Isolation and Characterization of Pseudobactin B: A Pseudobactin-Type Siderophore from *Pseudomonas* Species Strain PD 30†

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A simple method is discussed for the isolation of pseudobactin B, a new siderophore produced together with pseudobactin by plant growth promoting bacterium *Pseudomonas* PD 30. Pseudobactin B differs from the known pseudobactin only by the nature of the dicarboxylic acid attached to the chromophore, which is succinic acid instead of succinamide. The structure of pseudobactin B was determined by a comparison of its ^{1}H NMR, UV, and mass spectra with those of pseudobactin. An HPLC assay for pseudobactin B and pseudobactin, quantification in fermentation beer, and a new efficient method for isolating them are proposed.

INTRODUCTION

The *Pseudomonas* producing siderophores have been studied quite intensively for their ability to stimulate plant growth and to control phytopathogenic microorganisms (Neiland and Leong, 1986; Loper and Schroth, 1986; Schroth and Hancock, 1982; Schippers et al., 1987; Marugg et al., 1988). The function of siderophores is to scavenge Fe(III) and then transport it to the cell, in which a system for high-affinity uptake is present (De Weger et al., 1988; Hohnadel and Meyer, 1986; Leong, 1986; Kloepper et al., 1980).

In the course of our screening program to discover *Pseudomonas* species with beneficial activity on crop plants, one isolate, *Pseudomonas* species PD 30 (Donegani collection), produced two fluorescent siderophores when cultured in an iron-deficient medium. These were isolated and then identified as pseudobactin and a pseudobactin-type siderophore, called pseudobactin B, which differs from pseudobactin only by the nature of the dicarboxylic acid (Figure 1). Pseudobactin, a fluorescent siderophore produced by the plant growth promoting bacterium *Pseudomonas* B10 (Teintze et al., 1981; Teintze and Leong, 1989), has a structure formed by a linear hexapeptide, a substituted 2,3-diamino-6,7-dihydroxyquinoline nucleus as Fe(III) ligands, an α-hydroxycarboxylate function, and a terminal cyclic hydroxamic acid (Figure 1).

The method described by Teintze et al. (1981) for isolating pseudobactin required many purification steps and provided the complex of pseudobactin with Fe(III), which subsequently needed to be deferrated in a further step (Meyer and Abdallah, 1978).

This paper describes a new simple method for the recovery of these compounds from the fermentation broth and their structural characterization. This paper also describes a quantitative HPLC assay for pseudobactin B and pseudobactin, either in fermentation beer or in formulation samples.

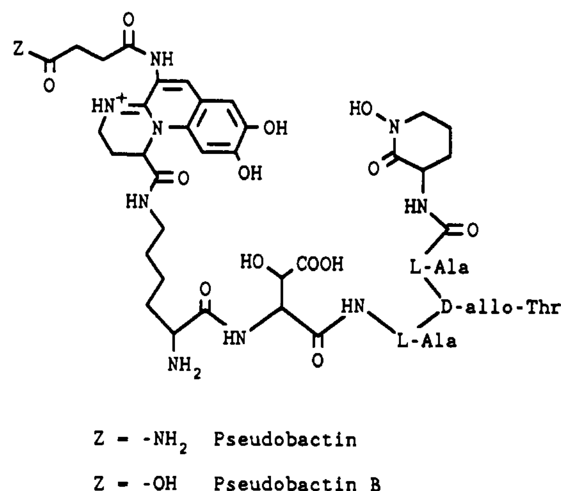

Figure 1. Structures of pseudobactin and pseudobactin B.

