Microbiology (1996), 142, 1191-1199

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

Marie-Christine Champomier-Vergès,¹ Alain Stintzi² and Jean-Marie Meyer²

Author for correspondence: Jean-Marie Meyer. Tel: +33 88 24 41 50. Fax: +33 88 35 84 84; e-mail: meyer@gem.u-strasbg.fr

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 ferrisiderophore 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 reported (O’Sullivan & 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. mendoza, 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 μM to about 0.5 μM (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 mM), FeCl₃ (20 mM) or EDDHA (50 mg ml⁻¹) after autoclaving.

Cultures in liquid media were prepared in 1 l Erlenmeyer flasks (500 ml) or in 180 x 18 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 OD₆₀₀ 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 (Sokol & Neillands, 1987) or directly from spent culture supernatants using the CAS or CAS-shuttle reagent (Schwyn & Neillands, 1987), or by adding an excess of iron (1 ml of 2 M FeCl₃ 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 μM), 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 mM NaCl, 10 mM NaHCO₃ in 10 mM HEPES, pH 7.4 (Morton & Williams, 1990). Iron-saturated human transferrin was purified according to Schmitthausner 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 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 x 50 mm column) equilibrated with the same buffer. Step elutions with 0.05 M Tris/HCl (pH 8.0) buffers containing increasing concentrations of NaCl (0, 0.04 and 0.5 M) permitted the isolation of ferritransferrin, which was eluted within a single peak with the 0.04 M NaCl-Tris buffer. The material recovered demonstrated a purity of 98-99% as judged by polyacrylamide gel electrophoresis, and an absence of haemoxepin 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 μg EDDHA l⁻¹. Siderophores used included enterobactin isolated from Escherichia coli AN51 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 27835, 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 desferriferrioxamine 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).
Iron acquisition by \textit{P. fragi}

RESULTS

High sensitivity of the \textit{P. fragi} strains to iron starvation

To assess the iron status of \textit{P. fragi}, growth of the bacterium in media containing various levels of available iron was studied and compared with growth of \textit{P. aeruginosa} ATCC 15692 in the same media. As previously shown for other \textit{Pseudomonas} species (Meyer et al., 1987), the succinate medium (Meyer & Abdallah, 1978) was iron-limiting for growth of \textit{P. fragi} since supplementation with iron (40 \text{mM}) 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 \textit{P. fragi} compared to \textit{P. aeruginosa}, whereas no significant differences in maximal cell yield were observed between the two species grown in succinate medium supplemented with 40 \text{mM} 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 \textit{P. fragi} than that of \textit{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 \text{mM}) did restore full growth in both cases (Table 1). The maximal cell yield as a function of the added iron concentration demonstrated that \textit{P. fragi} had a more pronounced iron requirement to reach full growth than \textit{P. aeruginosa}. \textit{P. aeruginosa} cells were no longer iron-limited for growth at iron concentrations higher than 4-5 \text{mM}, whereas \textit{P. fragi} strains were still iron-limited at 10 \text{mM} iron (data not shown).

The same conclusion was reached from experiments where contaminating iron in the medium was first chelated by EDDHA. Growth of \textit{P. fragi} on King’s B-agar medium supplemented with increasing amounts of EDDHA (0-1 \text{mg ml}^{-1}) was strongly affected for the 250 \text{mg ml}^{-1} EDDHA concentration and fully abolished at 500 \text{mg ml}^{-1}, a concentration which did not affect the growth of \textit{P. aeruginosa} or two other pyoverdine-producing \textit{Pseudomonas} spp., \textit{P. fluorescens} ATCC 17400 and \textit{P. putida} ATCC 12633. Similar data were obtained in EDDHA-supplemented (liquid) succinate medium, where 500 \text{mJ EDDHA ml}^{-1} fully inhibited the growth of \textit{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 \textit{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), \textit{P. fragi} supernatants from 0.5% Casamino-acid-supplemented succinate cultures were tested with the CAS and CAS-shuttle assays. Growth of all six \textit{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 \text{mJ}, 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium 1: succinate medium</th>
<th>Medium 2: Chelex-100-treated succinate medium</th>
<th>Medium 3: 8-OH-quinoline-treated succinate medium</th>
<th>Medium 1 or 2 or 3 supplemented with 40 μM iron*</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. aeruginosa PAO1</td>
<td>0.650</td>
<td>0.370</td>
<td>0.330</td>
<td>0.850</td>
</tr>
<tr>
<td>P. fragi ATCC 4973</td>
<td>0.500</td>
<td>0.200</td>
<td>0.080†</td>
<td>0.870</td>
</tr>
<tr>
<td>P. fragi 2.1</td>
<td>0.220</td>
<td>0.100</td>
<td>0.010†</td>
<td>0.830</td>
</tr>
<tr>
<td>P. fragi 6.1</td>
<td>0.360</td>
<td>0.140</td>
<td>0.070†</td>
<td>0.840</td>
</tr>
<tr>
<td>P. fragi 9.12</td>
<td>0.550</td>
<td>0.240</td>
<td>0.15†</td>
<td>0.820</td>
</tr>
<tr>
<td>P. fragi 82</td>
<td>0.220</td>
<td>0.165</td>
<td>0.070†</td>
<td>0.840</td>
</tr>
<tr>
<td>P. fragi 101</td>
<td>0.270</td>
<td>0.130</td>
<td>0.030†</td>
<td>0.800</td>
</tr>
</tbody>
</table>

