

Full Length Research Paper

Effect of iron and growth inhibitors on siderophores production by *Pseudomonas fluorescens*

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The ability of *Pseudomonas* to grow and to produce siderophores is dependent on the iron content and the type of carbon sources in the medium. Under conditions of low-iron concentration the *Pseudomonas* isolates studied produced yellow-green fluorescent iron-binding peptide siderophores and the biosynthesis of this siderophores was affected by several different environmental parameters. Four basal media, supplemented with different concentration of iron, were employed to study the effect of iron and different organic carbon sources on siderophore production in *Pseudomonas fluorescens*. The highest siderophores concentration was obtained in succinate medium. Ferric iron increased the growth yield and completely repressed siderophores production above 200 µg/l, but had a positive effect below 160 µg/l. Penicillin and lead elicited the production of siderophores in the presence of excess iron. Pre-treatment of the standard succinate medium with α,α -dipyridyl and 8-hydroxyquinoline to reduce the concentration of iron diminished both the growth yield and siderophore production, but α,α -dipyridyl increased significantly the amount of siderophores produced in the presence of 0.20 mg/l of the chelating ion.

Key words: *Pseudomonas fluorescens*, siderophores, iron, penicillin, lead, α,α -dipyridyl, 8-hydroxyquinoline.

INTRODUCTION

Under aerated conditions at neutral to alkaline pH, inorganic iron is extremely insoluble and its concentration is less than optimal for bacterial growth (Lindsay and Schwab, 1982; Schwyn and Neilands, 1986). *Pseudomonas fluorescens* is one of the fluorescent pseudomonads that secrete pyoverdins (Meyer, 2000) for its essential requirement for iron. Pyoverdin is a yellow-greenish fluorescent siderophore involved in high-affinity transport of iron into the cell (Budzikiewicz, 1992).

Iron is perhaps the most important micronutrient used by bacteria and is essential for their metabolism, being required as a cofactor for a large number of enzymes and iron-containing proteins (Escobar et al., 1999; Leong et al., 1990; Neilands, 1974). Interest in the pseudo-

monads has increased recently because of the possible use of siderophores as biopesticides (Wilson, 1997) and the possible use of pseudomonads in detoxifying chemical wastes through a wide range of enzymatic metabolic activities (Raaijmakers, 1995).

Iron metabolism has also been studied because of the potential role of microbial siderophores in facilitating uptake of heavy metals and their mobilisation under certain growth conditions (Chen et al., 1994; Mench et al., 1994). Here, we have investigated the specific stimuli for siderophores production in local strains of *P. fluorescens* under different Fe (III) concentrations in the presence of various carbon sources. The strains used were identified using phenotypic characters, G+C content and PCR amplification completed with a partial siderotyping. We also studied the influences of heavy metals (lead, mercury and cadmium) and antibiotics (penicillin and streptomycin) on siderophore production. Finally we inspected the effect of iron-depleted medium with two

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chelating iron (α,α -dipyridyl and 8-hydroxyquinoline) on siderophores yield.

MATERIAL AND METHODS

Isolation and identification of *P. fluorescens* strains

Samples of bacteria were collected from wheat rhizospheres cultivated in different regions of Northwest of Algeria. Pure cultures of fluorescent *Pseudomonas* species were obtained following successive selection of colonies on King B medium using ultraviolet light illumination to detect fluorescence.

Strains identification was done by biochemical analysis (Holt et al., 1994; Paleroni, 1984), coupled with determination of mole percent G+C of the DNA (Marmur and Doty, 1962), with *E. coli* DNA used as reference standard. Amplification of the 16S rRNA gene was carried out by PCR using the primers fD1 and rD1 corresponding to the 5' end (5'-AGAGTTTGATCCTGGCTCAG-3') and 3' end (5'-TAAGGAGGTGATCCAGGC-3') of the 16S rRNA gene (Weisburg et al., 1991). Amplification was confirmed by analysing 5 μ l of each PCR reaction mixture on a 0.9% agarose gel (Sambrook et al., 1989).

Purification of pyoverdins

Three strains (P1-14, P5-18 and P20-46) belonging to different biovars were selected for siderophores purification using the method of Meyer and Abdallah (1978). The pyoverdins produced in succinate medium were extracted with chloroform/phenol from the culture supernatants followed by separation in Sephadex CM25 column chromatography and eluting with 0.2 M pyridine/acetic acid buffer (pH 6.5). The compounds were rechromatographed twice on CM Sephadex. Iron-free pyoverdins were obtained by mixing the purified ferric pyoverdins with EDTA solution (1M) at pH 7 for 30 min. This was then applied to a Sephadex G25 gel filtration column to separate the pyoverdin from the ferric EDTA complex. The column was both equilibrated and eluted with 50 mM pyridine acetate buffer, pH 5 (Yvonne and Dennis, 1987).