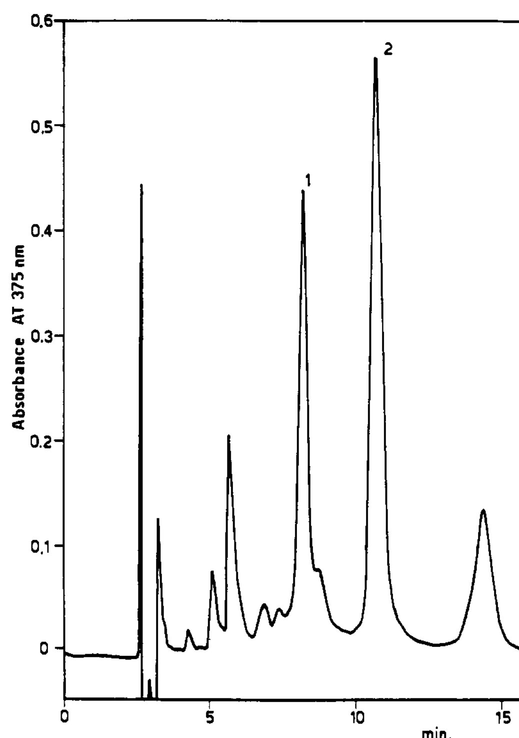

Figure 2. HPLC chromatogram of PD 30 cultured broth (10-μL injection): (1) pseudobactin; (2) pseudobactin B. Conditions are given under Materials and Methods.

MATERIALS AND METHODS

1. Isolation of *Pseudomonas* Species Bacteria Strain PD 30. The siderophore producing bacterial strain was isolated,

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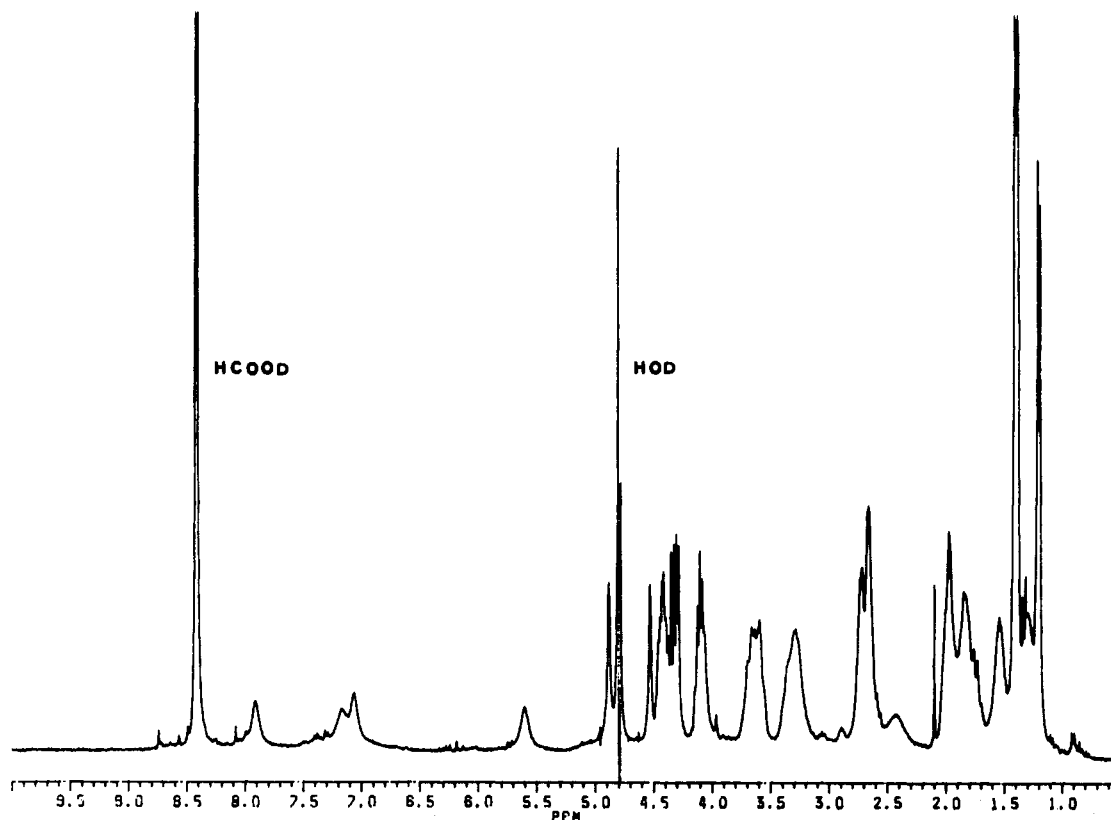

Figure 3. Proton NMR spectrum (300 MHz) of pseudobactin B in D_2O. Chemical shifts are relative to the HOD signal at 4.85 ppm.

