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Bradyrhizobium japonicum Mutants Defective in Nitrogen Fixation and Molybdenum Metabolism†

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Bradyrhizobium japonicum **JH mutants deficient in molybdenum metabolism into the enzymes nitrogenase and nitrate reductase were isolated by using the vector pSUP1011, which carries transposon Tn*5* (streptomycin and kanamycin resistance). Mutants in Mo metabolism were obtained at a frequency of 3.6×10^{-3} (per Kan^r Str^r colony). The mutants were detected by their poor ability to grow in nitrate-containing medium without added Mo. One of the mutant types required 10^5 times more molybdate than the wild type to obtain maximal nitrogen fixation activity. Double-reciprocal plots of Mo uptake versus concentration indicated that the wild-type strain had a high- and a lower-affinity component for Mo binding. Mutant strains JH-90 and JH-119 lacked the high-affinity Mo uptake component and were also clearly deficient in Mo accumulation into a nonexchangeable form. Nitrogenase activity as well as Mo uptake ability could be restored in strains JH-90 and JH-119 by the addition of the sterile supernatant fraction of the wild type. Therefore, mutant strains JH-90 and JH-119 appeared to be deficient in an extracellular Mo-binding factor produced by the wild type. Mutant strains JH-14 and JH-143 had Mo uptake kinetics like those of the wild type (both high- and low-affinity binding for Mo) and appeared to be deficient in intracellular Mo metabolism processes. The addition of the wild-type supernatant did not restore Mo uptake or nitrogenase activity in these strains.**

Bortels (3) discovered a connection between N_2 fixation and Mo when he showed that growth of *Azotobacter vinelandii* on N_2 required Mo, but growth of the cells on ammonium did not. Molybdenum is required as part of the nitrogenase enzyme (4) and is associated with an iron-molybdenum cofactor. The cofactor seems to be associated with the active site of the enzyme (30, 32). Additionally, for *A. vinelandii* Mo plays a role in the synthesis of nitrogenase (19).

Despite known cases in which Mo deficiency can limit the N_2 fixation process in natural environments (7, 16, 32), the uptake and intracellular metabolism of Mo by most N_2-fixing bacteria has been largely unstudied. The first studies on Mo uptake were performed on *Azotobacter* spp. by Keeler and Varner (21). They found that Mo uptake was not dependent on whether the cells were fixing N_2. Our knowledge of Mo uptake and metabolism into nitrogenase comes mostly from work on *Klebsiella pneumoniae* (17, 18, 29, 32) and *Clostridium pasteurianum* (8, 9, 14, 15). In *K. pneumoniae*, Mo metabolism mutants have been useful in identifying genes involved in transformations of Mo, whereas in *C. pasteurianum*, the Mo uptake process itself (8) has been characterized, and some intracellular molybdoproteins have been identified (14, 15). Interestingly, both *C. pasteurianum* and *A. vinelandii* are able to store Mo in a protein-associated form (26, 29), whereas *K. pneumoniae* does not (29, 32). The latter bacterium expresses a high-affinity Mo uptake system constitutively (17, 32); the results for *K. pneumoniae* indicate an efficient high-binding-affinity, energy-independent Mo uptake system at the surface of the cell (32).

Nothing is known about Mo transport, storage, incorporation, or metabolism in the N_2-fixing symbiotic bacterial genera *Rhizobium* and *Bradyrhizobium*. As a first step toward understanding Mo metabolism in *Bradyrhizobium japonicum*, we have isolated mutants in nitrogen fixation and nitrate reduction in which the phenotype of the mutation is suppressed by molybdate. Some of the mutants are probably deficient in intracellular Mo metabolism processes, whereas others appear to lack a functional extracellular factor involved in Mo binding or uptake.

MATERIALS AND METHODS

Chemicals and media. Ultrapure chemicals were puratonic grade 1 obtained from Johnson Matthey Chemicals, Ltd., Hertfordshire, England, and were obtained from Alfa Products, Danvers, Mass. They were certified to be 99.99% pure or greater. This included all trace elements and $MgSO_4$. Ultrex NaCl, also certified for 99.99% purity, was from J. T. Baker, Phillipsburg, N. J. For Mo-free medium, distilled water was further treated to remove traces of Mo by filtration through 8-hydroxyquinoline (8-HQ)(10). Sodium molybdate was from Alfa Products and was of the highest purity available. Controlled pore glass (CPG)-8-HQ was from Pierce Chemical Co. Stock cultures were inoculated by use of sterile plastic loops, and medium was kept in plastic flasks that were never contaminated with Mo-containing medium. Carrier-free $Na_2{}^{99}MoO_4$ (specific activity, ca. 100 mCi/ml) was obtained from Cintichem, Tuxedo, N.Y. One millicurie of carrier-free $Na_2{}^{99}MoO_4$ is equal to 0.021 nmol. Other chemicals were from J. T. Baker Chemical Co.

