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Structure of Pseudobactin A214, a Siderophore from a Bean-Deleterious *Pseudomonas*†

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ABSTRACT: Bean-deleterious *Pseudomonas* A214 produced the extracellular yellow-green, fluorescent siderophore [microbial iron(III) transport agent] pseudobactin A214 under iron-limiting conditions. Pseudobactin A214 has a molecular formula of $C_{46}H_{64}N_{13}O_{22}$ and a molecular mass of 1151 g/mol. Pseudobactin A214 contained an N-blocked linear octapeptide with the amino acid sequence Ser-Ala-Gly-Ser-Ala-*threo*-β-OH-Asp-L-*allo*-Thr-N^{δ}-OH-Orn with a yellow-green, fluorescent quinoline derivative attached via an amide bond to the amino terminus. A succinamide group was linked to carbon 3 of the quinoline derivative. Sequencing was accomplished by two-dimensional NMR spectroscopy and by Edman degradation of smaller peptides obtained from partial acid hydrolysis. Since pseudobactin A214 was not affected by nonspecific proteolytic enzymes, it might contain D-amino acids. The three bidentate iron(III)-chelating groups consisted of a 1,2-dihydroxy aromatic group in the quinoline chromophore, an α-hydroxy acid group present as β-hydroxyaspartic acid, and a hydroxamate group derived from N^{δ}-acetyl-N^{δ}-hydroxyornithine. The chemical structure of pseudobactin A214 is remarkably similar to those of pseudobactin and pseudobactin 7SR1, the siderophores of plant growth promoting and plant-deleterious *Pseudomonas* B10 and *Pseudomonas* 7SR1, respectively.

Specific root-colonizing members of the *Pseudomonas fluorescens–Pseudomonas putida* group (Schroth & Hancock, 1982) enhance the growth of a variety of crops in part by reducing rhizosphere populations of phytopathogenic fungi (Kloepper et al., 1980a) and deleterious rhizobacteria (Suslow & Schroth, 1982a). These beneficial fluorescent pseudomonads exert their plant growth promoting activity in part by producing under iron-limiting conditions extracellular siderophores [microbial iron(III) transport agents] (Neilands, 1981) that efficiently complex environmental iron, making it less available to certain endemic microorganisms, including phytopathogenic fungi, and thus inhibiting their growth (Kloepper et al., 1980a,b). The structure of pseudobactin, the yellow-green, fluorescent siderophore (Teintze et al., 1981) of plant growth promoting *Pseudomonas* B10, is shown in Figure 1.

Deleterious rhizobacteria, not previously recognized as plant pathogens, significantly decrease the growth of sugar beet, bean, or lettuce seedlings (Suslow & Schroth, 1982a). The

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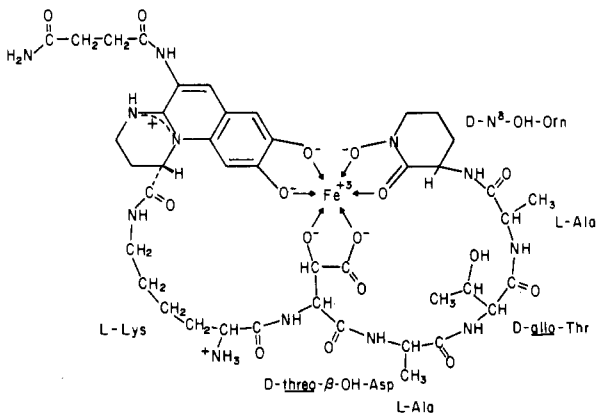

FIGURE 1: Schematic drawing of ferric pseudobactin.

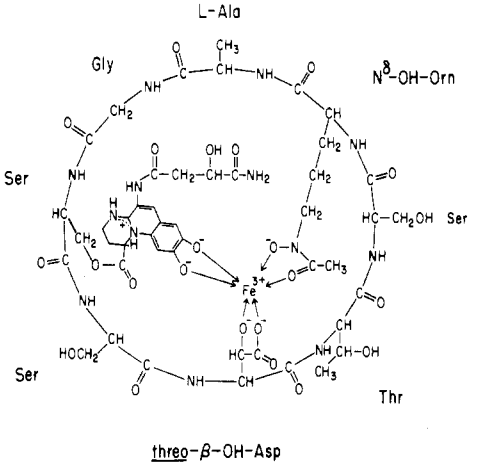

FIGURE 2: Schematic drawing of ferric pseudobactin 7SR1.

genera of deleterious rhizobacteria include *Pseudomonas*, *Enterobacter*, *Klebsiella*, *Citrobacter*, *Flavobacterium*, *Achromobacter*, and *Arthrobacter* (Suslow & Schroth, 1982a,b). Sugar beet deleterious *Pseudomonas* 7SR1 produces pseudobactin 7SR1, its yellow-green, fluorescent siderophore, under iron-deficient conditions (Figure 2) (Yang & Leong, 1984).

As part of a continuing effort to understand the biochemical basis for antagonism of beneficial fluorescent pseudomonad strains against deleterious fluorescent pseudomonad strains and to compare chemical structures of siderophores from these strains, we report here the structure of pseudobactin A214, the siderophore of *Pseudomonas* A214, a specific fluorescent pseudomonad deleterious to bean.

Experimental Procedures

Materials. Desferal (deferriferrioxamine B methanesulfonate) was a gift from Ciba-Geigy; pentaacetylglucose was obtained from E. Ng. The N^{δ}-acetyl-N^{δ}-hydroxyornithine was obtained from Dr. J. B. Neilands.

