Structure of Pseudobactin 7SR1, a Siderophore from a Plant-Deleterious *Pseudomonas*†

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ABSTRACT: When grown in iron-limiting culture medium, sugar beet deleterious *Pseudomonas* 7SR1 produced extracellularly the yellow-green, fluorescent siderophore pseudobactin 7SR1. Pseudobactin 7SR1 had a molecular formula of $C_{46}H_{63}N_{13}O_{23}$ and a molecular mass of 1166 g/mol. Pseudobactin 7SR1 contained a cyclic octapeptide with the amino acid sequence L-Ala-Gly-Ser-Ser-*threo*-β-OH-Asp-Thr-Ser-N^{δ}-OH-Orn. Since pseudobactin 7SR1 was not affected by nonspecific enzymes, it might contain D-amino acids. A yellow-green, fluorescent quinoline derivative is postulated to be attached via an ester bond to the serine residue following the glycine. A malamide group was attached to carbon 3 of the quinoline derivative. The three bidentate iron(III)-chelating groups consisted of an α-hydroxy acid group derived from β-hydroxyaspartic acid, an *o*-dihydroxy aromatic group derived from the yellow-green, fluorescent chromophore, and a hydroxamate group derived from N^{δ}-acetyl-N^{δ}-hydroxyornithine. The chemical structure of pseudobactin 7SR1 is remarkably similar to that of pseudobactin, the siderophore of plant growth promoting *Pseudomonas* B10.

Specific rhizosphere-colonizing strains of the *Pseudomonas fluorescens–Pseudomonas putida* group have recently been used as seed inoculants on crop plants to promote growth and increase yields. These fluorescent pseudomonads, generically termed plant growth promoting rhizobacteria (PGPR),[1] rapidly colonize plant roots of potato, sugar beet, and radish and cause statistically significant yield increases of up to 144% in field tests (Schroth & Hancock, 1982). Enhanced plant growth caused by PGPR is accompanied by reductions in root-zone fungal and bacterial populations. PGPR exert their plant growth promoting activity in part by depriving certain native microflora of iron and thereby reducing microbial root colonization (Kloepper et al., 1980b). PGPR produce under iron-limiting conditions extracellular siderophores (microbial iron-transport agents) (Neilands, 1981) that efficiently complex environmental iron, making it less available to certain endemic microorganisms and thus inhibiting their growth. In addition, plant growth promoting *Pseudomonas* B10 is an effective biological control agent of *Fusarium* wilt and take-all diseases, caused by the soil-borne fungi *Fusarium oxysporum* f. sp. *lini* and *Gaeumannomyces graminis* var. *tritici*, respectively (Kloepper et al., 1980a). Pseudobactin, the yellow-green, fluorescent siderophore of *Pseudomonas* B10, apparently denies iron(III) to these pathogens, thus inhibiting their growth.

The molecular structure of pseudobactin consists of a linear hexapeptide, L-Lys-D-*threo*-β-OH-Asp-L-Ala-D-*allo*-Thr-L-Ala-D-N^{δ}-OH-Orn, in which the N^{δ}-OH nitrogen of the ornithine is cyclized with the C-terminal carboxyl group and the N^{ϵ}-amino group of the lysine is linked via an amide bond to a fluorescent quinoline derivative (Teintze et al., 1981) (Figure 1). The iron-chelating groups consist of a hydroxamate group derived from N^{δ}-hydroxyornithine, an α-hydroxy acid from β-hydroxyaspartic acid, and an *o*-dihydroxy aromatic group derived from the quinoline moiety. The combination of metal-chelating ligands and the alternating L- and D-amino acids is unusual.

The microflora component that is inhibited by PGPR includes certain strains of root-colonizing bacteria that significantly decrease plant growth of sugar beet, bean, or lettuce seedlings and increase susceptibility of roots to infection by fungi (Suslow & Schroth, 1982a). These bacteria were termed deleterious rhizobacteria and were shown to be a major component of the bacterial microflora of field-grown sugar beet, bean, or lettuce roots. The genera of deleterious rhizobacteria include *Pseudomonas*, *Enterobacter*, *Klebsiella*, *Citrobacter*, *Flavobacterium*, *Achromobacter*, and *Arthrobacter* (Suslow & Schroth, 1982a,b). Enhanced plant growth by PGPR was related in part to inhibition of and reduced colonization of deleterious rhizosphere bacteria, not previously recognized as plant pathogens (Suslow & Schroth, 1982a,b). Thus, deleterious rhizobacteria may in some ways be as important as the widely recognized major root pathogens in reducing crop yield. Deleterious fluorescent pseudomonads, closely related to phytopathogenic *Pseudomonas syringae*, are of particular interest since they may produce phytotoxins (Leisinger & Magraff, 1979), which are responsible in part for disease. As part of a study to understand the microbial antagonism of PGPR against deleterious rhizobacteria, we describe the isolation and structural characterization of pseudobactin 7SR1, the siderophore of *Pseudomonas* 7SR1, a specific fluorescent pseudomonad deleterious to sugar beet.

Experimental Procedures

Materials. Phenyl isothiocyanate (PITC) (sequenation grade), polyamide sheets (Cheng Chin), dansyl chloride, DL-*threo*-β-hydroxyaspartic acid, and N^{δ}-hydroxyornithine

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[1] Abbreviations: PGPR, plant growth promoting rhizobacteria; β-OH-Asp, β-hydroxyaspartic acid; N^{δ}-OH-Orn, N^{δ}-hydroxyornithine; PITC, phenyl isothiocyanate; BSA, *N,O*-bis(trimethylsilyl)acetamide; KB, King's medium B; NMR, nuclear magnetic resonance; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography; GC–MS, gas chromatography–mass spectrometry; PTH, 3-phenyl-2-thiohydantoin; EDDA, ethylenediaminedi[(*o*-hydroxyphenyl)acetic acid]; R_f, rate of flow; Me_2SO, dimethyl sulfoxide; ppm, parts per million; NOE, nuclear Overhauser effect; Me_4Si, tetramethylsilane; DSS, 3-(trimethylsilyl)propanesulfonic acid sodium salt.

