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Characterization of the pyoverdines of *Azotobacter vinelandii* ATCC 12837 with regard to heterogeneity

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Summary. *Azotobacter vinelandii* strain ATCC 12837 produces peptide siderophores of the general class known as pyoverdines. In the past, it was assumed that a single well-defined pyoverdine was produced by each parent microorganism. However, there are a number of reports of incompletely characterized pyoverdines that demonstrate heterogeneity in pyoverdine preparations obtained from a single organism, but the nature of this phenomena has not been explained. This study shows that *A. vinelandii* does indeed produce more than one pyoverdine and that these compounds differ in their peptide components. The metabolism of these siderophores suggests that only one of them is a true siderophore while the others are metabolic byproducts. It was demonstrated that this phenomenon is likely due to intrinsic limitations of the synthetase complex involved in the biosynthesis of these compounds. Characterization of two of the major pyoverdines produced demonstrated that they are novel compounds, although they belonged to the *Azotobacter*-type family of pyoverdines.

Key words: Pyoverdines - Siderophores - *Azotobacter* - *N*-Hydroxy ornithine

Introduction

The pyoverdines have aroused much interest since Schroeter first reported a bacterium that produced a bright green fluorescence under certain growth conditions (Schroeter 1872). This colour was subsequently shown to be the result of the production of peptide siderophores (pyoverdines) which contained an unusual heterocyclic chromophore, the general structure of which is shown in Fig. 1. The *o*-catechol function of the chromophore serves as one of the three requisite bidentate Fe^{3+} ligands, with hydroxamates (provided by N^5-acyl-N^5-hydroxyornithine) and α-hydroxyacids (provided by β-hydroxyaspartic acid) commonly supplying the other two.

We have been studying the pyoverdines because they are unique in several respects. Firstly, they are relatively large siderophores, with molecular masses of the order of 1500 Da. Normally, siderophores have masses of the order of a few hundred daltons. Secondly, the diversity seen in the pyoverdine family is much greater than that observed in any of the other types of siderophores. Virtually every species so far studied that produces a pyoverdine-type siderophore does so with a unique peptide attached to the distinctive chromophore (with the single exception of *Pseudomonas chloraphis* 9446 and *Pseudomonas fluorescens* 13525, which appear to produce identical pyoverdines). In addition to this type of strain-specific structural variation, there have been a number of indications that the pyoverdines produced by individual strains are in some cases heterogeneous. Some reports have suggested that this phenomenon is an artifact of the procedure used to purify these siderophores (Philson and Llinas 1982; Palleroni 1984), an hypothesis that is in agreement with the demonstrated lability of the pyoverdines in the presence of light or extremes of pH (Meyer and Abdallah 1978). However, other reports indicated that in some cases a

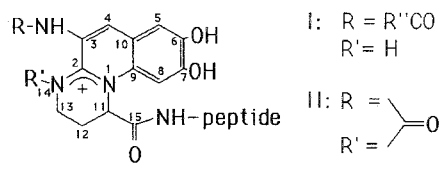

Fig. 1. Structure of the pyoverdines. *Pseudomonas* sp. produce type I pyoverdines, while *Azotobacter* sp. produce type II. The nature of the peptide chain is species-dependent and ranges in size over 6–11 amino acids. In the case of pyoverdine avi 12837 II it is an undecapaptide composed of β-hydroxyaspartate, serine, homoserine, glycine and *N*-hydroxyornithine in the ratios 1:4:3:2:1, while pyoverdine avi 12837 I has the same amino acids in the ratios 1:2:2:1:0. R'', found in the *Pseudomonas*-type siderophores, is usually a short-chain acid such as malate, succinate or acetate

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number of compounds could be detected in untreated culture supernatant, suggesting that some microorganisms did in fact produce more than one pyoverdine (MacDonald and Bishop 1979; Cox and Adams 1985). The relationships of these variants to each other and their biological roles have not been investigated, so whether or not all of these compounds function as true siderophores is unclear.

Thus, to determine the nature and causes of this heterogeneity we have investigated the pyoverdine production by *Azotobacter vinelandii* ATCC 12837. We have also investigated the stability of these compounds during purification steps, using procedures in the literature and a novel method presented here. The results indicate that, although breakdown during some reported purification procedures is significant, this microorganism does produce more than one pyoverdine. We have characterized two of the major species, and named them pyoverdines avi12837I and avi12837II. Only one of these compounds functions as a true siderophore.

Materials and methods

Pyoverdine characterization

Growth of cells and isolation of culture supernatant. A. vinelandii ATCC 12837 cultures were grown in minimal media containing (in g/l): D-mannitol 10.0; NaCl 0.2; K_2HPO_4 0.8; KH_2PO_4 0.2; $CaCl_2$ 0.1; $MgSO_4$ 0.2. Rich media contained in addition yeast extract (0.5 g/l) and had no NaCl. Unless otherwise stated, cultures (300 ml) were grown at 30° C in a rotary shaker at 150 rpm in 750-ml conical flasks. Cell- and polysaccharide-free supernatant was obtained by the addition of 10 ml 1.1 M $BaCl_2$ solution/l culture followed by centrifugation ($10000 \times g$; 30 min). All growth and purification procedures were carried out in the dark as far as possible, and samples were stored protected from light.

Florosil adsorption and conventional chromatography. Culture fluid was acidified with glacial acetic acid (1% by vol.) and florosil (5 g/l) was added with stirring for 30 min. The florosil was allowed to settle, the supernatant decanted and the resulting thick suspension poured into a scintered glass funnel and washed with 2 vol. water. Pyoverdines were eluted with water/acetone/concn. NH_4OH (900:100:1) and concentrated by rotary evaporation under reduced pressure at low heat (40° C). This crude pyoverdine fraction was applied to a column of DEAE-cellulose (2 cm × 25 cm; pyridine form) equilibrated with water. The column was washed with 1 vol. water and the pyoverdines were eluted with a gradient of pyridine (to 20%, over 1000 ml) adjusted to pH 6.0 with acetic acid. Two major fractions were detected by absorbance at 390 nm; these were pooled and concentrated separately. The fractions were further purified by desalting over Sephadex G-25 followed by chromatography over LH-20 resin in a column (1.5 cm × 130 cm) equilibrated and eluted with methanol/water (1:1). Two major peaks were evident for each of the fractions, these were pooled separately and lyophilized.

