Aminochelin, a Catecholamine Siderophore Produced by *Azotobacter vinelandii*

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A catecholamine siderophore, named aminochelin, produced by iron-limited *Azotobacter vinelandii* was purified and tentatively identified as 2,3-dihydroxybenzoylputrescine. This compound was first observed as an ethyl-acetate-insoluble catechol that accounted for 30 to 50% of the total catechol in iron-limited culture supernatant fluids. The purified compound was unstable at neutral to alkaline pH, bound Fe^{3+} , Fe^{2+} and molybdate, and promoted ^{55}Fe -uptake into iron-limited *A. vinelandii*. Aminochelin was induced and repressed coordinately with the other catechol siderophore azotochelin. The catechol siderophores were, however, less sensitive to repression by soluble iron than the yellow-green fluorescent peptide siderophore azotobactin.

INTRODUCTION

Optimal growth of *Azotobacter vinelandii* requires an adequate supply of iron. This iron is essential not only to support the obligate aerobic life of this bacterium, but also for nitrogen fixation and protection of the oxygen-labile nitrogenase enzyme (Post *et al.*, 1983). Although iron is plentiful in soil, the iron minerals that form in aerobic soil layers at neutral pH are very insoluble. *A. vinelandii*, like other bacteria (Neilands, 1981), can accumulate the very low levels of soluble iron by using siderophore-mediated transport systems (Page & Huyer, 1984; Knosp *et al.*, 1984). In addition to the deployment of these high affinity chelators, *A. vinelandii* also produces 2,3-dihydroxybenzoic acid (2,3-DHBA), which solubilizes iron minerals and may facilitate low-affinity iron uptake in the absence of true siderophores (Page & Huyer, 1984). This latter strategy appears to be common to the Azotobacteraceae as *Azomonas macrocytogenes* and *Azotobacter paspali* similarly use 3,4-dihydroxybenzoic acid (Westerveld *et al.*, 1985; Collinson *et al.*, 1987) and *Azotobacter chroococcum* employs citric acid to solubilize iron (Page, 1987).

When 2,3-DHBA is unable to provide sufficient iron for growth of *A. vinelandii*, the true siderophores are induced. This follows an unusual sequential derepression pattern, where the production of the catechol azotochelin [*N,N'*-bis(2,3-dihydroxybenzoyl)-L-lysine] precedes the production of the fluorescent yellow-green peptide azotobactin (Page & Huyer, 1984; Page & Grant, 1987). Certainly the nature of this sequential control is of interest as it will likely invoke additional levels of complexity on the model for iron-regulation of siderophore synthesis being developed in *Escherichia coli* (De Lorenzo *et al.*, 1987; Hantke, 1981, 1984). Before regulation can be addressed, all of the major components of the *A. vinelandii* siderophore system should be known. In this study, we report that a second catechol siderophore, not previously described and different from azotochelin, is produced by iron-limited *A. vinelandii*.

METHODS

Bacterial strains and growth conditions. The capsule-negative strain UW of *A. vinelandii* OP and a spontaneous variant which failed to produce azotobactin (strain UA1) were used. These strains were maintained on Burk medium, pH 7.2, containing 1% (w/v) glucose, 1.1 g ammonium acetate l^{-1} and 1.8% (w/v) agar, incubated at

Abbreviations: EA, ethyl acetate; 2,3-DHBA, 2,3-dihydroxybenzoic acid.

30 °C as described previously (Page & Huyer, 1984). Iron-limited Burk medium contained no added iron. The inoculum for each study was prepared from iron-starved cells (Page & Huyer, 1984). Soluble iron was supplied as ferric citrate. Insoluble iron was provided as finely ground iron minerals or chemicals (50 mg) added to 100 ml iron-limited medium in a 500 ml Erlenmeyer flask. Liquid medium was incubated at 28 to 30 °C with shaking at 225 r.p.m.

Siderophore analysis. After removal of cells by centrifugation, the culture supernatant fluid was acidified to pH 1.8 with HCl and scanned with a Perkin-Elmer Lambda 3 spectrophotometer. Absorbance at 310 nm was used to estimate total catechol and absorbance at 380 nm was used to estimate azotobactin (Page & Huyer, 1984). Azotochelin plus 2,3-DHBA were extracted from acidified culture supernatant fluid as previously described (Corbin & Bulen, 1969). Catechols were detected by the Arnov (1937) assay and hydroxamates were tested for by the Csaky (1948) assay.