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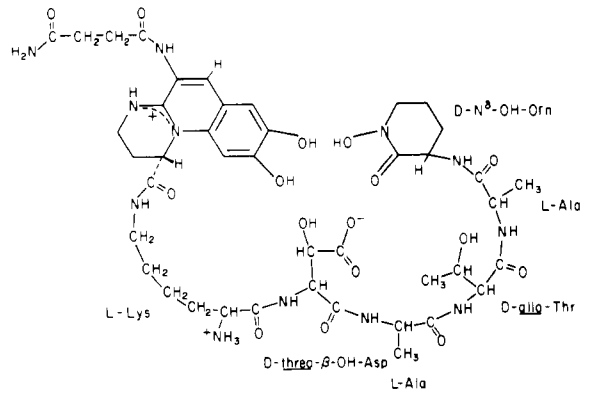

FIGURE 1: Schematic drawing of pseudobactin.

(N^{δ}-OH-Orn) were obtained as described previously (Teintze et al., 1981). Desferal (deferriferrioxamine B methanesulfonate) was a gift from Ciba-Geigy; pentaacetylglucose was obtained from E. Ng. *N,O*-Bis(trimethylsilyl)acetamide (BSA) was obtained from Applied Science Laboratories, Inc. Trifluoroacetic acid and pyridine were distilled prior to use.

Isolation of Ferric Pseudobactin 7SR1. *Pseudomonas* 7SR1 was obtained from Dr. M. N. Schroth and was maintained on King's medium B (KB) plates (King et al., 1954). An iron-deficient minimal medium consisting of 1.5 g of KH_2PO_4, 3 g of Na_2HPO_4, 1 g of NH_4Cl, and 10 mL of glycerol/L, and made 0.025% (w/v) in $MgSO_4 \cdot 7H_2O$ and 2% (w/v) in Casamino acids (Difco), was used for the production of pseudobactin 7SR1 from *Pseudomonas* 7SR1. A stock solution of Casamino acids was deferrated with 8-hydroxyquinoline as described previously (Bell et al., 1979).

A single colony of *Pseudomonas* 7SR1 was inoculated into 2 mL of KB medium, and the culture was shaken overnight at room temperature. Approximately 0.5 mL was transferred to 50 mL of minimal medium, and the culture was shaken overnight at room temperature. Approximately 5 mL each was transferred to eight 1-L portions of minimal medium, and the cultures were shaken at room temperature. The pH of the cultures was adjusted to 7 with 1 N HCl after 1 day, and all cultures were harvested by centrifugation after a total of 2 days. After approximately 1 g of $Fe(NO_3)_3 \cdot 9H_2O$ was added per L of yellow-green, fluorescent culture supernatant fluids, the red-brown suspension was saturated with ammonium sulfate. The resulting slurry was extracted with chloroform–phenol (1:1 v/v) and further treated by using a previously described procedure (Mullis et al., 1971). The red-brown aqueous extracts were concentrated to dryness in vacuo below room temperature. The residue was dissolved in approximately 50 mL of 10 mM acetic acid–pyridine buffer, pH 6.0. This solution was chromatographed at 4 °C on a column (2.5 × 25 cm) containing DEAE-Sephadex A-25, acetate form, equilibrated in the same buffer. Elution with 1 L of a linear gradient from 10 to 300 mM acetic acid–pyridine buffer, pH 6.0, yielded a predominant red-brown band consisting of ferric pseudobactin 7SR1. A red-brown band adhering to the top of the column was shown to be a degradation product of ferric pseudobactin 7SR1 (data not shown). The ferric pseudobactin 7SR1 containing fraction was subjected to column chromatography on Bio-Gel P-2 as described previously (Teintze et al., 1981). The yield after gel filtration was typically 250 mg/L of culture supernatant fluids. The extinction coefficient of ferric pseudobactin 7SR1 at its absorption maximum was determined as described earlier (Teintze et al., 1981).

Pseudobactin 7SR1. Pseudobactin 7SR1 was obtained by deferration of ferric pseudobactin 7SR1 with 8-hydroxyquinoline as described previously (Teintze et al., 1981). After the yellow-green, fluorescent pseudobactin 7SR1 containing band from gel filtration chromatography was concentrated to dryness in vacuo, the residue was dissolved in a small volume of water. This solution was chromatographed at 4 °C on a short column containing DEAE-Sephadex A-25, acetate form, equilibrated in water. After the pseudobactin 7SR1 containing band was eluted with 0.05 M acetic acid–pyridine buffer, pH 3.6, the eluate was concentrated to dryness in vacuo, and the residue was stored under high vacuum at −70 °C away from light.

For nuclear magnetic resonance (NMR) and mass spectrometric experiments with pseudobactin 7SR1, ferric pseudobactin 7SR1 (see above) was further purified by high-pressure liquid chromatography (HPLC) on a Spectra-Physics SP8700 system with an Altex C-18 ultrasphere-ODS column (1.0 × 25 cm). The elution buffer employed consisted of 15% 120 mM triethylamine–acetic acid buffer, pH 6.5, 82% water, and 3% acetonitrile. Ferric pseudobactin 7SR1 eluted ahead of a minor (less than 10%) red-brown band. After the ferric pseudobactin 7SR1 containing band was concentrated to a viscous residue in vacuo, it was deferrated with 8-hydroxyquinoline and further treated as described above with the following exception. In between the gel filtration and anion-exchange chromatography steps, an aqueous solution of pseudobactin 7SR1 was passed through a short column containing CM-Sephadex C-25, pyridinium form, equilibrated in water.