Hydrophobic interaction HPLC purification. NaCl (200 g/l) was added to the cell-free culture fluid, with stirring for 30 min. This solution was extracted with 0.2 vol. *n*-butyl acetate. The organic layer was discarded. Following saturation with $(NH_4)_2SO_4$ and centrifugation at $50000 \times g$ for 20 min to remove a white flocculation, the pyoverdines were adsorbed from the supernatant onto a Sep-Pak C_{18} (Waters) cartridge. The cartridge was washed immediately with a small volume of water (≈0.5 ml) and eluted with approximately 5 ml acetonitrile (30%) to recover the fluorescent material. The volume of this crude preparation was reduced by evaporation under reduced pressure at moderate temperature (40° C) and this fraction was applied to a Sephadex G-25 superfine column (1.0 × 30 cm) which was subsequently eluted with formic acid (0.5%) at a flow rate of 0.25 ml/min. Fractions emerging between 7.0–9.0 ml effluent volume were pooled and lyophilized. This material was then applied to an RP-300 HPLC column (4.6 × 200 mm) equilibrated with water at a flow rate of 1.0 ml/min. After washing with 10 ml water, a gradient of acetonitrile (to 50% over 25 ml) was used to elute the siderophores. Pyoverdines were detected by absorbance at 390 nm; components eluting separately were pooled individually and lyophilized. Each of these components was then separately rechromatographed under identical conditions on the RP-300 column to ensure homogeneity. Heterogeneity of the pyoverdines was examined by chromatography over the RP-300 column under the same conditions several times as described above, but with the aqueous solvent containing 100 mM potassium phosphate pH 5.8 and 1 mM EDTA. This buffered aqueous phase was essential for reproducibility of retention times between analyses.

Spectroscopic characterization. Spectral investigations were performed on the pyoverdine avi12837II isolated by the hydrophobic interaction procedure. ^1H- and ^{13}C-NMR spectra were recorded in D_2O, with dioxane (0.2%) as an internal standard for the ^{13}C spectra. Spectra were recorded using a Bruker AM-200 machine. Absorption spectra were recorded on a Varian 2200 spectrophotometer. Fluorescence spectra were recorded on a Perkin-Elmer HR1A fluorometer, with excitation and emission slit widths set at 2.5 nm. For fluorescence spectra, solutions with an absorbance at 390 nm of approximately 0.05 in 100 mM potassium acetate pH 4.20 were used. The effect of several metal ions (as shown in Table 4) on the absorption and fluorescence spectra was investigated at a metal ion concentration of 50 µM. Determination of the molar absorption coefficient of the chromophore and the binding constant for Fe^{3+} was performed by titration with Fe^{3+} while monitoring the spectral shifts in the 340–500-nm region. An aliquot (3.00 ml) of a pyoverdine solution with an absorbance at 390 nm of approximately 0.4 was prepared in 100 mM potassium acetate pH 4.20 and titration was performed with a solution of 174.80 mg $Fe(NO_3)_3 \cdot 9H_2O$ in 500.0 ml water (final concentration 0.853 mM) acidified with 100 µl HNO_3 to prevent precipitation of $Fe(OH)_3$. Determination of K_f and ε_M was achieved by least-squares fitting to the theoretical equilibrium of Eq. (1):

$$\frac{[\text{Pyoverdine}-Fe^{3+}][H^+]^n}{[\text{Pyoverdine}-H_n^+][Fe^{3+}]} = K \qquad (1)$$

when K_f is defined by Eq. (2) as K extrapolated to alkaline pH values ($n = 0$; all relevant protons dissociated).

$$\frac{[\text{Pyoverdine}-Fe^{3+}]}{[\text{Pyoverdine}][Fe^{3+}]} = K_f = K[H^+]^{-n} \qquad (2)$$

The value of n was determined by the amino acid composition of the pyoverdine, while $[H^+]$ is, of course, a function of the pH.

Amino acid analysis. Hydrolysis of pyoverdine peptides was performed in 6 M HCl containing *p*-cresol (0.05%) for 15 h at 105° C. Analyses were performed on a Beckman gold system. For the determination of N^5-hydroxyornithine, which is only poorly detected under these conditions, a reductive hydrolysis was performed in 57% HI for 24 h at 131° C. After precipitation of the HI with 1.5 mol silver acetate/mol, the excess Ag^+ ions were removed by addition of HCl. The solution was then filtered through Whatman no. 1 paper and the analysis continued as for the HCl-hydrolysed samples. Additionally, the presence of total partially oxygenated nitrogen (hydroxylamines and nitrones) in the HCl hydrolysates was determined by the iodine oxidation procedure (Parniak et al. 1979), which converts these species to NO_2^- and

detects this product by formation of a diazo dye. *N*-Methylhydroxylamine solutions served as standards for this procedure.

In vivo production of pyoverdines

Pyoverdine production of growing cultures of *A. vinelandii* was examined as a function of time. Furthermore, the effect of different carbon and nitrogen sources, vitamins (yeast extract), amino acids and temperature on the relative abundance of the two major pyoverdines was determined. For these studies, cultures (100 ml) in 250-ml conical flasks were grown in a rotary shaker at 150 rpm. Aliquots (1.0 ml) were removed periodically over a 5-day period, centrifuged (13000 × *g*; 2 min) to pellet the cells, and aliquots (250 µl) of supernatant were analyzed by the analytical HPLC protocol previously described. Individual pyoverdines were quantified by absorbance at 390 nm.

All cultures were grown at 30° C, except for a series of experiments where the temperature was kept at 24° C, 26° C, 28° C, 32° C, 34° C or 37° C. For the carbon source variation, the following compounds were used to replace the mannitol in the growth media, each at a concentration of 10.0 g/l: sodium glyoxylate, trisodium citrate, disodium succinate, 2-oxoglutaric acid, glucose, mannose, and sorbitol. The effect of nitrogen source supplementation was investigated by the addition (to mannitol basal medium) of the following compounds at a concentration of 2.0 g/l: sodium nitrate, sodium nitrite, glutamine, sodium glutamate, citrulline, urea, arginine, aspartic acid, and ammonium sulfate. Supplementation experiments with the amino acids incorporated into the pyoverdine (serine, glycine, homoserine, ornithine) were also conducted at 2.0 g/l.

^{14}C pulse labelling

The interconversion of individual siderophores in the growth media was studied by ^{14}C pulse labelling experiments. ^{14}C-labelled pyoverdines avi 12837 I and avi12837 II were produced by addition of 83 µCi [2-^{14}C]glycine to a 50-ml culture just after pyoverdine production had commenced. The labelled siderophores were purified by removing the cells by $BaCl_2$ precipitation and centrifugation, as previously described. In order to minimize the time and number of steps with this radioactive material, the bulk of the supernatant (40 ml) was at this stage applied directly to the RP-300 column and eluted using the unbuffered protocol as described. Siderophore was eluted with an acetonitrile gradient and rechromatographed under identical conditions to ensure homogeneity of each pyoverdine.

An aliquot (0.1 ml) of each [^{14}C]siderophore preparation (pyoverdine avi 12837 I: 50000 dpm; pyoverdine avi 12837 II: 62000 dpm) was then added to a culture (10 ml) in two separate experiments immediately after commencement of pyoverdine production. Aliquots (0.5 ml) were removed at intervals and the pyoverdine production was monitored by analytical HPLC as previously described. Fractions (0.3 ml) were collected from this chromatography and counted by liquid scintillation to determine ^{14}C content.

