Structures and Characteristics of Novel Siderophores from Plant Deleterious *Pseudomonas fluorescens* A225 and *Pseudomonas putida* ATCC 39167[†]

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ABSTRACT: When *Pseudomonas putida* ATCC 39167 and plant-deleterious *Pseudomonas fluorescens* A225 were grown in an iron-deficient culture medium, they each produced two different novel yellow-green fluorescent pseudobactins: P39167-I, II and PA225-I, II. Pseudobactin P39167-I has a molecular formula of $C_{46}H_{65}O_{23}N_{13}$ and is monoanionic at neutral pH. P39167-II has the molecular formula of $C_{46}H_{63}O_{22}N_{13}$ and no charge at neutral pH. Pseudobactin PA225-I has a molecular formula of $C_{46}H_{65}O_{24}N_{13}$ and is monoanionic at neutral pH whereas pseudobactin PA225-II has the molecular formula of $C_{46}H_{63}O_{23}N_{13}$ and no charge at neutral pH. All four of the pseudobactins contain a dihydroxyquinoline-based chromophore. The amino acid sequence for the octapeptide in case of pseudobactins from *P. putida* ATCC 39167 is Chr-Ser(1)-Ala(1)-AcOHOrn-Gly-Ala(2)-OHAsp-Ser(2)-Thr. In case of pseudobactins from *P. fluorescens* A225, the octapeptide has the sequence Chr-Ser(1)-Ala-AcOHOrn-Gly-Ser(2)-OHAsp-Ser(3)-Thr. For all four pseudobactins (P39167-I, II and PA225-I, II), the serine(1) residue of the octapeptide is attached to the carboxylic acid group on the C-11 of the fluorescent quinoline via an amide bond. Additionally, for pseudobactin P39167-II and PA225-II, the hydroxyl group of the serine(1) residue is also attached to the carboxyl group of threonine residue at the carboxy terminus of the peptide via an ester bond, resulting in a cyclic depsipeptide in contrast to the linear peptide chain of P39167-I and PA225-I. For all four pseudobactins, a malamide group is attached to the C-3 of the quinoline derived chromophore. The three bidentate iron(III) chelating groups in all four pseudobactins consist of a 1,2-dihydroxy aromatic group of the fluorescent chromophore, a hydroxy acid group of β-hydroxy aspartic acid, and a hydroxamate group from the acylated N^{δ}-hydroxyornithine. The amino acid constituents of the pseudobactins P39167 I, II are the same as those in pseudobactin A214, whereas those in A225 I, II are the same as in 7SR1, but in both cases the sequences are different. The uptake results indicate a single outer membrane receptor protein for ferric-pseudobactins in both organisms. The receptor proteins in the two species are similar but not identical.

Fluorescent pseudomonads are prominent members of the microflora in the rhizosphere of many plants. They produce a yellow-green pigment (Stanier et al., 1966), which was characterized as a siderophore, an iron transport agent, that contains a fluorescent chromophore (Abdallah, 1991; Meyer & Abdallah, 1978; Meyer & Hornsberger, 1978). Specific root colonizing members of *Pseudomonas* can be plant-growth promoting (PGP) or plant-growth deleterious (PGD)[1] (Loper & Schroth, 1986; Persmark et al., 1990; Schroth & Hancock, 1981). The deleterious organisms affect plant growth and crop yield adversely by inhibiting nutrient uptake by the roots or by producing phytotoxins or hormones (Becker et al., 1985; Gross & Cody, 1985; Persmark et al., 1990). PGP pseudomonads are presumed to function primarily by scavenging iron from the root environment and limiting the supply of this basic nutrient for deleterious fungi and bacteria (Kloepper et al., 1980; Schroth & Hancock, 1981, 1982; Suslow & Schroth, 1982). PGP may also contribute to production of plant hormones and antibiotics (Persmark et al., 1990; Schippers et al., 1987; Xu & Gross, 1986). The presence of pseudobactins (pyoverdines), the siderophores of fluorescent *Pseudomonas* species, have been reported to affect the crop yield of wheat, barley, maize, potato, flax, radish, bean, lettuce, and sugar beat (Leong, 1986; Schroth & Hancock, 1982; Suslow & Schroth, 1982; Xu & Gross, 1986). Among the members of PGP pseudomonads are *Pseudomonas* B10 (Teintze et al., 1981), *Pseudomonas putida* WCS358 (van der Hofstad et al., 1986), and *Pseudomonas* A589 (Persmark et al., 1990), A112, A215, A216, B122, B222, and B226 (Buyer & Leong, 1986). Some of the plant-deleterious strains are *Pseudomonas* 7SRI (Yang & Leong, 1984), A214, A225, and B117 (Buyer & Leong, 1986).

The three-dimensional structure of only one fluorescent siderophore, the one from *P.* B10, has been determined by

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[1] Abbreviations: PGP, plant growth promoting; PGD, plant growth deleterious; COSY, 2-dimensional $^1H-^1H$ correlated spectroscopy; OM, outer membrane.

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X-ray diffraction (Teintze et al., 1981). However, a number of other pseudobactins/pyoverdines have been isolated and characterized from other fluorescent *Pseudomonas* species (Budzikiewicz, 1993; Buyer et al., 1986; Demange et al., 1987; Persmark et al., 1990; Teintze & Leong, 1981; van der Hofstad et al., 1986). The chemical structure of these pseudobactins were elucidated by using chemical degradation and spectroscopic techniques. The pseudobactins have common structural features and are composed of either a cyclic or linear peptide chain to which is attached a chromophore derived from quinoline. The amino group at the C-3 position of the chromophore is commonly linked to a small dicarboxylic moiety. Pseudobactins represent a unique class of siderophores because they contain three different bidentate ligating groups for iron: hydroxamate from cyclic or linear $^{\delta}$N-acylated hydroxylated ornithine, an hydroxy acid from hydroxy aspartic acid, and an *o*-dihydroxy group on the fluorescent aromatic chromophore. Some pseudobactins contain two $^{\delta}$N-acylated hydroxy ornithines and an *o*-dihydroxy group for iron chelation (Budzikiewicz, 1993; Wendenbaum et al., 1983). The diversity in the structures of pseudobactins is larger than in any other family of siderophores and is due to the different number of amino acids in the peptide chain, their sequence, and chirality. Because there is diversity in their structures, it is interesting to pursue studies on the specificity of their uptake behavior.

The knowledge of the structures of pseudobactins from both PGP and PGD *Pseudomonas* species will contribute toward understanding the basics of iron competition in the rhizosphere as well as iron transport across the cell membrane. This structural knowledge will also be helpful in designing synthetic iron chelators for clinical and agricultural purposes.

