

## Role of Molybdate and Other Transition Metals in the Accumulation of Protochelin by *Azotobacter vinelandii*

ANTHONY S. CORNISH AND WILLIAM J. PAGE\*

Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

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**Both molybdate and iron are metals that are required by the obligately aerobic organism *Azotobacter vinelandii* to survive in the nutrient-limited conditions of its natural soil environment. Previous studies have shown that a high concentration of molybdate (1 mM) affects the formation of *A. vinelandii* siderophores such that the tricatecholate protochelin is formed to the exclusion of the other catecholate siderophores, azotochelin and aminochelin. It has been shown previously that molybdate combines readily with catecholates and interferes with siderophore function. In this study, we found that the manner in which each catecholate siderophore interacted with molybdate was consistent with the structure and binding potential of the siderophore. The affinity that each siderophore had for molybdate was high enough that stable molybdosiderophore complexes were formed but low enough that the complexes were readily destabilized by  $\text{Fe}^{3+}$ . Thus, competition between  $\text{Fe}^{3+}$  and molybdate did not appear to be the primary cause of protochelin accumulation; in addition, we determined that protochelin accumulated in the presence of vanadate, tungstate,  $\text{Zn}^{2+}$ , and  $\text{Mn}^{2+}$ . We found that all five of these metal ions partially inhibited uptake of  $^{55}\text{Fe}$ -protochelin and  $^{55}\text{Fe}$ -azotochelin complexes. Also, each of these metal ions partially inhibited the activity of ferric reductase, an enzyme important in the deferration of ferric siderophores. Our results suggest that protochelin accumulates in the presence of molybdate because protochelin uptake and conversion into its component parts, azotochelin and aminochelin, are inhibited by interference with ferric reductase.**

*Azotobacter vinelandii* is a gram-negative obligately aerobic bacterium that is commonly found in soil and aquatic environments. A novel feature of *Azotobacter* spp. is the ability of these organisms to fix nitrogen nonsymbiotically under aerobic conditions. *A. vinelandii* forms three nitrogenases that are differentiated on the basis of the metal cofactor (2), and nitrogenase activity is dependent on the acquisition of metals for cofactor synthesis. Both iron and molybdenum are found in the dominant nitrogenase, nitrogenase I; iron and vanadate are found in nitrogenase II; and only iron is present in nitrogenase III. Iron and molybdenum are similar chemically; both are large transition metals which can exist in a number of oxidation states, both are Lewis acids, and both can form six coordinate bonds at physiological pH values (33). In aqueous systems at a neutral pH molybdenum readily interacts with water and forms the highly soluble molybdate ( $\text{MoO}_4^{2-}$ ) ion. Under these conditions the molybdenum atom has an effective charge of +3.6 and, as a result, interacts with ligands like  $\text{Fe}^{3+}$  interacts with ligands (16). Iron, on the other hand, interacts with water to form ferric hydroxides and ferric oxyhydroxides that are very insoluble [ $K_{sp}$  of  $\text{Fe}(\text{OH})_3$ ,  $2 \times 10^{-39}$ ], so the concentration of free iron(III) is on the order of  $10^{-17}$  M, which is far too low to support bacterial growth (14).

High-affinity uptake systems for both iron and molybdate have been found in *Escherichia coli* and *A. vinelandii* (13, 26). High-affinity iron uptake is mediated by small organic molecules called siderophores which have high affinity for iron(III) but lower affinity for all other metal ions (14). *A. vinelandii* produces the catecholate siderophores azotochelin (5), aminochelin (31), and protochelin (6) (Fig. 1) and the pyoverdine-like siderophore azotobactin (29). In addition to iron, cat-

echolate siderophores bind molybdate to form complexes at a neutral pH. With both iron and molybdenum, this complex formation is the result of the presence of oxygen atoms within the 2,3-dihydroxybenzoic acid (2,3-DHBA) moieties of the catechol molecule, which are very electron dense. These groups displace the less electron-dense oxygen atoms of water and coordinate with either iron or molybdenum to form stable complexes, although the catecholate complexes formed with molybdenum are less stable than the catecholate complexes formed with iron (16). In the presence of a low molybdenum concentration, catecholate-like molybdate-coordinating compounds may be formed by a number of microorganisms. However, it is not clear whether these compounds are dedicated, true mediators of high-affinity molybdate uptake or are simply misidentified members of a high-affinity iron uptake system (20, 32, 35).

We have focused on the production of protochelin by *A. vinelandii* (6, 7). This tricatecholate siderophore has a high affinity for  $\text{Fe}^{3+}$  but is normally less abundant in iron-limited culture fluids than azotochelin and aminochelin. Protochelin is composed of one azotochelin molecule and one aminochelin molecule (Fig. 1), and it may be either the product of condensation or the progenitor of the other two catecholates. When *A. vinelandii* is grown in a medium containing a high concentration of molybdate (1 mM), protochelin is hyperproduced and is the only catecholate produced (6). At this high concentration molybdate interferes with the function of protochelin by competing with iron for binding to the siderophore (6). It has been proposed by Duhme et al. (10) that overproduction of protochelin is due to molybdate binding to azotochelin, which depletes the concentration of this siderophore available for iron transport and leads to hyperproduction of protochelin. These authors also speculated that formation of protochelin compensates for the use of azotochelin in high-affinity molybdate transport. The data presented here suggest that this probably does not occur and that protochelin accumulates in the

\* Corresponding author. Mailing address: Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9. Phone: (780) 492-4782. Fax: (780) 492-2216. E-mail: bill.page@ualberta.ca.