Isolation of Ferric Pseudobactin A214. Pseudomonas A214 was obtained from Dr. M. N. Schroth and was maintained on King's medium B plates (King et al., 1954). An iron-deficient minimal medium consisting of 3 g of KH_2PO_4, 6 g of K_2HPO_4, 1 g of NH_4Cl, and 10 mL of glycerol per liter 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 A214 from *Pseudomonas* A214. Stock solutions of phosphates and Casamino acids were deferrated with 8-hydroxyquinoline as described previously (Bell et al., 1979).

Cells were cultured, and ferric pseudobactin A214 was isolated as described previously (Yang & Leong, 1984). Ferric pseudobactin A214 was chromatographed on Bio-Gel P-2 as described previously (Teintze et al., 1981), and the resulting major red-brown band was chromatographed at 4 °C on a column (3.8 × 40 cm) containing DEAE-Sephadex A-25, acetate form, equilibrated with 5 mM acetic acid–pyridine buffer, pH 6. A linear gradient (1 L) from 5 mM to 1 M acetic acid–pyridine buffer, pH 6, was used to elute ferric pseudobactin A214 as a single band.

Ferric pseudobactin A214 was analyzed by high-pressure liquid chromatography (HPLC)[1] on a Varian Vista 54 system with an analytical column (4 × 250 mm) containing 5-μm LiChrosorb RP-18 (Merck). Isocratic elution with 10 mM tetraethylammonium acetate and 3% (v/v) acetonitrile at a flow rate of 1 mL/min yielded two peaks with approximately equal area when detected with a Varian UV-50 visible detector at 400 nm. HPLC analysis of cultures of *Pseudomonas* A214 harvested at various times and treated as described above indicated that the later-eluting peak was predominant at early time points (data not shown). Preparative HPLC was performed on a 10-μm LiChrosorb RP-18 column (10 × 250 mm) (Merck) with the same solvent system at 6 mL/min, and the second peak was collected. This ferric pseudobactin A214 was then chromatographed at 4 °C on a column containing DEAE-Sephadex A-25, acetate form, equilibrated with 10 mM acetic acid–pyridine buffer, pH 6, and eluted as described above. After the eluate was concentrated to dryness in vacuo, water was added, and the resulting solution was concentrated to dryness again. This procedure was repeated. The resulting ferric pseudobactin A214 was 90% pure as determined by HPLC analysis.

Pseudobactin A214. Pseudobactin A214 was obtained by deferration of ferric pseudobactin A214 with 8-hydroxyquinoline as described previously (Teintze et al., 1981). For nuclear magnetic resonance (NMR) and mass spectrometric experiments, pseudobactin A214 was further purified by chromatography at 4 °C on a column containing DEAE-Sephadex A-25, acetate form, equilibrated with 10 mM acetic acid–pyridine buffer, pH 6, and eluted with a linear gradient up to 500 mM buffer. For NMR experiments, pseudobactin A214 was passed through a short column containing CM-Sephadex C-25, sodium form, equilibrated in water.

Reversal of Iron Starvation of Pseudomonas A214. A literature procedure employing a plate bioassay was followed (Teintze & Leong, 1981).

Determination of Extinction Coefficients. After the visible absorption maximum of a pseudobactin A214 solution in 0.1 M sodium acetate, pH 5.2, was determined, the solution was spectrophotometrically titrated with 1.0 mM ferrous ammonium sulfate at 440 nm. The visible absorbance maximum of the resulting ferric pseudobactin A214 solution was recorded.

Paper Electrophoresis. Paper electrophoresis was performed as described earlier (Teintze et al., 1981). Standards included pseudobactin (1+ ionic charge) (Teintze et al., 1981), ferric pseudobactin (neutral) (Teintze et al., 1981), ferric pseudobactin 7SR1 (1− ionic charge) (Yang & Leong, 1984), and ferrichrome A (3− ionic charge) (Emery & Neilands, 1961). A pH 1.6 buffer consisted of 0.4 M formic acid adjusted to

[1] Abbreviations: HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; COSY, correlated spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; Me_2SO, dimethyl sulfoxide; GC–MS, gas chromatography–mass spectrometry; EDDA, ethylenediaminedi[(*o*-hydroxyphenyl)acetic acid]; β-OH-Asp, β-hydroxyaspartic acid; N^{δ}-OH-Orn, N^{δ}-hydroxyornithine; NOE, nuclear Overhauser effect; ppm, parts per million; PTH, 3-phenyl-2-thiohydantoin.

pH 1.6 with trifluoroacetic acid. Spots were visualized as described previously (Yang & Leong, 1984).

NMR Spectroscopy. Proton NMR spectra were obtained on a custom-designed 360-MHz spectrometer with a Nicolet computer. Conventional spectra were recorded in the Fourier-transform mode with quadrature detection. Resolution enhancement was accomplished by exponential multiplication with a negative line-broadening factor followed by Gaussian multiplication with a positive line-broadening factor. Each factor was determined empirically.

Two-dimensional correlated spectroscopy (COSY) employed the two pulse sequence $(90°-t_1-90°-t_2)_n$ (Aue et al., 1976). A 16-pulse phase cycle (Bax et al., 1981) was employed to suppress artifacts and to provide quadrature detection with the carrier frequency in the center of the spectrum. Thirty-two pulses were used for each of 512 t_1 values with 2048 points in t_2. Zero filling in t_1 gave a 1024 × 1024 point frequency domain matrix. Both t_1 and t_2 were multiplied with a sine-bell function to produce pseudoecho shaping (Bax & Freeman, 1981).

Two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) employed the three pulse sequence $(90°-t_1-90°-\tau_m-90°-t_2)_n$ (Jeener et al., 1979). A 32-pulse phase cycle was used to suppress artifacts, to produce absolute phase spectra, and to permit quadrature detection with the carrier frequency in the center of the spectrum (States et al., 1982). Thirty-two pulses were used for each of 512 t_1 values with 4096 points in t_2. Zero filling in t_1 gave a 1024 × 1024 point frequency domain matrix. Mixing times of 50–600 ms were tried with 200 ms found to be optimal. For resolution enhancement, both t_1 and t_2 were treated by Gaussian multiplication with a line-broadening factor of 1 Hz followed by multiplication with a linear function decreasing from 1 to 0 during the last 500 points of each data set. Both COSY and NOESY spectra were run at 9 °C because no NOESY cross-peaks were observed at room temperature; COSY and NOESY spectra were also examined before and after symmetrization.