We describe the isolation, purification, characterization, structures, and iron transport studies of four novel pseudobactins, P39167-I and P39167-II from *P. putida* ATCC 39167 (**1** and **2**) and PA225-I and PA225-II from the PGD *P. fluorescens* A225 (**3** and **4**).

EXPERIMENTAL PROCEDURE

Materials. Pseudobactin PWCS358, ferrichrome, ferrichrome A, and *N*-α-dimethyl coprogen were produced and purified in our laboratory. Pyridine, acetonitrile, and glacial acetic acid were purchased from Mallinckrodt. D_2O was purchased from Aldrich. Lichroprep C-18 material was purchased from EM Science corporation. $^{55}FeCl_3$(33.99 mCi/mg) in 0.5 N HCl was purchased from NEN Dupont. Ecolume scintillation cocktail was purchased from ICN Chemicals.

Bacteria and Growth Conditions. P. putida ATCC 39167 was obtained from American Type Culture Collection, and *P. fluorescens* A225 was provided by Dr. John Leong. Both the cultures were maintained on succinate minimal media with 10% glycerol at 4 °C. An iron-deficient minimal medium (Meyer & Abdallah, 1978) consisting of 6 g of K_2HPO_4, 3 g of KH_2PO_4, 5 g of succinic acid, 1 g of NH_4Cl, and 0.2 g of $MgSO_4 \cdot 7H_2O$ per liter was used for production of pseudobactins from the two organisms. The pH of the medium was adjusted to 7.0. A 250 mL conical flask containing 50 mL of the above medium was inoculated with 1 mL permanents, and the cultures were shaken overnight at room temperature. Approximately 30 mL of these cultures were used to inoculate 6 flasks, each containing 1 L of the sterilized medium. The cultures were shaken at 200 rpm at room temperature for 48 h. The pH of the cultures was checked occasionally and adjusted to 7.0 with 0.1 M HCl.

Isolation and Purification of Siderophores. Cells were separated from the supernatant by centrifugation at 3500 rpm for 40 min. The pH of the supernatant was adjusted to 4.0 with acetic acid, and the pseudobactins were extracted by hydrophobic chromatography on an octadecylsilane (Licroprep C-18) column that was previously equilibrated with 0.05 M pyridine−acetic acid buffer at pH 5.0. The column was washed with 10 bed volumes of 0.05 M pyridine−acetic acid buffer, and the pseudobactins were eluted with 1:1 v/v mixture of 0.05 M pyridine−acetic acid, pH 5.0, buffer and HPLC grade acetonitrile. The yellow-green pseudobactin bands, eluted from the Licroprep column, were concentrated under vacuum on a rotary evaporator and filtered through a 3 K molecular weight cutoff filter from Amicon in a centrifuge at 6000 rpm and 0 °C to remove high-molecular-weight peptide or protein. A Gilson HPLC System equipped with UV−vis detector was used for reverse phase chromatography for the final purification. The crude pseudobactin extract was applied to a Nova-pack HR C-18 (7.8 × 300 mm) column from Waters with 0.05 M pyridine−acetic acid at pH 5.8 and eluted with a linear gradient of 0−50% acetonitrile. The flow rate was 2 mL/min, and the detection wavelength was 380 nm. Major bands were rechromatographed to get maximum purity for mass spectrometry and nuclear magnetic resonance experiments. While working with the deferri pseudobactins, the column was periodically washed with 3 mM sodium EDTA solution to avoid possible iron contamination. Iron complexes of the purified pseudobactins were prepared by addition of equimolar amounts of $FeCl_3$. Complete complexation was ensured by adjusting the pH to 7.0. Purification of the Fe-pseudobactins was performed on HPLC-Nova-pack HR C-18 reverse phase column. The solvent system consisted of 18 mM triethylamine at pH 5.5 and eluted with a gradient of 0−50% acetonitrile in the same buffer. The purified pseudobactins and iron-pseudobactins were immediately dried on a freeze drier.

Paper Electrophoresis. Paper electrophoresis was performed on Whatman 3M paper at 1000-V for 1 h by using a buffer of pH 5.0 (pyridine:acetic acid:water, 14:10:930) (Jalal & van der Helm, 1991). Standards included ferrichrome A (Emery & Neilands, 1961), which has a triple negative charge at pH 5.0, ferrichrome (Neilands, 1952), which is neutral, and *N*-α-dimethyl coprogen (Jalal et al., 1988), which bears a positive charge at pH 5.0. Spots were identified directly by color, visualized by UV light, or by spraying with 0.1% $FeCl_3$ in 0.1 M HCl.

UV−Visible Absorption Spectrometry. UV−visible absorption spectra of the pseudobactins and Fe-pseudobactins from *P. putida* ATCC 39167 and *P. fluorescens* A225 were recorded in a 0.05-M sodium phosphate buffer at pH 5.5 on a HP-8452 spectrometer. Molar absorptivities were determined by obtaining the slope of a plot of absorbance at the absorption maxima at several concentrations.

Mass Spectrometry. Electrospray mass spectrometry was performed at the Molecular Biology Resource facility at the University of Oklahoma Health Science Center with a Sciex API III triple-quadruple mass spectrometer (Sciex, Inc. Toronto, Canada). Freeze-dried samples of pseudobactins

and ferric-pseudobactins from *P. putida* ATCC 39167 and *P. fluorescens* A225 were dissolved in acetic acid:water: methanol (0.5: 50:50) at approximately 0.4 mg/mL. This solution was introduced into an electrospray mass spectrometer at a rate of 5 μL/min with a syringe pump. Sample ions generated by the electrospray process were mass analyzed by scanning the first quadruple in 0.2-unit increments.

Electrospray tandem mass spectrometry (ESI-MS/MS) was performed at the Washington University Resource for Mass Spectrometry on a ZAB-T four-sector mass spectrometer (VG-Organic, Manchester, England) equipped with a VG electrospray source with pressure assisted nebulization. Samples were dissolved in water:methanol:acetic acid (50: 50:1) at approximately 50 pmol/μL and loop injected into the source using a running solution of the same composition at a rate of 10 μL/min with a syringe pump. The source was operated at an acceleration potential of 4 KeV. Collision activated dissociation (CAD) was performed with grounded cell and by using CH_4 at 30% transmission as collision gas.

Amino Acid Analysis. Amino acid analysis was performed at Molecular Biology Resource facility at the University of Oklahoma Health Science Center with an automated Beckman system Gold HPLC amino acid analyzer. Sample hydrolysis was performed in evacuated sealed tubes with 6 M HCl at 110 °C for 20−24 h. Amino acids were detected by an on-line post column reaction with ninhydrin (Troine Pickering Laboratories, Inc).

