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Characterization of Pyoverdin$_{pss}$, the Fluorescent Siderophore Produced by *Pseudomonas syringae* pv. *syringae*†

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***Pseudomonas syringae* pv. *syringae* B301D produces a yellow-green, fluorescent siderophore, pyoverdin$_{pss}$, in large quantities under iron-limited growth conditions. Maximum yields of pyoverdin$_{pss}$ of approximately 50 μg/ml occurred after 24 h of incubation in a deferrated synthetic medium. Increasing increments of Fe(III) coordinately repressed siderophore production until repression was complete at concentrations of ≥10 μM. Pyoverdin$_{pss}$ was isolated, chemically characterized, and found to resemble previously characterized pyoverdins in spectral traits (absorbance maxima of 365 and 410 nm for pyoverdin$_{pss}$ and its ferric chelate, respectively), size (1,175 molecular weight), and amino acid composition. Nevertheless, pyoverdin$_{pss}$ was structurally unique since amino acid analysis of reductive hydrolysates yielded β-hydroxyaspartic acid, serine, threonine, and lysine in a 2:2:2:1 ratio. Pyoverdin$_{pss}$ exhibited a relatively high affinity constant for Fe(III), with values of 10^{25} at pH 7.0 and 10^{32} at pH 10.0. Iron uptake assays with [^{55}Fe]pyoverdin$_{pss}$ demonstrated rapid active uptake of ^{55}Fe(III) by *P. syringae* pv. *syringae* B301D, while no uptake was observed for a mutant strain unable to acquire Fe(III) from ferric pyoverdin$_{pss}$. The chemical and biological properties of pyoverdin$_{pss}$ are discussed in relation to virulence and iron uptake during plant pathogenesis.**

Many pathogenic and saprophytic pseudomonads produce fluorescent, yellow-green pigments in iron-deficient environments. Recently, fluorescent pigments have been isolated from a few *Pseudomonas* species and found to be pyoverdin siderophores which function in scavenging Fe(III) and transporting it to the cell, wherein a system for high-affinity uptake is operative (7, 10, 18, 22, 26–28, 33, 36, 37, 40, 41). Little is known, however, about iron acquisition by the fluorescent phytopathogen *Pseudomonas syringae*, although it has been suggested that a pyoverdin siderophore is primarily responsible for iron acquisition during plant pathogenesis (13). Plant tissues contain considerable quantities of iron in various constituents, including iron proteins, phenolic compounds, heme, and citrate (23), but the iron may not be freely available to an invading bacterium unless there is an efficient means of sequestering it from plant reservoirs. Synthesis of a siderophore and expression of an outer membrane receptor occur when gram-negative bacteria are in an iron-deficient environment (8, 29, 30). Furthermore, virulence of some animal pathogens has been correlated with siderophore production and utilization (6, 8). For example, *P. aeruginosa* PAO1 produces two siderophores, pyoverdin$_{pa}$ and pyochelin (2, 7), during the infection process. Pyochelin has been shown (6) to have a dramatic effect on virulence of *P. aeruginosa*; the role of pyoverdin$_{pa}$ in infection is not as well characterized, although it has been shown (2) to stimulate growth of the bacterium in human serum and transferrin. It seems possible, therefore, that a siderophore from *P. syringae* would have a pivotal role in plant pathogenesis, sequestering Fe(III) from the host in a form which can be readily and specifically acquired by the pathogen.

The pyoverdins comprise a family of structurally related compounds which primarily differ in amino acid composition and configuration (1, 10); consequently, they often contain different Fe(III)-binding ligands. Pyoverdins form relatively stable iron chelates with binding constants of approximately 10^{32} at an alkaline pH (27, 37, 41). The structural feature common to pyoverdins is the fluorescent chromophore, which has been identified as 2,3-diamino-6,7-dihydroxyquinoline and which functions to bind Fe(III) by means of its *o*-dihydroxy aromatic group (1, 10, 22, 31, 36, 40, 41). Most pyoverdins also harbor hydroxamic acid groups which complex with Fe(III) due to the presence of N^{δ}-hydroxyornithine. Pyoverdin$_{pa}$ (41) and the pyoverdin produced by a strain (ATCC 13525) of *P. fluorescens* (33) have octapeptides and heptapeptides, respectively, which contain 2 mol of N^{δ}-hydroxyornithine, while pseudobactin, the pyoverdin produced by a root-colonizing pseudomonad (19, 36), has a hexapeptide that contains only 1 mol of this amino acid. An α-hydroxycarboxylate moiety supplied by β-hydroxyaspartic acid is the third ligand coordinating iron in pseudobactin (36); β-hydroxyaspartic acid is also found in several other pyoverdin siderophores (10, 22, 40), including azotobactin produced by *Azotobacter vinelandii* (10, 12). Ferric chelates of pyoverdin siderophores characteristically exhibit maximum absorbance between 400 and 410 nm, depending on the particular pyoverdin (10, 27, 33, 36, 37, 40).