For COSY and NOESY experiments, 15 mg of pseudobactin A214 was used. In order to avoid the difficulties of collecting spectra in pure H_2O, Me_2SO-d_6 solutions containing small amounts of water were employed. Dimethyl-d_6 sulfoxide solutions containing 4% D_2O or H_2O were found to improve the resolution of resonances corresponding to the α protons compared to Me_2SO solutions alone. Hence, deuterium-exchanged samples were run in Me_2SO-d_6–4% D_2O, whereas nonexchanged (protonated) samples were run in Me_2SO-d_6–4% H_2O. All samples were prepared under nitrogen gas. For samples in Me_2SO-d_6–D_2O, a sweep width of ±1600 Hz was employed, whereas for samples in Me_2SO-d_6–H_2O a sweep width of ±2500 Hz was used with the carrier frequency set on the H_2O peak. In Me_2SO-d_6–H_2O, the H_2O peak was suppressed by continuous irradiation at all times except t_1 and t_2.

Carbon-13 NMR spectra were obtained as described previously (Teintze & Leong, 1981) with chemical shifts expressed relative to an external standard of dioxane in D_2O equal to 67 ppm.

Mass Spectroscopy. Liquid secondary ion mass spectra were taken at the University of California San Francisco Mass Spectrometry Facility. Gas chromatography–mass spectrometry (GC–MS) was performed on a 3% Dexil 300 column followed by mass spectral analysis in the chemical ionization mode with ammonia or electron-impact mode at the University of California, San Diego.

Amino Acid Analyses. Analyses were performed on an Interaction amino acid column at 52 °C in Pico buffers (Pierce). A Varian 5040 HPLC and LDC Fluoromonitor III were employed with postcolumn detection by *o*-phthalaldehyde (Benson & Hare, 1975).

Partial Acid Hydrolysis. Partial acid hydrolysis of pseudobactin A214 was accomplished by two methods. In the first method, 30 mg of pseudobactin A214 was hydrolyzed in vacuo for 4.5 h at 105 °C in 10 mL of 0.05 N HCl. The hydrolysate was subjected to preparative paper electrophoresis on Whatman 3MM paper at pH 6.5; the neutral band, which was stained by both ninhydrin and *tert*-butyl hypochlorite, was cut out and eluted with water. This fraction was further purified by paper electrophoresis at pH 1.6 and then at pH 3.5 followed by electrophoresis twice more at pH 1.6. In each case, the slowest major, cathodic-migrating band that stained with ninhydrin and *tert*-butyl hypochlorite was eluted.

In the second method, six 5-mg portions of pseudobactin A214 were hydrolyzed in vacuo for 4 h at 110 °C in 1.5 mL aliquots of 0.03 N HCl. 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 *tert*-butyl hypochlorite, was eluted. The slowest cathodic-migrating band was eluted following electrophoresis at pH 1.6 and was further purified by electrophoresis at pH 3.5. The slowest anodic-migrating band was eluted and subjected to electrophoresis at pH 6.5. One band, migrating toward the anode, was observed and eluted.

Sequencing of Peptides. Hydrolytic peptides were sequenced on a Beckman 890C spinning-cup system with 3-phenyl-2-thiohydantoin (PTH) amino acids identified on a Beckman HPLC with a Hitachi UV detector. The PTH of β-OH-Asp was extracted with ethyl acetate.

Identification of Hydroxamate Acyl Group. The acetyl group was identified as described previously (Yang & Leong, 1984).

Identification of Succinic Acid. About 50 μg of pseudobactin A214 was hydrolyzed in vacuo for 18 h at 110 °C in 0.5 mL of 6 N HCl. After the hydrolysate was concentrated to dryness in vacuo, 0.10 mL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide–TRISIL (Pierce) (1:1 v/v) was added, and the solution was heated for 2 h at 60 °C. It was then analyzed by GC–MS and by gas chromatography on a SPB-5 capillary column (0.75 mm × 60 m) with flame ionization detection.

Results

Pseudomonas A214 produced a yellow-green, fluorescent siderophore, designated pseudobactin A214, when cultured in iron-limiting culture medium. Pseudobactin A214 possessed properties typical of siderophores, including complete repression of biosynthesis in culture media containing micromolar amounts of iron(III) (data not shown). Furthermore, pseudobactin A214 and ferric pseudobactin A214, both at 10 μM, were about equally effective in reversing iron starvation of strain A214 induced by the synthetic ferric complexing agent ethylenediaminedi[(*o*-hydroxyphenyl)acetic acid] (EDDA), the iron of which is not utilized by the cells in the plate bioassay. This stimulation of growth was evidenced by a halo of single colonies surrounding the paper disks containing the siderophore. 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. Hence, *Pseudomonas* A214 could utilize pseudobactin A214 to transport iron(III).