Modified Bergersen medium (BM)(2) was used for routine culture of *B. japonicum* as well as for screening mutants. LOKG medium for nitrogenase induction has been described (1). All medium was sterilized by pressure filtration through membrane filters (Membra-Fil; 0.22-μm pore size; Nucleopore Corp.).

Nitrogenase induction. The medium for nitrogenase induction was LOKG, as described by Agarwal and Keister (1). Nitrogenase induction in LOKG was done similarly to that

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described by Agarwal and Keister (1) with the minor modifications described by Graham et al. (13). All procedures were performed so as to minimize Mo contamination. This included the use of disposable plastic inoculating loops and plastic pipettes for transferring bacterial cultures. Additionally, polycarbonate flasks were used for growth of cultures. Cells were cultured on LOKG lacking molybdenum (see procedure described above for removing Mo) to a cell density of 1.5×10^8 viable cells per ml. Six milliliters of this suspension was dispensed into a 250-ml acid-washed serum bottle. The bottles were sealed with rubber serum stoppers, which were then wrapped with parafilm, and the bottle was sparged with argon for 15 min by use of an inflow and outflow needle injected through the stopper. Carbon dioxide and oxygen were injected to a final partial pressure of 0.5 and 0.1%, respectively. Two percent C_2H_2 was added (13), and the bottles were incubated for 48 h at 30°C with shaking at a speed of 100 rpm. Sodium molybdate was added as 0.1-ml amounts in H_2O from appropriate stock solutions. Nitrogenase was assayed by acetylene reduction as described previously (13).

Transposon mutagenesis and screening for mutants. Transposon Tn*5* was introduced by transfer of the Mob suicide plasmid pSUP1011 by conjugation from *Escherichia coli* to *B. japonicum* (33). The exact procedure has been described in detail (24) for isolation of Tn*5*-generated H_2-uptake-negative mutants of *B. japonicum*. The parent strain JH has been described (13) and was derived from strain USDA 110. After mating the strains on filters and plating (24), each kanamycin- and streptomycin-resistant colony was purified by streaking onto an agar plate of mannitol-yeast extract medium (24) containing rifampin, kanamycin, and streptomycin (each at 100 μg/ml).

Purified Tn*5*-containing strains were screened for the ability to grow on modified BM (2) with KNO_3 (0.5g/liter) as the sole N source, in the presence and absence of molybdate. The primary ingredients of BM (K_2HPO_4, glycerol, and KNO_3) were added to distilled water, and the solution was passed through a CPG-8-HQ column (Pierce Chemical Co.) to remove traces of Mo. This 8-HQ column has been shown to be effective in the removal of Zn and Ni (9), and it was also effective for Mo removal. Mo added to a liter of H_2O to a concentration of 400 ppm (400 μg/liter) and then passed through an 8-HQ column (2.5 by 10 cm) was reduced to less than 0.4 ppm, the minimum concentration detectable by our atomic absorption system. Also, N_2-fixing *K. pneumoniae* would not grow in N-free medium that had been passed through the 8-HQ column unless Mo was added. Ultrapure $MgSO_4$ and trace elements were then added to the 8-HQ-treated solution to complete the medium. Solid medium was solidified with 1.3% Noble agar (Difco Laboratories). Different lots of agar varied in their usefulness for screening mutants, probably because of variability in the amount of Mo in the agar. For the Mo-sufficient condition, Na_2MoO_4 was added to a concentration of 5 mM. Six thousand potential mutants were screened on BM plates with and without Mo, and the plates were incubated for 10 days at 30°C. Putative mutants were retested for verification of lack of ability or poor ability to grow in the Mo-deficient condition. Mutants in nitrate reductase were not sought; therefore, strains showing poor growth both with and without Mo were discarded.

From 6,000 individual Tn*5*-containing strains, 22 mutants were obtained. The 6,000 clones were from three independent matings for introducing Tn*5*; about 2,000 colonies from each mating were screened.

