Journal of General Microbiology (1978), **107**, 319–328. *Printed in Great Britain*

The Fluorescent Pigment of *Pseudomonas fluorescens*: Biosynthesis, Purification and Physicochemical Properties

By J. M. MEYER

Laboratoire de Biochimie Microbienne, 4 *rue Blaise Pascal*, *Université Louis Pasteur*, 67070 *Strasbourg Cedex*, *France*

AND M. A. ABDALLAH

Laboratoire no. 31 *associé au CNRS*, *Institut de Chimie*, 1 *rue Blaise Pascal*, *Université Louis Pasteur*, 67008 *Strasbourg Cedex*, *France*

(*Received* 7 *February* 1978; *revised* 4 *April* 1978)

The biosynthesis of a yellow–green, fluorescent, water-soluble pigment by *Pseudomonas fluorescens* occurred only when the bacteria were iron-deficient and was not directly influenced by the nature of the organic carbon source. The pigment formed a very stable Fe^{3+} complex and was purified in this form. *Pseudomonas fluorescens* produced only one molecular species of fluorescent pigment; however, its lability under mild alkaline conditions led to the formation of several pigmented decomposition products. The spectral properties of the pure pigment, its molecular weight (1500 ± 75) and its stability constant for Fe^{3+} (of the order of 10^{32}) were determined. Both its biosynthesis and its chemical properties (formation of a stable Fe^{3+} complex) suggest that the fluorescent pigment is a desferrisiderophore.

INTRODUCTION

The synthesis under certain growth conditions of yellow–green, fluorescent, water-soluble pigments is a characteristic property of some *Pseudomonas* spp. (Stanier *et al.*, 1966). These species are all members of the same intra-generic genetic homology group (Palleroni *et al.*, 1973). They include *P. aeruginosa*, *P. putida*, *P. fluorescens* and phytopathogens of the *P. syringae* type (Palleroni & Doudoroff, 1974).

Many different environmental factors affect the synthesis of these pigments, notably the chemical nature of the organic carbon and energy source (Lepierre, 1895; Sullivan, 1905; Blanchetière, 1920; Giral, 1936; Gouda & Greppin, 1965; Gouda & Chodat, 1963), the degree of aeration of the culture medium (Elliot, 1958; Lenhoff, 1963), pH and light (Greppin & Gouda, 1965) and the cations Mg^{2+} (Georgia & Poe, 1931), Zn^{2+} (Baghdiantz, 1952; Chakrabarty & Roy, 1964*a*) and Fe^{3+} (King *et al.*, 1948; Totter & Moseley, 1953; Lenhoff, 1963; Palumbo, 1972; Lluch *et al.*, 1973). No clear-cut physiological role has so far been assigned to this class of pigments. Various structures have been proposed for the pigments including pteridines (Giral, 1936; Chakrabarty & Roy, 1964*b*), flavines (Birkhoffer & Birkhoffer, 1948) and pyrroles (Lenhoff, 1963; Greppin & Gouda, 1965), but none is supported by unequivocal chemical evidence.

The observation that the fluorescent pigment synthesized by a strain of *P. fluorescens* can form a stable complex with Fe^{3+} has led us to re-examine the conditions that govern its formation, its chemical structure and its physiological role. We describe here the factors that affect pigment synthesis, the purification of the pigment and its physicochemical properties.

Terminology. Turfreijer (1942) proposed the term 'pyoverdine' for the yellow–green,

fluorescent, water-soluble pigment of *P. fluorescens*: he chose this name by analogy with that of the phenazine pigment, pyocyanine, produced by *P. aeruginosa*. This designation is preferable to those of 'bacterial fluorescein' or 'fluorescin' which have also sometimes been used in the literature (King *et al.*, 1948; Lenhoff, 1963). Other authors (Elliot, 1958; Hulcher, 1968) have extended the name of pyoverdine to include all pigments produced by fluorescent pseudomonads. However, since preliminary unpublished studies have shown that there are minor structural differences between the pigments of the different species of fluorescent pseudomonads, it seems desirable to designate the pigments of this class by a suffix indicating the species responsible for their production, e.g. pyoverdine$_{Pf}$ for the pyoverdine produced by *P. fluorescens*.

METHODS

Organism. The strain of *P. fluorescens* employed was isolated by Wurtz (1954) and is held in the Czechoslovak Collection of Microorganisms as CCM 2799. In terms of the taxonomic criteria proposed by Stanier *et al.* (1966), it is *P. fluorescens* biotype B.

Media and growth conditions. The standard succinate medium used for most experiments contained (g l^{-1} in distilled water): K_2HPO_4, 6·0; KH_2PO_4, 3·0; $(NH_4)_2SO_4$, 1·0; $MgSO_4.7H_2O$, 0·2; succinic acid, 4·0. The pH was adjusted to 7·0 by addition of NaOH prior to sterilization. For some experiments, citric acid (4·0 g l^{-1}) was used in place of succinic acid.

The standard medium described above contains no added source of iron. In some experiments, it was supplemented with $FeCl_3$ (1 mg Fe^{3+} l^{-1}, unless otherwise stated). In other experiments, traces of iron were removed from the standard medium by complexing with 8-hydroxyquinoline (Waring & Werkman, 1942) or by bathophenanthroline (Theodore & Schade, 1965), followed by chloroform extraction of the iron complex.

