plus fumarate medium. J. Gen. Microbiol. **93**:173–176.

36. **Lester, R. L., and J. A. DeMoss.** 1971. Effects of molybdate and selenite on formate and nitrate metabolism in *Escherichia coli*. J. Bacteriol. **105**:1006–1014.
37. **Ljungdahl, L. G.** 1980. Formate dehydrogenases: Role of molybdenum, tungsten and selenium, p. 463–486. *In* M. P. Coughlan (ed.), Molybdenum and molybdenum-containing enzymes. Pergamon Press, Inc., Elmsford, N.Y.
38. **Low, B.** 1973. Rapid mapping of conditional and auxotropic mutations in Escherichia coli K-12. J. Bacteriol. **113**:798–812.
39. **Low, K. B.** 1972. *Escherichia coli* K-12 F-prime factors, old and new. Bacteriol. Rev. **36**:587–607.
40. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
41. **MacGregor, C. H., and C. A. Schnaitman.** 1973. Reconstitution of nitrate reductase activity and formation of membrane particles from cytoplasmic extracts of chlorate-resistant mutants of *Escherichia coli*. J. Bacteriol. **114**:1164–1176.
42. **MacNeil, T., D. MacNeil, G. P. Roberts, M. A. Supiano, and W. J. Brill.** 1978. Fine-structure mapping and complementation analysis of *nif* (nitrogen fixation) genes in *Klebsiella pneumoniae*. J. Bacteriol. **136**:253–266.
43. **Márton, L., T. M. Dung, R. R. Mendel, and P. Maliga.** 1982. Nitrate reductase deficient cell lines from haploid protoplast cultures of *Nicotiana plumbaginifolia* Mol. Gen. Genet. **182**:301–304.
44. **Mendel, R. R., Z. A. Alikulov, N. P. Lvov, and A. J. Muller.** 1981. Presence of the molybdenum-cofactor in nitrate reductase-deficient mutant cell lines of *Nicotiana tabacum*. Mol. Gen. Genet. **181**:395–399.
45. **Mendel, R. R., R. J. Buchanan, and J. L. Wray.** 1984. Characterization of a new type of molybdenum cofactor-mutant in cell cultures of *Nicotiana tabacum*. Mol. Gen. Genet. **195**:186–189.
46. **Meyer, O., and K. V. Rajagopalan.** 1984. Molybdopterin in carbon monoxide oxidase from carboxydotrophic bacteria. J. Bacteriol. **157**:643–648.
47. **Miller, J. B., and N. K. Amy.** 1983. Molybdenum cofactor in chlorate-resistant and nitrate reductase-deficient insertion mutants of *Escherichia coli*. J. Bacteriol. **155**:793–801.
48. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
49. **Nason, A., K.-Y. Lee, S.-S. Pan, P. A. Ketchum, A. Lamberti, and J. DeVries.** 1971. *In vitro* formation of assimilatory reduced nicotinamide adenine dinucleotide phosphate: nitrate reductase from a *Neurospora* mutant and a component of molybdenum-enzymes. Proc. Natl. Acad. Sci. USA **68**:3242–3246.
50. **Nijkamp, H. J. J., and P. G. deHaan.** 1967. Genetic and biochemical studies of the guanosine monophosphate pathway in *Escherichia coli*. Biochim. Biophys. Acta **145**:31–40.
51. **Ordal, G. W., and J. Adler.** 1974. Isolation and complementation of mutants in galactose taxis and transport. J. Bacteriol. **117**:509–516.
52. **Pascal, M.-C., J.-F. Burini, J. Ratouchniak, and M. Chippaux.** 1982. Regulation of the nitrate reductase operon: Effect of mutations in *chl* A, B, D and E genes. Mol. Gen. Genet. **188**:103–106.
53. **Pateman, J. A., D. J. Cove, B. M. Rever, and D. B. Roberts.** 1964. A common co-factor for nitrate reductase and xanthine dehydrogenase which also regulates the synthesis of nitrate reductase. Nature (London) **201**:58–60.