Purification of aminochelin. Strain UW was grown in iron-limited Burk medium containing 50 mg micaceous haematite per 100 ml to enhance siderophore production (Page & Huyer, 1984). After 20 h incubation, the cells were removed by centrifugation and the culture supernatant fluid was sterilized by filtration. After ethyl acetate (EA) extraction, the aqueous phase was neutralized and concentrated twofold by rotary evaporation at 28 °C, with protection from the light.

The aqueous phase containing EA-insoluble catechol (aminochelin, $A_{310} = 62$ in 88 ml) was applied to a 2.6 × 40 cm column containing DEAE Sephadex A25 (Pharmacia). The column had been previously washed with 500 ml 3 mM-Tris/HCl, pH 7.5, followed by 250 ml distilled water. The sample was eluted with 100 ml distilled water followed by an 800 ml gradient formed with distilled water and 2 M-NaCl. Elution of aminochelin was followed by measuring A_{310} .

Pooled fractions containing aminochelin were diluted with distilled water to give an $A_{310} = 68.5$ in 50 ml which was loaded onto a 2.6 × 40 cm column of CM Sephadex (Pharmacia). The column had been equilibrated in 0.005 M-ammonium acetate, pH 6.8. The sample was washed with 50 ml of the same solution and then eluted with a 500 ml gradient formed from 0.005 M and 1.0 M-ammonium acetate.

Aminochelin fractions from this column were pooled and extracted with an equal volume of butanol. Aminochelin partitioned equally into butanol and water each time the extraction was repeated, so close to 100% recovery was possible after repeated extractions. The butanol fraction was reduced to dryness under an airstream and stored at -20 °C. The dry material was a clear whitish residue, with only a trace of salts, and was completely soluble in water.

Thin layer chromatography (TLC). Chromatography on silica gel G plates used the following solvent systems for catechol separations: (A) benzene/acetic acid/water (125:72:3, by vol.) and (B) butanol/acetic acid/water (150:30:30, by vol.). Amino compounds were separated using (C) 95% (v/v) ethanol/ammonium hydroxide/water (180:10:10, by vol.) and (D) propan-1-ol/ammonium hydroxide (63:33, w/w).

Spray reagents included ninhydrin in acetone for amines, potassium ferricyanide-ferric chloride and 2,2'-dipyridyl-ferric chloride for phenols (Krebs *et al.*, 1969) and the Arnov (1937) reagents for catechols.

Stability and hydrolysis of aminochelin. The stability of aminochelin was examined by spectrophotometric scanning and TLC after storage, for 4 and 20 h, at 20 °C and 4 °C, in the dark and in the light, in the following: 5 mM-HCl, pH 2.5; distilled water, pH 5.2; 5 mM-ammonium acetate, pH 5.5; 25 mM-potassium phosphate, pH 7.0; 25 mM-Tris/HCl, pH 7.5; and 5 mM-NaOH, pH 10.5.

Acid hydrolysis of aminochelin required at least 6 M-HCl at 90–100 °C for 25 h. Free catechol was removed from the hydrolysate by ethyl ether extraction. This extract was concentrated by evaporation and dissolved in 95% (v/v) ethanol before TLC analysis (Ong *et al.*, 1979). The presence of 2,3-DHBA was qualitatively determined by comparison with authentic standards.

The masses of aminochelin and its hydrolysis products were determined by positive ion fast atom bombardment spectroscopy, using samples dissolved in glycerol.

⁵⁵Fe-uptake measurement. The nitrogen-fixing strain UW, pregrown for 16 h in iron-limited Burk medium (lacking ammonium acetate), was used for ⁵⁵Fe-uptake measurement as described previously (Knosp *et al.*, 1984). Purified azotochelin and azotobactin were also prepared as described previously (Knosp *et al.*, 1984).