DNA hybridization analysis. Physical verification of Tn*5* insertion in the mutant strains was carried out by DNA hybridization analysis (25). *B. japonicum* genomic DNA was enzymatically digested to completion with *Eco*RI, electrophoresed in 1% agarose, and transferred to nitrocellulose paper as described (25). The Tn*5*-containing plasmid pSUP1011 was isolated from *E. coli* SM10 by CsCl gradient centrifugation as described previously (25). The probe used was isolated from low-melting-point agarose after pSUP1011 was cut with *Hpa*I. *Hpa*I cuts Tn*5* twice (and nowhere else in the plasmid) just within the outside ends of the inverted repeats (20), yielding a fragment approximately 5.1 kilobases (kb) long derived solely from Tn*5* DNA. This probe was radiolabeled with [^{32}P]CTP by using a random primer extension technique described elsewhere (11).

Kinetics of Mo uptake. Nitrogenase expression by *B. japonicum* requires a defined complex culture medium and precise environmental conditions (1, 13). The use of 0.1% partial pressure of 0_2, precise shaking speed, low cell density, and defined culture volume were essential parameters for successful nitrogenase induction. $Na_2{}^{99}MoO_4$ was injected directly into the sealed nitrogenase induction vial containing cells to initiate the assay, and each kinetic point (Mo concentration) tested used one induced culture. Cultures were induced for nitrogenase in the absence of molybdate for 48 h, and then $Na_2{}^{99}MoO_4$ (radioactive plus carrier) was added to initiate the assay. Molybdate concentrations of 0.01, 0.02, 0.03, 0.04, 0.05, and 0.10 μM contained 1% of the total Mo as $^{99}MoO_4{}^{2-}$. The 0.20 and 0.50 μM samples contained 0.1% of the molybdate as $^{99}MoO_4{}^{2-}$. After addition of Mo,the cultures were allowed to continue shaking for 20 min under the nitrogenase induction condition (30°C, shaking at 100 rpm; see above). After 20 min, three 0.5-ml samples were removed, and the cells were rapidly filtered onto Durapore (HVLP 02500; 0.45-μm pore size; Millipore Corp.) filters by use of a 12-sample filtering manifold (Millipore Corp.). The filters were previously saturated with LOKG medium containing 0.5 mM Na_2MoO_4. The cells on the filter were washed three times with 5 ml of ice-cold LOKG medium containing 0.5 mM Na_2MoO_4. After drying the filters overnight in the 6-ml scintillation vial, 5 ml of Aquasol 2 (New England Nuclear Corp.) was added, and each vial was capped and vortexed vigorously. After 30 min, vials were counted in an LKB model 1218 scintillation spectrometer on the ^{32}P setting. Background counts containing radioactive molybdate bound by filters without cells were subtracted from all values. Mo uptake was calculated, taking into account the short (66-h) half-life of ^{99}Mo. The cell number was determined by measuring optical density at 540 nm and compared with a standard curve prepared with cells from LOKG medium.

Molybdenum accumulation. Molybdenum accumulation over a 5-h period was determined for 10-ml cultures previously induced for nitrogenase for 24 h in the absence of Mo. Sodium molybdate was injected into the 10-ml culture to a final concentration of 20 nM, of which 0.002 nmol (0.10 mCi) was radioactive, and the bottle was returned to the shaker (nitrogenase induction conditions described above). Two 0.25-ml amounts were removed at 0, 1, 2, 3, 4, and 5 h for measurement of Mo accumulation. The samples were filtered and treated as described above. The exchangeability of the accumulated Mo in strain JH was determined by incubating 1 ml of the 5-h sample with 20 μM Na_2MoO_4 for 20 min and then filtering two 0.25-ml portions.

NO_3 reductase. NO_3 concentration was measured directly by nitration of salicylic acid as described by Cataldo et al. (5,

TABLE 1. Effect of MoO_4^{2-} on nitrogenase activity in wild-type and mutant *B. japonicum* strains[a]

Strain	Nitrogenase activity (nmol of C_2H_4 produced/24 h) at Mo concn (nM):								
	0	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8
JH (wild type)	30	35	205	160	80	60	42	0	0
JH-119	0	0	0	0	0	0	17	275	65
JH-90	0	0	0	0	0	0	85	442	8
JH-82	0	0	0	0	0	0	8	314	37
JH-112	0	0	0	0	0	0	0	19	21
JH-107	0	0	0	0	0	0	0	33	29
JH-14	0	0	25	20	50	150	28	0	0
JH-143	0	6	35	40	52	24	8	0	0
JH-312	0	30	35	42	65	12	0	0	0
JH-307	0	15	40	60	61	41	31	0	0
JH-13	0	22	25	25	25	25	27	426	10
JH-18	57	56	56	55	40	54	75	550	5

[a] Nitrogenase was induced as described previously (13) in LOKG medium (1). Each serum bottle contained 1.5×10^8 viable cells per ml in LOKG medium. Sodium molybdate and acetylene were added at the start of the induction period, and ethylene was quantitated at 48 h by gas chromatography.