NMR Spectroscopy. Proton (^{1}H, 500 MHz), ^{13}C (125 MHz), and two-dimensional ^{1}H−^{1}H correlated spectroscopy (COSY) were recorded at ambient temperature on a VXR500S NMR instrument. Samples of approximately 8 mg were initially deuterium exchanged by twice lyophilizing in D_2O (1 mL) and finally dissolved in 0.5 mL of D_2O. Resolution enhancement of regular ^{1}H spectra was achieved by Gaussian multiplication, and chemical shifts were referenced to the HOD signal at δ 4.9. All ^{1}H and COSY NMR spectra were obtained by using solvent suppression. An empirically determined low-power presaturation pulse, which gave optimal suppression of the HOD peak in the ^{1}D−^{1}H spectra, was then used in obtaining the COSY spectra. ^{13}C NMR spectra were referenced externally to a 1% CH_3OH in D_2O (49.0 ppm).

Uptake. The uptake studies were performed on the cells of *P. putida* ATCC 39167 and *P. fluorescens* A225 with five ferric-pseudobactins namely, PWCS358, **1**, **2**, **3**, and **4**. All glassware was washed with 6 M HCl, rinsed extensively with doubly distilled water, and autoclaved prior to use. The ^{55}Fe complexes were prepared by adding $^{55}FeCl_3$ to purified deferripseudobactin solutions, with a 20% excess of pseudobactin, and the pH was adjusted to 7.0 with 0.1 M NaOH solution. Iron-deficient standard succinate medium was prepared as descibed previously (Meyer & Abdallah, 1978). *Pseudomonas* cells were grown in this medium at room temperature. Cell growth was monitored by measuring the optical density at 650 nm with a HP-8452 spectrophotometer. Cells were harvested in the log phase, at the optical density of 0.2, by centrifugation at 3500*g* for 45 min at room temperature. The cell pellet was washed twice with half-strength standard succinate media and resuspended in fresh half strength deferriated standard succinate media to an optical density of 0.8 (approximately 0.6 mg of cell/mL).

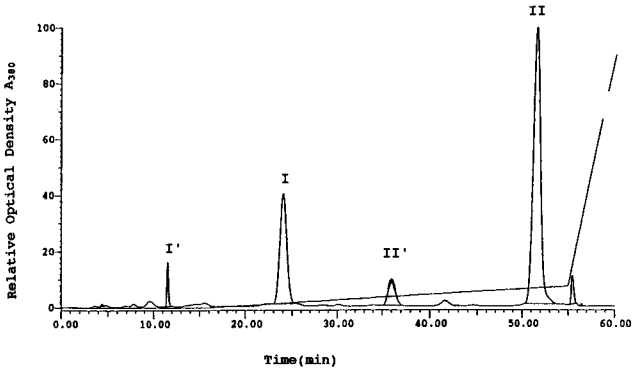

FIGURE 1: HPLC chromatogram for the pseudobactins from *P. putida* ATCC 39167.

This cell suspension was incubated for 1 h at room temperature on a rotary shaker at 200 rpm.

Time dependent uptake studies were started by the addition of ^{55}Fe-pseudobactins (final concentration of 1 μM) from the stock solution to 12 mL (final volume) of the cell suspension under continuous shaking. At regular intervals, 0.5 mL aliquots were withdrawn in duplicate and filtered under vaccum on 0.45 μm HAWP millipore filter. The cell pellets were washed with 10 mL of ice-cold, half-strength succinate media. Filters were dried in an oven at 70 °C for 30 min, weighed, and placed in a scintillation vial to which 6 mL of scintillation cocktail was subsequently added. Scintillation counting was done after 24 h of equilibration. The possibility of induced uptake of heterologous pseudobactin was checked by incubating the cells with that particular deferri heterologous pseudobactin for 4−6 h prior to uptake.

RESULTS

Both *P. putida* ATCC 39167 and *P. fluorescens* A225 produce yellow-green fluorescent pigments when cultured in an iron-limited medium. The pigment possesses the properties typical of pseudobactin siderophores including strong iron binding, a positive CAS assay (Schwyn & Neilands, 1987), and visible absorption maxima around 380−400 nm (Abdallah, 1991). The absorption maxima of the iron-free pseudobactins shifts to shorter wavelengths with a decrease in pH (data not shown). The absorption spectrum of the ferric-pseudobactins is insensitive to pH changes in the range 5−8 (data not shown). Both yellow-green pseudobactins and reddish-brown Fe-pseudobactins are very soluble in water.

The similarity of the visible spectra of pseudobactins from the two organisms and their iron complexes to those of pseudobactin B10, 7SRI, 589A, and others (Buyer et al., 1986; Demange et al., 1990; Persmark et al., 1990; Teintze et al., 1981; Yang & Leong, 1984) suggested that these pseudobactins contain a dihydroxyquinoline-derived aromatic chromophore.

P. putida ATCC 39167. The total siderophore concentration produced in the medium was approximately 125 mg/L. Reverse phase chromatography gave two major and two minor bands. The two major bands from reverse phase chromatography were designated as pseudobactins P39167-I (**1**) and P39167-II (**2**) whereas the minor bands were designated as P39167-I′ and P39167-II′ (Figure 1). Approximately 8 mg of P39167-I and 14 mg of P39167-II were obtained from 1 L of culture medium.

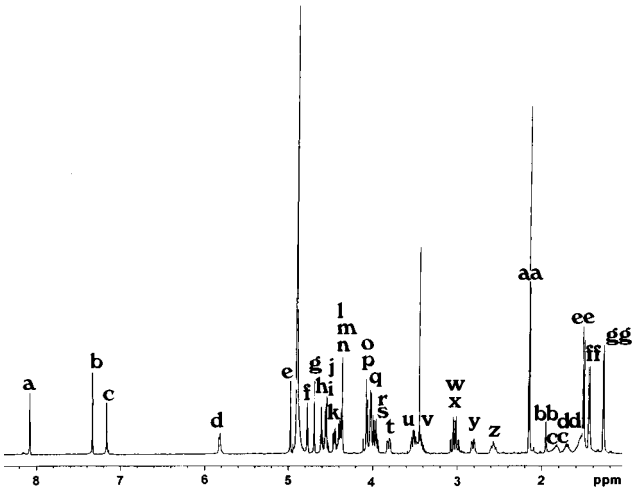

FIGURE 2: ^{1}H NMR spectrum of the linear pseudobactin (**1**) from *P. putida* ATCC 39167.

