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Ferripyoverdine-reductase activity in *Pseudomonas fluorescens*

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Summary. Enzymatic release of iron from ferripyoverdine through a reductive mechanism was demonstrated in cell-free extracts of *Pseudomonas fluorescens*. Ferripyoverdine reductase activity was localized primarily in the cytoplasm and/or periplasm and appeared not to be affected by the iron status of the cells. The reaction required a strict anaerobic environment and was fully inhibited by oxygen, whereas NADH was the most effective reductant. Ferripyoverdines from other bacterial sources (*P. aeruginosa* ATCC 15692, *P. fluorescens* ATCC 13525, *P. fluorescens* ATCC 17400) were able to serve as iron sources as well as ferric citrate. However, the activity with ferric citrate was not strongly affected by oxygen and did not display the characteristic lag phase observed with ferripyoverdines, suggesting the occurrence of a specific ferric citrate iron reductase. FMN should play a critical role in the reductive mechanism since it was absolutely required for the activity to occur with an intensively dialyzed cell-free extract, whereas it greatly stimulated (50-fold) the NADH-mediated activity of a crude extract.

Key words: Siderophore — Reductase — Iron — *Pseudomonas fluorescens*

Introduction

Under aerobic conditions, where iron is present as its highly insoluble ferric form (ferric hydroxides), bacteria and other microorganisms fulfill their iron requirement through iron(III) complexes of secondary metabolites termed 'siderophores', excreted usually in large amounts when cells are grown under iron deficiency. Translocation of iron through the cell membranes involves, for Gram-negative bacteria, an outer-membrane protein which acts as a receptor able to recognize specifically the iron(III)-siderophore complex (Neilands 1982). The extraordinarly high stability constant for iron of these complexes [10^{52} for ferrienterobactin (Harris et al. 1979), 10^{32} for pyoverdines (Meyer and Abdallah 1978)] stipulates, in order to allow the biosynthesis of the microbial metallo-compounds involving iron in their structures, the existence of an efficient mechanism of iron release from the cell-internalized ferrisiderophore. Since siderophores have usually no or only a very weak affinity for the ferrous ion, a reductive mechanism has been proposed (Neilands 1957) and effectively demonstrated through the description of several ferrisiderophore reductase activities detected in cell-free extracts of a variety of microorganisms (Brown and Ratledge 1975; Ernst and Winkelmann 1977; Straka and Emery 1979; Arceneaux and Byers 1980; Gaines et al. 1981; Lodge et al. 1982). Among the pyoverdine-producing pseudomonads, ferrisiderophore reductase activity has so far only been detected in the strain *Pseudomonas aeruginosa* ATCC 15692 (Cox 1980) and was restricted to the iron-pyochelin complex, thus related to the second siderophore, beside pyoverdine, known to be produced by this bacterium (Cox and Graham 1979) and some other pseudomonads (Sokol 1984).

A pyoverdine-related reductase activity in cell-free extract of *Pseudomonas fluorescens* is characterized in this paper and some of its properties are described and compared with the general features of other ferrisiderophore reductases.

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Materials and methods

Strains and growth conditions

Pseudomonas fluorescens W, a lab strain deposited in the Czechoslovak Collection of Microorganisms as *P. fluorescens* CCM 2799, was used throughout this work and grown in a synthetic medium (succinate medium) containing in 1 l distilled water: 6.0 g K_2HPO_4, 3.0 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 4.0 g succinic acid. The pH was adjusted to 7.0 by addition of saturated NaOH prior to sterilization by autoclaving (120° C, 30 min). Growth was performed at 25° C with vigorous shaking in 1-l conical flasks containing 500 ml medium. This medium, which allowed a maximal production of pyoverdine (the siderophore of this bacterium; Meyer and Hornsperger 1978), was considered as an iron-deficient growth medium (Meyer and Abdallah 1978). In order to obtain partially or fully iron-repleted cells, it was supplemented with a sterile 10 mM $FeCl_3$ solution just before inoculation, to reach final concentrations of 0.2 μM, 2 μM, and 20 μM in iron. The previous studies cited above demonstrated that cells grown in 0.2 μM iron-supplemented-media still produce pyoverdine and should be considered as iron-starved at the end of the exponential phase; 20 μM and even 2 μM iron supplementations led to cultures without production of pyoverdine and thus, fully iron-repleted cells were obtained. *P. aeruginosa* ATCC 15692 (PAO1 strain), *P. fluorescens* ATCC 13525, *P. fluorescens* ATCC 17400, were grown, in order to purify their respective pyoverdines, in iron-unsupplemented succinate medium under the same conditions as described for *P. fluorescens* W.