RESULTS

Observation of a second catechol siderophore

Spectrophotometric scans of acidified iron-limited *A. vinelandii* culture supernatant fluid revealed a peak at 380 nm which corresponded to azotobactin and a peak at 310 nm which corresponded to catechols (Fig. 1). EA extraction of this acidified culture fluid removed the catechols azotochelin and 2,3-DHBA, but approximately 37% of the original A_{310} material remained in the aqueous phase (Fig. 1). No further removal of the A_{310} material occurred after a

Fig. 1. Spectrophotometric scan of acidified iron-limited culture supernatant fluid before (---) and after (—) EA extraction.

Fig. 2. Yields of azotobactin (●), total catechols before EA extraction (○) and EA-insoluble catechol (□), after 22 h growth in medium containing various concentrations of ferric citrate. Siderophore yields were calculated as A_{380} (azotobactin) or A_{310} (catechols) ($\text{mg cell protein}^{-1}$). Cell yield is shown as $\mu\text{g protein ml}^{-1}$ (■).

third extraction with EA. When an aqueous solution of azotochelin plus 2,3-DHBA was similarly extracted with EA, less than 5% of the A_{310} material remained in the aqueous phase. Therefore, the remaining A_{310} material was not simply residual azotochelin plus 2,3-DHBA that was in equilibrium with the EA-saturated water.

The EA-insoluble material was strongly Arnow positive, which indicated the presence of catechol, but was negative in the Csaky test for hydroxamate. Addition of Fe^{3+} to this material resulted in a purple colour, as would be expected from iron chelation by a catechol.

In iron-limited culture, azotochelin was first detected after 8 h incubation (Page & Grant, 1987) which coincided with the first appearance of the EA-insoluble catechol (data not shown). The repression of the EA-insoluble catechol by iron also paralleled azotochelin repression and both were less tightly controlled by iron availability than azotobactin (Fig. 2). In iron-limited medium the EA-insoluble catechol accounted for approximately 50% of the total A_{310} material and this decreased to a constant 38 to 44% of the total in cultures containing 1.0 to 5.0 $\mu\text{M-Fe}^{3+}$.

Table 1. *Presence of EA-insoluble catechol in cultures grown with mineral iron sources*

Minerals were added at 50 mg per 100 ml of iron-limited medium and incubated at 28 °C with shaking at 225 r.p.m. for 22 h. The absorbance values of acidified culture fluids and the percentage of total A_{310} remaining in the aqueous phase after EA extraction are shown. Values shown are the means of duplicate experiments. Absorbance values were reproducible to ± 10 to 15% of the mean. Values for percentage of A_{310} insoluble in EA were reproducible to $\pm 2\%$ of the mean.

Mineral	Ideal formula	Strain UW			Strain UA1	
		A_{380}	A_{310}	Percentage A_{310} insoluble in EA	A_{310}	Percentage A_{310} insoluble in EA
None	—	0.340	0.370	53	0.410	41
Pyrrhotite	Fe_{1-x}S	0.966	1.860	44	2.175	35
Siderite	FeCO_3	0.810	1.300	36	2.160	35
Pyrite	FeS_2	0.240	1.230	44	0.980	37
Haematite	Fe_2O_3	0.190	0.620	39	2.175	31
Olivine	$(\text{Mg,Fe})_2\text{SiO}_4$	0.150	1.275	42	1.66	36
Glaucanite	$\text{K}_2(\text{Mg,Fe})_2\text{Al}_6(\text{Si}_4\text{O}_{10})_3(\text{OH})_{12}$	0	0.810	43	0.420	36
Fe_3O_4	Fe_3O_4	0	0.765	39	2.185	34

When *A. vinelandii* strain UW was grown in the presence of natural iron-containing minerals, the limited amounts of available iron caused hyperproduction or repression of part or all of the siderophore system (Page & Huyer, 1984; Table 1). The minerals used fell into three major classes: those which either enhanced the production of both azotobactin and catechols, enhanced the production of catechols only, or repressed azotobactin but allowed catechol production (Table 1). In each of these three cases the EA-insoluble catechol accounted for a constant 36 to 44% of the total catechol content. Similarly, strain UA1 hyperproduced catechols in response to the presence of the minerals and the EA-insoluble catechol remained at a constant 30 to 37% of the total catechol content (Table 1).