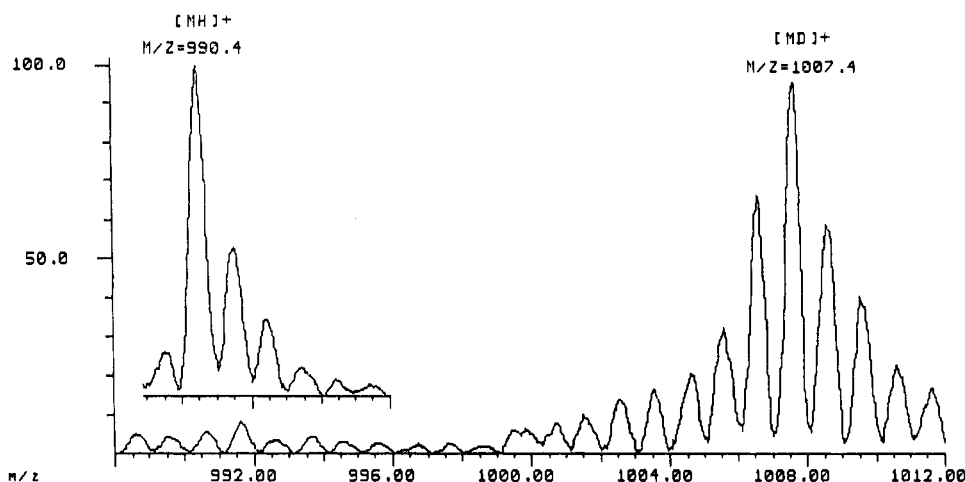

Figure 4. FAB spectra of pseudobactin B before and after hydrogen/deuterium exchange.

during the growing season, from the rhizosphere of maize grown in a field located in northern Italy. The isolation was carried out by plating serial dilutions of a suspension obtained by shaking a sample of small secondary roots and attached soil in a 0.01 M phosphate buffer at pH 7.0. The low-iron medium, *Pseudomonas* agar F (Difco Laboratories, Detroit, MI), selective for *Pseudomonas* species, was used. The PD 30 strain was selected for its in vitro high activity in controlling the growth of several plant pathogen fungi such as *Phytium ultimum*, *Sclerotium rolfsii*, and *Phoma betae* (data not published).

2. Growth Conditions for Siderophore Production. For the siderophore production, the *Pseudomonas* species strain PD 30 was grown on a synthetic succinate medium (Meyer and Abdallah, 1978) with the following composition: K_2HPO_4 (6.0 g/L), KH_2PO_4 (3.0 g/L), $(NH_4)_2SO_4$ (1 g/L), $MgSO_4{\cdot}7H_2O$ (0.2 g/L), succinic acid disodium salt (4.0 g/L). The pH was adjusted to 7.0 by addition of 1 M NaOH solution prior to sterilization. Five liters of medium was dispensed into 50 500-mL Erlenmeyer flasks, each containing 100 mL of medium. These were then inoculated, at a seed rate of 2%, with exponential phase cells grown in the same medium. Incubation was carried out at 28 °C on a rotary shaker at 180 rpm for 30 h. At harvesting time, batches were pooled together and centrifuged, and pelleted cells were discarded.

The siderophore production was estimated by absorbance of the supernatant liquid at 375 nm.

3. Isolation and Identification of Pseudobactin B. Cells of *Pseudomonas* species PD 30 were separated from the culture broth at pH 8.4 by centrifugation in a tubular bowl centrifuge. The clarified broth (4.5 L) was ultrafiltered through a membrane IRIS 3038 with a molecular weight cutoff of 20 000, mounted on a Pleiade UFP2 system (Rhone Poulenc), until only 0.4 L remained in the concentrate. The concentrate was diluted to 1.4 L with water and the ultrafiltration was repeated; the second concentrate was discarded.

The two filtrates (5.5 L) were acidified to pH 4.4 by phosphoric acid and then passed over a 4.4 × 30 cm column of XAD-4 resin (Rohm and Haas Co., Philadelphia) pre-equilibrated with a solution of H_3PO_4 (pH 4.0) at a flow rate of 5.5 mL/min.