54. **Puig, J., and E. Azoulay.** 1967. Étude génétique et biochimique des mutants resistant au $C103^-$ (genes *chlA*, *chlB*, *chlC*). C.R. Acad. Sci. **264**:1916–1918.
55. **Rajagopalan, K. V.** 1980. Sulphite oxidase, p. 241–272. *In* M. P. Coughlan (ed.), Molybdenum and molybdenum-containing enzymes. Pergamon Press, Inc., Elmsford, N.Y.
56. **Rajagopalan, K. V.** 1980. Xanthine oxidase and aldehyde oxidase, p. 295–309. *In* W. B. Jakoby (ed.), Enzymatic basis of detoxification, vol. 1. Academic Press, Inc., New York.
57. **Reeve, E. C. R., and P. Doherty.** 1968. Linkage relationships of two genes causing partial resistance to chloramphenicol in *Escherichia coli*. J. Bacteriol. **96**:1450–1451.
58. **Scazzochio, C.** 1980. The genetics of the molybdenum-containing enzymes, p. 487–516. *In* M. P. Coughlan (ed.), Molybdenum and molybdenum-containing enzymes. Pergamon Press, Inc., Elmsford, N.Y.
59. **Stewart, V., and C. H. MacGregor.** 1982. Nitrate reductase in *Escherichia coli* K-12: involvement of *chlC*, *chlE*, and *chlG* loci. J. Bacteriol. **151**:788–799.
60. **Taylor, J. L., J. R. Bedbrook, F. J. Grant, and A. Kleinhofs.** 1983. Reconstitution of plant nitrate reductase by *Escherichia coli* extracts and the molecular cloning of the *chlA* gene of *Escherichia coli* K12. J. Mol. Appl. Genet. **2**:261–271.
61. **Tomsett, A. B., and R. H. Garrett.** 1980. The isolation and characterization of mutants defective in nitrate assimilation in *Neurospora crassa*. Genetics **95**:649–660.
62. **Tomsett, A. B., and R. H. Garrett.** 1981. Biochemical analysis of mutants defective in nitrate assimilation of *Neurospora crassa*: evidence for autogenous control by nitrate reductase. Mol. Gen. Genet. **184**:183–190.
63. **Venables, W. A.** 1972. Genetic studies with nitrate reductase-less mutants of *Escherichia coli*. I. Fine structure analysis of the *NarA*, *NarB*, and *NarE* loci. Mol. Gen. Genet. **114**:223–231.
64. **Venables, W. A., and J. R. Guest.** 1968. Transduction of nitrate reductase loci of *Escherichia coli* by phages P1 and λ. Mol. Gen. Genet. **103**:127–140.
65. **Vogel, H. J., and D. M. Bonner.** 1955. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218**:97–106.
66. **Wadman, S. K., M. Duran, F. A. Beemer, B. P. Cats, J. L. Johnson, K. V. Rajagopalan, J. M. Saudubray, H. Ogier, C. Charpentier, R. Berger, G. P. A. Smit, J. Wilson, and S. Krywawych.** 1983. Absence of hepatic molybdenum cofactor: an inborn error of metabolism leading to a combined deficiency of sulphite oxidase and xanthine dehydrogenase. J. Inher. Metab. Dis. **6**(Suppl. 1):78–83.
67. **Wahl, R. C., R. V. Hageman, and K. R. Rajagopalan.** 1984. The relationship of Mo, molybdopterin, and the cyanolyzable sulfur in the Mo cofactor. Arch. Biochem. Biophys. **230**:264–273.
68. **Warner, C. K., and V. Finnerty.** 1981. Molybdenum hydroxylases in Drosophila. II. Molybdenum cofactor in xanthine dehydrogenase, aldehyde oxidase and pyridoxal oxidase. Mol. Gen. Genet. **184**:92–96.