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A simple assay for fluorescent siderophores produced by *Pseudomonas* species and an efficient isolation of pseudobactin

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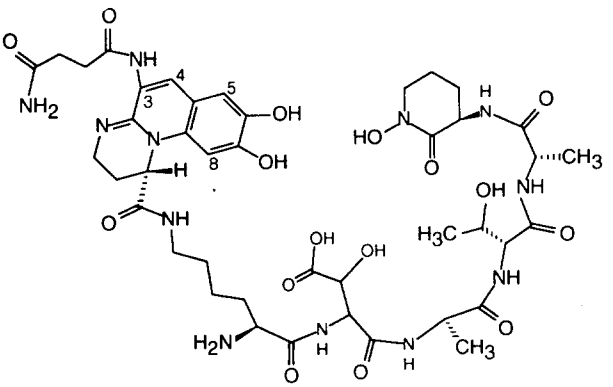
Several iron binding metabolites (siderophores) of *Pseudomonas fluorescens* B10 (JL-3133) have been detected using C_{18} reverse phase HPLC coupled with photodiode array detection methods. This analysis utilized a volatile mobile phase of 90% 20 mM NH_4HCO_3/10% MeOH, pH 6.5. It has been shown to be applicable to other *P. fluorescens* strains for the detection of related metabolites. Direct scale-up of the analytical HPLC conditions allowed for the efficient preparative isolation of pseudobactin, the principle siderophore produced by *P. fluorescens* B10 (JL-3133).

Keywords: HPLC, pseudobactin, *Pseudomonas fluorescens*, siderophores

Introduction

The iron-binding, fluorescent pigments produced by a number of *Pseudomonas* species (Budzikiewicz 1988, Abdallah 1991) have attracted attention for their possible role in the biological control of a number of plant pathogens (Kloepper *et al.* 1980, Kloepper and Schroth 1981a,b, Buyer & Leong 1986, Mishaghi *et al.* 1988). These metabolites are secreted by many Pseudomonads when cultures are deprived of available forms of iron (Stainer *et al.* 1966). The first of these metabolites to be completely characterized structurally was pseudobactin (Teintze *et al.* 1981), **1**, which was isolated from *Pseudomonas fluorescens* B10. Its structure was solved by X-ray crystallography, and it has since become the model structure for a number of related *Pseudomonas* metabolites, collectively referred to as the pyoverdins and pseudobactins. Numerous pseudobactins and pyoverdins have since been isolated and characterized (Budzikiewicz 1988, Demange *et al.* 1990, Persmark *et al.* 1990), and a variety of methods have been used for their isolation and purification (Yang & Leong 1984, Buyer *et al.* 1986, Demange *et al.* 1987, 1988, 1990, Persmark *et al.* 1990).



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In preparation for studies of pseudobactin biosynthesis, we found it advantageous to develop a rapid HPLC assay to monitor the number and quantities of fluorescent metabolites being produced by *P. fluorescens* B10. We now report results that demonstrate the general utility of this assay protocol for resolving mixtures of such compounds from B10 and a variety of other Pseudomonads. We also report an efficient and reliable purification of **1** which makes use of these HPLC conditions.

Materials and methods

General

HPLC analysis was performed using a Waters 600E chromatograph equipped with a Waters 990+ UV photo-

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diode array detector. Spectral data was processed using version 5.02 of Waters 990+ software. Analytical samples were centrifuged in an Eppendorf 5414 microcentrifuge. Water used for this study was deionized with a Milli-Q (Millipore) water purification system. All glassware used for fermentations or which came in contact with the deferrated pseudobactin was soaked in aqueous 6 M HCl (12–24 h). Chromatographic resins were obtained either from Sigma (St Louis, MO) or BioRad (Richmond, CA).

Maintenance of organism

P. fluorescens B10 (JL-3133) was obtained from Dr Joyce Loper (USDA-ARS, Corvallis, OR) and stored at −80 °C, in 1 ml suspensions of sterile Difco Nutrient Broth containing either 7% dimethylsulfoxide or 15% glycerol. Frozen cells were viable for at least 1 year.