* 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

<table>
<thead>
<tr>
<th><em>P. fragi</em> strains</th>
<th>Assay</th>
<th>Siderophores tested</th>
<th>Entero-</th>
<th>Ferri. A*</th>
<th>DfTB*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PVD&lt;sub&gt;PAO1&lt;/sub&gt;</td>
<td>PVD&lt;sub&gt;27853&lt;/sub&gt;</td>
<td>PVD&lt;sub&gt;17400&lt;/sub&gt;</td>
<td>PVD&lt;sub&gt;13&lt;/sub&gt;</td>
</tr>
<tr>
<td>ATCC 4973</td>
<td>Cross-feeding</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Uptake</td>
<td>±</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6.1</td>
<td>Cross-feeding</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Uptake</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9.12</td>
<td>Cross-feeding</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Uptake</td>
<td>±</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.1</td>
<td>Cross-feeding</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Uptake</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>82</td>
<td>Cross-feeding</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Uptake</td>
<td>±</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>101</td>
<td>Cross-feeding</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Uptake</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Ferri. A, ferrichrome A; DfTB, 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<sub>PAO1</sub> and PVD<sub>27853</sub> from *P. aeruginosa* ATCC 15692 (PAO1 strain) and *P. aeruginosa* ATCC 27853,
respectively, stimulated growth while the *P. fluorescens* pyoverdines PVD\textsubscript{17400} and PVD\textsubscript{1.3} inhibited the growth of *P. fragi* and PVD\textsubscript{18.1} and PVD\textsubscript{13525} stimulated growth of all strains except *P. fragi* ATCC 4973, whose growth was not influenced by PVD\textsubscript{13525}.

Consistent data were obtained for all the *P. fragi* strains from cross-feeding experiments done in liquid cultures. As an example, PVD\textsubscript{PAO}, PVD\textsubscript{27853} and to a lesser extent PVD\textsubscript{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\textsuperscript{-1}. PVD\textsubscript{17400}, PVD\textsubscript{1.3} and PVD\textsubscript{13525} 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 (FeCl\textsubscript{3}) 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 FeCl\textsubscript{3}-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, desferriferrioxamine B and ferrichrome A, together with coprogen and desferriferrioxamine E, were used as \textsuperscript{59}Fe-chelating agents for iron uptake experiments. Citrate, the presence of which was detected in the *P. fragi* culture supernatants (see above), 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, desferriferrioxamines B and E and coprogen, whereas iron incorporation was much lower, though still detectable, for \textsuperscript{59}Fe-pyoverdine from *P. aeruginosa* ATCC 15692. Ferrichrome A and additional pyoverdines (PVD\textsubscript{17400}, PVD\textsubscript{1.3}, PVD\textsubscript{19.1}) were inefficient in promoting \textsuperscript{59}Fe uptake. *P. fragi* ATCC 4973, unlike *P. fragi* 9.12, was, however, able to use PVD\textsubscript{27853} as an iron-transporter, whereas the latter strain incorporated \textsuperscript{59}Fe PVD\textsubscript{13525} and enterobactin, which were inefficiently transported by ATCC 4973. Studies with the other *P. fragi* strains revealed that all were able to use citrate, desferriferrioxamines B and E and coprogen, but not ferrichrome A, PVD\textsubscript{1.3} and PVD\textsubscript{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.
with PVD_{18,1}, for all the *P. fragi* strains, and with PVD_{27853} 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 PVD_{27853}. The iron uptake data in Table 3 for *P. fragi* 9.12 grown in succinate medium with or without PVD_{PAO} (or PVD_{27853}) 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 PVD_{27853} mediated iron uptake was observed for both strains grown in PVD_{27853}-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 PVD_{27853}-supplemented succinate medium. Attempts to detect siderophore-inducible IROMPs in *P. fragi* 82 were unsuccessful, although an inducible supplementary iron uptake was detected for PVD_{27853} 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-

