Acquisition of iron by the non-siderophore-producing *Pseudomonas fragi*

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The iron requirement, siderophore production and iron uptake mechanisms of the type strain *Pseudomonas fragi* ATCC 4973 and five *P. fragi* isolates from meat were analysed. The strains exhibited a high sensitivity to iron starvation: their growth was strongly inhibited in medium supplemented with the iron chelator ethylenediamine di(hydroxyphenylacetic acid) or in medium treated with 8-hydroxyquinoline to remove contaminating iron. No siderophores were detectable in the growth supernatants of iron-starved cells. Cross-feeding experiments in iron-depleted medium showed, however, that the bacterial growth could be strongly stimulated by siderophores of foreign origin including desferriferrioxamine B, enterobactin and some pyoverdines. Moreover, all the strains were capable of efficiently using the iron sources present in their natural environment, i.e. transferrin, lactoferrin and haemoglobin. Iron starvation led to the specific production of supplementary outer-membrane proteins of apparent molecular mass ranging from 80 to 88 kDa. Furthermore, growth in the presence of exogenous siderophores resulted, in some strains, in the induction of siderophore-mediated iron uptake systems. For one strain the concomitant synthesis of an iron-regulated, siderophore-inducible outer-membrane protein was observed.

Keywords: iron acquisition, *Pseudomonas fragi*, siderophore

INTRODUCTION

To fulfil their iron requirement, Gram-negative bacteria have evolved sophisticated iron uptake systems which usually involve the excretion, by iron-starved cells, of low-molecular-mass iron-sequestering compounds known under the generic name of siderophores (Neilands, 1981). The iron-siderophore complexes which form outside the cells are then taken up by outer-membrane proteins acting as specialized receptors (Neilands, 1982), while other proteins located in the periplasm and in the cytoplasmic membrane participate in the internalization of iron (Braun *et al.*, 1987).

Siderophore-mediated iron uptake systems are well documented for the so-called 'fluorescent' pseudomonads, particularly for strains implicated in human, animal or

plant pathogenicity, e.g. *Pseudomonas aeruginosa* (Cox, 1980; Cox & Adams, 1985) and *P. syringae* (Cody & Gross, 1987), or in biological control (de Weger *et al.*, 1988; O'Sullivan & O'Gara, 1992). These organisms all produce the structurally related pyoverdine siderophores (Budzikiewicz, 1993), responsible for the characteristic yellow-green fluorescent pigmentation observed during growth under conditions of iron stress (Meyer *et al.*, 1987). Supplementary siderophores, e.g. pyochelin (Cox, 1980) or salicylic acid (Meyer *et al.*, 1992; Visca *et al.*, 1993), may be produced together with pyoverdines. For some strains, pyoverdine- and pyochelin-related ferri-siderophore receptors have been characterized at the level of the outer membrane (Magazin *et al.*, 1986; Marugg *et al.*, 1989; Meyer *et al.*, 1990; Heinrichs *et al.*, 1991). Moreover, the existence of TonB and Fur homologues in *Pseudomonas* (Bitter *et al.*, 1993; Prince *et al.*, 1993) strongly suggests that substrate translocation and iron regulation involve mechanisms similar to those described in detail for *Escherichia coli* and other enterobacteria (Braun *et al.*, 1987; Bagg & Neilands, 1987). However, the involvement of additional positive regulatory factors has also

Abbreviations: EDDHA, ethylenediamine di(hydroxyphenylacetic acid); HDTMA, hexadecyltrimethylammonium bromide; IROMP, iron-regulated outer-membrane protein.

been reported (O'Sullivan & O'Gara, 1992; Venturi *et al.*, 1993).

Iron uptake by non-fluorescent pseudomonads, although they represent the majority of strains belonging to the *Pseudomonadaceae*, is not as well documented. The wide variety of chemically unrelated siderophores so far described, including desferrioxamine E (Meyer & Abdallah, 1980), cepabactin (Meyer *et al.*, 1989), pyochelin (Sokol, 1986), salicylic acid (Visca *et al.*, 1993) and ornibactins (Stephan *et al.*, 1993), involve only a few species of non-fluorescent *Pseudomonas*, i.e. *P. stutzeri* and *Burkholderia* (formerly *Pseudomonas*) *cepacia* or *B. cepacia*-related species. Preliminary investigations of other non-fluorescent pseudomonad species, including *P. diminuta*, *P. fragi* and *P. mendocina*, revealed that the ATCC type strains of these species do not appear to produce any siderophores (Meyer *et al.*, 1987). How these strains meet their iron requirements is not understood at all. Of these species, *P. fragi* is of particular interest since its natural environment includes meat or milk products (Gill & Newton, 1977; Molin & Ternström, 1982; Ternström *et al.*, 1993). In such environments iron is tightly bound to proteins like transferrin, ferritin, lactoferrin and haemoglobin, and should, therefore, be more difficult to access by bacteria as compared to other natural environments where iron is available as iron salts or iron hydroxides. Previous work on *P. fragi* meat isolates revealed growth inhibition of these bacteria when grown in the presence of some other *Pseudomonas* spp. Moreover, their sensitivity to iron-chelating compounds such as ethylenediamine di(hydroxyphenylacetic acid) (EDDHA) suggests that the growth inhibition of *P. fragi* observed in mixed cultures could result from the production of siderophores and/or bacteriocin-like substances by the antagonist bacteria (Champomier-Vergès & Richard, 1994). The present study was undertaken to define more precisely the iron requirement of *P. fragi* strains and to better understand the iron uptake mechanism used by this organism.

