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Purification of Pyoverdines of *Pseudomonas fluorescens* 2-79 by Copper-Chelate Chromatography

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Three pyoverdines, Pf-A, Pf-B, and Pf-C, were purified with copper-chelate Sepharose and Sephadex G-15 columns from *Pseudomonas fluorescens* 2-79, and the yields (per 100 ml of culture supernatant) were 2.8, 21.6, and 3.2 mg, respectively. The absorption and fluorescence spectra of these pyoverdines were strongly pH dependent. Characteristic changes in the maximal absorbance wavelengths were observed when Fe^{3+} or Cu^{2+} was added. The addition of Cu^{2+} shifted the pyoverdine Pf-B absorbance spectrum so that it exhibited a single peak at 410 nm but did not give rise to a new absorbance maximum at approximately 460 nm, which appeared when Fe^{3+} was added. Fluorescence quenching experiments revealed that the forward reaction rate constant with pyoverdines was much higher with Cu^{2+} (10^4 to 10^5 M^{-1} s^{-1}) than with Fe^{3+} (10^2 M^{-1} s^{-1}). However, Cu^{2+}-pyoverdine complexes were completely dissociated by EDTA at a low concentration (0.1 mM), while the level of Fe^{3+}-pyoverdine complex dissociation at the same EDTA concentration was relatively low. The dissociation of Fe^{3+}-pyoverdine complexes was EDTA concentration dependent. Formation of free pyoverdine was observed when the three types of Fe^{3+}-pyoverdine complexes were incubated separately with *P. fluorescens* 2-79 cells, thus demonstrating that pyoverdines Pf-A, Pf-B, and Pf-C mediate iron transport.

With the exception of the lactobacilli, all microorganisms require iron for growth and proliferation (36). Since the levels of ferric ion at pH 7.0 in aerobic aqueous environments are less than 10^{-17} M (35), most microorganisms respond to iron stress by producing iron transport agents called siderophores. These compounds chelate Fe^{3+} with a high specificity and serve as vehicles for transport of Fe^{3+} into microbial cells (24, 25).

The members of the fluorescent *Pseudomonas* group are the best-studied plant growth-promoting rhizobacteria. Some *Pseudomonas* species have been used as seed inoculants on crops to promote plant growth (6, 15, 19, 31). Enhanced plant growth caused by these strains is often accompanied by reductions in populations of deleterious microorganisms, the so-called minor pathogens (3, 16, 19, 21, 29, 30, 32). It has been suggested that pseudomonads exert their beneficial effects in part by producing yellow-green fluorescent siderophores, called pyoverdines (or pyoverdins or pseudobactins), under iron-deficient conditions (15, 30, 32). Pyoverdines are thought to chelate iron efficiently, making it less available to phytopathogens and thus promoting plant growth. Genetic evidence which supports the hypothesis that pyoverdines are involved in plant growth promotion and biological control has come from the results of experiments in which pyoverdine-negative mutants lost the ability to promote plant growth (12, 17). However, there have been reports of exceptions in which pyoverdine-negative mutants controlled plant diseases as effectively as their fluorescent parental strains (9). The role of pyoverdines in plant growth promotion is still very controversial. Our long-term goal is to determine why pyoverdines contribute to the biological control activity of *Pseudomonas* spp. in certain cases and not in others. As a first step, the objective of this study was to purify pyoverdines from *Pseudomonas fluorescens* 2-79 and characterize them. Pyoverdine-negative mutants of strain 2-79 have been shown to control plant diseases as effectively as the parental strain (9).

Direct use of purified pyoverdines in soil amendment studies to evaluate their role in plant growth promotion is important. However, very few soil amendment studies have been performed, and this has been attributed to difficulties in obtaining the large quantities of pyoverdine required (21). Most pyoverdines have been purified by the Fe^{3+} complexation-decomplexation method (23), which is based on the fact that Fe^{3+}-siderophore complexes can be extracted with organic solvents. This procedure is time consuming and may denature the pyoverdines because organic solvents and extreme pH conditions are used. Recently, a hydrophobic chromatography (octadecylsilane) method has been used for pyoverdine purification (8). This procedure is much faster than the complexation-decomplexation method, and its use resulted in a better yield of pyoverdines from *Pseudomonas aeruginosa* ATCC 15692. However, an artifact compound, pyoverdine Pa-C, appeared during the purification process.

