

## Fluorescent Pseudomonad Pyoverdines Bind and Oxidize Ferrous Ion

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Major pyoverdines from *Pseudomonas fluorescens* 2-79 (Pf-B), *P. aeruginosa* ATCC 15692 (Pa-C), and *P. putida* ATCC 12633 (Pp-C) were examined by absorption and fluorescence spectroscopic techniques to investigate the interaction between ferrous ion and the pyoverdine ligand. At physiological pH, ferrous ion quenched the fluorescence of all three pyoverdines much faster than ferric ion did. Also, increased absorbance at 460 nm was observed to be much faster for Fe<sup>2+</sup>-pyoverdine than for Fe<sup>3+</sup>-pyoverdine. At pH 7.4, about 90% of Fe<sup>3+</sup> was bound by pyoverdine Pa-C after 24 h whereas Fe<sup>2+</sup> was bound by the pyoverdine completely in only 5 min. The possibility that Fe<sup>2+</sup> underwent rapid autoxidation before being bound by pyoverdine was considered unlikely, since the Fe<sup>2+</sup> concentration in pyoverdine-free samples remained constant over a 3-min period at pH 7.4. Incubating excess Fe<sup>2+</sup> with pyoverdine in the presence of 8-hydroxyquinoline, an Fe<sup>3+</sup>-specific chelating agent, resulted in the formation of a Fe<sup>3+</sup>-hydroxyquinoline complex, suggesting that the iron in the Fe<sup>2+</sup>-pyoverdine complex existed in the oxidized form. These results strongly suggested that pyoverdines bind and oxidize the ferrous ion.

In the early history of the Earth, the appearance of photosynthesis and an oxidizing atmosphere caused the soluble ferrous ion to precipitate from solution (8). Due to the critical need for iron in aerobic metabolism and its tendency to form a highly insoluble ferric hydroxide, rendering it unavailable for transport in the ionic form, microorganisms have evolved special high-affinity systems for acquisition of the metal from the environment (8, 20). One of the systems involves low-molecular-mass secondary metabolites termed siderophores, which bind Fe<sup>3+</sup> with a high affinity and are excreted, usually in large amounts, when cells are grown under iron deficiency. Although this high-affinity iron uptake may vary among different microbial types, in gram-negative bacteria the general process involves an iron-regulated outer membrane protein which acts as a receptor that is able to recognize specifically the Fe<sup>3+</sup>-siderophore complex (19).

Three possible mechanisms have been proposed to explain the removal of iron from chelates with dissociation constants of the order 10<sup>-30</sup>: chelator hydrolysis (7), exchange with another chelator (18), and Fe<sup>3+</sup> reduction (7, 21). Based on qualitative observations (color changes) involving Fe<sup>2+</sup>-*o*-phenanthroline formation in a mixture containing reduced *Ustilago spargerana* siderophore, Neilands (17) concluded that the ferrous iron is bound only weakly, if at all, by siderophores and further pointed out that the extreme difference in the affinity of siderophores for ferrous and ferric ions offered a mechanism to remove Fe<sup>3+</sup> from siderophores. Subsequently, numerous laboratories have reported siderophore reductases in cell extracts of a variety of microorganisms (9–11). Similar reductase activities also have been found in cell extracts of *Pseudomonas fluorescens* (12), and *P. aeruginosa* (13).

The major exogenous siderophore of fluorescent pseudomonads is pyoverdine, a water-soluble yellow-green fluorescent peptide characteristically produced by iron-starved cells.

The binding of Fe<sup>3+</sup> by pyoverdine results in pyoverdine fluorescence quenching. In preliminary experiments, Xiao and Kisaalita (24) observed fast pyoverdine fluorescence quenching by Fe<sup>2+</sup>, indicating the possibility of high pyoverdine affinity for the ferrous ion and raising questions about Fe<sup>3+</sup> reduction as a possible mechanism of iron release from the iron-pyoverdine complex. The purpose of the present study was to use absorption and fluorescence spectroscopic techniques to investigate Fe<sup>2+</sup>-pyoverdine complex formation. We report evidence which strongly suggests that pyoverdines bind and oxidize ferrous ion. Implications regarding possible mechanisms of iron removal from iron-pyoverdine complexes by fluorescent pseudomonads are discussed.