Reversal of Iron Starvation of Pseudomonas 7SR1. A literature procedure was followed (Teintze & Leong, 1981).

Chromatography and Paper Electrophoresis. Thin-layer chromatography (TLC) of ferric pseudobactin 7SR1 and pseudobactin 7SR1 was performed on Polygram Cell 300 and Polyamid-11 plastic sheets (Brinkmann Instruments, Inc.) with 1-butanol–acetic acid–water (2:1:1.5 v/v/v) as the solvent system. Spots were visualized as described earlier (Teintze et al., 1981).

Descending paper chromatography and paper electrophoresis were performed according to earlier procedures (Teintze et al., 1981). Standards included ferrichrome (neutral) (Neilands, 1952), ferrichrome A (3− ionic charge) (Emery & Neilands, 1961), and ferric schizokinen (1− ionic charge) (Mullis et al., 1971). Spots were visualized as described previously (Teintze et al., 1981) and also with the *t*-butyl hypochlorite reagent (Easley, 1965). Although ferric pseudobactin 7SR1 and pseudobactin 7SR1 appeared to be homogeneous by TLC and electrophoresis of their solutions, only material obtained from HPLC was pure by NMR.

Spectroscopy. Visible spectra, infrared spectra, and proton and carbon-13 NMR spectra were obtained as described previously (Teintze & Leong, 1981). Mass spectra were obtained in the fast-atom mode in the Space Sciences Laboratory, University of California, Berkeley; gas chromatography–mass spectrometry (GC–MS) was performed on a 3% OV-17 or a 3% Dexil 300 column followed by mass spectral analysis in the chemical ionization mode with ammonia or electron-impact mode, respectively, at the University of California, San Diego.

Amino Acid Analyses. Analyses were carried out as described earlier (Teintze et al., 1981).

Partial Acid Hydrolysis. Partial acid hydrolysis of pseudobactin 7SR1 was accomplished by two methods. In the first method, 30 mg of pseudobactin 7SR1 was hydrolyzed in vacuo for 8 h at 110 °C in 10 mL of 0.03 N HCl. The hydrolysate was subjected to preparative paper electrophoresis on Whatman 3MM paper at pH 1.9; the two slowest migrating cathodic

bands, which were stained by both ninhydrin and *t*-butyl hypochlorite, were separately cut out and eluted with water. Each fraction was further purified by preparative paper electrophoresis, first at pH 3.5 and finally at pH 1.9.

In the second method, 40 mg of pseudobactin 7SR1 was hydrolyzed for 3 h at 37 °C in 2 mL of 10 N HCl–acetic acid (1:1 v/v). After the hydrolysate was subjected to preparative paper electrophoresis at pH 6.5, the slowest major, anodic-migrating band, which was stained by both ninhydrin and *t*-butyl hypochlorite, was eluted. This fraction was subjected to preparative paper electrophoresis at pH 3.5; the major cathodic band was eluted and further purified by electrophoresis at pH 1.9.

In addition, pseudobactin 7SR1 was first oxidized with performic acid and then partially hydrolyzed as follows. Performic acid was generated in situ from 2 mL of 30% hydrogen peroxide and 8 mL of 88% formic acid warmed at 37 °C for 15 min. After this solution was added to 60 mg of pseudobactin 7SR1, the reaction mixture was incubated at 37 °C for 3 h. Approximately 20 mL of water was added, and the solution was cautiously distilled to dryness under high vacuum. After 20 mL of water was added to the residue, the resulting solution was distilled to dryness again. This procedure was repeated. The residue was hydrolyzed for 3 h at 37 °C in 6 mL of 10 N HCl–acetic acid. The slowest migrating anodic band that was stained by both ninhydrin and *t*-butyl hypochlorite was eluted following preparative paper electrophoresis at pH 6.5. Subsequent preparative paper electrophoresis at pH 3.5 yielded a major cathodic band. Further purification by electrophoresis at pH 1.9 yielded two major cathodic bands, which were eluted separately and repurified at pH 6.5. The overall yield of each band was less than 0.1%.

Sequencing. Manual dansyl–Edman degradation was carried out as described earlier (Teintze et al., 1981; Edman & Henschen, 1975; Hartley, 1970). All thiazolinone amino acids were extracted with benzene except that of *threo*-β-hydroxyaspartic acid (see below), which was extracted with ethyl acetate. After each Edman degradation, the phenylthiocarbamoyl or 3-phenyl-2-thiohydantoin amino acid (PTH) derivatives were back-hydrolyzed with 47% HI in vacuo at 130 °C for 18 h; an aliquot of the remaining peptide was also hydrolyzed under the same conditions. Both samples were run on the amino acid analyzer prior to proceeding.

Reaction of Pseudobactin 7SR1 with Periodate. A solution containing 5 mg of pseudobactin 7SR1 in 0.5 mL of 0.05 M periodic acid and 0.5 M sodium formate buffer, pH 2.6, was incubated at room temperature for 10 min. After this solution was concentrated to dryness in vacuo, the residue was dissolved in D_2O. This solution was analyzed by proton NMR. As a control, Desferal was treated in an identical manner.

Reaction of Pseudobactin 7SR1 with Potassium Carbonate. A solution containing 5 mg of pseudobactin 7SR1 in 0.2 mL of CH_3OH–10% K_2CO_3 (1:1 v/v) was incubated for 2 h at room temperature. After this solution was adjusted to pH 2.6 with dilute HCl, it was concentrated to dryness in vacuo. The residue was dissolved in D_2O, and this solution was examined by proton NMR. As controls, Desferal and pentaacetylglucose were treated in identical fashion.