Additionally, the time course of [2-^{14}C]glycine incorporation into each of the two major pyoverdines was measured at the beginning, the end, and in the middle of pyoverdine production. [2-^{14}C]glycine (83 µCi) was added to 25-ml cultures of growing cells at the times indicated. Aliquots (1.2 ml) of the culture were removed at intervals and, after removal of the cells by centrifugation (2 min, 13000 × *g*), the supernatant (1.0 ml) was applied directly to the RP-300 column. The various pyoverdines were quantified by absorbance at 390 nm; fractions (0.3 ml) were collected and counted by liquid scintillation to determine ^{14}C content.

Results

Pyoverdine isolation and characterization

The conventional chromatography procedure as based on the literature (Fukasawa et al. 1972; Philson and Llinas 1982) resulted in two major components being resolved by ion-exchange chromatography (DEAE-cellulose; Fig. 2a) and hydrophobic interaction chromatography (Sephadex LH-20; Fig. 2b). A number of poorly resolved minor components also appeared to be present. However, when these conventionally purified preparations were examined by HPLC they were found to be grossly heterogeneous (Fig. 3A, B). Also, reliable amino acid stoichiometries were not obtainable for material purified in this manner because different batches yielded variable amounts of β-hydroxyaspartate, serine, homoserine, glycine and N^5-hydroxyornithine (data not shown). This irreproducibility, and the success of the analytical reverse-phase separation, prompted a preparative scale-up of this latter technique. Investigation of the culture supernatant by the same procedure showed that a number of pyoverdines were present initially (Fig. 3C) but not nearly as many as produced by the previously described isolation procedure. This alternative purification proved to be much less harsh, producing only one additional compound which was removable by rechromatography (Fig. 3D–F).

Homogeneous preparations of the two major pyoverdines evident in crude culture supernatant (named pyoverdines avi12837I and avi12837II in order of their elution during the reverse-phase chromatography) yielded much more reproducible amino acid composition stoichiometries. Analysis showed that they were

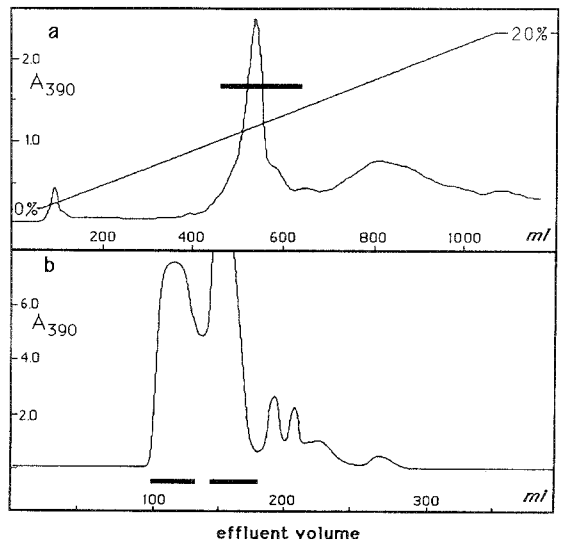

Fig. 2. Conventional chromatographic purification of the pyoverdines of *A. vinelandii*. Crude culture supernatant was first applied to a DEAE-cellulose column and then eluted with a gradient of pyridine/acetic acid (**a**) as described in Methods. A major peak was pooled as indicated (heavy line) and chromatographed over Sephadex LH-20 (**b**). Two major peaks were identified and pooled separately as shown

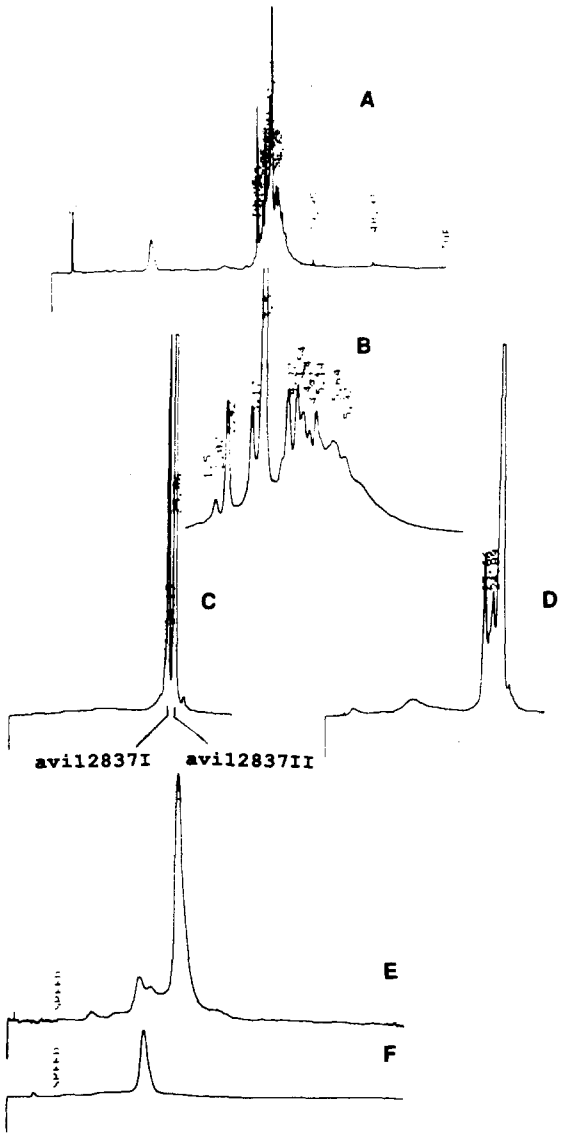

Fig. 3. HPLC chromatography of pyoverdines from *A. vinelandii*. Reverse-phase HPLC separation of various pyoverdine-containing fractions was performed as described. (**A**) Conventionally purified pyoverdines; (**B**) an expanded view of the major group of peaks in A; (**C**) pyoverdines initially present in the supernatant; (**D**) pyoverdines after elution from Sep-Pak cartridges; (**E**) RP-300-purified pyoverdine avi12837II; (**F**) RP-300 purified avi12837I. The scale is expanded in E and F as in B to indicate purity of the two fractions more easily