### MATERIALS AND METHODS

**Strains and growth conditions.** *P. fluorescens* 2-79, *P. aeruginosa* ATCC 15692, and *P. putida* ATCC 12633 were grown on a synthetic succinate medium made up of 6.0 g of K<sub>2</sub>HPO<sub>4</sub>, 3.0 g of KH<sub>2</sub>PO<sub>4</sub>, 1.0 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1 g of MgSO<sub>4</sub> · 7H<sub>2</sub>O, and 4.0 g of succinic acid per liter and adjusted to pH 7.0 by adding the required volume of 1 N NaOH prior to sterilization (15). Precultures were prepared by inoculating 125-ml flasks (working volume, 25 ml) with strain 2-79, ATCC 15692, or ATCC 12633 from slants and incubating them overnight. A 2-ml volume of the preculture broth was used to inoculate 500-ml flasks (working volume, 100 ml). Incubation was carried out with shaking at 200 rpm in a New Brunswick Innova 4000 shaker/incubator at 25°C for strains 2-79 and ATCC 12633 and at 37°C for strain ATCC 15692. The incubation was terminated at the end of the log phase (determined by a decrease in optical density), and the fermentation broth was centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was further membrane filtered (pore size, 0.25 μm; Amicon) to yield a cell-free solution of crude pyoverdine.

**Isolation and purification of pyoverdines.** Pyoverdine isolation and purification were carried out as previously described (25). Briefly, the cell-free supernatant was mixed with 1 M HEPES buffer (pH 7.0) (49:1, vol/vol) and applied to a chelating Sepharose Fast Flow column (1.5 by 25 cm; Pharmacia LKB Biotechnology). This column was presaturated with CuSO<sub>4</sub> and equilibrated with 20 mM HEPES buffer (pH 7.0) containing 100 mM NaCl. The eluent flow rate was set at 100 ml/h. Fractions (10 ml) were collected, and the absorbance at 400 nm (A<sub>400</sub>) of each fraction was determined. The first two peaks of 2-79 crude pyoverdine were eluted with the same HEPES buffer. The third peak was eluted with 20 mM acetate buffer (pH 5.0) containing 100 mM NaCl. All fractions for each peak were separately pooled and lyophilized. The dried material for each peak was dissolved in small volumes (approximately 1 ml) of distilled water containing 10 mM EDTA before being applied to a Sephadex G-15 column (1.5 by 100 cm) that had been preequilibrated with deionized water. The separation was carried out at eluent (deionized water) flow rate of 20 ml/h and monitored

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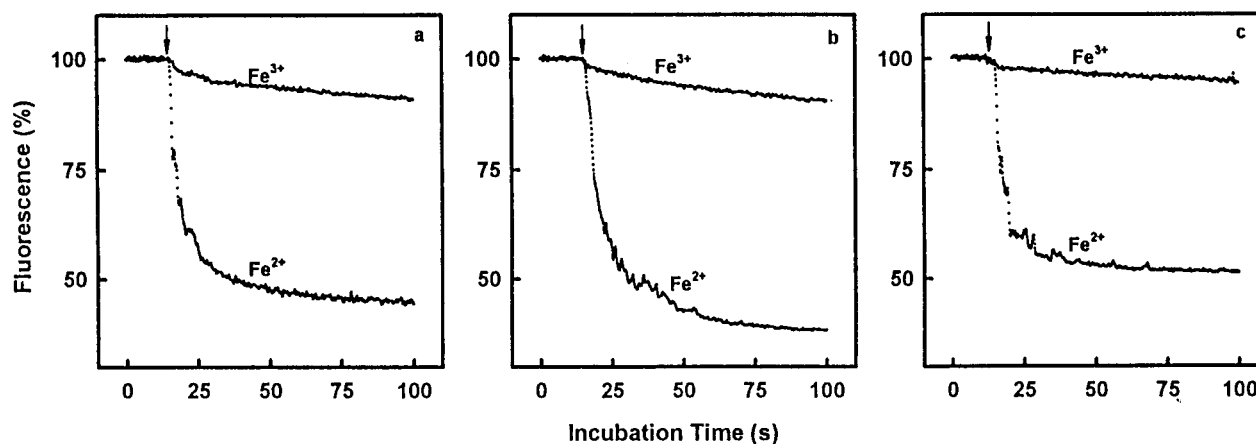


FIG. 1. Fluorescence quenching of pyoverdine Pf-B (a), Pa-C (b), and Pp-C (c) by  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  at physiological pH. Samples (3 ml) of pyoverdine solution (6.5  $\mu\text{M}$  Pf-B, 6.0  $\mu\text{M}$  Pa-C, and 6.0  $\mu\text{M}$  Pp-C) in 100 mM HEPES buffer (pH 7.4) were incubated at 25°C with stirring. A 10- $\mu\text{l}$  volume of  $\text{Fe}^{2+}$  or  $\text{Fe}^{3+}$  solution was added to the reaction mixture to a final concentration of 3.3  $\mu\text{M}$ . Fluorescence quenching was measured continuously at emission and excitation wavelengths of 460 and 400 nm, respectively.