A simple and rapid alternative method involving an immobilized metal affinity chromatography technique for the purification of fluorescent siderophores from *Pseudomonas* strains was developed recently in our laboratory. Immobilized metal affinity chromatography has been used widely to purify interferons, serum proteins, and other biomolecules on the basis of their affinity for metal ions (1, 10, 13, 14, 18, 22, 27, 28). In this paper, we describe purification of pyoverdines from *P. fluorescens* 2-79 by copper-chelate chromatography. Pyoverdines purified by this method were biologically active. The UV-visible absorption and fluorescence spectroscopic properties of these molecules were determined, and the Fe^{3+}- and Cu^{2+}-pyoverdine forward reaction rate constants (k_{+1}) were also determined.

MATERIALS AND METHODS

Microorganisms and pyoverdine production. *P. fluorescens* 2-79 (= NRRL-15132) was obtained from the Agricultural Research Service Culture Collection at the National Center for Agricultural Utilization Research in Peoria, Ill. *P.*

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fluorescens 2-79 was grown on a synthetic succinate medium containing (per liter) 6.0 g of K_2HPO_4, 3.0 g of KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, 0.1 g of $MgSO_4 \cdot 7H_2O$, and 4.0 g of succinic acid. The pH was adjusted to 7.0 by adding a 1 N NaOH solution prior to sterilization (23). The medium was dispensed into 500-ml Erlenmeyer flasks, each of which contained 100 ml of medium. The flasks were then inoculated, at a seed rate of 1.0%, with exponential-phase cells grown in the same medium. The cultures were incubated at 25°C and 200 rpm in a New Brunswick model Innova 4000 shaker-incubator for 48 h. Batches were pooled and centrifuged at 10,000 × *g* for 10 min at 4°C. The resulting supernatant was membrane filtered (pore size, 0.25 μm; Amicon) to yield a cell-free solution of crude pyoverdines. The amount of pyoverdine produced was estimated by determining the A_{400} of the supernatant.

Pyoverdine isolation and purification. All purification procedures were carried out at room temperature. The cell-free supernatant was buffered with 1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.0) and then applied to a Chelating Sepharose Fast Flow column (1.5 by 25 cm; Pharmacia LKB Biotechnology) that had been presaturated with $CuSO_4$. The column was equilibrated with 20 mM HEPES buffer (pH 7.0) containing 100 mM NaCl at a flow rate of 100 ml/h. The column was then washed with 200 ml of 20 mM HEPES buffer and eluted with 20 mM acetate buffer (pH 5.0) containing 100 mM NaCl. The A_{400} of each 10-ml fraction collected was determined. The pyoverdine-containing fractions (peaks FI, FII, and FIII) were pooled separately and lyophilized. The dried material from each of the fractions was dissolved in 1 ml of distilled water containing 10 mM EDTA and then applied to a Sephadex G-15 column (1.5 by 100 cm) that had been preequilibrated with deionized water. Elution was carried out with distilled water at a flow rate of 20 ml/h. Fractions (3 ml) were collected, and the A_{400} of each fraction was determined. In addition, the fluorescence of each fraction was determined at emission and excitation wavelengths of 460 and 400 nm, respectively. The pyoverdine-containing fractions were pooled, lyophilized, and stored at 4°C until they were needed. On the basis of the fraction fluorescence profiles after gel filtration, peaks FI, FII, and FIII each yielded one major fluorescent compound; since pyoverdines are the only fluorescent siderophores produced by *P. fluorescens* 2-79, the three compounds were designated Pf-A, Pf-B, and Pf-C, respectively. The concentrations of pyoverdines Pf-A, Pf-B, and Pf-C were estimated by using an extinction coefficient of 20,000 M^{-1} cm^{-1} (23).

Fe^{3+}-pyoverdine complex preparation. Purified pyoverdine and $FeCl_3$ were added to 100 mM HEPES buffer (pH 7.0) at a ratio of 1:2. The mixture was incubated at room temperature for 30 min and then applied to a Sephadex G-15 column (1.5 by 100 cm) that had been preequilibrated with the same buffer. The free Fe^{3+} ions were separated from the Fe^{3+}-pyoverdine complexes by using the same buffer at a flow rate of 20 ml/h. Elution of the Fe^{3+}-pyoverdine complexes was monitored by measuring the A_{400}. Fractions (3 ml) were collected, and the fractions containing Fe^{3+}-pyoverdine complexes were pooled and stored at 4°C until they were needed.