Red-brown ferric pseudobactin A214 was very soluble in water and had a migration upon electrophoresis at pH 6.5

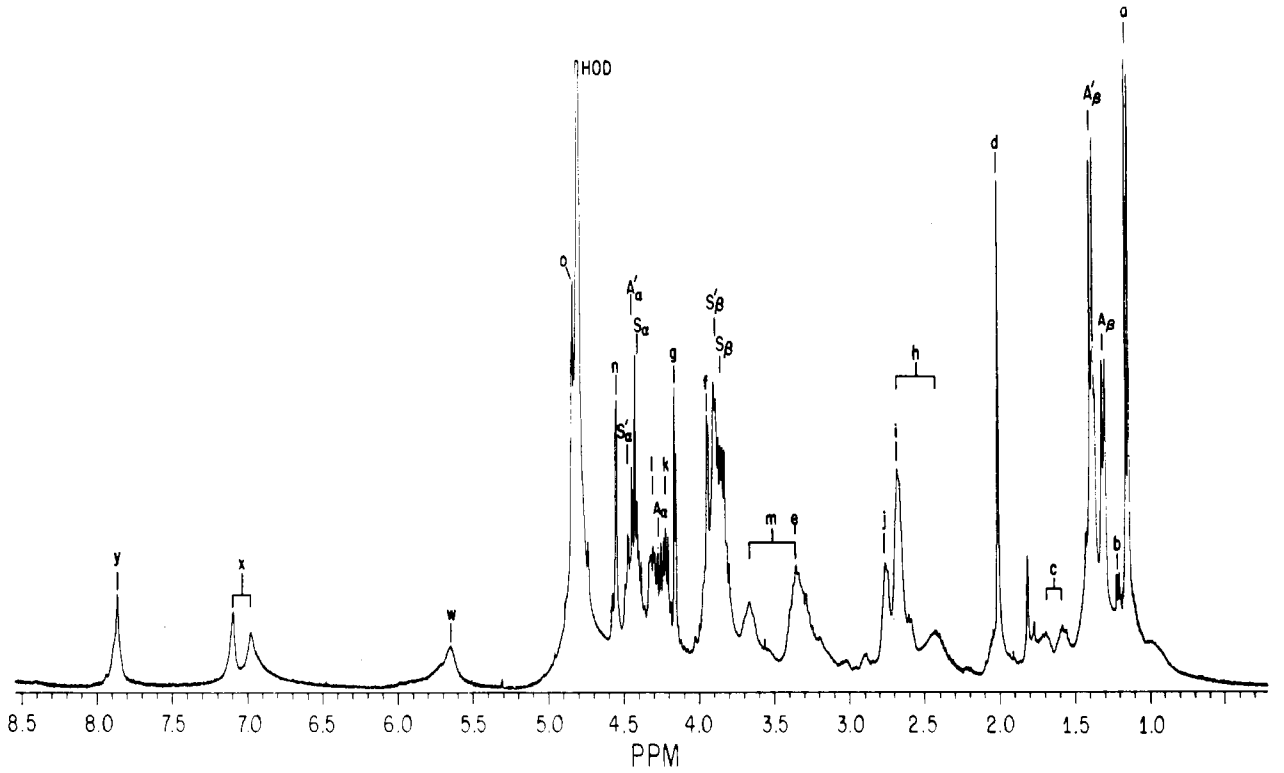

FIGURE 3: ^{1}H NMR spectrum (360 MHz) of pseudobactin A214 in D_2O at room temperature. Chemical shifts are in ppm from internal sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$. Since the alanine and serine resonances could not be assigned to a specific alanine (Ala_1 or Ala_2) or serine (Ser_1 or Ser_2), they are indicated in this figure as A and A′ and S and S′, respectively. S_β is apparently an ABX octet whose two furthest downfield peaks are obscured by S'_β, which appears as a degenerate ABX system. The peak at 1.82 ppm was determined to be an impurity.

intermediate between that of ferric pseudobactin 7SR1 (1− ionic charge) and that of ferrichrome A (3− ionic charge), indicating that it was dianionic. The extinction coefficient, ϵ_{405}, was 2.3×10^4 L mol^{-1} cm^{-1} (data not shown), on the basis of one iron(III) per molecule of ferric pseudobactin A214.

Yellow-green, fluorescent pseudobactin A214 was very soluble in water and had a migration similar to that of ferric pseudobactin 7SR1 upon electrophoresis at pH 6.5, indicating that it was monoanionic. Its extinction coefficient, ϵ_{381}, was 1.6×10^4 L mol^{-1} cm^{-1} (data not shown). Mass spectral analysis of the pyridinium salt of pseudobactin A214 yielded an *m/e* peak at 1150.4 in the anionic detection mode and at 1152 in the cationic detection mode, which were assigned to M^- and MH_2^+, respectively, where M^- is the parent anion. Thus, the molecular mass of pseudobactin A214 according to mass spectrometry was 1150 g/mol.

Amino acid analysis of a 6 N HCl hydrolysate of pseudobactin A214 yielded 2 mol of alanine, 2 mol of serine, 1 mol of glycine, 1 mol of threonine, and 1 mol of *threo*-β-hydroxyaspartic acid (β-OH-Asp) (Teintze et al., 1981). Analysis of a 47% HI hydrolysate revealed 1 mol of ornithine in addition to the above analysis. The presence of ornithine in the reductive hydrolysate but not in the HCl hydrolysate indicated that pseudobactin A214 contained 1 mol of N^δ-hydroxyornithine (N^δ-OH-Orn) (Emery & Neilands, 1961). The threonine residue was determined to have the L-allo configuration by Dr. J. L. Bada at the Amino Acid Dating Laboratory, Scripps Institution of Oceanography, University of California, San Diego, by a modification of a previously published method (Aswad, 1984), which permitted the separation of the four possible stereoisomers of threonine.