P. syringae pv. *syringae* is a pathogen of several crop plants that invades parenchymatous tissues, causing leaf spots and stem cankers. Production of the necrosis-inducing toxin syringomycin has been related to virulence (14, 16), although ample supplies of iron are needed to deregulate phytotoxin synthesis to yield physiologically significant quantities (13). Under defined cultural conditions, strain B301D of *P. syringae* pv. *syringae* requires at least 2 μM Fe(III) for a maximum yield of syringomycin. This implies that the pathogen procures relatively high concentrations of iron during plant pathogenesis for expression of full virulence. *P. syringae* pv. *syringae* produces a yellow-green, fluorescent pigment, but it has not yet been shown to be a pyoverdin siderophore which functions in Fe(III) uptake. Only recently has a pyoverdin been isolated from an unidentified pathovar of *P. syringae* and partially characterized

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(37). It resembled previously described pyoverdins in absorbance characteristics, in iron-binding affinity, and by the presence of key structural components. However, it was not shown to supply iron to the cell. In a genetic study (25), synthesis of an uncharacterized fluorescent pigment by *P. syringae* pv. *syringae* was found to be controlled by at least four genes or gene clusters, which in turn controlled the ability to grow when iron was deficient.

This study shows that *P. syringae* pv. *syringae* produces copious quantities of a pyoverdin siderophore that functions in iron acquisition and uptake to permit growth in iron-deficient environments. We also establish that the siderophore, called pyoverdin$_{pss}$, has unique chemical and physical properties. In addition, the synthesis of pyoverdin$_{pss}$ together with its iron-chelating attributes are discussed in relation to virulence and iron uptake in the plant environment.

MATERIALS AND METHODS

Bacterial strains. *P. syringae* pv. *syringae* B301D was originally isolated from a diseased pear (*Pyrus communis* L.) flower in England and is highly virulent (15). A mutant (strain W4N2225) of B301D incapable of iron uptake (Iu$^-$) when supplied as ferric pyoverdin$_{pss}$ was obtained after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis (Y. S. Cody and D. C. Gross, J. Bacteriol., in press). Strains were grown on nutrient broth-yeast extract agar (38) slants and then stored at 4°C. Strains were lyophilized for long-term storage.

Media and glassware. In all experiments bacteria were grown in N minimal (NM) salts medium (38). Siderophore production was achieved in NM liquid medium in which the salts and glucose were previously deferrated with 8-hydroxyquinoline (39). After autoclaving, 1 ml of a filter-sterilized salts solution (200 mg of $MgSO_4 \cdot 7H_2O$, 10 mg of $CaCl_2 \cdot 2H_2O$, 2 mg of $ZnSO_4 \cdot 7H_2O$ per liter) was added to each liter of deferrated medium. Iron-free glassware was used in all experiments and was prepared by the procedure of Waring and Werkman (39).

Bacterial growth and pyoverdin$_{pss}$ synthesis. To test the effects of iron on growth and pyoverdin$_{pss}$ synthesis, strain B301D was grown to log phase in NM liquid medium at 25°C and then transferred (~5 × 10^6 CFU/ml) into 20 ml of deferrated NM liquid medium. The culture medium was then supplemented with filter-sterilized $FeCl_3$ to one of the following final concentrations: 100, 10, 1, 0.5, 0.1, or 0.05 μM or no iron added. Duplicate cultures were grown in Erlenmeyer flasks (250 ml) with side arms at 25°C on a rotary shaker (250 rpm). Bacterial growth was determined by monitoring changes in A_{420} over a period of 36 h; ferric pyoverdin$_{pss}$ did not interfere with growth measurements at 420 nm since unchelated pyoverdin$_{pss}$ was the major form accumulated in culture fluids. Fluorescent siderophore synthesis was measured over the same time period by taking samples (1 ml) from each replicate culture, removing the cells by centrifugation in a Microfuge (Beckman Instruments, Inc., Fullerton, Calif.) at 13,000 × *g*, and measuring A_{360} of the supernatant with a double-beam spectrophotometer. Yields of pyoverdin$_{pss}$ were corrected by subtracting the absorbance of culture fluids containing 100 μM iron, in which pyoverdin$_{pss}$ synthesis is completely repressed.

Chemical assays for detecting siderophores. The ability of *P. syringae* pv. *syringae* B301D to produce phenolate or hydroxamate siderophores was assayed at intervals throughout the growth cycle in deferrated NM liquid medium. Strain B301D was grown to log phase in NM liquid medium, transferred (~5 × 10^6 CFU/ml) into deferrated NM liquid medium, and then incubated at 25°C on a rotary shaker (250 rpm). The supernatant fluid (1-ml samples) from duplicate cultures was tested for extracellular siderophores after removal of the cells by centrifugation (15 min, 11,700 × *g*). The presence of a phenolate group was assayed by the procedure of Arnow (3), with catechol as the standard. The method of Csaky (9) was used to assay for the presence of hydroxamate compounds, with hydroxylamine hydrochloride as the standard. The method described by Leong and Neilands (24) was also used to assay for the presence of hydroxamic acids. The test sample (0.8 ml) was mixed with 0.1 ml each of $FeClO_4 \cdot 6H_2O$ (75 mM) and sodium phosphate buffer (1.0 M, pH 7.0) and centrifuged to clarify the supernatant, and the A_{400} was read. The standard used again was hydroxylamine hydrochloride.

