JOURNAL OF BACTERIOLOGY, Oct. 1991, p. 6499–6506
0021-9193/91/206499-08$02.00/0

Vol. 173, No. 20

Isolation of *Escherichia coli* Mutants Defective in Uptake of Molybdate

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Received 29 October 1990/Accepted 19 July 1991

For the study of molybdenum uptake by *Escherichia coli*, we generated Tn*5lac* transposition mutants, which were screened for the pleiotropic loss of molybdoenzyme activities. Three mutants A1, A4, and M22 were finally selected for further analysis. Even in the presence of 100 μM molybdate in the growth medium, no active nitrate reductase, formate dehydrogenase, and trimethylamine-*N*-oxide reductase were detected in these mutants, indicating that the intracellular supply of molybdenum was not sufficient. This was also supported by the observation that introduction of plasmid pWK225 carrying the complete *nif* regulon of *Klebsiella pneumoniae* did not lead to a functional expression of nitrogenase. Finally, molybdenum determination by induced coupled plasma mass spectroscopy confirmed a significant reduction of cell-bound molybdenum in the mutants compared with that in wild-type *E. coli*, even at high molybdate concentrations in the medium. A genomic library established with the plasmid mini-F-derived *cop*(ts) vector pJE258 allowed the isolation of cosmid pBK229 complementing the molybdate uptake deficiency of the *chlD* mutant and the Tn*5lac*-induced mutants. Certain subfragments of pBK229 which do not contain the *chlD* gene are still able to complement the Tn*5lac* mutants. Mapping experiments showed that the Tn*5lac* insertions did not occur within the chromosomal region present in pBK229 but did occur very close to that region. We assume that the Tn*5lac* insertions have a polar effect, thus preventing the expression of transport genes, or that a positively acting regulatory element was inactivated.

In *Escherichia coli*, the trace element molybdenum is essential for a variety of bacterial enzymes including nitrate reductase (NR), formate dehydrogenase (FDH), and trimethylamine-*N*-oxide reductase (TOR).

These enzymes are induced under anaerobic conditions and contain molybdenum bound to a low-molecular-weight cofactor (Mo-co) (19). The cofactor present in NR, FDH, and TOR has been shown to consist of molybdenum bound to a modified pterin (19), which is loosely associated with a carrier protein of approximately 40 kDa (1) and is produced constitutively (26).

Defects in Mo-co synthesis lead to a pleiotropic loss of all molybdoenzymes. Most mutants deficient in Mo-co synthesis have been isolated by their resistance to chlorate under anaerobic conditions because of a loss of NR activity. In the absence of oxygen, chlorate is reduced to the toxic hypochlorite by NR (24).

Several *chl* genes which are involved in synthesis, insertion, and processing of the Mo-co are known (13, 24, 39). The *chlD* gene product is probably required for the insertion of molybdenum into a precursor, Mo-X, or directly involved in the uptake of molybdate. Addition of molybdate (0.1 to 1 mM) to the growth medium suppresses the *chlD* mutation and results in a complete restoration of molybdoenzyme activity. This effect may be due to either nonenzymatic processing of molybdate (13) or use of an unspecific uptake mechanism at such unphysiologically high concentrations of molybdate. In contrast to other *chl* mutations, the *chlD* mutation also affects nitrogenase activity (20), indicating the involvement of *chlD* in the very early steps of molybdenum metabolism. A similar phenotype is described for mutations in the *molR* locus mapping at 65.3 min (*chlD*, 17 min), which is also suppressed by high molybdate concentrations in the medium (22).

Recently, Johann und Hinton (18) have reported the nucleotide sequence of the *chlD* gene. In the sequence, *chlD* is flanked by two open reading frames, suggesting that *chlD* is part of an operon. The predicted sequence of the *chlD* protein reveals extensive homologies to components of periplasmic transport systems like the gene products of *hisP* and *malK*. On the basis of these analyses, the *chlD* protein seems to be a hydrophilic membrane-associated protein with a nucleotide binding site.

Accordingly, one can assume that in *E. coli* several genes are involved in the transport of molybdate. As a first step in the identification of these genes, we have isolated mutants of *E. coli* defective in molybdate uptake. A fast and highly sensitive determination of molybdenum by induced coupled plasma mass spectroscopy (ICP-MS) allowed a direct screening of Tn*5lac* transposition mutants. By this approach, we were able to isolate several new mutants which are defective in the uptake of molybdate and behave differently from the known *chlD* mutants.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. Strains, plasmids, and bacteriophages used in this study are listed in Table 1. Mutants M22, A1, and A4 were constructed by transposon mutagenesis with λ Tn*5lac*-B20 and selection for resistance against kanamycin and chlorate. Resistant clones were subsequently tested for the activity of molybdoenzymes, and the uptake of molybdate was analyzed directly by ICP-MS.

