

## PYOVERDINE, FLUORESCENT PIGMENT OF *PSEUDOMONAS AERUGINOSA*: PRODUCTION, PURIFICATION AND PHYSICOCHEMICAL PROPERTIES

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SIDEROPHORES have specific affinity for  $\text{Fe}^{3+}$ , and are synthesized and excreted into the culture medium by microorganisms in iron-deficient conditions. Pyoverdine  $P_f$ , the yellow-green fluorescent pigment is the physiological iron carrier of *Pseudomonas fluorescens*<sup>1</sup>. In this paper we describe the conditions controlling the formation of a yellow-green fluorescent compound by *P. aeruginosa* PAO<sub>1</sub>, along with its purification and physicochemical properties.

*Pseudomonas aeruginosa* PAO<sub>1</sub> used in the present studies was a gift from Dr Holloway, Department of Genetics, Monash University, Clayton, Victoria, Australia. Media, bacterial growth conditions, stoichiometry of the  $\text{Fe}^{3+}$  pigment complex and its stability constant were determined as described<sup>2</sup>. IR spectrum was recorded on a Perkin Elmer 398 instrument.

### Effects of $\text{Fe}^{3+}$ concentration on bacterial growth and production of pyoverdine $P_a$

Growth of *P. aeruginosa* was accompanied by the excretion of a fluorescent compound in standard succinate medium (containing no added iron), which formed a deep brown-red complex on addition of  $\text{FeCl}_3$  solution to the culture medium. The excretion stopped as the culture entered the stationary phase of growth. Addition of  $\text{Fe}^{3+}$  (1 mg/l) to the culture medium increased the growth yield significantly but repressed the production of the iron-chelating compound (figure 1).

Pretreatment of the standard succinate medium with hydroxyquinoline to reduce the iron content of the medium diminished the growth but increased the pigment formation (point Q in figure 1). Extrapolation to zero of the experimentally determined iron-limited growth yields indicated that the standard succinate medium without added iron contained  $100 \mu\text{g Fe}^{3+}/\text{l}$ , in reasonable agreement with a value of  $115 \mu\text{g Fe}^{3+}/\text{l}$  calculated by the chemical analysis of the medium<sup>3</sup>. As the standard succinate medium showed the formation of greater amounts of pyoverdine than iron-depleted medium Q (figure 1), it

