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Transport of molybdate in the cyanobacterium *Anabaena variabilis* ATCC 29413

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Abstract Heterocyst-forming filamentous cyanobacteria, such as *Anabaena variabilis* ATCC 29413, require molybdenum as a component of two essential cofactors for the enzymes nitrate reductase and nitrogenase. *A. variabilis* efficiently transported ^{99}Mo (molybdate) at concentrations less than 10^{-9} M. Competition experiments with other oxyanions suggested that the molybdate-transport system of *A. variabilis* also transported tungstate but not vanadate or sulfate. Although tungstate was probably transported, tungsten did not function in place of molybdenum in the Mo-nitrogenase. Transport of ^{99}Mo required prior starvation of the cells for molybdate, suggesting that the Mo-transport system was repressed by molybdate. Starvation, which required several generations of growth for depletion of molybdate, was enhanced by growth under conditions that required synthesis of nitrate reductase or nitrogenase. These data provide evidence for a molybdate storage system in *A. variabilis*. NtcA, a regulatory protein that is essential for synthesis of nitrate reductase and nitrogenase, was not required for transport of molybdate. The closely related strain *Anabaena* sp. PCC 7120 transported ^{99}Mo in a very similar way to *A. variabilis*.

Keywords Cyanobacteria · *Anabaena* · Molybdate · Molybdenum · Metal transport

Introduction

Cyanobacteria live in disparate environments requiring adaptation to rapid changes in macronutrients and micronutrients. *Anabaena variabilis* is a filamentous, heterocyst-forming cyanobacterium that grows well with either fixed nitrogen, such as nitrate or ammonium, or with N_2 .

It has three systems for nitrogen fixation, two mediated by different Mo-nitrogenases (Brusca et al. 1989; Schrautemeier et al. 1995; Thiel et al. 1995, 1997) and one by a V-nitrogenase (Thiel 1993; Lyons and Thiel 1995). One Mo-nitrogenase (Nif1) functions under oxic growth conditions in heterocyst cells that are specialized for nitrogen fixation (Wolk 1996), while the other (Nif2) functions in vegetative cells and heterocysts under anoxic growth conditions (Thiel et al. 1995, 1997). V-nitrogenase, like Nif1 Mo-nitrogenase, functions under oxic conditions in heterocysts (Thiel 1993).

Molybdenum is an essential component of two cofactors that are required for nitrogen metabolism in many bacteria, including cyanobacteria. Mo-nitrogenases require an iron-molybdenum cofactor that is made by the products of some of the *nif* genes (Newton 1992; Rangaraj et al. 2001). The assimilatory nitrate reductase requires a molybdopterin cofactor, made by the products of the *moe-moa* genes (Rajagopalan and Johnson 1992; Rajagopalan 1996). In *Anabaena* sp. PCC 7120, a mutant in *moeA* (whose product is involved in biosynthesis of molybdopterin) cannot use nitrate as a source of fixed nitrogen and cannot synthesize nitrate reductase, but grows well diazotrophically using nitrogenase (Ramaswamy et al. 1996). Similarly, in *Synechococcus* sp. strain PCC 7942, mutants in *moeA*, *moaC*, *moaE*, and *moaA* fail to grow on nitrate or to express nitrate reductase (Rubio et al. 1998).

The transport of molybdate has been characterized best in *Escherichia coli*, which has a high-affinity ABC transport system for molybdate encoded by the *modABCD* genes (Maupin-Furlow et al. 1995; Rech et al. 1995; Grunden and Shanmugam 1997). ModA is the periplasmic Mo-binding protein, ModB is the membrane-spanning component, and ModC provides the energizer function on the cytoplasmic side of the membrane. Upstream of *modA* and divergently transcribed in *E. coli* is the gene encoding the regulatory protein, ModE. Dimers of ModE bind molybdate and repress transcription of *modABCD* (Grunden et al. 1996, 1999; Anderson et al. 1997). Thus, growth with molybdate represses transport of this anion. The diazotrophic bacteria, *Rhodobacter capsulatus* and