6). The cultures were inoculated into Mo-free BM in polycarbonate flasks by use of sterile plastic inoculating loops and grown to an OD_{540} of Ca. 0.6 (6.3×10^8 viable cells per ml). The medium for the mutants also contained streptomycin and kanamycin (each at 100 μg/ml). The cells were harvested by centrifugation in polystyrene tubes (Corning no. 25310) and suspended to a concentration of 5×10^8 cells per ml in Mo-free BM containing 10 mM KNO_3 as the sole N source. The 15-ml polystyrene tubes containing 5 ml of cell suspension were incubated at 30°C with shaking at 50 cycles per min for 24 h. The cells were pelleted by centrifugation, and 0.5-ml samples of the supernatant solution were assayed for NO_3. The activity was calculated as micromoles of NO_3 utilized per 10^9 cells during the 24-h incubation.

Addition of spent supernatant to cultures. For experiments in which the spent supernatant was added to the cell suspensions, the wild-type JH or mutant JH-119 or JH-90 was first placed under nitrogenase induction conditions in LOKG medium containing 100 nM Mo for 48 h. The cell yield from one of these induced cultures was approximately 2×10^9 viable cells per 10 ml. The cell suspension was centrifuged at $10,400 \times g$ for 12 min, and the supernatant was removed from the pellet. The supernatant was passed through a Millex-GS filter unit (0.22-μm pore size; Millipore Corp.) and placed into a sterile polystyrene tube. Portions of this were injected with a syringe into the LOKG-incubated cultures, which were placed under nitrogenase induction conditions in 100 nM Mo (as sodium molybdate) for 24 h. Then the sterile supernatant from the indicated strain was injected through the serum stopper, the culture was returned to the shaker, and the nitrogenase activity was measured at 48 h. Mo accumulation for these cultures was measured by adding 5.0 nM $^{99}MoO_4^{2-}$ (carrier free) at 36 h to replicate cultures and determining total picomoles of Mo accumulated at 48 h.

RESULTS

Isolation of mutants. Individual colonies containing Tn*5* were screened for ability to grow with NO_3 as the sole N source on agar plates with and without molybdate. From screening about 6,000 such colonies (see Materials and Methods), 22 mutants were obtained that showed poor growth on the Mo-free medium, but good growth on the Mo medium. Genomic digests of the 22 mutants were probed with Tn*5*. All of the strains described in this manuscript contained a single Tn*5* insertion except strain JH-18, which had two Tn*5*-hybridizing bands by Southern blot analysis (data not shown). Not all of the Mo was removed from the medium, as the wild-type strain JH was able to grow well in medium that had been treated to remove Mo. However, this facilitated the screening for mutants, as strains growing poorly in the treated medium could be readily distinguished from the wild type.

Screening strains for free-living nitrogenase activity. The 22 mutant strains were screened for nitrogenase activity in free-living culture with various molybdate concentrations to determine whether their Mo metabolism deficiency affected nitrogenase activity as well as nitrate-dependent growth. The results for 11 mutant strains and the wild type are shown in Table 1. The parent strain, JH, had optimal nitrogenase activity at 100 nM Mo. Most of the mutant strains were poor in nitrogenase activity compared with the wild type in the lower molybdate concentrations (0 to 10^3 nM).

All of the mutant strains had restored nitrogenase activity at levels of molybdate that were greater than optimal for strain JH. The phenotypes of the mutants could be grouped into three classes, based on restoration of nitrogenase activity by added Mo. The first group (exemplified by strains JH-90 and JH-119, Table 1) had no nitrogenase activity until 1 mM molybdate was added. The nitrogenase activity of these mutants was optimal at 10^5-fold-higher molybdate concentrations than for the wild type. The second type of mutant, exemplified by strains JH-14, JH-143, JH-312, and JH-307, had optimal nitrogenase activity at 10^4 to 10^5nM Mo and, like the wild type, had no activity above 10^6 nM Mo. The final type of mutant, exemplified by JH-13 and JH-18, had nitrogenase activity at low molybdate concentrations like the wild type, but their activity was optimal at 10^7 nM, a concentration at which the wild type exhibited no activity. The differences between these strains did not appear to be generally due to a toxic effect of Mo on cell growth. In the free-living nitrogenase assay the cells were initially suspended to the same cell concentration in a nongrowing condition, and lost cell viability was observed only at $\geq 10^7$ nM molybdate.