The presence of β-OH-Asp and N^δ-OH-Orn in pseudobactin A214 implicated an α-hydroxy acid group and a hydroxamate group, respectively, as two iron-binding bidentate ligands. The

Table I: Proton NMR Chemical Shifts and Multiplicities of Pseudobactin A214 in D_2O[a]

resonance	δ (ppm)	multiplicity	resonance	δ (ppm)	multiplicity
a	1.19	d	S'_β	3.94	br m
b	1.25	m	f	3.98	br s
A_β	1.35	d	g	4.19	d
A'_β	1.43	d	k	4.21	m
c	1.61	br m	A_α	4.30	q
	1.73		l	4.33	m
d	2.05	s	S_α	4.45	m
h	2.47	br m	A'_α	4.48	q
	2.71		S'_α	4.51	m
i	2.72	m	n	4.59	d
j	2.80	m	o	4.88	d
e	3.36	br m	w	5.69	br s
m	3.41	br m	x	7.02	br s
	3.72			7.13	br s
S_β	3.87	m	y	7.91	br s
	3.92				

[a] Abbreviations: s, singlet; d, doublet; q, quartet; m, multiplet; br, broad.

similarity of the visible absorption spectra of pseudobactin A214 and ferric pseudobactin A214 to the respective spectra of pseudobactin (Teintze et al., 1981) and pseudobactin 7SR1 (Yang & Leong, 1984) and their ferric complexes suggested that pseudobactin A214 contained a 1,2-dihydroxy aromatic group derived from the yellow-green, fluorescent chromophore as the third iron-binding bidentate ligand. The structure of pseudobactin A214 including the nature of the acyl group of the hydroxamate group and the fluorescent group and its location were determined from its ^{1}H and ^{13}C NMR spectra in conjunction with its mass spectral molecular weight of 1150.

The ^{1}H NMR spectrum of pseudobactin A214 in D_2O is shown in Figure 3 with the resonances summarized in Table I. The proposed structure of pseudobactin A214 is shown in

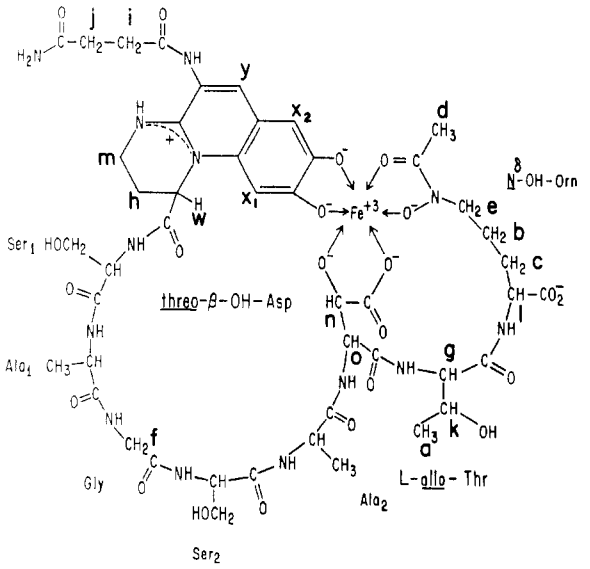

FIGURE 4: Proposed structure of ferric pseudobactin A214. The letters refer to the assignments in the ^1H NMR spectrum of pseudobactin A214 in D_2O. A rationale for the structure shown here is given in the text.

Figure 4; the letters assigned to the hydrogen atoms in Figure 4 refer to the proton resonances in Figures 3, 5, and 6. Assignments in Figure 3 were made by comparison with spectra of pseudobactin (Teintze & Leong, 1981) and pseudobactin 7SR1 (Yang & Leong, 1984) and by decoupling experiments. The proton-decoupled ^{13}C resonances of pseudobactin A214 in D_2O are summarized in Table II (Table II is included in the supplementary material; see paragraph at end of paper regarding supplementary material.) A total of 46 carbon resonances was observed: 13 carbonyl carbon resonances between 170 and 178 ppm, 9 aromatic carbon resonances between 100 and 155 ppm, and 24 aliphatic carbon resonances between 16 and 72 ppm from tetramethylsilane. The structure shown in Figure 4 is consistent with the total number of carbon resonances and the number of carbon resonances within each family.

The fluorescent chromophore of pseudobactin A214 was readily identified from its characteristic ^1H and ^{13}C resonances. The chemical shifts of the nine aromatic carbon resonances (Table II) were very similar to those of pseudobactin (Teintze & Leong, 1981) and pseudobactin 7SR1 (Yang & Leong, 1984). The chemical shifts for protons h, m, w, x_1, x_2, and y (Table I) were remarkably similar to those of pseudobactin and pseudobactin 7SR1. The substituent attached to carbon 3 of the quinoline chromophore was determined to be derived from succinamide. Succinic acid was identified as its bis(trimethylsilyl) derivative after trimethylsilylation of acid-hydrolyzed pseudobactin A214 by its retention time from gas chromatography and its electron-impact mass spectral fragmentation pattern from GC–MS. Chemical-ionization mass spectrometry yielded an m/e peak at 263, which was assigned as MH^+, where M is the parent. The mass spectral molecular weight of 1150 for pseudobactin A214 required that both carboxylic acid groups of succinic acid be amides. The chemical shifts (Table I) for protons i and j of the succinamide moiety were similar to those of pseudobactin (Teintze & Leong, 1981).

The hydroxamate acyl group was determined to be acetyl in the same manner described previously for pseudobactin 7SR1 (Yang & Leong, 1984). Pseudobactin A214 therefore contained 1 mol of N^δ-acetyl-N^δ-hydroxyornithine. As expected, the acetyl group of pseudobactin A214 had a proton resonance (2.05 ppm) virtually identical with that of pseudobactin 7SR1 (Yang & Leong, 1984).

