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Isolation and Characterization of an Fe(III)-Chelating Compound Produced by *Pseudomonas syringae*

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The phytopathogenic bacterium *Pseudomonas syringae* produces a fluorescent pigment when it is grown in iron-deficient media. This pigment forms a very stable Fe(III) complex that was purified in this form by using a novel procedure based on ultrafiltration and column chromatography. The Fe(III) complex has a molecular weight of 1,100 and contains 1 mol of Fe(III). The pigment is composed of an amino acid moiety with three threonines, three serines, one lysine, δ-*N*-hydroxyornithine, and a quinoline-type fluorescent chromophore. These features and its stability constant (in the range of 10^{32}) suggest that the fluorescent pigment of *P. syringae* is related to the siderophores produced by another *Pseudomonas* species.

Iron exists mainly as insoluble complexes of Fe(III) in aerobic conditions. Most fungi and bacteria respond to this low iron availability by secreting siderophores, a class of high-affinity ferric-specific chelating compounds. The involvement of siderophores in iron transport has been demonstrated in *Escherichia coli* (14, 15), *Pseudomonas fluorescens* (6), and other microorganisms (7).

Iron availability is very important for both pathogenic and nonpathogenic pseudomonads. The production of siderophores by pathogenic pseudomonads allows them to grow in iron-deficient environments and is probably related to their virulence (16). On the other hand, disease-suppressive soils are known in which plants are resistant to diseases produced by several fungi such as *Fusarium oxysporum*. These soils possess a microflora rich in *Pseudomonas* species that belong to the plant growth-promoting rhizobacteria group (12). These bacteria increase the growth of plants because they produce siderophores that efficiently chelate the iron present in the soil, making it less available to the plant pathogens (2, 3).

The siderophores produced by fluorescent pseudomonads are small molecules (M_r 1,000 to 1,500) composed of a quinoline-type chromophore and a linear peptide whose C-terminal amino acid is δ-*N*-hydroxyornithine (9, 10, 13, 17). *Pseudomonas syringae* is a phytopathogenic bacterium that produces a siderophorelike material (4) which has not been characterized to date. Our aim has been the purification of the fluorescent pigment produced by *P. syringae* as well as the study of some of its physicochemical properties.

MATERIALS AND METHODS

The *P. syringae* used was obtained from G. A. Strobel and is kept as strain M27 in the Department of Plant Pathology, Montana State University at Bozeman.

Media and growth conditions. The culture medium utilized was the synthetic medium of Dye [1.3 g of $(NH_4)_2HPO_4$ per liter, 0.26 g of KCl per liter, 0.26 g of $MgSO_4 \cdot 7H_2O$ per liter, and 1% glycerol adjusted to pH 7 with NaOH]. Cultures were grown at 28°C in 2-liter flasks containing 1 liter of medium with gentle stirring. The bacterial growth was measured by turbidometry at 600 nm. The production of pigment was measured as A_{407} of the cell-free medium.

Purification of the iron complex. Bacteria were grown for 72 h. Then 100 mg of $FeCl_3$ per liter was added to the medium. Cells were eliminated by centrifugation (21,000 × *g*, 10 min), and the supernatant was filtered through a 0.45-μm membrane (Millipore Corp.). The iron complex was purified from the filtrate by two alternative procedures. (i) The first was based on the previously published procedure of Meyer and Abdallah for the purification of pyoverdine$_{Pf}$ (5). The filtrate was concentrated under low pressure to 50 ml, saturated with NaCl, and extracted with 25 ml of phenol-chloroform (1:1, vol/vol). Two volumes of ethyl ether was added to the organic phase, and the mixture was extracted with 5 ml of distilled water. The pH of the aqueous phase was lowered to 6 with HCl, and the volume was reduced to 1 ml by evaporation under low pressure. The sample was diluted with 2 ml of 0.1 M pyridine–acetic acid (pH 6.5) and chromatographed on a CM-Sephadex C-25 column (1.2 by 48 cm) equilibrated and eluted with the same buffer. Fractions containing the iron complex were identified by measuring the absorbance at 407 nm, pooled, concentrated under low pressure, and chromatographed in the same conditions. Samples were then lyophilized and kept at 4°C. (ii) In the second procedure, the filtrate was concentrated to 15 ml by means of a Nuclepore cell with a membrane with a molecular weight cutoff of 1,000 and replacing the buffer with 0.1 M pyridine–acetic acid (pH 6.5). The sample was loaded on a Bio-Gel P4 column (3 by 52 cm; Bio-Rad Laboratories) equilibrated and eluted with the same buffer. Fractions containing the iron complex were pooled, concentrated under low pressure, and chromatographed twice on a CM-Sephadex C-25 column under the same conditions as for the first procedure.