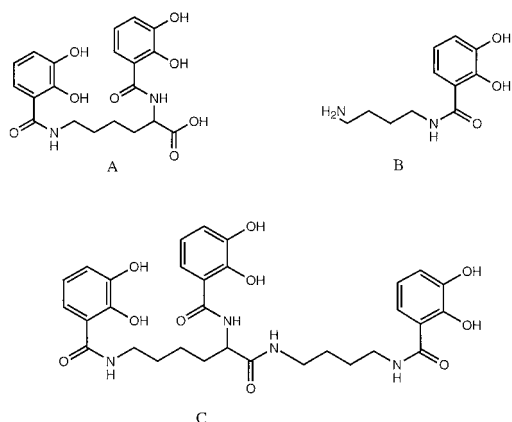


FIG. 1. Catecholate siderophores of *A. vinelandii*. (A) Azotochelin. (B) Aminochelin. (C) Protochelin.

presence of molybdate as a result of inhibition of ferric siderophore uptake, in which a ferric reductase is the most likely target for inhibition by molybdate and other divalent metal ions that increase protochelin accumulation.

#### MATERIALS AND METHODS

**Strains and growth conditions.** *A. vinelandii* capsule-negative strain UW (= OP = ATCC 13705) was used in this study. The mutants derived from strain UW included azotobactin-deficient strain UA1 (30), ferredoxin I (*fdxA*)-negative strain LM100 (25), and strain RP40, which is defective in both high- and low-affinity molybdate uptake (26). Cultures were grown in Burk's medium, which contained 1% (wt/vol) glucose, 15 mM ammonium acetate, 1  $\mu$ M  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.81 mM  $\text{MgSO}_4$ , and 0.58 mM  $\text{CaSO}_4$  in 6 mM potassium phosphate buffer (pH 7.6) (37). The iron-sufficient medium used for culture maintenance contained 50  $\mu$ M ferric citrate, while the iron-limited medium contained 1  $\mu$ M ferric citrate. The effects of metals other than molybdate on accumulation of protochelin were examined by growing strain UW in iron-limited Burk's medium (20-ml portions in 50-ml Erlenmeyer flasks) at 28°C and 225 rpm. The following divalent metals were used at concentrations ranging from 10 to 1,000 mM:  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{NaVO}_3 \cdot 2\text{H}_2\text{O}$ ,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{Na}_2\text{WO}_3$ ,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , and  $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$ . All glassware was acid washed with 4 M HCl and then rinsed with 50 mM EDTA (pH 7.0) and Milli-Q deionized water (Millipore) to remove contaminating iron (27).

**Spectrophotometric and colorimetric analyses.** Pure preparations of azotochelin, aminochelin, and protochelin were obtained from iron-limited strain LM100 culture supernatant as previously described (7). Siderophores were detected spectrophotometrically by scanning iron-limited culture supernatant fluid which had been acidified to pH 1.8 with HCl. Absorption peaks were measured with a Hitachi model U-2000 recording spectrophotometer at  $A_{310}$  for catechols and at  $A_{380}$  for azotobactin (30). Catecholate siderophore concentrations in stock solutions were quantified by using a molar absorptivity for 2,3-DHBA of  $3.26 \times 10^3 A_{310} \text{ M}^{-1} \text{ cm}^{-1}$ , corrected for the number of 2,3-DHBA moieties per siderophore (7). Catechol was also quantified by using the colorimetric assay of Barnum (1). The identities of catecholate siderophores were determined by performing thin-layer chromatography (TLC) as described by Cornish and Page (6) and comparing data with data for authentic standards. Total cellular protein contents were determined by the method of Lowry et al. (24).

**Molybdo-siderophore molar binding and affinity determination.** The molar binding ratios of each catecholate siderophore and molybdate were determined by the continuous-variation method of Job (4). Each siderophore was mixed with  $\text{MoO}_4^{2-}$  (as sodium molybdate) at various ratios in 100 mM MOPS (morpholinepropanesulfonic acid) (pH 7.0) buffer; protochelin was also examined in 100 mM MES (morpholineethanesulfonic acid) (pH 6.0) buffer. The absorbance of each of the mixtures was measured. As the absorbance spectra of all of the molybdo-siderophore complexes exhibited a broad peak at wavelengths from 300 to 500 nm, all measurements were taken at 375 nm. The solution with the highest absorbance at equilibrium was used to represent the correct molar binding ratio of molybdate and the siderophore.

The affinity of each siderophore for molybdate binding was determined by using a modification of the method used by Cornish and Page to determine siderophore-iron affinity (7). To determine the conditional formation constant of a molybdo-siderophore complex, competition between the formation of a ferric siderophore complex and the formation of a molybdo-siderophore complex was analyzed. In this analysis, a ferric siderophore complex (final concentration, 0.1 mM) was allowed to form for 72 h before 0.05 to 2 mM molybdate was added.