METHODS

Bacterial strains, growth conditions and siderophore detection. *Pseudomonas fragi* ATCC 4973 (the type strain) and five other meat isolates identified as *P. fragi* were used, together with five fluorescent pseudomonads, *P. aeruginosa* ATCC 15692 (PAO1 strain), *P. putida* ATCC 12633, *P. fluorescens* ATCC 17400, and two fish isolates identified as *P. fluorescens* (strains 18.1 and 1.3) and previously shown to have antagonistic properties towards *P. fragi* (Champomier-Vergès & Richard, 1994). Two of the *P. fragi* isolates, strains 82 and 101, were kindly provided by J. C. Labadie (INRA, Theix, France). The three others, strains 9.12, 6.1 and 2.1, were isolated and characterized at the Laboratoire de Recherches sur la Viande, INRA, Jouy-en-Josas, France. Strains were maintained at -80°C in a 1:1 (v/v) mixture of 50% glycerol and LB medium and routinely grown at 30°C on solid (12 g agar l^{-1}) or liquid LB medium, or on solid King's B medium (King *et al.*, 1954). Succinate medium (Meyer & Abdallah, 1978) was used for studies requiring a chemically defined medium. When necessary, the contaminating iron in succinate medium was lowered from $2.5\ \mu\text{M}$ to about $0.5\ \mu\text{M}$ (Meyer & Abdallah, 1978) by treatment with 8-hydroxyquinoline (Waring & Werkman, 1942), yielding

the so-called iron-depleted succinate medium. For some experiments iron-depleted medium was produced by treatment of succinate medium with Chelex-100 as described by the manufacturer (Bio-Rad). Decrease of the available contaminating iron was also achieved by supplementing succinate medium, or the LB- or King's B-agar media, with EDDHA at various concentrations as indicated in the text. The EDDHA (Sigma) was purified before use by the procedure of Rogers (1973). Where indicated, growth media were supplemented with freshly prepared, filter-sterilized aqueous solutions of siderophore ($1\ \text{mM}$), FeCl_3 ($20\ \text{mM}$) or EDDHA ($50\ \text{mg ml}^{-1}$) after autoclaving.

Cultures in liquid media were prepared in 1 l Erlenmeyer flasks (500 ml) or in $180 \times 18\ \text{mm}$ capped test tubes (7.5 ml) and incubated at 30°C with shaking (200 r.p.m.). Growth in test tubes was estimated directly by measuring the turbidity in a colorimeter (Photovolt) with a filter at 650 nm, while spectrophotometric measurements of the OD_{620} were obtained for flask cultures using a Uvikon-930 spectrophotometer (Kontron Instruments).

Siderophore production was detected by growing the bacteria as single colonies on Chrome-Azurol-S (CAS) agar medium (Schwyn & Neilands, 1987) or directly from spent culture supernatants using the CAS or CAS-shuttle reagent (Schwyn & Neilands, 1987), or by adding an excess of iron (1 ml of $2\ \text{M FeCl}_3$ per 500 ml growth supernatant) to visualize the formation of siderophore-iron complexes.

Cross-feeding experiments. The influence of siderophores or iron-containing proteins on bacterial growth was studied in liquid cultures by adding purified siderophores ($50\ \mu\text{M}$), or haemoglobin, iron-saturated human transferrin or lactoferrin ($0.04\ \%$ w/v), to iron-depleted succinate medium. Haemoglobin and iron-saturated human lactoferrin were of commercial origin (Sigma); the latter was used after extensive dialysis against $150\ \text{mM NaCl}$, $10\ \text{mM NaHCO}_3$ in $10\ \text{mM HEPES}$, pH 7.4 (Morton & Williams, 1990). Iron-saturated human transferrin was purified according to Schmithaeusler *et al.*, (1984). Briefly, human serum Cohn's fraction IV, kindly provided by the Centre de Transfusion Sanguine, Strasbourg, France, was suspended in $0.05\ \text{M Tris/HCl}$, pH 8.4 ($10\ \%$, w/v), and the soluble part, after saturation with iron according to Sawatzki *et al.* (1981), was chromatographed on DEAE-Trisacryl-M (IBF, France; $400 \times 50\ \text{mm}$ column) equilibrated with the same buffer. Step elutions with $0.05\ \text{M Tris/HCl}$ (pH 8.0) buffers containing increasing concentrations of NaCl (0 , 0.04 and $0.5\ \text{M}$) permitted the isolation of ferritransferrin, which was eluted within a single peak with the $0.04\ \text{M NaCl-Tris}$ buffer. The material recovered demonstrated a purity of 98–99% as judged by polyacrylamide gel electrophoresis, and an absence of haemopexin contamination as observed by immunoelectrophoresis.

Cross-feeding experiments involving siderophores were conducted using a plate bioassay as described previously (Hohnadel & Meyer, 1988) using King's B agar medium supplemented with $250\ \mu\text{g EDDHA l}^{-1}$. Siderophores used included enterobactin isolated from *Escherichia coli* AN311 by the procedure of Young & Gibson (1979) and pyoverdines from *P. fluorescens* strains ATCC 13525, ATCC 17400, 1.3 and 18.1, and *P. aeruginosa* strains ATCC 15692 and ATCC 27853, purified according to previously published procedures (Meyer & Abdallah, 1978; Hohnadel & Meyer, 1988; Cornelis *et al.*, 1989). Desferrioxamine B was of commercial origin (Desferal, Ciba-Geigy), whereas desferrioxamine E was purified from *P. stutzeri* (Meyer & Abdallah, 1980). Ferrichrome A and coprogen were gifts from Dr G. Winkelmann (Tübingen University, Germany) and Dr N. A. C. Curtis (ICI, UK),

respectively. These compounds, supplied as iron complexes, were deferrated by the method of Wiebe & Winkelmann (1975).

⁵⁹Fe uptake. The siderophores described above were tested for their capacity to mediate iron uptake into cells grown in succinate medium or in 100 μ M iron-enriched succinate medium. The labelling mixture was composed of the following: 5 μ l commercial ⁵⁹FeCl₃ solution (25 mCi mg⁻¹, 925 MBq mg⁻¹; Amersham), 50 μ l of a 1 mM siderophore solution or 10 mM citrate solution, and 945 μ l uptake medium (succinate medium with the nitrogen source omitted). The first two ingredients were mixed and allowed to react for 10 min to ensure complete complexation of the iron before adding the uptake medium. Cells harvested by centrifugation from late-exponential-phase cultures and washed twice with distilled water were suspended in uptake medium to a final OD₆₀₀ of 0.3. Cell suspensions (9 ml) were preincubated for 10 min at 30 °C in 250 ml Erlenmeyer flasks with agitation (100 r.p.m.) before the addition of 1 ml of the labelling mixture. Samples of 1 ml were taken at 2, 5, 10 and 15 min, rapidly filtered onto membranes (HAWP, pore size 0.45 μ m; Millipore) and washed twice with 2 ml uptake medium. Cell-associated radioactivity which remained on the filters was measured in a Beckman Gammamatic 4000 counter. Assays without cells were performed as controls for the full solubilization of iron during the 15 min incubation period. The data, expressed as pmol ⁵⁹Fe incorporated per mg (dry weight) of cells, are means of at least duplicate experiments.