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Azotobacter vinelandii, have a similar high-affinity molybdate-transport system with homologues of the genes found in *E. coli* (Luque et al. 1993; Wang et al. 1993; Mouncey et al. 1995, 1996). Little is known about the mechanism of molybdate transport in cyanobacteria. However, in *A. variabilis* molybdate at very low concentrations represses V-nitrogenase, suggesting that there is a high-affinity molybdate-transport system (Thiel 1993). We demonstrate here that *A. variabilis* has a molybdate-transport system that appears similar to those of *E. coli* and *A. vinelandii*.

Materials and methods

Strains and growth conditions

Strains used in this study were *A. variabilis* FD (a derivative of *A. variabilis* ATCC 29413 that can grow at 40 °C and can support the growth of bacteriophages better than the parent strain; Currier and Wolk 1979), MM3 (a *ntcA* mutant of FD; Thiel and Pratte 2001), *Anabaena* sp. PCC 7120, and CSE2 (a *ntcA* mutant of *Anabaena* sp. PCC 7120; Frías et al. 1994). Strains were grown photoautotrophically in liquid cultures in AA/8, an eight-fold dilution of the medium of Allen and Arnon (1955), in some instances supplemented with 2.5 mM NaNO₃ and 2.5 mM KNO₃ (AA/8+nitrate), or with 5.0 mM NH₄Cl and 10 mM *N*-Tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), pH 7.2, at 30 °C with illumination at 50–80 μE m⁻² s⁻¹. Mo-free medium was prepared from stocks scrubbed free of contaminating Mo, using activated charcoal (Schneider et al. 1991). The microelement stock was prepared without Mo, but was not treated with activated charcoal. To remove traces of Mo, glassware was treated with 1% Count-Off (New England Nuclear) and 10 mM ethylene diamine tetraacetic acid for 24 h and then thoroughly rinsed with deionized water, purified through a Millipore water purification system. Cyanobacteria were subcultured in Mo-free medium for at least 15 generations to deplete internal molybdate reserves. Cyanobacterial cultures were maintained on AA or on BG-11 (Allen 1968) media solidified with 1.5% Difco Bacto agar (Thiel et al. 1989).

⁹⁹Mo (molybdate) transport

Cultures for ⁹⁹Mo (molybdate) transport assays were grown for at least 15 generations in Mo-free AA/8, in some instances supplemented with 5.0 mM NH₄Cl and 10 mM TES, pH 7.2. Cultures were grown to an optical density at 720 nm (OD₇₂₀) of 0.12–0.20, washed three times in Mo-free AA/8 and resuspended at OD₇₂₀=0.10–0.17, as indicated in the figure legends. The only exception to this was the experiment shown in Fig. 2 where, because of the very low concentrations of molybdate in the medium, cells were diluted to OD₇₂₀=0.015–0.017 in order to ensure incorporation for the 5-min time-course of the experiment. Under the conditions of these experiments, the generation time was about 24 h; and, therefore, there was no significant change in cell numbers over the time-course of the experiments. Washed cells were transferred to 50-ml plastic tissue culture flasks for transport assays. ⁹⁹Mo (7,000 Ci mg⁻¹; kindly provided by Mallinckrodt, St. Louis) and cold Na₂MoO₄ were mixed to give a specific activity of 6.25 × 10³ Ci mol⁻¹. The labeled stock solution (8 × 10⁻⁷ M molybdate), diluted into the culture to give the final concentration of molybdate indicated in each experiment, was added to prewarmed cells at time 0 and the cells were incubated at 30 °C in the light. Duplicate 0.5-ml samples were removed at the times indicated in the graphs to 25 mm, 0.45 μm nitrocellulose filters where the samples were washed five times with 50 mM Tris, pH 7.5, 50 mM NaCl by vacuum filtration. In the exchange experiments, some samples were washed with 1.0 mM Na₂MoO₄. Non-specific binding to cells was measured 1, 2, 3, 4, and 5 min after the addition of various con-

centrations of ⁹⁹Mo to cells that had been heated to 70 °C for 15 min. In the competition experiments, the competing metal was added to the cells 1 min before the labeled molybdate. All assays were performed 3–5 times and representative data are provided.