Each reaction mixture was incubated for 96 h at room temperature in the dark under a nitrogen atmosphere to maintain anaerobic conditions before the equilibrium concentration of the ferric siderophore complex was determined spectrophotometrically (7). Conversely, a molybdo-siderophore complex (final concentration, 0.1 mM) was allowed to form for 72 h before 0.05 to 2 mM ferric nitrate was added. Each reaction mixture was allowed to reach equilibrium (96 h), under the anaerobic conditions described above, before the concentration of the ferric siderophore complex was determined (7). A conditional formation constant for a molybdo-siderophore complex was then calculated as described for the calculation of a ferric siderophore complex (34). All reactions were carried out in 96-well microtiter plates, and absorbance was measured with a Biotek Instruments model EL311 microplate reader; a minimum of two duplicate reaction series, each containing six replicates of each reaction mixture, were used (7).

**Competition between molybdate and iron(III) for siderophore binding.** To determine if the presence of molybdate affected the formation of a ferric siderophore complex, ferric nitrate, sodium molybdate, and a purified siderophore were combined, and formation of the metal-siderophore complexes was monitored at  $A_{490}$  for ferric protochelin and ferric aminochelin complexes and at  $A_{570}$  for ferric azotochelin complexes (7). Protochelin was mixed 1:1 with iron and 1:1 with molybdate, and the mixture contained 0.20  $\mu$ mol of siderophore, 0.20  $\mu$ mol of molybdate, and 0.20  $\mu$ mol of iron(III). Alternatively, protochelin was mixed 1:1 with iron and 2:3 with molybdate, and the resulting preparation contained 0.16  $\mu$ mol of siderophore, 0.24  $\mu$ mol of molybdate, and 0.16  $\mu$ mol of iron(III). Azotochelin was mixed 3:2 with iron and 1:1 with molybdate, and the resulting mixture contained 0.20  $\mu$ mol of siderophore, 0.20  $\mu$ mol of molybdate, and 0.13  $\mu$ mol of iron(III). Finally, aminochelin was mixed 3:1 with iron and 2:1 with molybdate, and the resulting preparation contained 0.30  $\mu$ mol of siderophore, 0.15  $\mu$ mol of molybdate, and 0.10  $\mu$ mol of iron(III). In each case, the total volume of the reaction mixture was brought to 2 ml with 6 mM potassium phosphate buffer (pH 7.6).

The stability of a ferric siderophore complex in the presence of equimolar molybdate or excess molybdate was also investigated. A solution containing 12.5  $\mu$ M ferric protochelin or ferric azotochelin was challenged with either 12.5  $\mu$ M or 1 mM molybdate, and formation of molybdo-protochelin or molybdo-azotochelin complexes was monitored at 375 nm.

Each reaction mixture was placed in a disposable plastic cuvette (catalog no. 223-9955; Bio-Rad) and was incubated under nitrogen gas using buffers, sparged with nitrogen gas for 10 min, in the dark in an air-tight chromatography tank flushed with nitrogen under high-humidity conditions. The formation of a ferric siderophore complex was monitored for up to 338 h. The percentage of a metal-siderophore complex present was determined by determining the ratio of the concentration of the metal-siderophore complex present to the theoretically maximum possible concentration of the metal-siderophore complex that could be formed, based on the amount of free metal and siderophore added. The concentration of ferric siderophore present was calculated by using the following molar absorptivities: ferric protochelin,  $5.45 \times 10^3 A_{310} \text{ M}^{-1} \text{ cm}^{-1}$ ; ferric azotochelin,  $1.01 \times 10^4 A_{310} \text{ M}^{-1} \text{ cm}^{-1}$ ; and ferric aminochelin,  $4.76 \times 10^3 A_{310} \text{ M}^{-1} \text{ cm}^{-1}$ . Each reaction was examined three times.

**CX preparation.** Strain UW was grown for 24 h in 200 ml of iron-limited Burk's medium containing 1  $\mu$ M molybdate and 3  $\mu$ M ferric citrate. Cell extract (CX) was prepared by the method of Page and von Tigerstrom (31), except that lysis in the French press was performed in 50 mM potassium phosphate buffer (pH 7.6) containing 2 mM dithiothreitol. The cell lysate was cleared by centrifugation (40,000  $\times$  g, 1 h), and the resulting CX was stored at  $-20^\circ\text{C}$ .

**Ferric reductase assay.** The ferric reductase activity in strain UW CX was measured by monitoring  $\text{Fe}^{2+}$  binding to ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine] (catalog no. P-9762; Sigma) as previously described (28). The 1.6 mM iron(III) source used was either ferric citrate, ferric protochelin, ferric azotochelin, or ferric aminochelin complexes formed as described by Cornish and Page (7). Rates of conversion of iron(III) to iron(II) were calculated in the first 2 min of the assay by using a molar absorptivity for the  $\text{Fe}^{2+}$ -ferrozine complex of  $2.79 \times 10^4 A_{562} \text{ M}^{-1} \text{ cm}^{-1}$  (38). Ferric reductase activity was expressed in nanomoles of  $\text{Fe}^{2+}$  per minute per milligram of CX protein. Each assay was performed at least twice.