Preparation of extracts

P. fluorescens W cells grown in succinate medium were harvested by centrifugation (15 min, $20000 \times g$) when they had reached the end of the exponential phase (40 h of growth). After two washes with distilled water, the cells (20–30 g, wet mass) were resuspended in 25 ml 50 mM Tris/HCl pH 7.4 supplemented with 0.1 M KCl. Sonication was performed at 0° C by six 30-s pulses at 30-s intervals. The suspension was centrifuged 15 min at $20000 \times g$ to remove unbroken cells. The soluble cell-free extract usually used in the reductase assays was obtained as the supernatant of a $300000 \times g$ ultracentrifugation of the crude extract. For some experiments the soluble extract was dialyzed against 0.1 M sodium phosphate pH 7.4 during 48 h at 4° C and then dialyzed for 12 h against the 25 mM Tris/HCl pH 7.4 used for activity measurements. Protein content of the extracts was measured according to Lowry et al. (1951) using bovine serum albumin as a standard.

Preparation of substrates

Ferripyoverdines. The supernatant of a 40-h culture in succinate medium was supplemented with 4 mM $FeCl_3$ (final concentration) and the ferripyoverdine material, extracted by the chloroform/phenol method (Meyer and Abdallah 1978), was purified through a column (100×2.5 cm) of carboxymethyl-Sephadex (CM-Sephadex). The column was eluted with 0.1 M pyridine/acetic acid pH 6.5 for the elution of the ferripyoverdine of *P. fluorescens* W, and the same buffer but adjusted to pH 5.0 for the other ferripyoverdines (Demange et al. 1986). The ferripyoverdine solutions were quantified by a spectrophotometric method based on the well defined molar absorption coefficient of the *P. aeruginosa* ATCC 15692 ferripyoverdine which is 19000 $M^{-1} \cdot cm^{-1}$ at 403 nm (Demange et al. 1986). Solutions of ferripyoverdines for the reductase assays were made 5 mM in 25 mM Tris/HCl pH 7.4.

Other substrates. NADH, NADPH, FMN and FAD were from Boehringer (Mannheim, FRG) and prepared as 3.9 mM solutions for NADH and NADPH and 1.25 mM for FMN and FAD, all in 25 mM Tris/HCl pH 7.4. Ferrozine [3-(2-pyridyl)5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine], which reacts specifically with ferrous iron to give a purple-colored complex with a molar absorption coefficient of 28000 $M^{-1} \cdot cm^{-1}$ at 562 nm (Carter 1971), was from Sigma Chemical Co. and used as a 20.8 mM solution in the same buffer. Ferric citrate (2 mM) was prepared by mixing vigorously equimolar ferrous ammonium sulfate and sodium citrate, in 25 mM Tris/HCl pH 7.4, as described by Dailey and Lascelles (1977). The iron(III)-EDTA complex was prepared by mixing 10 mM EDTA (Titriplex III from Merck) and $FeCl_3$ solutions in 25 mM Tris/HCl pH 7.4.