Acetylene reduction assays

Cultures were concentrated to approximately OD₇₂₀=0.8 and added to 10-ml serum bottles in 1.0-ml aliquots. The bottles were sealed with gas-tight serum stoppers, injected with 1.0 ml acetylene gas and placed in an illuminated 30 °C shaking water bath for 30 min. Then, 1.0-ml samples of gas were removed via a hypodermic needle and injected into a Shimadzu gas chromatograph equipped with a 183-cm Poropak N column. The column temperature was 75 °C. Chlorophyll *a* determinations for each 1.0-ml aliquot were made from methanol extracts, according to MacKinney (1941).

Results

⁹⁹Mo transport

Transport of molybdate was measured in the wild-type strain FD. In preliminary experiments, we determined that cells did not transport ⁹⁹Mo unless they were starved for molybdate, suggesting that the transport system was repressed by molybdate. Cells were starved in Mo-free medium for several generations and then supplemented at the

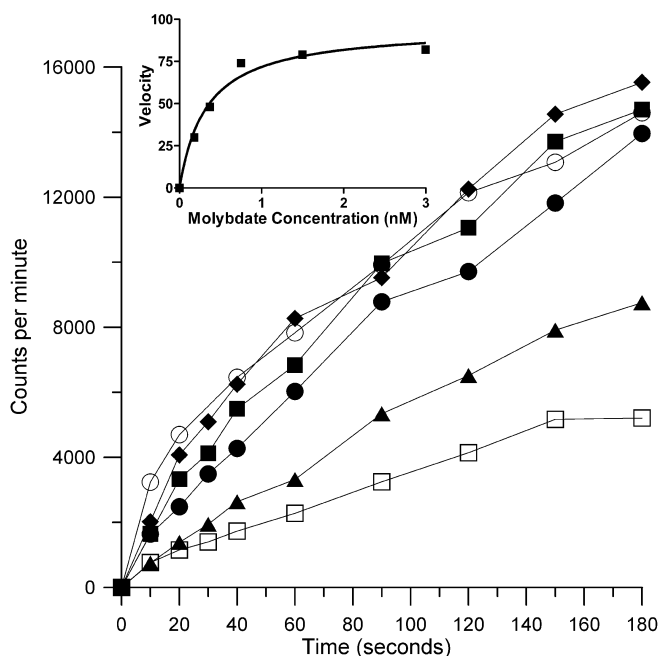


Fig. 1 Kinetics of transport. Accumulation of ⁹⁹Mo (molybdate) in strain FD with an optical density at 720 nm (OD₇₂₀) of 0.017 ± 0.002 was measured at various concentrations of molybdate, as described in Materials and methods: 6 × 10⁻⁹ M (white circles), 3 × 10⁻⁹ M (black diamonds), 1.5 × 10⁻⁹ M (black squares), 7.5 × 10⁻¹⁰ M (black circles), 3.75 × 10⁻¹⁰ M (black triangles), 1.8 × 10⁻¹⁰ M (white squares). Inset Rates of transport determined from linear regression analysis of incorporation data after 40 s were plotted against the concentration of molybdate (nM) using a non-linear regression analysis

start of the experiment with ^{99}Mo at various concentrations, keeping the specific activity constant. The time-course for molybdate transport at several very low concentrations was measured in order to determine the approximate K_m for transport (Fig. 1). There was an initial high rate of molybdate uptake that was complete in less than 1 min, followed by a slower, long-term rate. Using rate data from a linear regression analysis of uptake after 40 s, a non-linear regression plot (Fig. 1, inset) gave an estimated K_m for transport of 3.3×10^{-10} M. The counts associated with cells at very early time-points were not the result of non-specific binding. Heat-killed cells incorporated only about 1% of the counts observed in living cells, even at high concentrations of ^{99}Mo (8×10^{-9} M). Heat-killed cells showed no increase in incorporation over time (data not shown).