Absorption spectrum measurements. Pyoverdine absorption spectra were determined with a Beckman model DU 650 spectrophotometer. The effects of different pH values on the spectra of iron-free pyoverdines Pf-A, Pf-B, and Pf-C were determined as follows: pyoverdines were dissolved in 0.1 M citric acid-phosphate buffers (pH 3.0, 5.0, 7.0, and 8.0) and 0.1 M Tris-HCl buffer (pH 9.0), and then the absorption spectra from 300 to 500 nm were determined and compared. To study the interaction of Cu^{2+} with pyoverdine Pf-B, 10.0 μM $CuSO_4$ or 10.0 μM $FeCl_3$ was added to 100 mM acetate buffer (pH 5.0) containing 3.0 μM pyoverdine Pf-B. The reaction mixture was kept at room temperature for 30 min, and then the absorption spectra between 340 and 500 nm for free pyoverdine Pf-B, Cu^{2+}-pyoverdine Pf-B complexes, and Fe^{3+}-pyoverdine Pf-B complexes were determined and compared.

Association and dissociation of pyoverdine with Fe^{3+} and Cu^{2+}. Association and dissociation of pyoverdine with Fe^{3+} and Cu^{2+} were measured by continuously monitoring the fluorescence changes at emission and excitation wavelengths of 460 and 400 nm, respectively. Portions (3 ml) of a pyoverdine solution (5.0 μM in 100 mM HEPES buffer [pH 7.0]) were incubated at 25°C, and association was initiated by adding an Fe^{3+} or Cu^{2+} solution to a final concentration of 3.0 μM. Dissociation of Fe^{3+}- or Cu^{2+}-pyoverdine complexes was initiated by adding EDTA (final concentration, 0.1 mM) to the reaction mixture.

Determination of Fe^{3+}- and Cu^{2+}-pyoverdine k_{+1}. Fe^{3+}- or Cu^{2+}-pyoverdine k_{+1} was determined as follows. Since neither Fe^{3+}-pyoverdine complexes nor Cu^{2+}-pyoverdine complexes exhibit fluorescence, the concentration of Fe^{3+}- or Cu^{2+}-pyoverdine formed at time (*t*) during the reaction, $P + \text{Met} \rightleftharpoons P\text{:Met}$, was calculated from the change in fluorescence; k_{+1} was determined by calculating the slope of the linear plot of $\ln([P]_0[\text{Met}]_t)/([\text{Met}]_0[P]_t)$ $([\text{Met}]_0 - [P]_0)$ versus reaction time *t*, where the subscripts 0 and *t* refer to the initial time and an arbitrary time, respectively, and P and Met represent pyoverdine and Fe^{3+} (or Cu^{2+}), respectively.

Pyoverdine-mediated iron transport. To demonstrate that iron transport is mediated by pyoverdines, *P. fluorescens* 2-79 cells were harvested from a mid-exponential-phase culture grown in iron-deficient succinate medium. The cells were washed three times with sterile distilled water at 4°C and were suspended in 100 mM HEPES buffer (pH 7.0) at an optical density at 600 nm of 0.05 U. Portions (3 ml) of this cell suspension were dispensed into cuvettes and incubated at 25°C with stirring for 10 min before 10-μl portions of Fe^{3+}-pyoverdine complex solutions were added so that the final Fe^{3+}-pyoverdine complex concentrations were 0.01, 0.05, and 0.1 mM. The fluorescence due to free pyoverdine formation was monitored continuously for 20 min at emission and excitation wavelengths of 460 and 400 nm, respectively. Two controls were included. In the first control, the Fe^{3+}-pyoverdine complex was replaced with HEPES buffer; in the second control, cells grown in succinate medium supplemented with 50 μM $FeCl_3$ were used instead of iron-starved cells. The cells were suspended in 100 mM HEPES buffer (pH 7.0) at an optical density at 600 nm of 0.24 U and were incubated with 0.1 mM Fe^{3+}-pyoverdine complex as described above.