Fermentation conditions

To initiate growth of *P. fluorescens* B10 (JL-3133), a frozen culture was plated on sterile Kings Medium B agar (King *et al.* 1954) and incubated for 2–3 days at 26 °C. The culture was transferred to seed broth (2 ml of Kings Medium B) contained in a 17 × 150 mm culture tube and incubated at 21 °C in an orbital shaker with a 1 inch throw (150 r.p.m.) for 24 h. A 250 ml Erlenmeyer flask containing sterile minimal medium (Loper & Lindow 1987) (50 ml of the following solution: 0.13% $(NH_4)_2SO_4$, 0.025% $MgSO_4 \cdot 7H_2O$, 1.0% glycerol, 1.0% Difco casamino acids, 0.40% HEPES buffer, 0.95% K_2HPO_4, 0.30% KH_2PO_4, pH 6.9) was then inoculated with 0.5 ml of the seed culture and incubated as specified above (24 h). Aliquots of this culture (1 ml) were subsequently added to the minimal medium (300 ml) contained in several 2 l Erlenmeyer flasks and incubated as above (42 h). Pseudobactin was first detected in the culture 12 h after inoculation, and reached maximum production (45–55 mg l^{-1}) between 42 and 45 h.

Pseudobactin assay

Pseudobactin was detected as the ferric complex by HPLC analysis on a Waters Nova-Pak C_{18} radial compression cartridge (0.8 × 10 cm, 4 μm particle size) eluted with a buffer comprised of 90% 20 mM NH_4HCO_3/10% MeOH, adjusted to pH 6.5 with acetic acid. The flow rate was 1.25 ml min^{-1} (t_R = 11.2 min). The UV absorbance of the column effluent was monitored at both 254 and 400 nm (2 nm interval, sampled at 2 s intervals). Samples were compared to a standard curve constructed using five serial dilutions of purified ferric pseudobactin (30, 60, 90, 120 and 150 mg l^{-1}).

Production profiles of related Pseudomonads

Cultures of *P. fluorescens* strains JL-2000, JL-3551, and JL-4312 were grown using identical media formulations and conditions as described above. However, aliquots (1 ml) of the minimal media culture contained in the 250 ml flasks were added to minimal medium (200 ml) contained in 1 l Erlenmeyer flasks. Analytical samples were prepared from aliquots of broth (1 ml). The sample was ferrated with $Fe(NO_3)_3 \cdot 9H_2O$ solution (10 μl, 500 mg in 5 ml H_2O), vortexed, allowed to settle (5 min) and centrifuged (2 min). Analysis of the supernatant was performed using the HPLC conditions cited above.

Isolation of ferric pseudobactin from strain JL-3133

$FeCl_3$ (1 g l^{-1}) was added to the yellow-green production broth (1 l) 42 h after inoculation, and the mixture was stirred for 5 min. Removal of solids by centrifugation (10 min, 13 800 × *g*, 4 °C) afforded a clear, red-brown supernatant. Amberlite XAD-4 resin (100 g l^{-1} of broth) previously rinsed with 50% aqueous ethanol (200 ml) followed by H_2O (200 ml) was added to the supernatant and the mixture slowly stirred. After almost all color had been absorbed onto the resin (4–9 h), the slurry was poured into a glass column (5 cm diameter) and washed with H_2O (about 750 ml). The colored pigments were then eluted from the resin with an 80% acetone/20% water solution (about 400 ml). The solvent was removed by rotary evaporation (25–30 °C) and lyophilization, which provided a dark brown powder (yield about 350 mg l^{-1}).

The brown powder was dissolved in a minimal volume (about 12 ml) of 0.1 M aqueous pyridinium acetate buffer, pH 6.5. This concentrate was loaded onto Sephadex CM-25 resin (pyridinium form, 2.5 × 100 cm) and eluted isocratically in the same buffer at a flow rate of 20–30 ml h^{-1}. Ferric pseudobactin was contained in the last brown band to elute from the column. This band was distinguished by the presence of a partially resolved, forerunning purple component. The red-brown fractions were collected and the solvent was removed by high vacuum rotary evaporation (25–30 °C) followed by lyophilization (yield about 50 mg l^{-1}).