Cultures were grown at 25 °C in 1 litre Erlenmeyer flasks containing 500 ml medium and subject to mechanical agitation.

Measurement of growth and pigment synthesis. Bacterial growth was estimated turbidimetrically at 600 nm; from these measurements cell dry weights were determined using an appropriate calibration curve. To determine the concentration of fluorescent pigment in culture media, bacteria were removed by centrifugation and the absorbance of the supernatant liquid was measured at 400 nm; measurements were converted to a weight basis using the known absorption coefficient.

Isolation and purification of the fluorescent pigment. Bacteria were grown for 40 h at 25 °C in standard succinate medium, the pH being maintained in the range of 7·0 to 7·3 by periodic addition of HCl. At the time of harvesting, batches were pooled to a volume of 20 l; $FeCl_3$ (200 mg Fe^{3+} l^{-1}) was then added to the culture, which was centrifuged, the pelleted cells being discarded. The supernatant was concentrated to approximately 1 litre under reduced pressure, saturated with NaCl, and extracted with 0·5 vol. $CHCl_3$/phenol (1:1, v/w). The aqueous phase was discarded, and the organic phase was treated with 2 vol. diethylether and 100 ml distilled water. The aqueous phase, containing the pigment–iron complex, was re-extracted three times with ether in order to remove phenol completely, and then evaporated under reduced pressure. This solution (20 ml) was applied to a column of CM-Sephadex C25 (2·5 × 90 cm) eluted with 0·1 M-pyridine/acetic acid buffer (pH 6·5). A front-running minor fraction, generally representing less than 10% of the total, was shown by electrophoresis to contain a mixture of pigmented degradation products. The major fraction consisted of the native Fe(III)–pigment complex. Repetition of the CM-Sephadex C25 step showed this fraction to be 99% pure, in agreement with the results of electrophoretic analysis. The yield (after lyophilization) was about 130 mg per litre of the initial culture medium.

Preparation of the iron-free pigment. The Fe(III)–pigment complex (500 mg) was suspended in 50 ml distilled water, and the pH was adjusted to 4·0 with 10% (v/v) aqueous acetic acid. A solution (5%, w/v) of 8-hydroxyquinoline in chloroform (150 ml) was added, and the mixture was stirred vigorously in a stoppered flask. The pH of the aqueous phase was re-adjusted to 4·0, and this phase was re-extracted four times with 150 ml of 8-hydroxyquinoline/chloroform solution. The aqueous phase was washed with chloroform to remove 8-hydroxyquinoline, concentrated to 20 ml and applied to a column of Sephadex G25 (2·5 × 90 cm), from which it was eluted with distilled water as a single peak. The yield was 450 mg of pure iron-free pigment. This treatment was performed as rapidly as possible to minimize decomposition of the pigment.

Electrophoretic analysis. Electrophoresis of the pigment (300 V, 30 min) was done in cellulose acetate sheets using two buffer systems: 0·1 M-pyridine/acetic acid (pH 5) and sodium veronal (pH 10). Spots were visualized with ultraviolet light from a lamp having a major emission at 350 nm.

Tonometry. Measurements were made with a vapour pressure osmometer model 302 (Mechrolab, Moun-

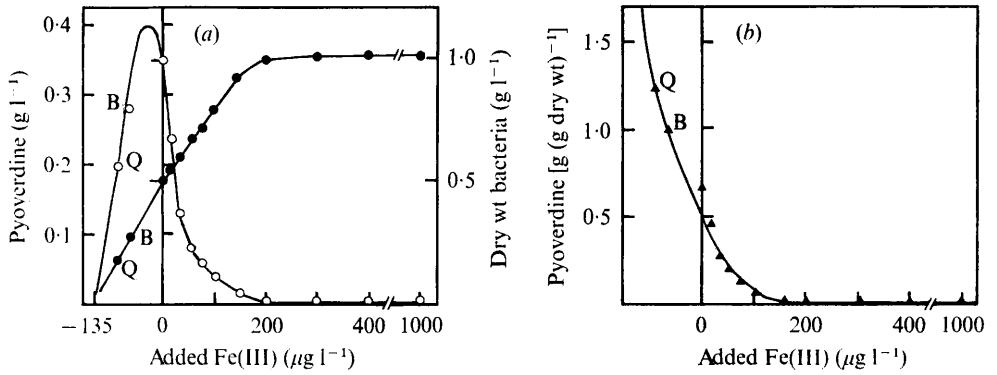

Fig. 1. Growth and pigmentation of *P. fluorescens* as a function of Fe(III) present in the succinate medium. (*a*) Growth (●) and pigmentation (○) were determined as described in Methods. Points B and Q correspond to the standard media pretreated with bathophenanthroline (B) or 8-hydroxyquinoline (Q) (see Methods). Negative values on the abscissa correspond to the amount of Fe(III) present in the media as a contaminant. (*b*) Amounts of pyoverdine$_{Pf}$ (g) synthesized from 1 g dry wt bacteria (derived from Fig. 1*a*).

tain View, California, U.S.A.), using a Hewlett-Packard probe for aqueous solutions at 37 °C, with streptomycin sulphate as a standard. Aqueous solutions containing 15 to 60 mg pigment ml^{-1} were examined.