by measuring the  $A_{400}$ . The ATCC 15692 and ATCC 12633 pyoverdines were purified in a similar manner, except that for the ATCC 15692 pyoverdine, acetate buffers (pH 6.0 and 5.0) were used to elute fractions B, C, and D. Purified pyoverdines from consecutive peaks were designated Pf-A, Pf-B, and Pf-C (2-79 strain); Pa-A, Pa-B, Pa-C, and Pa-D (ATCC 15692 strain); and Pp-A, Pp-B, and Pp-C (ATCC 12633 strain). The three main pyoverdines used in this study were Pf-B, Pa-C, and Pp-C. With the exception of Pf-B, the chemical structures of all the pyoverdines used have been previously published (5, 6).

**Determination of iron-pyoverdine complex formation.** (i) **Fluorimetric method.** A 3-ml volume of pyoverdine solution (6.0  $\mu\text{M}$  Pa-C, 6.5  $\mu\text{M}$  Pf-B, or 6.0  $\mu\text{M}$  Pp-C) in 100 mM HEPES buffer (pH 7.4) was incubated at 25°C with stirring. Then 10  $\mu\text{l}$  of  $\text{Fe}^{3+}$  or  $\text{Fe}^{2+}$  solution (freshly prepared with 20 mM HCl) was added to a final concentration of 3.3  $\mu\text{M}$ . Fluorescence quenching due to iron-pyoverdine complex formation was continuously monitored with a Perkin-Elmer fluorometer (LD 50) at excitation and emission wavelengths of 400 and 460 nm, respectively.

(ii) **Spectrophotometric method.** Iron-pyoverdine complex formation also was investigated by measuring absorbance changes. Pyoverdine Pa-C (30  $\mu\text{M}$ , 1.6 ml) in various buffers (100 mM acetate [pH 5.0 and 6.0] and HEPES [pH 7.4]) was incubated at 25°C with stirring. The reaction was initiated by adding 5  $\mu\text{l}$  of  $\text{Fe}^{3+}$  or  $\text{Fe}^{2+}$  solution to a final concentration of 20  $\mu\text{M}$ . The change in  $A_{460}$  due to pyoverdine-iron complex formation was measured with a Beckman DU 650 spectrophotometer continuously or at desired time intervals.

(iii) **Determination of ferrous ion concentrations.** The  $\text{Fe}^{2+}$  concentration was determined by the ferrozine method (2). The ferrozine reagent was obtained from Sigma. There was no statistically significant difference between iron concentration profiles (ferrozine method) in samples that were deoxygenated and continuously flushed with argon and those that were not. This showed that oxidation of  $\text{Fe}^{2+}$  under our experimental conditions was negligible.

(iv) **Investigation of  $\text{Fe}^{3+}$ -Pa-C complex formation.**  $\text{Fe}^{3+}$ -Pa-C complex formation at pH 7.4 was investigated by combining 5  $\mu\text{l}$  of  $\text{Fe}^{3+}$  (final concentration, 20  $\mu\text{M}$ ) with pyoverdine Pa-C (final concentration, 30  $\mu\text{M}$ ) in 100 mM HEPES buffer (pH 7.4 with or without 10 mM ascorbic acid). Ascorbic acid was added 30 s after the  $\text{Fe}^{3+}$  and pyoverdine were mixed. In both cases, changes in the  $A_{460}$  were measured continuously. The oxidation of  $\text{Fe}^{2+}$  was confirmed by using 100 mM HEPES buffer (pH 7.4) with or without 10 mM ferrozine. Ferrozine was added 30 s after the  $\text{Fe}^{2+}$  addition. Changes in the  $A_{562}$  were measured continuously. The effect of ascorbic acid on  $\text{Fe}^{3+}$  reduction in the absence of pyoverdine also was investigated with ferrozine in a similar manner.