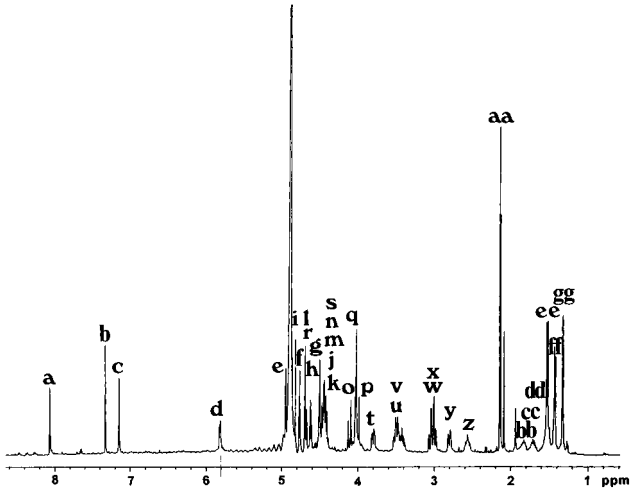

FIGURE 3: ^{1}H NMR spectrum of the cyclic pseudobactin (**2**) from *P. putida* ATCC 39167.

Amino acid analysis of the 6 M HCl hydrolysate of both pseudobactins **1** and **2** revealed the presence of four amino acids Ser, Ala, Gly, and Thr, with the molar ratio of 2:2:1:1. This analysis also indicated that two other, unidentified, amino acids were present.

Upon electrophoresis at pH 5.0, pseudobactin **1** had a migration of $^1/_3$ the distance in between ferrichrome A (-3 ionic charge) and ferrichrome (neutral), revealing itself to be monoanionic, and the Fe complex of **1** moved $^2/_3$ the distance, indicating it was dianionic. Pseudobactin **2** did not migrate from its original position, which indicated it to be neutral, whereas its iron complex **2** migrated $^1/_3$ of the distance between ferrichrome (neutral) and ferrichrome A (-3), suggesting a single negative charge. Molar absorptivities of **1** and **2** were determined to be 1.6×10^4 L $M^{-1}cm^{-1}$ and 1.8×10^4 L $M^{-1}cm^{-1}$, respectively, at 380 nm.

The ^{1}H NMR spectrum of **1** and **2** in D_2O are shown in Figures 2 and 3, and the resonances are summarized in Table 1. The numbering scheme for protons on the chromophore is presented in Figure 4. The chemical shifts of three aromatic protons (labeled as 4, 5, and 8) at δ 8.08, 7.33, and 7.17 are similar to the ones reported in literature for pseudobactins B10, A214, and 7SRI (Buyer et al., 1986; Teintze et al., 1981; Yang & Leong, 1984). The proton at the carbon (C11) to which the carboxyl group is attached in the heterocyclic six-membered ring is accounted for by the characterstic broad doublet at δ 5.82. It was determined by analysis of 2D COSY that this doublet is coupled to the two mutually coupled protons at δ 2.82 [H12 (eq)] and δ 2.57 [H12 (ax)]. These, in turn, are further coupled to the two mutually coupled protons at δ 3.81 [H13 (eq)] and δ 3.52 [H13 (ax)]. Also, these signals are analogous to the ones found for the chromophore in pseudobactins B10, 7SR1, A214, and other pyoverdines (Budzikiewicz, 1993).

A malamide residue was identified as the substituent at the C-3 position of the quinoline ring on the basis of the characteristic chemical shifts and the ABX coupling pattern, which is almost identical to that reported for 7SRI (Yang & Leong, 1984). An AB quartet centered at δ 3.03 (Mal-x_1, x_2) is further coupled to a methylene triplet at δ 4.78 (Mal-y). The chemical shift of this triplet indicates a proton on a carbon bearing an oxygen atom.

The two amino acids that remained unidentified by amino acid analysis were also characterized by ^{1}H NMR (Figures 2 and 3) and COSY spectra. The presence of OH−aspartic acid was evident from two characteristic fine doublets at 4.98 and δ 4.70, which were assigned to β and α protons of hydroxy aspartic acid. An ornithine residue was also identified by inspection of ^{1}H NMR COSY spectra, which reveals the expected spin system: a doublet of doublets resonating at δ 4.46 (Orn-α). This spin system is coupled to two mutually coupled protons at δ 1.83 and 1.71(Orn-β). These, in turn, are coupled to a two-proton multiplet at δ 1.54 (Orn-γ), which is further coupled to a two proton multiplet centered at δ 3.47 (Orn-ϵ). The chemical shift of this multiplet indicates a methylene bearing a nitrogen atom. The ornithine nitrogen is acylated by acetic acid rather than cyclized. This was concluded on the basis of the presence of a three-proton singlet at δ 2.15, which is identical to the one present in the NMR spectra of pseudobactin 7SRI and A214 (Buyer et al., 1986; Yang & Leong, 1984).

The presence of amino acids that were identified by amino acid analysis was also confirmed by means of NMR spectroscopy. The assignments of the three methyl doublets (δ 1.51, 1.45, and 1.28) are based on the analysis of the individual spin systems identified from the COSY spectrum. The two methyl doublets at δ 1.51 and 1.45 show coupling to two quartets at δ 4.55 and 4.39. As no further coupling is evident, two alanines are indicated, in which the resonances at δ 4.55 and 4.39 are assigned to the α protons of two alanines, respectively. The third methyl doublet at δ 1.28 was found to be coupled to a two proton multiplet centered at δ 4.39. As no further coupling was evident, both the α and β of a threonine residue must be superimposed. This is further confirmed by resolution enhancement of the ^{1}H NMR spectrum, which shows the methyl doublet possessing virtual second-order coupling as a result of the close chemical shift proximity of the α and β protons. A two proton doublet at δ 4.03, which is coupled to a triplet at δ 4.61, indicates serine(2), and an AB quartet centered at δ 3.98, which is further coupled to a triplet at δ 4.56, is assigned to serine(1). The numbering of 1 and 2 for these two serines is arbitrary. The remaining spin system, an AB quartet, being centered at δ 4.07 is consistent with α-protons of glycine.