Spectra. Absorption spectra in the visible and ultraviolet regions were determined with a Leres Spila 50 or a Cary 118 (Varian) spectrophotometer. Fluorescence spectra were determined with a Zeiss ZFM4C instrument.

Stoicheiometry of the Fe(III)–pigment complex. A cuvette (1 cm light path) was supplied with 0·15 ml of an aqueous solution (0·65 mM) of the iron-free pigment and 2·85 ml of 0·1 M-acetate buffer (pH 5·2). Successive additions (5 μl) of a freshly prepared aqueous solution (3·25 mM) of $FeCl_3$ were then made, and the absorption spectrum of the mixture was measured after each addition.

Determination of the apparent stability constant of the Fe(III)–pigment complex. Solutions containing 50 μM Fe(III)–pigment complex and 15 mM-EDTA were prepared in a series of buffers (0·1 M): acetate buffer (pH 5·4), phosphate buffer (pH 6·0 to 8·0) and glycine/NaOH buffer (pH 10·0). Known amounts of the solutions of EDTA and Fe(III)–pigment complex in each buffer were added to a 50 μM solution of the Fe(III)–pigment complex dissolved in similar buffer, the total volume of the system being maintained constant at 6·0 ml. Under these conditions, the initial concentration of the Fe(III)–pigment complex was constant. Absorbances at 450 nm were determined after they had reached constant values.

Radioactivity. Radioactivity of ^{59}Fe was measured with a Gammamatic instrument (SAIP-CGR, 75015 Paris, France).

Chemicals. Succinic acid and citric acid (analytical reagent grade) were purchased from Merck, and ^{59}Fe (as ferric citrate) from CEA (91190 Gif-sur-Yvette, France).

RESULTS

Influence of Fe^{3+} concentration on growth and production of pyoverdine$_{Pf}$

Growth of *P. fluorescens* in standard succinate medium (which contained no added iron) was accompanied by excretion of pyoverdine; excretion ceased as the culture entered the stationary phase. Addition of 1 mg Fe^{3+} l^{-1} to the culture medium almost doubled the growth yield but completely repressed formation of pyoverdine. When Fe^{3+} was growth-limiting (< 200 μg Fe^{3+} l^{-1}), there was an inverse relationship between the iron content of the medium and the amount of pigment synthesized after entry into the stationary phase (Fig. 1).

Pretreatment of the standard succinate medium to reduce its content of contaminating iron (see Methods) diminished the growth yield, while significantly increasing the amount of pigment produced (points B and Q in Fig. 1*a*). The amount of pigment synthesized per unit of cell mass was inversely related to the initial Fe^{3+} concentration of the medium under

Table 1. *Growth and pigmentation of P. fluorescens cultures in iron-deficient or iron-sufficient citrate media*

The measurements were made after the cultures had entered the stationary phase. Iron-supplemented citrate medium was standard citrate medium supplemented with 1 mg Fe^{3+} l^{-1} (added as $FeCl_3$). Iron-depleted medium was obtained by treating standard citrate medium with 8-hydroxyquinoline (see Methods).

Growth medium	Growth (mg dry wt bacteria l^{-1})	Pigment (mg l^{-1})
Standard citrate medium	844	traces
Citrate medium + Fe^{3+}	850	0
Iron-depleted citrate medium	523	160

all conditions where this cation was growth-limiting (Fig. 1*b*). Extrapolation to zero of the experimentally determined iron-limited growth yields indicated that the standard succinate medium without added iron contained 135 μg Fe l^{-1}, in reasonable agreement with a value of 160 μg l^{-1} calculated from the manufacturers' analytical data for the chemicals used in the medium.

As the standard succinate medium without added iron permitted the synthesis of greater amounts of fluorescent pigment (Fig. 1*a*) than did the more severely iron-depleted media B and Q, this standard medium was subsequently used to grow cultures for the isolation and purification of the pigment.

Some authors (Gouda & Chodat, 1963) have attributed an important role in pyoverdine synthesis to the nature of the organic carbon and energy source; depending on their presumed influence on pigment synthesis, such substrates have been classified as either 'chromogenic' or 'anti-chromogenic'. We observed that when succinic acid was replaced by citric acid (or malic acid) at the same concentration (4 g l^{-1}) in the standard medium, growth of *P. fluorescens* was not accompanied by pigment production. Addition of 1 mg Fe^{3+} l^{-1} did not increase the growth yield. Thus the citrate medium, unlike the standard succinate medium, was not iron-deficient. When the standard citrate medium was pretreated with 8-hydroxyquinoline to reduce its iron content, the growth yield of *P. fluorescens* was diminished and considerable amounts of fluorescent pigment were produced (Table 1). An 'anti-chromogenic' substrate can thus be converted into a 'chromogenic' one by a specific reduction of the iron content of the medium.