Growth conditions. Bacterial cultures were grown at 37°C

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TABLE 1. Bacterial strains, plasmids, and bacteriophages used in this study

Strain, plasmid, or phage	Relevant genotype	Source or reference
Strains		
MC1000	F⁻ *araD139* Δ(*araABC-leu*) *7679 galU galK* Δ(*lac*)*X74 rpsL thi*	33
C600	*thr-1 leuB6 thi-1 supE44 lacY1 tonA21*	2
M22	MC1000::Tn*5lac*	This work
A1	MC1000::Tn*5lac*	This work
A4	MC1000::Tn*5lac*	This work
JRG94	F⁻ *leuB6 gal31 chlD4 bioA2 rpsL129 thi-1*	B. Bachmann CGSC strain 4458
S17.1	*recA thi pro hsdR*	35
RK5200	RK4353 *chlA200*::Mu *cts araD139* Δ(*argF-lac*)*U169 deoC1 flbB5201 gyrA219 non-9 ptsF25 relA1 rpsL150*	30
RK5201	RK4353 *chlE201*::Mu *cts*	30
RK5204	RK4353 *chlG*::Mu *cts*	V. Stewart via J. Reiss
RK5208	RK4353 *chlB207*::Mu *cts*	30
BHB2688	N205 *recA* λ^r (λ*D*am4 *b2 red3 imm*^{434} *c*Its *S*am7)	17
BHB2690	N205 *recA* λ^r (λ*D*am15 *b2 red3 imm*^{434} *c*Its *S*am7)	17
Phages		
λ::Tn*5lac*-B20	λ*b*221 *c*I857 *P*am80 Tn*5lac*	35
P1 Vir^s	*virB*	32
Plasmids		
pJR1	pUC18 derivative, *chlA*	30
pJR2	pUC18 derivative, *chlB*	30
pJR3	pUC18 derivative, *chlE*	30
pJR42	pUC18 derivative, *chlG*	30
puc13		40
pSUP205		34
pWK225	pSUP401*nif*[a]	W. Klipp
pJE258	Derivative of pJE253; Cm^r Tet^r λ *cos*, mini-F (43.915–47.629) *cop*(ts)	9, 10, 16
pBK229	pJE258:chromosomal insert	This work
pBK2	*Eco*RI/*Bam*HI fragment of pBK229 in pJE258	This work
pSK10	Deletion derivative of pBK2	This work
pSK19	Deletion derivative of pBK2	This work
pSK143	Deletion derivative of pBK2	This work
pSK22	Deletion derivative of pBK2	This work
pSUPA1	pSUP205:chromosomal fragment of mutant A1	This work
pSUPA4	pSUP205:chromosomal fragment of mutant A4	This work
pSUPM22	pSUP205:chromosomal fragment of mutant M22	This work

[a] pWK225 carries the complete *nif* regulon of *K. pneumoniae* (26.5-kb insert comprising a *Hin*dIII-*Hin*dIII and a *Hin*dIII-*Xho*I fragment) cloned into vector pSUP401 (34).

in L broth medium or on agar plates containing 15 g of agar per liter. Selective media contained 15 μg of kanamycin per ml or 15 mM potassium chlorate or both. For conjugational transfer, streptomycin and chloramphenicol at concentrations of 60 and 30 μg/ml, respectively, were used for selection.

Cultures were grown anaerobically in L broth medium supplemented with 0.2% glucose and 1% potassium nitrate (LB_a) in anaerobic jars equipped with a palladium catalyst and GasPak envelopes (Becton Dickinson and Co., Paramus, N.Y.). Anaerobic 1.5-liter cultures were grown in a Biostat M fermentor (Braun Diessel Biotech, Melsungen, Germany) under dinitrogen at 30°C to a titer of approximately 1.5×10^8 cells per ml.

When necessary, great care was taken to reduce the molybdenum contamination of the media. All reagents were the purest that could be obtained commercially. In order to further reduce molybdenum contamination, glassware was treated with 0.1 N NaOH and rinsed at least 10 times with doubly distilled water.

Southern hybridization. For Southern hybridization, DNA was digested with appropriate restriction enzymes, separated by gel electrophoresis, and transferred to nylon membranes via blotting with an LKB 2016 Vakugene apparatus. Hybridization probes were labeled by nick translation with biotin-7-ATP according to the method of Maniatis et al. (25). Hybridizations were performed at 68°C, and the results were visualized by use of a nonradioactive detection kit (BluGENE) from Bethesda Research Laboratories, Inc.

Construction of an *E. coli* genomic library. *E. coli* chromosomal DNA was isolated and partially digested with the restriction endonuclease *Sau*3A. By centrifugation for 2 h at 250,000 × *g* in a 10 to 30% NaCl gradient, restriction fragments were separated by their sizes. The NaCl gradient was formed by freezing (−20°C) and thawing 20% NaCl in a 2 mM EDTA solution. Fractions containing DNA fragments of approximately 40 kb were identified by gel electrophoresis, precipitated, and washed four or five times with 70% ethanol. DNA fragments of 30 to 40 kb were ligated with the *Bam*HI-linearized cosmid vector pJE258 (9, 10, 16). After in vitro packaging of the genomic library of *E. coli* MC1000 into bacteriophage λ heads (17), an *E. coli chlD* mutant was infected. Clones complementing the *chlD* mutant were selected by assaying for Cm^r bacteria and for the activity of NR and FDH on agar plates. Plasmid DNA of Cm^r, NR$^+$, and FDH$^+$ clones was isolated and analyzed by gel electrophoresis.