**Determination of  $\text{Fe}^{2+}$  oxidation.** Addition of aqueous solution of ferric ion to 8-hydroxyquinoline produces a black complex with a maximal absorption at 600 nm. The specificity of this reaction was confirmed by adding 1 mM  $\text{Fe}^{2+}$  or  $\text{Fe}^{3+}$  (in 20 mM HCl) to a cuvette containing 1 mM 8-hydroxyquinoline in 100 mM acetate buffer (pH 4.0) to a final volume of 1 ml. After incubation for 10 min at room temperature, the  $A_{600}$  was measured. This reaction was used to determine the extent of  $\text{Fe}^{2+}$  oxidation to  $\text{Fe}^{3+}$  during the  $\text{Fe}^{2+}$ -pyoverdine reaction. A pyoverdine- $\text{Fe}^{2+}$  reaction mixture containing 0.5 mM  $\text{Fe}^{2+}$  and 50  $\mu\text{M}$  pyoverdine Pa-C in 100 mM acetate buffer (pH 4.0) was preincubated at room temperature for 20 min. Then 8-hydroxyquinoline was added to a final concentration of 1.0 mM, and the increase in the  $A_{600}$  was monitored continuously. One of the reactants ( $\text{Fe}^{2+}$  or pyoverdine) was omitted in each of the two controls included in each experiment.

## RESULTS

**Comparison of  $\text{Fe}^{2+}$ - and  $\text{Fe}^{3+}$ -pyoverdine complex formation.** Figure 1 shows fluorescence quenching of pyoverdine Pf-B, Pa-C, and Pp-C by  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  at physiological pH. As previously reported by Xiao and Kisaalita (24),  $\text{Fe}^{2+}$  quenched the pyoverdine fluorescence much faster than  $\text{Fe}^{3+}$  did in all cases. At 10 s after the  $\text{Fe}^{2+}$  addition, the fluorescence of pyoverdine Pf-B, Pa-C, and Pp-C was quenched to approximately 50% (Fig. 1). In comparison,  $\text{Fe}^{3+}$  quenched the fluorescence of the three pyoverdines to less than 10% of their maximum fluorescence after 100 s of incubation. It is well known that the formation of the  $\text{Fe}^{3+}$ -pyoverdine complex results in a shift in the maximum absorption of the free pyoverdine absorption spectrum as well as in the appearance of a pronounced shoulder at 460 nm (15). The increase in  $A_{460}$  was used to further confirm  $\text{Fe}^{2+}$ - and  $\text{Fe}^{3+}$ -pyoverdine complex formation. As shown in Fig. 2a, at physiological pH, the  $A_{460}$  increased rapidly after  $\text{Fe}^{2+}$  was added to the pyoverdine Pa-C solution and reached a steady state after only 6 min of incubation, indicating that  $\text{Fe}^{2+}$  was completely bound to pyoverdine Pa-C in a very short period. However, less than 10% of  $\text{Fe}^{3+}$  was bound by pyoverdine (Fig. 2b) at pH 7.4 after 10 min of incubation. When the incubation was carried out over 24 h, about 90% of  $\text{Fe}^{3+}$  was bound by pyoverdine (data not shown). Figure 2 also shows the effect of pH on the iron-pyoverdine complex formation rate. The  $\text{Fe}^{2+}$ -pyoverdine formation was pH independent, while the  $\text{Fe}^{3+}$ -pyoverdine formation was dramatically increased when the pH was decreased from 7.4 to 5.0.

The slower association between pyoverdine and  $\text{Fe}^{3+}$  at pH 7.4 was attributed to limited  $\text{Fe}^{3+}$  solubility. As shown in Fig. 3, addition of  $\text{Fe}^{3+}$  to a reaction mixture containing a reducing agent (ascorbic acid) resulted in a sudden increase in  $A_{460}$ , which was similar to that obtained with  $\text{Fe}^{2+}$ . When ascorbic acid was added to the same reaction mixture 30 s after  $\text{Fe}^{3+}$  addition, a meager change in  $A_{460}$  was observed in comparison to the control (no ascorbic acid). The effect of ascorbic acid addition on  $\text{Fe}^{3+}$  reduction in the absence of pyoverdine was further investigated by using ferrozine. A significant amount of  $\text{Fe}^{2+}$  was detected in the reaction mixture when  $\text{Fe}^{3+}$  was added to an ascorbic acid-containing solution (Fig. 4). A