**Uptake of  $^{55}\text{Fe}^{3+}$ -siderophore complexes.** Uptake of  $^{55}\text{Fe}$ -siderophore complexes was examined as described previously (6, 21), with the following modifications. Pure siderophore stock preparations and  $^{55}\text{FeCl}_3$  (7  $\mu\text{g}$  of  $^{55}\text{FeCl}_3 \text{ ml}^{-1}$ ; specific activity, 35  $\mu\text{Ci ml}^{-1}$ ) were incubated for 72 h in the dark under high-humidity conditions (7) in order to obtain  $^{55}\text{Fe}$ -siderophore complexes with a final  $^{55}\text{Fe}^{3+}$  concentration of 12.5  $\mu\text{M}$ . The effect that metal ion addition had on the uptake of a  $^{55}\text{Fe}$ -containing complex was expressed as the ratio of the control uptake rate in the absence of the metal ion from 0 to 8 min to the treated uptake rate in the presence of the metal ion from 8 to 16 min, expressed as a percentage. Rates were calculated by using data obtained from a single assay or duplicate assays, as indicated.

## RESULTS

**Affinities of catecholate siderophores for molybdate.** Each catecholate siderophore bound molybdate in a manner which

TABLE 1.  $\text{Log}_{10} K_{\text{Form}}$  for molybdo-siderophore complexes in 100 mM MOPS buffer (pH 7.0)

Reaction	$\text{Log}_{10} K_{\text{Form}}$	Avg $\text{log}_{10} K_{\text{Form}}$	$\text{pMoO}_4^{2-}$ ( $-\text{log}_{10} [\text{MoO}_4^{2-}]$ )	$\text{pFe}^{3+}$ ( $-\text{log}_{10} [\text{Fe}^{3+}]$ ) <sup>a</sup>
Protochelin (1:1) complex				
Molybdo-protocelin + $\text{Fe}^{3+}$	$24.2 \pm 0.2^b$	$22.7 \pm 1.7$	26	27.7
Ferric protocelin + $\text{MoO}_4^{2-}$	$21.1 \pm 0.5$			
Protochelin (2:3) complex				
Molybdo-protocelin + $\text{Fe}^{3+}$	$48.0 \pm 0.6$	$49.1 \pm 1.3$	16	NA <sup>c</sup>
Ferric protocelin + $\text{MoO}_4^{2-}$	$50.2 \pm 1.2$			
Azotochelin (1:1) complex				
Molybdo-azotochelin + $\text{Fe}^{3+}$	$3.6 \pm 0.05$	$3.6 \pm 0.06$	6.1	23.1
Ferric azotochelin + $\text{MoO}_4^{2-}$	$3.5 \pm 0.02$			
Aminochelin (2:1) complex				
Molybdo-aminochelin + $\text{Fe}^{3+}$	$3.9 \pm 0.01$	$3.9 \pm 0.01$	5.6	22.0
Ferric aminochelin + $\text{MoO}_4^{2-}$	ND <sup>d</sup>			

<sup>a</sup> Data from reference 7.

<sup>b</sup> Average  $\pm$  standard deviation based on multiple determinations.

<sup>c</sup> NA, not applicable.

<sup>d</sup> ND, not determined.

was consistent with the fact that at a neutral pH molybdate has four sites at which water is coordinated and may be displaced by a more electronegative ligand (9, 16). Although a yellow catecholate siderophore complex was formed immediately after molybdate was added, formation of fully coordinated molybdo-siderophore complexes took time. Azotochelin chelated molybdate immediately at a molar binding ratio of 1:1, forming a complex which was stable for up to 191 h. A 2:1 molybdo-aminochelin complex was not observed until 28 h and was stable only until 98 h, after which it was replaced by other, undefined molybdo-aminochelin complexes. The interaction between protocelin and molybdate was not as easy to define. The predicted stoichiometry of a molybdo-protocelin complex was 2:3. This type of complex was observed at pH 6.0 in MES buffer. However, data obtained at pH 7.0 with MOPS buffer indicated that a complex with a 1:1 ratio of protocelin to molybdate was formed. This implies that at equilibrium protocelin had two empty coordination sites, a condition that would not be expected considering the amount of free molybdate in the test solution. As a result of this ambiguity, which we could not immediately explain, both 1:1 and 2:3 complexes were considered in subsequent calculations of the affinity of protocelin for molybdate. The 1:1 molybdo-protocelin complex was formed when molybdate was added and was stable for 167 h at pH 7.0, while the 2:3 molybdo-protocelin complex formed after 22 h and was stable for 376 h at pH 6.0.

Using the molar binding ratios described above, we determined the proton-dependent formation constant ( $K_{\text{Form}}$ ) for each siderophore with molybdate. Equilibrium was approached from either direction as the ferric siderophore complex was challenged with molybdate and the molybdo-siderophore complex was challenged with iron(III). The  $K_{\text{Form}}$  values for each mixture were similar (Table 1), which indicated that equilibrium could be approached from either direction. The  $K_{\text{Form}}$  for molybdo-aminochelin was approached only by competition between the ferric siderophore complex and molybdate due to a lack of pure aminochelin.