Outer membrane purification and SDS-PAGE. The outer membranes from cells grown in succinate medium or in iron- or siderophore-supplemented succinate medium (50 ml) were prepared by the method of Mizuno & Kageyama (1978). A simpler procedure (Pugsley & Oudega, 1987) involving the preparation of outer membranes as Triton-X-100-insoluble cellular material from 5 ml cultures was used to analyse the outer-membrane protein content of cells grown in the presence of ferritransferrin or haemoglobin. Briefly, the cells were harvested by centrifugation and resuspended in a microcentrifuge tube in 1 ml 50 mM Tris/HCl, 0.5 mM MgCl₂ buffer, pH 7.4. The cells were then broken by three cycles of 30 s sonication at 4 °C using a microsonicator probe. The supernatant of a 30 s centrifugation at 15000 *g* (repeated twice) was transferred to a fresh tube and supplemented with Triton X-100 to a final concentration of 2% (v/v). After vigorous mixing, the extract was kept on ice for 5–10 min and then centrifuged at 15000 *g* for 30 min. The Triton-insoluble material, rinsed quickly with 70% (v/v) ethanol, was resuspended in 100 μ l SDS-PAGE sample buffer and analysed by electrophoresis on 10% (w/v) polyacrylamide gels (Laemmli, 1970).

RESULTS

High sensitivity of the *P. fragi* strains to iron starvation

To assess the iron status of *P. fragi*, growth of the bacterium in media containing various levels of available iron was studied and compared with growth of *P. aeruginosa* ATCC 15692 in the same media. As previously shown for other *Pseudomonas* species (Meyer *et al.*, 1987), the succinate medium (Meyer & Abdallah, 1978) was iron-limiting for growth of *P. fragi* since supplementation with iron (40 μ M) resulted in a substantial increase in the cell yield (Table 1). The maximal cell yield in succinate medium was consistently lower (as much as one-third lower), for *P. fragi* compared to *P. aeruginosa*, whereas no significant differences in maximal cell yield were observed

between the two species grown in succinate medium supplemented with 40 μ M iron (Table 1). Decreasing the contaminating iron content of the growth medium by treatment with 8-hydroxyquinoline (Waring & Werkman, 1942), or by adsorption with Chelex-100 resin, had a more marked effect on the growth of *P. fragi* than that of *P. aeruginosa*. The Chelex-100-treated succinate medium permitted a maximal cell yield which was not more than half that reached in untreated medium (Table 1). The most efficient removal of iron, however, was obtained by 8-hydroxyquinoline treatment, which resulted in little or no growth, although supplementation of iron (40 μ M) did restore full growth in both cases (Table 1). The maximal cell yield as a function of the added iron concentration demonstrated that *P. fragi* had a more pronounced iron requirement to reach full growth than *P. aeruginosa*. *P. aeruginosa* cells were no longer iron-limited for growth at iron concentrations higher than 4–5 μ M, whereas *P. fragi* strains were still iron-limited at 10 μ M iron (data not shown).

The same conclusion was reached from experiments where contaminating iron in the medium was first chelated by EDDHA. Growth of *P. fragi* on King's B-agar medium supplemented with increasing amounts of EDDHA (0–1 mg ml⁻¹) was strongly affected for the 250 μ g ml⁻¹ EDDHA concentration and fully abolished at 500 μ g ml⁻¹, a concentration which did not affect the growth of *P. aeruginosa* or two other pyoverdine-producing *Pseudomonas* spp., *P. fluorescens* ATCC 17400 and *P. putida* ATCC 12633. Similar data were obtained in EDDHA-supplemented (liquid) succinate medium, where 500 μ g EDDHA ml⁻¹ fully inhibited the growth of *P. fragi*, with growth being restored following an iron enrichment of the medium.

Search for siderophores

All attempts to detect siderophores in iron-deficient culture supernatants of *P. fragi* strains were unsuccessful. Addition of iron chloride to the supernatants never resulted in the formation of a coloured iron complex. Furthermore, no iron-chelating compounds were detectable by the highly sensitive CAS assays defined by Schwyn & Neilands (1987). Since amino acid supplementation of a synthetic growth medium can increase siderophore production (Meyer *et al.*, 1995), *P. fragi* supernatants from 0.5% Casamino-acid-supplemented succinate cultures were tested with the CAS and CAS-shuttle assays. Growth of all six *P. fragi* strains was slightly stimulated by the amino acid supplementation, although no evidence for siderophore production was observed.

The CAS assay performed on 25-fold freeze-dried concentrated supernatants reacted positively, however. As detected by a citrate lyase assay (Moellering & Gruber, 1966) performed on the culture supernatants, the production of citrate during growth (2–7 μ M, depending on the strain) might explain this CAS-positive reaction, which could also have been due to the high phosphate concentrations reached in the concentrated supernatants (Schwyn & Neilands, 1987). Citrate production was detectable in

Table 1. Maximal growth reached by *P. fragi* strains and *P. aeruginosa* PAO1 in synthetic media with various iron contents

Strain	Maximal growth (OD ₆₅₀) expressed in			
	Medium 1: succinate medium	Medium 2: Chelex-100-treated succinate medium	Medium 3: 8-OH-quinoline-treated succinate medium	Medium 1 or 2 or 3 supplemented with 40 µM iron*
<i>P. aeruginosa</i> PAO1	0.650	0.370	0.330	0.850
<i>P. fragi</i> ATCC 4973	0.500	0.200	≤0.080†	0.870
<i>P. fragi</i> 2.1	0.220	0.100	≤0.010†	0.830
<i>P. fragi</i> 6.1	0.360	0.140	≤0.070†	0.840
<i>P. fragi</i> 9.12	0.550	0.240	≤0.115†	0.820
<i>P. fragi</i> 82	0.220	0.165	≤0.070†	0.840
<i>P. fragi</i> 101	0.270	0.130	≤0.030†	0.800

* Mean values for the three different media (standard error ≤ ±0.020).

† The value scored represents the highest value reached in a set of three experiments for which independent batches of 8-hydroxyquinoline-treated succinate medium were used.

Table 2. Siderophore-mediated cross-feeding and ⁵⁹Fe uptake in *P. fragi* strains

Key: 0, no effect on growth or no iron incorporation; +, stimulation of growth or iron incorporation ≥100 pmol ⁵⁹Fe (mg dry wt cells)⁻¹; ±, slight iron incorporation [<100 pmol ⁵⁹Fe (mg dry wt cells)⁻¹]; -, inhibition of growth.