The purified pyoverdins were analysed by isoelectric focusing (IEF) according to the method described by Koedam et al. (1994) and Meyer et al (1998).

Minimal inhibitory concentrations of some bacterial growth inhibitors

The liquid King B medium was modified by inclusion of inhibitors at different concentration and was inoculated with 100 μ l of cell suspensions from pre-culture of 24 h diluted to 1%. The following concentration of inhibitors were tested: lead (200, 500, 700, 1000, 1200, 1600, 2000, 4000, 6000, and 8000 μ M), mercury (250, 350, 400, 500, 600, 800, and 1200 μ M), cadmium (200, 500, 700, 1000, 1200, 1600, 2000, 4000, 6000, 8000 μ M), penicillin (200, 500, 1000 units/30 ml) and streptomycin (0.10, 0.20, 0.40, 0.60, and 0.80 mg/l). Only the most resistant strain (P5-18) was used in subsequent studies.

Effect of iron concentration and various carbon sources on siderophore production

Cultures were grown for 40 h at 25°C with shaking (200 rpm) in 500 ml Erlenmeyer flasks containing 125 ml medium, with the pH adjusted 7. To remove traces of iron, glassware was cleaned with 6 M HCl and with double distilled water. Four basal media were

employed with FeCl₃ added in increasing amounts (5, 10, 50, 100, 150, 200, 250, and 300 μ g/ml). The media contain the following components:

Asparagine medium: Asparagine 5 g/l, MgSO₄ 0.1 g/l, and K₂HPO₄ 0.5 g/l.

King B: Glycerine 10, Proteose-peptone 20 g/l, and MgSO₄ 1.5 g/l.

Glycerol medium: Glycerol 10 g/l, (NH₄)₂SO₄ 1 g/l, MgSO₄.7H₂O 1 g/l, K₂HPO₄ 4 g/l.

Succinate medium: KH₂PO₄ 6 g/l, K₂HPO₄ 3 g/l, (NH₄)₂SO₄ 1 g/l, MgSO₄.7H₂O 0.2 g/l, sodium succinate.

Measurement of growth and siderophore assay

Both were determined according to the method of Meyer and Abdallah (1978). Bacterial growth was estimated turbidimetrically at 600 nm, the amount of siderophore secreted into the culture medium was determined by removing bacteria by centrifugation and measuring the absorbance of the supernatant at 400 nm. Concentration was calculated using absorption maximum (λ =400nm) and molar extinction coefficient ϵ = 20000.

Effect of the bacterial growth inhibitors on siderophores production

We studied the influence of bacterial growth inhibitors on siderophores production under different concentration of Fe³⁺ in succinate medium. The strains were grown in succinate medium with different concentrations of the following inhibitors: lead and cadmium (500 μ M), mercury (250 μ M), Penicillin (30 units/ml), streptomycin (0.40 mg/l).

Effect of iron-depleted medium on siderophore production

Two strong iron chelators were studied for their influence in increasing or decreasing siderophore production. The strain was grown in succinate medium without adding iron and in the presence of α,α -dipyridyl (0.1, 0.15, 0.20, 0.25, 0.30, 0.35, and 0.40 mg/l) and 8-hydroxyquinoline (0.10, 0.20, 0.40, 0.60, 0.80, 1 mg/l).

RESULTS AND DISCUSSION

Strain characterisation

The five strains selected for this study appeared very similar to *P. fluorescens*. They produced a fluorescent yellow-green pigment on King B medium and did not produce pyocyanin on King A medium. They were also capable of growth on cetrimide agar. Isolates were positive for catalase, lipase, arginine dihydrolase, gelatinase, urease and did not hydrolyse starch. They could grow at 4°C but not at 41°C; their sensitivity to antibiotics as well as their nutritional requirements also matched those reported for *P. fluorescens*. The mole percent G+C varied between 64.0 and 67.3 (Doudoroff and Palleroni, 1974).

The isolate P1-14 was characterised by the presence of a blue non-fluorescent pigment (biovar IV); strains P10-22 and P20-46 were differentiated by negative denitrification and negative egg yolk reactions (biovar V).