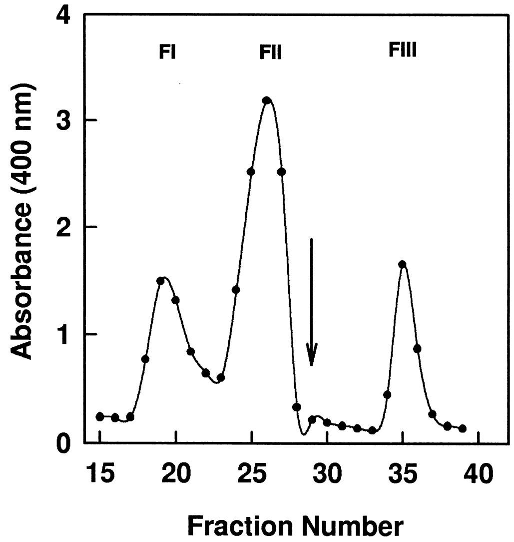

FIG. 1. Copper-chelate chromatography of the pyoverdines from *P. fluorescens* 2-79. Supernatant (100 ml) from a *P. fluorescens* 2-79 culture was mixed with 2 ml of 1.0 M HEPES buffer (pH 7.0) and applied to a copper-chelate Sepharose Fast Flow column (1.5 by 25 cm) that had been equilibrated with 20 mM HEPES buffer (pH 7.0) containing 100 mM NaCl. The first two peaks (peaks FI and FII) were eluted with 200 ml of the equilibration buffer. Elution of the third peak with 20 mM acetate buffer (pH 5.0) containing 100 mM NaCl started at the arrow.

RESULTS

Purification of the pyoverdines from *P. fluorescens* 2-79. As shown in Fig. 1, chromatographic separation of *P. fluorescens* 2-79 pyoverdines on a copper-chelate affinity column resulted in three peaks. The first two peaks (peaks FI and FII) were eluted with approximately 200 ml of equilibration buffer. The third peak (peak FIII) was bound more tightly to the column and was subsequently eluted with 100 ml of 20 mM acetate buffer (pH 5.0) containing 100 mM NaCl.

The fractions containing peaks FI, FII, and FIII were pooled separately and lyophilized. The dried material from each of the fractions was dissolved in 1 ml of distilled water containing 10 mM EDTA and then applied to a Sephadex G-15 column (1.5 by 100 cm). Three major fluorescent siderophores were purified from peaks FI, FII, and FIII; since pyoverdines are the only fluorescent siderophores produced by *P. fluorescens* 2-79, the three compounds were designated pyoverdines Pf-A, Pf-B, and Pf-C, respectively. The yields obtained from 100 ml of cell-free fermentation broth were 2.8 mg of pyoverdine Pf-A, 21.6 mg of pyoverdine Pf-B, and 3.2 mg of pyoverdine Pf-C.

Characterization of the pyoverdines. The effects of different pH values on the absorption spectra of iron-free pyoverdines Pf-A, Pf-B, and Pf-C are shown in Fig. 2. The absorption spectra of the three pyoverdines were pH sensitive. At low pH values (pH 3 and 5), the spectra had double peaks (366 and 384 nm for pyoverdine Pf-A, 368 and 384 nm for pyoverdine Pf-B, and 369 and 385 nm for pyoverdine Pf-C). However, at higher pH values (pH 7, 8, and 9), the pyoverdine spectra had single