The mass spectral analysis of the pseudobactin **1** yielded a parent ion peak at 1168.4 which represented the $[M + 2H]^+$ species since **1** has a single negative charge. The

Table 1: ^{1}H NMR Chemical Shift and Coupling Constant in D_2O for Pseudobactins **1** and **2**

		δ (ppm) **1**	multiplicity (J)	coupling constant (Hz)	δ (ppm) **2**	multiplicity (J)	coupling constant (Hz)
a.	H4	8.08	s		8.08	s	
b.	H5	7.33	s		7.34	s	
c.	H8	7.17	s		7.16	s	
d.	H11	5.82	d	*J* 5.6	5.83	bs	
e.	β-OH-Asp-β	4.98	d	*J* 2.5	4.96	d	*J* 2.5
f.	mal-y	4.78	t	*J* 5.0	4.78	t	*J* 4.8
g.	β-OH-Asp-α	4.70	d	*J* 2.5	4.51	d	*J* 2.5
h.	Ser2-α	4.61	t	*J* 6.2	4.63	t	*J* 6.1
i.	Ser1-α	4.56	t	*J* 4.9	4.88	m	
j.	Ala-1-α	4.55	q	*J* 7.2	4.46	m	
k.	Orn-α	4.46	dd	*J* 9.5, 5.0	4.46	m	
l.	Thr-α	4.39	m		4.71	d	*J* 2.4
m.	Thr-β	4.39	m		4.46	m	
n.	Ala-2-α	4.39	m		4.46	m	
o.	Gly-α_1	4.10	d	*J* 17.4	4.12	d	*J* 16.1
p.	Gly-α_2	4.05	d	*J* 17.4	4.01	d	*J* 16.1
q.	Ser2-β_{1and2}	4.03	d	*J* 6.2	4.04	d	*J* 6.1
r.	Ser1-β_1	4.00	dd	*J* 11.7, 4.9	4.69	dd	*J* 12.0, 3.0
s.	Ser1-β_2	3.95	dd	*J* 11.7, 4.9	4.46	m	
t.	H13 (eq)	3.81	dd	*J* 13.6, 5.0	3.81	dd	*J* 14.3, 4.7
u.	H13 (ax)	3.52	ddd	*J* 13.6, 13.6, 4.4	3.47	m	
v.	Orn ϵ_{1and2}	3.47	m		3.47	m	
w.	Mal-x_1	3.06	dd	*J* 15.6, 5.0	3.07	dd	*J* 15.7, 5.0
x.	Mal-x_2	3.00	dd	*J* 15.6, 5.0	3.00	dd	*J* 15.7, 5.0
y.	H12 (eq)	2.82	bd	*J* 13.7	2.81	bd	*J* 13.8
z.	H12 (ax)	2.57	m		2.58	m	
aa.	Orn-N-$COCH_3$ (CH_3)	2.15	s		2.15	s	
bb.	Orn-β_1	1.83	m		1.84	m	
cc.	Orn-β_2	1.71	m		1.72	m	
dd.	Orn-γ_{1and2}	1.54	m		1.54	m	
ee.	Ala-1-β-(CH_3)	1.51	d	*J* 7.2	1.54	d	*J* 7.2
ff.	Ala-2-β-(CH_3)	1.45	d	*J* 7.4	1.44	d	*J* 7.2
gg.	Thr-γ-(CH_3)	1.28	d	*J* 6.4	1.34	d	*J* 6.2

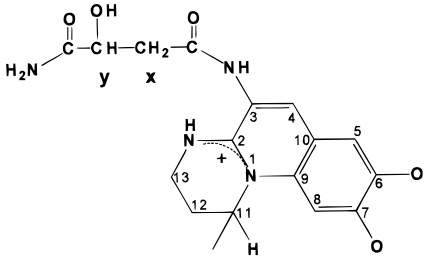

FIGURE 4: Atom numbering scheme of the chromophore in the pseudobactins.

molecular weight of **1** was thus calculated to be 1166.4. The parent ion in the mass spectrum of the corresponding ferric complex is at *m*/*z* 1221.4. Since this complex is dianionic, this peak corresponds to the $[M + 3H]^+$ species and indicates a molecular weight of 1218.4. The mass spectral analysis of pseudobactin **2** shows the parent ion peak at *m*/*z* 1150.4, which is the $[M + H]^+$ species and suggests the molecular weight is 1149.4. The parent ion in the mass spectrum of ferric complex $[M + 2H]^+$ is at *m*/*z* 1203.2 and indicates the molecular weight of 1201.2. The difference in mass between the ligand and the chelate in each case is 52 units and allows for the ionization of four protons and their replacement with a ferric ion.

To complete the structure elucidation, it was necessary to sequence the peptide portion of pseudobactins **1** and **2**. The sequence was determined by means of CAD-MS (Collisionally Activated Decomposition Mass Spectrometry). The ESI tandem mass spectrum for **1** is shown in Figure 5. Peptide fragments were identified and specified according to accepted nomenclature. The differences in molecular weights of consecutive fragments are the molecular weights of different

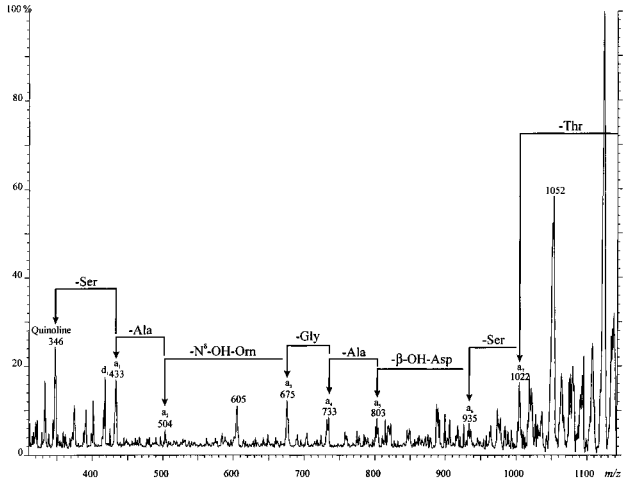

FIGURE 5: ESI-MS/MS spectrum of the linear pseudobactin (**1**) from *P. pustida* ATCC 39167.

amino acids identified by NMR and amino acid analysis. The positive charge on the quinoline moiety causes a simple sequence ion series to form. Most are a-type ions (Biemann, 1990). The CAD fragmentation pattern establishes the sequence of this peptide to be Chr-Ser(1)-Ala(1)-AcOHOrn-Gly-Ala(2)-βOH Asp-Ser(2)-Thr. Most of the sequence for **2** was also found by means of mass spectrometry (Supporting Information).