As a result of the different stoichiometries of the molybdate-binding reactions, it was not possible to directly compare the  $K_{\text{Form}}$  values generated for each siderophore as the different constants had different units. To directly compare the abilities of different siderophores to bind molybdenum, the amount of free  $\text{MoO}_4^{2-}$  in a theoretical molybdo-siderophore system at pH 7.4 was calculated, as described by Harris et al. (15). This required calculation of the proton-independent  $K_{\text{Form}}$  for each

siderophore by using the proton-dependent  $K_{\text{Form}}$  determined at pH 7.0 (Table 1) and the pKa values for the model compound *N,N*-dimethyl-2,3-dihydroxybenzamide (23), as described by Reid et al. (34) and Cornish and Page (7). Using this method, we determined the concentration of free molybdate in the system of Harris et al. (15) and expressed it as  $\text{pMoO}_4^{2-}$  ( $-\text{log}_{10} [\text{MoO}_4^{2-}]$ ). Thus, the larger the  $\text{pMoO}_4^{2-}$  value, the lower the free  $[\text{MoO}_4^{2-}]$  at equilibrium and the higher the affinity of a siderophore for molybdenum (Table 1).

**Molybdate and iron siderophore complex competition.** To determine if molybdate could interfere with formation of a ferric siderophore complex, molybdate,  $\text{Fe}^{3+}$ , and a purified siderophore were incubated together under the anaerobic conditions described above at the correct molar binding ratios, and formation of the ferric siderophore complex was monitored spectrophotometrically. The results of one experiment showed that formation of a ferric protocelin complex was not affected by the presence of molybdate at either a 1:1 ratio (Fig. 2a) or a 3:2 ratio (data not shown) and are representative of the results of multiple experiments in which data were obtained at different times. Formation of a ferric azotochelin complex and formation of a ferric aminochelin complex were both affected to small degrees by the presence of molybdate, and formation of a ferric azotochelin complex was more sensitive to the presence of molybdate (Fig. 2b) than formation of a ferric aminochelin complex (Fig. 2c). The results obtained for formation of a ferric azotochelin complex in the presence of molybdate were consistent with the results of Duhme et al. (9). Ferric siderophore complexes that were already formed were not affected by the presence of 12.5  $\mu\text{M}$  or 1 mM molybdate.

**Effects of other metal ions on the accumulation of protocelin.** Divalent metal ions other than molybdate were tested to determine whether they could increase protocelin accumulation. We found that vanadate, tungstate,  $\text{Mn}^{2+}$ , and  $\text{Zn}^{2+}$  increased protocelin accumulation (Table 2),  $\text{Co}^{2+}$  and  $\text{Ni}^{2+}$  were toxic to *A. vinelandii*, and  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ , and  $\text{Sr}^{2+}$  had no effect on protocelin accumulation. The minimum concentrations of the metals required to increase protocelin accumulation were determined by TLC analysis of culture fluids to be 70  $\mu\text{M}$  molybdate, 60  $\mu\text{M}$  vanadate, 30  $\mu\text{M}$  tungstate, 70  $\mu\text{M}$   $\text{Mn}^{2+}$ , and 500  $\mu\text{M}$   $\text{Zn}^{2+}$  (data not shown). At these concentrations, the metals were not detrimental to *A. vinelandii* growth. In addition, strain RP40, which is defective in both high- and low-affinity molybdate transport, was found to form protocelin in response to different molybdate concentrations