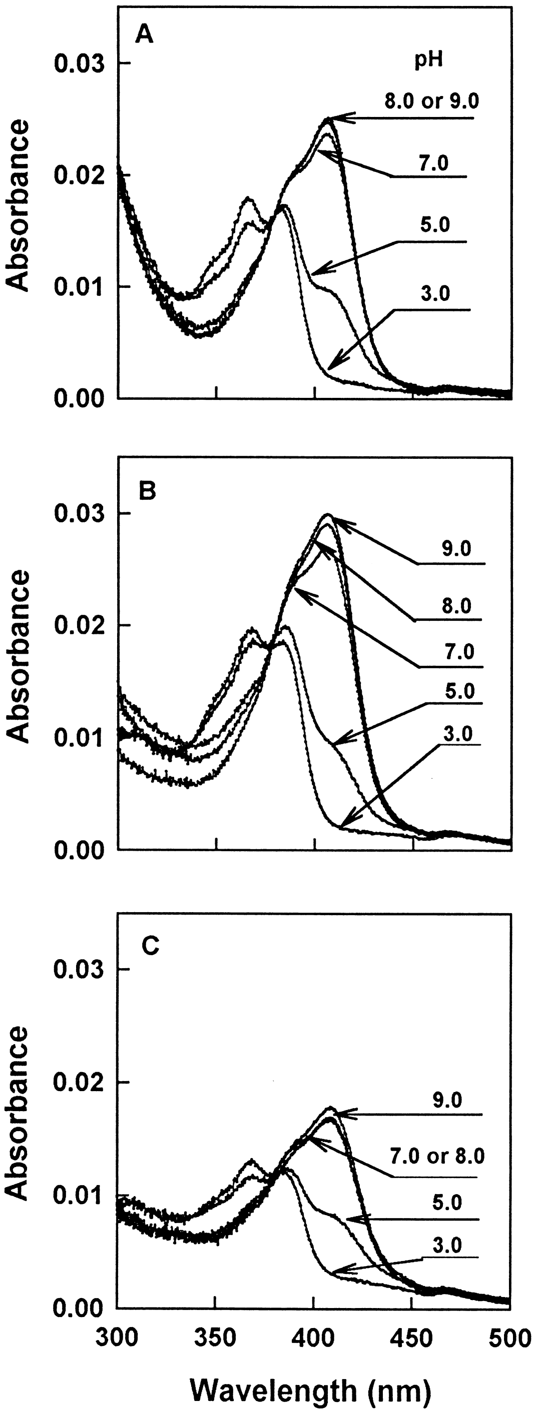

FIG. 2. Effects of pH on absorption spectra of *P. fluorescens* 2-79 pyoverdines Pf-A (A), Pf-B (B), and Pf-C (C). Citric acid-phosphate buffers (0.1 M; pH 3.0, 5.0, 7.0, and 8.0) and Tris-HCl buffer (0.1 M; pH 9.0) were used. The pH values are indicated beside the spectra.

peaks (407 nm for pyoverdines Pf-A and Pf-B and 408 nm for pyoverdine Pf-C). These results are consistent with previous findings obtained with other pyoverdines (23, 26, 33). Fluorescence of the three pyoverdines was strongly pH dependent, and maximum fluorescence was observed at pH 7.0 (data not shown).

The absorption spectrum of the Fe^{3+}-pyoverdine Pf-B complex (Fig. 3) had a maximum at 398 nm and a pronounced shoulder at 460 nm. Addition of Cu^{2+} to pyoverdine Pf-B at pH 5.0 resulted in a change in the absorption spectrum to a single peak at 410 nm, but the pronounced shoulder observed with the Fe^{3+}-pyoverdine Pf-B complex was not formed (Fig. 3). Similar results were obtained with Cu^{2+}-pyoverdine Pf-A and Cu^{2+}-pyoverdine Pf-C complexes. The results of a comparison of the differences between the free and metal-bound pyoverdine spectra in Fig. 3 strongly suggested that pyoverdines bind Cu^{2+}, as well as Fe^{3+}.

As shown in Fig. 4, Cu^{2+} quenched pyoverdine Pf-B fluorescence much faster than Fe^{3+} did. The extent of fluorescence quenching due to Cu^{2+} was not affected by preincubating pyoverdine with Fe^{3+} for 1 min, and as shown in Fig. 4, the fluorescence quenching due to Cu^{2+} was completely restored by adding EDTA to a final concentration of 0.1 mM. However, the level of Fe^{3+}-pyoverdine complex dissociation at the same EDTA concentration was relatively much lower. Dissociation of the Fe^{3+}-pyoverdine Pf-B complex was found to be EDTA concentration dependent (Fig. 5).

The Fe^{3+}-pyoverdine k_{+1} for the three pyoverdines were determined as described in Materials and Methods (Table 1). The three pyoverdines exhibited greater affinity for Cu^{2+} (the k_{+1} ranged from 10^4 to 10^5 M^{-1} s^{-1}) than for Fe^{3+} (the k_{+1} was approximately 10^2 M^{-1} s^{-1}).

Pyoverdine-mediated iron transport in *P. fluorescens* 2-79. As shown in Fig. 6A, addition of Fe^{3+}-pyoverdine Pf-B complexes to an iron-starved mid-log-phase *P. fluorescens* 2-79 cell suspension resulted in a significant increase in fluorescence. This increase depended on the Fe^{3+}-pyoverdine concentration. Similar results were obtained with Fe^{3+}-pyoverdine Pf-A and Fe^{3+}-pyoverdine Pf-C complexes. In contrast, no changes in fluorescence were observed when Fe^{3+}-pyoverdine complexes were added to a suspension of mid-log-phase *P. fluorescens* 2-79 cells grown in an iron-rich succinate medium (Fig. 6B).

