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Production of the triacetecholate siderophore protochelin by *Azotobacter vinelandii*

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Azotobacter vinelandii **grown in iron-limited medium containing 1 μM molybdate released the catecholate siderophores azotochelin and aminochelin [bis(2,3-dihydroxybenzoyl-lysine) and 2,3-dihydroxybenzoyl-putrescine, respectively] into the culture fluid. However these catecholates were not observed when the medium contained 1 mM molybdate, but were replaced by another catecholate compound. The appearance of this new compound was not an artifact of extraction of the catecholates from the culture fluid in the presence of high molybdate. Full and partial acid hydrolysis and fast atom bombardment mass spectroscopy showed that the new compound was the tricatecholate protochelin, a product of the condensation of azotochelin and aminochelin. The production of protochelin was iron-repressible and protochelin very rapidly decolorized the Chrome Azurol-S assay. Protochelin promoted the growth of the siderophore-deficient *A. vinelandii* strain P100 under iron-restricted conditions and promoted ^{55}Fe uptake into iron-limited cells, confirming that protochelin can be used as a siderophore by *A. vinelandii*.**

Keywords: siderophore, protochelin, tricatecholate, molybdate, iron uptake

Introduction

Azotobacter vinelandii is a Gram negative, obligately aerobic soil bacterium that plays an important role in the environmental nitrogen cycle as it is one of the few bacteria that can fix nitrogen aerobically (Brill 1980). In order to fix nitrogen, *Azotobacter* must accumulate molybdenum and iron for use as co-factors in the nitrogenase enzyme (Robson & Postgate 1980, Yates & Jones 1974). However, iron in the soil is present as oxyhydroxides or oxides under aerobic, neutral pH conditions and is extremely insoluble. In order to scavenge, bind and promote the uptake of this scarce essential nutrient, *A. vinelandii* employs a variety of iron uptake systems (Neilands 1981, Page & Huyter 1984). When the iron is relatively aundant (>7 μM) or when it is bound to the cell surface, the cells use 2,3-dihydroxybenzoic acid (2,3-DHBA) as a low-affinity ligand to facilitate iron uptake (Page & Huyer 1984). High-affinity uptake systems are induced under more iron-deficient conditions, where true siderophores are excreted into the surrounding environment to solubilize and chelate iron (Page & Huyer 1984). *A. vinelandii* produces the catecholate siderophores, azotochelin (N,N^1-bis(2,3-dihydroxy-benzoyl)-L-lysine, Corbin *et al.* 1969) and aminochelin (2,3-dihydroxybenzoyl-putrescine, Page & von Tigertstrom 1988) (Figure 1) when iron concentrations are below about 7 μM. These simple dicatecholate and monocatecholate compounds are expected to have a relatively low affinity for iron (Hider 1984), but are produced in abundance and effectively extract iron from a variety of insoluble iron minerals (Page & Huyer 1984). At iron concentrations below 3 μM, the high-affinity, pyoverdin-type siderophore azotobactin is formed (Page & Huyer 1984, Demange *et al.* 1988, Page *et al.* 1991).

In this study we report the identification of a third catecholate siderophore. This siderophore called protochelin (Figure 1) is a tricatecholate compound that has never before been identified as a natural product of *A. vinelandii*.

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Materials and methods

Bacterial strains and growth conditions

The capsule-negative *A. vinelandii* strain UW (OP, ATCC 13705) and siderophore-defective mutants derived from this strain were used. The mutants included the azotobactin-deficient strain UA1 (Page & Huyer 1984), the catecholate-deficient strain F196 and the siderophore-deficient strain P100 (Sevinc & Page 1992).