Measurement of the reductase activity

The iron reductase activity in extracts of *P. fluorescens* was determined by a procedure previously described by Dailey and Lascelles (1977) based on the spectrophotometric measurement of the increase in absorbance at 562 nm of the assay, due to the formation of the ferrozine-iron(II) complex resulting from the reduction of the ferric ion provided by the ferriside-rophore. The standard assay mixture for a final volume of 1 ml in a spectrophotometric cuvette contained: 0.8 mM ferrozine, 0.2 mM ferripyoverdine, 0.15 mM NADH, 0.05 mM FMN in 25 mM Tris/HCl pH 7.4, and bacterial extract containing 0.2–0.8 mg protein. The assay cuvette was read against a reference cuvette containing all the constituents except the extract which was replaced by Tris buffer. Assays were usually started by adding the bacterial extract, and the variations in OD_{562} followed on a Pye-Unicam (Philips) spectrophotometer with a recorder at 0.1 or 0.5 full-scale in absorbance, over a period of 30 min. Unless otherwise stated, the assays were performed under strict anaerobic conditions by bubbling argon (Argon U from Air Liquide, France; <5 ppm O_2, 5 ppm H_2O as contaminants) through all the solutions, kept in serum-capped vials, for 10 min before use. Extracts were also maintained under argon by flushing the gas just at the surface of the preparations during 10 min before use. Injections of the different solutions in the assay cuvette, closed with a serum cap, were made as quickly as possible by using microsyringes (Hamilton), while a continuous argon flush (5 ml/min) was passed through the cuvette and maintained over the liquid level during all the time of the measurement. Controls for chemical reduction by NADH of all the iron compounds used in this study were followed in a similar way but without extract and with NADH omitted in the reference cuvette. The reaction was initiated by the addition of NADH in the assay cuvette. Except for $FeCl_3$, the non-enzymatic reduction process was negligible or nonexistent with assays conducted at 25° C. This temperature was chosen for all the assays by using a thermostatted jacket adapted to the spectrophotometric cuvettes. Activity was expressed as the rate of change of absorbance at 562 nm measured during the linear increasing phase following the lag phase ($\Delta OD_{562} \cdot min^{-1}$). Specific activity was expressed as rate of formation of ferroferrozine per mass of protein of the bacterial extract introduced in the spectrophotometric assay ($nmol \cdot min^{-1} \cdot mg^{-1}$).

Results

Ferripyoverdine reductase activity in cell-free extracts of Pseudomonas fluorescens

The formation of ferrozine-iron(II) complex during a spectrophotometric assay performed under strict anaerobic conditions and containing NADH (0.15 mM), ferrozine (0.8 mM), ferripyoverdine (0.2 mM), and cell-free soluble extract of *P. fluorescens* W grown in succinate medium (0.44 mg protein), resulted in curve (a) of Fig. 1. The addition of the bacterial extract, added at zero time to the assay cuvette, was followed by a 12-min period without any change in the absorbance at 562 nm of the assay (lag phase). Then, a regular increase of OD_{562} occurred, which was linear during the next 10 min. The same assay, but conducted in aerobic conditions, revealed no change in OD_{562} as shown in Fig. 1, curve b, which was unchanged even after a 1-h course. The same result was observed when cell-free extract was omitted in the assay (curve c, Fig. 1), conducted with or without air, demonstrating that reduction of iron was not due to a chemical, but to an enzymatic process. Supplementation of FMN (0.05 mM) to the assay mixture before starting the reaction resulted in a shorter lag phase (6 min instead of 12 min) followed by a much faster increase in OD_{562} (curve d, Fig. 1). However, in the presence of air, the addition of FMN did not change the profile described in Fig. 1, curve b, and no activity at all was visible even after a 1-h assay. Moreover, injection of 0.5 ml air in a standard, FMN-added, anaerobic assay during the phase of increase in OD_{562} (arrow, curve d, Fig. 1), had a drastic effect by preventing completely the increase in OD_{562} (dashed line, Fig. 1).