Mutant strains JH-119 and JH-90 were chosen for further study, as these two strains differed the most from the wild type in the molybdate concentration required for maximal nitrogenase activity. A plot of nitrogenase activity over a wide range of molybdate concentrations is shown for these two strains in Fig. 1. It is clear that these two strains required 10^5-fold more molybdate for maximum nitrogenase activity than was required by the parent strain, JH. The

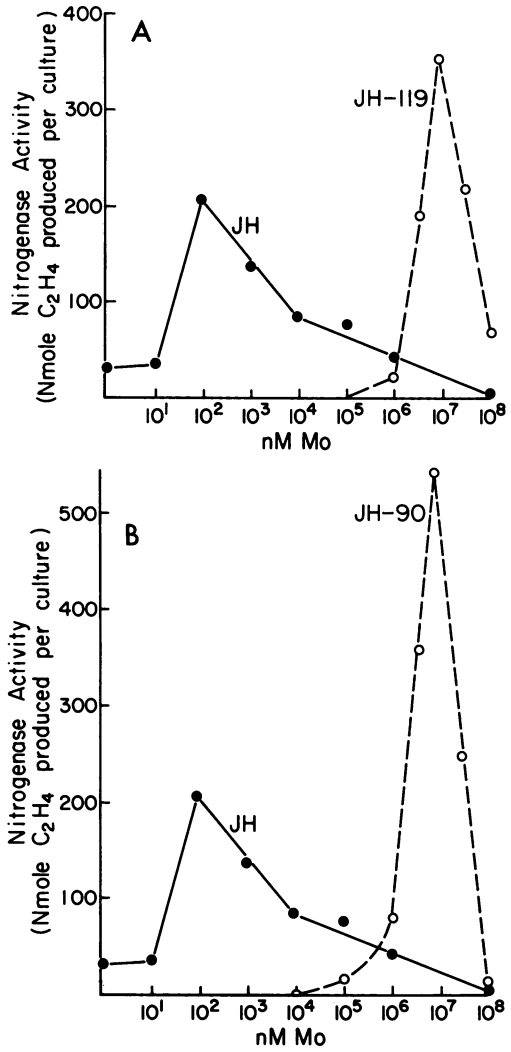

FIG. 1. Effect of Mo concentration on nitrogenase induction in strains JH and mutants JH-119(A) and JH-90(B). Strains at 1.5×10^8 to 2.0×10^8 viable cells per ml were induced for nitrogenase in LOKG medium (1) as described in the text. Sodium molybdate was added from appropriate stock solutions, acetylene (2%, (vol/vol) was added at the start of the 48-h induction period, and nitrogenase activity (ethylene formed) was measured at 48 h.

maximum amount of activity for both strains was also higher than for the wild type (Fig. 1).

Mo uptake rates. Mo uptake rates of strains JH-90 and JH-119 were measured to determine whether these mutants were deficient in an Mo uptake process or in an intracellular Mo metabolism step. The Mo uptake showed saturation kinetics, permitting a double-reciprocal plot of velocity versus substrate (Fig. 2). The Mo uptake system of the wild type was biphasic, exhibiting a high-affinity Mo uptake component at low Mo concentrations and another component with lower affinity at high concentrations. A major difference was observed between the wild type and the mutant strains JH-119 and JH-90. These two mutants (data for JH-119 are shown in Fig. 2) lacked the high-affinity portion of the Mo uptake system. Therefore, the inability of these strains to fix N_2 at low molybdate levels (such as 1 μM) was correlated with a much lower affinity for molybdenum. Mutant strains JH-143 and JH-14 were similarly tested for