Production and purification of pyoverdin$_{pss}$. Pyoverdin$_{pss}$ was purified by a modification of the methods of Meyer and Abdallah (27) and Philson and Llinas (33) for pyoverdin siderophores. Strain B301D was grown from an initial concentration of ~5 × 10^6 CFU/ml in 1-liter flasks containing 250 ml of deferrated NM liquid medium. Cells were grown at 25°C with rotary shaking (250 rpm); after 24 h, 4 ml of an $FeCl_3$ stock (1 M) was added per liter of medium. After the mixture was stirred for 20 min, cells and precipitate were removed by centrifugation at 10,000 × *g* for 20 min. The culture supernatant was concentrated 50-fold by flash evaporation at 55°C, and then contaminating proteins were precipitated by saturation with NaCl. After removal of the precipitate by centrifugation, the concentrated culture supernatant was extracted with an equal volume of chloroform-phenol (1:1 [vol/wt]). The organic phase was mixed with 3 volumes of diethyl ether, and the pigment then was partitioned into deionized water (~20 ml) until color could no longer be extracted from the organic phase. The resulting aqueous extract was washed three times with diethyl ether to remove phenol and reduced to dryness by lyophilization.

The crude siderophore extract, 50 to 150 mg, was further purified through a carboxymethyl (CM)-Sephadex (Pharmacia, Piscataway, N.J.) cation-exchange column (1.5 by 90 cm) equilibrated with 50 mM pyridine-acetate buffer, pH 5.0. The column was eluted with the same buffer. Absorbance was monitored at 405 nm and all peaks were collected. The predominant reddish-brown peak was nonfluorescent and consisted of ferric pyoverdin$_{pss}$. The ferric pyoverdin$_{pss}$ was lyophilized, suspended in pyridine-acetate buffer (1 ml), and again passed through the CM-Sephadex column to increase its homogeneity. The purity of the pyoverdin$_{pss}$ was determined by thin-layer chromatography, using Silica Gel G (Merck & Co., Inc., Rahway, N.J.) Uniplates (Analtech, Newark, Del.) developed with an ethanol-water (70:30 [vol/vol]) solvent system. A homogeneous preparation of pyoverdin$_{pss}$ was noted by the presence of only one sharp reddish-brown spot; no fluorescent spots were observed when plates were illuminated with UV light (366 nm).

Biological activity of lyophilized column fractions was measured by reversal of iron starvation of strain B301D. Iron starvation was induced by spotting a 5-μl droplet of a cell suspension (3 × 10^8 CFU/ml grown in NM liquid medium) onto NM agar containing 200 μg of purified (35) ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDA) per ml. After absorption of the liquid into the agar, the cells were overlaid with a 5-μl droplet of the test sample dissolved in sterile, deionized water. Plates were incubated for 48 h at 25°C, and the reversal of iron starvation was observed as

visible growth of strain B301D. If siderophore activity was observed, the sample was serially diluted (1:2 [vol/vol]) with sterile, deionized water and bioassayed to measure the endpoint of biological activity.

Iron-free pyoverdin$_{pss}$. To obtain iron-free pyoverdin$_{pss}$, purified ferric pyoverdin$_{pss}$ was mixed for 30 min with EDTA (1.0 M) at pH 7. This was then applied to a Sephadex G-25 (Pharmacia) gel filtration column to separate the pyoverdin$_{pss}$ from the ferric EDTA. The column was both equilibrated and eluted with 50 mM pyridine-acetate buffer, pH 5.0. Peaks were monitored as described above; the fluorescent peak was lyophilized and stored at −20°C.

Spectroscopy. Samples (200 μg/ml) were dissolved in deionized water and adjusted to pH 7.0. Optical spectra of pyoverdin$_{pss}$ and ferric pyoverdin$_{pss}$ were determined with a Beckman model 24 double-beam spectrophotometer.

Fe(III)-binding constants of pyoverdin$_{pss}$. The apparent stability constant of ferric pyoverdin$_{pss}$ was determined at pH 7.0 and 10.0, using EDTA as a competitive chelator of Fe(III) (27). Solutions of 50 μM ferric pyoverdin$_{pss}$ and 15 mM EDTA were prepared in either 0.1 M phosphate buffer (pH 7.0) or 0.1 M glycine-NaOH buffer (pH 10.0). Known amounts of EDTA ranging from 0 to 15 mM were added to a 50 μM solution of ferric pyoverdin$_{pss}$ in the respective buffer to a final volume of 6 ml. Absorbancies were determined at 450 nm after constant values had been reached. The formula described by Meyer and Abdallah (27) was used to calculate Fe(III)-binding constants.