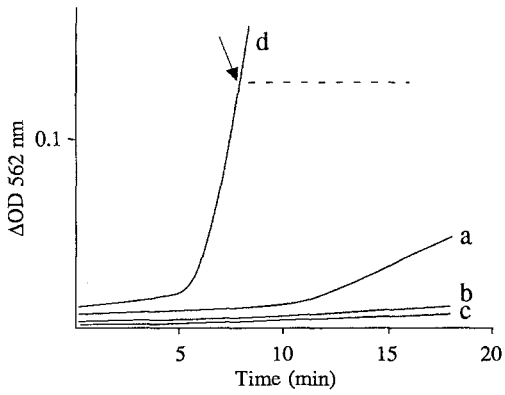

Fig. 1. Ferripyoverdine reductase activities in cell-free extract of *Pseudomonas fluorescens* W. Assays were as described in Materials and methods with a protein content per assay of 0.44 mg. Curve (a) corresponded to an assay conducted in anaerobic conditions without FMN; curve (b) was the same as for curve (a), but under aerobic condition; curve (d) was the same as for curve (a), but with supplementation of FMN (0.05 mM) in the assay; curve (c) represented the chemical reduction without extract (see Materials and methods). The dashed line corresponded to the activity following the injection of 0.5 ml air to the assay (arrow, curve d)

All the experiments described above were performed with a soluble, cell-free extract, i.e. the supernatant of a high-speed centrifugation ($300\,000 \times g$) of the sonicated cells obtained from an iron-deficient medium (succinate medium). Ferripyoverdine reductase activities from extracts of cells grown in succinate media supplemented with 0.2 μM, 2 μM or 20 μM iron, were not significantly different when compared to the activity of iron-starved cells. The specific activities of such extracts were 11, 10 and 12.5 $nmol \cdot min^{-1} \cdot mg^{-1}$ respectively, whereas the standard assay as described in Fig. 1, curve d, resulted in a specific activity of 12 $nmol \cdot min^{-1} \cdot mg^{-1}$.

Omission of the high-speed centrifugation during the preparation of the bacterial extracts did not change the reductase activity (data not shown), suggesting that the corresponding enzyme was localized inside the cells in the cytoplasm and/or in the periplasm. Moreover, the activity found in the high-speed centrifugation pellets when resuspended and homogenized in Tris buffer was found to be insignificant. Depending on the assays, between 2%–4% of the total activity was found for the uncentrifuged extract, and decreased to values not detectable in the standard conditions after one or two washes of the membrane material.

Measuring the ΔOD_{562} during the linearly increasing portion of the curves (a) and (d) in Fig. 1 led to the activities expressed in Table 1. It can be shown that the addition of FMN to the assay resulted in a 46-fold increase of the reductase activ-

Table 1. Effect of reductants in combination with FMN or FAD on ferripyoverdine reductase activity of *P. fluorescens*

Reductant	Enzyme activity[a]
None	n.d.
NADH 0.15 mM	1.3
NADH 0.15 mM, FMN 0.05 mM	60
NADH 0.15 mM, FAD 0.05 mM	53
NADPH 0.15 mM	n.d.
NADPH 0.15 mM, FMN 0.05 mM	36
NADPH 0.15 mM, FAD 0.05 mM	25

[a] Expressed in $\Delta OD_{562} \cdot min^{-1}$. Assays were performed as described in Materials and methods; n.d. = not detectable

ity compared to the assay with NADH alone. These differences in activity, resulting from the supplementation of the assay with FMN, were even greater if the cell-free extract was first dialyzed for 48 h against phosphate buffer and then for 12 h against the Tris buffer used for the assays (see Materials and methods). After these treatments no activity was detectable in an assay with NADH alone, even after a 30-min time course. Addition of FMN (0.05 mM) at that time restored the activity and resulted, after a 7-min lag phase, in a linear progression of OD_{562}, identical to that represented in Fig. 1, curve d.