Incubation of strains UW or UA1 in iron-limited medium for up to 36 h, with or without added minerals, did not alter the percent of EA-insoluble catechol in the culture. Therefore, there was no simple conversion of azotochelin to the EA-insoluble catechol or vice versa in response to iron-starvation or after the use of the siderophores during iron transport. The EA-insoluble catechol also has been observed in iron-limited cultures of *A. vinelandii* ATCC 12837, 13705 and 12518. The production of only azotochelin or only the EA-insoluble catechol has not yet been observed with any strain or cultural condition.

Purification of the EA-insoluble catechol

Batch treatment of iron-limited culture supernatant fluid with DEAE Sephadex A25 indicated that 2,3-DHBA and azotochelin were firmly bound, but the EA-insoluble catechol was easily removed by washing with aqueous NaCl. This observation indicated that the EA-insoluble catechol was not simply an artefact created by the acidification of the culture fluid before EA extraction.

Large scale fractionation of the iron-limited culture supernatant fluid, however, usually involved the EA extraction step, to avoid permanent contamination of the column material. The EA-insoluble catechol was eluted from the DEAE Sephadex A25 column as a sharp peak with the initial distilled water wash, just before the start of the NaCl gradient (data not shown). The azotobactin also present in the aqueous phase was eluted with the NaCl; further characterization of these fractions (W. J. Page, S. K. Collinson & M. A. Abdallah) will appear elsewhere. The yield of A_{310} material after this step was 60% of the starting material.

The fractions containing the EA-insoluble catechol were pooled, applied to a CM Sephadex column and eluted with an ammonium acetate gradient. Minor coloured impurities in the sample were removed by this step and the A_{310} material eluted as a sharp peak at approximately 0.8 M-ammonium acetate (data not shown). The A_{310} material in this peak accounted for 80% of

the material applied to the column. The EA-insoluble catechol was found to be soluble in butanol, so extraction into butanol was used as a final purification step.

The spectrum of an aqueous solution of the purified EA-insoluble catechol had a maximum at 310 nm, minimum at 275 nm and a maximum of 265 nm, with an $A_{265}:A_{310}$ ratio of 2.7. Acidification resulted in a slight variation in this spectrum with a clear minimum developing at 235 nm. Basic pH adjustment resulted in a complex spectrum having a strong absorption in the range 280 to 360 nm and a visible yellow colouration, characteristic of 2,3-DHBA at alkaline pH (Pittard *et al.*, 1961). Acidification of an ethanolic solution of the EA-insoluble catechol did not result in an increased absorbance at 280 nm, which suggested that an oxazoline ring was not present (Ong *et al.*, 1979; Griffiths *et al.*, 1984).

Properties of the EA-insoluble catechol

TLC in solvent system (A), used previously to resolve *A. vinelandii* catechols (Knosp *et al.*, 1984), separated 2,3-DHBA from azotochelin but the EA-insoluble catechol did not move from the origin. In solvent system (B), the EA-insoluble catechol moved as a single spot (R_F 0.42 to 0.47) with white-blue fluorescence under 366 nm light. The EA-insoluble catechol was also strongly reactive with ninhydrin, which indicated the presence of a primary amine. Therefore, the EA-insoluble catechol, presumed to be a siderophore of *A. vinelandii*, was named aminochelin.

Stability and hydrolysis of aminochelin

Aminochelin was notably unstable at alkaline pH at 20 °C in the light. This was evident after 4 h in alkaline solution (5 mM-NaOH, pH 10.5) but was also noticeable in buffer at pH 7.5 and to a lesser extent in buffer at pH 7.0, after 20 h. The instability in these solutions was diminished at 4 °C but the trend towards a loss of the A_{310} peak and development of a strong yellow colouration was apparent. Only solutions stored in distilled water, pH 5.2, or ammonium acetate buffer, pH 5.5, in the dark at 4 °C, or in 5 mM-HCl, pH 2.5, were stable.

When aminochelin that had been held in NaOH overnight at 20 °C in the light was subjected to TLC in solvent system (B), the R_F 0.47 spot disappeared and was replaced by material which remained near the origin. Native aminochelin moved as a streak upon TLC in the alkaline solvent system (C), while NaOH-treated aminochelin produced a fluorescent spot just off the origin and a ninhydrin-positive spot at $R_F \sim 0.2$. Less severe alkaline treatment of aminochelin resulted in less intense spots and more streaking in solvent system (C). These results suggested that alkaline treatment resulted in hydrolysis of aminochelin.