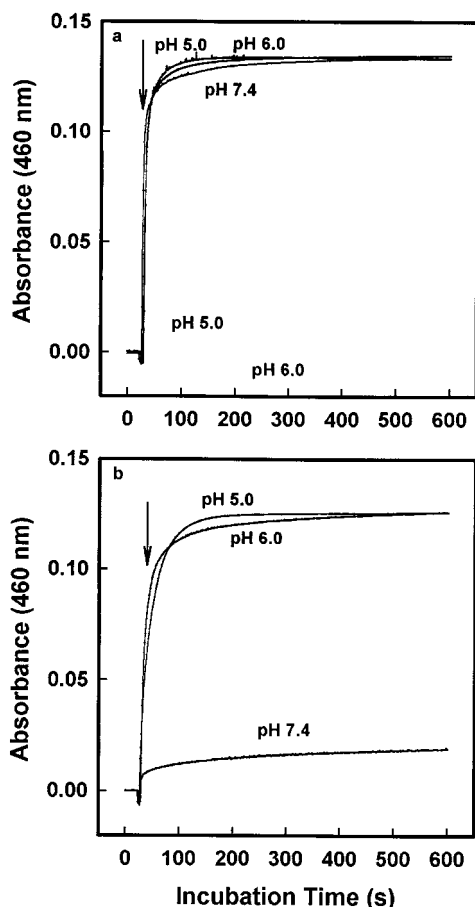


FIG. 2. Effect of pH on iron-pyoverdine Pa-C complex formation. Pyoverdine Pa-C (30  $\mu\text{M}$ , 1.6 ml) in various buffers (100 mM acetate buffer [pH 5.0 and 6.0] and HEPES buffer [pH 7.4]) was incubated at 25°C with stirring. The reaction was initiated by adding 5  $\mu\text{l}$  of  $\text{Fe}^{2+}$  (a) or  $\text{Fe}^{3+}$  (b) solution to a final concentration of 20  $\mu\text{M}$ . The change in  $A_{460}$  due to pyoverdine-iron complex formation was measured continuously.

smaller amount of  $\text{Fe}^{2+}$  was detected when ascorbic acid was added to an  $\text{Fe}^{3+}$ -containing reaction mixture.

**$\text{Fe}^{2+}$ -pyoverdine interactions.** The possibility that  $\text{Fe}^{2+}$  underwent rapid autoxidation before being bound by pyoverdine was investigated. As shown in Fig. 5, the profile of  $A_{562}$  plotted against time for pyoverdine-free samples was independent of the order of  $\text{Fe}^{2+}$  and ferrozine addition at pH 7.4, indicating that  $\text{Fe}^{2+}$  autoxidation was negligible under these experimental conditions.

To find out whether  $\text{Fe}^{2+}$  remains in its reduced state or is oxidized to  $\text{Fe}^{3+}$  after being bound by pyoverdine, two approaches were used. First, the rates of iron removal from  $\text{Fe}^{2+}$ - or  $\text{Fe}^{3+}$ -pyoverdine complexes by EDTA were measured. These removal rates were almost identical (Fig. 6), suggesting that either  $\text{Fe}^{2+}$  was oxidized on being bound by Pa-C or there was no difference in the iron dissociation rates between  $\text{Fe}^{2+}$ - and  $\text{Fe}^{3+}$ -pyoverdine complexes. Second, 8-hydroxyquinoline, a chelator that binds  $\text{Fe}^{3+}$  but has negligible  $\text{Fe}^{2+}$  affinity (Fig. 7), was used to confirm whether  $\text{Fe}^{3+}$  can be detected in iron-pyoverdine complexes formed in  $\text{Fe}^{2+}$ -pyoverdine samples. Figure 8 shows that there was no change in  $A_{600}$  when 8-hydroxyquinoline was reacted with pyoverdine Pa-C and a relatively small increase when 8-hydroxyquinoline was reacted with 0.5 mM  $\text{Fe}^{2+}$  (attributed to  $\text{Fe}^{3+}$  contamination, also seen

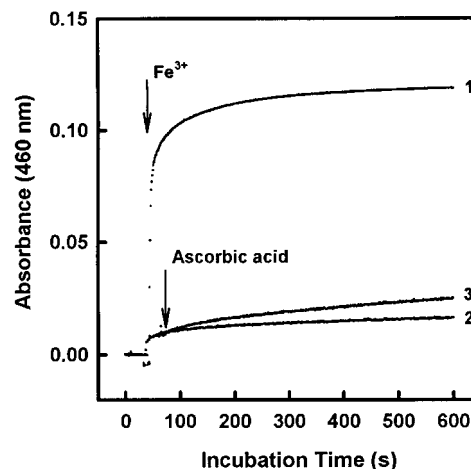


FIG. 3. Effect of ascorbic acid on the  $\text{Fe}^{3+}$ -pyoverdine reaction. Pyoverdine Pa-C (30  $\mu\text{M}$ ) in 100 mM HEPES buffer (pH 7.4) (1.6 ml) was incubated at 25°C with stirring. The change in  $A_{460}$  was measured continuously.  $\text{Fe}^{3+}$  (5  $\mu\text{l}$ ) was added to a final concentration of 20  $\mu\text{M}$  in the presence of 10 mM ascorbic acid (curve 1), in the absence of ascorbic acid (curve 2), or 30 s before addition of ascorbic acid (10 mM) (curve 3).