Cofactors and determination of the standard assay conditions

Table 1 also gives the reductase activities obtained with assays where the different combinations between NADH, NADPH, and FMN, FAD, were analyzed. The concentration of the other constituents (ferrozine, ferripyoverdine, bacterial extract) remained unchanged during these assays. When NADPH was used as the reductant instead of NADH, no activity at all was detectable in the soluble cell-free extract of iron-starved cells, unless FMN was present in the assay. The resulting activity was decreased by a factor of 2 compared to the NADH plus FMN assay. Replacement of FMN by FAD at the same molar concentration resulted in a slightly lower value of activity, whatever the reductant source. Measurements in NADH-mediated assays of the activity as a function of FMN or FAD concentrations are shown in Fig. 2. The K_m value for FMN was found to be 8.9 μM with a V_{max} of 0.065 $\Delta OD_{562} \cdot min^{-1}$, whereas FAD was characterized by a K_m value of 25.4 μM (V_{max}: 0.049 $\Delta OD_{562} \cdot min^{-1}$). Thus, the chosen standard assay conditions were 0.05 mM FMN, 0.15 mM NADH, 0.2 mM ferripyoverdine and 0.8 mM ferrozine, since it was verified that at the concentrations used and for a quantity of 0.2–0.8 mg extract protein in the assay, NADH, ferripyoverdine and ferrozine were saturating (data not shown). The effect of pH on the activity was determined between pH 6.8–8.0. A maximal activity was observed at pH 7.4, the activities at pH 6.8 and pH 8.0 representing 79% and 36% respectively of the maximal activity.

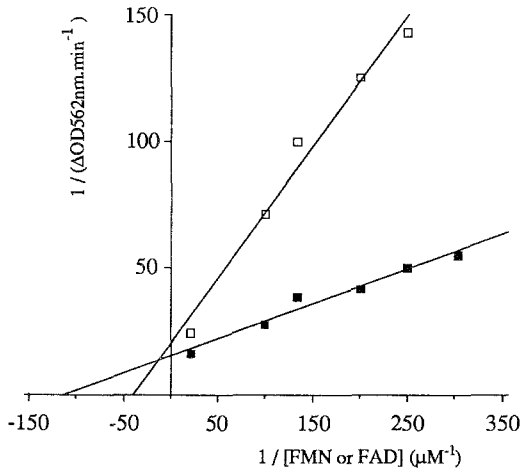

Fig. 2. Lineweaver-Burk plot of FMN (■) or FAD (□) concentration and ferripyoverdine reductase activity. Assays conditions were as described in the text with a cell-free extract containing 0.44 mg protein

Iron sources

Reductase activity of the *P. fluorescens* cell-free extract was tested when replacing ferripyoverdine by $FeCl_3$, iron citrate, iron-EDTA, or iron complexed to pyoverdines from different origins (pyoverdines from *P. aeruginosa* ATCC 15692, *P. fluorescens* ATCC 13525, *P. fluorescens* ATCC 17400). All substrates were tested before measuring the reductase activities for their potential chemical reduction by NADH. Among the iron compounds used, only $FeCl_3$ was subject to a marked chemical reduction. In the standard assay in presence of the enzyme, the very small increase or decrease in OD_{562} which was observed when $FeCl_3$ was the iron source (± 0.006 in ΔOD_{562}) was thought more likely to be due to a difference in the chemical reduction rate within the two cuvettes, rather than to an enzymatic process. When the iron source was the EDTA-Fe(III) complex almost no chemical reduction occurred, whereas the enzymatic assay revealed a slight reductase activity (Fig. 3, curves a and a′ respectively). The citrate-Fe(III) was subject to a pronounced enzymatic reduction (Fig. 3, curve b′), whereas chemical reduction was not observed (Fig. 3, curve b). Compared to the activity obtained with ferripyoverdine (Fig. 1), the main difference was the absence of the lag phase. The increase in OD_{562} started immediately after initiating the reaction with the cell extract. Another difference appeared when the citrate-mediated assay was performed under aerobic conditions: in that case the presence of oxygen did not completely inhibit the reductase activity but slightly affected the rate of reduction (curve c, Fig. 3). The iron complexes of pyoverdines isolated from *P. aeruginosa* ATCC