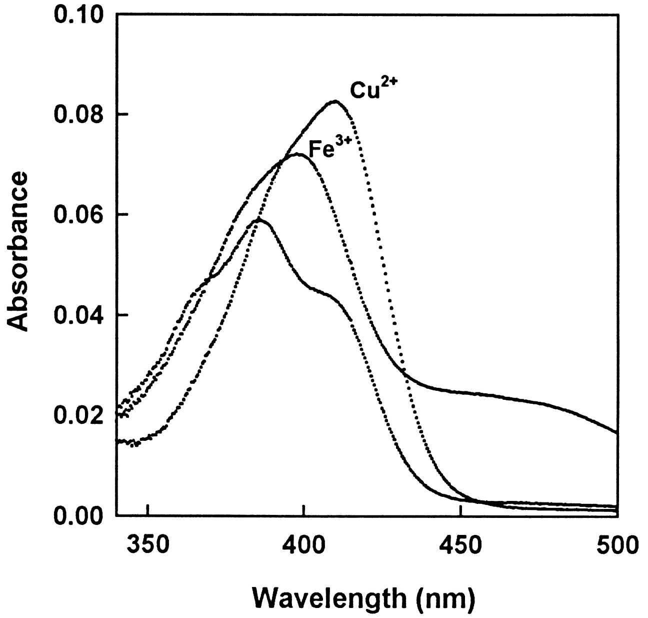

FIG. 3. Absorption spectra of free pyoverdine Pf-B and its Fe^{3+} and Cu^{2+} complexes at pH 5.0. Pyoverdine Pf-B (3.0 μM) in 100 mM acetate buffer (pH 5.0) was incubated with either 10.0 μM $CuSO_4$ or 10.0 μM $FeCl_3$ for 30 min at room temperature.

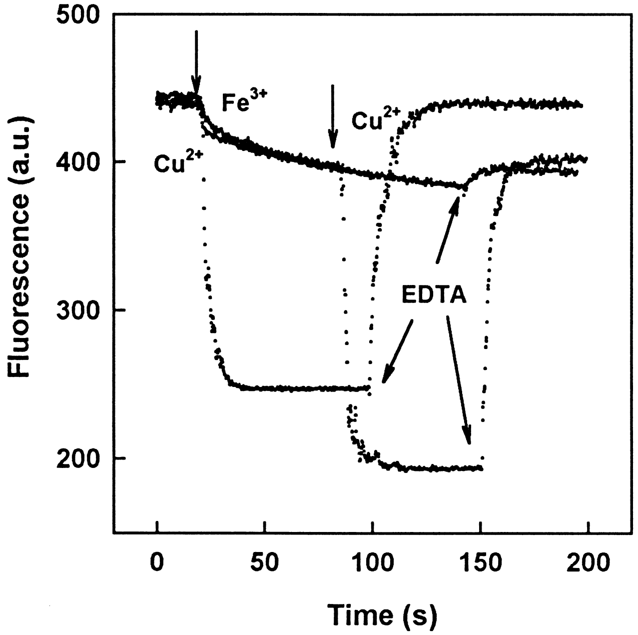

FIG. 4. Fluorescence quenching of pyoverdine Pf-B by Cu^{2+} and Fe^{3+}. Pyoverdine Pf-B was added to 3 ml of 100 mM HEPES buffer (pH 7.0) to a final concentration of 5.0 μM. Standard solutions of $FeCl_3$, $CuSO_4$, and EDTA were added at the times indicated by the arrows to final concentrations of 3.0 μM, 3.0 μM, and 0.1 mM, respectively. Fluorescence was measured at an excitation wavelength of 400 nm and an emission wavelength of 460 nm. The slit widths for excitation and emission were 5.0 and 2.5 nm, respectively. a.u., arbitrary units.

