Siderophore-specific induction of iron uptake in *Pseudomonas aeruginosa*

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*Pseudomonas aeruginosa* has two siderophore-based high-affinity iron-uptake systems utilizing pyoverdin and pyochelin. Using strain IAl, a mutant deficient in production of both siderophores, we have shown that addition of purified siderophore to the growth medium induces expression of specific iron-regulated outer-membrane proteins and increases $^{55}$Fe-siderophore transport. Addition of pyoverdin from the parent strain PA01 or from a clinical strain 0:12 induced expression of an 85 kDa IROMP and increased the rate of $^{55}$Fe-pyoverdin transport. Transport rates for $^{55}$Fe-PA01 pyoverdin increased from 1.27 to 3.57 pmol Fe min$^{-1}$ per 10$^9$ cells. Addition of purified pyochelin induced expression of a 75 kDa IROMP accompanied with increased $^{55}$Fe-pyochelin uptake without affecting $^{55}$Fe-pyoverdin transport. $^{55}$Fe-pyochelin transport increased from 0.3 to 10.6 pmol min$^{-1}$ per 10$^9$ cells. Addition of pyoverdin from the parent strain or a chromatographically distinct pyoverdin caused increased reactivity with an anti-85 kDa mAb in Western blotting, indicating that the same receptor is being induced. These results suggest that *P. aeruginosa* can respond specifically to the presence of siderophore and moreover that not only can the pyoverdin receptor transport its cognate ferri-pyoverdin but also different ferri-pyoverdins, albeit at a reduced rate.

**Introduction**

*Pseudomonas aeruginosa* has two siderophore-mediated iron uptake systems based on pyochelin and pyoverdin. These systems will operate both in the organism's natural habitat, soil and water, where the solubility of iron at neutral pH is extremely low, and in the human host where the availability of free iron is too low to sustain bacterial growth due to the iron-binding glycoproteins transferrin and lactoferrin (Griffiths, 1991).

Pyochelin comprises a salicyl ring bonded to a thiazoline ring, which is itself bonded to a N-methylthiazolidine ring (Cox et al., 1981), and appears to be produced by all strains. Two iron-regulated outer-membrane protein (IROMP) uptake systems have been identified for this siderophore. Firstly, a 14 kDa ferri-pyochelin binding protein was detected (Sokol & Woods, 1983) and mutants deficient in surface expression of this protein showed reduced ferri-pyochelin uptake (Sokol, 1987). Secondly, a 75 kDa IROMP has been shown to be associated with ferri-pyochelin uptake (Heinrichs et al., 1991) using protease protection and labelled-iron transport studies. Mutants deficient in expression either of the 14 kDa protein or the 75 kDa IROMP were still able to transport ferri-pyochelin, indicating that both systems are operative. Heinrichs et al. (1991) suggested that the 14 kDa system operated in exponential phase cells, whereas the 75 kDa IROMP transporter was of greater importance in late exponential and early stationary phase.

*P. aeruginosa* also produces pyoverdins, which are structurally related to the pseudobactins from other fluorescent pseudomonads. These are highly water-soluble yellow-green compounds which all share a dihydroxyquinoline chromophore and two N-hydroxyornithines attached to a varying amino acid backbone of 6–10 residues (Demange et al., 1987). Evidence is now emerging that transport of different ferri-pyoverdin and ferri-pseudobactin complexes is mediated via various IROMPs, some of which have broad specificity for several complexes and some of which are specific for a single complex. It seems likely that these interactions are controlled possibly by subtle differences in the amino acid backbone of the siderophore. Amongst *P. aeruginosa* strains, Cornelis et al. (1989) noted three pyoverdin uptake groups and polyclonal antisera raised against an 80 kDa IROMP reacted only against strains producing the same siderophore. This antiserum was subsequently shown to inhibit ferri-pyoverdin transport in this strain, confirming that the 80 kDa IROMP was a transporter.
(Meyer et al., 1990). Poole et al. (1991) have identified a 90 kDa IROMP ferri-pyoverdin transporter in the pyoverdin-deficient strain 6609. A mutant deficient in expression of this protein still showed low uptake of ferri-pyoverdin, providing evidence for a second transport system. We have obtained similar evidence in a strain made resistant to P. aeruginosa pyocin Sa. This mutant lacked an 85 kDa IROMP and was deficient in ferri-pyoverdin, providing evidence for a second transport system.

Although it is now becoming clear that P. aeruginosa has multiple iron transport systems, the contribution of these systems to growth in vivo is unclear. Ankenbauer et al. (1985) obtained mutants deficient in one or both siderophores and concluded that pyoverdin was more important for growth in the presence of serum or purified transferrin. However, the inability of the pyoverdin-deficient (pvd-) strain to grow in serum was attributed to growth having multiple iron transport systems, the contribution of pyoverdin transport, but it did still show 5% residual uptake (Smith et al., 1992).

In an attempt to understand the regulation of expression of iron-uptake systems in P. aeruginosa, we have been investigating iron uptake in a mutant deficient in both pyoverdin and pyochelin production. In this report, we demonstrate induction of specific IROMPs in response to growth either in the presence of pyoverdin or pyochelin and show that induction of iron uptake is siderophore-specific.