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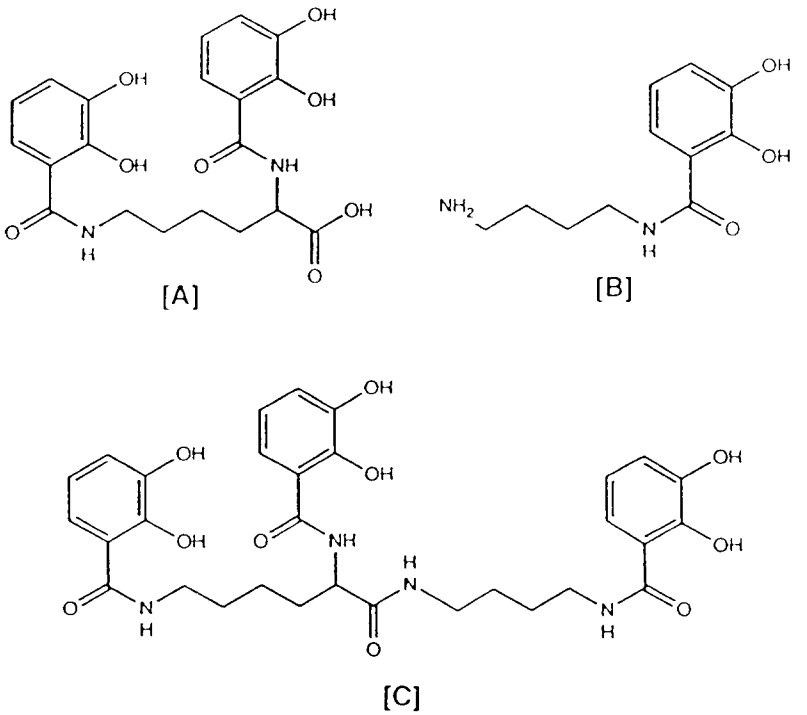


Figure 1. Catechol siderophores produced by *A. vinelandii*. (**A**) Azotochelin, (**B**) aminochelin and (**C**) protochelin.

The basic medium used in this study was Burk's medium, pH 7.2, containing 1% glucose, 12.5 μM $FeSO_4 \cdot 7H_2O$, 15 mM CH_3COONH_4 and 1 μM $Na_2MoO_4 \cdot 2H_2O$ (Sevinc & Page 1992). This medium was modified such that it lacked the calcium sulfate normally present (modified Burk's medium) or contained 1 μM ferric citrate as the sole ion source (iron-limited medium) or contained excess 1 mM molybdate. All glassware used in these studies was acid-washed with 4 M HCl and rinsed with 50 mM EDTA (pH 7.0) and deionized water to remove contaminating iron (Page 1993).

The strains were maintained on slants of modified Burk's medium containing 1.8% agar and incubated for 72 h at 30°C. Cells were washed from slants with 2.5 ml of 6 mM potassium phosphate buffer, pH 7.2, and were used to inoculate 20 ml medium in a 50 ml Erlenmeyer flask (2.5% v/v). Cultures were incubated for 20–24 h at 28°C with shaking at 225 r.p.m. in a New Brunswick model G-76 gyratory water bath shaker. If larger quantities of cells and culture fluid were required, cells were grown in iron-limited medium in a 5.0 l Bioflo II fermentor (New Brunswick Scientific, Edison, NJ) at 30°C with agitation (air flow 5 l min^{-1} and mixing at 500 r.p.m.). Ferric citrate (1 μM) was added to promote growth, but was still at a limiting concentration for *A. vinelandii* (Page von Tigerstrom 1988).

Spectrophotometric and colorimetric analyses

Siderophores were detected spectrophotometrically by scanning iron-limited culture supernatant fluid which had been acidified to pH 1.8 with 6 N HCl. Absorption peaks were measured using a Hitachi U-2000 recording spectrophotometer at 310 nm for catechols and 380 nm for azotobactin (Page & Huyer 1984). Catechol also was quantitated by the colorimetric assay of Barnum (1977). Total cellular protein was determined by the method of Lowry *et al.* (1951) after digestion of the cell pellet in 0.1 N NaOH for 60 min at 80°C.