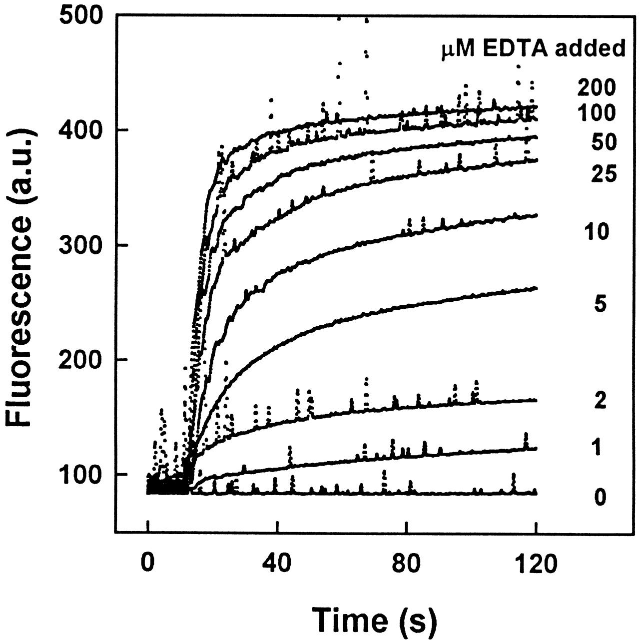

FIG. 5. EDTA titration of Fe^{3+}-pyoverdine complexes. Iron-pyoverdine Pf-B complexes were prepared as described in Materials and Methods. A 10-μl portion of an EDTA solution was added to 3 ml of 100 mM HEPES buffer (pH 7.0) containing iron-pyoverdine Pf-B complexes at a concentration of 1 μM. The final concentrations of EDTA are indicated beside the titration curves. Fluorescence was measured at an excitation wavelength of 400 nm and an emission wavelength of 460 nm. The slit widths for excitation and emission were 10.0 and 10.0 nm, respectively. a.u., arbitrary units.

DISCUSSION

In this study, we developed a simple and rapid method for purifying the pyoverdines secreted by *P. fluorescens* 2-79; this method was based on the affinity of pyoverdines for copper (Fig. 1). The procedure described above required only two column chromatographic steps, copper-chelate Sepharose chromatography and Sephadex G-15 chromatography. The use of copper-chelate Sepharose ensured that all compounds with no affinity for Cu^{2+} were removed, and this allowed us to isolate the pyoverdines and obtain excellent yields. As Fig. 2 shows, the three purified pyoverdines, pyoverdines Pf-A, Pf-B, and Pf-C, produced absorption spectra similar to previously published spectra for *P. fluorescens* ATCC 13525 (26) and *Pseudomonas syringae* M 27 (33) pyoverdines, and this attested to the high levels of pyoverdine purity obtained when we used our purification procedure.

It has been reported that fluorescent *Pseudomonas* species usually produce multiple pyoverdines. For example, numerous pyoverdines have been purified from *P. fluorescens* ATCC 13525 (20, 26), *P. aeruginosa* ATCC 15692 (8), and other species (7). The three purified compounds obtained from strain 2-79 (pyoverdines Pf-A, Pf-B, and Pf-C) were considered true pyoverdines for several reasons. First, they were fluorescent. Second, they mediated Fe^{3+} transport into the cells, as shown in Fig. 6. And third, their three corresponding peaks after ion-exchange chromatography were observed in both young cultures (cultures incubated for 36 h) and old cultures (cultures incubated for 48 h). This precluded the possibility that any of the compounds was a degradation product of the other compounds. It should also be pointed out that the supernatant pH values for the young and old cultures were approximately 7.40 and 8.05 and that the level of supernatant fluorescence increased fivefold over the 12-h period from 36 to 48 h. On the basis of these results, it is not likely that pyoverdine was degraded since the change in pH over the 12-h period was modest. The copper-chelate chromatography technique has been used to purify pyoverdines from other fluorescent pseudomonads in our laboratory. For example, four pyoverdines from *P. aeruginosa* ATCC 15692, three pyoverdines from *Pseudomonas putida* ATCC 12633, and one azotobactin from *Azotobacter vinelandii* have been purified (unpublished data); this suggests that the copper-chelate affinity approach is indeed a useful technique for isolating fluorescent siderophore.

Until now, direct determination of Fe^{3+}-siderophore k_{+1} has not been possible. We propose that pyoverdines should be used to determine Fe^{3+}-pyoverdine and Cu^{2+}-pyoverdine k_{+1} values as described in Materials and Methods. Since k_{+1} indicates of how fast pyoverdine acquires iron from the environment, it can be argued that k_{+1} is a more accurate indicator of the biological effectiveness of a given pyoverdine than the stability constant (K_s) is. We found that Cu^{2+} bound pyoverdine at a much faster rate than Fe^{3+} did (Fig. 4 and Table 1). However, we could not determine if there were any Fe^{3+} solubility limitations. It is unclear why pyoverdines bind Cu^{2+}; however, it has been reported that the siderophore schizokinen

TABLE 1. Fe^{3+}- and Cu^{2+}-pyoverdine k_{+1} values for *P. fluorescens* 2-79 pyoverdines

Ligand	k_{+1}(M^{-1} s^{-1})		
	Pf-A	Pf-B	Pf-C
Iron(III)	0.88×10^2	1.27×10^2	1.06×10^2
Copper(II)	6.52×10^4	1.47×10^5	2.15×10^4