**Methods**

**Bacterial strains and culture conditions.** P. aeruginosa IA1, a Pvd "Pch" mutant of PAO1 (ATCC 15692) obtained from Dr C. D. Cox (Ankenbauer et al., 1985) was used. PAO1 (ATCC 15692) was obtained from the Aston Collection and 0.0025% (w/v) 4-chloro-1-naphthol (Sigma) in 0.01 M-Tris/HCl, pH 7.4) to block non-specific antibody binding. The nitrocellulose paper was incubated for 4 h at 37 °C with hybridoma supernatant 1 : 50 in TBS/milk. The blot was washed three times in TBS and incubated with 1 : 1000 protein A (from *Staphylococcus aureus*) conjugated to horseradish peroxidase (Sigma) in TBS/milk for 2 h at 37 °C. The antigenic components were visualized with 0.0025% (w/v) 4-chloro-1-naphthol (Sigma) in 0.01% (v/v) H₂O₂ in 10 mM-Tris/HCl, at pH 7-4.

**Purification of pyoverdin.** The method was adapted from Poole et al. (1991). Cells were grown to early stationary phase in 500 ml of succinate medium at 37 °C and removed by centrifugation at 10000 g for 10 min. The supernatant was freeze-dried, resuspended in 12 ml of water and any undissolved matter removed by centrifugation. This solution was extracted with an equal volume of ethyl acetate and then saturated with NaCl prior to extraction with 0.5 vol. phenol/chloroform (in equal parts). The aqueous phase was re-extracted and the pooled organic phases were mixed with 2 vol. diethyl ether. The precipitated pyoverdin was recovered by centrifugation at 10000 g and washed three times with ether. Finally, the pellet was dried and dissolved in water to 20 mg ml⁻¹.

**55-Fe-Pyoverdin uptake.** The general procedure of Poole et al. (1991) was used. Cells were grown in succinate medium to late exponential phase and harvested by centrifugation at 4 °C. The cells were washed twice with succinate medium, resuspended to an OD₆₀₀ of 1.0, and equilibrated at 37 °C for 15 min prior to transport assays. During equilibration, no observable increase in OD₆₀₀ was observed. Pyoverdin (80 pg) and 115 nM-55FeCl₃ in 0.5 ml of 55Fe-Pyoverdin uptake. This was performed on 12% (w/v) acrylamide gels (Lugtenberg et al., 1975) in a Mini-Protein apparatus (Bio-Rad). OM samples were purified on an equal volume of sample buffer (50 mM-Tris/HCl, pH 6-8, 2% (w/v) SDS, 10% (v/v) glycerol and 1% (v/v) β-mercaptoethanol) and boiled at 100 °C for 10 min prior to electrophoresis. Gels were stained with Coomassie Brilliant blue R-250 in methanol/water/acetic acid (50:40:10, by vol.) and destained in water/methanol/acetic acid (83:10:7, by vol.).

**Immunoblotting procedure.** Proteins separated by SDS-PAGE were electroblotted to nitrocellulose by the method of Towbin et al. (1977) using a Transblot apparatus (Bio-Rad). Transfer of proteins was confirmed by staining with 1% (w/v) amido black in water/methanol/acetic acid (83:10:7, by vol.). The nitrocellulose was incubated at 37 °C for 1 h in 5% (w/v) skimmed milk in Tris-buffered saline (TBS; 0.9% w/v, NaCl in 10 mM-Tris/HCl, pH 7-4) to block non-specific antibody binding. The nitrocellulose paper was incubated for 4 h at 37 °C with hybridoma supernatant 1 : 50 in TBS/milk. The blot was washed three times in TBS and incubated with 1 : 1000 protein A (from *Staphylococcus aureus*) conjugated to horseradish peroxidase (Sigma) in TBS/milk for 2 h at 37 °C. The antigenic components were visualized with 0.0025% (w/v) 4-chloro-1-naphthol (Sigma) in 0.01% (v/v) H₂O₂ in 10 mM-Tris/HCl, at pH 7-4.

**Purification of pyochelin.** The method was adapted from Poole et al. (1991). Cells were grown to early stationary phase in 500 ml of succinate medium at 37 °C and removed by centrifugation at 10000 g for 10 min. The supernatant was freeze-dried, resuspended in 12 ml of water and any undissolved matter removed by centrifugation. This solution was extracted with an equal volume of ethyl acetate and then saturated with NaCl prior to two extractions with 0.5 vol. phenol/chloroform (in equal parts). The aqueous phase was re-extracted and the pooled organic phases were mixed with 2 vol. diethyl ether. The precipitated pyochelin was recovered by centrifugation at 10000 g and washed three times with ether. Finally, the pellet was dried and dissolved in water to 20 mg ml⁻¹.
Induction of iron uptake in P. aeruginosa

The cells were harvested and washed as described above for the 55Fe-pyoverdin uptake studies. Pyochelin (10 mM in methanol) and 55FeCl₃ were added to final concentrations of 20 µg ml⁻¹ and 115 nM, respectively, in succinate medium 15 min prior to the start of an uptake experiment. Uptake was initiated by addition of cells at 37 °C to a final OD₄₇₀ of 1.0. Cell filtration and scintillation counting were as described above, except that cells were washed with 0.5 M-HCl.