Tn*5lac* transposition mutagenesis. Since phage λ Tn*5lac*-B20 cannot propagate in Sup$^-$ strains such as *E. coli* MC1000, this strain was chosen for transposon mutagenesis. MC1000 was grown at 30°C with vigorous aeration to a titer of 5×10^8 cells per ml. This culture (1 ml) was infected with λ Tn*5lac*-B20 (multiplicity of infection of 2 to 3) and incubated at 37°C for 30 min without shaking. After dilution by the addition of 10 ml of L broth medium supplemented with 1% $MgCl_2$, incubation was continued for another 60 min. Cells were then collected by centrifugation, resuspended in 1 ml of L broth medium, and plated on selective media containing 15 μg of kanamycin per ml or 15 μg of kanamycin per ml plus 15 mM $KClO_3$. Transposition frequencies were generally in the range of 10^{-4} to 10^{-5}.

Selection and screening procedure for defects in molydoenzymes. Agar plate assays with color indicators for NR and FDH were used by a modified procedure as described by Begg et al. (4). Clones to be tested for NR activity were transferred to LB_a agar plates and incubated anaerobically for 1 to 2 days. These plates were then overlaid with 1 ml each of 60% lactic acid and 2 M potassium nitrate and incubated for 15 min at room temperature. After the supernatant was carefully removed, plates were overlaid with 1.5 ml of a solution containing 1 ml of 4% sulfanilic acid in 25% HCl and 0.5 ml of 0.08% *N*-1-naphthylethylene diamine dihydrochloride in ethanol. Colonies able to reduce nitrate to

nitrite turned red almost immediately after the addition of the color reagent.

FDH was assayed after anaerobic growth of colonies on LB agar plates supplemented with 1 μM Na_2SeO_3 and 0.2 M potassium formate. Plates were overlaid with a mixture of 1.5 ml of liquid soft agar, 1.5 ml of 0.1 M KPO_4 (pH 6.2), and 50 μl of 3% phenol red. Bacterial clones that cannot metabolize formate turn yellow, while FDH-positive colonies have a pink color (4).

TOR activity was assayed on MacConkey nitrate agar plates according to the method of Stewart and MacGregor (37).

Fumarate reductase activity was tested with minimal medium containing 0.04 M glycerol and 0.04 M sodium fumarate (GF medium) (21). Growth of bacteria in this medium requires an active fumarate reductase (11).

Enzyme assays. Assays for NR and nitrogenase were performed with intact cells. NR activity was determined as described by Garrett and Nason (12). The reaction mixture contained 0.1 ml of 50 mM methylviologene, 0.6 ml of 0.2 M $NaHCO_3$-Na_2CO_3 (pH 9.0), 0.1 ml of sodium dithionite (14.7 mg/ml in 0.3 M $NaHCO_3$), 0.1 ml of 0.5 M KNO_3, and 0.1 ml of cell suspension (optical density at 580 nm, 0.4 to 0.7). The reaction was stopped after definite intervals by vortexing the sample, and then the concentration of nitrite was determined with 1.5 ml of color reagent as described for the agar plate assay. After 10 min at room temperature, the optical density at 530 nm was measured.

For the nitrogenase assay, bacteria were grown anaerobically at 30°C in 100 ml of LB medium supplemented with 100 μM Na_2MoO_4. Cells were collected by centrifugation, washed, and resuspended in 70 ml of N-free medium containing mannitol instead of glucose as the carbon source (15). Derepression of nitrogenase was achieved by growth overnight under argon. Acetylene reduction was determined as described by Dilworth (8).

Measurement of molybdenum uptake. Cultures (1.5 liters) were grown anaerobically under dinitrogen in a Biostat M fermentor at 30°C and constant pH (7.0) to a titer of approximately 1.5×10^8 cells per ml. Cells were harvested by centrifugation in 250-ml plastic tubes, washed with doubly distilled water, resuspended in 0.2 N NaOH to give a final titer of 2×10^9 cells per ml, boiled for 10 min, and stored at −20°C until assayed.

Molybdenum concentrations were determined by ICP-MS (VG PlasmaQuad; VG Elemental, Windsford, United Kingdom). Argon carrier gas was regulated to 20 liters/min at a pressure of 80 lb/in^2 (5.5 bars [5.5×10^5 Pa]). The sample was introduced into the carrier gas by a Meinhardt nebulizer. Each sample was measured at least twice, with an uptake period of 90 s followed by a washing period of an additional 90 s.

Kinetics of molybdate uptake. Cells were grown anaerobically in a fermentor with low molybdenum LB_a medium. Samples of 10^{10} cells were collected, and Na_2MoO_4 was added to a final concentration of 0.1 mM. After certain time intervals, as indicated in Fig. 1, cells were sedimented by centrifugation and processed for molybdenum determination as described above.

Other techniques. Hydrolysis of DNA with restriction endonucleases, DNA ligation with T4 DNA ligase, transformation of bacteria, conjugational transfer of plasmids, and agarose gel electrophoresis as described by Maniatis et al. (25) were performed. Isolation of phage P1-transducing particles and generalized transduction were carried out according to the procedure of Miller (28). Site-directed deletions were created by using a nested deletion kit (Pharmacia).