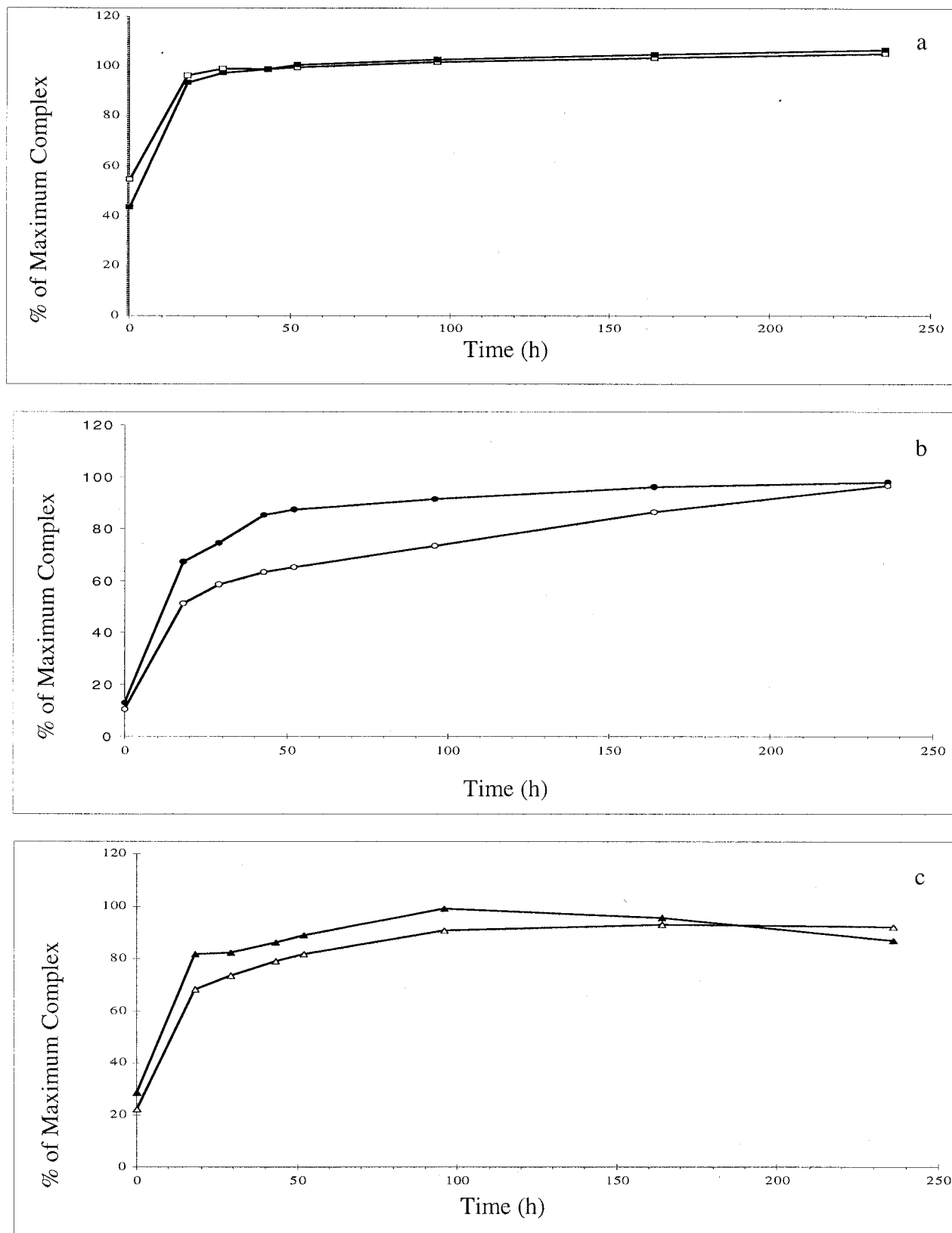


FIG. 2. Effect of molybdate competition on formation of a ferric siderophore complex. (a) Formation of ferric protochelin in the absence (■) and in the presence (□) of 0.2 mM molybdate. (b) Formation of ferric azotochelin in the absence (●) and in the presence (○) of 0.2 mM molybdate. (c) Formation of ferric protochelin in the absence (▲) and in the presence (△) of 0.2 mM molybdate. Data were obtained from a single experiment and are representative of the data obtained in multiple assays.



TABLE 2. Effects of metals that increase protochelin accumulation on the uptake of ferric protochelin and ferric azotochelin complexes

Metal	Concn ( $\mu\text{M}$ )	Avg % decrease in $^{55}\text{Fe}$ -protochelin uptake	Avg % decrease in $^{55}\text{Fe}$ -azotochelin uptake
None	NA <sup>a</sup>	0	0
Molybdate	70	40 $\pm$ 7 <sup>b</sup>	75 $\pm$ 8
Vanadate	100	55 $\pm$ 2	27 $\pm$ 15
Tungstate	30	51 $\pm$ 6	74 $\pm$ 6
Mn <sup>2+</sup>	70	29 $\pm$ 17	71 $\pm$ 15
Zn <sup>2+</sup>	500	56 $\pm$ 37	90 $\pm$ 31

<sup>a</sup> NA, not applicable.

<sup>b</sup> Range of values obtained in duplicate assays.

in a manner which was consistent with formation by wild-type strain UW (data not shown).

**Ferric reductase activity.** It has been shown previously that Mn<sup>2+</sup> and Zn<sup>2+</sup> are inhibitors of the *A. vinelandii* ferric reductase, an enzyme important in ferric siderophore uptake (18, 28). *A. vinelandii* cells grown in medium containing 1  $\mu\text{M}$  molybdate and 3  $\mu\text{M}$  ferric citrate were used to prepare CX. This level of iron repressed azotobactin production (31), and we expected that it would limit our examination of ferric reductases to the enzymes important in catechol siderophore use. Each metal that increased the accumulation of protochelin was found to be an inhibitor of ferric reductase activity (Table 3). Molybdate, vanadate, and tungstate appeared to have greater inhibitory effects on ferric citrate complex reduction than on ferric siderophore complex reduction. Mn<sup>2+</sup> had a greater effect on ferric aminochelin or ferric citrate complex reduction, while Zn<sup>2+</sup> appeared to have a greater effect on reduction of high-affinity chelates. It has been shown previously that Ca<sup>2+</sup>, Sr<sup>2+</sup>, and Mg<sup>2+</sup>, which had no effect on protochelin accumulation, activate ferric reductase activity (18).

**Inhibition of ferric siderophore complex uptake.** Ferric protochelin was taken up by Fe-limited *A. vinelandii* at a rate of 6.0 ng of  $^{55}\text{Fe}^{3+}$   $10^8$  cells<sup>-1</sup> min<sup>-1</sup>, and ferric azotochelin was taken up at a rate of 7.0 ng of  $^{55}\text{Fe}^{3+}$   $10^8$  cells<sup>-1</sup> min<sup>-1</sup>. Uptake continued with no decrease in the rate for the full 16 min of the assay. When 1 mM molybdate was added to cells which had been grown in Fe-limited medium containing 1  $\mu\text{M}$  molybdate, the rate of  $^{55}\text{Fe}$ -protochelin uptake decreased by 89% and the rate of  $^{55}\text{Fe}$ -azotochelin uptake decreased by 68%. When excess molybdate was removed by washing the cells in uptake buffer, the rates of uptake of  $^{55}\text{Fe}$ -protochelin and  $^{55}\text{Fe}$ -azotochelin increased by 10%. This indicated that the effect of molybdate was in part transient and could be reduced by removing free molybdate.