<i>P. fragi</i> strains	Assay	Siderophores tested							
		PVD _{PAO1}	PVD ₂₇₈₅₃	PVD ₁₇₄₀₀	PVD _{1.3}	PVD _{18.1}	PVD ₁₃₅₂₅	Entero- bactin	Ferri. A* DffB*
ATCC 4973	Cross-feeding	+	+	-	-	+	0	+	0
	Uptake	±	+	0	0	0	0	0	0
6.1	Cross-feeding	+	+	-	-	+	+	+	0
	Uptake	+	0	0	0	0	+	+	0
9.12	Cross-feeding	+	+	-	-	+	+	+	0
	Uptake	±	0	0	0	0	+	+	0
2.1	Cross-feeding	+	+	-	-	+	+	0	0
	Uptake	+	0	0	0	0	+	+	0
82	Cross-feeding	+	+	-	-	+	+	+	0
	Uptake	±	0	0	0	0	+	0	0
101	Cross-feeding	+	+	-	-	+	+	+	0
	Uptake	+	0	0	0	0	+	0	0

* Ferri. A, ferrichrome A; DffB, desferriferrioxamine B.

both iron-limited and iron-supplemented conditions of growth (data not shown).

Growth stimulation of *P. fragi* strains by siderophores and iron-proteins

Cross-feeding experiments were performed on King's B agar medium supplemented with EDDHA at 250 µg ml⁻¹, a concentration which allowed a residual lawn growth of the *P. fragi* strains. Thus, the effect of various siderophores (stimulation, inhibition or no effect) on bacterial growth could be determined around

siderophore-impregnated paper disks placed on the top of the bacteria-seeded agar plates. The siderophores tested (iron-free forms) were enterobactin, pyoverdines originating from six different fluorescent *Pseudomonas* strains, desferriferrioxamine B in its commercially available form (Desferal, Ciba-Geigy), and ferrichrome A of fungal origin (*Ustilago sphaerogena*, Neilands, 1952). As shown in Table 2, ferrichrome A had no effect on growth of any of the *P. fragi* strains, while desferriferrioxamine B stimulated their growth markedly. Interestingly, of the pyoverdines, PVD_{PAO1} and PVD₂₇₈₅₃, from *P. aeruginosa* ATCC 15692 (PAO1 strain) and *P. aeruginosa* ATCC 27853,

Fig. 1. Growth (OD₆₅₀) of *P. fragi* 6.1 in succinate medium (□) or in the same medium supplemented with 40 μM FeCl₃ (●), 0.04% (w/v) iron-saturated human ferritransferrin (○), 0.04% (w/v) iron-saturated human lactoferrin (■), or 0.04% (w/v) haemoglobin (◇).

respectively, stimulated growth while the *P. fluorescens* pyoverdines PVD₁₇₄₀₀ and PVD_{1.3} inhibited the growth of *P. fragi* and PVD_{18.1} and PVD₁₃₅₂₅ stimulated growth of all strains except *P. fragi* ATCC 4973, whose growth was not influenced by PVD₁₃₅₂₅.

Consistent data were obtained for all the *P. fragi* strains from cross-feeding experiments done in liquid cultures. As an example, PVD_{PAO}, PVD₂₇₈₅₃ and to a lesser extent PVD_{18.1} reversed the growth-inhibitory effect of EDDHA on growth of *P. fragi* ATCC 4973 when added at 50 μM final concentration to succinate medium supplemented with 500 μg EDDHA ml⁻¹. PVD₁₇₄₀₀, PVD_{1.3} and PVD₁₃₅₂₅ had no such effect.

To determine if the *P. fragi* strains were able to use the iron forms available in their natural (meat or milk product) environment, i.e. transferrin, lactoferrin and haemoglobin, their growth response to the supplementation of such compounds in succinate medium was recorded and compared to the effect of an iron (FeCl₃) supplementation. As illustrated in Fig. 1 for *P. fragi* strain 6.1, haemoglobin and fully iron-saturated transferrin and lactoferrin efficiently stimulated growth. The lactoferrin-supplemented medium allowed better growth than the transferrin- and haemoglobin-supplemented media, equivalent to the growth in FeCl₃-supplemented medium. Only one strain, *P. fragi* 2.1, proved to be better stimulated by haemoglobin (data not shown).

Siderophore-mediated iron uptake

Pyoverdines, enterobactin, desferrioxamine B and ferrichrome A, together with coprogen and desferrioxamine E, were used as ⁵⁹Fe-chelating agents for iron uptake experiments. Citrate, the presence of which was detected in the *P. fragi* culture supernatants (see above),

Fig. 2. ⁵⁹Fe uptake in *P. fragi* ATCC 4973 (a) and *P. fragi* 9.12 (b) mediated by citrate (○), desferrioxamine B (●), desferrioxamine E (□), coprogen (■), enterobactin (*), PVD₂₇₈₅₃ (+), PVD₁₃₅₂₅ (x), and PVD_{PAO} (◇). Siderophores which mediated no iron uptake (◆) were PVD₁₃₅₂₅, PVD₁₇₄₀₀, PVD_{1.3}, PVD_{18.1}, enterobactin and ferrichrome A for *P. fragi* ATCC 4973 (a), and PVD₂₇₈₅₃, PVD₁₇₄₀₀, PVD_{1.3}, PVD_{18.1} and ferrichrome A for *P. fragi* 9.12 (b).

was also included in this study. As shown in Fig. 2, which details the kinetics of siderophore-mediated iron uptake obtained with *P. fragi* ATCC 4973 and *P. fragi* 9.12, the efficiency of iron uptake was siderophore- and strain-dependent. Both strains efficiently incorporated the iron complexed by citrate, desferrioxamines B and E and coprogen, whereas iron incorporation was much lower, though still detectable, for ⁵⁹Fe-pyoverdine from *P. aeruginosa* ATCC 15692. Ferrichrome A and additional pyoverdines (PVD₁₇₄₀₀, PVD_{1.3}, PVD_{18.1}) were inefficient in promoting ⁵⁹Fe uptake. *P. fragi* ATCC 4973, unlike *P. fragi* 9.12, was, however, able to use PVD₂₇₈₅₃ as an iron-transporter, whereas the latter strain incorporated ⁵⁹Fe-PVD₁₃₅₂₅ and enterobactin, which were inefficiently transported by ATCC 4973. Studies with the other *P. fragi* strains revealed that all were able to use citrate, desferrioxamines B and E and coprogen, but not ferrichrome A, PVD_{1.3} and PVD_{18.1}. Some of these data, as well as the results obtained with the other siderophores, are summarized in Table 2, where cross-feeding and uptake capacities of the iron chelators are compared. These data relate to iron-starved cells only. No siderophore-mediated iron uptake was detectable with cells harvested from succinate medium supplemented with 40 μM iron, which, however, were still able to incorporate the iron chelated by citrate, as efficiently as iron-starved cells (data not shown).