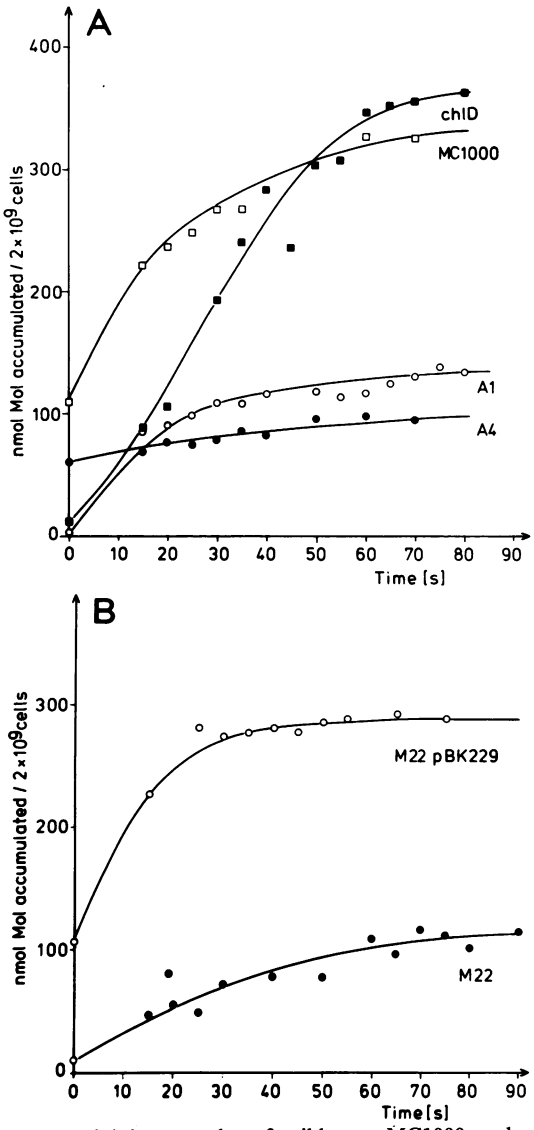

FIG. 1. Molybdate uptake of wild-type MC1000 and mutants JRG94 *chlD*, A1, A4 and M22 as determined by ICP-MS. Cells were grown anaerobically in LB_a medium without molybdenum supplementation. To samples of 10^{10} cells, 0.1 mM $NaMoO_4$ was added, and cells were collected by centrifugation after certain time intervals. Cells were washed with doubly distilled water and hydrolyzed by incubation in 5 ml of 0.2% NaOH at 100°C for 10 min. The kinetics of molybdate uptake by mutants A1 and A4, wild-type MC1000, and mutant *chlD* (A) and by mutant M22 and M22 complemented by plasmid pBK229 (B) are shown.

RESULTS

Isolation of pleiotropic Tn*5lac* mutants. After infection of *E. coli* MC1000 with λ Tn*5lac*-B20, Tn*5lac* transposition mutants were selected by their resistance against kanamycin. Those clones which do not contain an intact Mo-co (mutants in uptake and processing of molybdate) or which lack an active NR were identified by their abilities to grow on chlorate. Approximately 1,500 independent Km^r and Chl^r

TABLE 2. Determination of NR activity

Strain	Mean concn of nitrite (nmol/10^7 cells/min) ± SD in medium with Mo concn of[a]:	
	<50 nM	100 μM
MC1000	2,900 ± 190	2,930 ± 90
JRG94 *chlD*	26 ± 3	3,070 ± 210
A1	35 ± 8	9 ± 0.03
A4	64 ± 8	26 ± 5
M22	15 ± 3	9 ± 3

[a] NR activity was determined by methylviologene-linked reduction of nitrate to nitrite. Bacteria were grown anaerobically in LB_a medium with molybdenum concentrations as indicated. Data are calculated from 10 independent determinations.

TABLE 3. Comparison of cell-bound molybdenum in wild-type and mutant cells, plasmid free and harboring cosmid pBK229

Strain	Mean cell-bound Mo concn (nmol/2 × 10^9 cells) ± SD in[a]:			
	Plasmid-free cells in medium with Mo concn of:		Cells harboring cosmid pBK229 in medium with Mo concn of:	
	<50 nM	100 μM	<50 nM	100 μM
MC1000	93 ± 24	324 ± 62	ND	ND
JRG94 *chlD*	9 ± 2	367 ± 40	96 ± 1	467 ± 31
M22	10 ± 5	105 ± 7	91 ± 5	318 ± 23
A1	1 ± 1	156 ± 8	82 ± 5	252 ± 55
A4	64 ± 5	87 ± 10	91 ± 7	316 ± 24

[a] Data are calculated from 8 to 14 independent determinations. ND, not done.

mutants were isolated this way. Since defects in molybdate uptake or cofactor synthesis cause a pleiotropic loss of all molybdoenzyme activities, Km^r and Chl^r mutants were screened for NR, FDH, and TOR on special agar indicator plates. Finally, about 160 pleiotropic mutants which were defective in all three molybdoenzymes tested were isolated. Three mutants were selected for further analysis after ICP-MS analysis.

Characterization of pleiotropic mutants. In the following experiments, the pleiotropic Tn*5lac* transposition mutants were tested for (i) phenotypic suppression of the mutation by high molybdate concentrations in the medium, (ii) a defect in the *fnr* gene, (iii) molybdate uptake, and (iv) nitrogenase activity in the presence of the *nif* plasmid pWK225.

NR activity at different molybdate concentrations. Mutations in the *chlD* gene are classical examples of mutations resulting in pleiotropic phenotype, as observed here. Since suppression of the mutant phenotype by high molybdate concentrations (36) is typical for all types of *chlD* mutations described in the literature, including point mutations (14), deletions (31), and phage Mu or transposon integrations (27, 37), a *chlD* mutant can be detected by assaying NR after growth at different molybdate concentrations in the medium. In subsequent tests, three mutants, A1, A4, and M22, which had a residual NR activity of only 1 to 2% as compared with the wild type, were obtained. In contrast to what occurred with *chlD* mutant JRG94, a suppression of the phenotype (restoration of the enzyme activity) after growth in the presence of 100 μM molybdate was not observed (Table 2).