Nevertheless, the specific iron requirements of *P. fluorescens* may vary as a function of the organic carbon and energy source. To investigate this, we determined the quantity of iron incorporated by bacteria growing with succinate and citrate as carbon sources, both in the presence of excess iron. The standard culture media were supplemented prior to inoculation with 200 μg Fe^{3+} l^{-1} plus ^{59}Fe citrate at 0·4 μg $^{59}Fe^{3+}$ l^{-1}. The cultures were harvested after 40 h and the cells were washed three times with the corresponding media without added iron. Measurements of radioactivity in the cell pellets showed that succinate-grown cells had incorporated 228 μg Fe (g dry wt)$^{-1}$, and citrate-grown cells only 140. As a result of the lesser iron requirement of *P. fluorescens* when growing at the expense of citrate, pigment excretion (the visible manifestation of iron deficiency) occurs only when the absolute iron level in a citrate medium is substantially lower than that in a succinate medium.

Preliminary observations on the properties of the Fe(III)–pigment complex

Addition of an aqueous solution of $FeCl_3$ (200 mg Fe^{3+} l^{-1}) to the pigment-containing supernatant from a culture of *P. fluorescens* caused an immediate colour change to brown–red accompanied by a total disappearance of fluorescence. These changes, characteristic of the complexation of $pyoverdine_{Pf}$ by Fe^{3+} were not observed with other cations tested (Al^{3+}, Cr^{3+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+}). Free pigment, characterized by its yellow–green colour

and strong fluorescence, could be recovered from the Fe(III)–pigment complex by any of the following treatments: (i) Reduction with sodium dithionite: this liberates Fe^{2+}, which was identified as the ferricyanide complex (Charlot, 1961). (ii) Acidification to pH 1: this liberates Fe^{3+} which was identified as the sulphocyanide complex (Charlot, 1961). (iii) Treatment with other strong chelators of Fe^{3+}: 8-hydroxyquinoline, EDTA, citric acid.

The Fe(III)–pigment complex and its decomposition products

Chromatography on CM-Sephadex of the Fe(III)–pigment complex prepared from relatively young (24 to 40 h) cultures revealed two peaks, A and B. The minor peak (A), which eluted first, was heterogeneous, as revealed by electrophoresis at two pH values (see Methods). It contained a mixture, in roughly equal quantities, of two pigments a and b. Electrophoretic analysis of the major peak (B) showed that it consisted of a single component c, accompanied by only traces of components a and b.

Attempts to purify further the pigment eluted in peak B, whether by repeated chromatography on CM-Sephadex, or by repeated electrophoresis and subsequent elution of the resulting spots, were not successful: electrophoretic analysis showed that peak B, although consisting largely of component c, was always contaminated by traces of components a and b. The two latter components accounted at most for 1 % of the total absorbance. These results suggested that the material of peak B, which is the major pigment in the supernatant from young cultures, is slightly unstable in aqueous solution.

Analogous chromatographic analyses were conducted on the Fe(III)–pigment complex isolated from older (70 to 120 h) cultures. The amount of pigment associated with peak B diminished progressively with time and was replaced by the heterogeneous pigment fraction associated with peak A. The pH of the standard succinate medium, initially at pH 7, rose after 40 h to approximately pH 9, as a result of the utilization of the organic anion by the bacteria. Since the evidence previously described suggested that pyoverdine was slightly unstable in aqueous solution, the extensive decomposition observed in alkaline culture media could reflect a chemical, rather than a biological degradation. Therefore solutions of purified pyoverdine and of its Fe(III) complex were prepared in water adjusted to pH 5, 7 and 9, by adding either acetic acid or Na_2CO_3, and agitated at 25 °C for 70 h. After complexing the free pyoverdine with Fe^{3+}, each fraction was subjected to chromatography on CM-Sephadex, followed by electrophoresis. The residual desferri pigment decreased from 94 % (at pH 5) to 60 % (at pH 9) whereas the Fe(III)–pigment complex itself decreased from 97 % to 86 % at these pH values. Pyoverdine and, to a lesser degree, the complex were evidently alkali-labile. Furthermore, the degradation both of the free pigment and of the Fe(III) complex under mild alkaline conditions yielded a mixture of pigments indistinguishable in their chromatographic and electrophoretic behaviour from those of fraction A, as initially characterized in the supernatant of culture media.

To summarize, the available evidence strongly suggests that *P. fluorescens* synthesizes only one molecular species of the pyoverdine type. However, the native pigment is labile in aqueous solution, particularly under mildly alkaline conditions, and gives rise to several pigmented decomposition products. Therefore to minimize the chemical decomposition of pyoverdine, cultures were maintained throughout growth at a pH value below 7·3 and were harvested shortly after entry into the stationary phase.

Physicochemical properties of the pigment and its Fe(III) complex

The molecular weight of the free pigment was determined by tonometry as 1500 ± 75. The absorption spectrum in water of the free pigment showed two main bands: one at 230 nm ($\epsilon = 32000$) with a shoulder at 255 nm, the other at 385 nm ($\epsilon = 16500$) with shoulders at 365 and 400 nm (Fig. 2). As shown in Fig. 3, at pH values $\leqslant 5$, there were two peaks with maxima at 365 and 380 nm in the visible region; at pH values $\geqslant 7$, there was only one peak