Siderophore-inducible, siderophore-mediated iron uptake

Surprisingly, some siderophores which stimulated bacterial growth during cross-feeding experiments did not facilitate the uptake of iron. This was particularly clear

Table 3. Pyoverdine-inducible, pyoverdine-mediated ^{59}Fe uptake in *P. fragi* 9.12 and *P. fragi* 82

<i>P. fragi</i> strain	Iron uptake mediated by	Iron uptake* for cells grown in succinate medium with supplementation indicated		
		None	PVD _{PAO}	PVD ₂₇₈₅₃
9.12	PVD _{PAO}	92	1357	34
	PVD ₂₇₈₅₃	0	0	199
82	PVD _{PAO}	74	657	24
	PVD ₂₇₈₅₃	0	0	197

* pmol ^{59}Fe incorporated after 15 min incubation (mg dry wt cells)⁻¹.

Fig. 3. IROMPs and siderophore-inducible IROMPs in *P. fragi* 9.12. Outer membranes from cells grown in succinate medium (lane 1), or in succinate medium supplemented with 50 μM PVD_{PAO} (lane 2) or PVD_{18.1} (lane 3), or 40 μM FeCl₃ (lane 4) were prepared as described in Methods and analysed by SDS-PAGE on a 10% polyacrylamide gel (70 μg protein per slot) with Coomassie Blue staining (Laemmli, 1970). The positions of standard proteins are shown on the left: phosphorylase b (94 kDa), albumin (67 kDa) and ovalbumin (43 kDa).

with PVD_{18.1} for all the *P. fragi* strains, and with PVD₂₇₈₅₃ for all strains except *P. fragi* ATCC 4973. PVD_{PAO} and enterobactin stimulated the growth of the latter strain, but did not facilitate iron uptake (Table 2). To resolve these discrepancies, pyoverdine-mediated iron uptake capacities of two strains (9.12 and 82) were analysed for cells grown in succinate medium with and without supplementation with pyoverdines PVD_{PAO} and PVD₂₇₈₅₃. The iron uptake data in Table 3 for *P. fragi* 9.12 grown in succinate medium with or without PVD_{PAO} (or PVD₂₇₈₅₃) supplementation clearly demonstrate that PVD_{PAO} strongly stimulated iron uptake only in cells grown in the presence of PVD_{PAO}. Similar results, though less pronounced,

were obtained with *P. fragi* 82, whereas a weak PVD₂₇₈₅₃-mediated iron uptake was observed for both strains grown in PVD₂₇₈₅₃-supplemented medium (Table 3). Among the other siderophores where discrepancies between cross-feeding and uptake data was observed, only PVD_{18.1} induced ^{59}Fe -PVD_{18.1} uptake and then only for strain 9.12 (data not shown).

IROMPs and siderophore-inducible IROMPs

The outer-membrane protein patterns of the six *P. fragi* strains grown in iron-depleted or iron-enriched media were compared on Coomassie-Blue-stained, SDS-PAGE gels. As illustrated in Fig. 3 for strain 9.12, two major protein bands characterized the iron-starved cells (lane 1) compared to fully iron-replete cells (lane 4). The apparent molecular mass of these proteins was 80 kDa for the lowest band and 88 kDa for the upper band, a size range which is in agreement with a ferrisiderophore receptor function (Neilands, 1982). The other strains showed similar IROMPs, with the exception of strain 101, which apparently lacked the 80 kDa protein (data not shown). Since light protein bands migrated at the same molecular mass range for iron-replete cells (Fig. 3, lane 4), it was not possible to conclude whether or not intermediate bands, e.g. the 85 kDa band visible in lane 1 of Fig. 3, could be considered as IROMPs.

The comparison of IROMP patterns for iron-starved cells grown in the presence or absence of pyoverdines showed that additional IROMPs appeared in pyoverdine-fed, iron-starved cells. Strain 9.12 grown in the presence of PVD_{PAO} (Fig. 3, lane 2) or PVD_{18.1} (lane 3) produced siderophore-inducible IROMPs of 84 kDa (PVD_{PAO}) and 86 kDa (PVD_{18.1}). However, no supplementary protein band was detectable for cells of strain 9.12 grown in PVD₂₇₈₅₃-supplemented succinate medium. Attempts to detect siderophore-inducible IROMPs in *P. fragi* 82 were unsuccessful, although an inducible supplementary iron uptake was detected for PVD₂₇₈₅₃ and for PVD_{PAO} (Table 3). Analysis of the outer-membrane protein pattern for cells grown with (ferri)transferrin or haemoglobin as iron sources did not result in the induction of transferrin- or haemoglobin-inducible outer-membrane proteins (data not shown).

DISCUSSION

A large majority of micro-organisms have evolved siderophore excretion and siderophore-mediated uptake systems to acquire traces of iron when grown in an iron-poor environment. The synthesis of not only one but sometimes two or even more chemically unrelated siderophores by a single strain, e.g. pyoverdine and pyochelin for *P. aeruginosa* ATCC 15692 (Cox, 1980; Cox & Adams, 1985), or pyochelin, cepabactin and ornibactins for *Burkholderia (Pseudomonas) cepacia* (Sokol, 1986; Meyer *et al.*, 1989, 1995), emphasizes the importance of iron as a nutrient in the development of the microbial cell.

A few micro-organisms do, however, grow very well in iron-poor medium apparently without producing sidero-

phores. This is the case for the lactic acid bacteria, which have resolved the problem of iron nutrition by completely abolishing any need for iron (Archibald, 1983; Pandey *et al.*, 1994). Similarly, some pathogens like *Haemophilus* (Schryvers, 1988; Morton & Williams, 1990) or *Neisseria* spp. (Simonson *et al.*, 1982; Schryvers & Morris, 1988; Martel & Lee, 1994), which require iron for growth, access it directly from host transferrin or haemoglobin in a siderophore-independent manner. Other micro-organisms, e.g. *Saccharomyces cerevisiae* (Lesuisse & Labbe, 1989) and *Legionella pneumophila* (Johnson *et al.*, 1991), incorporate iron via a surface-associated reductive mechanism, again in the absence of siderophores. *Listeria monocytogenes* also accesses iron by a reductive mechanism (Adams *et al.*, 1990) although it is additionally able to utilize exogenous siderophores (Simon *et al.*, 1995).