Mo-exchange

The biphasic transport kinetics suggested that the initial high rate of transport might be attributable to relatively weak binding of molybdate to a periplasmic protein, similar to the ModA protein of *E. coli*. In order to determine whether the bound ^{99}Mo was periplasmic and could exchange with cold molybdate outside the cell, we added excess cold molybdate to cells of strain FD after 15 min incubation with ^{99}Mo (Fig. 2). About 10% of the cell-associated counts were lost within 30 s and there was no further incorporation of ^{99}Mo into the cells, suggesting that some of the ^{99}Mo was associated with the outer surface of the cell and the periplasm. In a similar experiment, dupli-

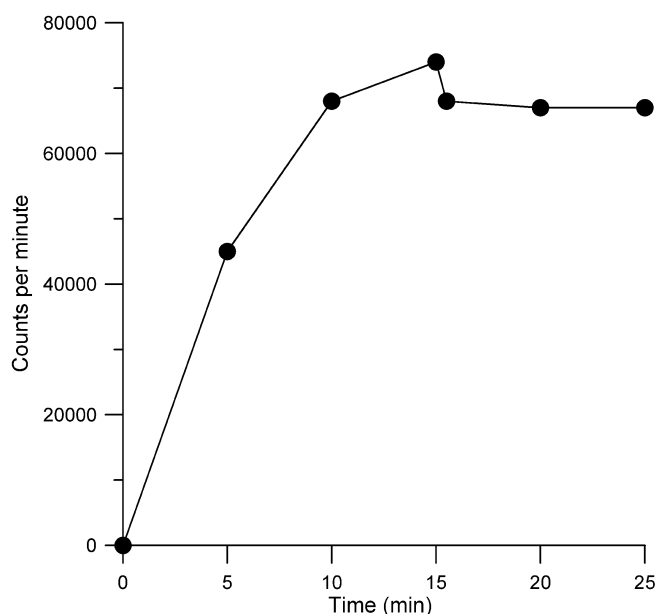


Fig. 2 Exchange of ^{99}Mo with cold molybdate. The accumulation of ^{99}Mo (concentration of ^{99}Mo in the assay was 1.6×10^{-8} M) in strain FD at $\text{OD}_{720} = 0.14 \pm 0.01$ was measured before and after the addition of 1.0 mM cold molybdate at 15 min, as described in Materials and methods

cate ^{99}Mo -labeled samples were diluted at various times (5, 10, 15, 20 min) after the start of the experiment into Tris/NaCl wash solution or into 1.0 mM molybdate wash solution. If the bound ^{99}Mo could exchange with cold molybdate, we would expect the samples washed with 1.0 mM molybdate to have lower incorporation of ^{99}Mo than the cells washed with Tris/NaCl. We observed lower levels of accumulation of ^{99}Mo in cells washed with 1.0 mM molybdate (10–20% lower), compared with cells washed with Tris/NaCl (data not shown), supporting the hypothesis of some reversible molybdate-binding in the periplasm.

Competition with other anions

In *A. vinelandii*, the high-affinity molybdate-transport system also transports tungstate, but not vanadate or sulfate (Mouncey 1995). We determined that 1.0 μM tungstate or 1.0 μM cold molybdate inhibited transport of ^{99}Mo in *A. variabilis*, whereas the same concentrations of sulfate or vanadate had little or no effect on ^{99}Mo transport (Fig. 3A). At concentrations of 10 μM , the effect of these oxyanions was similar to the effect of 1.0 μM , although there was some inhibition of molybdate transport by vanadate (Fig. 3B). It is not clear whether this was actually competition for binding, or trace amounts of molybdate in the vanadate that could compete. Thus, the high-affinity molybdate-transport system of *A. variabilis* transports molybdate and tungstate, but not vanadate or sulfate.