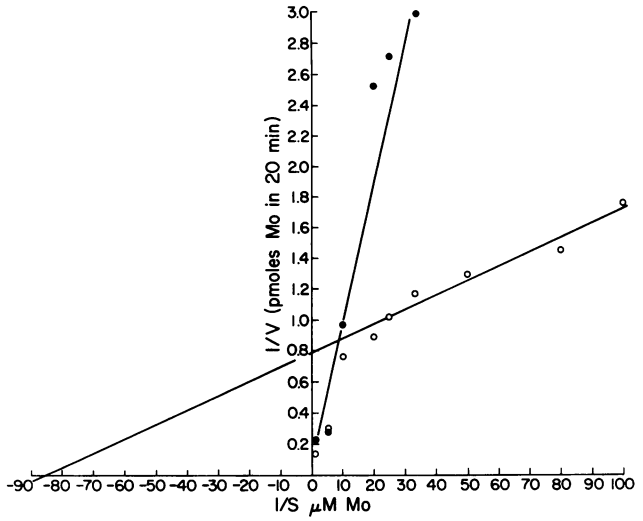

FIG. 2. Double-reciprocal plot of Mo uptake rate versus substrate (Mo) concentration. Cultures were induced for nitrogenase in the absence of Mo for 48 h, and $Na_2{}^{99}MoO_4$ was added to initiate the assay. After 20 min in a shaker bath, three 0.5-ml samples were removed, the cells were harvested by filtration, and the filters were counted for radioactivity. Each point is the average of the three determinations. Symbols: ○, strain JH; ●, strain JH-119.

Mo uptake ability (Fig. 3). Strain JH143 contained both the high- and low-affinity uptake components, like the wild type. Strain JH-14 gave results similar to those for JH-143 (data not shown). These two mutant strains were therefore able to transport Mo like the wild type and possibly were deficient in an intracellular Mo metabolism step. These mutants may be similar to the *K. pneumoniae nifQ* or Mol$^-$ mutants described previously (17, 18). The Mol$^-$ mutants were not deficient in Mo uptake but required higher levels of Mo than the wild type for expression of nitrogenase and nitrate reductase.

Effect of Mo on nitrate reductase activity. To determine the

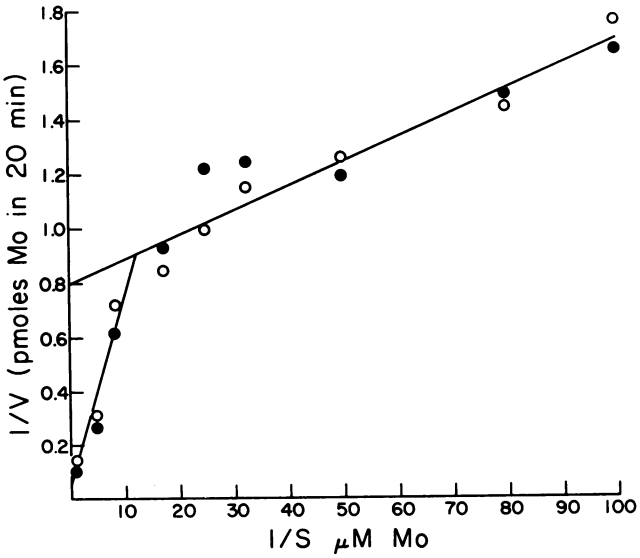

FIG. 3. Double-reciprocal plot of Mo uptake rate versus Mo concentration for strains JH (○) and JH-143 (●). Conditions were as described in the Legend to Fig. 2.

TABLE 2. Effect of added MoO_4^{2-} on nitrate reductase activity in JH and mutant strains[a]

Strain	Mo added (nM)	Nitrate reductase activity (μmol of NO_3 utilized/10^9 cells per 24 h)
JH	None	3.1
	10^3	4.4
	10^6	4.7
JH-119	None	0.06
	10^3	2.3
	10^6	7.4
JH-90	None	0.06
	10^3	1.5
	10^6	6.8
JH-14	None	0.26
	10^3	1.8
	10^6	6.7
JH-112	None	0.0
	10^3	0.04
	10^6	1.4
JH-143	None	0.53
	10^3	2.3
	10^6	7.5

[a] The nitrate concentration of the supernatant after removing cells was determined by nitration of salicylic acid (5, 6).

effect of Mo on nitrate reductase, the strains were grown in medium treated to remove Mo and then incubated in NO_3-containing medium with 0, 10^3, or 10^6 nM sodium molybdate. All five mutants tested had no or low activity with no molybdate, whereas the wild type had considerable activity (Table 2). Similar to the effect of added Mo on nitrogenase activity, nitrate reductase activity was restored by the addition of Mo to all five mutants tested. However, less Mo was required to restore nitrate reductase activity than nitrogenase activity in these mutants. As pointed out by Shah et al. (32), probably more Mo is needed for nitrogen fixation than for nitrate reduction because the catalytic efficiency of the latter is greater.