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

<table>
<thead>
<tr>
<th><em>P. fragi</em> strain</th>
<th>Iron uptake mediated by</th>
<th>Iron uptake* for cells grown in succinate medium with supplementation indicated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>PVD_{PAO}</td>
</tr>
<tr>
<td>9.12</td>
<td>92</td>
<td>1357</td>
</tr>
<tr>
<td>PVD_{27853}</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>82</td>
<td>74</td>
<td>657</td>
</tr>
<tr>
<td>PVD_{PAO}</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PVD_{27853}</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* 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 FeCl$_3$ (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).
Iron acquisition by \textit{P. fragi}

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; Pankey \textit{et al.}, 1994). Similarly, some pathogens like \textit{Haemophilus} (Schryvers, 1988; Morton & Williams, 1990) or \textit{Neisseria} \textit{spp.} (Simonson \textit{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 microorganisms, e.g. \textit{Saccharomyces cerevisiae} (Lesuisse & Labbe, 1989) and \textit{Legionella pneumophila} (Johnson \textit{et al.}, 1991), incorporate iron via a surface-associated reductive mechanism, again in the absence of siderophores. \textit{Listeria monocytogenes} also accesses iron by a reductive mechanism (Adams \textit{et al.}, 1990) although it is additionally able to utilize exogenous siderophores (Simon \textit{et al.}, 1995).

\textit{Pseudomonas fragi}, as shown in the present study, exhibits a major iron requirement for full growth, needing at least twice as much iron as \textit{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 \textit{P. fragi} strains, which showed little or no growth in the same iron-depleted medium or in succinate medium supplemented with EDDHA (500 \textmu g ml$^{-1}$). This apparently poor ability to acquire trace amounts of iron is consistent with the apparent lack of siderophore production by \textit{P. fragi}. However, growth of \textit{P. fragi} strains was highly stimulated by a 2 \textmu 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 \textmu M (Schwyn & Neilands, 1987). How the \textit{P. fragi} strains remove this tightly bound iron, and how they capture the directly supplemented 2 \textmu M iron, presumably present in the succinate medium as highly insoluble phosphate salts of hydroxides, remains unclear. The yellowish-coloured appearance of \textit{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 \textit{Mycobacterium} \textit{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 \textit{E. coli} (Frost & Rosenberg, 1973) and for \textit{P. aeruginosa} (Cox, 1980; Harding & Royt, 1990), and appears to function as a true siderophore in \textit{Bradyrhizobium}, where it is specifically produced during iron starvation (Guerinot \textit{et al.}, 1990; Lesueur \textit{et al.}, 1993). However, citrate is produced by \textit{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 \textit{P. fragi} strains (Fig. 2).

In its natural environment, mainly meat and milk products (Gill & Newton, 1977; Molin & Ternström, 1982; Ternström \textit{et al.}, 1993), \textit{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, \textit{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 \textit{Haemophilus} cell surface was unsuccessful in the present study. \textit{P. fragi} growth was unexpectedly stimulated in the control themselves, i.e. EDTA-bicarbonate-treated (Sambrook \textit{et al.}, 1989) dialysis bags filled with medium instead of ferritansferrin.

As shown previously for other \textit{Pseudomonas} \textit{spp.}, including \textit{P. aeruginosa} (Meyer, 1992), \textit{P. putida} (Jarkevitch \textit{et al.}, 1992) and \textit{B. (P.) cepacia} (Meyer \textit{et al.}, 1989), \textit{P. fragi} is also able to use siderophores of foreign origin. Consistent with this, \textit{P. fragi} synthesizes a number of IRMOps including, at least for one strain, siderophore-inducible IRMOps, which probably act as ferrisiderophore receptors. \textit{P. fragi} 9.12 for example, expressed two IRMOps during iron-depleted growth and produced a novel IROMP during growth in iron-depleted pyoverdin (PVD$\text{P}_{\text{PAO}}$)-supplemented medium, which probably functions as a (ferrisiderophore) receptor. Such siderophore-inducible iron uptake systems could be a common feature among \textit{Pseudomonas} \textit{spp.}, since similar mechanisms have already been described for \textit{P. aeruginosa} and \textit{P. putida} (Poole \textit{et al.}, 1990; Gensberg \textit{et al.}, 1992; Koster \textit{et al.}, 1993). Preliminary data from DNA-hybridization studies using the PVD$\text{P}_{\text{PAO}}$ receptor gene from \textit{P. aeruginosa ATCC 15692} as a probe (Poole \textit{et al.}, 1993) strongly favour the existence of a homologous gene in \textit{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.

ACKNOWLEDGEMENTS

We are grateful to Nigel Curtis, Jean-Claude Labadie, Keith Poole and Günther Winkelmann for supplying strains and siderophores, and to Gérard Seyer for technical assistance. Critical comments and improvement of the manuscript by Keith Poole and Ashok Pankey are gratefully acknowledged. M.-C.C.-V. is indebted to Jean Richard and Dominique Expert for advice and encouragement at the beginning of this work and to Jean-Claude Hubert for hospitality in his laboratory.

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Received 20 December 1995; accepted 4 January 1996.