Identification of Malic Acid. About 20 mg of pseudobactin 7SR1 was hydrolyzed in vacuo for 18 h at 110 °C in 1.8 mL of 6 N HCl. After this solution was subjected to continuous extraction with diethyl ether for 4 days, the resulting ether solution was dried with sodium sulfate and concentrated to dryness in vacuo. About 0.2 mL of BSA was added to the residue, and this solution was allowed to stand at room temperature for 10 min and was then analyzed by GC–MS.

Preparation of DL-*Malamide.* The DL-malamide was prepared from DL-malic acid according to literature procedures (Grewe & Rockstroh, 1957; Arakawa, 1963).

Results

When grown in iron-limiting culture medium, *Pseudomonas* 7SR1 produced a yellow-green, fluorescent iron-binding substance, which was different from pseudobactin (see below). We assigned to this new substance the trivial name pseudobactin 7SR1. Pseudobactin 7SR1 exhibited properties typical of a siderophore, including complete repression of production in various culture media containing micromolar amounts of iron(III) (data not shown). Furthermore, pseudobactin 7SR1 and ferric pseudobactin 7SR1, both at 10 μM, were about equally effective in reversing iron starvation of strain 7SR1 induced by the synthetic ferric-complexing agent ethylenediaminedi[(*o*-hydroxyphenyl)acetic acid] (EDDA), the iron of which is not utilized by the cells. This stimulation of growth was evidenced by a halo of single colonies surrounding the disks. In contrast, $FeCl_3 \cdot 6H_2O$ at 10 mM was apparently required to saturate the EDDA in the medium, thereby producing similar-sized growth halos as the above compounds.

Red-brown ferric pseudobactin 7SR1 was very soluble in water and had a migration similar to that of ferric schizokinen upon electrophoresis at pH 6.5, indicating that it was monoanionic. An aqueous solution of the pyridinium salt had an absorption maximum at 401 nm, which was relatively insensitive to pH. The extinction coefficient, ϵ_{401}, was 2.1×10^4 L mol^{-1} cm^{-1} (data not shown), on the basis of one iron(III) per molecule of ferric pseudobactin 7SR1. Its molecular weight on the basis of the extinction coefficient would be 1300 g/mol.

Yellow-green, fluorescent pseudobactin 7SR1 was neutral upon electrophoresis at pH 6.5. Pseudobactin 7SR1 was very soluble in water; an aqueous solution at pH 7.2 had an absorption maximum at 401 nm with a shoulder at 385 nm (data not shown). The extinction coefficient, ϵ_{401}, was 1.9×10^4 L mol^{-1} cm^{-1}, on the basis of a molecular weight of 1166 g/mol as determined from its molecular formula (see below). The absorption maximum shifted to longer wavelengths and the extinction coefficient became slightly greater as the pH increased from 3 to 9. Spectrophotometric titration of a solution of pseudobactin 7SR1 in 0.1 M sodium acetate buffer, pH 5.2, at 460 nm with 19.4 mM ferrous ammonium sulfate gave an equivalent weight of 1260 g of ligand/mol of iron(III). This value suggested a ligand to iron(III) ratio of 1:1 for ferric pseudobactin 7SR1. Mass spectral analysis of pseudobactin 7SR1 yielded *m/e* peaks at 1166.3 ± 0.2 and 1188.3 ± 0.2, which were assigned to MH^+ and MNa^+, respectively, where M is the parent. Therefore, the molecular weight of pseudobactin 7SR1 according to mass spectrometry was 1165 g/mol.

Amino acid analysis of a 6 N HCl hydrolysate of pseudobactin 7SR1 yielded 1 mol of alanine, 1 mol of glycine, 3 mol of serine, 1 mol of threonine, and 1 mol of *threo*-β-hydroxyaspartic acid (β-OH-Asp) (Teintze et al., 1981; Ikegami, 1975). Analysis of a 47% HI hydrolysate yielded 1 mol of alanine, 1 mol of glycine, 3 mol of serine, 1 mol of threonine, 1 mol of *threo*-β-hydroxyaspartic acid, and 1 mol of ornithine. The presence of ornithine in the reductive hydrolysate but not in the HCl hydrolysate indicated that pseudobactin 7SR1 contained 1 mol of N^δ-hydroxyornithine (N^δ-OH-Orn) (Emery & Neilands, 1961). The stereochemistry of the threonine, isolated by preparative paper electrophoresis at pH 6.5 of a 6 N HCl hydrolysate of pseudobactin 7SR1, was determined