We also measured the effect of tungstate on nitrogenase. Nitrogenase activity can be readily measured by its ability to reduce acetylene to ethylene. Although both Mo-nitrogenase and V-nitrogenase reduce acetylene, Mo-nitrogenase reduces acetylene much more efficiently than does V-nitrogenase (Bishop and Premakumar 1992). Thus, addition of molybdate to cells already using V-nitrogenase results in an increase in acetylene reduction, as the molybdate both represses synthesis of V-nitrogenase and stimulates synthesis of Mo-nitrogenase. If tungstate competes for molybdate transport, it should inhibit the stimulation of acetylene reduction by the addition of molybdate to cells already using V-nitrogenase. Cultures of FD were grown with vanadate in the absence of molybdate to induce V-nitrogenase and then either molybdate, or tungstate, or a mixture of both was added to the cells. Within 2 h after addition of molybdate, there was stimulation of acetylene reduction, resulting in activity that increased linearly over 6 h (Fig. 4). Cultures containing tungstate showed no such effect. Addition of tungstate with molybdate inhibited the stimulation of acetylene reduction by molybdate, supporting the hypothesis that tungstate competed for the transport of molybdate. Because of the similarity of molybdate and tungstate, it seemed very likely that tungstate was transported; however, it did not function in place of Mo in Mo-nitrogenase, since it did not stimulate acetylene reduction.

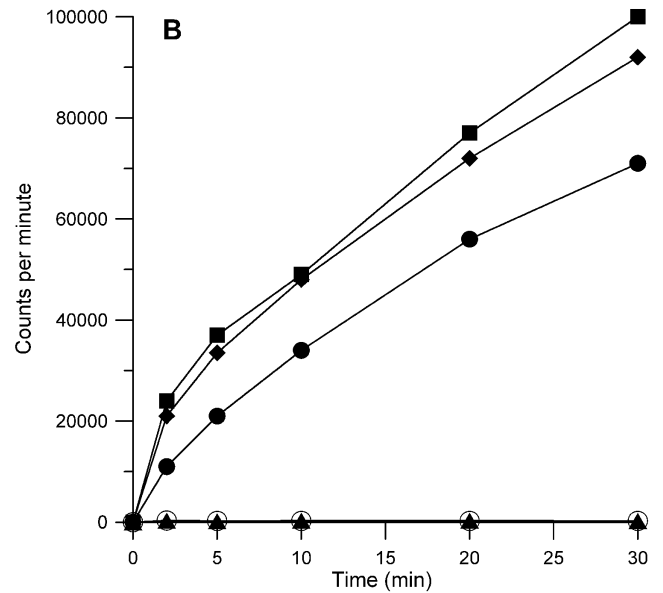
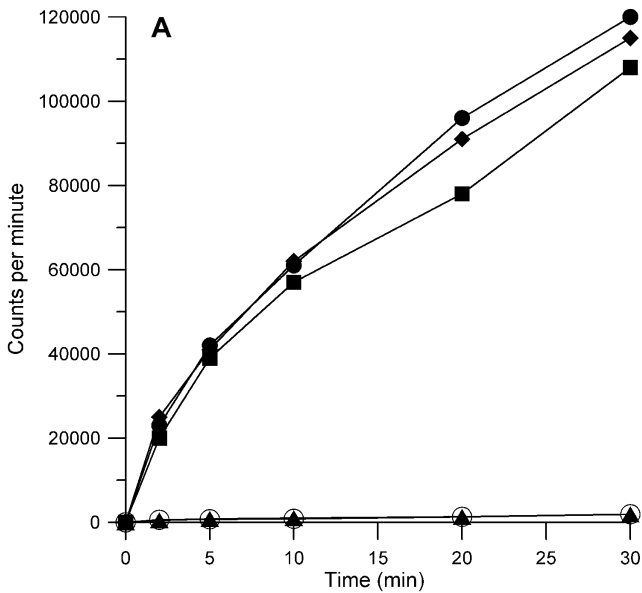