Pseudomonas fragi, as shown in the present study, exhibits a major iron requirement for full growth, needing at least twice as much iron as *P. aeruginosa* ATCC 15692. While the maximal cell yield of the latter micro-organism is halved in severely iron-starved media (e.g. 8-hydroxyquinoline-treated succinate medium), the iron-limitation had a much more drastic effect on *P. fragi* strains, which showed little or no growth in the same iron-depleted medium or in succinate medium supplemented with EDDHA ($500 \mu\text{g ml}^{-1}$). This apparently poor ability to acquire trace amounts of iron is consistent with the apparent lack of siderophore production by *P. fragi*. However, growth of *P. fragi* strains was highly stimulated by a $2 \mu\text{M}$ iron supplementation of the succinate medium and these strains developed well on CAS-agar medium, whose iron content, in the form of a HDTMA-CAS-iron complex, is $10 \mu\text{M}$ (Schwyn & Neilands, 1987). How the *P. fragi* strains remove this tightly bound iron, and how they capture the directly supplemented $2 \mu\text{M}$ iron, presumably present in the succinate medium as highly insoluble phosphate salts of hydroxides, remains unclear. The yellowish-coloured appearance of *P. fragi* on CAS-agar medium, despite the absence of a yellow halo, may result from a very low level of siderophore production, or from the presence of membrane-associated siderophore(s) as has been reported for mycobactin and *Mycobacterium* spp. (Ratledge, 1987). Alternatively, these cells may be able to directly reduce iron at the membrane level or access the iron from the HDTMA-CAS-Fe complex using citrate which is produced during growth. Citrate is reportedly an efficient iron carrier for *E. coli* (Frost & Rosenberg, 1973) and for *P. aeruginosa* (Cox, 1980; Harding & Royt, 1990), and appears to function as a true siderophore in *Bradyrhizobium*, where it is specifically produced during iron starvation (Guerinot *et al.*, 1990; Lesueur *et al.*, 1993). However, citrate is produced by *P. fragi* independently of the iron concentration of the medium. Therefore, it could not be considered a siderophore *per se*, although it appeared to be the most efficient iron transporter for a majority of *P. fragi* strains (Fig. 2).

In its natural environment, mainly meat and milk products (Gill & Newton, 1977; Molin & Ternström, 1982; Ternström *et al.*, 1993), *P. fragi* grows well and should, therefore, compete successfully with the other bacterial

species, such as fluorescent pseudomonads, which also participate in meat spoilage (Molin & Ternström, 1982). As demonstrated here, *P. fragi* strains are well equipped to acquire iron even if they do not apparently produce siderophores themselves. They can use natural iron sources present in their environment (e.g. transferrin, lactoferrin and haemoglobin), although the mechanism and identity of any outer-membrane receptors remains to be elucidated. Unfortunately, the dialysis bag system used by Morton & Williams (1990) for demonstrating the requirement for a direct interaction between transferrin and the *Haemophilus* cell surface was unsuccessful in the present study. *P. fragi* growth was unexpectedly stimulated in the control themselves, i.e. EDTA-bicarbonate-treated (Sambrook *et al.*, 1989) dialysis bags filled with medium instead of ferritransferrin.

As shown previously for other *Pseudomonas* spp., including *P. aeruginosa* (Meyer, 1992), *P. putida* (Jurkevitch *et al.*, 1992) and *B. (P.) cepacia* (Meyer *et al.*, 1989), *P. fragi* is also able to use siderophores of foreign origin. Consistent with this, *P. fragi* synthesizes a number of IROMPs including, at least for one strain, siderophore-inducible IROMPs, which probably act as ferrisiderophore receptors. *P. fragi* 9.12 for example, expressed two IROMPs during iron-depleted growth and produced a novel IROMP during growth in iron-depleted, pyoverdine (PVD_{PAO})-supplemented medium, which probably functions as a (ferri)PVD_{PAO} receptor. Such siderophore-inducible iron uptake systems could be a common feature among *Pseudomonas* spp., since similar mechanisms have already been described for *P. aeruginosa* and *P. putida* (Poole *et al.*, 1990; Gensberg *et al.*, 1992; Koster *et al.*, 1993). Preliminary data from DNA-hybridization studies using the PVD_{PAO} receptor gene from *P. aeruginosa* ATCC 15692 as a probe (Poole *et al.*, 1993) strongly favour the existence of a homologous gene in *P. fragi* 9.12. Cloning of this gene, as well as more detailed characterization of the other constitutive and siderophore-inducible iron uptake systems suggested by the present study, will be our next goal.

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REFERENCES

- Adams, T. J., Vartivarian, S. & Cowart, R. E. (1990). Iron acquisition of *Listeria monocytogenes*. *Infect Immun* **58**, 2715–2718.
- Archibald, F. (1983). *Lactobacillus plantarum*, an organism not requiring iron. *FEMS Microbiol Lett* **19**, 29–32.
- Bagg, A. & Neilands, J. B. (1987). Molecular mechanism of regulation of siderophore-mediated iron assimilation. *Microbiol Rev* **51**, 509–518.