Amino acid analysis. In the first analysis, ferric pyoverdin$_{pss}$ (1 mg/ml) was hydrolyzed in vacuo for 24 h at 110°C by HCl (6 N) and analyzed with a Beckman 121 MB amino acid analyzer (Bioanalytical Center, Washington State University, Pullman) with a type AA-10 resin column and a lithium citrate buffer (0.2 M, pH 2.2) system. In a second analysis, ferric pyoverdin$_{pss}$ (1 mg/ml) was hydrolyzed in vacuo for 24, 48, or 72 h at 110°C by 47% hydriodic acid (HI) and analyzed on a Beckman 6300 amino acid analyzer (Bioanalytical Laboratory, University of California, Davis) with the lithium citrate buffer described above. The internal standard used in this analysis was α-amino-β-guanidino propionic acid (200 nmol/ml). To aid in determining the possible presence of N^{δ}-hydroxyornithine, a hydrolysate of rhodotorulic acid ([cyclo N^{δ}-acetyl-N^{δ}-hydroxyl-L-ornithine]$_2$; Sigma Chemical Co., St. Louis, Mo.) in 47% HI for 24 h was prepared as described above. An authentic sample of DL-*threo*-β-hydroxyaspartic acid (Calbiochem-Behring, La Jolla, Calif.) was analyzed separately in each analysis. The extinction coefficient was determined for ferric pyoverdin$_{pss}$ based on the molecular weight calculated from the results of the amino acid analysis.

Uptake of ^{55}Fe(III). Cells of strain B301D and its Iu$^-$ mutant (strain W4N2225) were grown in NM (10 ml) to mid-log phase (~5 × 10^8 CFU/ml) and harvested by centrifugation (11,700 × *g*, 15 min at room temperature). Under sterile conditions, cells were washed three times in deferrated NM, suspended to their original cell density in deferrated NM, and incubated in Erlenmeyer flasks (250 ml) for 10 min with rotary shaking (250 rpm) at 25°C. A 5-ml mixture of purified pyoverdin$_{pss}$ and [^{55}Fe]pyoverdin$_{pss}$ was then added to each culture flask to initiate ^{55}Fe(III) uptake. The filter-sterilized stock solution of ferric pyoverdin$_{pss}$ was prepared after mixing [^{55}Fe]Cl$_3$ (New England Nuclear Corp., Boston, Mass.) with pyoverdin$_{pss}$ to yield 5 and 10 μM stocks, respectively.

Uptake of ^{55}Fe(III) was measured at intervals of up to 60 min. Cells were collected by filtering samples (0.5 ml) through nitrocellulose membranes (0.22 μm) by vacuum followed by washing with deionized water (20 ml). Filters were air dried and then immersed in scintillation fluid (Scinti Verse II; Fischer Scientific Co., Pittsburgh, Pa.). After 24 h, samples were counted in the tritium channel of a Minaxi Tri-Carb 4,000 counter (Packard Instrument Co., Inc., Downers Grove, Ill.). The amount of cellular iron uptake was calculated by subtracting background ^{55}Fe(III) associated with the membrane filters, which was determined by filtering uninoculated medium. Duplicate cultures were sampled at each interval.

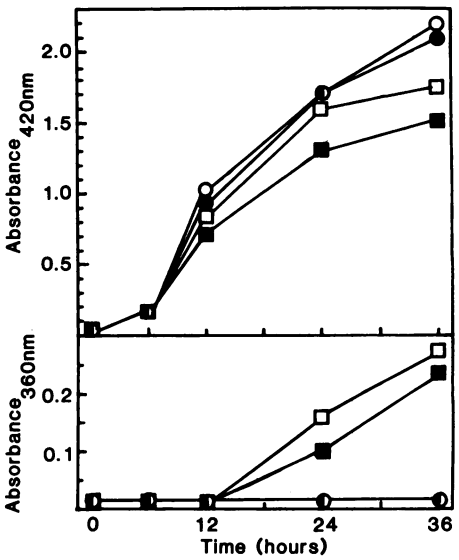

FIG. 1. Relationship between growth (top) and pyoverdin$_{pss}$ synthesis (bottom) of cells of *P. syringae* pv. *syringae* B301D cultured in deferrated NM broth (20 ml) amended with filter-sterilized $FeCl_3$ as follows: ○, 100 μM; ●, 10 μM; □, 1 μM; ■, no iron added. Over a period of 36 h, growth at 25°C was measured as the increase in A_{420}; pyoverdin$_{pss}$ synthesis was measured as the increase in A_{360} of a cell-free supernatant, corrected for yield.

RESULTS

Effect of Fe(III) on growth and siderophore synthesis. The concentration of Fe(III) markedly affected the growth of *P. syringae* pv. *syringae* B301D in an iron-limited medium and inversely regulated the synthesis of the fluorescent siderophore pyoverdin$_{pss}$ (Fig. 1). Growth of strain B301D by 24 h in deferrated culture medium not supplemented with Fe(III) was less relative to all cultures which were supplemented with varying quantities of Fe(III). An increase in growth was observed with increasing quantities of Fe(III) until maximum growth was achieved at a concentration of ≥10 μM. Synthesis of pyoverdin$_{pss}$ was inversely proportional to the concentration of Fe(III) such that synthesis was repressed at high and derepressed at low concentrations. For example, the maximum yield obtained was achieved after 24 h of incubation when the medium was supplemented with ≤1 μM Fe(III); yields were slightly less for cultures which had not been supplemented with Fe(III), reflecting suppressed growth due to iron deprivation. At concentrations of 0.05, 0.1, and 0.5 μM Fe(III), pyoverdin$_{pss}$ yields (approximately 50 μg/ml after 36 h of incubation) were similar to those obtained for cultures supplemented with 1 μM Fe(III). Further increases in Fe(III) progressively suppressed