The ^{1}H and 2 D COSY spectra are nearly identical for **1** and **2** (Table 1, Figures 2 and 3), and this is evidence that the chromophore, side chain, and amino acid sequence are the same for the two compounds. Despite the identification of identical consituents, the difference in molecular weights, charge, and HPLC retention times indicate that **1** and **2** are

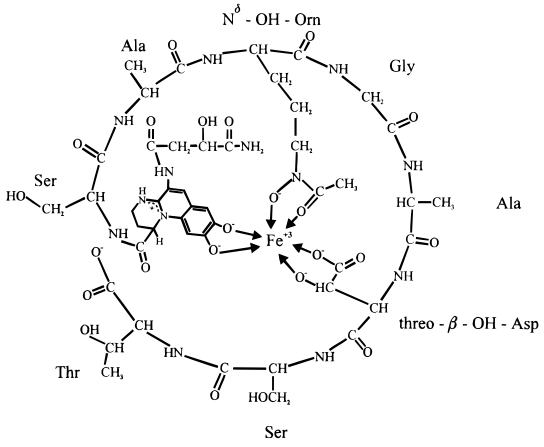

FIGURE 6: Molecular structure of the Fe complex **1**. In the structure of **2**, the acid group of Thr and the hydroxyl group of Ser have formed an ester bond.

different. From the fragmentation pattern, it was apparent that the attachment of the chromophore to the peptide was via one of the serine residues in both compounds, and the following argument showed this to be the serine(1) residue. Upon inspection of the NMR spectra, it was noticed that, among the two serine residues, serine(2) appears to be in the same environment in **1** and **2**. The significant difference between the ^{1}H NMR spectra of **1** and **2** is the downfield shift of the β protons for the serine(1) residue in case the of **2**. The signals at δ 4.56, 4.00, and 3.95 were assigned to the α and β protons of serine(1) in the case of **1**. This serine proton pattern was reported for other pseudobactins, indicating that there is an amide bond between the carboxylic group of the chromophore and NH_2 of serine(1). However, in the case of **2**, all the serine(1) protons are shifted downfield: Ser1-α, δ 4.88; β, δ 4.69, 4.46. Such a downfield shift of protons was reported for one of the serines in the cyclic pseudobactin 7SRI (Yang & Leong, 1984) and was interpreted by those authors to be due to an ester linkage, instead of an amide bond, between the serine β-OH group and the chromophore, while cyclization was believed to have occurred with an amide bond formed by the amino and carboxy terminii, respectively, of the first and third serine.

However, an alternative interpretation is possible for these observations in contrast to those reported for 7SR1. Which is, that, for **2**, the NH_2 of serine(1) forms an amide linkage with the chromophore and that the β-OH of serine(1) forms a depsipeptide (ester) bond with the carboxy terminal threonine residue. Both possibilities would give rise to a structure that corroborated the mass spectral, electrophoresis, chromatographic, and IR data. A conclusion was reached by hydrolyzing the ester bond in **2** by adjusting the siderophore solution to pH 9.0 with 0.1 M NaOH. The hydrolization product exhibited the same retention time and molecular weight as **1**. Therefore, the ester bond in **2** must be between serine(1) and threonine rather than between serine(1) and the chromophore, and by breaking that bond, **2** can converted into **1**, as is observed, instead of forming a mixture of peptide and chromophore. The results also show that serine(1) is the N-terminal residue in **1**.

The proposed structures of the ferric complex of **1** and **2** are shown in Figure 6. The difference in the molecular weights of **1** and **2** is 17 units, which accounts for the loss of a water molecule during cyclization. The molecular weight of a water molecule is 18, but the threonine residue involved in cylization (for **2**) exists in the deprotonated form at pH 5.5 in the case of the linear pseudobactin **1**. These structures are also consistent with the observed difference in charges. In the case of the Fe complex of **1**, five negative charges surround the iron and one more negative charge is on the deprotonated threonine residue. The total number of positive charges is four, three from Fe^{3+} and one on the heterocyclic six membered ring of the chromophore. Therefore, two negative charges remain unbalanced, making the compound dianionic at neutral pH. Consequently, the deferri form carries a single unbalanced negative charge. The Fe complex of **2** was observed to be monoanionic, which is consistent with the proposed structure because there is no charge on threonine in the cyclized form. This is also supported by the fact that **2** is found to be neutral.

A total of 46 carbon resonances were observed for both compounds: 13 carbonyl resonances between 170 and 180 ppm, 9 aromatic resonances between 100 and 152 ppm, and 24 aliphatic carbon resonances between 15 and 71 ppm (data not shown). The proposed structures are consistent with the total number of carbon resonances and the number of carbon resonances in each category. The NMR spectra of the two minor pseudobactins P39167-I′ and P39167-II′ are identical to those of **1** and **2**, respectively. The molecular weight of P39167-I′ was determined to be 1167.4, which is 1 unit higher than the molecular weight of **1**. Similarly, the molecular weight of P39167-II′ was 1150.4, which is 1 unit higher than the molecular weight of corresponding major pseudobactin **2**. In the light of literature on pseudobactin structures, it is reasonable to conclude that the minor compounds actually are only modified in the side chain and carry a malic acid side chain instead of malamide. P39167-I′ and P39167-II′ carry one extra negative charge relative to the corresponding major compounds consistent with the deprotonated form of malic acid side chain at neutral pH.

In summary, the molecular formulas of **1** and **2** and their ferric complexes are $C_{46}H_{64}O_{23}N_{13}^-$, $C_{46}H_{62}O_{22}N_{13}$, $FeC_{46}H_{60}O_{23}N_{13}^{2-}$, and $FeC_{46}H_{59}O_{22}N_{13}^-$ at neutral pH.

Pseudomonas fluorescens A225. The total siderophore concentration produced in the medium was approximately 100 mg/L. Reverse phase chromatography of the crude pseudobactin mixture gave two major and two minor bands. The two major bands were designated as pseudobactins PA225-I (**3**) and PA225-II (**4**) whereas the minor bands were designated as PA225-I′ and PA225-II′. After purification, 6 mg of PA225-I and 10 mg of PA225-II were obtained from 1 L of culture medium. Electrophoretic mobility data for **3** and **4** and their iron complexes are nearly identical to those for the corresponding compounds from *P. putida* ATCC 39167. Molar absorptivities of the yellow-green **3** and **4** are $\epsilon_{380} = 1.4 \times 10^4$ and 1.6×10^4 L M^{-1} cm^{-1}, respectively.

The amino acid analysis of 6 M HCl hydrolysate for both pseudobactins **3** and **4** revealed the presence of four amino acids: Ser, Ala, Gly, and Thr in the molar ratio of 3:1:1:1. This analysis also showed that two other amino acids are present, which could not be identified. The ^{1}H NMR spectra of pseudobactin **3** and **4** are very similar to those of **1** and **2**. The presence of chromophore, malamide side chain, and six amino acid, which were already apparent from amino acid analysis, are also confirmed by means of ^{1}H NMR. The presence of β-OH aspartic acid and N$^{\delta}$-acetylated hydroxylated ornithine residue in **3** and **4** were confirmed from the characteristic NMR signals as in the case of pseudobactins

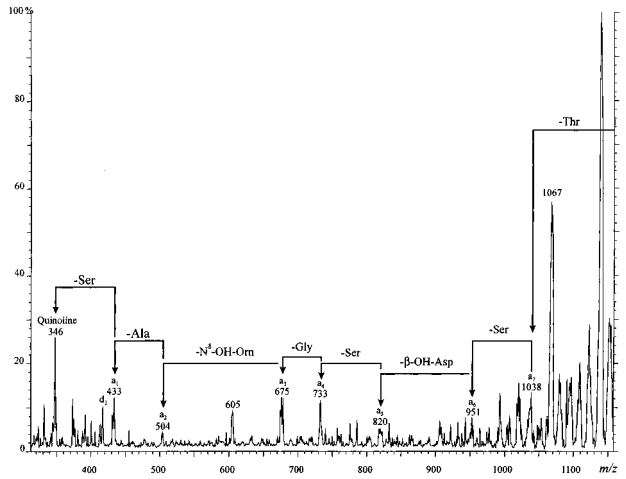

FIGURE 7: ESI MS/MS spectrum of the linear pseudobactin (**3**) from *P. fluorescens* A225.