This sample was dissolved in a minimal amount (about 30 ml) of 90% 20 mM NH_4HCO_3/10% MeOH buffer, pH 6.5 (adjusted with CO_2) and filtered (0.45 μm). Pure ferric pseudobactin was isolated by preparative HPLC using a Waters Nova-Pak C_{18} radial compression cartridge (2.5 × 10 cm, 6 μm particle size) eluted with the same buffer. The flow rate was maintained at 14 ml min^{-1}. The effluent was monitored for absorbance at 254 nm and fractions were collected manually. Ferric pseudobactin was eluted in the last, red-brown band (t_R = 14 min). The pooled HPLC fractions were rotary evaporated and lyophilized (yield about 25–35 mg l^{-1}).

Isolation of pseudobactin

In a 125 ml Erlenmeyer flask fitted with a ground glass stopper and a stir bar, purified ferric pseudobactin (25–35 mg) was dissolved in deionized water (about 10–15 ml) and the mixture extracted following a reported method (Meyer & Abdallah 1978). The 8-hydroxyquinoline used in this procedure was first recrystallized from hot ethanol and water, dried, dissolved in $CHCl_3$ (5% w/v),

and further purified by repeated extraction with a 10% volume of deionized water. Extraction of the ferric pseudobactin was repeated until the chloroform solution ceased to turn green. The bright yellow, UV-active aqueous solution was then rotary evaporated and lyophilized with protection from light (aluminum foil) during lyophilization.

A glass column packed with BioRad P-2 size exlusion gel (1.5 × 50 cm) equilibrated in water was first treated with aqueous 1% EDTA (200 ml) and then rinsed with Milli-Q water (1 l). The pseudobactin was dissolved in a minimal volume of water (about 1 ml), applied to the BioGel P-2 resin and eluted with 500 ml of Milli-Q water at a flow rate of 5 ml h^{-1}. The yellow–green, UV-active fractions were combined and lyophilized to yield pure pseudobactin (typical yield 10–15 mg/l).

Results and discussion

For biosynthetic feedings, it was necessary first to develop a fast and reliable assay to monitor the timing of pseudobactin production. This assay could also then be used to monitor its purification. To avoid possible problems stemming from the iron scavenging ability of pseudobactin, an analysis was developed for the ferrated siderophore. Analytical HPLC using C_{18} reverse phase silica and spectrometric detection at both 254 and 400 nm provided for resolution and detection of ferric pseudobactin from the sample matrix. Reverse phase C_{18} HPLC using triethylamine acetate buffer and acetonitrile as the mobile phase (Yang & Leong 1984) was tried first. This solvent system did resolve ferric pseudobactin from the sample matrix, but the analysis time was in excess of 30 min. However, isocratic elution with a mobile phase of 20 mM ammonium bicarbonate:methanol (9:1) adjusted to pH 6.5 with CO_2 was found to provide base-line resolution of ferric pseudobactin and an analysis time of less than 12 min. While this gave reproducible retention times over short periods, over the course of several hours the retention times of late eluting compounds changed significantly (less than 2 min). This was attributed to the observed increase in the buffer pH (from 6.5 to 7.3) resulting from sparging of the solvent reservoir. The retention times were stabilized by adjusting the pH to 6.5 using acetic acid and by decreasing the sparging rate. However, for preparative work, adjustment of the pH with CO_2 was preferable (see below).

Samples from a *P. fluorescens* B10 fermentation were ferrated with a solution of $Fe(NO_3)_3$ and centrifuged to remove suspended solids. Aliquots of the supernatant were then analyzed directly by HPLC. Results from HPLC analysis of a typical supernatant are illustrated in Figure 1. Using this protocol it was found that *P. fluorescens* B10 produced more metabolites which contained the pseudobactin chromophore than were anticipated from the assays using triethylamine acetate buffer. By using a photodiode array HPLC detector, the complete UV spectrum of each metabolite was obtained, and the metabolites which eluted at 5.18, 7.02, 7.95 and 9.15 min all exhibited UV spectra similar to ferric pseudobactin, which was the major component, eluting at 11.22 min.