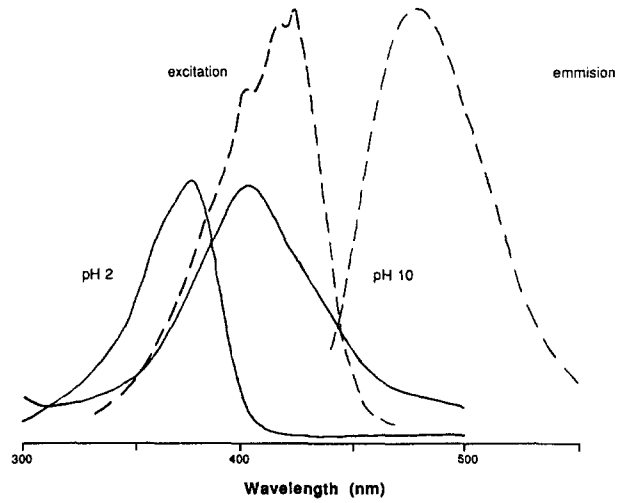

Fig. 4. Absorption and fluorescence spectra of pyoverdine avi12837II. The absorption (—) and fluorescence (---) spectra of pyoverdine avi12837II was determined (pyoverdine avi12837I exhibited identical spectra)

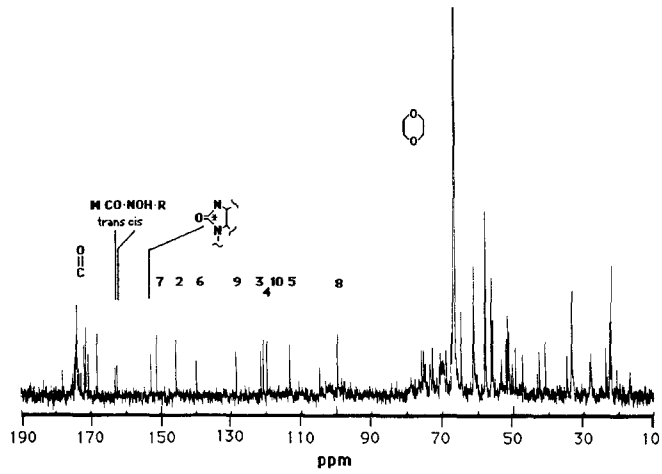

Fig. 5. ^{13}C-NMR spectrum of pyoverdine avi12837II. The signals resulting from the carbons of the chromophore are labelled following the numbering scheme of Fig. 1. Also indicated are the carbonyl signals, and two peaks resulting from the two regio-isomers of the formyl hydroxamate. An internal standard of dioxane was used

composed of the amino acids β-hydroxyaspartic acid, serine, homoserine, glycine, and N^5-hydroxyornithine in the ratios 1:2:2:1:0 and 1:4:3:2:1 for avi12837I and avi12837II respectively.

Both compounds exhibited identical absorption and fluorescence spectra (Fig. 4) that were similar to analogous spectra reported for other pyoverdines. Metal ions were found to alter these spectra dramatically (Table 1). The ^{1}H- and ^{13}C-NMR spectra (Figs. 5 and 6) were very complex and did not allow unambiguous assignment of all the peaks in the aliphatic region. However, comparison of the aromatic region peaks with published spectra of *Azotobacter* (Demanage et al. 1988) and *Pseudomonas* (Philson and Llinas 1982) compounds allowed unambiguous confirmation of the chromophore as the former type. As well, two peaks for the *cis* and *trans* isomers of a formyl hydroxamate were detected. These peaks were labile to mild acid or IO_4^- treatment, confirming their assignment as resulting from hydroxamates.

Titration of the desferripyoverdine with Fe^{3+} ions (Fig. 7) allowed the following molar absorption coefficients to be determined: $\varepsilon_{410} = 25\,700\ M^{-1}\,cm^{-1}$ (basic or Fe^{3+} form λ_{max}), $\varepsilon_{378} = 25\,400\ M^{-1}\,cm^{-1}$ (acidic form λ_{max}), $\varepsilon_{390} = 19\,500\ M^{-1}\,cm^{-1}$ (isosbestic λ). The strength of Fe^{3+} ligation was determined by nonlinear least-squares fitting to the points near the titration end point that deviated from the strict linearity (where significant amounts of desferri and ferri forms exist in

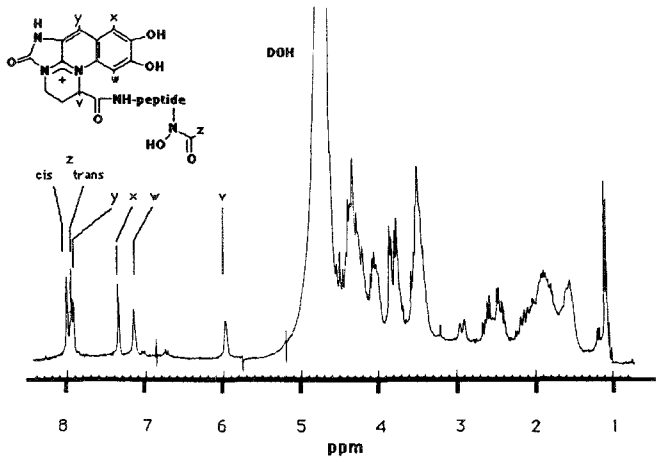

Fig. 6. ^{1}H-NMR spectrum of pyoverdine avi 12837 II. The aromatic region signals have been assigned as indicated, as well as the two formyl hydroxamate regio-isomer signals

Table 1. Metal-ion-induced spectral perturbations

Ion	Ground state d^n	Ionic radius (pm)	Absorption shift	Fluorescence (%)
Al^{3+}	0	510	+	100
Ga^{3+}	10	620	+	155
Cr^{3+}	3	630	−	82
Co^{3+}	6	640	−	83
Fe^{3+}	5	650	+	0.0
Rh^{3+}	6	680	−	85
V^{3+}	2	740	+	50
In^{3+}	10	810	+	120
Mo^{6+}	4	620	+	0.5
Mg^{2+}	0	660	−	126
Co^{2+}	7	720	−	78
Mn^{2+}	7	800	−	78

The effect of various metal ions (50 μM) on the absorption spectra of pyoverdine aviUWII at pH 4.25 was measured. The absorption shift refers to the presence (+) or absence (−) of a shift of the λ_{max} of the chromophore from 378 nm to 410 nm, which is the result of ionization of the catecholate function. The fluorescence reported is relative to a sample with no metal ion

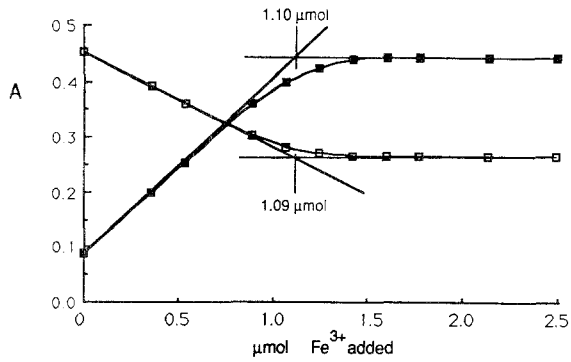

Fig. 7. Fe(III) titration of desferri-pyoverdine avi 12837 II. Titration of desferri-pyoverdine avi 12837 II while monitoring absorbance at 378 nm, □ (protonated chromophore λ_{max}) and 410 nm, ■ (deprotonated chromophore λ_{max}) allowed determination of the absorption coefficient of the chromophore and the pK_f of Fe(III) binding. A 1:1 stoichiometry of binding was assumed

equilibrium) yielded the value $pK_f = 24.5$ (pH adjusted).