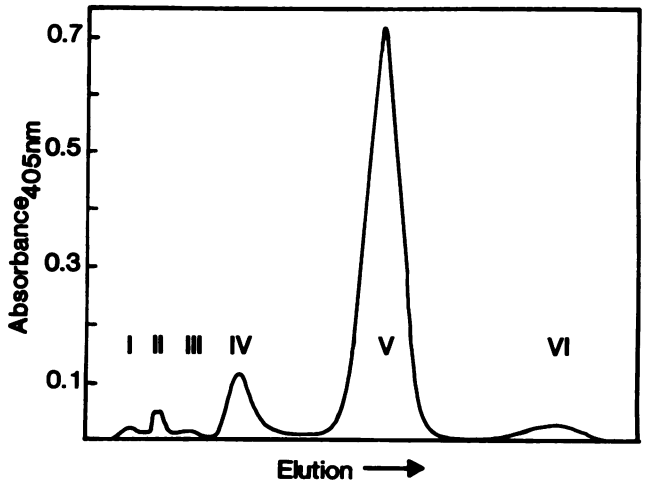

FIG. 2. Purification of ferric pyoverdin$_{pss}$ on a CM-Sephadex column eluted with 50 mM pyridine-acetate buffer (pH 5.0). Peaks were detected at an A_{405} and are labeled with Roman numerals. The partially purified siderophore sample applied to the column in a volume of 1 ml contained approximately 50 mg of ferric pyoverdin$_{pss}$.

pyoverdin$_{pss}$ yields until Fe(III) concentrations of $\geq$10 μM were reached, when pyoverdin$_{pss}$ synthesis ceased. Detectable quantities of pyoverdin$_{pss}$ were not obtained until after about 12 h of incubation regardless of the specific Fe(III) concentration.

Phenolate compounds were not detected in low-iron supernatant fluids from cultures of strain B301D (3). Companion tests for hydroxamic acids, using the Csaky (9) and perchlorate (24) assays, also were negative despite the production of copious quantities of the fluorescent siderophore. The ability of supernatant fluids to reverse EDDA-induced iron starvation of strain B301D demonstrated that a siderophore was produced in low-iron cultures; fresh medium itself did not reverse iron deprivation.

Purification and spectral characteristics of the fluorescent siderophore. The fluorescent siderophore produced by strain B301D was readily purified by using the basic procedures developed for other pyoverdin siderophores (27, 33, 36). This siderophore was named pyoverdin$_{pss}$ according to the convention of nomenclature established by Meyer and Abdallah (27) and modified by Cox and Adams (7). Because the iron-chelated form of pyoverdin$_{pss}$ is less susceptible to decomposition, the siderophore was ferrated before it was extracted from 24-h cultures of strain B301D. One liter of NM culture fluid usually yielded about 50 mg of the ferric siderophore, based on lyophilized weights of the extract. Ferric pyoverdin$_{pss}$ was then separated from possible precursors and breakdown products on a column of CM-Sephadex, resulting in one major peak and five minor peaks (Fig. 2). The first three peaks to elute (I, II, and III) contained no detectable biological activity and represented a very small proportion of the sample; peak II was distinctly purple. In contrast, peak IV was nonfluorescent, reddish-brown, and biologically active since it was able to reverse iron starvation at a concentration of 1,250 μg/ml. Ferric pyoverdin$_{pss}$ formed the major peak detected at 405 nm and was labeled peak V. It represented over 80% of the crude extract, was reddish-brown and nonfluorescent, and reversed EDDA-induced iron starvation of strain B301D at a concentration of 160 μg/ml. Peak VI, which was concluded to be iron-free pyoverdin$_{pss}$, was fluorescent and biologically active.

The absorption maximum for ferric pyoverdin$_{pss}$ at pH 7.0 occurred near 410 nm, an absorbance which is characteristic of fluorescent aromatic groups (33), with a small shoulder at 330 nm (Fig. 3). The molar extinction coefficient, ε_{400}, was 2.8×10^{-3} mol^{-1} cm^{-1}, based on an estimated molecular weight of 1,175 (see Discussion). Ferric pyoverdin$_{pss}$ (peak V) was yellow-green and fluorescent when iron was removed and was able to reverse EDDA-induced iron starvation. Removal of the iron from ferric pyoverdin$_{pss}$ with EDTA caused the spectrum at pH 7.0 to shift, resulting in an absorption maximum of 365 nm for pyoverdin$_{pss}$ (Fig. 3). However, peak V, as well as the other peaks collected from the CM-Sephadex column (Fig. 2), did not give reactions indicative of the presence of either a phenolate (3) or a hydroxamate (9) moiety.

Fe(III)-binding constants. Pyoverdin$_{pss}$ exhibited a relatively high affinity constant for Fe(III) at neutral pH with a mean value of 1.1×10^{25} (pH 7.0). At pH 10.0 the stability constant was 1.9×10^{32}, showing that the affinity of the siderophore for Fe(III) was highly pH dependent.