in the  $\text{Fe}^{2+}$  curve in Fig. 7). However, a relatively larger increase in the  $A_{600}$  was observed when 8-hydroxyquinoline was added to the  $\text{Fe}^{2+}$ -pyoverdine sample. The increase in  $A_{600}$  observed in  $\text{Fe}^{2+}$ -pyoverdine samples suggested that  $\text{Fe}^{2+}$  was oxidized after being bound by pyoverdine Pa-C.

## DISCUSSION

Two types of evidence support the conclusion that pyoverdines have affinity for  $\text{Fe}^{2+}$  as well as  $\text{Fe}^{3+}$ . First, incubation of  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  with the three pyoverdines at physiological pH resulted in faster fluorescence quenching by  $\text{Fe}^{2+}$  than by  $\text{Fe}^{3+}$  (Fig. 1). Second, a faster increase in  $A_{460}$  was observed when  $\text{Fe}^{2+}$  was added to the pyoverdine solutions than when  $\text{Fe}^{3+}$  was added (Fig. 2). Pyoverdines seem to be the most complex siderophores described to date. The chemical structure of the

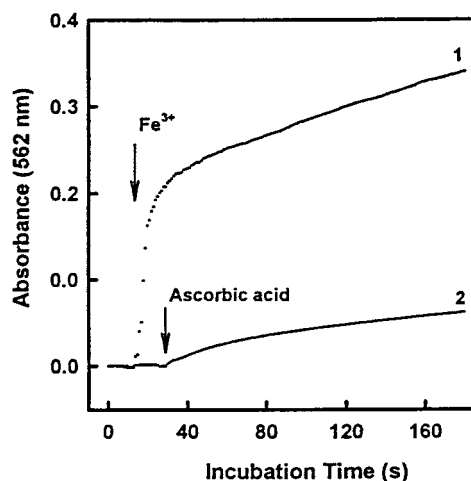


FIG. 4. Effect of ascorbic acid on  $\text{Fe}^{3+}$  reduction. Ferrozine (10 mM) in 100 mM HEPES buffer (pH 7.4) (1.6 ml) was incubated at 25°C with stirring. The  $A_{562}$  was continuously measured.  $\text{Fe}^{3+}$  was added to the reaction mixture to a final concentration of 20  $\mu\text{M}$ , either in the presence of 10 mM ascorbic acid (curve 1) or 30 s before ascorbic acid addition (curve 2).

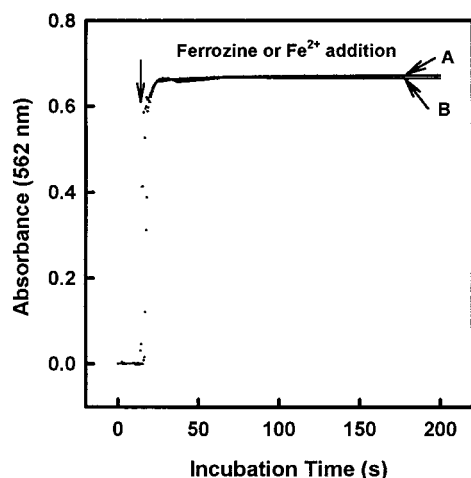


FIG. 5. Determination of  $\text{Fe}^{2+}$  by using ferrozine.  $\text{Fe}^{2+}$  ( $10 \mu\text{l}$ ) was added to a ferrozine solution ( $10 \text{ mM}$  in  $100 \text{ mM}$  HEPES buffer [ $\text{pH } 7.4$ ]) to a final concentration of  $25 \mu\text{M}$ , and the  $A_{562}$  was monitored continuously (curve A). The oxidation of  $\text{Fe}^{2+}$  was investigated by adding ferrozine to the  $100 \text{ mM}$  HEPES buffer ( $\text{pH } 7.4$ )  $30 \text{ s}$  after  $\text{Fe}^{2+}$  addition and monitoring the  $A_{562}$  (curve B).