Inhibition of  $^{55}\text{Fe}$ -siderophore uptake was also observed in the presence of 70  $\mu\text{M}$  molybdate, the minimal concentration of molybdate required to increase protochelin accumulation (Table 2). Under these conditions, the uptake rate without

molybdate was 4.9 ng of  $^{55}\text{Fe}^{3+}$   $10^8$  cells<sup>-1</sup> min<sup>-1</sup> ( $R^2 = 0.90$ ), which decreased by 33% to 3.3 ng of  $^{55}\text{Fe}^{3+}$   $10^8$  cells<sup>-1</sup> min<sup>-1</sup> ( $R^2 = 0.80$ ) after 5 min of incubation with molybdate. The uptake rate in a siderophore-free uptake buffer control was 0.42 ng of  $^{55}\text{Fe}^{3+}$   $10^8$  cells<sup>-1</sup> min<sup>-1</sup> ( $R^2 = 0.91$ ), indicating that molybdate did not eliminate  $^{55}\text{Fe}^{3+}$  transport. Similarly,  $^{55}\text{Fe}$ -azotochelin uptake (4.0 ng of  $^{55}\text{Fe}^{3+}$   $10^8$  cells<sup>-1</sup> min<sup>-1</sup>;  $R^2 = 0.99$ ) decreased by 35% to 2.6 ng of  $^{55}\text{Fe}^{3+}$   $10^8$  cells<sup>-1</sup> min<sup>-1</sup> ( $R^2 = 0.82$ ) in the presence of 70  $\mu\text{M}$  molybdate.

Other metals that were found to increase protochelin accumulation and to decrease ferric reductase activity were examined to determine whether they had this effect on uptake of  $^{55}\text{Fe}$ -protochelin and  $^{55}\text{Fe}$ -azotochelin (Table 2). Although the effects of some of the metals, such as Zn<sup>2+</sup>, on  $^{55}\text{Fe}$ -siderophore uptake were quite variable, the results revealed the following trend: the metals that increased protochelin accumulation also decreased the uptake of  $^{55}\text{Fe}$ -siderophore complexes.

## DISCUSSION

Since it was first observed that high concentrations of molybdate result in accumulation of protochelin in *A. vinelandii* (6), the manner in which molybdate exerts this effect has been an unresolved question. Molybdate does not spontaneously promote condensation of aminochelin and azotochelin under aqueous conditions (6). Protochelin is a natural product of *A. vinelandii*; small amounts of it are produced by strain UW (6), and larger amounts are produced by strain LM100 (7) in low-molybdate media. Since higher levels of protochelin are formed in the presence of higher molybdate concentrations, an easy interpretation is that molybdate catalyzes formation of protochelin from its component parts, azotochelin and aminochelin. However, this probably does not occur, since strain RP40, which cannot transport molybdate, continues to form protochelin in the presence of increased molybdate concentrations.

Molybdate appears to impair the function of protochelin as a siderophore. It prevents decolorization of the ferric iron-Chrome Azurol S complex (6) in the universal assay for siderophore activity (36). It also prevents protochelin from promoting the growth of the siderophore-deficient strain *A. vinelandii* P100 in iron-restricted medium (6). By using a combination of molar binding ratio data, affinity data, and iron-molybdate competition data (this study) it was possible to ascertain, in an indirect manner, how protochelin and molybdate interact. Using affinity data, we calculated that under the hypothetical equilibrium conditions described by Harris et al. (15) the concentrations of free molybdate were  $10^{-26}$  M for the 1:1 molybdo-protochelin complex and  $10^{-16}$  M for the 2:3 molybdo-protochelin complex (Table 1). By comparison, the amount of free iron(III) under the same concentration and pH conditions, as determined by using ferric siderophore  $K_{\text{Form}}$

TABLE 3. Effects of metals that increase protochelin accumulation on ferric reductase activity

Iron(III) source	% of control ferric reductase activity in the presence of:				
	Molybdate (1,000 $\mu\text{M}$ )	Vanadate (100 $\mu\text{M}$ )	Tungstate (30 $\mu\text{M}$ )	Mn <sup>2+</sup> (70 $\mu\text{M}$ )	Zn <sup>2+</sup> (500 $\mu\text{M}$ )
Ferric protochelin	86 $\pm$ 6 <sup>a</sup>	42 <sup>b</sup>	64 $\pm$ 8	77 $\pm$ 7	32 $\pm$ 3
Ferric azotochelin	71 $\pm$ 4	66 $\pm$ 3	75 $\pm$ 6	87 $\pm$ 5	61 $\pm$ 1
Ferric aminochelin	60 $\pm$ 1	44 $\pm$ 5	83 $\pm$ 5	46 $\pm$ 4	94 $\pm$ 1
Ferric citrate	53 $\pm$ 5	36 $\pm$ 6	51 $\pm$ 3	36 $\pm$ 2	78 $\pm$ 5

<sup>a</sup> Mean  $\pm$  standard deviation based on values from at least two replicates.