Although only pseudobactin and pseudobactin A (Teintze & Leong 1981), in which the $\Delta^{3,4}$-double bond is saturated, were reported to be produced by *P. fluorescens* B10, it appears that several other related compounds are produced, with the structural differences probably residing in the peptide chain. In a recent study on ferrioxamine siderophores (Konetschny-Rapp *et al.* 1992), homologs of biosynthetic precursors administered to the fermentation induced structural variations in the siderophores. A similar enzymatic flexibility in *P. fluorescens* could account for the various siderophores.

The versatility of this photodiode array HPLC analysis methodology was demonstrated by screening fermentations of several *P. fluorescens* strains. Strains JL-4312, JL-3551 and JL-2000 were grown for 42 h, and aliquots treated as described above. The results are shown in Figure 2 as chromatograms detected at 400 nm. All of the prominent peaks (Abs. > 0.009) in these chromatograms had UV spectra virtually identical to that of ferric pseudobactin. Thus, this methodology should be useful in screening other Pseudomonads for production of pseudobactin/pyoverdin-type siderophores.

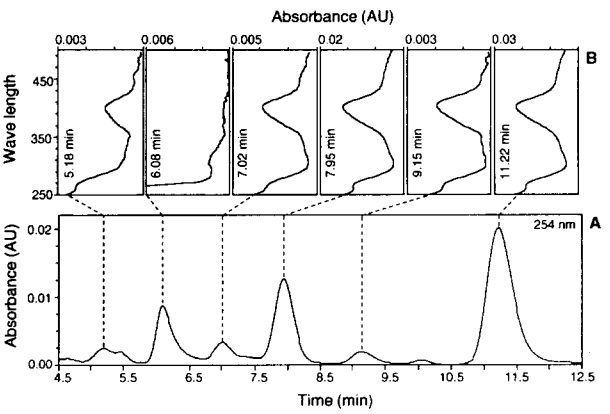

Figure 1. (**A**) HPLC production profile detected at 254 nm for *P. fluorescens* B10. The peak at 11.2 min is ferric pseudobactin. (**B**) UV spectra of individual components, obtained with photodiode array detection.

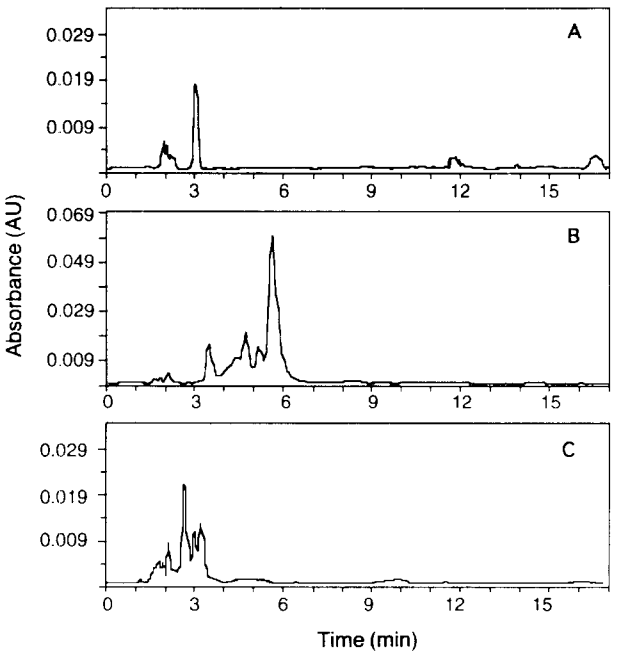

Figure 2. HPLC chromatograms detected at 400 nm for *P. fluorescens* strains JL-2000 (**A**) JL-3551 (**B**) and JL-4312 (**C**). HPLC conditions were identical to those used for the chromatogram shown in Figure 1.