Strain P10-22 produced weak fluorescence, and the two others isolates (P12-10 and P5-18) produced negative denitrification and positive egg yolk reactions (biovar I). Strain P5-18 produced a visible high fluorescence (Holt et al., 1994; Paleroni, 1984).

PCR amplification of the 16S rRNA gene (Figure 1) showed the same fragment for all five samples confirming that they belong to the same species and form one 16S rRNA halotype (Sambrook et al., 1989).

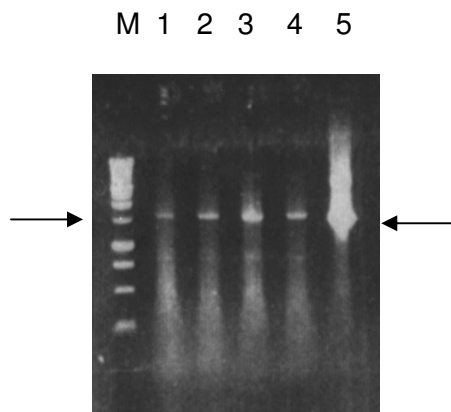


Figure 1. Agarose gel electrophoresis of the polymerase chain reaction (PCR) amplified 16S-rRNA gene and digestion products for *Pseudomonas fluorescens* strains 1 (P1-14) , 2 (P10-22) , 3 (P12 -10), 4 (P5-18), 5 (P20-46).

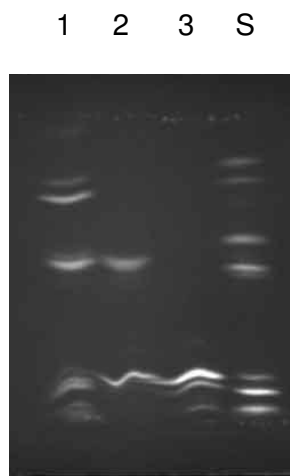


Figure 2. IEF profiles of pyoverdins from *P. fluorescens*: P1-14 (lane 1), P5-18 (lane 2) and P20-46 (lane 3) with isoelectric pH (pI) internal standards revealed under UV light following electrophoresis (see Meyer et al., 1998).

Electrophoretic analysis of pyoverdins

As shown in Figure 2, strains P1-14, P5-18 and P20-46 show diversity in the pyoverdins. Pyoverdin from P1-14

(lane 1) produced four bands characterised by pI values of 8.75, 8.42, 7.30 and 4.83. Pyoverdin of P5-18 (lane 2) produced two bands, of pI 7.30 and 5.13. The third strain, P20-46 showed three bands with pI values of 5.27, 5.07 and 4.01. The three strains produced compounds belonging to three different siderovars indicating the presence of a novel pyoverdin structure. Further studies using ^{59}Fe incorporation (Meyer et al., 1998) will be done to confirm this data. The detection of different siderovars confirms the diversity of the three strains used.

Minimal inhibitory concentration of bacterial growth inhibitors

Minimal inhibitory concentrations of the three heavy metals and the two antibiotics are shown in Table 1. The results indicate that these strains showed multiple resistance compared to others reported by Booth and Williams (1984) and Filali et al. (2000). Strain P5-18 was found to be the most resistant strain to the metals followed by the strain P1-14.

Influence of iron and medium content on siderophore production

Tables 2 and Figure 3 show that high siderophore production was obtained with a standard succinate medium. Meyer and Abdallah (1978) used the same medium to produce siderophores from *P. fluorescens*. Chodat and Gouda (1961) have attributed an important role in pyoverdin synthesis to the nature of the carbon source for growth and succinate is classified as a chromogenic substrate increasing siderophore yields. The lowest production was in a glycerol medium, and King et al. (1948) found noproduct of fluorescent pigment with a similar medium.

The results in and Figure 3 showed that cell growth and siderophores production were inversely proportional responses, and Meyer and Abdallah (1978) had previously shown that the amount of pigment synthesized per unit of cell mass was inversely related to the concentration of the factor limiting growth.