<sup>b</sup> The standard deviation was not determined.

values, was  $10^{-27.7}$  M (Table 1) (7). This implies that in a competition assay, protochelin would have approximately equal affinities for molybdate and iron(III) in a 1:1 complex. If this were the case, there should be some inhibition of formation of the ferric protochelin complex in the presence of stoichiometrically balanced amounts of molybdate. This was not observed. Therefore, these data suggest that protochelin and molybdate do not form a 1:1 complex but interact to form a 2:3 complex, as predicted by the structure of protochelin.

Although catecholates and molybdate interact to quickly form a colored complex, it is thought that  $\text{Fe}^{3+}$  should displace molybdate bound to protochelin over the course of a 24-h growth period because of the much greater affinity of catecholates for iron ( $\text{pFe}^{3+}$  is 27.7 and  $\text{pMoO}_4^{2-}$  is 16 in a 2:3 complex). Also, competition assays revealed that molybdate cannot displace iron in a preformed ferric protochelin or ferric azotochelin complex. Thus, interference with ferric siderophore complex formation by molybdate may be minimized over time, suggesting that molybdate affects protochelin accumulation through another site of action.

Molybdate interferes with catecholate siderophore-mediated  $^{55}\text{Fe}$  uptake in *A. vinelandii*. Similarly, vanadate, tungstate,  $\text{Zn}^{2+}$ , and  $\text{Mn}^{2+}$  increase protochelin accumulation and decrease rates of  $^{55}\text{Fe}$  uptake (Table 2). While molybdate, vanadate, and tungstate are chemically related,  $\text{Zn}^{2+}$  and  $\text{Mn}^{2+}$  are not. A common feature of these metal ions is that they all inhibit ferric reductase activity. This has been observed previously in *A. vinelandii* with  $\text{Mn}^{2+}$  and  $\text{Zn}^{2+}$  (18, 28) but not with the other ions. In *A. vinelandii* two ferric reductase enzymes have been localized to either the cytoplasm or the periplasm. It is possible that ferric siderophore reduction, mediated by ferric reductase, takes place at the cell surface and is affected by high concentrations of metal ions (18). In studies performed with *A. vinelandii*, it was found that ferric reductase activity could not be completely inhibited by high concentrations of metal ions. The pattern of inhibition observed was characteristic of a mixed or partial type of inhibitor, so that  $\text{Zn}^{2+}$  and  $\text{Mn}^{2+}$  act as both competitive and noncompetitive inhibitors of ferric reductase activity (18, 28). As a result, the cells continue to grow in the presence of these metal ions. Inhibition of ferric reductase may have a direct effect on iron uptake but also may have an indirect effect, as activity of this enzyme affects iron speciation within the cell. It is the ratio of  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  in the cell that controls siderophore production through the Fur repressor (8). Subtle changes in  $\text{Fe}^{2+}$  corepressor availability could result in overproduction of protochelin, as observed previously for azotobactin hyperproduction in the presence of  $\text{Zn}^{2+}$  (17).

We believe that protochelin is the true siderophore of *A. vinelandii*. As a result of the high affinity of protochelin for iron, uptake of this siderophore probably involves ferric reduction, as well as cleavage, like enterobactin uptake in *E. coli* (3). The cleavage products (azotochelin and aminochelin) are recycled as siderophores and as reducing agents for iron mineral solubilization (30). Preliminary experiments in our laboratory have revealed that a CX of iron-limited *A. vinelandii* can cleave ferric protochelin into azotochelin and aminochelin (unpublished data). The much greater abundance of the cleavage products than of protochelin (7) suggests that protochelin is used and turned over rapidly, as expected for a very effective, high-affinity siderophore.

The conclusions described above do not preclude the possibility that azotochelin plays a role in high-affinity molybdate transport (10), but they do raise conflicting issues. The cells must be iron limited to form catecholate siderophores, yet high-affinity molybdate transport operates well in iron-suffi-

cient medium (26). Duhme et al. (10) suggested that protochelin replaces azotochelin in medium containing molybdate at a concentration of 70  $\mu\text{M}$  or more, because azotochelin is depleted during high-affinity molybdate transport. However, high-affinity molybdate transport in *A. vinelandii* is repressed in the presence of 10  $\mu\text{M}$  molybdate (26). In the presence of 1 mM molybdate, ferric protochelin uptake is reduced by 89% and ferric azotochelin uptake is reduced by 68%. Thus, it seems that little is gained by replacing azotochelin with a chelator that functions less well in the presence of high concentrations of molybdate.

Finally, our results suggest that metals like molybdate may be useful in studies of microorganisms that produce small, low-affinity siderophores which appear to be ineffective in iron chelation but at the same time have been shown to be virulence factors. These siderophores include chrysobactin produced by *Erwinia chrysanthemi* (12), anguibactin produced by *Vibrio anguillarum*, (19), myxochelin A isolated from *Angiococcus disciformis* (22), and serratiochelin produced by *Serratia marcescens* (11). It may be possible to determine if these molecules are the primary siderophores produced by these microorganisms or if there are larger, more effective siderophores that do not accumulate in culture fluid and are not detected. Metal ions like molybdate could be used to "trap" the parent compounds and determine more about their roles in iron acquisition and virulence.

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