1 and **2**. However, some differences in the spectrum were noticed. The presence of only one alanine in **3** and **4** was evident compared to two alanines in **1** and **2**. On the other hand, the signals of the α and β protons of one additional serine was observed for **3** and **4**.

The mass spectral analysis of **3** established the molecular weight to be 1182.5, and the molecular weight of the Fe complex to be 1234.6, while the molecular weights of pseudobactin **4** and the Fe complex of **4** were observed to be 1165.4 and 1217.1, respectively, all measurements at neutral pH.

The tandem (CAD) mass spectrum for **3** is shown in Figure 7. This fragmentation pattern revealed the sequence of the peptide to be Chr-Ser(1)-Ala -Ac OH Orn-Gly-Ser(2)-β OH Asp-Ser(3)-Thr. The mass spectrometry evidence also proved that one of the serine residues is attached to the chromophore.

When a solution of **4** was left at pH 9 for several hours and the solution chromatographed, only **3** was observed. This behavior proves that the relationship between **3** and **4** is the same as between **1** and **2** and that **4** is the cyclic depsipeptide of **3**.

Chr-Ser(1)-Ala-Ac OH Orn-Gly-Ser(2)-b OH Asp-Ser(3)-Thr

This structure is in agreement with the MS and electrophoresis data. The proposed structures of the Fe complexes of **3** and **4** are shown in Figure 8. Their formulas at neutral pH are $FeC_{46}H_{60}O_{24}N_{13}^{2-}$, and $FeC_{46}H_{59}O_{23}N_{13}^{-}$.

Uptake Results. The results of the time dependent uptake of the ferric-pseudobactins (Fe-PWCS 358 and the ferric complexes of **1**, **2**, **3** and **4**) by the cells of *P. putida* ATCC 39167 and *P. fluorescens* A225, are given in Figures 9 and 10, respectively. It is apparent from these results that both *Pseudomonas* species assimilate iron bound to the native or homologous pseudobactin very efficiently. It is also clear that the cells of *P. putida* ATCC 39167 and *P. fluorescens* A225 are also able to transport iron bound to heterologous pseudobactin from each other in an efficient manner. However, both organisms do not acquire iron bound to the pseudobactin from the PGP *P. putida* WCS358. Pseudobactin PWCS358 is structurally very different from the **1**, **2**, **3**, and **4** (van der Hofstad et al., 1986)

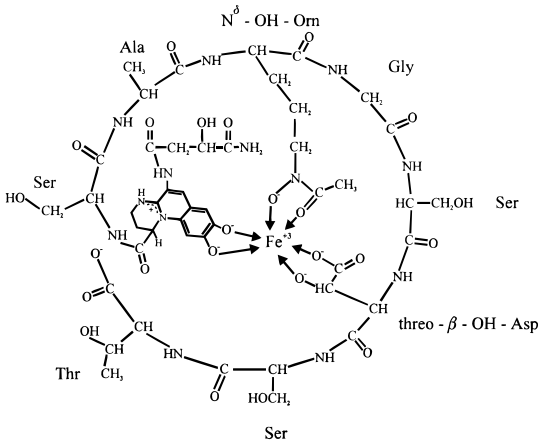

FIGURE 8: Molecular structure of the Fe complex **3**. In the structure of **4** the acid group of Thr and the hydroxyl group of Ser have formed an ester bond.

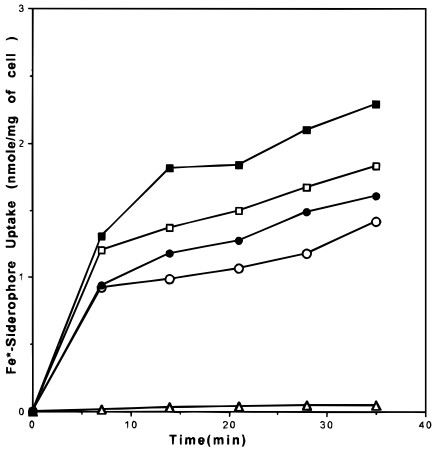

FIGURE 9: Time dependent uptake by *P. putida* ATCC 39167 of the ^{55}Fe complexes of **1** (□), **2** (■), **3** (○), **4** (●), PWCS 358 (△).

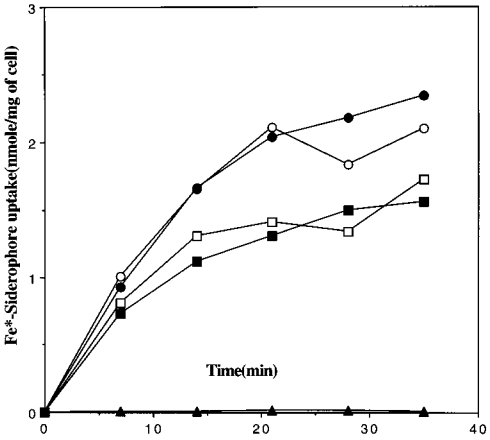

FIGURE 10: Time dependent uptake by *P. fluorescens* A225 of the ^{55}Fe complexes of **3** (○), **4** (●), **1** (□), **2** (■), PWCS 358 (▲).

DISCUSSION

The siderophores of fluorescent pseudomonads are commonly referred to as pseudobactins or pyoverdines and are composed of a peptide chain attached to a quinoline-derived chromophore. A small dicarboxylic moiety is attached at the C-3 position of the chromophore (Budzikiewicz, 1993). The structural diversity of the yellow-green fluorescent siderophores from different *Pseudomonas* species seems to be primarily due to the difference in the length, amino acid content, chirality, and sequence of the peptide chain (Pers-

mark, 1991). Diversity among the structures of pseudobactins produced by one strain have, so far, only been reported due to differences in the dicarboxylic side chain attached to C-3 of the chromophore.