Test for an intact *fnr* gene. A mutation in the *fnr* gene would produce a pleiotropic phenotype similar to that observed in the mutants, since several genes involved in anaerobic electron transport are not expressed (6, 23, 29). Therefore, mutants were tested for their abilities to grow anaerobically in GF medium containing only glycerol and fumarate as carbon sources. Bacterial growth in this medium depends on *fnr*-mediated induction of fumarate reductase, which is not a molybdoenzyme (21). Mutants A1, A4, and M22 grew as well as wild-type *E. coli* in this medium, excluding a possible defect in the *fnr* gene.

Determination of cell-bound molybdenum. The cell-bound molybdenum was determined by ICP-MS. For this purpose, a protocol for sample preparation allowing a fast analysis of the mutants was developed. For rapid screening, cultures of 100 ml were grown anaerobically in 250-ml Erlenmeyer flasks; smaller volumes of culture gave unsatisfying results with respect to reproducibility. Contamination of flasks with molybdenum should be avoided, since even extensive NaOH treatment does not remove all molybdenum bound to glass or plastic surfaces. Although cell suspensions can be directly assayed by ICP-MS, it is advantageous to use cells which have been treated with 0.2 N NaOH at 100°C for 10 min, since this improves the homogeneity of the sample. An acid hydrolysis of the bacteria by HNO_3, which leads to the formation of molybdic acid, reduced the accuracy of the measurements.

The concentration of cell-bound molybdenum was measured in the wild type (MC1000), the *chlD* mutant JRG94, and in our Tn*5lac* mutants. The data are summarized in Table 3. Mean values and standard deviations for 8 to 14 samples taken from a 1.5-liter culture grown anaerobically in a Biostat M fermentor were calculated. For the ICP-MS analysis, 5-ml samples with 2 × 10^9 cells per ml were used. In order to determine the kinetics of molybdate uptake, the same cell titers were used to make both methods comparable. Kinetic measurements were performed up to 90 s, since the same level of molybdate accumulation is reached at that time as in bacteria grown overnight in media with 0.1 mM molybdate (Fig. 1; Table 3).

While wild-type MC1000 is able to accumulate 93 nmol of molybdenum per 2 × 10^9 cells when grown in media with low molybdenum concentrations (<50 nM), the *chlD* mutant JRG94 and mutants A1, A4, and M22 exhibit significant reductions in molybdate accumulation. When grown in LB_a supplemented with 100 μM molybdate, the *chlD* control JRG94 accumulated as much molybdate as the wild type, while mutants A1, A4 and M22 had only between 30 and 50% of the molybdenum bound by the wild type under these conditions and exhibited completely different uptake kinetics (Fig. 1). The accumulation of molybdate in the mutants seems not to represent real uptake but rather is due to unspecific binding to the cell surface or cytoplasmic membrane. This is also supported by the fact that cell-bound molybdenum does not allow for molybdoenzyme activity in mutants A1, A4, and M22 and, thus, is not available in the cytoplasm.

Determination of nitrogenase activity in the presence of plasmid pWK225. Since it is entirely possible that mutations in genes involved in cofactor synthesis directly or indirectly affect molybdate uptake, we tested this possibility by introducing the complete *nif* regulon of *Klebsiella pneumoniae* into the *E. coli* mutants and assaying for nitrogenase activity. An appropriate hybrid plasmid, pWK225, was transferred into our mutants by conjugation from *E. coli* S17.1, and transconjugants were tested for nitrogenase activity by monitoring acetylene reduction. The *chlD* mutant JRG94 and the Mo-co mutant RK5200 *chlA* were taken as controls and

TABLE 4. Activity of nitrogenase determined by reduction of acetylene

Strain carrying plasmid pWK225	Acetylene (nmol/2 × 10^9 cells/h) in medium with Mo concn of[a]:	
	50 nM	100 μM
MC1000	89	91
JRG94 *chlD*	11.6	84
RK5200 *chlA*	93	79
M22	2.6	6.1
A1	9.8	23.7
A4	4.5	9.1

[a] The amount of acetylene reduced to ethylene by nitrogenase was determined according to the procedure described by Dilworth (8). Cells were grown anaerobically in LB_a medium with molybdenum concentrations as indicated.

demonstrated that a functional nitrogenase was produced, since plasmid pWK225 carries the genes required for the synthesis of the nitrogenase-specific FeMo cofactor. Table 4 shows the nitrogenase activities of the wild-type MC1000 and the mutants when they carry plasmid pWK225. In the wild type and the *chlA* mutant, nitrogenase activity is also observed at low molybdenum concentrations (<50 nM), while a *chlD* mutant required 100 μM molybdate in the medium. None of our Tn*5lac* mutants reduced acetylene under these conditions, indicating that the mutants do not contain sufficient intracellular molybdenum.

Establishment of an *E. coli* genomic library and isolation of a *chlD*-complementing cosmid clone. The cloning of genes with transport functions into high-copy-number vectors is often accompanied by lethal effects on the recipient cell. This seems to be caused by disorder of the membrane when cloned genes are expressed and their gene products are inserted into the membrane. Although the *chlD* gene was cloned recently (18), all attempts to establish a cosmid with *chlD* and the adjacent genes have failed (30). In our experiments, we have used the cosmid vector pJE258 *cop*(ts).