Preparation of the iron-free pigment. To obtain iron-free pigment, 8-hidroxyquinoline was used as described by Meyer and Abdallah (5).

Paper electrophoresis. Paper electrophoresis of the different fractions was carried out on Whatman no. 1 or no. 3 paper in 0.1 M pyridine–acetic acid (pH 3.9) for 16 h at 400 V. The spots were localized by fluorescence, by spraying with ninhydrine (0.2% in ethanol), or by their own color.

Molecular weight determination. A Bio-Gel P2 column (1.8 by 48 cm) equilibrated with 0.1 M phosphate buffer (pH 7) was utilized. The column was calibrated with the following

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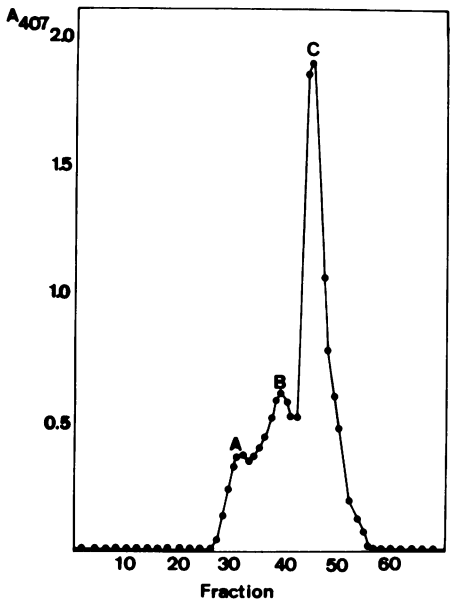

FIG. 1. Elution profile of Bio-Gel P4 column loaded with crude Fe(III)-pigment complex in 0.1 M pyridine–acetic acid (pH 6.5). Fractions contained 4.5 ml. A_{407}, Absorbance at 407 nm.

standards: reduced glutathione (M_r 307), oxidized glutathione (M_r 613), vitamin B_{12} (M_r 1,355), and serotransferrin (M_r 78,000).

Absorption and fluorescence spectra. Absorption spectra of the fluorescent pigment and its Fe(III) complex were measured in a Beckman 25 spectrophotometer. The following buffers were used: 0.1 M sodium acetate (pH 3), 0.1 sodium phosphate (pH 6, 7, and 8), and 0.1 M glycine (pH 10). Fluorescence spectra of the pigment in 0.1 M sodium phosphate buffer (pH 7) were made with a Perkin-Elmer MPH HHB spectrofluorimeter.

Stability constants of the Fe(III) complex. To determine the stability constants of the Fe(III) complex, EDTA was used as a competing ligand; increasing amounts of EDTA (from 9.4×10^{-4} to 6×10^{-2} M) were added to series of Fe(III) complex solutions (1.5×10^{-4} M) (1). The remaining Fe(III) complex concentration at equilibrium was calculated from the A_{450} of the solutions. The buffers used were as described above for the determination of absorption spectra.

The iron content of the samples was measured by the spectrophotometric method of Ovenston and Parker (8).

Amino acid analysis of the iron complex. Iron complex isolated by preparative electrophoresis was hydrolyzed with 6 N HCl at 105°C for 48 h or with 6 N HI in the same conditions. The hydriodic hydrolysate was washed with water until the red color was completely removed. The hydrochloric hydrolysate was analyzed in a Durrum D-500 automatic amino acid analyzer. The hydriodic hydrolysate was analyzed by high-pressure liquid chromatography as described by Portolés et al. (11).