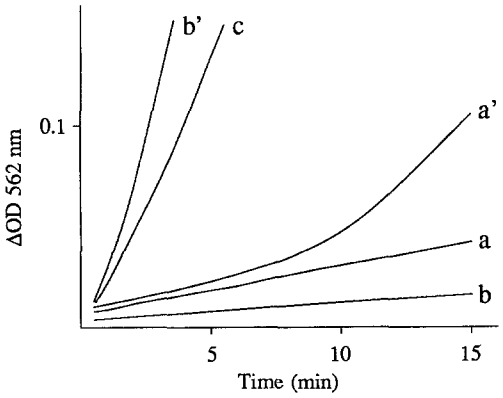

Fig. 3. Iron reductase activities with EDTA-Fe(III) and ferric citrate as iron sources. Assays for detection of chemical (curves a and b) or enzymatic (curves a′ and b′) activities with EDTA-Fe(III) (curves a and a′) or ferric citrate (curves b and b′) as iron sources were performed as described in the text. Curve (c) resulted from an enzymatic assay with ferric citrate as iron source conducted under aerobic conditions. Enzymatic assays were performed with an extract containing 0.8 mg protein

15692, *P. fluorescens* ATCC 13525 and *P. fluorescens* ATCC 17400, when tested with the *P. fluorescens* W extracts, led to results very similar to those obtained with the ferripyoverdine from *P. fluorescens* W. A lag phase was always observed, FMN stimulated greatly the activities, and no reaction occurred in aerobic conditions (data not shown). However, saturation kinetic studies with the different ferripyoverdines revealed some differences between these compounds, as shown by the Lineweaver-Burk diagrams represented in Fig. 4. The

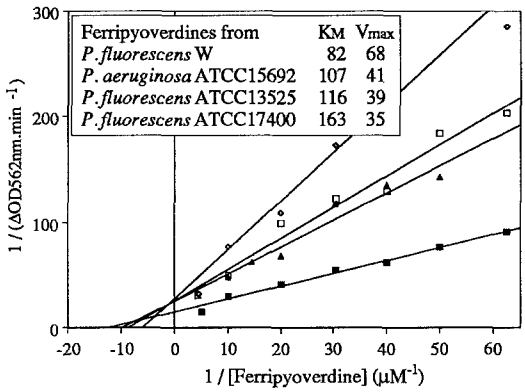

Fig. 4. Lineweaver-Burk diagrams and kinetic parameters (insert) of ferripyoverdine reductase activity expressed with ferrisiderophores of different bacterial sources. (■) Ferripyoverdine from *P. fluorescens* W; (▲) ferripyoverdine from *P. aeruginosa* ATCC 15692; (□) ferripyoverdine from *P. fluorescens* ATCC 13525; (◇) ferripyoverdine from *P. fluorescens* ATCC 17400. The values of V_{max} in the insert are expressed as $\Delta OD_{562} \cdot 10^{-3} \cdot min^{-1}$

values of K_m and V_{max} which resulted are compared in the insert of Fig. 4.

Discussion

As has been shown in these studies, the ferripyoverdine reductase activity found in cell-free extracts of *P. fluorescens* is characterized by some properties which have already been recognized for other ferrisiderophore reductases of various microorganisms. The activity was detected whatever the iron concentration in the growth medium and, thus, appeared to be constitutive, a feature common to all bacteria or fungi so far analyzed with the exception, however, of *Neurospora crassa* where some induction of sideramine reductase activity occurred under iron deficiency (Ernst and Winkelmann 1977). Another similarity concerns the intracellular localization of the *P. fluorescens* enzyme, which was found to be cytoplasmic or periplasmic or both, since all the activity detected in crude extracts was still present in the supernatant of a $300000 \times g$ centrifugation. Conversely, the membrane fraction revealed no significant activity. Ferrisiderophore reductases of *Bacillus megaterium* (Arceneaux and Byers 1980), *Bacillus subtilis* (Gaines et al. 1981), and *Agrobacterium tumefaciens* (Lodge et al. 1982), together with the ferric citrate reductase of *Rhodopseudomonas sphaeroides* (Moody and Dailey 1985), have also been recognized as soluble enzymes, located in the cytoplasm. However, the ferripyochelin reductase of *Pseudomonas aeruginosa* PAO1 appeared to be distributed in the cytoplasm as well as in the periplasm, the ferric citrate reductase being localized specifically in the cytoplasm (Cox 1980). In *Mycobacterium smegmatis* the reductase has been described as a soluble enzyme although the siderophore (mycobactin) is localized in the membranes (McCready and Ratledge 1979).