In vivo pyoverdine production

Maximal pyoverdine production was observed at 28–30° C (Fig. 8). The time course of the production of each pyoverdine in both minimal media and media supplemented with yeast extract was similar (Fig. 9). Pyoverdine avi 12837 II was the dominant siderophore produced early, but accumulation of this compound slowed at approximately 75 h. Production of pyoverdine avi 12837 I continued unabated, however.

Supplementation of minimal media with various carbon sources was shown to affect the total amount of pyoverdine produced, but not the pyoverdine avi 12837 II/I ratio (Table 2). Addition of certain ni-

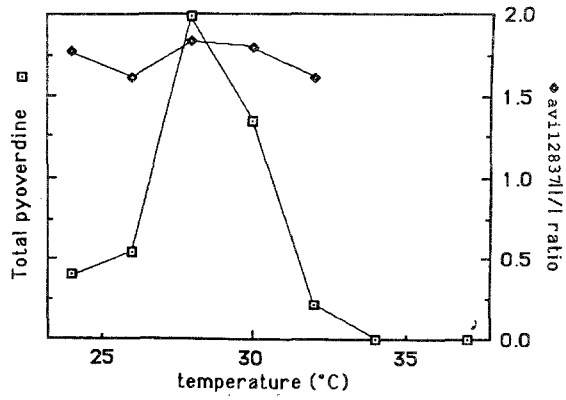

Fig. 8. Temperature dependence of pyoverdine production. The temperature dependence of total pyoverdine production and relative ratio of pyoverdines avi 12837 I and avi 12837 II was examined in cultures grown in minimal media. Pyoverdines were quantified by HPLC directly from the culture supernatant as described in Methods

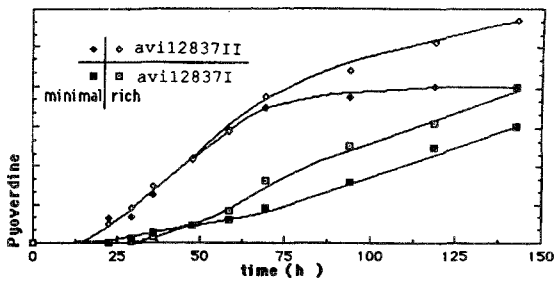

Fig. 9. Time course of pyoverdine production. The time course of production of pyoverdines avi 12837 I and avi 12837 II, the two major biosynthetic products, was examined in cultures grown in minimal media and a rich medium which contained 0.5% yeast extract. Pyoverdines were quantified by HPLC directly from the culture supernatant as described in Methods. Similar amounts of each siderophore were produced initially, but it can be seen that pyoverdine avi 12837 II production completely ceased in minimal media after about 75 h, while in rich media the production slowed but did not stop completely. Pyoverdine avi 12837 I synthesis seemed to have commenced slightly earlier in minimal media when compared to rich media

Table 2. Pyoverdine production as a function of growth media composition

Media	Growth (A_{600})	Pyoverdine production (A_{390} relative area)	avi 12837 II/I ratio
Mannitol	0.950	119	1.85
Mannose	n.g.		
Glucose	0.770	47	1.64
Sorbitol	0.880	140	1.74
Succinate	n.g.		
2-Oxoglutarate	n.g.		
Glyoxylate	n.g.		
Citrate	n.g.		
Mannitol +			
$NaNO_3$	0.770	70	0.60
$NaNO_2$	n.g.		
NH_4Cl	1.050	31	4.11
glutamine	0.930	112	0.48
glutamate	n.g.		
serine	1.530	31	1.50
homoserine	n.g.		
ornithine	0.850	64	1.90
glycine	1.750	0	

Cultures were grown in a basal medium containing mannitol as a carbon source as described in the Methods. For C source studies, the indicated compounds replaced the mannitol, while for the N source and amino acid supplementation experiments were carried out by addition of the indicated compounds at 2 g/l. The cultures were grown until stationary phase, and the pyoverdines quantified by HPLC. Total growth was quantified by nephelometry at 600 nm. n.g., no growth was observed

Table 3. The effect of nitrogen source on pyoverdine avi 12837 II/ avi 12837 I ratio

N source	avi 12837 II/avi 12837 I			Mean ± SD
None	1.80,	1.60,		1.7 ±0.10
NO_3^-	0.373,	0.578,	0.420	0.47 ± 0.15
NH_4^+	++,	++,	++	>5.0
Ornithine	2.55,	1.72,	2.08	2.1 ±0.3
Arginine	1.28,	1.71,	1.36	1.5 ±0.2
Citrulline	9.40,	6.00,	4.60	6.7 ±2.5
Glutamine	0*,	0*,	0*	<0.1
Urea				n.d.
Aspartate				n.d.
Glutamate				n.g.
NO_2^-				n.g.

Cultures were grown in basal media supplemented with 0.2% of each of the nitrogen sources until stationary phase and the pyoverdines quantified by HPLC. Cultures were grown in triplicate to examine the variability of the pyoverdine avi 12837 II/I ratio.
(++) Very small amounts of pyoverdine avi 12837 I were detected. Because the overall siderophore production was reduced a reliable estimate of the ratio in this case was not possible, although pyoverdine avi 12837 II predominated.
(0*) Very small amounts of pyoverdine avi 12837 II were found but avi 12837 I predominated. n.d., no pyoverdine production detected; n.g., no growth was observed

trogen sources proved to be quite effective in manipulating this ratio, so a more thorough examination of this phenomena was undertaken (Table 3). It was found that citrulline and NH_4^+ caused a relative increase in