RESULTS AND DISCUSSION

Purification of the Fe(III)-pigment complex. When grown in iron-deficient media, the bacterium *P. syringae* produces a fluorescent pigment. The maximum yield of this compound is obtained after 70 h of culture. The pigment produced by *P. syringae* is very unstable, as in the case of pyoverdine$_{Pf}$ (5); therefore, attempts to purify it in the free ligand form were unsatisfactory. When Fe(III) is added to the pigment-containing medium, the color changes from pale green to red and the fluorescence is lost. The Fe(III) complex is very stable and thus easier to purify. The usual approach in the purification of siderophores is an extraction with organic solvents. However, organic extractions of the Fe(III)-chelating compound produced by *P. syringae* gave a very low yield (7%). This result is consistent with results obtained with pigments from other *Pseudomonas* species (4, 5, 9, 10, 14). For this reason we developed a different extraction procedure. Ultrafiltration under pressure at 4°C separated salt from pigment and introduced the desired buffer, allowing pigment concentration under mild conditions. It was then possible to load the sample directly on the Bio-Gel P4 column. The elution profile of the P4 column is shown in Fig. 1. Peak C was the only one in which the free pigment absorption spectrum was restored after treatment with EDTA. The relative amounts of peaks A and B increased when the extract was heated or stored (results not shown), suggesting that they were probably degradation products.

Peak C was concentrated by ultrafiltration and chromatographed on a CM-Sephadex column (Fig. 2A). The main peak (II) was rechromatographed under the same conditions (Fig. 2B). The elution profile was similar, but the proportion of peak II_2 was increased in this case. The compound present in peak II_2 produced a single band in analytical paper electrophoresis. The electrophoretic band obtained was isolated by preparative paper electrophoresis and characterized by absorption spectroscopy.

The yield obtained with this method was three times higher (20%) than those obtained by means of extraction with organic solvents.

Physicochemical properties of the pigment and its Fe(III) complex. The approximate molecular weight of the Fe(III) complex, calculated by gel exclusion chromatography in a previously calibrated Bio-Gel P2 column, was 1,200. Ac-

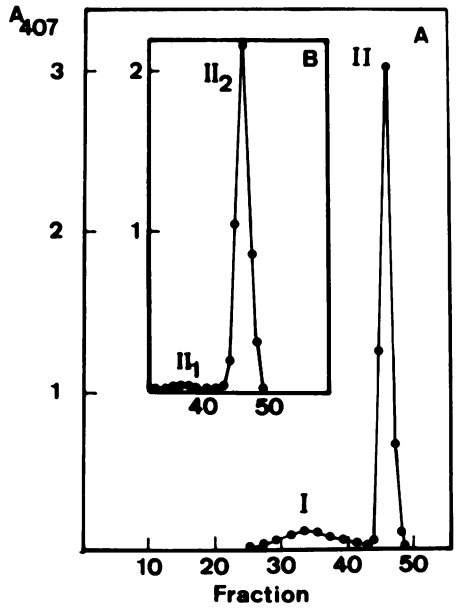

FIG. 2. Elution profile of a CM-Sephadex column loaded with Fe(III)-pigment complex in 0.1 M pyridine–acetic acid (pH 6.5). Fractions contained 2 ml. (A) First chromatography with Bio-Gel P4-purified complex. (B) Second chromatography with peak II from the first chromatography. A_{407}, Absorbance at 407 nm.

cording to this value, we estimated the stoichiometry of the complex Fe(III) pigment by calculating the molar ratio between the complex and the Fe(III) that it contains. This was 1.07 mol of Fe(III) per mol of complex, or one ion of Fe(III) per molecule of complex. The molecular weight of the complex could be recalculated with this ratio. The new value obtained was 1,060.

Absorption and fluorescence spectra of the free ligand and its ferric complex. Figure 3A shows the absorption spectrum of an aqueous solution of the fluorescent ligand and its ferric complex at pH 6. The complex showed two main absorption bands: one in the UV region at 230 nm, the other one in the visible region with a maximum at 407 nm (ε, 2,560 liters mol^{-1} cm^{-1}). This spectrum did not change with the pH between pH 3 and 10. The fluorescent ligand had an absorption spectrum with two main bands as its ferric complex, one at 230 nm and the other at 380 nm with two shoulders at 370 and 400 nm, respectively. The ligand did not absorb at 450 nm. The spectrum of the fluorescent ligand changed with the pH, being identical to the spectrum of the complex at pH 10 (Fig. 3B). These results were similar to those obtained by other authors (5, 9, 13) for siderophores of *P. fluorescens*.

The fluorescence spectrum of the free ligand had an excitation maximum at 400 nm, with a shoulder at 360 nm and an emission maximum at 460 nm. This result was similar to that obtained by Meyer and Abdallah (5) for pyoverdine$_{Pf}$. The ferric complex was not fluorescent.