The column was washed with 0.5 L of deionized water and eluted with a linear gradient of acetonitrile/water from 0:100 to

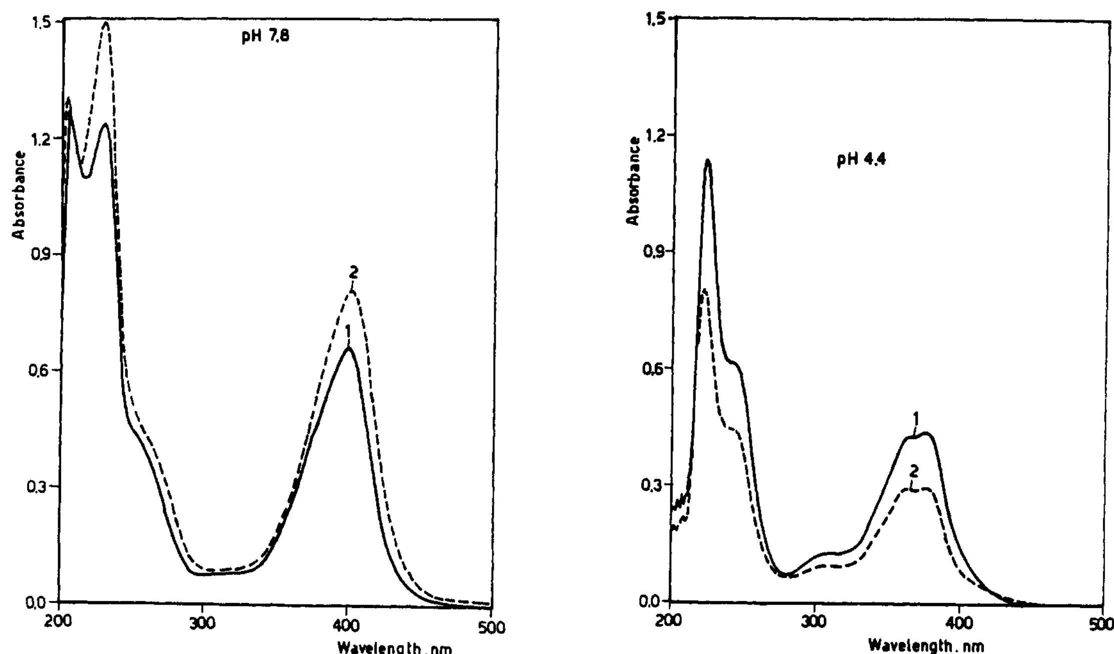

Figure 5. UV spectra of pseudobactin (1) and pseudobactin B (2) at pH 7.8 and 4.4.

60:40 (total volume of eluent was 2.5 L). The fractions containing the siderophores, selected on the basis of their UV spectra, were pooled together and concentrated under vacuum to dryness. The residue was dissolved in water (5 mL), and the siderophores were precipitated from the solution by dilution with acetone (15 mL). The precipitate was separated by centrifugation. The resulting residue was dissolved in 2 mL of 0.02 M ammonium formate and then loaded onto a 3.1 × 45 cm column of Fraktogel TSK HW 40(F) (E. Merck) pre-equilibrated with the same solution which was used for the elution at a flow rate of 0.2 mL/min. Six-milliliter fractions were collected, and those containing pseudobactins (determined by HPLC) were pooled and dried under vacuum. The dark brown residue was taken up in 2 mL of 0.02 M ammonium formate and the chromatography repeated. Pseudobactin B was eluted in 24 mL in the fractions from 18 to 21, while pseudobactin was eluted in 24 mL in the fractions from 28 to 31.

The pooled fractions were lyophilized to produce 100 mg of pseudobactin B and 55 mg of pseudobactin, both >95% pure by HPLC. ^{1}H NMR spectra were obtained at 300 MHz on a Bruker AM 300 using D_2O as solvent. FAB-MS spectra were recorded on a Finnigan 8400 double-focusing mass spectrometer operating at an accelerating potential of 3 kV. Xenon was used as the bombarding gas with a M-SCAN atom gun operated at 9 kV and 1 mA. Glycerol was used as the FAB matrix. The H/D exchange experiments were performed by adding a drop of D_2O (99.9% atom deuterium, Aldrich Chemical Co.) to the sample on the FAB tip under a gentle stream of argon. The water was then allowed to evaporate in the forevacuum chamber of the instrument. The entire operation was repeated three times before analysis. The actual level of deuterium incorporation in the sample, calculated according to the method of Verma et al. (1986), was 96%.