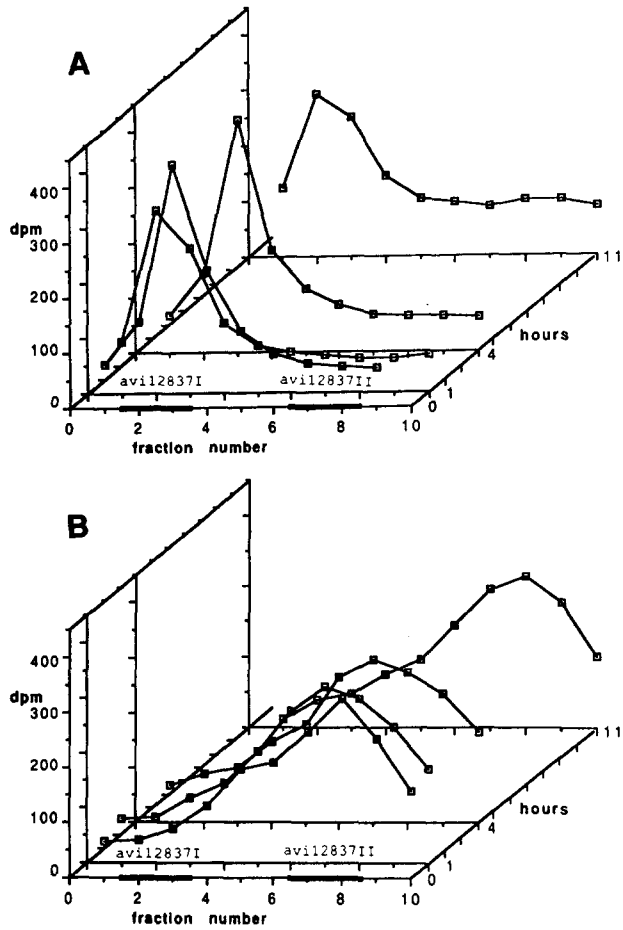

Fig. 10. [^{14}C]Pyoverdine label exchange experiment. [^{14}C]Pyoverdine avi 12837 I (**A**) and avi 12837 II (**B**) were added to growing cultures of *A. vinelandii* in two separate experiments as described in Methods. Aliquots were removed at the indicated intervals (z dimension) and the pyoverdines separated by HPLC. Fractions of eluant (x dimension) in the region in which the pyoverdines emerged were collected and counted by liquid scintillation to determine ^{14}C content (y dimension). Although the resolution of the two pyoverdines is poor, it can be seen that no exchange of label occurred because the shape of the profiles remained similar. The total radioactivity in the fractions expected for each pyoverdine (as indicated by the heavy bars) were averaged for each experiment, and this is indicated in the y/z plane. A fairly constant value is obtained, indicating that neither pyoverdine is significantly destroyed

pyoverdine avi 12837 II production, while NO_3^- and glutamine increased pyoverdine avi 12837 I production. The nature of this change was not solely due to the presence of fixed nitrogen but was specific to certain compounds, as indicated both by the opposite effects of the above-mentioned compounds and the fact that some readily utilizable nitrogen sources had no effect on this ratio.

The biosynthesis and metabolism of the two siderophores was shown to be independent by ^{14}C-labelling experiments. Purified ^{14}C-labelled pyoverdines avi 12837 I and avi 12837 II were shown to be nonin-terconvertible by growing cultures of the organism (Fig. 10). Furthermore, [2-^{14}C]glycine was incorporated into both pyoverdines simultaneously, and in relative pro-

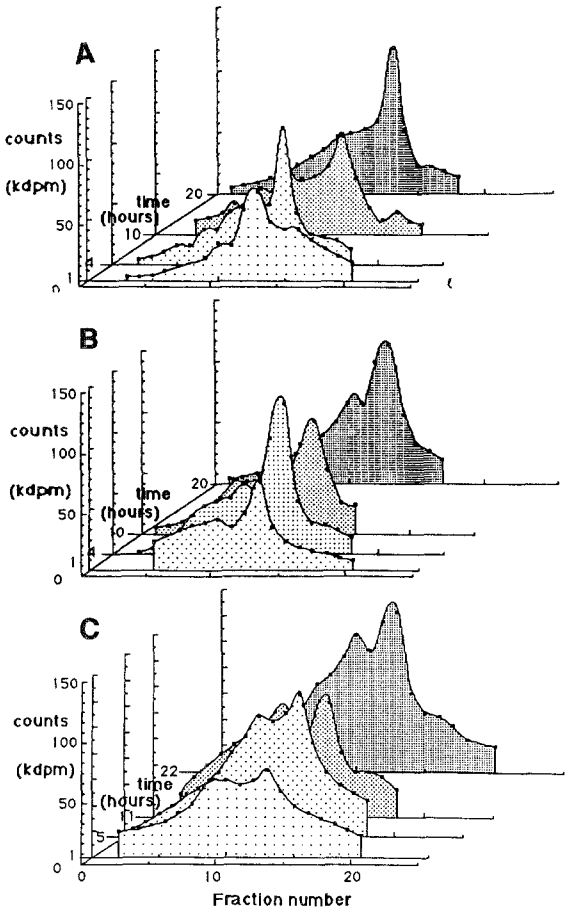

Fig. 11. Incorporation of [2-^{14}C]glycine into pyoverdines. [2-^{14}C]Glycine was added to growing cultures of *A. vinelandii* in early (**A**), middle (**B**) and late (**C**) stages of exponential growth as described in Methods. Aliquots were removed at the indicated intervals (*z* dimension) and the pyoverdines separated by HPLC. Fractions of eluant (*x* dimension) in the region in which the pyoverdines emerged were collected and ^{14}C content determined by liquid scintillation (*y* dimension). Two peaks of label incorporation coinciding with the emergence of pyoverdines avi 12837 I (first peak) and avi 12837 II (second peak; as detected by simultaneous monitoring of absorbance at 390 nm) are clearly seen in each case. ^{14}C incorporated into each pyoverdine is stable and does not appear to move from one siderophore to the other. Also, the relative amounts of label initially incorporated into each of the two peaks is in agreement with the observed relative rates of synthesis of pyoverdines avi 12837 I and avi 12837 II (Fig. 9) at each stage of growth

portion to the overall rate of biosynthesis of each siderophore (Fig. 11). This is in agreement with the relative rates of formation of each siderophore shown in Fig. 9.

Experiments to determine the efficiency of uptake of each of these two pyoverdines indicated that pyoverdine avi 12837 II was taken up by cells much more rapidly than pyoverdine avi 12837 I (Table 4). The import was also strongly dependent on the presence of Fe^{3+} ions. Cells grown to middle exponential growth phase showed maximal rates of uptake.

Table 4. Uptake of pyoverdines avi 12837 I and avi 12837 II by cells

Stage of exponential growth	^{14}C incorporation					
	avi 12837 I			avi 12837 II		
	Φ	Δ	Fe^{3+}	Φ	Δ	Fe^{3+}
Early	0.36	0.08	0.43	0.47	0.13	0.61
Middle	0.27	0.60	0.88	1.69	6.09	7.77
Late	0.47	0.83	1.26	0.44	1.50	2.45

The ability of each pyoverdine to be transported into cells of *A. vinelandii* ATCC 12837 was measured by incubating cells with ^{14}C-labelled pyoverdines and determining the extent (expressed as a percentage of added label) of label that was pelletted out with the cells in the presence and absence of Fe^{3+} (50 μM). Approximately 30000 dpm of each labelled pyoverdine was used in each experiment, which were conducted in triplicate ($s \approx 0.1\%$). A fairly constant blank value (Φ) bound to the cells in a nonspecific manner, but the difference (Δ) between this value and the amount incorporated in the presence of Fe^{3+} (Fe^{3+}) is strongly dependent upon which pyoverdine is used

Discussion

The purification procedure presented in the literature for the isolation of the pyoverdine from *A. vinelandii* O (Fukusawa et al. 1972) was found to be inadequate for the isolation of homogeneous pyoverdine avi 12837 due to the extreme lability of these compounds. They appear to be very sensitive to extremes of pH. The hydrophobic interaction purification procedure seems to be the best method for isolation of these compounds as it can be conducted exclusively at neutral pH.