Acid hydrolysis of aminochelin resulted in the disappearance of the R_F 0.47 spot in solvent system (B) and the appearance of a fluorescent spot (R_F 0.70), a weakly ninhydrin-positive, weakly fluorescent, yellowish spot (R_F 0.43), and a strong ninhydrin-positive spot (R_F 0.15). The spectrum of the R_F 0.43 material resembled that of unhydrolysed aminochelin. Extraction of the hydrolysate with ether and TLC of the extract revealed that the R_F 0.70 spot was 2,3-DHBA.

The ninhydrin-positive spot was not lysine, as shown by TLC in solvent system (C). In this solvent, lysine moved off the origin (0.7 cm) while the hydrolysis product remained at the origin. TLC in solvent system (D) confirmed that the hydrolysis product (R_F 0.08) was not asparagine, ornithine, arginine or lysine (R_F 0.20 to 0.24). The hydrolysis product did not correspond to a common amino acid, and notably threonine, which forms an oxazoline ring in several siderophores (Neilands, 1981), was absent. The low mobility of the hydrolysis product in solvent (D) indicated that it was more basic than lysine. Accordingly, the amino compound in the hydrolysate was found to have a mobility that corresponded to putrescine but not spermidine or spermine.

The mass of aminochelin was determined to be 225, which corresponded to the protonated form of 2,3-dihydroxybenzoylputrescine. Sodium was present in the sample as evidenced by its association with the glycerol carrier. Similarly the ether-extracted hydrolysate contained a small amount of unhydrolysed material and masses at 110 and 131, which corresponded to the mono- and di-sodium forms of putrescine.

Table 2. ^{55}Fe -uptake promoted by aminochelin and other siderophores produced by *A. vinelandii*

Iron transport activity was assayed using 16 h iron-limited cells suspended in iron-limited Burk medium containing 10 mM-sodium citrate (Knosp *et al.*, 1984) plus siderophore solutions with the absorbance characteristics indicated, in duplicate 10 min assays. Values for ^{55}Fe uptake have been corrected for ^{55}Fe bound to cells in the same solutions, but incubated on ice for 10 min; reproducibility between duplicates was accurate to $\pm 5\%$ of the mean.

Siderophore	A_{410}	A_{310}	^{55}Fe -uptake (d.p.m.)	Percentage of maximum ^{55}Fe -uptake
Culture supernate	0.770	0.345	43 390	90
	0.036	0.150	47 980	100
Azotobactin	0.227	0	39 660	83
	0.082	0	46 050	96
Azotochelin	0	0.215	21 510	45
	0	0.080	20 500	43
Aminochelin	0	0.210	37 780	79
	0	0.083	31 240	65
Buffer only	0	0	8 680	18

Iron uptake promoted by aminochelin

Aminochelin ($A_{310} = 0.3$) formed a blue-purple coloured complex within 30 s with either $5 \times 10^{-5} \text{ M-Fe}^{2+}$ ($A_{\text{max.}} 500 \text{ nm}$) or Fe^{3+} ($A_{\text{max.}} 485 \text{ nm}$) in distilled water, iron-limited Burk medium, or iron-limited Burk medium containing 10 mM-sodium citrate, the latter to be used in ^{55}Fe -uptake assays. Addition of 0.4 mM-dithiothreitol to prevent oxidation of the Fe^{2+} ion did not alter this result. Aminochelin also reacted within 30 s with molybdate ($5 \times 10^{-5} \text{ M}$), forming a yellow complex.

Aminochelin was an effective siderophore in iron-limited *A. vinelandii*, although the majority of the ^{55}Fe -uptake in the unfractionated culture supernatant fluid was accounted for by azotobactin (Table 2). Maximum ^{55}Fe -uptake by azotobactin was observed when the purified siderophore was diluted, possibly because salts that were inhibitory to siderophore-mediated transport (Knosp *et al.*, 1984) were in the purified preparation. Azotochelin-mediated (Knosp *et al.*, 1984) and aminochelin-mediated ^{55}Fe -uptake were also salt sensitive; the latter was decreased 55% by the addition of 40 mM-NaCl to the uptake medium (data not shown).