Mo accumulation. The accumulation of Mo over a 5-h period was determined in strain JH and mutant strains JH-90 and JH-119. The wild type was able to steadily accumulate Mo from a low-Mo environment (20 nM) over this period (Fig. 4). Only 15% of the accumulated Mo was exchangeable

TABLE 3. Evidence for an extracellular molybdenum transport factor produced by strain JH[a]

Strain	Addition	Nitrogenase activity (nmol of C_2H_4/h per 10^9 cells)	^{99}Mo accumulation (pmol/10^9 cells per 12 h)
JH	None	5.1	7.4
JH-90	None	0.0	<0.2
JH-119	None	0.0	<0.2
JH-143	None	0.0	1.2
JH	JH SS	7.6	8.9
JH-90	JH SS	4.9	6.0
JH-119	JH SS	5.6	8.5
JH-143	JH SS	0.0	1.6
JH-90	JH-90 SS	0.0	<0.2
JH-119	JH-119 SS	0.0	<0.2

[a] Strains were induced for nitrogenase in 100 nM Mo for 24 h, and then no addition was made or 0.3 ml of the sterile supernatant (SS) was added as indicated. The supernatant was obtained from cells harvested at 2× 10^8 cells per ml as described in the text. Nitrogenase activity was determined at 48 h by gas chromatography (13). $^{99}MoO_4^{2-}$ (carrier free) (5 nM) was added at 36 h, and the amount of Mo accumulated was determined at 48 h. All values are averages for at least three samples.

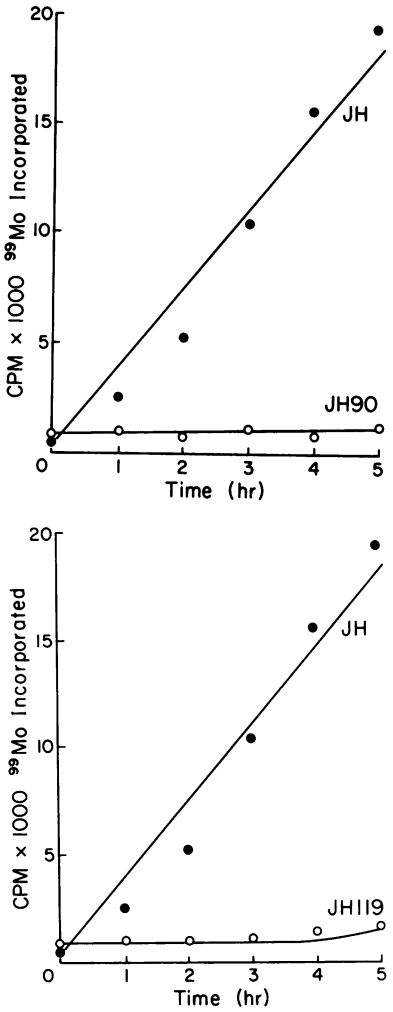

FIG. 4. Molybdenum accumulation in strain JH and mutants JH-119 and JH-90. Sodium molybdate (20 nM, of which 0.002 nmol was radioactive) was injected into 10-ml cultures subjected to nitrogenase induction conditions (see text). Two 0.25-ml samples were removed hourly for measurement of Mo accumulation.

with nonradioactive molybdate (20 μM over 20 min), which indicated that it was firmly associated with the cells. The mutant strains, however, did not accumulate Mo from the medium, even over a 5-h incubation period (Fig. 4).

Extracellular Mo uptake factor produced by the wild type. The properties of Mo transport by *K. pneumoniae* are consistent with the involvement of extracellular Mo-binding chelators for binding and temporarily storing Mo (32). Extracellular siderochromes produced by *A. vinelandii* in response to iron deprivation are capable of binding molybdate (28, 34). Therefore, the *B. japonicum* wild type was tested to determine whether a similar extracellular diffusible factor was produced that could scavenge Mo. Strain JH was induced for nitrogenase in medium containing 100 nM Mo for 48 h. The spent supernatant from JH was filter sterilized and then added to strains JH-90, JH-119, and JH-143 incubated under derepressing conditions with 100 nM molybdate. The mutant strains JH-90 and JH-119 were then able to take up Mo and express nitrogenase activity (Table 3). This activity was not due to Mo contamination from handling the spent supernatant, since addition of the spent supernatant from each mutant (strains JH-90 and JH-119) back to the mutant

TABLE 4. Effect of amount of added wild-type spent supernatant on JH-119 nitrogenase activity[a]

Strain JH spent supernatant added (ml)	Nitrogenase activity (nmol of C_2H_4 produced/h per 10^9 cells)	^{99}Mo accumulation (pmol/10^9 cells per 12 h)
0	0.0	0.2
0.05	1.6	0.7
0.10	5.0	7.2
0.20	4.6	7.4
0.30	5.1	7.7
1.0	1.2	6.4