The lack of specificity for the iron source appeared to be another feature of the ferripyoverdine reductase enzyme of *P. fluorescens*, which fits with the general rule usually recognized among different microorganisms so far tested (Emery 1987). Although some differences appeared in K_m and V_{max} values when the endogenous ferripyoverdine was replaced by similar compounds produced by other strains, a far from negligible activity was always found with these compounds. It is now well established that the pyoverdines produced by different strains of fluorescent pseudomonads usually differ in their structure. The chromophore part, constituted by a 2,3-diamino-6,7-

dihydroxyquinoline derivative, appears to be identical among all the pyoverdines so far identified, but differences have been found at the level of the peptide moiety, especially concerning the amino acids not involved in iron complexation. These few differences in structure resulted, however, in a strong effect on the pyoverdine-mediated iron uptake, indicating a strict strain specificity at the level of the iron translocation through the bacterial membranes (Hohnadel and Meyer 1988). As shown in the present investigation, such a strict specificity was not observed for ferripyoverdine reductase. More accuracy, especially in the determination of the molar concentrations of pyoverdine and ferripyoverdine solutions, is needed before deciding whether the differences in affinity and velocity found with the different pyoverdines used in this study are significant.

The absence of specificity of the ferripyoverdine reductase was apparently confirmed by the results obtained with ferric citrate as the iron(III) source. However, this activity was, in many aspects, very different from the standard assay with ferripyoverdines. The outstanding feature of the ferric-citrate-mediated activity was indeed that it worked in the presence of oxygen, whereas the ferripyoverdine-mediated assay needed a strict anaerobic environment. Moreover, no lag phase was observed in the ferric citrate assay. Such differences suggested that the ferric-citrate-mediated reductase activity was due to another enzymatic system and not to ferripyoverdine reductase itself. The purification of the reductase is needed to clarify this point, already supported by the results of Cox (1980) who found in *P. aeruginosa* two different reductases, one specific for ferripyochelin and the other for ferric citrate.

To explain the inhibitory effect of oxygen on ferrisiderophore reductase activity, Straka and Emery (1979) suggested the possibility of a partial reoxidation by oxygen of the native ferrous iron before being complexed by ferrozine. However, this hypothesis does not fit very well with our results when one consider that ferric citrate reductase and ferripyoverdine reductase display such marked differences in the presence of oxygen while being measured by the same ferrozine assay. A relationship between the strong inhibitory effect of oxygen and the strong stimulation effect of FMN is probable but needs to be elaborated in detail for a better understanding of the reductive mechanism of ferrisiderophore.

FMN has been shown to stimulate actively the enzymatic reduction by NADH of some ferrisiderophores. This was shown for the *Bacillus subtilis* enzyme (Gaines et al. 1981) and for the ferric citrate reductase described by Moody and Dailey (1983) in *Rhodopseudomonas sphaeroides*. Usually, the stimulation was seen to be 2–4-fold. It has to be emphasized that, with the *P. fluorescens* soluble extract, the stimulation factor of FMN was much greater since it was almost 50-fold. Moreover, when using an intensively dialyzed extract, no activity at all occurred with NADH alone, unless FMN was added. The NADPH-mediated ferriagrobactin reductase of *Agrobacterium tumefaciens* was shown to possess a similar absolute requirement for FMN (Lodge et al. 1982). It may be that FMN not only stimulates, but is in fact absolutely necessary for, the ferrisiderophore reductases. More experiments are needed to confirm this view. A purification of the ferripyoverdine reductase is presently in progress in our laboratory.

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