DISCUSSION

A. vinelandii produces three siderophores in response to iron-limitation. These include the yellow-green fluorescent compound azotobactin, the catechol azotochelin and the newly discovered EA-insoluble catechol which we have named aminochelin. Aminochelin has a simple structure which appears to be 2,3-dihydroxybenzoylputrescine (Fig. 3). This is the first report of a siderophore of this composition and containing this polyamine. As would be expected, the free amine of aminochelin makes it more hydrophilic than azotochelin and it is not extracted into EA. This property also distinguishes aminochelin from other catechol siderophores containing polyamines. For example, *Paracoccus denitrificans* produces N^1, N^8 -bis(2,3-dihydroxybenzoyl)spermidine (compound II), an EA-soluble siderophore lacking a primary amine (Trait, 1975). The EA-soluble siderophores parabactin and agrobactin also contain compound II but are acylated at the secondary amide-N with a residue of salicylate or 2,3-DHBA, respectively, via an oxazoline ring (Neilands, 1981). Similarly, vibriobactin is a tricatechol with two oxazoline rings and a norspermidine backbone (Griffiths *et al.*, 1984). These latter three siderophores have high affinities and specificity for Fe^{3+} , a characteristic that was not found for aminochelin nor would it be expected from its simple structure.

Although aminochelin promotes ^{55}Fe -uptake into *A. vinelandii*, it appears to be less efficient than azotochelin. The amount of ^{55}Fe transported by the two siderophores was $1.8 \times 10^5 \text{ d.p.m.}$

Fig. 3. Proposed aminochelin structure (2,3-dihydroxybenzoylputrescine).

(A_{310} aminochelin) $^{-1}$ versus 1.0×10^5 d.p.m. (A_{310} azotochelin) $^{-1}$ (Table 2). Considering that azotochelin is a dicatechol, the amount of ^{55}Fe transported per mol of each siderophore would be approximately equal. However, a 2.7-fold dilution of the siderophores resulted in a 2% decline in azotochelin-mediated transport versus a 14% decline in aminochelin-mediated transport. Therefore, relatively high levels of aminochelin appear to be required to promote its maximal level of iron uptake.

Aminochelin is very stable at acidic pH and is increasingly less stable as the pH increases. Even at pH 7.0 there is evidence of breakdown. This raises questions about its role in the alkaline environments preferred by *A. vinelandii* (Thompson & Skerman, 1979). This siderophore and the others, however, are produced in vast excess of that required to promote rapid iron uptake, so continuous destruction of the siderophore may be tolerated provided an amount of active material remains.

One also must wonder why *A. vinelandii* produces three siderophores, two of which are not specific for Fe^{3+} and are incapable of high affinity binding of Fe^{3+} . *A. vinelandii* has been isolated from mineral and calcareous soils (Thompson & Skerman, 1979; Rennie, 1980) where the ability to solubilize mineral forms of iron, and perhaps other ions, would be advantageous. Catechols are effective agents in the solubilization of these minerals (this study; Page & Huyer, 1984; Page & von Tigerstrom, 1982) and it may be desirable not to have this nutritional system tightly controlled by iron. Aminochelin and azotochelin are produced coordinately, along with major iron-repressible outer membrane proteins of molecular mass 93000, 85000 and 81000, followed by azotobactin and a molecular mass 77000 iron-repressible outer membrane protein (Page & Huyer, 1984). This suggests that there are two regulons affected by iron availability in *A. vinelandii*. The azotobactin regulon is most sensitive to iron availability, whereas the catechol regulon is expressed under conditions of iron repletion that are repressive to the azotobactin regulon. In *E. coli*, siderophore expression is negatively regulated by the Fur protein (Hantke, 1981, 1984). The Fur protein is activated by iron, which acts as a corepressor, promoting the binding of the Fur:Fe complex to the promotor regions of iron-regulated genes (De Lorenzo *et al.*, 1987). If a parallel system of regulation exists in *A. vinelandii*, differences between the promotor regions of the catechol and azotobactin regulons may similarly provide a basis for differential control by a single repressor complex. This possibility is presently under investigation.

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