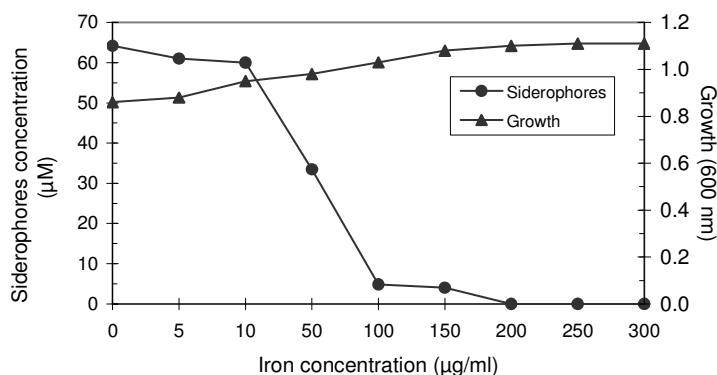
Siderophores are iron-specific compounds which are secreted under low iron stress and we found that production of siderophores in the medium employed was inversely proportional to the iron concentration in the medium (Budzikiewicz, 1993). The standard succinate medium without added iron permitted the synthesis of greater amounts of pyoverdins (Meyer and Abdallah, 1978). As shown in Figure 2, although cell growth reached a maximal value with 200 $\mu\text{g/l}$ Fe (III), siderophore production was lower at this iron concentration. The optimal iron concentration for high siderophore production was in the succinate medium

Table 1. Minimal inhibitory concentration of some growth inhibitors.

Inhibitor	MIC (μM) for the following strains of <i>P. fluorescens</i>				
	P1-14	P10-22	P12-10	P5-18	P20-46
Pb ²⁺	4000	2800	3000	6000	3046
Hg ²⁺	1200	900	853	1200	1000
Cd ²⁺	3000	2500	3000	6000	2000
Penicillin	ND	ND	ND	ND	ND
Streptomycin (mg/l)	0.70	0.65	0.50	0.80	0.66

Table 2. Influence of iron and medium content on siderophores production by *P. fluorescens* strain P5-18.

MEDIUM Fe ³⁺ added $\mu\text{g/l}$	King B		Glycerol		Asparagine	
	Growth (OD _{600nm})	Siderophore concentration (μM)	Growth (OD _{600nm})	Siderophore concentration (μM)	Growth (OD _{600nm})	Siderophore concentration (μM)
00	0.98	34.70	1.54	29.55	0.91	29.6
5	0.98	34.66	1.55	29.56	0.98	27.15
10	1.02	34.61	1.81	29.04	1.25	28.01
50	1.02	33.9	1.84	29.13	1.24	15.12
100	1.12	14.31	1.98	27.20	1.42	15.02
150	1.3	14.02	2.01	13.33	1.78	10.11
200	1.38	2.25	1.77	0.5	1.14	0.45
250	1.22	1.22	0.92	0.0	0.45	00
300	0.97	0.33	0.11	0.0	0.16	00

**Figure 3.** Siderophore production by *P. fluorescens* P5-18 as a function of Fe (III) added to the succinate medium.

without any added iron but which was calculated from the chemicals used to prepare the medium to contain 160 $\mu\text{g/l}$ (Meyer and Abdallah, 1978). De Villegas et al. (2002) found that concentrations of iron $>10 \mu\text{M}$ had a negative effect on siderophores production, and Manninen and Sandholm (1993) reported that

siderophores production occurred only at an iron concentration of $>50 \mu\text{M}$. Therefore, we confirm that *P. fluorescens* growing in iron-limited conditions (less than 160 $\mu\text{g/l}$) should induce siderophore production (Colquhoun and Sorum, 2001).

Effect of bacterial growth inhibitors on siderophore production

Streptomycin and penicillin added to succinate medium acts differently on the siderophores production. Streptomycin reduced siderophores production below 10 μM in different iron concentrations. While penicillin, as shown in Figure 4, increased the production of siderophores in the presence of excess iron (above 100 $\mu\text{g/ml}$). Totter and Mosley (1952) found similar results and suggested that penicillin exerts its effect on *P. aeruginosa* by interfering with iron-containing enzymes.

Growth and siderophore production were both inhibited by the heavy metals (lead, mercury and cadmium) especially under iron limitation (Figures 5 and 6). An exception was found with lead which increased siderophore yield under excess of iron. The role of lead

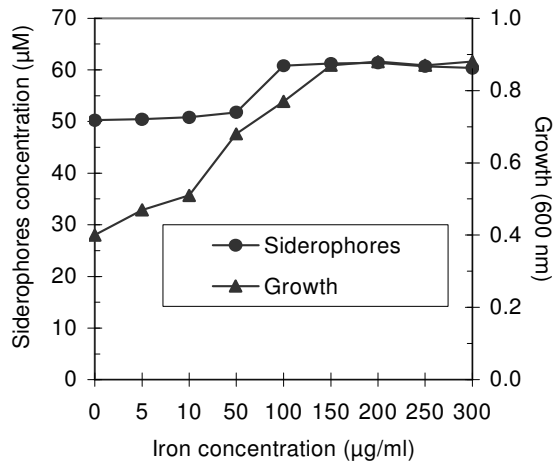


Figure 4. Siderophore production by *P. fluorescens* P5-18 as a function of Fe (III) added to the succinate medium with 30 units /ml of penicillin.