A. vinelandii produces two major pyoverdines as well as a number of other less abundant forms. Only one of these, pyoverdine avi 12837 II, appears to be biologically active in mediating Fe^{3+} uptake. Spectral evidence (^{1}H nd ^{13}C NMR and absorption) indicates that all of these pyoverdines contain an identical *Azotobacter*-type chromophore, while amino acid composition values suggest that their differences may be ascribed to the peptide moieties. The absence of an accessible amino terminus, as well as the unusual nature and lability of the constituent amino acids, have prevented the identification of the complete amino acid sequence of the peptide moiety at this time.

During purification by HPLC, it was possible to monitor these siderophores by their absorbance at both 390 nm and 440 nm. The ratio of the absorbance at these two wavelengths was found to be approximately 4; it was the same for all of the pyoverdines observed (even the minor components which were not characterized by other means). In contrast, oxidation of the chromophore was found to lower this ratio to 1.5 (data not shown) so it would seem that other alterations to the chromophore might also change this ratio. Thus, it appears likely that the situation observed with the pyover-

dines avi 12837 I and avi 12837 II is paralleled with all of the components observed: the chromophores are the same, while the peptide portions are different.

Pyoverdine avi 12837 II appeared similar to other pyoverdines in most other respects. It is the largest such siderophore currently known, being composed of 11 amino acid residues. The iron-binding strength determined ($pK_f = 24.5$) is between those determined for pyoverdines from ATCC 13525 ($pK_f = 19$; Philson and Linas 1982) and ATCC 12799 ($pK_f = 32$; Meyer and Abdallah 1978). Several metal ions induced spectral shifts, with no definitive pattern emerging. More than one metal binding site may be implicated, as some ions (e.g. Mg^{2+}) were able to alter the fluorescence spectra without changing the absorption spectrum, whereas others (e.g. Al^{3+}) had the opposite effect (Table 1).

Heterogeneity of the pyoverdines

Heterogeneity of the pyoverdines might be due to either peptide sequence modifications or alteration of the chromophore moiety. However, this present study suggests that the chromophore is the same in all native pyoverdine species and demonstrates that the differences between at least the major pyoverdine forms resides in the peptide portions of the molecule.

Three hypotheses to account for peptide sequence heterogeneity were explored. (a) The pyoverdine produced might be extremely labile, breaking down to form an array of fragments and byproducts. (b) The organism might produce several pyoverdines *de novo* as a result of lack of stringency of the synthetase complex or for some, as yet unknown, purpose. (c) The ferripyoverdine complex may be specifically cleaved to facilitate release of iron (as in the case of enterobactin; Hider 1984).

These hypotheses were tested by examining the time course of pyoverdine production, as well as by the ^{14}C pulse labelling experiments. The first hypothesis, postulating breakdown of a single product, would predict initial production of only one component, followed by gradual accumulation of degradation byproducts. ^{14}C label in the siderophore should move from precursor to product but not in the opposite direction. Initial incorporation of ^{14}C label should be specific for one of the pyoverdines (i.e. the true biosynthetic product) and then gradually appear in the breakdown products. Because pyoverdine avi 12837 contains numerous relatively labile amino acids such as homoserine, serine, hydroxyaspartate and glycine, breakdown is quite plausible.

The second hypothesis predicts that the labelled pyoverdines would not exchange in either direction and that many components should appear almost simultaneously in the growth media. If amino acid activation is rate-limiting in the biosynthetic pathway, manipulation of the media composition might be expected to alter cytosolic amino acid levels and hence the pyoverdine ratios. That is, creating a deficiency of an amino acid specific to one of the two pyoverdines might be expected to limit preferentially the production of that particular siderophore. A variant on this hypothesis, that the various pyoverdines are the result of a simple lack of stringency and not due to absence of some key intermediate, would be expected to result in a fairly constant pyoverdine avi 12837 I/II ratio with variation in growth media. However, temperature should strongly affect this ratio, with more of the intended or favoured product being formed at lower temperatures.

The third hypothesis, that cleavage of an initially produced larger pyoverdine is intentional, postulates that there should be great specificity in flow of label from a large pyoverdine into a unique smaller form. If recycling occurs, regenerating the larger siderophore from the fragments (as in enterobactin), label should also move in the other direction. If no recycling occurs, all of the label in the larger pyoverdine would be chased into the smaller fragment where it would remain. In this case, media composition should have little effect on relative abundances of the pyoverdines produced.

The observations in this study favour the second of these hypotheses. Pulse labelling with [^{14}C]pyoverdine avi 12837 I and avi 12837 II, in separate experiments, indicated that no label exchange occurred in either direction (Fig. 10). The absolute levels of label in each compound appear to be relatively stable showing that, at least in the growth media, breakdown and conversion to products of different chromatographic mobilities is not significant. Incorporation of [2-^{14}C]glycine into each of the two major pyoverdines appeared to proceed in relative proportion to the rate of synthesis of each of these siderophores. At later stages of growth, the efficiency of label incorporation into pyoverdine avi 12837 I was increased relative to pyoverdine avi 12837 II, reflecting increased production of this siderophore. Initially the situation was exactly the converse with more pyoverdine avi 12837 II being produced and labelled (Fig. 11).

Several pyoverdines do appear simultaneously in the growth media although in most experiments pyoverdine avi 12837 II appeared initially and at the highest concentration. In minimal media production of this siderophore stops almost completely near the end of exponential growth while production of pyoverdine avi 12837 I continues, along with production of several less abundant pyoverdines. In rich media this phenomenon is not as clearly observed; only a minor reduction in pyoverdine avi 12837 II production is observed. This finding is consistent with the hypothesis that the production of the smaller siderophore is the result of limitation of certain biosynthetic precursors, which is expected to be more severe in minimal media.

The conditions of growth did not seem to have a great effect on the pyoverdine avi 12837 I/II ratio. The optimum growth temperature for pyoverdine production was found to be 28–30° C. Production ceased at temperatures above 34° C, although growth was slower but appeared normal in other respects. There did not appear to be a low temperature limit of pyoverdine production *per se*, but a drop in production which paral-

leled the lower rate of growth was seen below 28° C. The ratio of the two major pyoverdines was fairly constant in the temperature range studied.