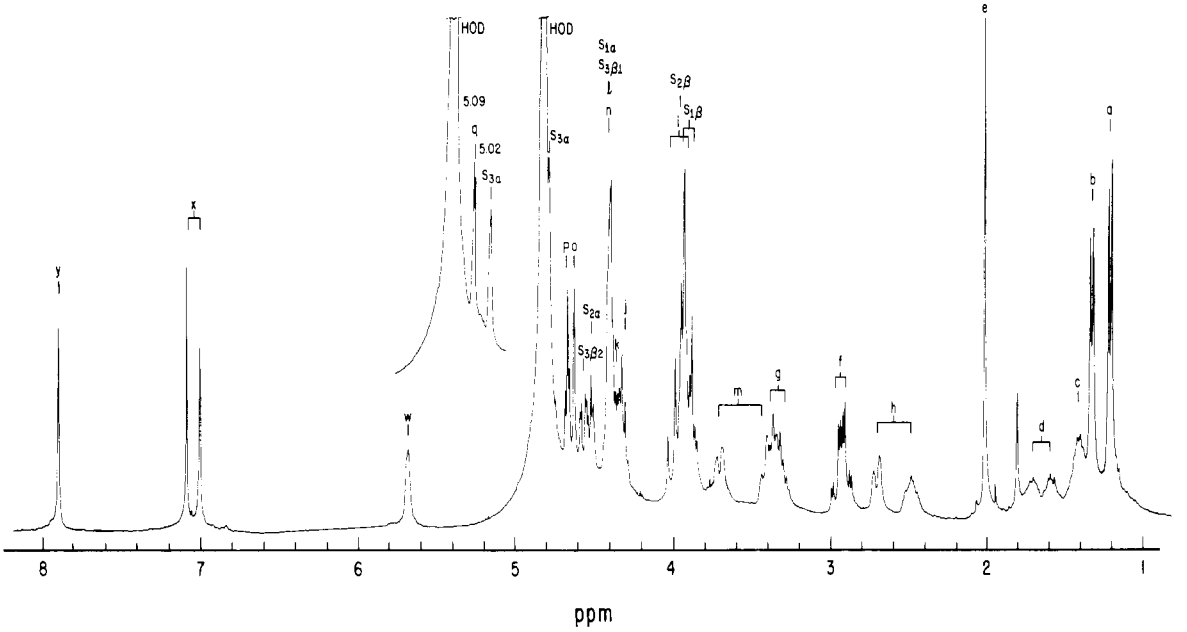

FIGURE 2: ^{1}H NMR spectrum (360 MHz) of pseudobactin 7SR1 in D_2O at room temperature. Chemical shifts are in parts per million (ppm) from internal 3-(trimethylsilyl)propanesulfonic acid sodium salt (DSS). The ^{1}H resonances $S_3\alpha$ and q were obscured by the HOD peak in this particular NMR spectrum; the insert shows these resonances more clearly as part of an NMR spectrum taken at 7 °C. The HOD peak moved from 4.83 ppm at room temperature to 5.20 ppm at 7 °C. The small singlet at 1.82 ppm was determined to be an impurity.

by descending paper chromatography. Its R_f was identical to that of DL-threonine but different from that of DL-*allo*-threonine. The alanine residue was determined to have the L configuration by Dr. J. L. Bada at the Amino Acid Dating Laboratory, Scripps Institution of Oceanography, University of California, San Diego, as follows. A 6 N HCl hydrolysate of pseudobactin 7SR1 was reacted first with acidic methanol and then with *N*-(trifluoroacetyl)-L-prolyl chloride, and the alanine enantiomeric ratio was determined by gas–liquid chromatography of the resulting *N*-(trifluoroacetyl)-L-prolyl peptide methyl esters (Hoopes et al., 1978). Pseudobactin 7SR1 therefore contained 1 mol of L-alanine, 1 mol of glycine, 3 mol of serine, 1 mol of threonine, 1 mol of *threo*-β-hydroxyaspartic acid, and 1 mol of N^δ-hydroxyornithine.

Pseudobactin 7SR1 did not react with ninhydrin or dansyl chloride, indicating the absence of a free amino terminus. Pseudobactin 7SR1 was not affected by chymotrypsin or Pronase under conditions where control peptides were completely digested (data not shown). The amino acid sequence of pseudobactin 7SR1 was finally obtained from overlap of amino acid sequences of smaller peptides obtained from partial acid hydrolysis. Two peptides, L-Ala-Gly-Ser and L-Ala-Gly-Ser-Ser, were obtained from preparative paper electrophoresis of a 0.03 N HCl hydrolysate of pseudobactin 7SR1; the latter peptide was the slower migrating band of the two at pH 1.9. The peptide Ser-Ser-*threo*-β-OH-Asp-Thr was obtained from a 10 N HCl–acetic acid (1:1) hydrolysate of pseudobactin 7SR1. Since no N^δ-OH-Orn-containing peptide could be isolated from partial acid hydrolysis, this residue in pseudobactin 7SR1 was first oxidized to a glutamic acid residue with performic acid (Mikěs & Turkovâ, 1962). Subsequent 10 N HCl–acetic acid hydrolysis of Glu-containing pseudobactin 7SR1 followed by preparative paper electrophoresis yielded two peptides, Ser-Glu-L-Ala and Ser-Glu-L-Ala-Gly; the latter peptide migrated slower than the former at pH 1.9. Hence, pseudobactin 7SR1 contained a cyclic octapeptide with the amino acid sequence L-Ala-Gly-Ser-Ser-*threo*-β-OH-Asp-Thr-Ser-N^δ-OH-Orn. Although no overlap was obtained between the Thr and Ser, the presence of one or more amino acids between these two residues was ruled out by the subsequent structure determination (see below).

The amino acid sequence of pseudobactin 7SR1 did not fully account for its structure, namely, its iron(III)-binding ligands and its yellow-green, fluorescent chromophore. Our working hypothesis was that the iron(III) coordination octahedron of ferric pseudobactin 7SR1 consists of three bidentate groups, specifically an α-hydroxy acid group derived from β-OH-Asp, a hydroxamate group derived from N^δ-OH-Orn, and an *o*-dihydroxy aromatic group derived from the yellow-green, fluorescent chromophore. The presence of these three ligands, which also appear in pseudobactin (Teintze et al., 1981), seemed justified since the visible absorption spectra of pseudobactin 7SR1 and ferric pseudobactin 7SR1 were almost identical with the respective spectra of pseudobactin and ferric pseudobactin (Teintze et al., 1981). Also, the ionization of four protons from the above ligands in electrically neutral pseudobactin 7SR1 upon coordination to iron(III) would account for the monoanionic charge of ferric pseudobactin 7SR1. Hence, the remaining structural features that required elucidation included the acyl group of the hydroxamate group and the fluorescent group and its location.