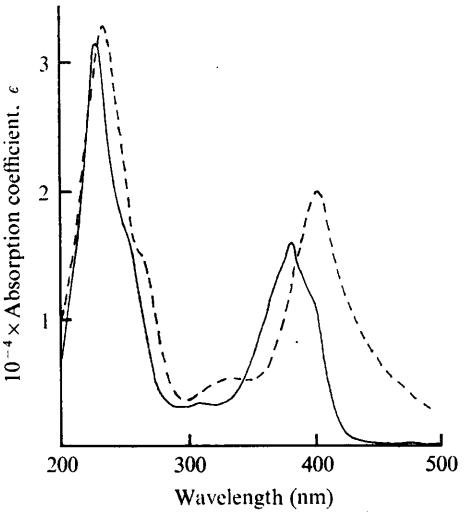

Fig. 2

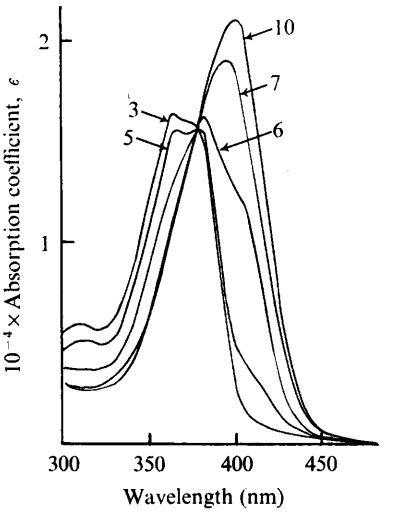

Fig. 3

Fig. 2. Absorption spectra of pyoverdine$_{Pf}$ (——) and its Fe(III) complex (– – –) in aqueous solution.

Fig. 3. Visible absorption spectrum of pyoverdine$_{Pf}$ as a function of pH. pH values are indicated beside the spectra.

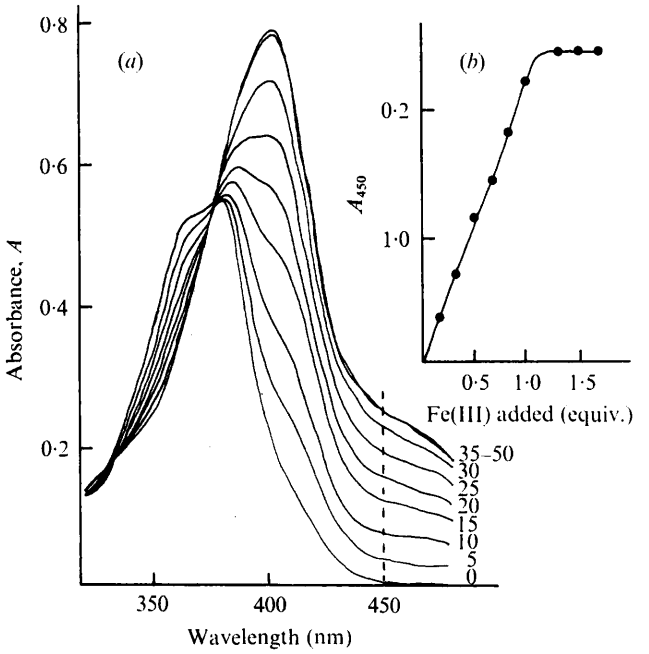

Fig. 4. Determination of the stoicheiometry of the Fe(III)–pyoverdine$_{Pf}$ complex. (*a*) Change in the absorption spectrum of pyoverdine$_{Pf}$ as a function of the amount of Fe(III) added. The numbers beside the spectra indicate the volumes (μl) of 3·25 mM-$FeCl_3$ solution added (see Methods). (*b*) Increase in absorption at 450 nm (derived from Fig. 4*a*) as a function of the equivalent amounts of added Fe(III).

in this region, which shifted from 402 nm (pH 7) to 410 nm (pH 10). The spectrum in the ultraviolet region was essentially invariant as a function of pH.

The absorption spectrum of the Fe(III)–pigment complex (Fig. 2) had maxima at 235 nm (ϵ = 33000) and at 403 nm (ϵ = 20000) with a pronounced shoulder at 450 nm (ϵ = 6800). It was pH-invariant.

The large difference in absorbance at 450 nm between the Fe(III)–pigment complex (ϵ = 6800) and the free pigment (ϵ = 0) made it possible to determine spectrophotometrically the concentration of the Fe(III)–pigment complex present in a mixture of both species. As shown in Fig. 4, the stoicheiometry of the complex is 1:1.

The uncorrected fluorescence spectrum of pyoverdine$_{Pf}$, measured at pH 7·0 (0·1 M-phosphate buffer), had a maximum of excitation at 398 nm, and of emission at 470 nm. At lower pH (1 % acetic acid), the maxima were shifted to 384 nm and 510 nm, respectively. The Fe(III)–complex was non-fluorescent.