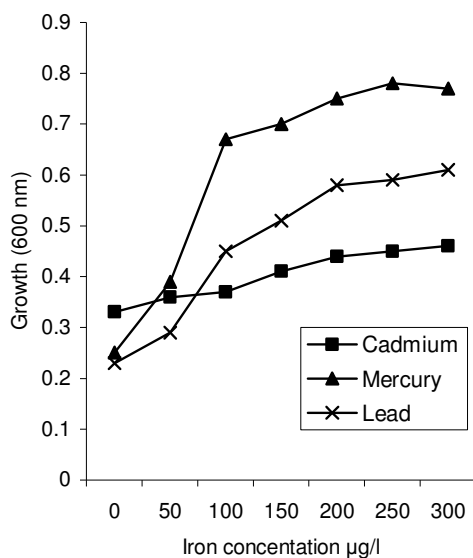


Figure 5. Effect of iron on the growth of *P. fluorescens* P5-18 in the presence of some heavy metals.

in this experiment is not well understood but we propose that lead affected function(s) in iron metabolism when it was transferred into the cell through siderophore-

mediated iron uptake. Baysse et al. (2000) reported the repression of pyoverdine production by vanadium and explained that uptake of several metals by siderophores was possible. Duhme et al. (1998) have also demonstrated that catechol siderophores have been suggested to participate in molybdenum acquisition. These results are encouraging as they suggest it is possible to utilise bacteria to protect and stimulate plant growth in soils which are polluted with pesticides and

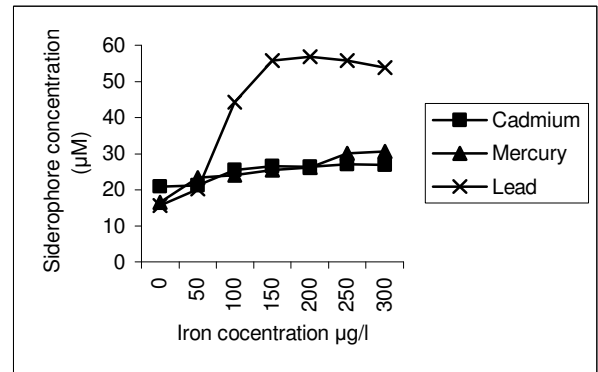


Figure 6. Effect of iron on siderophore production by *P. fluorescens* P5-18 in the presence of some heavy metals.

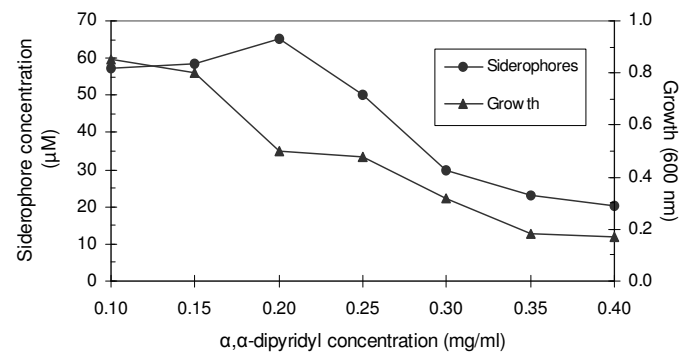


Figure 7. Effect of α,α -dipyridyl on siderophore production by *P. fluorescens* P5-18.

agrochemicals (Vivas et al., 2003).

The iron chelator, α,α -dipyridyl, diminished the contaminating iron content of the medium and growth yield increased significantly with the amount of siderophores produced at 0.20 mg/l α,α -dipyridyl (Figure 7). With 8-hydroxyquinoline both parameters were diminished. Growth (optical density) was decreased to 0.35 and siderophore production to 20.1 μ M. Others have also used strong iron chelators such as 8-hydroxyquinoline (Meyer and Abdallah, 1978) and EDTA (Manninen and Sandholm, 1993) to show growth inhibition, and Totter and Mosley (1952) confirmed the negative effect of 8-hydroxyquinoline on siderophore production.

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