Amino acid composition of pyoverdin$_{pss}$. Amino acid analysis of a 24-h HCl hydrolysate of ferric pyoverdin$_{pss}$ showed four amino acids to be present; β-hydroxyaspartic acid, serine, threonine, and lysine were in about a 2:2:2:1 molar ratio, with a lysine content of <1 mol. The presence of β-hydroxyaspartic acid was verified by using DL-*threo*-β-hydroxyaspartic acid as the standard. Hydrolysis of ferric pyoverdin$_{pss}$ in 47% HI was performed to ascertain whether N^{δ}-hydroxyornithine was present. N^{δ}-hydroxyornithine, which is degraded by HCl hydrolysis, would be expected to occur as ornithine in the HI-reductive hydrolysate (11). Analysis of a 24-h HI hydrolysate yielded the same amino acids at the expected molar ratios as the 24-h HCl hydroly-

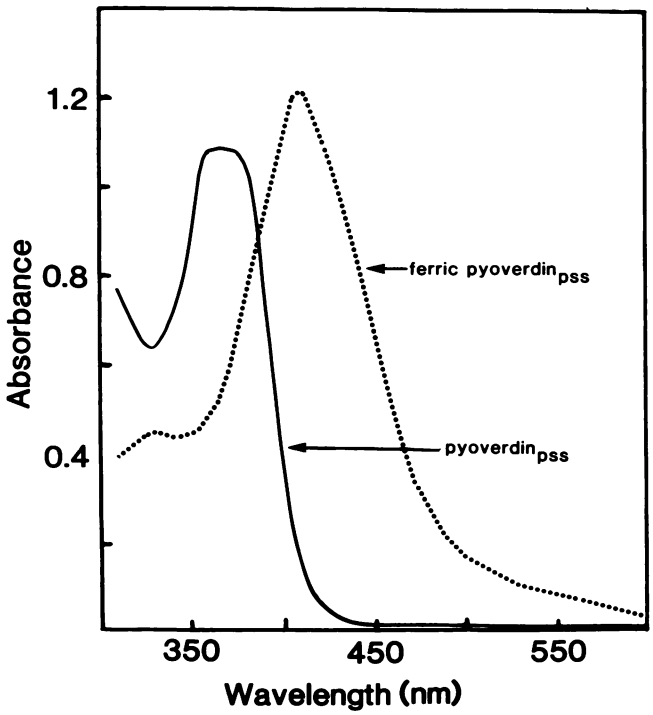

FIG. 3. Absorption spectra of pyoverdin$_{pss}$ (200 μg/ml) and ferric pyoverdin$_{pss}$ (200 μg/ml) in aqueous solution at pH 7.0.

TABLE 1. Amino acid analysis of ferric pyoverdin$_{pss}$ from *P. syringae* pv. *syringae* B301D[a]

Amino acid	Normalized[b] content (nmol) after hydrolysis for:			Molar ratio
	24 h	48 h	72 h	
β-Hydroxyaspartic acid	0.98	0.81	0.72	2
Serine	1.03	0.89	0.80	2
Threonine	1.00	1.00	1.00	2
Lysine	0.21	0.40	0.48	1

[a] Analysis of the ferric siderophore was made after hydrolysis in 47% HI at 110°C.
[b] Normalized to threonine content.

sate (Table 1). However, prolonged hydrolysis up to 72 h resulted in the recovery of 1 mol of lysine. No ornithine was detected in the ferric pyoverdin$_{pss}$ despite the fact that only ornithine was recovered from a 24-h hydrolysate of rhodotorulic acid in 47% HI. The preparations of ferric pyoverdin$_{pss}$ usually showed a high degree of purity with no discernible contaminating amino acids.

Uptake of iron from [^{55}Fe]pyoverdin$_{pss}$. The uptake of iron from [^{55}Fe]pyoverdin$_{pss}$ was demonstrated for cells of *P. syringae* pv. *syringae* B301D grown to mid-log phase in deferrated NM liquid medium (Fig. 4). All iron supplied to the culture was in the form of [^{55}Fe]pyoverdin$_{pss}$. Uptake of ^{55}Fe(III) by iron-starved cells of strain B301D was biphasic and could be detected 5 min after addition of [^{55}Fe]pyoverdin$_{pss}$. The rate of uptake within the first 10 min of incubation was rapid; subsequently, the rate of uptake was slightly reduced but linear up to 60 min. Strain W4N2225, which is an uptake mutant of strain B301D originally selected for inability to acquire Fe(III) from ferric pyoverdin$_{pss}$, did not accumulate ^{55}Fe(III). Nonspecific binding of ^{55}Fe(III) to cells was therefore considered to be negligible, uptake being specifically mediated by pyoverdin$_{pss}$.