Stability constant of the Fe(III)–pigment complex

The stability constant K_2 of the complex was measured as described by Rosotti & Rosotti (1961) using EDTA as a competitive chelator of Fe^{3+}. In the presence of EDTA, the Fe(III)–pyoverdine$_{Pf}$ complex (PFe) was partly decomplexed:

$$PFe + EDTA \rightleftharpoons P + EDTA\text{–}Fe \tag{1}$$

the equilibrium constant K_1 being

$$\frac{[PFe][EDTA]}{[P][EDTA\text{–}Fe]}$$

Equation (1) represents two equilibria:

$$PFe \rightleftharpoons P + Fe \tag{2}$$

and

$$EDTA + Fe \rightleftharpoons EDTA\text{–}Fe \tag{3}$$

with equilibrium constants:

$$K_2 = \frac{[PFe]}{[P][Fe]} \quad \text{and} \quad K_3 = \frac{[EDTA\text{–}Fe]}{[EDTA][Fe]}$$

The stability constant of PFe is thus:

$$K_2 = K_1 \times K_3$$

The value of K_3 as a function of pH is known (Anderegg *et al.*, 1963). If we neglect the concentration of free iron, then [P] = [EDTA–Fe]. Since $[P] = [PFe]_{initial} - [PFe]$ and $[EDTA] = [EDTA]_{initial} - [EDTA\text{–}Fe]$, K_1 could be calculated by determination of the values at equilibrium of [PFe]. These are directly proportional to the absorbance at 450 nm, since none of the other reacting species absorbs significantly at this wavelength.

In the experiment illustrated in Fig. 5, where all solutions were buffered at pH 7·0 with 0·1 M-phosphate, a range from 0·125 to 15 mM-EDTA was necessary to achieve satisfactory decomplexation of the pigment. The mean value of the stability constant, $K_{PFe,\,pH7}$, was found to be equal to $189 \times K_{EDTA,\,pH7}$. Since the stability constant of EDTA at pH 7·0 is 10^{22} (Anderegg *et al.*, 1963), a value for K_2 of $1{\cdot}9 \times 10^{24}$ could be deduced at pH 7·0.

K_2 was a function of pH, and determinations of the apparent stability constants at a series of pH values (Table 2) permitted a calculation by extrapolation to alkaline pH values of the real stability constant which was of the order of 10^{32} (Fig. 6), characteristic of a highly stable Fe^{3+} complex (Anderegg *et al.*, 1963).

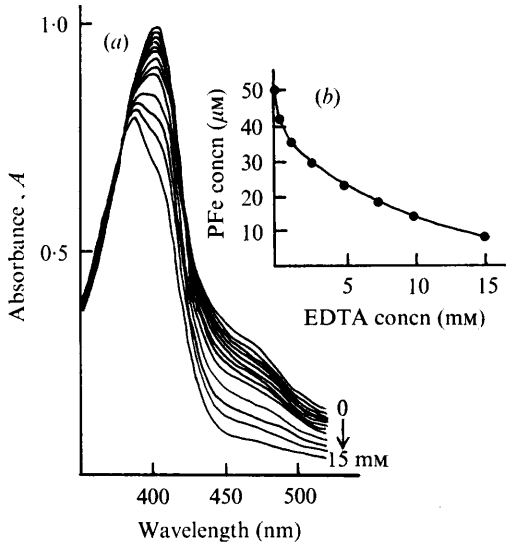

Fig. 5. Decomplexation of the Fe(III)–pyoverdine$_{Pf}$ complex by EDTA (pH 7·0). (*a*) Absorption spectra of a 50 μM solution of Fe(III)–pyoverdine$_{Pf}$ complex in the presence of increasing amounts (0 to 15 mM) of EDTA, prepared as described in Methods. (*b*) Decrease in the concentration of the Fe(III)–pyoverdine$_{Pf}$ complex (measured by absorption at 450 nm) as a function of added EDTA.

Table 2. *Variation of the apparent stability constant (K_2) of the iron(III)–pyoverdine$_{Pf}$ complex as a function of pH*

For definitions of K_1, K_2 and K_3, see text.

pH	K_1	K_3	K_2	log K_2
5	0·27	10^{18}	$0{\cdot}27\times10^{18}$	17·44
6	3	10^{20}	$3{\cdot}00\times10^{20}$	20·47
7	189	10^{22}	$1{\cdot}89\times10^{24}$	24·26
8	5000	10^{23}	$5{\cdot}00\times10^{26}$	26·70
10	10600	10^{27}	$1{\cdot}06\times10^{31}$	31·02

DISCUSSION

Many investigators have shown that the synthesis of pyoverdines in fluorescent pseudomonads is inhibited by adding Fe^{3+} to cultures (King *et al.*, 1948; Koepsell, 1950; Totter & Moseley, 1953; Lenhoff, 1963; Love & Hulcher, 1964; Palumbo, 1972). Nevertheless, the specific role of iron as a regulator of fluorescent pigment synthesis has remained unclear, since the synthesis appeared also to be regulated by other factors, notably the nature of the organic substrate (Gouda & Greppin, 1965; Gouda & Chodat, 1963).

The experiments described here reveal that the concentration of Fe^{3+} is the sole factor that regulates pyoverdine synthesis by *P. fluorescens*. The observation that no fluorescent pigment was produced when citric acid or malic acid was used as the substrate was due to these cells not being iron-limited. Succinate-grown cells had a specific iron requirement of about 1·6 times that of citrate-grown cells and thus readily became iron-deficient. The classification of organic substrates for fluorescent pseudomonads into the categories of 'chromogens' and 'anti-chromogens' is, accordingly, unjustified. Our observations suggest that any organic substrate can support pyoverdine synthesis but also show that the absolute level of Fe^{3+} at which growth becomes limited may vary significantly with the nature of the substrate.