The cosmid vector pJE258 (9) is a derivative of the mini-F vector pJE253 (10, 16), which carries a 0.6-kb DNA fragment with the λ *cos* site from plasmid pHC79 (7) inserted into the *Kpn*I site (47.274 F) of the mini-F entity of the vector. Plasmid pJE258 is 7.3 kb in size and carries the resistance genes against chloramphenicol and tetracycline from pBR325 (5). A map of cosmid vector pJE258 is presented in Fig. 2. The *cop*(ts) mutation of pJE258 (16) allows modulation of the plasmid copy number by varying the growth temperature. The copy number of about 40 at 25°C is reduced to about 4 at 42°C. Thus, growth of *E. coli* harboring pJE258 at a slightly elevated temperature (39 to 40°C) reduces the copy number of pJE258 to a level at which gene dosage effects are much less pronounced; thus, the lethal effects of cloned genes are prevented.

By transfection of a *chlD* mutant with the genomic library and subsequent selection for Cm^r, NR^+, and FDH^+ clones, the cosmid pBK229 with a chromosomal insert of 36.8 kb was isolated. This cosmid was introduced into mutants A1, A4, and M22, and assays for molybdoenzyme activities were performed. Cosmid pBK229 (Fig. 3) was able to complement all three Tn*5lac*-induced mutants. The NR activity was fully restored in the mutants, even at low concentrations of molybdate in the medium (Table 5). Consequently, it was observed that the cell-bound molybdenum was reaching the wild-type level when mutants harbored cosmid pBK229 (Table 3) and that the uptake kinetics was the same as in the wild-type strain (Fig. 1B). An 18-kb *Hin*dIII-*Bam*HI subfragment of pBK229 in plasmid pBK2 (Fig. 3B) had the same properties with respect to complementation of *chlD* and the Tn*5lac* mutants as the parental cosmid pBK229.

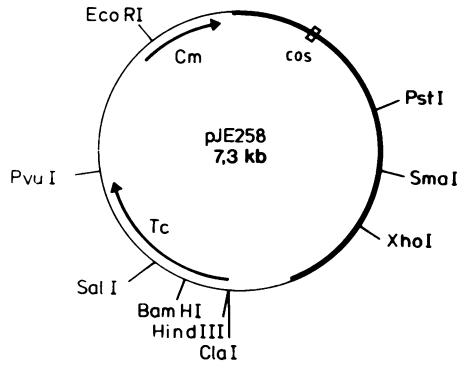

FIG. 2. Physical map of cosmid vector pJE258. The chloramphenicol (Cm) and tetracycline (Tc) resistance genes from pBR325 with their direction of transcription are indicated. The λ *cos* site was derived from pHC79 (7). The thick line indicates the replicon region derived from plasmid mini-F.

Test for complementation of other *chl* mutants by pBK229. Cosmid pBK229 was introduced into several mutants carrying Mu *cts* integrations in genes *chlA*, *chlB*, *chlE*, and *chlG* (30). Only complementation of the *chlA* mutant was observed, since the *chlA* locus maps very close to *chlD* (approximate distance, 0.5 min). As expected, none of the other *chl* mutants were complemented, as they map too far

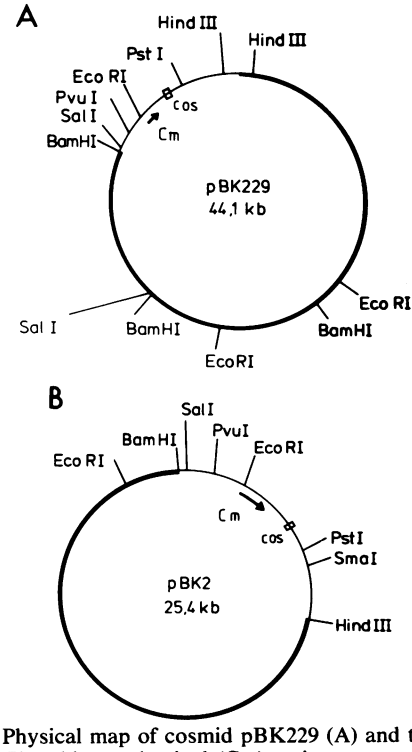

FIG. 3. Physical map of cosmid pBK229 (A) and the derivative pBK2 (B). The chloramphenicol (Cm) resistance gene is indicated by an arrow pointing in the direction of transcription; the thick line represents the chromosomal fragment carried by the cosmid, inserted into the tetracycline resistance gene of the vector. pBK2 contains an 18-kb *Hin*dIII-*Bam*HI subfragment of pBK229.

TABLE 5. NR activity of wild type and mutants carrying cosmid pBK229

Strain carrying plasmid pBK229	Mean nitrite concn (nmol/10^7 cells/min) ± SD in medium with Mo concn of[a]:	
	50 nM	100 μM
MC1000	2,900 ± 190	2,930 ± 90
JRG94 *chlD*	2,660 ± 340	3,364 ± 261
A1	2,869 ± 440	2,944 ± 341
A4	2,712 ± 225	2,716 ± 246
M22	2,166 ± 272	2,212 ± 326

[a] Nitrate reductase activity was determined by methylviologene-linked reduction of nitrate to nitrite. Bacteria were grown anaerobically in LB_a medium with molybdenum concentrations as indicated. Data represent mean values and standard deviations calculated from 10 independent determinations.

from *chlD*. The 18-kb subfragment present in pBK2 did not complement the *chlA* mutant. In a reciprocal experiment, complementation of mutant A1, A4, or M22 by plasmids harboring the gene *chlA* (pJR1), *chlB* (pJR2), *chlE* (pJR3), or *chlG* (pJR42) (30) was tested, but no complementation was observed.