Extraction of catechol siderophores

Azotochelin and 2,3-DHBA present in an acidified (pH 1.8) iron-limited *A. vinelandii* culture supernatant fluid were extracted into ethylacetate leaving the aminochelin in the acidic aqueous phase (Page & von Tigerstrom 1988). The ethylacetate fraction was pooled, evaporated to dryness, then dissolved in 200 μl of ethylacetate. Aminochelin was extracted from the acidic aqueous phase using butanol (Page & von Tigerstrom 1988). The catecholate components of these extracts were resolved by thin layer chromatography (TLC) using silica gel G plates (Brinkman) and a benzene:acetic acid:water (125:72:3) solvent system (Sevinc & Page 1992). Standards included 2,3-DHBA (Sigma Chemical Co, St Louis, MO), azotochelin and aminochelin prepared from strain UA1 (Page & von Tigerstrom 1988), and protochelin from the methylotrophic organism DSM 5746 (Taraz *et al.* 1990). Catecholates were visualized by spraying the TLC plate with a dipyridyl-ferric chloride solution (Krebs *et al.* 1969).

Purification of protochelin

The culture supernatant fluid from strain UA1 grown overnight in iron-limited medium containing 1 mM molybdate

was acidified and extracted with ethylacetate. The solvent fraction was evaporated to dryness, dissolved in ethylacetate to give an $A_{310}=67.5$ and 5 ml of this was applied to a silicic acid column. The column was formed in a 2.1×25 cm all glass Kontes column loaded with 9.0 g of silicic acid suspended in ethylacetate:benzene (1:4) saturated with 0.5 mM formic acid and was equilibrated overnight. Fractions (3 ml) were collected and the absorbance at 310 nm was used to determine the location of catechol-containing compounds.

Partial and total hydrolysis of protochelin

Partial and total acid hydrolysis of protochelin (final $A_{310}=10$) was performed as previously described (Page & von Tigerstrom 1988). A time course showed that partial acid hydrolysis of protochelin took about 8 h in 1 mM HCl at 100°C in a boiling water bath. Total acid hydrolysis of protochelin required 25 h in 6 mM HCl at 100°C. Full and partial hydrolysis products were resolved and visualized by TLC.

Amino acid standards including lysine and putrescine (0.5 μmol ml^{-1}) and products from the total hydrolysis of YP1 were resolved by TLC on silica gel G plates (Brinkman) using a propan-1-ol:ammonium hydroxide (63:33) solvent system. The plates were sprayed with 0.2% ninhydrin in acetone to visualize the resolved amino-N compounds (Page & von Tigerstrom 1988).

Fast atom bombardment (FAB) spectroscopy

The mass of purified *A. vinelandii* protochelin and the protochelin standard was determined by FAB mass spectroscopy (Department of Chemistry, University of Alberta) using methanol as a solvent on a dithiothreitol:dithioerythritol (6:1) matrix.

Chrome Azurol-S (CAS) siderophore assay

The CAS assay was used as a universal assay to detect siderophore activity by monitoring the deferration of the synthetic, chromogenic iron chelator CAS (Schwyn & Neilands 1987). The CAS assay did not include sulfosalicyclic acid (Schwyn & Neilands 1987). When protochelin was assayed in the CAS assay, it was dissolved in a small volume of 95% ethanol then diluted in distilled water to a final concentration of 0.12 μmol catechol ml^{-1}. One unit of siderophore activity was calculated as the amount of sample required to decrease the CAS A_{630} by 0.001 s^{-1}. Total siderophore specific activity was calculated as siderophore units (mg cell protein)$^{-1}$ in 1.0 ml of culture fluid.

Siderophore bioassay using strain P100

Plates of Burk's medium containing 50 μM ferric citrate and 50 μg ml^{-1} of the artificial iron chelator ethylenediamine di-(*o*-hydroxyphenylacetic acid) (EDDHA) were spread with a lawn of strain P100. Sterile, iron-limited supernatant fluid from strains UA1 (catecholates $A_{310}=0.500$) and F196 (azotobactin $A_{380}=0.750$) and purified protochelin ($A_{310}=0.500$) were absorbed onto 0.5 cm sterile paper disks and dried. These disks were placed aseptically onto the lawn of strain P100 and moistened with 15 μl of sterile 6 mM potassium phosphate buffer, pH 7.2, which also was added to a control disk. The plates were incubated for 5 days at 30°C and zones of strain P100 growth around each disk were measured.