main pyoverdine used in this study, Pa-C, was published by Demange et al. (4). Like most pyoverdines, Pa-C possesses the same type of chromophore, derived from 2,3-diamino-6,7-dihydroxyquinoline, linked to a small peptide which differs among strains by the number and composition of amino acids. The three bidentate chelating groups that bind  $\text{Fe}^{3+}$  are the catechol group of the chromophore, the hydroxamate group of  $N^6$ -hydroxyornithine, and either an  $\alpha$ -hydroxy acid of hydroxy-aspartic acid or the hydroxamate group of a second  $N^6$ -hydroxyornithine (4, 6, 22). Structurally, pyoverdines are intermediate between the strict hydroxamates and catechols found in the majority of microorganisms. Hider (14) showed that electrostatic interactions dominated the interactions between several divalent metal ions and two (catechol and hydroxamate) ligands. It is therefore possible that the  $\text{Fe}^{2+}$  complexation is coordinated by two of the three pyoverdine bidentate

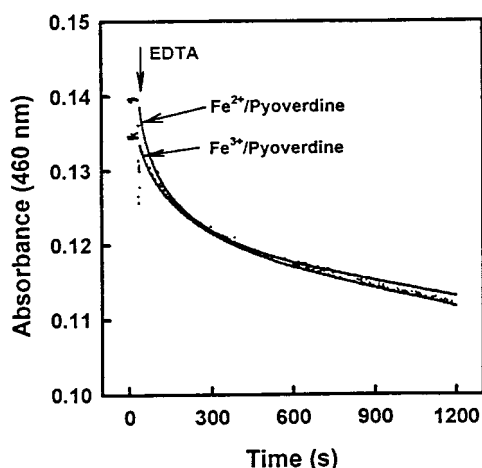


FIG. 6. Iron dissociation from iron-pyoverdine complexes by EDTA. EDTA was added to  $\text{Fe}^{2+}$ - or  $\text{Fe}^{3+}$ -pyoverdine solution ( $20 \mu\text{M}$  in  $100 \text{ mM}$  HEPES buffer [ $\text{pH } 7.4$ ]) to a final concentration of  $10 \text{ mM}$ . The decrease in  $A_{460}$  was monitored continuously.

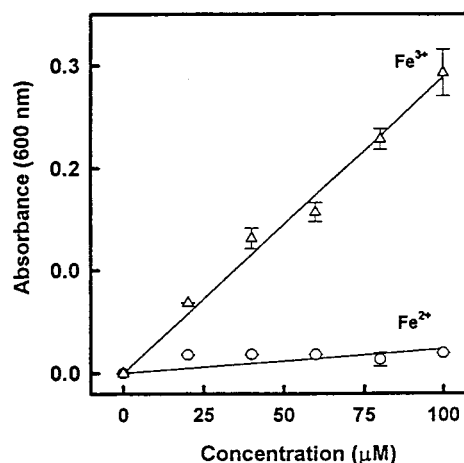


FIG. 7. Standard curve for determination of  $\text{Fe}^{3+}$  and  $\text{Fe}^{2+}$  by using 8-hydroxyquinoline. The experiments were carried out in triplicate, and error bars represent the standard error. The samples were prepared by adding  $1 \text{ mM}$   $\text{Fe}^{2+}$  or  $\text{Fe}^{3+}$  (in  $20 \text{ mM}$  HCl) to a cuvette containing  $1 \text{ mM}$  8-hydroxyquinoline in  $100 \text{ mM}$  acetate buffer ( $\text{pH } 4.0$ ) to a final volume of  $1 \text{ ml}$ . After incubation for  $10 \text{ min}$  at room temperature, the  $A_{600}$  was measured.

chelating groups. It should also be pointed out that other investigators have observed ion-pyoverdine complex formation with  $\text{Fe}^{2+}$  and other divalent transition metal ions such as  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Mo}^{2+}$ , and  $\text{Ni}^{2+}$  (16, 23).

Unlike  $\text{Fe}^{2+}$ -pyoverdine, the  $\text{Fe}^{3+}$ -pyoverdine reaction was pH dependent. The reaction rate increased with decreasing pH from  $7.0$  to  $5.0$  (Fig. 2b). This observation is consistent with the fact that simple ferric salts are hydrolyzed at neutral pH to rapidly form extremely insoluble  $\text{Fe}(\text{OH})_3$ . Since  $\text{Fe}(\text{OH})_3$  has a solubility product of  $4 \times 10^{-36}$ , any free  $\text{Fe}^{3+}$  in excess of  $2.5 \times 10^{-18} \text{ M}$  would be precipitated as the hydroxide (1, 20). The slow change in fluorescence quenching or absorption at  $460 \text{ nm}$  observed with  $\text{Fe}^{3+}$  in Fig. 1 and 2 was attributed to the low availability of free  $\text{Fe}^{3+}$ . Applying a reducing agent (ascorbic acid) before  $\text{Fe}^{3+}$  addition resulted in a significant rise in