In order to complete the structure determination, it was necessary to sequence the peptide portion of pseudobactin A214. Pseudobactin A214 did not react with ninhydrin or dansyl chloride, indicating the absence of a free amino terminus. The ionic charge of pseudobactin A214 (1−) (see below) and its mass spectral molecular weight ruled out the possibility of a cyclic peptide. Hence, pseudobactin A214 must be an N-blocked peptide. It was not affected by Nagarse, Pronase, or thermolysin under conditions where control peptides were completely digested (data not shown). We therefore attempted to sequence the peptide by ^1H NMR using methods developed by Wüthrich et al. to study protein conformation (Wider et al., 1984). Our strategy was to assign proton resonances for all the amino acids and then use the nuclear Overhauser effect to determine which protons were spatially near each other (less than 5 Å). NOE's would be expected to be observed between the amide proton of one amino acid residue and either the α, β, or amide proton of the preceding amino acid (Billeter et al., 1982). The resonances corresponding to the side-chain protons were assigned by decoupling and COSY experiments in Me_2SO-d_6–D_2O (data not shown). The amide protons were then assigned from COSY spectra taken in Me_2SO-d_6–H_2O. An example of a COSY spectrum is shown in Figure 5. The resonances for the N^δ-OH-Orn protons and the Gly protons could not be identified with certainty.

A NOESY spectrum of pseudobactin A214 is shown in Figure 6. NOESY cross-peaks were observed between Ser_1 NH and proton w and between Ser_1 NH and proton x_1, indicating that Ser_1 NH was spatially close to both protons w and x_1. In addition, the amide (9.3 ppm) and α (4.5 ppm) proton resonances of Ser_1 occurred further downfield than the corresponding proton resonances (8.5 and 4.3 ppm, respectively) of Ser_2, suggesting that Ser_1 was closer to the quinoline aromatic system than Ser_2. A similar effect was observed in pseudobactin 7SR1 for the α and β proton resonances of the serine residue attached to the fluorescent chromophore via an ester linkage (Yang & Leong, 1984). Since pseudobactin A214 must be an N-blocked peptide, the above NOESY cross-peaks, the downfield chemical shifts of the amide and α proton resonances of Ser_1 compared to those of Ser_2, and the fact that the amide proton resonance of Ser_1 occurred further downfield than any other amide proton suggest that the fluorescent chromophore is attached via an amide bond to N-terminal Ser_1.

NOESY cross-peaks between Ser_1 $C_\alpha H$ and Ala_1 NH, between Ala_1 $C_\alpha H$ and Ser_2 NH, and between Ala_1 $C_\beta H$ and Ser_2 NH suggest the sequence Ser_1-Ala_1-Ser_2. There was a break in the sequence because no cross-peak was observed with Ser_2 $C_\alpha H$ or $C_\beta H$. Cross-peaks between Ala_2 $C_\alpha H$ and β-OH-Asp NH and between β-OH-Asp $C_\alpha H$ and Thr NH suggest the sequence Ala_2-β-OH-Asp-Thr.

The remainder of the amino acid sequence of pseudobactin A214 was obtained from overlap of amino acid sequences of smaller peptides obtained from partial acid hydrolysis. The peptide Ala-Gly-Ser-Ala was obtained from preparative paper electrophoresis of a 0.05 N HCl hydrolysate of pseudobactin A214, whereas partial acid hydrolysis of pseudobactin A214 with 0.03 N HCl yielded the peptide Ala-Gly-Ser-Ala-β-OH-Asp. Since the N-terminal Ser was attached to the fluorescent chromophore, N^δ-OH-Orn must be the C-terminal amino acid.[2] Hence, pseudobactin A214 contained an N-blocked

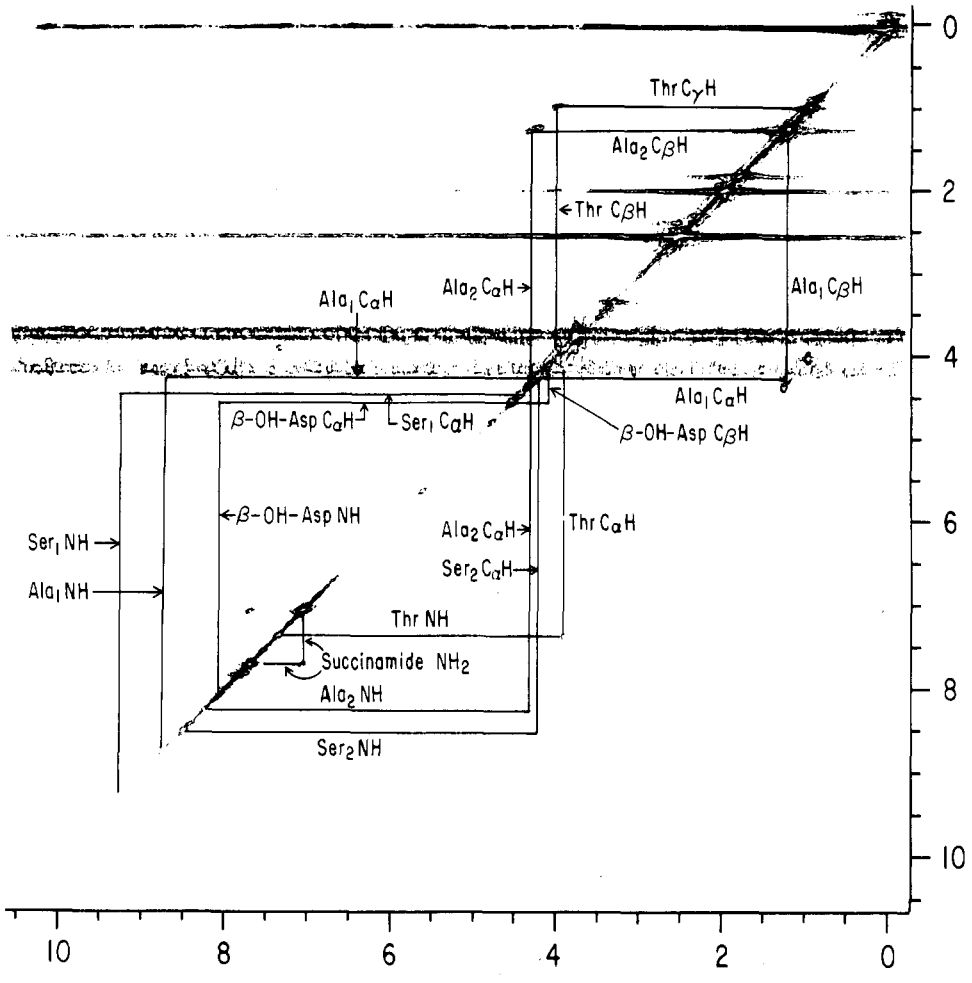

FIGURE 5: COSY spectrum of pseudobactin A214 in Me_2SO-d_6–4% H_2O at 9 °C. Chemical shifts are in ppm from internal tetramethylsilane. COSY cross-peaks occur symmetrically about the main diagonal. Only one set of lines joining the main diagonal to cross-peaks has been drawn; these lines trace out connectivities for each amino acid.