Fig. 3A, B Competition for ^{99}Mo transport. Accumulation of ^{99}Mo in strain FD at $\text{OD}_{720}=0.14\pm 0.01$ was measured using a concentration of molybdate of 4×10^{-9} M, with the addition of either 1 μM (A) or 10 μM (B) vanadate (black circles), sulfate (black squares), tungstate (black triangles), cold molybdate (white circles), or no added competitor (black diamonds)

1997). We observed that induction of V-nitrogenase (which is repressed by molybdate) in Mo-grown cells requires growth for several generations in Mo-free medium to deplete internal molybdate reserves, suggesting that *A. variabilis* can store molybdate. In order to determine how quickly internal molybdate reserves decreased to a level that allowed expression of the molybdate-transport system, cells were grown with 0.1 μM molybdate, washed free of molybdate and then assayed daily for ^{99}Mo trans-

Molybdate storage

Mo-binding proteins may serve as molybdate-storage proteins, allowing cells to continue to make Mo-enzymes when external molybdate becomes limiting (Pau et al.

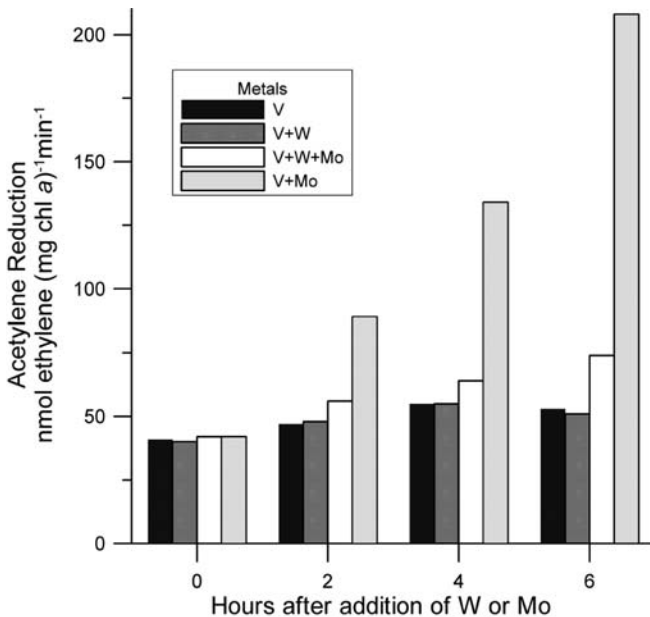


Fig. 4 Effect of tungstate on induction of Mo-nitrogenase. Cells of FD were grown in Mo-free AA/8 medium (Allen and Arnon 1955) with 0.1 μM vanadate prior to addition of molybdate (0.01 μM), tungstate (10 μM), or both. At various times samples were removed for acetylene reduction assays