Complementation properties of deletion derivatives of pBK2. To map the gene(s) responsible for the complementation of the mutants, the *Hin*dIII-*Bam*HI fragment of pBK2 was cloned into pUC13, and deletions progressing from the *Bam*HI site were introduced. The deletion derivatives of pBK2 were transformed into *chlD*, A1, A4, and M22, and then complementation was assayed on trimethylamine-*N*-oxide plates. Four such deletion derivatives are shown in Fig. 4. Plasmids pSK10 and pSK19 can complement all mutants, whereas pSK143 and pSK22 have lost the ability to complement *chlD* but still complement mutants A1, A4, and M22. These findings are also consistent with preliminary sequence data locating the *chlD* operon on pBK2 as indicated in Fig. 4. Thus, the mutant phenotype caused by Tn*5lac* integration is complemented by a gene or genes located upstream of *chlD*.

Subcloning of the Tn*5lac* integration sites. In order to map the Tn*5lac* integration sites in relation to the chromosomal region present on cosmid pBK229, *E. coli* MC1000 was transformed with an *Eco*RI library prepared from chromosomal DNA of all mutant strains. Since Tn*5lac* has a single

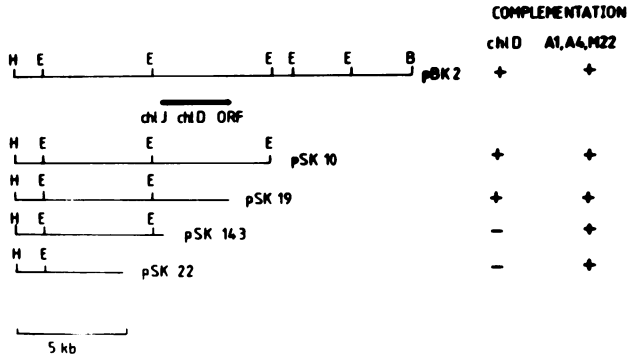

FIG. 4. Deletion derivatives of pBK2. The chromosomal *Hin*dIII-*Bam*HI insert of pBK2 was inserted into pUC13, and nested deletions progressing from the *Bam*HI site were created. The deletion derivatives were tested for complementation of mutants by assaying for TOR activity. The presence (+) or absence (−) of TOR activity is indicated on the left. H, *Hin*dIII; E, *Eco*RV; B, *Bam*HI.

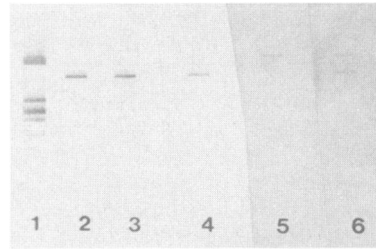

FIG. 5. Southern hybridization analysis of DNA of strain MC1000. *Hin*dIII-digested chromosomal DNA was probed with plasmids pSUPA1, pSUPA4, and pSUPM22, a *chlD*-specific probe, and pBK229-labeled with biotin-7-ATP by nick translation. The specific *chlD* probe was a polymerase chain reaction product labeled accordingly. Lane 1; λ *Eco*RI-*Hin*dIII size standard. Chromosomal DNA of the wild-type MC1000 was digested with *Hin*dIII and hybridized against pSUPA1 (lane 2), pSUPA4 (lane 3), pSUPM22 (lane 4), the *chlD* probe (lane 5), and pBK229 (lane 6).

*Eco*RI site (35), it is possible to clone *Eco*RI fragments carrying the kanamycin resistance gene of Tn*5lac* and a segment of chromosomal DNA representing one border of the Tn*5lac* integration site. Selection for kanamycin resistant clones yielded plasmids pSUPA1, pSUPA4, and pSUPM22 with chromosomal inserts of 2 to 4 kb.

Hybridization of pSUPA1, pSUPA4, and pSUPM22 against pBK229 and pBK2 revealed no homology, indicating that the region in which Tn*5lac* insertions occurred was not represented in the complementing cosmid pBK229. Since the chromosomal fragment of pBK229 carries a *Hin*dIII site very close to one border of the insert, we tested the possibility of whether Tn*5lac* insertions were located on the other side of this *Hin*dIII site, thus outside of the pBK229 insert but on the adjacent *Hin*dIII fragment.