octapeptide with the amino acid sequence Ser-Ala-Gly-Ser-Ala-*threo*-β-OH-Asp-L-*allo*-Thr-N^δ-OH-Orn. However, these results contradict the sequence Ser_1-Ala_1-Ser_2 predicted from the NMR data. In retrospect, the cross-peaks between Ala_1 C_αH and Ser_2 NH and between Ala_1 C_βH and Ser_2 NH may be artifacts (see Discussion).

The proposed structure of pseudobactin A214 (Figure 4) is consistent with its ionic charge of 1−. This is easily explained by the 1+ from the quinoline group, the 1− from the β-OH-Asp group, and the 1− from the C-terminal carboxyl group. Loss of four protons (two from the 1,2-dihydroxy aromatic group, one more from the α-hydroxy acid group, and one from the hydroxamate group) upon binding to iron(III) would result in a net charge of 2− for ferric pseudobactin A214. The proposed structure, which has a molecular formula of $C_{46}H_{64}N_{13}O_{22}$ and an accompanying calculated mass spectral molecular mass of 1150.4 g/mol, was consistent with its ^1H and ^{13}C NMR spectra and its observed mass spectral molecular weight of 1150.4 in the anionic mode and 1152 in the cationic mode. Protonation of the two carboxylic acid groups of pseudobactin A214 would produce a cationic species with a calculated mass spectral molecular weight of 1152.4.

Discussion

Pseudobactin A214 is the third siderophore from root-colonizing fluorescent pseudomonads whose structure has been determined. The chemical structures of pseudobactin (Teintze et al., 1981), pseudobactin 7SR1 (Yang & Leong, 1984), and pseudobactin A214 are remarkably similar. The three bidentate iron(III)-chelating groups consisting of a 1,2-dihydroxy aromatic group in the fluorescent quinoline chromophore, an α-hydroxy acid group present at β-OH-Asp, and a hydroxamate group derived from N^δ-OH-Orn are conserved in each structure. The fluorescent chromophore is nearly identical in all three compounds, differing only in the succinamide or malamide side chains. Pseudobactin has been shown to contain D-amino acids (Teintze et al., 1981), and pseudobactin 7SR1 (Yang & Leong, 1984) and pseudobactin A214 probably also contain D-amino acids because they were both unaffected by a variety of nonspecific proteolytic enzymes.

A number of comparisons can be made among the structures of these three siderophores. Pseudobactin contains a C-blocked peptide; pseudobactin A214 contains an N-blocked peptide, whereas pseudobactin 7SR1 consists of a cyclic peptide. The quinoline derivative is attached via an amide bond to the peptide in pseudobactin and pseudobactin A214, whereas an

[2] Since no N^δ-OH-Orn-containing peptide could be isolated from partial acid hydrolysis, we attempted to confirm that N^δ-OH-Orn was the C-terminal amino acid by hydrazinolysis of pseudobactin A214 (Narita et al., 1975). No free amino acid could be identified. When N^δ-acetyl-N^δ-hydroxyornithine was treated with hydrazine as a positive control, no N^δ-OH-Orn could be likewise isolated. These results were not surprising considering the oxidative instability of N^δ-OH-Orn. We next attempted to convert the N^δ-OH-Orn residue in pseudobactin A214 into Glu by oxidation with performic acid (Mikeš & Turková, 1962). However, the resulting compound contained little β-OH-Asp, Thr, or Ser, and only about half of the N^δ-OH-Orn was converted to Glu (data not shown). This compound was not studied further.