The remaining structure of pseudobactin 7SR1 was determined from its ^{1}H and ^{13}C NMR spectra in conjunction with its mass spectral molecular weight of 1165. The proton NMR spectrum of pseudobactin 7SR1 in D_2O is shown in Figure 2; the resonances are also summarized in Table I. The proposed structure of pseudobactin 7SR1 is shown in Figure 3; the letters assigned to the hydrogen atoms in Figure 3 refer to the above ^{1}H NMR resonances. Assignments were made by comparison with previously reported chemical shifts of amino acid protons (Wüthrich, 1976), by comparison with spectra of pseudobactin (Teintze & Leong, 1981), and by irradiation of each of the resonances in Figure 2 to determine which signals were coupled. Some of the chemical shifts and some of the geminal coupling constants listed in Table I were obtained from decoupling experiments (data not shown). The ^{1}H NMR spectrum of pseudobactin 7SR1 in Me_2SO-d_6 from 6.5 to 10

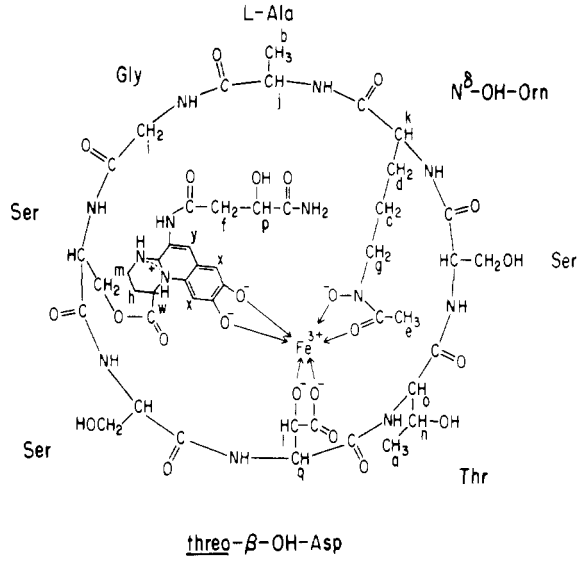

FIGURE 3: Proposed structure of ferric pseudobactin 7SR1. The letters refer to the assignments in the ^{1}H NMR spectrum of pseudobactin 7SR1. The attachment of the yellow-green, fluorescent quinoline derivative to the peptide is not known with certainty. A rationale for the structure shown here is given in the text.

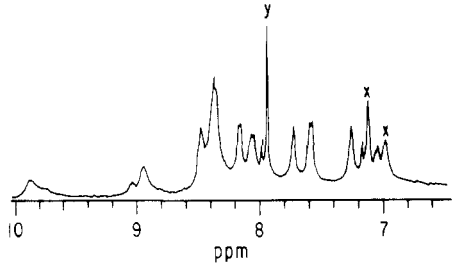

FIGURE 4: ^{1}H NMR spectrum (360 MHz) of pseudobactin 7SR1 in Me_2SO-d_6 at room temperature. Chemical shifts are in parts per million (ppm) from internal tetramethylsilane (Me_4Si). Only the region from 6.5 to 10 ppm is shown.

ppm is shown in Figure 4. The resonances labeled x and y were assigned by titrating the sample with D_2O; all other resonances in this region disappeared upon addition of sufficient D_2O. The high-field region of the Me_2SO-d_6 spectrum was similar to that in D_2O. The proton-decoupled ^{13}C NMR spectrum of pseudobactin 7SR1 in D_2O is shown in Figure 5. A total of 46 carbon resonances was observed: 13 carbonyl carbon resonances between 170 and 177 ppm, 9 aromatic carbon resonances between 99 and 152 ppm, and 24 aliphatic carbon resonances between 16 and 71 ppm from tetramethylsilane. The peaks at 173.14 and 173.32 ppm, those at 171.25, 171.41, 171.56, and 171.63 ppm, those at 170.01 and 170.13 ppm, those at 56.80 and 56.85 ppm, and those at 18.64 and 18.91 ppm might be too close together to be distinguishable in the figure. The structure shown in Figure 3 was consistent with the total number of carbon resonances and the number of carbon resonances within each family.

Table I: Proton NMR Chemical Shifts and Coupling Constants of Pseudobactin 7SR1 in D_2O

resonance	δ (ppm)	coupling constants (Hz), $\|J\|$
a	1.23	$J_{an} = 6.4$
b	1.34	$J_{bj} = 7.2$
c	1.42	
d	1.61	$J_{d_1k} = 8.6$, $J_{d_2k} = 6.7$
	1.72	
e	2.03	
h	2.50	$J_{hh} = 12.2$, $J_{h_1w} = 4.9$
	2.71	
f	2.91	$J_{ff} = 15.7$, $J_{f_1p} = 4.7$, $J_{f_2p} = 4.7$
	2.96	
g	3.31	$J_{gg} = 14.6$
	3.39	
m	3.41	$J_{mm} = 11.2$
	3.71	
i	3.92	$J_{ii} = 16.3$
	4.01	
$S_{1\beta}$	3.90	$J_{S_{1\beta}S_{1\beta}} = 12.5$
	3.96	
$S_{2\beta}$	3.95	$J_{S_{2\beta}S_{2\alpha}} = 6.0$
j	4.32	$J_{jb} = 7.2$
k	4.35	$J_{kd_1} = 8.6$, $J_{kd_2} = 6.7$
$S_{1\alpha}$	4.41	
$S_{3\beta}$	4.41	$J_{S_{3\beta}S_{3\beta}} = 11.6$, $J_{S_{3\beta 2}S_{3\alpha}} = 2.6$, $J_{S_{3\beta 1}S_{3\alpha}} = 1.8$
	4.58	
l	4.41	$J_{lq} = 2.5$
n	4.42	$J_{na} = 6.4$, $J_{no} = 2.3$
$S_{2\alpha}$	4.53	$J_{S_{2\alpha}S_{2\beta}} = 6.0$
o	4.64	$J_{on} = 2.3$
p	4.68	$J_{pf_1} = 4.7$, $J_{pf_2} = 4.7$
$S_{3\alpha}$	4.80	$J_{S_{3\alpha}S_{3\beta 1}} = 1.8$, $J_{S_{3\alpha}S_{3\beta 2}} = 2.6$
q	4.84	$J_{ql} = 2.5$
w	5.68	$J_{wh_1} = 4.9$
x	7.00	
	7.09	
y	7.90	