^{55}Fe-uptake assay

Iron-limited culture supernatant fluid from strains UA1 (azotochelin, aminochelin and DHBA present) and F196 (azotobactin present), purified *A. vinelandii* protochelin, and the protochelin standard (adjusted to an A_{310} or A_{380} of 0.300, as appropriate) were ferrated with $^{55}FeCl_3$ (10 μCi ml^{-1} in 0.1 N HCl stock obtained from Amersham, Oakville Ontario). These ^{55}Fe-siderophore solutions were used in ^{55}Fe-uptake assays as described by Knosp *et al.* (1984). When ^{55}Fe-uptake rates were compared, the iron-limited strain UW cells used in the assays were all from the same culture and hence were identical.

Results and discussion

Production of protochelin by A. vinelandii

In the course of studying the effect of molybdenum on the iron-limited growth of *A. vinelandii* UA1, it was observed that 1 mM molybdate caused a brilliant orange color to form in the culture fluid. Although this was spectacular, it was not surprising as it is well documented that molybdenum will complex with catechol-containing compounds to give a yellow–orange color (Hider 1984). When the catechols from this iron-limited culture fluid were extracted into ethylacetate and examined by TLC, it was observed that the pattern of catecholate spots normally produced by *A. vinelandii* UA1 had changed. The production of 2,3-DHBA ($R_f=0.85$) was unaffected, but the azotochelin spot ($R_f=0.45$) was not present and an unknown catecholate spot migrating at $R_f=0.27$ was formed (Figure 2). Spectrophotometric analysis of the acidified aqueous supernatant left after ethylacetate extraction showed that aminochelin also was absent.

This growth condition was repeated using the parent strain UW with identical results. However, a spot coinciding with the unknown catecholate spot was faintly visible after ethylacetate extraction of the culture fluid of cells grown in iron-limited medium containing 1 μM molybdate (Figure 2). This suggested that the unknown catecholate was a natural product of a wild-type *A. vinelandii*. It appeared to be formed in very small amounts in normal iron-limited Burk's medium, but its production was increased in medum containing higher concentrations of molybdate. While the wild-type strain UW formed this new compound in the presence of 1 μM molybdate, strain UA1 required about 70 μM molybdate for production of this new compound (TLC data not shown).

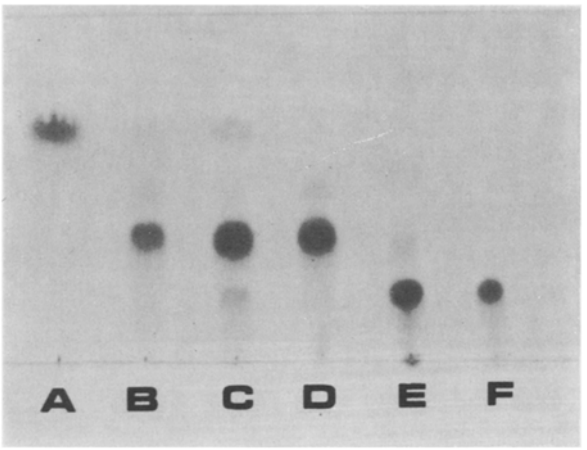


Figure 2. Thin layer chromatography of catecholate compounds. Lane (A) 2,3-DHBA standard, (B) purified azotochelin standard, (C) ethylacetate extract of culture fluid of strain UW grown in iron-limited culture containing 1 μM molybdate, (D) ethylacetate extract of culture fluid of strain UA1 grown in iron-limited culture containing 1 μM molybdate, (E) ethylacetate extract of culture fluid of strain UA1 grown in iron-limited culture containing 1 mM molybdate and (F) authentic protochelin standard.