[a] The sterile supernatant from the wild type derepressed for nitrogenase activity for 48 h was added after 24 h of incubation of JH-119 in LOKG medium (1) containing 100 nM Mo. At 36 h, 0.05 nmol of carrier-free $^{99}MoO_4^{2-}$ was added to each 10-ml culture under nitrogenase induction conditions, and the nitrogenase activity and Mo accumulated were determined at 48 h. The nitrogenase activities and Mo accumulation values are the averages of three determinations.

incubated under nitrogenase induction conditions did not restore activity (Table 3). The addition of JH spent supernatant to JH-143 did not restore nitrogenase activity or significantly increase the Mo accumulation ability of that strain. Therefore, this mutant was probably deficient in an intracellular Mo metabolism step, consistent with its Mo uptake properties (Fig. 3).

Only a small amount of the spent supernatant from strain JH was required to restore the ability of the mutants JH-90 and JH-119 to express nitrogenase activity in 100 nM Mo. Only 0.1 ml of the spent supernatant was required to give optimal nitrogenase activity in strain JH-119 (Table 4). This amount (0.1 ml) was also sufficient to saturate the Mo accumulation ability of the strain over a 12-h period (Table 4). Mo accumulation by strain JH-90 was also restored by adding 0.1 ml of the spent supernatant from strain JH (data not shown). The results indicated that an extracellular component was required in relatively small amounts from the wild type that could restore both Mo accumulation ability and nitrogenase activity in these mutants.

DISCUSSION

A connection between Mo and N_2 fixation was discovered in 1930 by Bortels (3), who showed that growth of *A. vinelandii* on N_2 required Mo. Now we know the biochemical basis for the connection with Mo, as purified nitrogenase from most sources contain Mo. Despite known instances of Mo inaccessibility or limitation to the N_2 fixation process in natural conditions, little is known of Mo transport and its incorporation into nitrogenase. It is likely that many Mo metabolism steps are involved, including Mo binding at the cell surface, sequestering from the free molybdate pool, and synthesis of intermediates such as the iron-molybdenum cofactor (32). The isolation and study of mutants deficient in Mo metabolism into nitrogenase is a logical step in elucidating these problems.

Mutants deficient in N_2 fixation in which the mutation is suppressed by molybdate have been described for *K. pneumoniae*. These mutants include those that are specific for N_2 fixation, $NifQ^-$ mutants, and those which are deficient in Mo metabolism for both nitrogenase and nitrate reductase, called Mol^- mutants (17, 18). The latter mutants are analogous to *chlD* mutants of *E. coli* (12), in which nitrate reductase activity is restored by molybdate. In fact *chlD* mutants containing *K. pneumoniae nif* genes show pleiotropic dependence on Mo for both nitrogenase and nitrate reductase activity (22). All of these mutants are normal in Mo uptake; the defect is apparently in an intracellular Mo metabolism process. Also, mutants of *Rhodopseudomonas capsulata* have been described that are phenotypically similar to the *nifQ* mutants of *K. pneumoniae* (35). The phenotypes of some of the *B. japonicum* mutants described here are similar to those of *K. pneumoniae* Mol^- mutants (18). Like the Mol^- mutants, *B. japonicum* mutant strains JH-143 and JH-14 did not seem to be deficient in short-term uptake of exogenous Mo, yet the deficiencies in both nitrogenase and nitrate reductase activity were suppressed by molybdate. It is likely that these strains are defective in an intracellular Mo metabolism process. In N_2-fixing *C. pasteurianum*, Mo is associated with four molybdoproteins prior to incorporation of Mo into nitrogenase and formate dehydrogenase (14, 15).

On the other hand, mutant strains JH-119 and JH-90 were clearly deficient compared with the wild type in their affinity for Mo. Furthermore, the mutant phenotype of these two strains was suppressed by an extracellular factor produced by the wild type. It is possible that strains JH-90 and JH-119 lack a molybdate chelator or chelate transport system component. Although such processes are well documented for ferric iron (27), few cases of molybdate chelation are documented. *A. vinelandii* produces siderochromes which bind MoO_4^{2-} as well as Fe^{3+} (28, 34), and 44,000- and 77,000-dalton outer membrane proteins are produced in response to Mo-deficient growth conditions (28). *Bacillus thuringiensis* synthesizes a peptide chelator in response to iron starvation that is also capable of binding molybdate (23). Further characterization of Mo-binding factors from *B. japonicum* and their role in suppressing the phenotype of mutants JH-119 and JH-90 are needed. The possible role of an Mo chelation system functioning in *B. japonicum* under Mo-deficient symbiotic N_2 fixation conditions will be especially interesting.

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