The specific derepression of pyoverdine synthesis that results from iron limitation suggested that the pigment might play a role in either the transport or the metabolism of iron. This

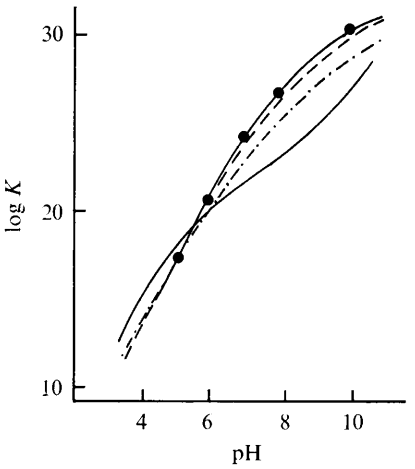

Fig. 6. Apparent stability constants of the Fe(III)–pyoverdine$_{Pf}$ complex as a function of pH (●), compared with reported values (Anderegg *et al.*, 1963) for other siderophores: ferroxamine B (---); ferrichrome (-·-·-); EDTA–Fe(III) complex (——).

hypothesis was strengthened by the discovery that the fluorescent pigment is a powerful chelator of Fe^{3+}, with an affinity constant for this cation of about 10^{32}. Formation of the Fe(III)–pigment complex permitted the purification of the fluorescent pigment by a method analogous to that used by Zähner *et al.* (1963) for the purification of the microbial iron chelators known as desferrisiderochromes (Keller-Schierlein *et al.*, 1964).

Pyoverdine was shown to be chemically unstable under mild alkaline conditions. The mixture of pigments reported by several workers (Chodat & Gouda, 1961; Favre & Greppin, 1971; Michea & Greppin, 1974) is probably an artefact caused by the lability of the native pigment at pH values above 7·0.

The properties of pyoverdine$_{Pf}$ are consistent with its role as a siderophore (Neilands, 1973). They include specific derepression under conditions of Fe^{3+} deficiency, and a very high affinity for Fe^{3+}, together with a lack of affinity for Fe^{2+}. Moreover, the apparent stability constants of the Fe(III)–pigment complex are comparable to those of two other siderophores (Fig. 6). The role of pyoverdine in facilitating the transport of Fe^{3+} into the cell of *P. fluorescens* is described in the following paper (Meyer & Hornsperger, 1978).

We wish to thank Professor R. Y. Stanier for invaluable advice and discussion, and Professor B. Wurtz in whose laboratory this work was performed. We also gratefully acknowledge the interest shown by Dr J. F. Biellmann in this work.

REFERENCES

ANDEREGG, G., L'EPLATTENIER, F. & SCHWARZENBACH, G. (1963). Hydroxamatkomplexe: III. Eisen(III)-Austausch zwischen Sideraminen und Komplexonen. Diskussion der Bildungskonstanten der Hydroxamatkomplexe. *Helvetica chimica acta* **46**, 1409–1422.

BAGHDIANTZ, A. (1952). Role of zinc in appearance of component II of the pigment of *Pseudomonas fluorescens* (Flügge-Migula). *Archives des sciences, Genève* **5**, 47–48.

BIRKHOFFER, L. & BIRKHOFFER, A. (1948). Riboflavine, a component of 'bacterial fluorescein'. *Zeitschrift für Naturforschung* **3b**, 136.

BLANCHETIÈRE, A. (1920). Action du *Bacillus* fluorescent liquefiant de Flügge sur l'asparagine en milieu chimiquement défini. *Annales de l'Institut Pasteur* **34**, 392–411.

CHAKRABARTY, A. M. & ROY, S. C. (1964*a*). Effect of trace elements on the production of pigments by a pseudomonad. *Biochemical Journal* **93**, 228–231.

CHAKRABARTY, A. M. & ROY, S. C. (1964*b*). Characterization of a pigment from a pseudomonad. *Biochemical Journal* **93**, 144–148.

CHARLOT, G. (1961). *Analyse Qualitative Rapide des Cations et des Anions*. Paris: Dunod.

CHODAT, F. & GOUDA, S. (1961). Contribution à l'étude du pigment de *Pseudomonas fluorescens* Migula. *Pathologia et microbiologia* **24**, 840–847.

ELLIOT, R. P. (1958). Some properties of Pyoverdine, the water-soluble pigment of the pseudomonads. *Applied Microbiology* **6**, 241–246.

FAVRE, J. & GREPPIN, H. (1971). Séparation de l'extrait pigmentaire de *Pseudomonas fluorescens* sur gel de Sephadex G25. *Saussurea* **2**, 25–28.

GEORGIA, F. R. & POE, C. F. (1931). Study of bacterial fluorescence in various media. I. Inorganic substances necessary for bacterial fluorescence. *Journal of Bacteriology* **22**, 349–361.

GIRAL, F. (1936). Sobre los liocromos característicos del grupo de bacterias fluorescentes. *Anales de la Sociedad española de física y química* **34**, 667–693.

GOUDA, S. & CHODAT, F. (1963). Glyoxylate et succinate, facteurs déterminant respectivement l'hypochromie et l'hyperchromie des cultures de *Pseudomonas fluorescens*. *Pathologia et microbiologia* **26**, 655–664.