The unknown catecholate compound was purified from strain UA1 iron-limited, high molybdate culture fluid by chromatography on a silicic acid column (see Materials and methods). Under these growth conditions, the majority of the ethylacetate extractable catecholate was eluted as a single band from the silicic acid column. This catecholate compound was subjected to partial acid hydrolysis with 1 M HCl over 8 h, which resulted in a gradual decrease in the intensity of the $R_f = 0.27$ catecholate spot and an increase in intensity spot and an increase of azotochelin and 2,3-DHBA spots. Scans of the acidified aqueous digestion mixture that remained after ethylacetate extraction showed an increase in A_{310} during partial digestion. Extraction of this aqueous layer with butanol and subsequent TLC showed that aminochelin was being formed. Total hydrolysis of the $R_f = 0.27$ catecholate in 6 M HCl and TLC showed the presence of lysine, putrescine and 2,3-DHBA. Thus the new catecholate compound was most likely formed through the condensation of azotochelin and aminochelin.

The predicted molecular weight for the new catecholate compound was 624 g mol^{-1}, this being the sum of azotochelin (418 g mol^{-1}) and aminochelin (224 g mol^{-1}) molecular weights minus 16 g mol^{-1} for the water lost during the condensation reaction. Indeed, the mass obtained for the purified compound was 625 g mol^{-1} (624 + 1 hydrogen) (Figure 3). Also indicated in the FAB mass spectra were peaks at 418, 224 and 154 g mol^{-1}, representing the major breakdown products azotochelin, aminochelin and 2,3-DHBA, respectively.

A tricatecholate compound with this structure (Figure 1C, protochelin) has already been isolated from an unrelated methylotrophic bacterium DSM 7546 (Taraz *et al.* 1990). An authentic sample of protochelin was subjected to partial and full acid digestion, as well as FAB mass spectroscopy, and was shown to have characteristics identical to the new catecholate from *A. vinelandii*. Thus protochelin (Figure 1C) is also a natural product formed by iron-limited *A. vinelandii*.

Effect of molybdate on protochelin stability and production

High levels of molybdate were definitely required to obtain high yields of protochelin from iron-limited *A. vinelandii* culture, but were not required for protochelin production by strain DSM 5746 (Taraz *et al.* 1990). It was possible that molybdate was required for the cellular synthesis of protochelin, promoted chemical synthesis of protochelin in the culture fluid or stabilized protochelin during the ethylacetate extraction procedure.

However, purified protochelin dissolved in growth medium or water was extracted intact into ethylacetate at pH 1–2 without molybdate present. Protochelin was stable in ethylacetate, ethanol or distilled water without molybdate present and survived repeated freeze–thaw cycles. Thus molybdate was not required for protochelin stability or for its extraction into ethylacetate. When 1 mM molybdate was added to iron-limited culture supernatant from strain UA1 the fluid turned bright yellow–orange. When this colored fluid was extracted with ethylacetate and examined by TLC, only 2,3-DHBA and azotochelin were present. Therefore protochelin did not form chemically because 1 mM molybdenum was present during ethylacetate extraction of iron-limited culture fluid. Rather it appeared that molybdate was required during *A. vinelandii* growth for protochelin to be produced.

Iron repressibility of protochelin production

Protochelin production in *A. vinelandii* was iron-repressible. As the ferric citrate content of the iron-limited medium (containing 1 mM molecular weight molybdate) was increased from 1 to 10 μM, the intensity of the protochelin spot seen on a TLC plate was decreased. Catechol values dropped from 0.22 to 0.03 mM as did the catechol (cell protein)$^{-1}$ values (Figure 4). Parallel results were seen in medium containing 1 μM molybdate (Figure 4). TLC showed that protochelin in the high molybdate medium and azotochelin and aminochelin in the low molybdate medium were all repressed at about 9 μM ferric citrate, with the remaining catechol content accounted for by constitutive production of DHBA. In addition to affecting the form of catecholate released from the cell, 1 mM molybdate also promoted about 2-fold greater catecholate production than 1 μM molybdate, at iron concentrations of 4–8 μM (Figure 4).