Isolation of pseudobactin

Virtually all pseudobactins and pyoverdins have been first isolated as the ferric complex and then deferrated as the last step in the purification. The propensity of pseudobactins to scavenge iron would complicate isolation of the desferric siderophore; furthermore, the ferrated siderophore is more stable to a variety of manipulations.

Initial attempts to extract ferric pseudobactin from *P. fluorescens* B10 (JL-3133) using previously reported methods with either a chloroform:phenol mixture (Philson & Llinás 1982) (1:1, v/v) or benzyl alcohol (Teintze *et al.* 1981) were not sufficiently selective for the ferrated complexes and, in addition, the mixtures tended to emulsify. Amberlite XAD resins which have recently been used for isolating fungal siderophores (Konetschny-Rapp *et al.* 1988) were tested. Passage of the ferrated broth from *P. fluorescens* B10 through a column containing Amberlite XAD-4 resin provided low recovery of the iron complexes. However, if the resin was stirred with the ferrated broth (100 $g l^{-1}$) for several hours, almost all of the pigments were adsorbed. The XAD resin exhibited a high specificity towards the complexed siderophores, and the red-brown ferric complexes were subsequently eluted from the resin with an acetone:water mixture (8:2, v/v).

The material obtained from the XAD-4 was resolved into four distinct fractions with Sephadex CM-25 ion exchange resin (pyridinium form) eluted with 0.1 M pyridinium acetate buffer, pH 6.5. The first fraction was red-brown and contained compounds with UV spectra virtually identical to the published spectrum of ferric pseudobactin. Analysis by HPLC determined this fraction to be enriched in the metabolite eluting at 7.95 min in the original assay. The second red-brown fraction was a minor component and therefore was not analyzed by HPLC. The third fraction consisted of two partially resolved bands: a purple front-running region and a red-brown tailing region. The red-brown tailing region was shown to consist primarily of ferric pseudobactin by analytical HPLC co-injection with an authentic sample and subsequently by ^{1}H and ^{13}C NMR analysis of the deferrated product after further purification (see below). It was inferred from the original isolation procedure (Teintze *et al.* 1981, Teintze & Leong 1981) that the front-running purple component contained ferric pseudobactin A. The fourth fraction collected was yellow; it eluted from the ion exchange column well behind the others. Although ion exchange did not completely resolve ferric pseudobactin and ferric pseudobactin A, it did provide an efficient method for removing other siderophores from the sample.

Final purification of ferric pseudobactin was obtained by scale-up of the analytical HPLC conditions. In this case, pH adjustment with CO_2 did not present problems and it provided a more volatile buffer for recovery of ferric pseudobactin. Preparative HPLC was the only method found which provided efficient separation of ferric pseudobactin (t_R = 11.22 min) and ferric pseudobactin A (t_R = 6.08 min).

Ferric pseudobactin was deferrated with 8-hydroxyquinoline using a literature procedure (Meyer & Abdallah 1978). However, maintenance of the pH between 3.5 and 4.0 and vigourous stirring were found to be critical factors for efficient removal of iron from the ferrated complex. Purification of 8-hydroxyquinoline was required prior to the deferration step either by recrystallization of the 8-hydroxyquinoline from hot ethanol and water, or by repeated extraction of the chloroform solution with water. Final purification of **1** was obtained by chromatography of the sample on a BioRad P-2 size exclusion column eluted with water. Removal of water by lyophilization afforded pure pseudobactin, adequate for spectroscopic analysis.

Conclusions

The HPLC analysis for pseudobactin described herein provides advantages over other techniques in that it requires minimal sample preparation, and is efficient and reliable. It has also been shown that when this analytical procedure is coupled with photodiode array detection methods, other *P. fluorescens* strains may be successfully screened for the production of pseudobactin/pyoverdin-type compounds. The HPLC conditions were easily scaled up for preparative isolation of pseudobactin. Persmark *et al.* (1990) recently reported a similar method in the isolation of pseudobactin 589A from a *P. putida* strain. Thus the protocol may be useful for isolation of other *Pseudomonas* siderophores, although adjustments in the strength of the mobile phase used in the preparative HPLC step may be necessary.

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