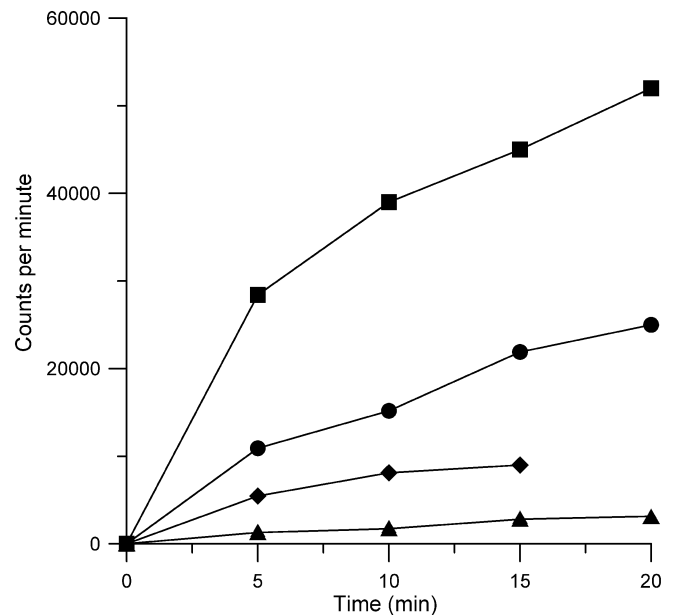


Fig. 5 Starvation for molybdate. Cells of FD were grown for 3 days with 0.1 μM molybdate and then washed free of molybdate at the start of the experiment. On day 0 and each subsequent day, cells were washed and adjusted to $\text{OD}_{720}=0.14\pm 0.01$ and assayed for ^{99}Mo transport. Cells were diluted two-fold with fresh Mo-free growth medium every day to maintain exponential growth of the stock culture. Day 0 (triangles), day 1 (diamonds), day 2 (circles), day 3 (squares)

port. Within 24 h after removal of molybdate, there was some induction of ^{99}Mo transport. However, high levels of transport required 3 days of starvation, which corresponded to about four generations (Fig. 5). There was no increase in transport after day 3 (data not shown). These data suggest that *A. variabilis* has a mechanism for the storage of molybdate in the cells. Depletion of these reserves occurred most quickly in cells grown under nitrogen-fixing conditions, under which the cells appeared to require large amounts of molybdate. Cells grown with nitrate, in a Mo-free medium, required more generations of growth to deplete internal molybdate reserves. The evidence for molybdate depletion was the inability of these cells to use nitrate as sole nitrogen source and the induction in these cells of V-nitrogenase that is repressed by molybdate (data not shown). Ammonia-grown cells required many generations of growth to reduce molybdate reserves to a level that prevented synthesis of Mo-enzymes and derepressed V-nitrogenase.

Role of NtcA in molybdate transport

NtcA is required for growth of *Anabaena* sp. PCC 7120 with nitrate or with N_2 (Frías et al. 1994). Since the enzymes that are essential for utilization of nitrate or N_2 are the only ones known in cyanobacteria to require molybdate, it seemed possible that transport of molybdate might also require NtcA. We determined the ability of the *ntcA* mutant of *A. variabilis* to transport ^{99}Mo . Because the mutant requires NH_4Cl for growth, we also determined, in the wild-type strain, whether growth with NH_4Cl affected transport of ^{99}Mo . Both the wild type and the *ntcA* mutant transported ^{99}Mo well. Growth with NH_4Cl had no significant effect on transport in the wild-type strain (data not shown); and, thus, it appears that neither NtcA nor ammonia regulates molybdate transport.

Molybdate transport in *Anabaena* sp. PCC 7120

Using the conditions described here for transport of ^{99}Mo , we determined that *Anabaena* sp. PCC 7120 transported molybdate in a way similar to *A. variabilis*. There was no significant difference in transport either in terms of rate or affinity for molybdate for these two strains. In addition, the *ntcA* mutant of *Anabaena* sp. PCC 7120 transported molybdate at a rate similar to the wild-type strain and similar to the *ntcA* mutant of *A. variabilis* (data not shown). The only difference in transport observed between these two strains was that depletion of internal molybdate reserves took longer in *Anabaena* sp. PCC 7120 than in *A. variabilis*. However, that is likely due to its longer generation time.

Discussion

High-affinity ABC-type molybdate-transport systems have been identified and characterized genetically in *E. coli*,