Protochelin reaction in the CAS assay

Protochelin reacted very rapidly in the CAS universal assay for siderophores (Schwyn & Neilands 1987). Iron-limited culture supernatant fluid from strains F196 (azotobactin present) and UA1 (azotochelin, aminochelin and DHBA present) decolorized the CAS assay solution

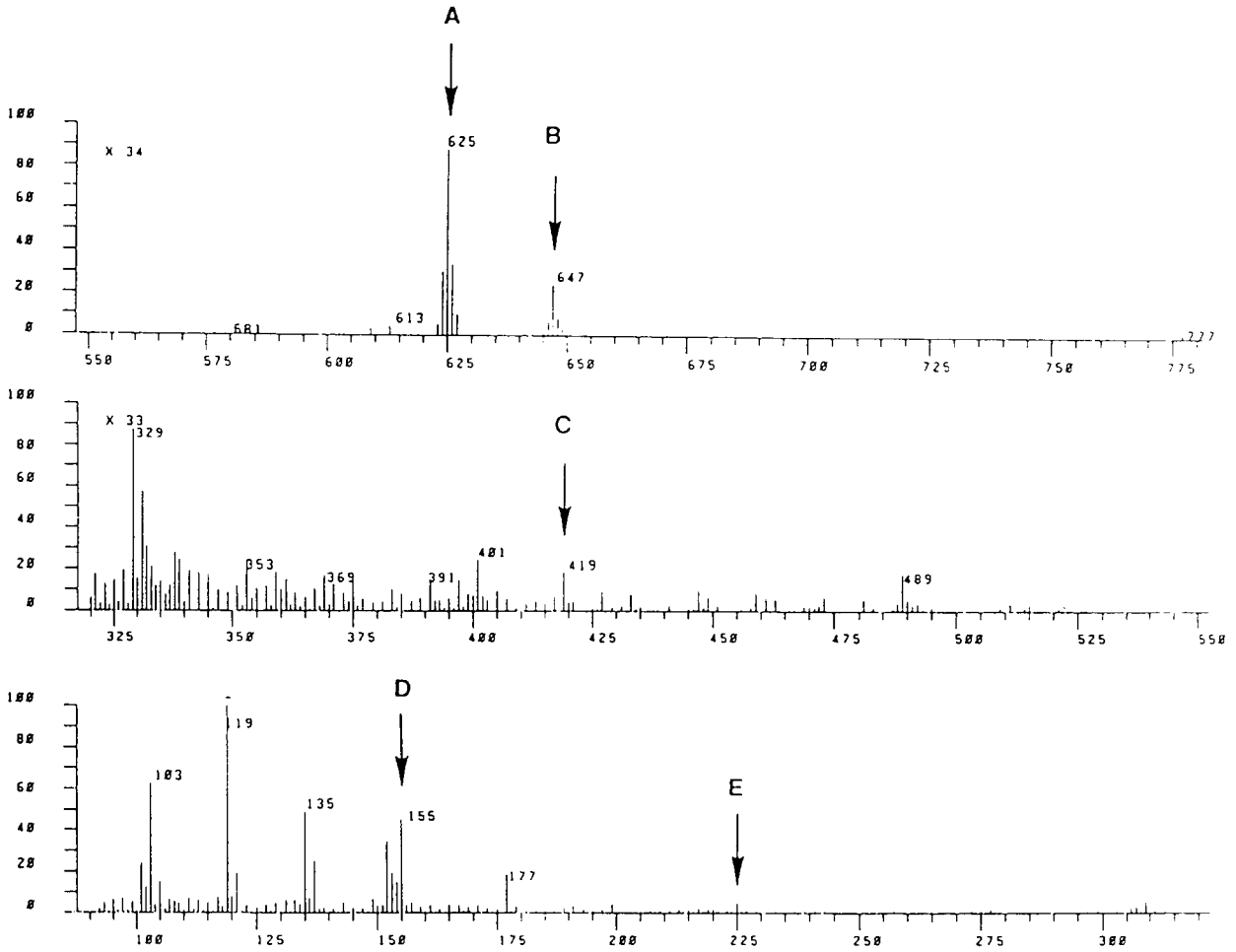
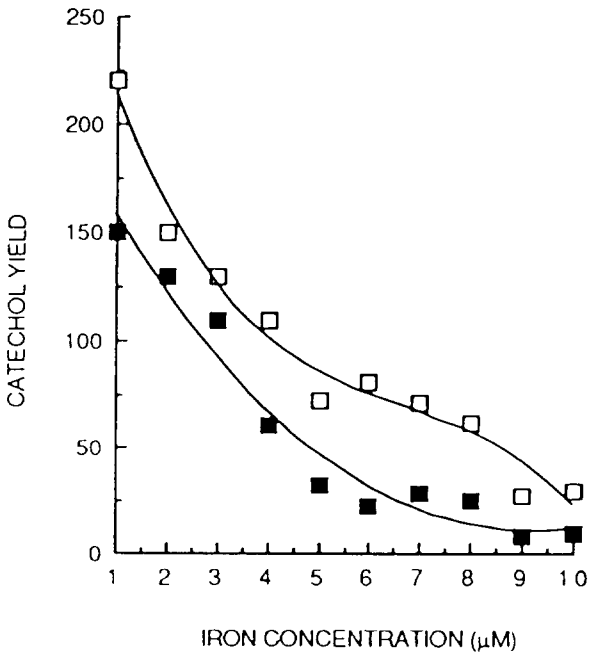