For this, chromosomal DNA of the wild-type MC1000 was digested with *Hin*dIII and subsequently hybridized against pBK229. Two *Hin*dIII fragments with sizes of 14 and >35 kb hybridized with pBK229 (Fig. 5, lane 6). The >35-kb *Hin*dIII fragment comprises most of the insert DNA of pBK229 with the *chlD* gene, as confirmed by hybridization with a *chlD*-specific probe (Fig. 5, lane 5). Plasmids pSUPA1, pSUPA4, and pSUPM22 hybridized only with the smaller *Hin*dIII fragment of 14 kb (Fig. 5, lanes 2 through 4). Therefore, we assume that in all cases Tn*5lac* integration occurred in the same region which is located to the left of the region present in pBK229 and pBK2. This was also supported by cotransduction experiments. The *galK* gene is located at map position 17 min on the *E. coli* chromosome (3). A Tn*5lac* integration between *chlD* and *galK* should result in high cotransduction frequencies of *galK* and Tn*5lac*. Phage P1 lysates were prepared from MC1000 mutants A1, A4, and M22, which are *galK*, and lysates were used to infect *E. coli* C600. Cotransduction frequencies of 87 to 92% for *galK* and Tn*5lac* were observed, indicating that the *galK* locus and the Tn*5lac* integration sites are very closely linked in all three mutants.

DISCUSSION

The isolation of mutants defective in the uptake of molybdate has been hampered by difficulties in the determination of molybdenum accumulation by the bacteria, since ^{99}Mo had to be used. However, ICP-MS solved this problem and allowed the inclusion of the determination of molybdate uptake in the screening for mutants. Although traces of

molybdenum in the range of parts per trillion can be detected by ICP-MS, the determination of molybdenum in cell suspensions or cell extracts has its difficulties because of the formation of aggregates or adsorption of molybdate to glass and plastic tubes. For an accurate determination, the molybdenum concentration should be in the parts-per-billion range (corresponding to >10 nM molybdenum). Reproducible results with standard deviations below 10% were obtained with bacterial titers of 2×10^9 cells per ml in the sample and hydrolysis of the cells by treatment with NaOH prior to ICP-MS analysis. Among all conditions tested (e.g., washing with 0.1 M Tris-HCl [pH 7.5 and 9.5] or with equimolar $NaWO_4$ solutions), washing with doubly distilled water gave the best results (data not shown).

After transposon mutagenesis and mutant screening, we identified three mutants which showed reduced abilities for molybdate accumulation. The absence of a phenotypic suppression by molybdate in our mutants suggests that they are not defective in the *chlD* gene or in the recently discovered *molR* gene, which behaves like *chlD* in this respect (22). In addition to a complete loss of activity of the molybdoenzymes NR, FDH, and TOR, mutants M22, A1, and A4 when carrying plasmid pWK225 are also not able to form an active nitrogenase. Thus, the possibility that the mutations do reside in genes involved in Mo-co synthesis can be excluded, since plasmid pWK225 carries all the genes required for the synthesis of the FeMo-co of nitrogenase.

Finally, the determination of molybdate uptake by mutants M22, A1, and A4 demonstrates that they are indeed defective in the uptake of molybdate.

A cellular concentration of about 100 nM molybdenum (accumulated at a low molybdenum concentration) is sufficient for maximum activity of molybdoenzymes in the wild type. Such levels of cell-bound molybdenum are only reached by the mutants at an unphysiologically high molybdate concentration in the medium (100 μM); however, no molybdoenzyme activity is observed. Thus, the behavior of our mutants at a high molybdate concentration (100 μM) can be explained by the unspecific binding of molybdate to the cell surface. Neither protoplasting of the bacteria (31) nor extensive washing of cells prior to molybdenum determinations significantly reduced the amount of unspecifically bound molybdate (data not shown). Kinetic studies show that binding of molybdate by the mutants is very rapid and is completed after 20 to 30 s, suggesting ionic interactions with the cell surface.

The fact that there is no phenotypic suppression of the mutations at high molybdate concentrations indicates that only a single high-affinity uptake system for molybdenum exists and that unspecific uptake by other transport systems obviously does not occur under these conditions.

The isolation of cosmid pBK229 based on the mini-F *cop*(ts) cosmid vector pJE258 and the subcloning of an 18-kb *Hin*dIII-*Bam*HI fragment, both of which complement the *chlD* mutation as well as the Tn*5lac*-inactivated genes, support the hypothesis of Johann and Hinton (18) suggesting a close linkage of these genes. The analysis of deletion derivatives of pBK2 showed that *chlD* and the gene(s) complementing A1, A4, and M22 represent distinct genetic loci.

Interestingly, the Tn*5lac* integrations are not located within the pBK229 chromosomal insert but on the adjacent *Hin*dIII fragment, of which only a small entity is present on pBK229. The fact that complementation of the mutants by pBK229 and pBK2 is possible, although Tn*5lac* insertions responsible for the mutant phenotype occurred in a region not present in pBK229, allows the following conclusions. (i) Tn*5lac* integrations occurred in the promoter region or within structural genes of an operon essential for the uptake of molybdate, causing a strong polar effect on distally located genes present on pBK229. Complementation is possible by expression of the uptake genes carried by pBK229 from the promoter of the tetracycline resistance gene of the cosmid vector and pUC13. (ii) It is also possible that Tn*5lac* insertions inactivated an essential, positively acting control element which is required for the expression of the uptake genes. As discussed above, expression of the uptake genes on pBK229 from the tetracycline promoter would circumvent this regulation and allow complementation. In order to decide which possibility is correct, we are currently determining the nucleotide sequence of the whole region upstream and downstream of *chlD*.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (DFG).

We are grateful to B. Bachmann, W. Klipp, J. Reiss, and R. Simon for supplying strains, plasmids, and phages and to B. Namuth and E. Diemann for the ICP-MS analysis. The Biostat M fermentor was a generous gift from the Ministerium für Wissenschaft und Forschung NRW (grant no. IV A6-102 031 87).

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