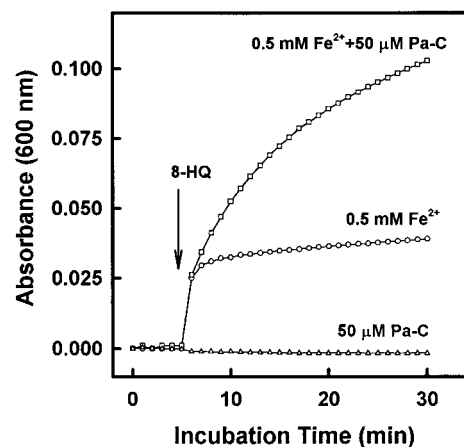


FIG. 8.  $\text{Fe}^{3+}$  dissociation from the  $\text{Fe}^{2+}$ -pyoverdine Pa-C mixture by 8-hydroxyquinoline (8-HQ). A reaction mixture containing  $\text{Fe}^{2+}$  ( $0.5 \text{ mM}$ ) and pyoverdine Pa-C ( $50 \mu\text{M}$ ) in  $100 \text{ mM}$  acetate buffer ( $\text{pH } 4.0$ ) was preincubated at room temperature for  $20 \text{ min}$ , and 8-hydroxyquinoline was then added to a final concentration of  $1 \text{ mM}$ . The increase in  $A_{600}$  was monitored continuously. Two control experiments, where either  $\text{Fe}^{2+}$  or pyoverdine Pa-C was omitted, were also performed.

absorption due to  $\text{Fe}^{3+}$  reduction to the more soluble  $\text{Fe}^{2+}$  (Fig. 3). Since  $\text{Fe}^{3+}$  was precipitated as  $\text{Fe}(\text{OH})_3$ , it was not surprising that the addition of ascorbic acid after  $\text{Fe}^{3+}$  application did not result in significant iron-pyoverdine complex formation.

The possibility that  $\text{Fe}^{2+}$  underwent autoxidation before reacting with pyoverdine was considered unlikely because the change in absorption was independent of the order in which  $\text{Fe}^{2+}$  and ferrozine were added (Fig. 5), suggesting that the  $\text{Fe}^{2+}$ -autoxidation reaction under the experimental conditions was insignificant. In addition, the higher pyoverdine binding rate exhibited with  $\text{Fe}^{2+}$  than with  $\text{Fe}^{3+}$  conclusively ruled out the possibility of  $\text{Fe}^{2+}$  autoxidation followed by  $\text{Fe}^{3+}$ -pyoverdine complex formation.

Given that EDTA has a higher association rate with  $\text{Fe}^{3+}$  ( $\log K_1 = 24.23$ ) than with  $\text{Fe}^{2+}$  ( $\log K_1 = 14.33$ ) (3), it was hypothesized that differences in EDTA-iron titration from  $\text{Fe}^{2+}$ - and  $\text{Fe}^{3+}$ -pyoverdine complexes would suggest that the iron in the iron-pyoverdine complex formed from the  $\text{Fe}^{2+}$ -pyoverdine reaction existed in its reduced form. However, identical EDTA-iron titration rates from iron-pyoverdine complexes formed from  $\text{Fe}^{2+}$ -pyoverdine and  $\text{Fe}^{3+}$ -pyoverdine reaction mixtures (Fig. 6) suggest that the iron in the complex formed from the  $\text{Fe}^{2+}$ -pyoverdine mixture existed as  $\text{Fe}^{3+}$ . This was further confirmed by the 8-hydroxyquinoline assay, which showed the presence of  $\text{Fe}^{3+}$  in this complex (Fig. 8).

The results reported in this study have important implications for possible mechanisms of iron removal from iron-pyoverdine complexes by fluorescent pseudomonads in natural environments. In view of the observed spontaneous complexation and oxidation of the ferrous ion by pyoverdine in this study, it can be suggested that successful release of ferric iron from the iron-pyoverdine complex by ferripyoverdine reductases, as previously reported (12), would require an  $\text{Fe}^{2+}$  chelator. Further, a strong case can be made that the iron exchange and reduction mechanisms in fluorescent pseudomonads may not be mutually exclusive.

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