GOUDA, S. & GREPPIN, H. (1965). Biosynthèse pigmentaire chez *Pseudomonas fluorescens* en fonction de la concentration du substrat hydrocarboné ou aminé. *Archives des sciences, Genève* **18**, 716–721.

GREPPIN, H. & GOUDA, S. (1965). Action de la lumière sur le pigment de *Pseudomonas fluorescens* Migula. *Archives des sciences, Genève* **18**, 721–725.

HULCHER, F. H. (1968). Activation of 6-phosphogluconate dehydrase by Pyoverdine. *Biochemical and Biophysical Research Communications* **31**, 247–251.

KELLER-SCHIERLEIN, W., PRELOG, V. & ZÄHNER, H. (1964). Siderochrome. (Natürliche Eisen(III)–trihydroxamat-Komplexe). *Fortschritte der Chemie organischer Naturstoffe* **22**, 279–322.

KING, J. V., CAMPBELL, J. J. R. & EAGLES, B. A. (1948). Mineral requirements for fluorescin production by *Pseudomonas*. *Canadian Journal of Research* **26 C**, 514–519.

KOEPSELL, J. (1950). Gluconate oxidation by *Pseudomonas fluorescens*. *Journal of Biological Chemistry* **186**, 743–751.

LENHOFF, H. M. (1963). An inverse relationship of the effects of oxygen and iron on the production of fluorescin and cytochrome *c* by *Pseudomonas fluorescens*. *Nature, London* **199**, 601–602.

LEPIERRE, C. (1895). Recherches sur la fraction fluorescinogène des microbes. *Annales de l'Institut Pasteur* **8**, 643–663.

LLUCH, C., CALLAO, V. & OLIVARES, J. (1973). Pigment production by *Pseudomonas reptilivora*. I. Effect of iron concentration in culture media. *Archiv für Mikrobiologie* **93**, 239–243.

LOVE, S. H. & HULCHER, F. H. (1964). Green fluorescent pigment accumulated by a mutant of *Cellvibrio gilvus*. *Journal of Bacteriology* **87**, 39–45.

MEYER, J. M. & HORNSPERGER, J. M. (1978). Role of pyoverdine$_{Pf}$, the iron-binding fluorescent pigment of *Pseudomonas fluorescens*, in iron transport. *Journal of General Microbiology* **107**, 329–331.

MICHEA, M. & GREPPIN, H. (1974). Separation et évolution du complexe pigmentaire de *Pseudomonas fluorescens* Migula. *Comptes rendus de la Société d'histoire naturelle de Genève* **8**, 19–31.

NEILANDS, J. B. (1973). Microbial iron transport compounds (siderochromes). In *Inorganic Biochemistry*, vol. I, pp. 167–209. Edited by G. L. Eichhorn. Amsterdam: Elsevier.

PALLERONI, N. J. & DOUDOROFF, M. (1974). The genus *Pseudomonas*. In *Bergey's Manual of Determinative Bacteriology*, 8th edn, pp. 217–243. Edited by R. E. Buchanan & N. E. Gibbons. Baltimore: Williams & Wilkins.

PALLERONI, N. J., KUNISAWA, R., CONTOPOULOU, R. & DOUDOROFF, M. (1973). Nucleic acid homologies in the genus *Pseudomonas*. *International Journal of Systematic Bacteriology* **23**, 333–339.

PALUMBO, S. A. (1972). Role of iron and sulfur in pigment and slime formation by *Pseudomonas aeruginosa*. *Journal of Bacteriology* **111**, 430–436.

ROSOTTI, J. C. & ROSOTTI, H. (1961). *The Determination of Stability Constants*. New York: McGraw Hill.

STANIER, R. Y., PALLERONI, N. J. & DOUDOROFF, M. (1966). The aerobic pseudomonads: a taxonomic study. *Journal of General Microbiology* **43**, 159–271.

SULLIVAN, M. X. (1905). Synthetic culture media and the biochemistry of bacterial pigments. *Journal of Medical Research* **14**, 109–160.

THEODORE, J. S. & SCHADE, A. L. (1965). Growth of *Staphylococcus aureus* in media of restricted and unrestricted inorganic iron availability. *Journal of General Microbiology* **39**, 75–83.

TOTTER, J. R. & MOSELEY, F. T. (1953). Influence of the concentration of iron on the production of Fluorescin by *Pseudomonas aeruginosa*. *Journal of Bacteriology* **65**, 45–47.

TURFREIJER, A. (1942). *Pyoverdinen de groene fluorescende Kleurstoffen van Pseudomonas fluorescens*. Thesis, University of Amsterdam. *British Abstracts* **16**, 16578.

WARING, W. S. & WERKMAN, C. H. (1942). Growth of bacteria in an iron-free medium. *Archives of Biochemistry* **1**, 303–310.

WURTZ, B. (1954). Antagonisme entre les bacteries lactiques et protéolytiques des ensilages. *Conférence Européenne des Herbages, Paris*, 288–297.

ZÄHNER, H., KELLER-SCHIERLEIN, W., HUTTER, R., HESS-LEISINGER, K. & DEER, A. (1963). Siderамine aus Aspergillaceen. *Archiv für Mikrobiologie* **45**, 119–135.