Figure 3. FAB mass spectrometry of purified *A. vinelandii* protochelin. The peaks indicated are (A) protonated protochelin, (B) sodium protochelin, (C) protonated azotochelin, (D) protonated 2,3-DHBA and (E) protonated aminochelin.



within 2 min, but purified protochelin decolorized the solution in less than 5 s (Table 1). Dilution of the protochelin solution prior to CAS assay did not slow the progress of decolorization, but only limited the amount of CAS decolorized.

If protochelin was incubated with 1 mM molybdate before its addition to the CAS solution, decolorization did not occur. Thus the binding of molybdate to the hydroxyl groups of protochelin (Hider 1984) effectively prevented its iron binding activity. This observation may also explain the increased yield of catecholate (cell protein)$^{-1}$ observed in the presence of 1 mM molybdate (Figure 4). The presence of 1 mM molybdate in the medium must hinder the binding of iron to protochelin, such that the cell fails to correctly detect the level of exogenous iron in the medium, which results in

Figure 4. Iron-repressible production of catecholate compounds. Strain UA1 was incubated for 24 h in medium containing varied iron concentrations and 1 μM molybdate (■) or 1 mM molybdate (□) before quantitation of total catechol yield (nmol catechol mg^{-1} cell protein).

Table 1. Activity of siderophore preparations in the CAS assay[a]

Siderophore preparation[b]	Siderophore units[c]	Total siderophore activity[d]	CAS reaction end point (s)
Catecholates	82	470	60 120
Azotobactin	37	220	60 120
Protochelin	64	120	<5

[a] Assays were conducted at 25 C.
[b] Used as unacidified iron-limited culture supernatant fluid from strain UA1 (catecholates, $A_{310}=0.310$), strain F196 (azotobactin, $A_{380}=0.246$) grown in shake flasks or as protochelin ($A_{310}=0.350$) purified from strain UA1 grown with 1 mM molybdate in a fermentor.
[c] One unit is a change in A_{630} of 0.001 s^{-1}.
[d] Total siderophore activity, as units (mg cell protein)$^{-1}$ present in 1.0 ml of culture.

continued production of the chelator. At higher iron concentrations ($\geq 9\ \mu M$, Figure 4) the ligand's affinity for iron may promote ligand saturation in the presence of molybdate, iron transport and repression of ligand production. Alternatively, iron may enter the cell through the constitutive low-affinity uptake route, leading to repression of ligand formation.

Protochelin promotes iron uptake in A. vinelandii

The function of protochelin as an iron carrier was tested in a bioassay using the siderophore-deficient strain P100. When this strain is placed in a medium where the available iron is complexed in a non-useable form, in this case as FeEDDHA, growth will be dependent on added siderophores (Sevinc & Page 1992). The promotion of strain P100 growth in the plate bioassay was only observed around disks containing protochelin or strain F196 culture fluid containing azotobactin (halo diameters 10 and 12 mm, respectively). Growth promotion was not observed around the strain UA1 culture fluid disk or the control disk impregnated with phosphate buffer. These results were somewhat unexpected since the siderophores formed by strain UA1 growing on an EDDHA plate appeared previously to promote the growth of strain P100 (Sevinc & Page 1992). The results may be reconciled if the growth promotion previously observed was caused by small amounts of protochelin formed during the 8 day incubation of strain UA1 on EDDHA plates, while growth promotion was not observed in the current study using the culture fluid from strain UA1 pregrown 24 h in iron-limited medium, where no detectable protochelin was formed.