- Bitter, W., Tommassen, J. & Weisbeek, P. J. (1993). Identification and characterization of *exbB*, *exbD* and *tonB* genes of *Pseudomonas putida* WCS358: their involvement in ferric-pseudobactin transport. *Mol Microbiol* **7**, 117–130.
- Braun, V., Hantke, K., Eick-Helmerich, K., Köster, W., Pressler, U., Sauer, M., Schäffer, S., Schöffler, H., Staudenmaier, H. & Zimmermann, L. (1987). Iron transport systems in *Escherichia coli*. In *Iron Transport in Microbes, Plants and Animals*, pp. 35–51. Edited by G. Winkelmann, D. van der Helm & J. B. Neilands. Weinheim: VCH Verlagsgesellschaft.
- Budzikiewicz, H. (1993). Secondary metabolites from fluorescent pseudomonads. *FEMS Microbiol Rev* **104**, 209–228.
- Champomier-Vergès, M. C. & Richard, J. (1994). Antibacterial activity among *Pseudomonas* strains of meat origin. *Lett Appl Microbiol* **18**, 18–20.
- Cody, Y. S. & Gross, D. C. (1987). Characterization of pyoverdine_{PS8}, the fluorescent siderophore produced by *Pseudomonas syringae* pv. *syringae*. *Appl Environ Microbiol* **53**, 928–934.
- Cornelis, P., Hohnadel, D. & Meyer, J. M. (1989). Evidence for different pyoverdine-mediated iron uptake systems among *Pseudomonas aeruginosa* strains. *Infect Immun* **57**, 3491–3497.
- Cox, C. D. (1980). Iron uptake with ferripyochelin and ferric citrate by *Pseudomonas aeruginosa*. *J Bacteriol* **142**, 581–587.
- Cox, C. D. & Adams, P. (1985). Siderophore activity of pyoverdine for *Pseudomonas aeruginosa*. *Infect Immun* **48**, 130–138.
- Frost, G. & Rosenberg, H. (1973). The inducible citrate-dependent iron transport system in *Escherichia coli* K12. *Biochim Biophys Acta* **330**, 90–101.
- Gensberg, K., Hughes, K. & Smith, A. W. (1992). Siderophore-specific induction of iron uptake in *Pseudomonas aeruginosa*. *J Gen Microbiol* **138**, 2381–2387.
- Gill, C. O. & Newton, K. G. (1977). The development of aerobic flora on a meat stored at chill temperature. *J Appl Bacteriol* **43**, 189–195.
- Guerinot, M. L., Meidl, E. J. & Plessner, O. (1990). Citrate as a siderophore in *Bradyrhizobium japonicum*. *J Bacteriol* **172**, 3298–3303.
- Harding, R. A. & Royt, P. (1990). Acquisition of iron from citrate by *Pseudomonas aeruginosa*. *J Gen Microbiol* **136**, 1859–1867.
- Heinrichs, D. E., Young, L. & Poole, K. (1991). Pyochelin-mediated iron transport in *Pseudomonas aeruginosa*: involvement of a high-molecular-mass outer membrane protein. *Infect Immun* **59**, 3680–3684.
- Hohnadel, D. & Meyer, J. M. (1988). Specificity of pyoverdine-mediated iron uptake among fluorescent *Pseudomonas* strains. *J Bacteriol* **170**, 4865–4873.
- Johnson, W., Varner, L. & Poch, M. (1991). Acquisition of iron by *Legionella pneumophila*: role of iron reductase. *Infect Immun* **59**, 2376–2381.
- Jurkevitch, E., Hadar, Y. & Chen, Y. (1992). Differential siderophore utilization and iron uptake by soil rhizosphere bacteria. *Appl Environ Microbiol* **58**, 119–124.
- King, E. O., Ward, M. K. & Raney, D. F. (1954). Two simple media for the demonstration of pyocyanin and fluorescein. *J Lab Clin Med* **44**, 301–307.
- Koster, M. C., van de Vossenbergh, J., Leong, J. & Weisbeek, P. J. (1993). Identification and characterization of the *pupB* gene encoding an inducible ferric-pseudobactin receptor of *Pseudomonas putida* WCS358. *Mol Microbiol* **8**, 591–601.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227**, 680–685.
- Lesueur, D., Diem, H. J. & Meyer, J. M. (1993). Iron requirements and siderophore production in *Bradyrhizobium* strains isolated from *Acacia mangium*. *J Appl Bacteriol* **74**, 675–682.
- Lesuisse, E. & Labbe, P. (1989). Reductive and nonreductive mechanisms of iron assimilation by the yeast *Saccharomyces cerevisiae*. *J Gen Microbiol* **135**, 257–263.
- Magazin, M. D., Moores, J. C. & Leong, J. (1986). Cloning of the gene coding for the outer membrane receptor for ferric pseudobactin, a siderophore from a plant growth promoting *Pseudomonas* strain. *J Biol Chem* **261**, 795–799.
- Marugg, J. D., de Weger, L. A., Nielander, H. B., Oorthuizen, M., Recourt, K., Lugtenberg, B., van der Hofstad, G. A. J. M. & Weisbeek, P. J. (1989). Cloning and characterization of a gene encoding an outer membrane protein required for siderophore-mediated uptake of Fe³⁺ in *Pseudomonas putida* WCS358. *J Bacteriol* **171**, 2819–2826.
- Martel, N. & Lee, B. C. (1994). Acquisition of heme iron by *Neisseria meningitidis* does not involve meningococcal transferrin-binding proteins. *Infect Immun* **62**, 700–703.
- Meyer, J. M. (1992). Exogenous siderophore-mediated iron uptake in *Pseudomonas aeruginosa*. Possible involvement of porin OprF in iron translocation. *J Gen Microbiol* **138**, 951–958.
- Meyer, J. M. & Abdallah, M. A. (1978). The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physico-chemical properties. *J Gen Microbiol* **107**, 319–328.
- Meyer, J. M. & Abdallah, M. A. (1980). The siderochromes of non-fluorescent pseudomonads: production of nocardamine by *Pseudomonas stutzeri*. *J Gen Microbiol* **118**, 125–129.
- Meyer, J. M., Hallé, F., Hohnadel, D., Lemanceau, P. & Ratelafiarivelo, H. (1987). Siderophores of *Pseudomonas* – biological properties. In *Iron Transport in Microbes, Plants and Animals*, pp. 188–205. Edited by G. Winkelmann, D. van der Helm & J. B. Neilands. Weinheim: VCH Verlagsgesellschaft.
- Meyer, J. M., Hallé, F. & Hohnadel, D. (1989). Cepabactin from *Pseudomonas cepacia*, a new type of siderophore. *J Gen Microbiol* **135**, 1479–1487.
- Meyer, J. M., Hohnadel, D., Kahn, A. & Cornelis, P. (1990). Pyoverdine-facilitated iron uptake in *Pseudomonas aeruginosa*: immunological characterization of the ferripyoverdine receptor. *Mol Microbiol* **4**, 1401–1405.
- Meyer, J. M., Azelvandre, P. & Georges, C. (1992). Iron metabolism in *Pseudomonas*: salicylic acid, a siderophore of *Pseudomonas fluorescens* CHA0. *Biofactors* **4**, 23–27.
- Meyer, J. M., Tran Van, V., Stintzi, A., Berge, O. & Winkelmann, G. (1995). Ornibactin production and transport properties in strains of *Burkholderia vietnamiensis* and *Burkholderia cepacia* (formerly *Pseudomonas cepacia*). *BioMetals* **8**, 309–317.
- Mizuno, T. & Kageyama, A. (1978). Separation and characterization of outer membrane of *Pseudomonas aeruginosa*. *J Biochem* **84**, 179–191.
- Moellering, H. & Gruber, W. (1966). Determination of citrate with citrate lyase. *Anal Biochem* **17**, 369–376.
- Molin, G. & Ternström, A. (1982). Numerical taxonomy of psychrotrophic pseudomonads. *J Gen Microbiol* **128**, 1249–1264.
- Morton, D. J. & Williams, P. (1990). Siderophore-independent acquisition of transferrin-bound iron by *Haemophilus influenzae* type b. *J Gen Microbiol* **136**, 927–933.
- Neilands, J. B. (1952). A crystalline organo-iron pigment from a rust fungus (*Ustilago sphaerogena*). *J Am Chem Soc* **74**, 4846–4847.
- Neilands, J. B. (1981). Microbial iron compounds. *Annu Rev Biochem* **50**, 715–731.
- Neilands, J. B. (1982). Microbial envelope proteins related to iron. *Annu Rev Microbiol* **36**, 285–309.