All of these results suggest a quinolinic type structure for the moiety of the pigment, as in the case of siderophores pyoverdine *Pa* (17), pseudobactine (13), or pyoverdine$_{Pf}$ (9, 10).

Amino acid analysis. The hydrochloric hydrolysis of the complex released serine, threonine, and lysine in a molar ratio of 3:3:1. The hydriodic hydrolysis also released ornithine. The lack of ornithine in the hydrochloric hydrolysates may be explained as described by Zahner et al. (18) by its degradation under these hydrolysis conditions. According to the same authors, the ornithine present in the hydriodic hydrolysates can be a degradation product of δ-*N*-hydroxyornithine. The amino acid composition is different from that reported previously for other pigments produced by *Pseudomonas* species (13, 17).

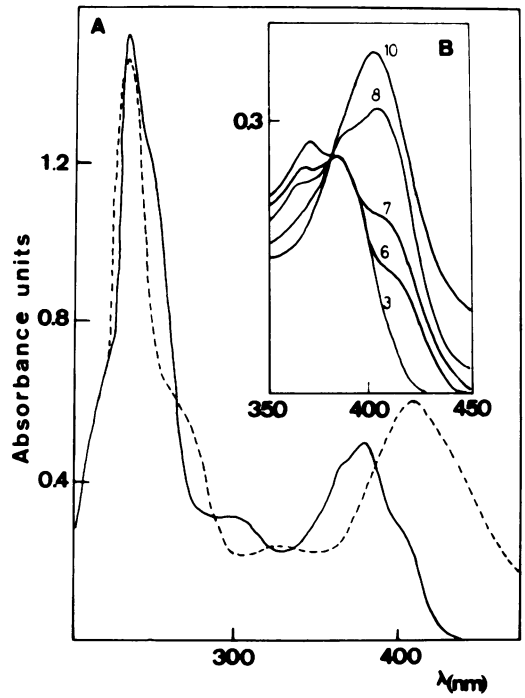

FIG. 3. (A) Absorption spectra of fluorescent pigment produced by *P. syringae* (——) and its Fe(III) complex (– – –) in 0.1 M phosphate buffer (pH 6). (B) Visible absorption spectrum of fluorescent pigment produced by *P. syringae* as a function of the pH. pH values are indicated beside the spectra.

TABLE 1. Variation of the apparent stability constant of the Fe(III)-pigment complex as a function of pH

pH	$K_{Fe(III)\text{-pigment}}/K_{Fe(III)\text{-EDTA}}$	$K_{Fe(III)\text{-EDTA}}$	$K_{Fe(III)\text{-pigment}}$
5	0.35	10^{18}	3.50×10^{17}
6	37.00	10^{20}	3.70×10^{21}
7	3.52×10^{3}	10^{22}	3.52×10^{25}
8	6.80×10^{3}	10^{23}	6.80×10^{26}
10	2.78×10^{3}	10^{27}	2.78×10^{30}

Stability constant of Fe(III)-pigment complex. The apparent stability constant (*K*) of the complex was determined by displacing the iron from the complex with EDTA (1) and taking advantage of the lack of absorption at 450 nm of the fluorescent ligand. A *K* of 3.52×10^{25} was obtained at pH 7. To compare *K*s of siderophores produced by different bacteria, values at pH 14 are frequently used (1). This takes into account the competition established between Fe(III) and H^+ for several of the chelating groups of siderophores. Table 1 shows *K* values at different pHs (it is impossible to carry out determinations of *K* above pH 10 due to the lability of the complex at basic pH). A *K* of 1.9×10^{32} was obtained by extrapolation to pH 14. This value was of the same order of those obtained for other nonphytopathogenic pseudomonads (5, 9). Nevertheless, the comparison of *K* at pH 14 may sometimes lead to misinterpretation of the data. It is more intresting to compare *K*s of the siderophores at physiological pHs. Thus, *K*s at pH 14 of the pigment produced by *P. syringae* and by the siderophore produced by *P. fluorescens* were identical; however, at pH 7 the *P. syringae* pigment had an apparent stability constant 10 times higher than that of pyoverdine$_{Pf}$ (5). It may well be that the affinity for iron of the siderophores at physiological pHs is especially important in the case of *Pseudomonadaceae*. Competition for iron may have great ecological importance in this genus, which includes such phytopathogenic species as *P. syringae*, and others that are beneficial to crops.

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