A. vinelandii, and *R. capsulatus* (for a review, see Pau et al. 1997). Molybdate is an essential anion for cyanobacteria, such as *A. variabilis* and *Anabaena* sp. PCC 7120, since it is required for growth with either nitrate or N_2 . The high-affinity transport of ^{99}Mo (molybdate) in these strains suggests the presence of a system similar to the ABC transport system for molybdate in other bacteria. Concentrations of $2\text{--}3\times 10^{-9}$ M molybdate saturated the transport system in *A. variabilis*. This is about 5-fold lower than the concentration (about 10^{-8} M) that saturated the system in *A. vinelandii* (Mouncey et al. 1995). In *E. coli*, the molybdate-transport system has a K_m of 2.5×10^{-8} M (Lopez Corcuero et al. 1993), while that of *Clostridium pasteurianum* has a K_m of about 5×10^{-5} M (Elliot and Mortenson 1975). As is true for the molybdate-transport systems of *A. vinelandii* (Mouncey et al. 1995) and *C. pasteurianum* (Elliot and Mortenson 1975), tungstate inhibited the molybdate-transport system in *A. variabilis*, but vanadate did not. While sulfate failed to inhibit transport in *A. variabilis* and in *A. vinelandii* (Mouncey et al. 1995), it did so in *E. coli* (Lopez Corcuero et al. 1993) and in *C. pasteurianum* (Elliot and Mortenson 1975), suggesting a difference in the anion specificity of these transport systems. Assuming that tungstate was transported in *A. variabilis*, tungsten did not function in the place of molybdenum in Mo-nitrogenase.

In *A. variabilis*, as is true in *E. coli* (Lopez Corcuero et al. 1993) and *A. vinelandii* (Mouncey et al. 1995), molybdate transport was biphasic, with a very rapid initial phase of uptake followed by slower accumulation. This suggested the presence of a periplasmic binding protein as did the exchange of some bound ^{99}Mo with cold molybdate in the medium. A *modA* (*sll0738*) homologue has been identified in the genome of *Synechocystis* sp. PCC 6803; and that gene is followed by a gene (*sll0739*) that appears to be in the class of ABC transporters (Kaneko et al. 1996). There are genes similar to *sll0738* and *sll0739* in the genome of *Anabaena* sp. PCC 7120 (Kaneko et al. 2001). However, they are not contiguous. We have evidence that the open reading frame (ORF) in *Anabaena* sp. PCC 7120 that is the homologue of *sll0739* of *Synechocystis* sp. PCC 6803 is involved in the high-affinity transport of molybdate (data not shown).

A. variabilis and *Anabaena* sp. PCC 7120 maintained sufficient stores of molybdate to grow with nitrate or N_2 for several generations in a medium lacking molybdate. In *A. vinelandii* and *C. pasteurianum*, stored molybdate is probably bound to small proteins that share a conserved Mo-binding domain. A Mo-binding domain is present in several proteins involved in molybdate transport (for a review, see Pau et al. 1997). The regulatory protein ModE binds molybdate, as do the small proteins called Mop. In addition, ModC, part of the ABC transport system for molybdate, has a Mo-binding domain. Recently another protein, ModG, in *A. vinelandii* has been shown to bind molybdate as a trimer that can bind eight molybdate molecules (Delarbre et al. 2001). It appears likely that Mop or ModG serve in homeostasis of molybdate (Delarbre et al. 2001). There is a single *mop* gene in *Anabaena* sp. PCC

7120, located within the cluster of nitrogenase genes. A mutation in that region (which also inactivated nearby ORFs) had a minor effect on nitrogen fixation in *Anabaena* sp. PCC 7120 (Masepohl et al. 1997). There is no *mop* gene present in *Synechocystis* sp. PCC 6803 (Kaneko et al. 1996) or *Synechococcus* WH8102 (<http://bahama.jgi-psf.org/prod/bin/microbes/syn/home.syn.cgi>), both of which have nitrate reductase, suggesting that the role of *mop* genes in cyanobacteria may be primarily in support of nitrogen fixation.

NtcA has an important role in the expression of the genes for nitrate reductase, nitrite reductase, and nitrogenases, although the effect on nitrogenase may be an indirect effect of the requirement of NtcA for heterocyst differentiation (Wei et al. 1994; Herrero et al. 2001). Since the enzymes that require Mo cannot be made in the absence of NtcA and NtcA is now known to control diverse genes in cyanobacteria (Herrero et al. 2001), it seemed reasonable that this protein would also control molybdate transport. However, we determined that the *ntcA* mutants in both *A. variabilis* and *Anabaena* sp. PCC 7120 were capable of normal, inducible molybdate uptake; and, therefore, it does not appear likely that NtcA is involved in regulating the genes involved in molybdate transport.

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