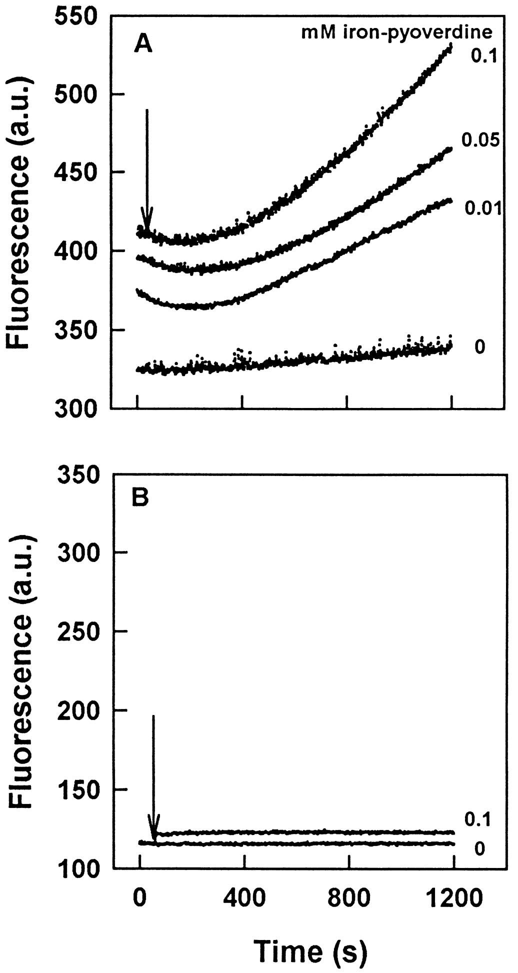

FIG. 6. Formation of iron-free pyoverdine from Fe^{3+}-pyoverdine complexes which were incubated with *P. fluorescens* 2-79 cells grown in iron-limited medium (A) and iron-rich medium (B). (A) *P. fluorescens* 2-79 cells harvested from an iron-limited culture were suspended in 3 ml of HEPES buffer (pH 7.0) at a final A_{600} 0.05 U and incubated at 25°C for 10 min. Then, 10-μl portions of an Fe^{3+}-pyoverdine Pf-B solution were added so that the final concentrations of Fe^{3+}-pyoverdine Pf-B were 0.01, 0.05, and 0.1 mM. (B) Cells harvested from an iron-rich culture were suspended in 3 ml of HEPES buffer (pH 7.0) at a final A_{600} of 0.24 U and incubated at 25°C for 10 min, and Fe^{3+}-pyoverdine Pf-B was added to a final concentration of 0.1 mM. Fluorescence was measured at an excitation wavelength of 400 nm and an emission wavelength of 460 nm. The slit widths for excitation and emission were 10.0 and 10.0 nm, respectively. a.u., arbitrary units.

produced by *Anabaena* spp. and *Bacillus megaterium* binds Cu^{2+}, resulting in decreased copper toxicity in *Anabaena* spp. (5) and increased toxicity in *B. megaterium* (2). Chakrabarty and Roy showed that the presence of copper in the growth medium promoted the production of a yellow pigment by *P. fluorescens-putida* (4). On the other hand, Cu^{2+} did not affect pyoverdine synthesis by *P. aeruginosa*; however, Cu^{2+}-pyoverdine complexes significantly stimulated growth when their effects were compared with the effects of other metal salts (34). Also, according to Hider (11), Cu^{2+} exhibits considerable affinity for synthetic hydroxamate and catechol ligands.

Fluorescence quenching due to Cu^{2+} was completely restored by adding EDTA to a final concentration of 0.1 mM. However, the level of Fe^{3+}-pyoverdine complex dissociation at the same EDTA concentration was relatively much lower (Fig. 4). Since the Fe^{3+}-EDTA stability constant ($\log K_s = 24.23$) is greater than the Cu^{2+}-EDTA stability constant ($\log K_s = 18.7$), we found no explanation for the difference between the ability of EDTA to dissociate Cu^{2+}-pyoverdine complexes and the ability of EDTA to dissociate Fe^{3+}-pyoverdine complexes.

In this study, pyoverdines were used successfully to continuously monitor association and dissociation of pyoverdines with Fe^{3+} or Cu^{2+} (Fig. 4). The results shown in Fig. 6A suggest that pyoverdine mediates iron transport to iron-starved *P. fluorescens* 2-79 cells, since an increase in fluorescence could only come from free pyoverdine. Whether pyoverdine was dissociated directly into the extracellular medium or in the periplasmic and/or cytoplasmic spaces is still not clear.

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