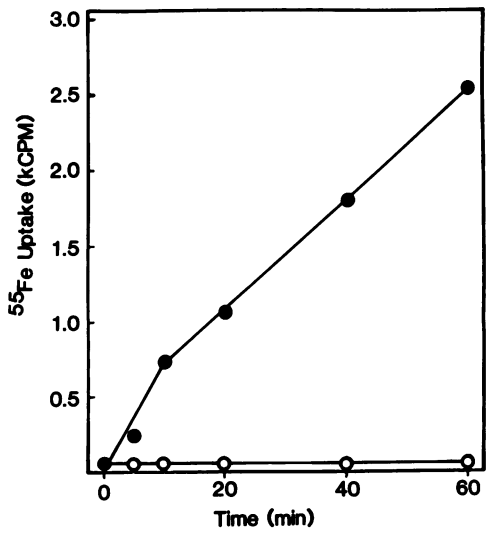

FIG. 4. Uptake of [^{55}Fe]pyoverdin$_{pss}$ by *P. syringae* pv. *syringae* B301D (●) and its Iu$^-$ mutant W4N2225 (○). Cells were grown in NM broth (10 ml) at 25°C to approximately 5 × 10^8 CFU/ml and washed three times in deferrated NM broth. Cells were suspended in deferrated NM broth to the original cell density, and after 10 min of incubation transport was started by addition of 1.67 μM ^{55}Fe(III) to 3.33 μM pyoverdin$_{pss}$ (final concentrations). Samples of 0.5 ml were made at the times indicated; cells were collected, washed by vacuum filtration onto nitrocellulose membranes, and air dried, and the cell-associated radioactivity was determined in a liquid scintillation counter. Results shown are averages from duplicate cultures.

DISCUSSION

When exposed to iron-deficient environments, *P. syringae* pv. *syringae* synthesizes and excretes large quantities of a fluorescent siderophore, pyoverdin$_{pss}$, which scavenges Fe(III) to form a highly stable chelate that can be assimilated via a high-affinity transport system. The conclusion that pyoverdin$_{pss}$ is the primary, if not sole, siderophore produced by strain B301D is based on the following observations: pyoverdin$_{pss}$ synthesis and growth of strain B301D were regulated by the concentration of iron; neither phenolate nor hydroxamate compounds were detected in fluids from cultures grown in a medium deficient in iron; and biological activity was largely associated with a single column fraction (peak V) consisting of ferric pyoverdin$_{pss}$ and a small auxiliary fraction (peak VI) consisting of unchelated pyoverdin$_{pss}$ (Fig. 2).

The crude siderophore extract when fractionated on a CM-Sephadex column yielded several minor substances (peaks I to IV) which appeared to be either breakdown products or precursors of pyoverdin$_{pss}$. Peak IV, which displayed some biological activity, resembled peak A from preparations of pyoverdin$_{pf}$, which was consistently obtained by Meyer and Abdallah (27). These authors (27) concluded that peak A resulted from pyoverdin$_{pf}$ lability in aqueous solutions near neutrality. Peak II resembled the purple compound contained in a ferric pyoverdin preparation from *P. fluorescens* (34). Philson and Llinas (34) noted that the purple compound appeared to have an absorbance at around 320 nm; similar small absorbancy peaks can be observed in preparations of pyoverdins from other sources (27), including *P. syringae* (37). No red-orange compound resembling ferribactin, which was originally isolated from a strain of *P. fluorescens* (34), was produced by *P. syringae* pv. *syringae* B301D.

The lack of either a hydroxamate or a phenolate group which can be detected in culture fluids of *P. syringae* pv. *syringae* B301D makes it difficult to detect and monitor siderophore production. The negative reaction of pyoverdin$_{pss}$ in the Arnow (3) test for phenolates, however, was not unexpected as it corresponds with the result reported for pyoverdin$_{pa}$ (7), despite the contribution of a phenolate ligand by the *o*-dihydroxyquinoline moiety (36, 41). That strain B301D does not produce compounds that give positive reactions to these tests despite production of a pyoverdin siderophore in high quantities underscores the need, as suggested by Neilands (30), for an efficient and general method of detecting iron-complexing agents.

The molecular weight of ferric pyoverdin$_{pss}$ was estimated to be 1,175. This was based on the known amino acid composition (Table 1) of pyoverdin$_{pss}$, the presence of 1 mol of iron, and the speculation that the fluorescent chromophore 2,3-diamino-6,7-dihydroxyquinoline is present since it is found in all structurally characterized pyoverdins (e.g., pseudobactin [22, 36], pyoverdin$_{pa}$ [10, 41], and azotobactin [1, 10, 12]).

Pyoverdin$_{pss}$ resembles previously characterized pyoverdins (2, 10, 18, 27, 37) in its ability to chelate Fe(III) at a relatively high binding constant and transport Fe(III) to cells in a form which permits rapid uptake via a high-affinity system. In addition, pyoverdin$_{pss}$ is similar to other pyoverdins in spectral traits (e.g., maximum absorbance of