When strain P100 was incubated in iron-limited liquid medium containing 50 μg EDDHA ml^{-1} and 1 μM molybdate, growth was promoted by the addition of protochelin (Table 2). However, when 1 mM molybdate was added to the medium, protochelin did not promote strain P100 growth (Table 2), presumably because the binding of molybdate to the siderophore prevented its extraction of Fe^{3+} from the EDDHA complex, as observed in the CAS assay. After the 24 h incubation, the culture fluids were acidified and extracted with ethylacetate. In all cases, the catecholates present were extracted completely by ethylacetate and only protochelin was present on the TLC chromatogram.

Table 2. The effect of protochelin on the growth of *A. vinelandii* strain P100 in iron-restricted liquid medium

Molybdate (μM)	Inoculated	Protochelin added[a]	Catechol (nmol ml^{-1})	Cell protein (mg ml^{-1})
1.0	+	–	37	0.42
1.0	+	–	3	0.20
1.0	–[b]	+	42	0.00
1000	+	+	46	0.25
1000	+	-	2	0.29
1000	–[b]	+	45	0.00

[a] Added to a final A_{310} of about 0.40.
[b] Uninoculated control.

Uptake of the ^{55}Fe protochelin complex by wild-type strain UW was tested using experimental techniques described in Knosp *et al.* (1984). The wild-type strain was used in uptake assays to establish that protochelin use was a normal process in *A. vinelandii*. Ferriprotochelin was taken up by iron-limited cells at a rate of 1.26 ng ^{55}Fe min^{-1}, compared with 0.725 ng ^{55}Fe min^{-1} for the ferriazotobactin complex (from strain F196), 0.496 ng ^{55}Fe min^{-1} for a mixture of ferricatecholates (azotochelin, aminochelin and 2,3-DHBA from strain UA1) and 0.091 ng ^{55}Fe min^{-1} seen with ^{55}Fe citrate in uptake buffer alone. In all cases the uptake of ^{55}Fe has been corrected for non-specific binding of ^{55}Fe to the cell by subtraction of control values obtained for each mixture incubated on ice. Furthermore, these results indicate that the production of the receptor for ferriprotochelin binding and ^{55}Fe uptake into the cell does not require the presence of high molybdate, as the UW cells used in these assays were grown in medium containing only 1 μM molybdate. Thus the ferriprotochelin receptor is likely one of the *A. vinelandii* iron-repressible outer membrane proteins that have already been described (Page & von Tigerstrom 1982).

Conclusion

From the data presented here there should be no doubt that protochelin functions as a siderophore in *A. vinelandii*. However, it remains a mystery why such an excellent siderophore is barely present in normal, iron-limited low-molybdate medium. Indeed very little protochelin should be produced in the soil environment where the molybdate concentration is usually below 1 μM, which often limits nitrogen fixation or nitrate reduction (Brady 1984). However, there may be other conditions which stimulate protochelin formation in the soil and these are not duplicated in the Burk's medium commonly used for *Azotobacter* spp. culture in the laboratory.

The results of this study demonstrate that a factor other than iron can have a profound effect on the type of siderophore produced by *A. vinelandii*. Although the hyperproduction of protochelin under high molybdate conditions appears to be a product of an altered or aberrant physiology, it will be very interesting to determine how molybdate promotes these events. It also will be interesting to determine if excess molybdate has an effect on the formation of siderophores by other organisms. There are a number of organisms that appear to form only simple catecholate siderophores under laboratory conditions (Persmark *et al.* 1989), but these compounds appear to act as virulence factors or high-affinity iron chelators when the organism is grown within a host. It may be that the true siderophores of these organisms remain to be identified.

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