We observe a similar diversity for both *P. fluorescens* A225 and *P. putida* ATCC 39167: both organisms produce pseudobactins with a side chain derived from malamide (**1**, **2**, **3**, and **4**) while minor quantities of pseudobactins are observed for which the side chain is malic acid. It is not known if this diversity is biosynthetic in nature or an artifact of the purification procedure. Most important, however, is that, for the first time a particular fluorescent *Pseudomonas* species is reported to produce both a pseudobactin with a linear peptide chain together with a cyclic pseudobactin, (i.e., **1** and **2** and **3** and **4**, respectively) with the same amino acid sequence. The concentration of **1** and **3**, with respect to **2** and **4**, increases on prolonged growth. However, the medium for normal growth is adjusted regularly to pH 7.0, and never exceeds pH 7.4, well below the pH to hydrolyze the ester bond. Also, during the purification, the pH never falls below 4.0. The production of a linear and a cyclic pseudobactin therefore seems to be a biosynthetic phenomenon that allows the organism to acquire iron under different environmental conditions in competition with other organisms. Other examples of the ability by microorganisms to produce more than one siderophore are known in the literature. An extreme example of this phenomenon is the large number (more than 10) of ferrichromes (asperchromes) produced by *Aspergillus ochraceous* (Jalal et al., 1984). One can, therefore, predict that the present observation is not an isolated one and that other fluorescent *Pseudomonas* species will also be shown to produce more than one pseudobactin (besides differences in the side chain) and that these will be observed when minor products in the pseudobactin mixture are purified and investigated.

The pseudobactins isolated from *P.* A225 and *P.* ATCC 39167 are closely similar to one another, differing only in position "five" of the octapeptide where a serine in **3** and **4** is replaced by alanine in **1** and **2**. It is important as well to note that the amino acids in **1** are identical to those found in PA214 (Buyer et al., 1986) but that the sequence for both is completely different: Ser-Ala-AcOHOrn-Gly-Ala-OHAsp-Ser-Thr (in **1**) and Ser-Ala-Gly-Ser-Ala-OHAsp-Thr-AcOHOrn (in PA214). The same is the case for the cyclic pseudobactin P7SR1 (Yang & Leong, 1984) and **4**. Again, their amino acid constituents are the same but the sequences are quite different: Ser-Ala-AcOHOrn-Gly-Ser-OHAsp-Ser-Thr in **4** and Ser-Gly-Ala-AcOHOrn-Ser-Thr-OHAsp-Ser in P7SR1. The data for 7SRI are consistent with an ester bond cyclizing the structure. However, the authors concluded that the ester bond was formed between the Ser and the chromophore. A hydrolysis experiment could give a resolution between these two possibilities.

Pseudobactins always have the fluorescent chromophore attached to the peptide chain. This attachment is via a lysine, serine, aspartic acid, aspargine, or alanine residue (Persmark, 1991). Among the plant-related *Pseudomonas* species, it seems that, in all pseudobactins from PGPs for which structures are known, the chromophore is attached through lysine as in B10 (Teintze et al., 1981) and WCS358 or aspartic acid as in A589 (Persmark et al., 1990). A common factor among PGD pseudobactins, for which structures are known, is the attachment of the chromophore to a serine residue such as for pseudobactins from *P.* A214 and 7SRI (Buyer et al., 1986; Yang & Leong, 1984). Whether this structural difference between pseudobactins from PGP and PGD organisms is coincidental or characteristic is not known at the present time.

In Gram-negative bacteria, ferric siderophores are transported through the outer membrane, periplasm, and inner membrane by a set of proteins (Neilands, 1982). In *P.* WCS358, an OM protein PupA has been identified (Marugg et al., 1989), and it is specific for the ferric-pseudobactin produced by this organism. In addition, however, a second OM protein has been characterized, PupB, which is induced by the pseudobactin ligand, produced by *P.* BN7, which is specific for ferric-pseudobactin BN7 (Koster et al., 1993, 1994). The ability of *P.* WCS358 to exploit many heterologous ferric-pseudobactins is related to the presence of multiple OM receptor proteins (Koster et al., 1995).

The organism *P.* WCS358 is a PGP whereas *P.* A225 is PGD, and *P.* ATCC 39167 might be a PGD organism. It is, therefore, informative to study the uptake behavior of these PGD organisms and compare it with that of PGP organisms. Because the chromophore is conserved in all fluorescent pseudobactins, it is clear that the peptide moiety should play an important role in the specificity of the OM protein and the resultant uptake. The results shown in Figures 9 and 10 allow a number of conclusions to be drawn for the organisms used in this study.

(a) For *P.* ATCC 39167, there seems to be a slight advantage for the homologous (**2**) and heterologous (**4**) cyclic pseudobactins over the linear ones in the uptake by the organism, but this trend is not obvious for *P.* A225. Still, it may be that the cyclic pseudobactins fit the OM receptor protein somewhat better while conformational adjustments are required for the linear ferric-pseudobactins to fit these proteins. (b) For *P.* ATCC 39167, the homologous ferric-pseudobactins (**1** and **2**) are taken up most efficiently, but also the heterologous ferric-pseudobactins from *P.* A225 (**3** and **4**) are taken up albeit with about 25% lower efficiency. Precisely the same is true for *P.* A225 where the homologous ferric-pseudobactins are also taken up more efficiently than those from *P.* ATCC 39167. The consistency is remarkable and seems to imply that the Ser for Ala substitution has a small but significant effect on recognition. It also implies that the OM receptor proteins in the organisms *P.* A225 and *P.* ATCC 39167 are similar but not identical. (c) The ferric-pseudobactin WCS358, which is structurally very different (van der Hofstad et al., 1986) from those of *P.* A225 and *P.* ATCC 39167, is not taken up at all by these two organisms. Induction of the two organisms by pseudobactin WCS358 does not alter this observation.

The evidence which is presented indicates that *P.* A225 and *P.* 39167 seem not to be able to produce more than one OM receptor protein for ferric-pseudobactins either by iron restriction or induction, but, instead produce two differnt siderophores for iron acquisition, in contrast to the PGP organism *P.* WCS358, which is able to produce many different ferric-pseudobactin OM receptor proteins to obtain iron from the environment.

ACKNOWLEDGMENT

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ogy Resource Facility at the Univeristy of Oklahoma, Health Sciences Center, Oklahoma City, Oklahoma.

SUPPORTING INFORMATION AVAILABLE

Figures of the COSY spectra of **1**, the ESI tandem mass spectrum of **2**, and the chromatogram for *P. fluorescens* A 225, the ^{1}H NMR spectra and assignments for **3** and **4** (8 pages). Ordering information is given on any current masthead page.

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