410 nm for ferric pyoverdin$_{pss}$), size (~1,200 molecular weight), and overall amino acid composition (seven amino acids with abundant serine and threonine residues) (10, 22, 27, 33, 36, 37, 40, 41). Such similarities made it possible to produce and isolate large, homogeneous quantities of the pyoverdin siderophore with only minor modifications of existing purification protocols (24, 30, 33). Nevertheless, pyoverdin$_{pss}$ exhibits unique features, the most notable being the absence of a hydroxamate group as contributed by the presence of N^{δ}-hydroxyornithine, which is found in all other pyoverdins (10) except azotobactin (1). Pyoverdin$_{pss}$ contains 2 mol of β-hydroxyaspartic acid, which probably provides four of the six coordination sites required to complex with Fe(III). This amino acid, however, is not unique to pyoverdin$_{pss}$ since it is also found in pseudobactin (22, 36), azotobactin (10, 12), and other pyoverdins (10, 22, 40). The final two coordination sites for Fe(III) in pyoverdin$_{pss}$ are probably supplied by the fluorescent chromophore, which is present in all fully characterized pyoverdins and has been found to be an *o*-dihydroxyquinoline moiety (1, 10, 22, 31, 40). It is further speculated that lysine is linked to the fluorescent quinoline derivative since prolonged acid hydrolysis was needed for its release. A precedent for the attachment of the N^{δ}-amino group of lysine to an amide group of the fluorescent quinoline is observed for pseudobactin (22, 36), produced by *Pseudomonas* sp. strain B10, and pseudobactin 358 (40). Other pyoverdins contain amino acids, in addition to N^{δ}-hydroxyornithine, which are not found in pyoverdin$_{pss}$, such as arginine in pyoverdin$_{pa}$ (10, 41), alanine in pseudobactin (22, 36), and glycine in the pyoverdin produced by *P. fluorescens* ATCC 13525 (33) and other pseudomonads (10, 22). It also appears that the amino acid composition of pyoverdin$_{pss}$ is distinct from that of the pyoverdin produced by an unidentified pathovar of *P. syringae* (37). These structural differences in pyoverdins are believed (18, 22) to be important in uptake since protein receptors for ferric siderophores appear to recognize specifically the geometry of the siderophore surrounding the Fe(III) atom (17). An alternative explanation is that the structural properties can enhance the ability to remove iron from specific reservoirs of Fe(III) (8). This phenomenon is exemplified by aerobactin, which is adept at removing iron from transferrin; thus, aerobactin significantly contributes to iron acquisition during pathogenesis despite a relatively low stability constant (21).

The Fe(III) stability constant of pyoverdin$_{pss}$ is a function of pH; consequently, at a physiological pH of 7.0 its value is approximately 10^{25} compared with the much higher value of 10^{32} at pH 10.0. These formation constants are 10 to 100 times greater than those reported for pyoverdin$_{pf}$ isolated from *P. fluorescens* (10^{24} at pH 7.0) (27) and the pyoverdin isolated from a strain of *P. syringae* (10^{25} and 10^{30}, respectively, at pH 7.0 and 10.0) (37). The higher Fe(III) stability constants of pyoverdin$_{pss}$ probably reflect the different combination of ligands which coordinate the iron atom, namely, the presence of an α-hydroxy acid group from each of the β-hydroxyaspartate residues in addition to the *o*-dihydroxy aromatic group from the fluorescent quinoline. The stability constant of pyoverdin$_{pss}$ also shows it to be a highly competitive chelator of Fe(III) relative to various siderophores produced by a wide array of bacteria (30). Therefore, it would seem to be highly proficient in extracting Fe(III) from plant constituents.

Iron uptake, as mediated by [^{55}Fe]pyoverdin$_{pss}$ in iron-starved cells of *P. syringae* pv. *syringae* B301D, was rapid and appeared to proceed in two phases. Initially, uptake of ^{55}Fe(III) occurred at a very rapid rate, but after a few minutes a slower, linear rate was maintained. Similar biphasic rates of iron uptake have been shown for ferripyochelin in *P. aeruginosa* (5) and azotobactin in *A. vinelandii* (20).

The rapid synthesis of large quantities of pyoverdin$_{pss}$ by *P. syringae* pv. *syringae* when iron is deficient, along with the high stability constant of pyoverdin$_{pss}$ for Fe(III), suggests that the siderophore may have a distinct role in extracting iron from plant constituents, thereby enhancing pathogenesis in a manner analogous to bacteria-animal interactions (8, 31). Indeed, the requirement for large quantities of iron during pathogenesis is underscored by the positive regulation of phytotoxin synthesis by iron (13). The concentration of iron has been shown to affect the production of toxin A, alkaline protease, elastase, and various extracellular proteins in *P. aeruginosa* (4). Similar wide-spectrum iron effects may also occur in *P. syringae* pv. *syringae*, affecting growth and virulence in the plant environment. Evidence (14; Cody and Gross, in press) indicates that uptake of iron from ferric pyoverdin$_{pss}$ is mediated by the presence of a 74,000-molecular-weight protein which is a major protein in the outer membrane of iron-starved cells. However, since pyoverdin$_{pss}$ does not appear to supply crucial quantities of iron during plant pathogenesis, pyoverdin$_{pss}$-mediated iron uptake may be operative only under extreme iron limitation, as is the case for azotobactin produced by *A. vinelandii* (32). The exact role of pyoverdin$_{pss}$ in the plant-bacteria interaction is obscure (Cody and Gross, in press), therefore requiring considerable work before its ramifications are fully known. Siderophore production, nevertheless, raises several intriguing questions relative to iron acquisition and the formation of iron-regulated metabolites, such as syringomycin, during phytopathogenesis.

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