4. **Liquid Chromatography.** HPLC assays were performed on a cartridge column (Aquapore octyl RP 300, 220 × 4.6 mm, Pierce) equipped with a guard cartridge RP 8 (15 × 3.2 mm) maintained at 40 °C. A mobile phase consisting of 95% (v/v) 0.1% (v/v) aqueous trifluoroacetic acid and 5% (v/v) acetonitrile was used at a flow rate of 1 mL/min. The chromatograms were monitored at 375 nm, and the spectra were analyzed with a photodiode array detector (Waters); samples were filtered through a 0.45-μm disposable cellulose acetate filter FP 030/20 (Schleicher & Schuell, Dassel, FRG) and then injected into the HPLC. The instrumentation included two M510 pumps, a U6K injector, a 990 S photodiode array detector, an RCM-100 column chamber (all from Millipore/Waters, Milford, MA), and an APC IV computer (NEC Corp., Tokyo).

RESULTS AND DISCUSSION

1. **Isolation of Pseudobactin and Pseudobactin B.** The recovery method was very efficient and supplied the pseudobactin B and pseudobactin with an overall yield of 50% and a purity over 95%. The ultrafiltration step was used to eliminate high molecular weight proteins, polysaccarides, and cell debris and also to remove a considerable amount of the dark brown pigment associated with the fermentation product. Such a step of cleaning the fermentation broth definitely extends column life and reproducibility in the chromatographic procedures and often leads to a better resolution of desired compounds.

The chromatography media can be reused for many runs without loss of efficiency with just a final washing with an appropriate solution, especially during the size exclusion chromatographic step.

2. **HPLC Assay.** A typical HPLC chromatogram of pseudobactin B and pseudobactin in the cultured broth of *Pseudomonas* PD 30 is shown in Figure 2. Detector response was linear over a 0.0–20 mg/mL range (injections of 10 μL), and the response vs concentration curve passed through the origin.

3. **Characterization of Pseudobactin and Pseudobactin B.** Pseudobactin was unequivocally characterized by ^{1}H NMR and UV spectra (Figure 5) and both positive and negative ion FAB-MS mass spectra. These data were consistent with the structure of the known antibiotic pseudobactin cited above (Teintze and Leong, 1981; MacDonald and Bock, 1987; Meyer and Abdallah, 1978). Fast atom bombardment mass spectrometry of pseudobactin B showed ions at m/z 990 $[M + H]^+$ and at m/z 988 $[M - H]^-$, as the base peaks, respectively, in the positive and negative ion spectra, while pseudobactin showed ions at m/z 989 $[M + H]^+$ and at m/z 987 $[M - H]^-$. These results established a monoisotopic molecular mass of 989 Da for pseudobactin B, which is 1 mass unit greater than that for pseudobactin. The ^{1}H NMR spectrum of pseudobactin B, measured in D_2O, showed the same chemical shifts and multiplicity of the corresponding unexchangeable hydrogens of pseudobactin (Figure 3); this similarity implies that the structural difference between pseudobactin and

pseudobactin B must involve a portion of the molecule bearing only exchangeable hydrogens.

This assumption was confirmed by FAB-MS experiments, carried out after the sample (previously dissolved in glycerol) was treated with D_2O (Guarini et al., 1992). Because of the H/D exchange of labile hydrogens, the base peak in the positive ion spectrum of pseudobactin B shifted to m/z 1007 $[M + D]^+$ (Figure 4). This result clearly points out that only 16 exchangeable hydrogens are present in the neutral molecule, while pseudobactin has 17.

The UV spectra of pseudobactin B (Figure 5) were very similar to that of pseudobactin (MacDonald and Bock, 1987; Meyer and Abdallah, 1978) and pyoverdine (Briskot et al., 1989) and confirmed the presence of the same chromophore group.

Because of the similarity of the analytical data, the most consistent hypothesis for the structure of pseudobactin B is the replacement of an NH_2 group (16 mass units) in pseudobactin with an OH group (17 mass units) in pseudobactin B. This accounts for both the molecular weight and the number of labile hydrogens. Also, this modification is possible only at the terminal succinamide group ($-CONH_2$) of pseudobactin. Therefore, pseudobactin B is a new siderophore different from the known pseudobactin only in the nature of the terminal dicarboxylic acid residue.

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Registry No. Pseudobactin B, 138145-39-8; pseudobactin, 76975-04-7.