The hydroxamate acyl group was determined to be acetyl from ^{1}H NMR experiments as follows. Oxidation of hydroxamate-containing compounds with periodate is known to release the acyl group as the corresponding carboxylic acid (Emery & Neilands, 1960). The acetyl resonance at 2.03 ppm (Figure 2) disappeared following workup of the reaction mixture containing pseudobactin 7SR1 and periodate, suggesting loss of acetic acid. Desferal, which also contains an acetyl hydroxamate group, behaved similarly (data not shown). Moreover, the acetyl group of pseudobactin 7SR1 was precluded from participating in an ester linkage from ^{1}H NMR experiments as follows. The acetyl resonance at 2.03 ppm persisted even after workup of the reaction mixture containing pseudobactin 7SR1 in aqueous potassium carbonate, which is known to result in ester hydrolysis. As controls, Desferal behaved similarly, whereas pentaacetylglucose, which contains acetyl ester linkages, displayed loss of its acetyl resonances (data not shown).

The substituted quinoline chromophore of pseudobactin 7SR1 was readily identified from its characteristic ^{1}H and ^{13}C NMR resonances. The ^{13}C chemical shifts for the nine aro-

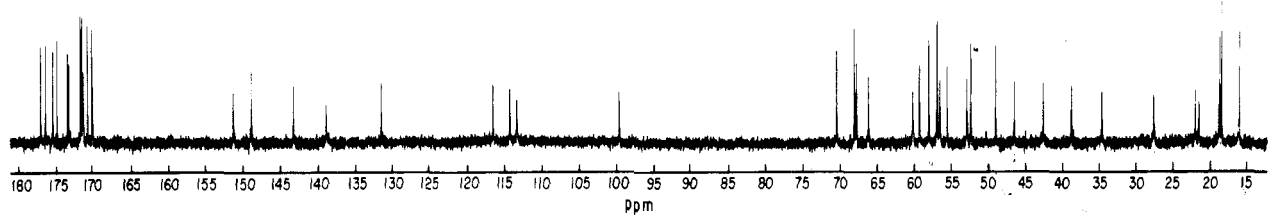

FIGURE 5: ^{1}H broad-band-decoupled ^{13}C NMR spectrum of pseudobactin 7SR1 at 125.76 MHz in D_2O at 7 °C. Chemical shifts are relative to Me_4Si.

matic carbons (Figure 5) were remarkably similar to those reported for pseudobactin (Teintze & Leong, 1981). The chemical shifts (7.00, 7.09, and 7.90 ppm) of the three aromatic proton singlets for pseudobactin 7SR1 were virtually identical with those of pseudobactin (Teintze & Leong, 1981) and suggested a substitution pattern for the quinoline chromophore reminiscent of that of pseudobactin.

The six-membered heterocyclic ring containing both nitrogen atoms and its substitution pattern in pseudobactin 7SR1 were ascertained from comparison of its ^{1}H NMR resonances with those of pseudobactin (Teintze & Leong, 1981). Reexamination of the ^{1}H NMR spectrum of pseudobactin in D_2O by use of decoupling indicated that the resonances assigned to protons h should be centered at 2.34 ppm and *also* at 2.69 ppm. The chemical shifts of the resonances assigned to protons m, h, and w and their splitting patterns in pseudobactin 7SR1 were very similar to those observed for the analogous protons in pseudobactin (Teintze & Leong, 1981). Hence, pseudobactin 7SR1 also had a six-membered heterocyclic ring with proton w and a carboxylic acid group attached to the same carbon. The absolute configuration of this carbon is not known.

The substituent attached to carbon 3 of the quinoline chromophore was determined to be derived from malamide. Malic acid was identified as its tris(trimethylsilyl) derivative by GC–MS after trimethylsilylation of the ether extract of acid-hydrolyzed pseudobactin 7SR1. The major peak obtained from gas chromatography had a retention time and an electron-impact mass spectral fragmentation pattern identical with those of authentic DL-malic acid, which had been treated identically first with 6 N HCl and then with BSA (data not shown). Chemical-ionization mass spectrometry of these two samples yielded the same *m/e* peak at 351, which was assigned as MH^+, where M is the parent. The mass spectral molecular weight of 1165 for pseudobactin 7SR1 required that both carboxylic acid groups of malic acid be amides. The absolute configuration of the malamide group is not known. The two malyl protons f, which appeared as two superimposed AB quartets centered at 2.909 and 2.964 ppm, were both coupled to proton p, which appeared as a triplet centered at 4.676 ppm. The orientation of the malamide group with respect to the quinoline chromophore was tentatively formulated from comparison of the chemical shifts of protons f and p in pseudobactin 7SR1 with those of the corresponding protons in DL-malamide. The ^{1}H NMR resonances of protons f and p of malamide in D_2O were centered at 2.605 and 2.758 ppm and 4.484 ppm, respectively (data not shown). Protons f in pseudobactin 7SR1 and malamide were treated as single resonances with average chemical shifts of 2.937 and 2.682 ppm, respectively. Since the magnitude of the downfield chemical shift difference was larger for protons f than that for proton p in pseudobactin 7SR1, protons f were postulated to be closer to the quinoline aromatic system than proton p. Similarly, the α-proton resonance at 2.35 ppm of *N*-phenylpropionamide in $CDCl_3$ is shifted more downfield than the β resonance at 1.19 ppm when compared with the corresponding resonances at 2.23 and 1.12 ppm, respectively, of propionamide (Sadtler Research Laboratories, 1980).