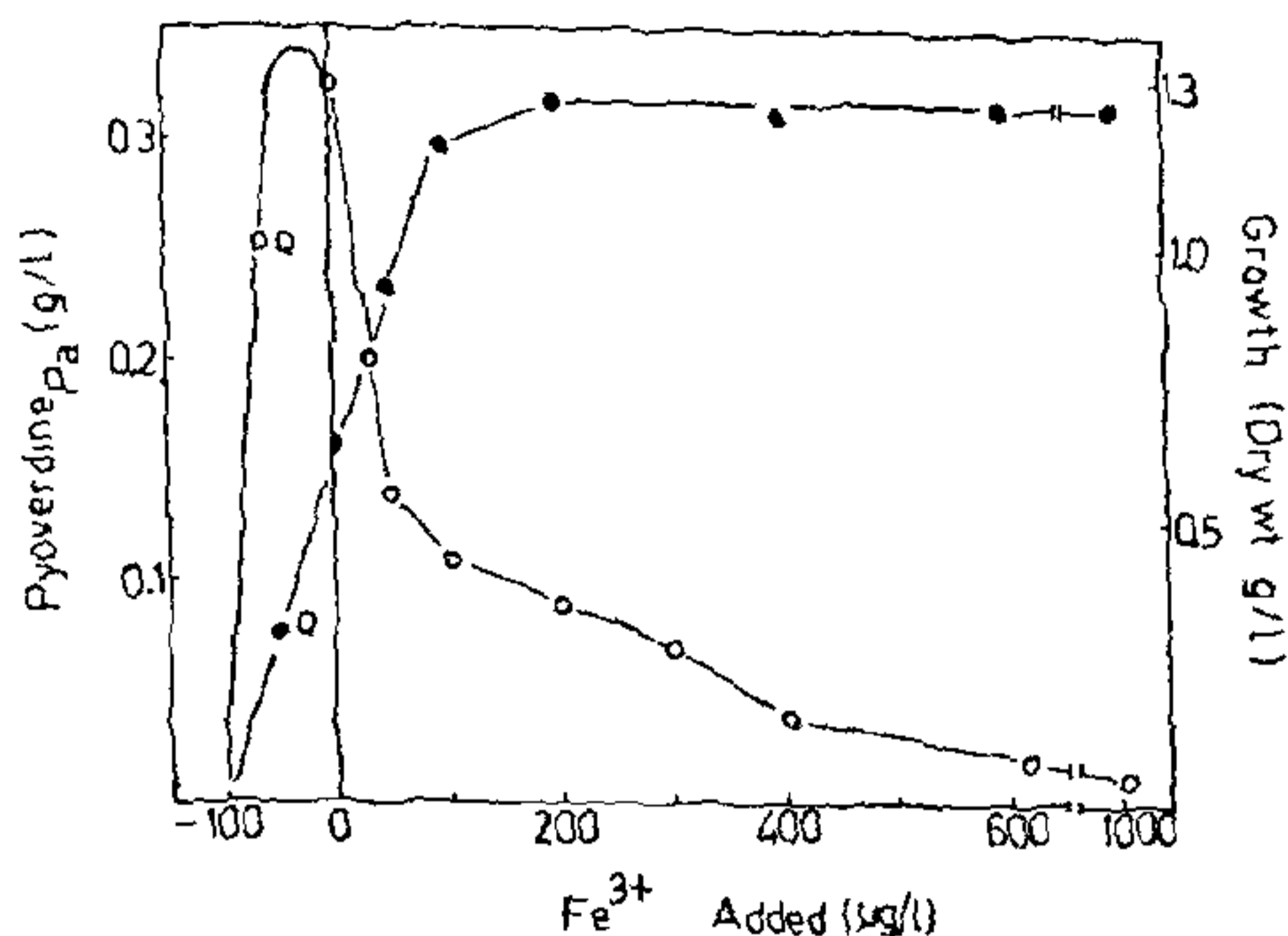


Figure 1. Pigmentation (O) and growth (●) of *P. aeruginosa* PAO<sub>1</sub> as a function of  $\text{Fe}^{3+}$  present in the succinate medium. Point Q corresponds to the standard medium pretreated with hydroxyquinoline. Negative value on the abscissa corresponds to the amount of  $\text{Fe}^{3+}$  present in the medium as a contaminant.

was subsequently used to grow cultures for the isolation and purification of the pigment. Gouda and Chodat<sup>4</sup> have presumed the influence of organic carbon and energy source in pyoverdine synthesis; such substrates have been classified as either 'chromogenic' or 'antichromogenic'. We observed that when succinic acid was replaced by citric acid (or malic acid) at the same concentration (4 g/l) in the standard medium, the growth of *P. aeruginosa* was not accompanied by pigment production. Addition of  $\text{Fe}^{3+}$  (1 mg/l) did not increase the growth significantly. Standard citrate medium gave traces of pigment with an increase in the bacterial growth. When iron was removed from citrate medium, considerable amount of pigmentation was found with decrease in bacterial growth. Thus the citrate medium, unlike the standard succinate medium, was not iron-deficient.

### Physicochemical properties of the pigment and its complex

The absorption spectrum of the free pigment showed two main bands: one at 306 nm with a shoulder at 316 nm, the other at 365 nm with a shoulder at 380 nm. The absorption spectrum of the  $\text{Fe}^{3+}$ -pigment complex had a maximum at 403 nm with a pronounced shoulder at 450 nm. The change in the pH of the iron-pyoverdine complex does not show any effect on the spectrum of pigment complex. Infrared spectrum of



pyoverdine showed major bands at 3450, 1590, 1435 and  $940\text{ cm}^{-1}$ . The stability constant  $K_2$  of the  $\text{Fe}^{3+}$ -pigment complex was measured<sup>5</sup> using EDTA as a competitive chelator of  $\text{Fe}^{3+}$ . In the experiment where all solutions were buffered at pH 7.0 with 0.1 M phosphate, a range from 2 to 14 mM EDTA was necessary to achieve satisfactory decomplexation of the pigment. The mean value of the stability constant ( $K_{\text{PF}_2}$ ), was found to be  $18.26 \times \text{EDTA}$ , at pH 7. Since the stability constant of EDTA at pH 7.0 is  $10^{22}$ , a value for  $K_2$  of  $2.85 \times 10^{23}$  could be deduced at pH 7.0.  $K_2$  was a function of pH, and the determination of the apparent stability constant at a series of pH values (table 1) permitted a calculation by extrapolation to alkaline pH values of the real stability constant which was of the order of  $10^{23}$ , characteristic of a highly stable  $\text{Fe}^{3+}$ -complex<sup>6</sup>.

Earlier studies have shown that the synthesis of pyoverdine in fluorescent pseudomonas is inhibited by adding  $\text{Fe}^{3+}$  to culture medium<sup>7,8</sup>. Nevertheless, the specific role of iron as a regulator of pyoverdine synthesis has remained unclear, since the synthesis appeared also to be regulated by other factors, notably the nature of the organic substrates<sup>9</sup>. The specific depression 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 proposition was strengthened by the fact that the pyoverdine is a strong chelator of  $\text{Fe}^{3+}$ , with an affinity constant for this cation of about  $10^{28}$ . Pyoverdine<sub>pa</sub> and siderophores in general, are characterized by (i) their synthesis is derepressed only when microbial cells are iron-deficient, (ii) the  $\text{Fe}^{3+}$  complexes have very high stability constants, (iii) they specifically complex  $\text{Fe}^{3+}$  and have a weak affinity for  $\text{Fe}^{2+}$ . The present investigation shows that pyoverdine is a typical microbial iron chelator *i.e.* it is a siderophore.<sup>10</sup> Our preliminary observations of infrared and NMR studies indicated that pyoverdine contains four functional groups, OH, COOH, CH<sub>2</sub> and NH<sub>2</sub>. Although the siderophores as chemical entities

display considerable structural variation, a majority of them are either hydroxamates or phenolates-catecholates.<sup>11-13</sup>

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## A RAPID DIAGNOSTIC TEST FOR Q FEVER EMPLOYING IMMUNOFLOUORESCENCE AND THE MOSQUITO INOCULATION TECHNIQUES

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It has been shown that the mosquito inoculation technique can be successfully utilized for detecting *Coxiella burnetii* by employing guinea pig as an animal

**Table 1** Variation of the apparent stability constant ( $K_2$ ) of the iron (III)-pyoverdine<sub>pa</sub> complex as a function of pH.

pH	$K_1$	$K_3$	$K_2$	$\log K_2$
5	6.28	$10^{18}$	$6.28 \times 10^{18}$	18.79
6	15.19	$10^{20}$	$1.51 \times 10^{21}$	21.17
7	18.26	$10^{22}$	$1.82 \times 10^{23}$	23.26
8	44.19	$10^{23}$	$4.41 \times 10^{24}$	24.64
10	49.11	$10^{27}$	$4.91 \times 10^{28}$	28.69