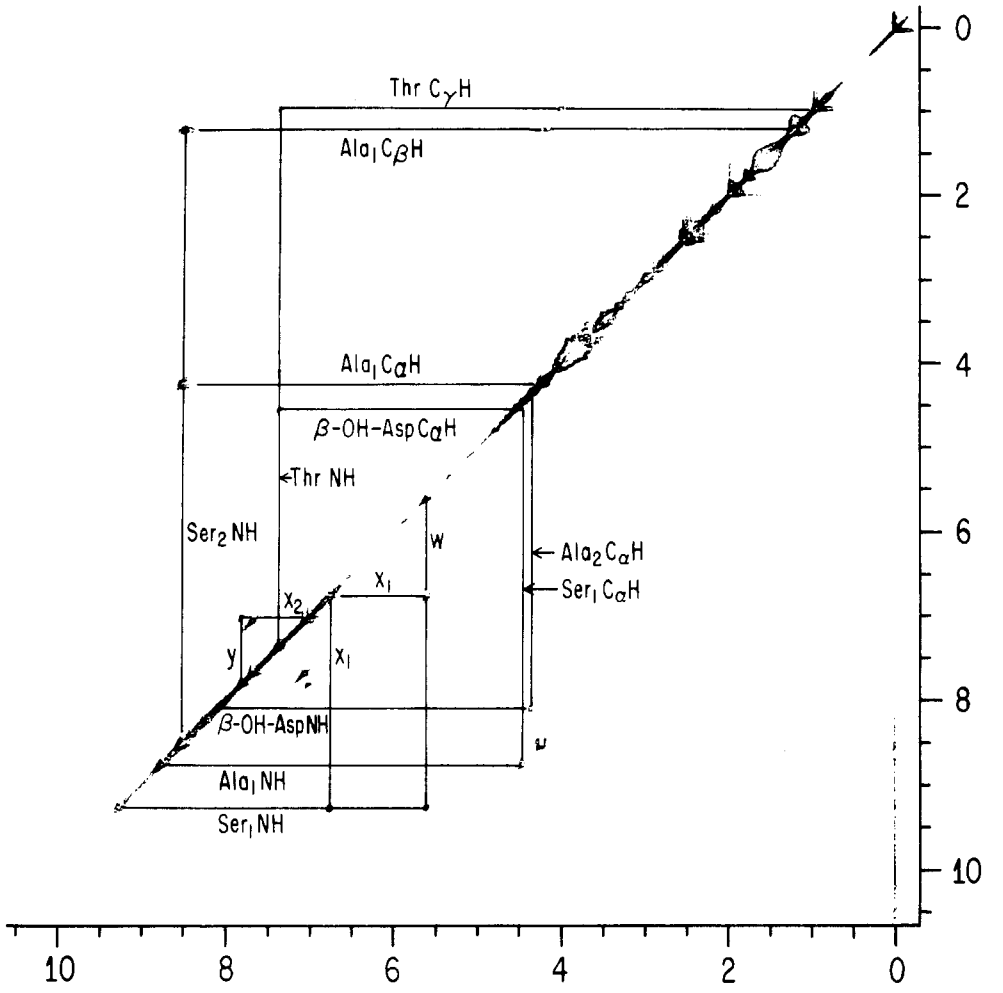

FIGURE 6: Symmetrized NOESY spectrum of pseudobactin A214 in Me_2SO-d_6–4% H_2O at 9 °C. Chemical shifts are in ppm from internal tetramethylsilane. Both COSY and NOESY cross-peaks occur symmetrically about the main diagonal, but for the sake of clarity only one set of NOESY cross-peaks is connected with lines drawn to the main diagonal, indicating protons spatially near each other.

ester linkage is used in pseudobactin 7SR1. Pseudobactin 7SR1 and pseudobactin A214 are octapeptides with amino acid compositions differing in only one amino acid and have the same hydroxamate group present as N^δ-acetyl-N^δ-hydroxyornithine. Pseudobactin is a hexapeptide with the hydroxamate group formed from a cyclized carboxy terminal N^δ-OH-Orn. Pseudobactin 7SR1 and pseudobactin A214 have very similar sequences; the stretch of six amino acids from Ala_1 to Thr in pseudobactin A214 is identical with that of pseudobactin 7SR1 except that the latter contains a Ser instead of Ala_2.

Pseudomonas fluorescens ATCC 13525 produces a siderophore designated pyoverdine with a fluorescent chromophore similar to that of pseudobactin (Philson & Llinâs, 1982 a,b). The same chromophore occurs in pyoverdine *Pa* isolated from *Pseudomonas aeruginosa* PAO 1 (ATCC 15692) (Wendenbaum et al., 1983). These siderophores were both reported to contain N^δ-OH-Orn but not β-OH-Asp.

Root-colonizing fluorescent pseudomonads produce siderophores with apparently diverse structures, yet the three bidentate iron-chelating ligands appear to be conserved thus far. Hence, these siderophores might be expected to have similar equilibrium binding constants for iron(III); however, the kinetics of bacterial iron transport from these ferric siderophores might vary considerably. We have found that the iron starvation of certain bean-deleterious fluorescent pseudomonads by specific bean-beneficial fluorescent pseudomonads is due in part to the inability of susceptible strains to utilize siderophores from beneficial strains to transport iron(III) (Buyer & Leong, 1986). Conversely, deleterious strains that were able to utilize siderophores from beneficial strains were not inhibited. Hence, the advantage gained by any strain that produces a siderophore that no other strain can use for iron transport may account for the structural diversity of fluorescent siderophores. Structural variations in a siderophore might affect the ability of a ferric siderophore transport system to recognize and hence utilize a particular ferric siderophore. It might also be advantageous for a strain to possess an iron transport system capable of recognizing and utilizing as many different siderophores as possible. We have found that the ability of a given fluorescent pseudomonad to utilize another fluorescent pseudomonad's siderophore appears to depend upon its possessing an outer membrane receptor protein for that pseudomonad's ferric siderophore (Magazin et al., 1986). Since iron starvation is one mechanism by which beneficial fluorescent pseudomonads inhibit the growth of deleterious fluorescent pseudomonads, the most effective beneficial strain might be expected to produce a siderophore that is not utilized by any deleterious strains present in the rhizosphere yet possesses an iron transport system capable of utilizing siderophores from deleterious strains.

Finally, it should be noted that amino acid sequencing by NMR will clearly not replace Edman degradation as a general method. The erroneous sequence Ser_1-Ala_1-Ser_2 as deduced from NMR cross-peaks between Ala_1 and Ser_2 may be attributed in part to an unusual conformation of pseudobactin A214 bringing Ala_1 $C_\alpha H$ and Ala_1 $C_\beta H$ in close spatial proximity to Ser_2 NH. We conclude that great caution should be exercised in deducing sequences from NMR data. How-

ever, NMR sequencing may be useful in certain cases such as cyclic or N-blocked peptides as a supplement to sequencing by more traditional methods.

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SUPPLEMENTARY MATERIAL AVAILABLE

Table II listing the ^{13}C NMR chemical shifts of pseudobactin A214 in D_2O (2 pages). Ordering information is given on any current masthead page.

Registry No. Fe, 7439-89-6; pseudobactin A214, 99332-36-2.

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