The site of attachment of the fluorescent chromophore via its carboxyl group to the cyclic octapeptide is not known with certainty. None of the smaller peptides obtained from pseudobactin 7SR1 by partial acid hydrolysis contained the fluorescent group, indicating that it had been removed. Since only hydroxyl groups from the peptide are available for bonding, the fluorescent group must be linked via an ester bond to one of the three serines or the threonine. Nevertheless, the extremely broad and intense carbonyl absorption observed in the infrared spectrum of pseudobactin 7SR1 precluded the unequivocal location of a specific carbonyl stretch attributable to the ester group (data not shown). An examination of CPK molecular models indicated that octahedral coordination in ferric pseudobactin 7SR1 was possible with the fluorescent derivative bonded to any of the above hydroxyl groups. The ^{1}H NMR spectrum of pseudobactin 7SR1 (Figure 2) provided a partial answer. The serine resonances in Figure 2 and Table I could not be assigned to the specific serines shown in Figure 3. The three serines, arbitrarily designated S_1, S_2, and S_3, exhibited ABX, A_2X, and AMX (Wüthrich, 1976) ^{1}H NMR coupling patterns, respectively. In particular, the resonances of the two β protons of S_3 were widely separated at 4.41 and 4.58 ppm (Table I), indicating that these two protons were the most nonequivalent of all three serines. Also, the chemical shifts of the β protons of S_1 and S_2 were nearly identical whereas the β-proton resonance of S_3 occurred further downfield. A similar trend was observed with the α protons. We suggest that these perturbations of S_3 were due to the fact that it was bonded to the fluorescent chromophore. NMR studies involving one- and two-dimensional nuclear Overhauser effect (NOE) spectroscopy of pseudobactin 7SR1 in Me_2SO-d_6 suggested that serine S_3 was one of the two adjacent serines depicted in Figure 3 (J. S. Buyer, unpublished experiments). It is tempting to assign S_3 to the particular serine shown in Figure 3 for the following reason. In this particular configuration, the number of intervening atoms (14) separating the *o*-dihydroxy group from the α-hydroxy group and the number of atoms (13) separating the latter group from the hydroxamate group are remarkably the same as those in pseudobactin (Teintze et al., 1981).

In summary, the proposed structure of pseudobactin 7SR1 (Figure 3), which has a molecular formula of $C_{46}H_{63}N_{13}O_{23}$ and an accompanying calculated mass spectral molecular weight of 1165.4 g/mol, was consistent with its ^{1}H and ^{13}C NMR spectra and its observed mass spectral molecular weight of 1165.3 g/mol.

Discussion

The chemical structures of pseudobactin 7SR1, the siderophore of plant-deleterious *Pseudomonas* 7SR1, and pseudobactin, the siderophore of plant growth promoting *Pseudomonas* B10, are remarkably similar. The combination of three different chelating groups found in pseudobactin 7SR1 is identical with that of pseudobactin (Teintze et al., 1981). The yellow-green, fluorescent group of pseudobactin 7SR1 contained a malamide group instead of a succinamide group; the replacement of a hydrogen with a hydroxyl group in pseudobactin 7SR1 accounts for the only difference in the fluorescent chromophore. Since pseudobactin 7SR1 was not affected by the nonspecific enzyme Pronase, pseudobactin 7SR1 might contain D-amino acids like pseudobactin (Teintze et al., 1981). However, differences between the two siderophores include the acyl moiety of the hydroxamate group; pseudobactin 7SR1 had an acetyl group whereas pseudobactin has the C-terminal carboxyl group. Also, pseudobactin 7SR1 contained a cyclic octapeptide whereas pseudobactin contains a linear hexapeptide. A key difference between the two siderophores is that the fluorescent group of pseudobactin 7SR1 was attached via an ester bond to the peptide whereas that of pseudobactin is attached via an amide bond. Therefore, pseudobactin 7SR1 might be more susceptible to hydrolysis than pseudobactin. Such hydrolysis might be expected to diminish the ability of pseudobactin 7SR1 to bind iron(III)

and, hence, might adversely affect the growth of *Pseudomonas* 7SR1 in iron-limiting environments. Whether or not other siderophores from plant-deleterious fluorescent pseudomonads also contain ester linkages remains to be determined.

The present study and others in progress in our laboratory demonstrate that all sugar beet deleterious and bean-deleterious fluorescent pseudomonads examined thus far produce siderophores (C.-C. Yang & J. S. Buyer, unpublished results). In fact, siderophore production in deleterious strains often equaled or surpassed that of plant growth promoting strains. Hence, the siderophore-mediated growth inhibition of certain deleterious pseudomonads by certain PGPR cannot be explained by the lack of or diminished production of siderophores by the former.

We are presently isolating and structurally characterizing siderophores from a number of bean growth promoting and bean growth inhibiting fluorescent pseudomonads as part of an effort to understand the iron-repressible antagonism between beneficial and deleterious strains. We plan to determine if siderophore-mediated iron deprivation is responsible for the observed patterns of antagonism.

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