The carbon source for growth was also found to affect the quantity, but not the type, of pyoverdine produced. As reported (Demanage et al. 1988), D-mannitol appears to be the preferred substrate for pyoverdine production, with glucose and sucrose found to be not quite as effective. Simple carbon compounds such as acetate or 2-oxoglutarate either did not allow growth, or did not favour pyoverdine production (Table 2). Variation in carbon source is known to greatly affect the demands on the cell for iron, the primary determinant of which is the extent to which the metabolism of the particular compound requires membrane-bound electron transport, which is heavily dependent on Fe-containing enzymes. Mannitol is a good carbon source for this organism and must first be oxidized and phosphorylated to mannose 1-phosphate prior to fermentation; this is reflected in the greater pyoverdine production when grown on this carbon source. However, the above manipulations did not affect the pyoverdine avi 12837 I/II ratio but merely served to modulate the total pyoverdine production. Supplementation with various nitrogen sources was found to be effective in this regard. This observation suggests that the determining factor in the production of the two pyoverdines may be related to the availability of N^5-hydroxyornithine, an amino acid which is a constituent of pyoverdine avi 12837 II, but not avi 12837 I.

Determination of the ratios of the two major pyoverdines in this series of experiments was complicated by the fact that addition of available nitrogen to the growth media reduced the organism's iron requirements by eliminating the nitrogenase system. In addition to eliminating electron transport flux necessary to reduce dinitrogen, the flux is further reduced by the elimination of futile cycles involved in keeping the cytosol microaerobic (Yates 1988), a condition necessary for nitrogenase to be active. As such, a number of nitrogen sources did not allow any pyoverdine production.

Presumably, N^5-hydroxyornithine is produced from ornithine by a monooxygenase, in analogy to the lysine N^6-hydroxylase system involved in aerobactin production (Viswanatha et al. 1987). The existence of such an ornithine-specific enzyme has been demonstrated by the cloning of the gene for this activity from *Ustilago maydis*, a fungus that produces N^5-hydroxyornithine as a component of ferrichrome (Wang et al. 1989). It is not unreasonable to hypothesize that the availability of N^5-hydroxyornithine in the cell might therefore be dependent on the availability of ornithine.

Addition of ammonia would be expected to result in an overall higher nitrogen charge of the cell and greater availability of all nitrogenous compounds, including N^5-hydroxyornithine. Addition of ammonia to the media resulted in a rise in the pyoverdine avi 12837 II/I ratio, but total pyoverdine production was repressed so that this ratio could not be accurately determined. Ornithine also produced a slight relative increase in pyoverdine avi 12837 II, in this case without affecting the overall siderophore production. However the effect of citrulline was most pronounced, resulting in a substantial increase in pyoverdine avi 12837 II and a slight overall increase in total pyoverdine production. Curiously, arginine had virtually no effect.

These results may be explained by examining the regulation of the pathways involved in the utilization of the above-mentioned compounds. In the Pseudomonads and Azotobacteracea arginine catabolism may be carried out by two pathways: the arginase pathway which produces urea and ornithine and the arginine deiminase pathway which converts arginine to citrulline and then to ornithine yielding a molecule each of ammonia and carbamoyl phosphate. The arginase pathway is the normal metabolic pathway responsible for arginine depletion necessary for a dynamic equilibrium to be established. The arginine deiminase pathway is strictly catabolic and is normally not expressed, but may be induced by high concentrations of arginine. Because of these dual pathways, supplementation with arginine may not be effective in raising the cytosolic levels of ornithine. Any ornithine produced by this catabolism could be rapidly depleted by the induced levels of catabolic enzymes.

With citrulline supplementation, the arginine catabolic enzymes are not derepressed and ornithine produced from the excess of citrulline cannot be readily metabolized. Because the equilibrium of the ornithine carbamoyltransferase reaction lies very much in favour of ornithine, ornithine is expected to build up substantially. Pyoverdine avi 12837 II is produced in great excess over avi 12837 I upon citrulline supplementation, lending credence to this hypothesis.

A number of substances served to lower the pyoverdine avi 12837 II/I ratio. Glutamine and nitrate were most effective in this regard. Both of these compounds are effective in manipulating the regulation of the nitrogen assimilation pathways in *Azotobacter* [glutamine (Toukdarian and Kennedy 1986; Voldmy and Leidinger 1978); nitrate (Luque et al. 1987)] which is similar to the classical *ntr* system characterized in enteric bacteria. The Azotobacteracea system appears to be substantially more complex and is as yet only partially characterized (Luthi et al. 1986; Picard and Dillon 1989) so full interpretation of the pyoverdine avi 12837 II/I ratio changes is quite speculative at the moment. However, the fact that nitrate and glutamine are extremely powerful modulators of this system does in itself implicate nitrogen regulation and associated metabolic pathways as responsible for the relative production of two pyoverdines.

These observations taken together suggest that the true siderophore of *A. vinelandii* ATCC 12837 is pyoverdine avi 12837 II. This siderophore, and not pyoverdine avi 12837 I, is efficiently taken up by the cells in the presence of Fe^{3+} (Table 4). Pyoverdine avi 12837 I appears to be an inevitable biosynthetic byproduct produced due to the lack of some amino acid, probably N^5-hydroxyornithine. This is consistent with the observation that pyoverdine avi 12837 II contains three Fe^{3+}

ligands and is suitable as a siderophore, while pyoverdine avi 12837 I has only two and is not likely to chelate Fe^{3+} as effectively.

We have previously shown (Menhart and Viswanatha 1990) that production of pyoverdines in *A. vinelandii* ATCC 12837 involves a non-ribosomal peptide synthetase. This class of enzymes utilizes a mechanism that involves storage of the growing peptide chain as a thioester, pending activation (as adenylylates or thioesters) and transfer of each subsequent amino acids in order of their occurrence in the completed peptide (Kleinkauf and von Dohren 1987). Because thioesters are relatively labile compounds, if a particular amino acid is in short supply, growth of the peptide chain may be temporarily halted and hydrolysis of the thioester may occur. Amino acid limitation has been shown to result in premature termination of peptides synthesized by gramicidin S synthetase (EC 5.1.1.11) in vitro (Vater et al. 1987), although no analogous in vivo process has been detected with that system.

In conclusion, we have partially characterized the pyoverdines produced by *A. vinelandii* ATCC 12837. Although this organism produces multiple pyoverdines, only one of them (pyoverdine avi 12837 II) is a true siderophore in the sense that it is able to mediate Fe^{3+} uptake by the organism. Another major pyoverdine was produced along with several minor pyoverdines, and these appear to be produced *de novo* by the organism although no biological role for them has been identified. The available evidence suggests that they are metabolic artifacts: nutrient limitation or other growth stresses appear to act to increase the normally low level of premature release and infidelity of synthesis of the growing peptide chain on the peptide synthetase complex and produce these other truncated pyoverdines. We have also demonstrated that a more careful examination of the pyoverdines produced by the organism directly is necessary, particularly due to the substantial alterations seen with commonly employed isolation procedures.

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