- O'Sullivan, D. J. & O'Gara, F. (1992). Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. *Microbiol Rev* **56**, 662–676.
- Pandey, A., Bringel, F. & Meyer, J. M. (1994). Iron requirement and search for siderophores in lactic acid bacteria. *Appl Microbiol Biotechnol* **40**, 735–739.
- Poole, K., Young, L. & Neshat, S. (1990). Enterobactin-mediated iron transport in *Pseudomonas aeruginosa*. *J Bacteriol* **172**, 6991–6996.
- Poole, K., Neshat, S., Krebes, K. & Heinrichs, D. E. (1993). Cloning and nucleotide sequence analysis of the ferripyoverdine receptor gene *fvpA* of *Pseudomonas aeruginosa*. *J Bacteriol* **175**, 4597–4604.
- Prince, P. W., Cox, C. D. & Vasil, M. L. (1993). Coordinate regulation of siderophore and exotoxin A production: molecular cloning and sequencing of the *Pseudomonas aeruginosa fur* gene. *J Bacteriol* **175**, 2589–2598.
- Pugsley, A. P. & Oudega, B. (1987). Methods for studying colicins and their plasmids. In *Plasmids, a Practical Approach*, pp. 105–161. Edited by K. G. Hardy. Oxford: IRL Press.
- Ratledge, C. (1987). Iron metabolism in mycobacteria. In *Iron Transport in Microbes, Plants and Animals*, pp. 207–221. Edited by G. Winkelmann, D. van der Helm & J. B. Neilands. Weinheim: VCH Verlagsgesellschaft.
- Rogers, H. J. (1973). Iron-binding catechols and virulence in *Escherichia coli*. *Infect Immun* **7**, 445–456.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sawatzki, G., Anselstetter, V. & Kubanek, B. (1981). Isolation of mouse transferrin using salting-out chromatography on Sepharose CL6B. *Biochim Biophys Acta* **667**, 132–138.
- Schmithaesler, R., Ratefiarivelo, H. & Meyer, J. M. (1984). Purification de transferrine (Tsf) humaine par chromatographie d'échange d'ion à partir de la fraction IV de Cohn. In *Premier Colloque National de Technologies de Purification de Protéines, Paris, Oct. 1984*.
- Schryvers, A. B. (1988). Characterization of the human transferrin and lactoferrin receptors in *Haemophilus influenzae*. *Mol Microbiol* **2**, 467–472.
- Schryvers, A. B. & Morris, L. J. (1988). Identification and characterization of the transferrin receptor from *Neisseria gonorrhoeae*. *Mol Microbiol* **2**, 281–288.
- Schwyn, B. & Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* **160**, 47–56.
- Simon, N., Coulanges, V., André, P. & Vidon, D. J.-M. (1995). Utilization of exogenous siderophores and natural catechols by *Listeria monocytogenes*. *Appl Environ Microbiol* **61**, 1643–1645.
- Simonson, C., Brenner, D. & Devoe, I. W. (1982). Expression of a high-affinity mechanism for acquisition of transferrin iron by *Neisseria meningitidis*. *Infect Immun* **36**, 107–113.
- Sokol, P. A. (1986). Production and utilization of pyochelin by clinical isolates of *Pseudomonas cepacia*. *J Clin Microbiol* **23**, 560–562.
- Stephan, H., Freund, S., Beck, W., Jung, G., Meyer, J. M. & Winkelmann, G. (1993). Ornibactins – a new family of siderophores from *Pseudomonas*. *BioMetals* **6**, 93–100.
- Ternström, A., Lindberg, A. M. & Molin, G. (1993). Classification of the spoilage flora of raw and pasteurized bovine milk with special reference to *Pseudomonas* and *Bacillus*. *J Appl Bacteriol* **75**, 25–34.
- Venturi, V., Ottevanger, C., Leong, J. & Weisbeek, P. J. (1993). Identification and characterization of a siderophore regulatory gene (*pfrA*) of *Pseudomonas putida* WCS358: homology to the alginate regulatory gene *algQ* of *Pseudomonas aeruginosa*. *Mol Microbiol* **10**, 63–73.
- Visca, P., Ciervo, A., Sanfilippo, V. & Orsi, N. (1993). Iron-regulated salicylate synthesis by *Pseudomonas* spp. *J Gen Microbiol* **139**, 1995–2001.
- Waring, W. S. & Werkman, C. H. (1942). Growth of bacteria in an iron-free medium. *Arch Biochem* **1**, 303–310.
- de Weger, L. A., von Arendonk, J. C. H. M., Recourt, K., van der Hofstad, G. A. J. M., Weisbeek, P. J. & Lugtenberg, B. (1988). Siderophore-mediated iron uptake in the plant-stimulating *Pseudomonas putida* WCS358 and other rhizosphere microorganisms. *J Bacteriol* **170**, 4693–4698.
- Wiebe, C. & Winkelmann, G. (1975). Kinetics studies on the specificity of chelate iron uptake in *Aspergillus*. *J Bacteriol* **123**, 837–842.
- Young, I. G. & Gibson, F. (1979). Isolation of enterochelin from *Escherichia coli*. *Methods Enzymol* **56**, 394–398.

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