Results

Analysis of IROMPs

Preliminary experiments in which crude supernatants from cultures of 17 other cystic-fibrosis-derived clinical isolates of P. aeruginosa were added to IA1 indicated that growth could be either increased or inhibited depending on the strain, suggesting that IA1 could be cross-fed from some siderophores, but not from others. In order to assess whether these effects were reflected in changes in OM protein profiles, siderophores were purified and added to cultures of IA1 in succinate medium. The IROMP profiles are shown in Fig. 1. In succinate medium alone, IA1 strongly expressed three IROMPs of 81, 83 and 96 kDa (lane 1), whereas addition of pyoverdin from the parental strain PAO1 (lane 2) caused induction of an 85 kDa IROMP and partially repressed the 96, 83 and 81 kDa IROMPs. Addition of chromatographically distinct pyoverdin from strain 0:12 (lane 3) induced the 85 kDa IROMP, and partially repressed the 81 kDa IROMP. Addition of purified pyochelin increased expression of a 75 kDa IROMP only (lane 4). Growth in iron-replete succinate medium (lane 5) repressed all high molecular mass IROMPs.

Fig. 2 shows a Western blot of the IROMPs reacted with mAb C108, which was raised against an 85 kDa IROMP from strain AK1282, an LPS-defective derivative of PAO1 (Smith et al., 1991). No reaction was seen against IROMPs prepared from iron-replete cells (lane 5). Weak activity was seen against IROMPs of IA1 alone (lane 1) or supplemented with pyochelin (lane 4), whereas a strong reaction with some cross-reactivity with lower molecular mass proteins was seen against IROMPs from cells grown in the presence of added pyoverdins (lanes 2 and 3).

Ferri-pyoverdin uptake after growth with pyochelin or pyoverdin

Since cross-feeding with purified pyoverdins caused changes in IROMP profiles, labelled-iron transport studies were performed to determine whether ferrisiderophore uptake was affected. Fig. 3 shows iron uptake from ferri-pyoverdin after growth in succinate medium with and without addition of pyoverdin or pyochelin. Growth in the presence of pyoverdin caused a 2.8-fold increase in the initial rate of iron uptake from ferri-pyoverdin. The rate increased from 1.27 pmol Fe min⁻¹ per 10⁹ cells without induction to 3.57 pmol Fe min⁻¹ per 10⁹ cells with growth in the presence of pyoverdin. Growth in medium supplemented with pyochelin had no effect on ferri-pyoverdin uptake.Preincubation of cells with mAb C108 hybridoma supernatant diluted 1:50 in succinate medium during the 15 min equilibration period at 37 °C did not affect uptake (data not shown).

![Fig. 1. SDS-PAGE of OMs prepared from P. aeruginosa IA1 grown in iron-deficient succinate medium (lane 1), supplemented with 50 µg PAO1 pyoverdin ml⁻¹ (lane 2), 50 µg 0:12 pyoverdin ml⁻¹ (lane 3), 10 µg pyochelin ml⁻¹ (lane 4) and 100 µM-FeCl₃ (lane 5). Each lane contained 5 µg protein.](image1)

![Fig. 2. Immunoblot probed with mAb C108. Lanes are as described in Fig. 1.](image2)
Fig. 3. PAO1-pyoverdin-mediated iron ($^{55}$Fe$^{3+}$) uptake by *P. aeruginosa* IA1 grown in iron-deficient succinate medium (■), supplemented with 50 μg PAO1 pyoverdin ml$^{-1}$ (■) or supplemented with 10 μg pyochelin ml$^{-1}$ (▲). The uptake mixture contained pyoverdin (80 μg ml$^{-1}$), $^{55}$FeCl$_3$ (115 nM) and 1 ml of cells at an OD$_{470}$ of 1.0. Experiments were performed in triplicate and the results expressed as the mean ± SD.

Fig. 4. Pyochelin-mediated iron ($^{55}$Fe$^{3+}$) uptake by *P. aeruginosa* IA1 grown in iron-deficient succinate medium (■), supplemented with 50 μg PAO1 pyoverdin ml$^{-1}$ (▲) or supplemented with 10 μg pyochelin ml$^{-1}$ (■). The uptake mixture contained pyochelin (20 μg ml$^{-1}$), $^{55}$FeCl$_3$ (115 nM) and 1 ml of cells at an OD$_{470}$ of 1.0. Experiments were performed in triplicate and the results expressed as the mean ± SD.

Fig. 5. 0:12-pyoverdin-mediated iron ($^{55}$Fe$^{3+}$) uptake by *P. aeruginosa* IA1 grown in iron-deficient succinate medium alone (○) or supplemented with 50 μg 0:12 pyoverdin ml$^{-1}$ (●). The uptake mixture contained pyoverdin (80 μg ml$^{-1}$), $^{55}$FeCl$_3$ (115 nM) and 1 ml of cells at an OD$_{470}$ of 1.0. Experiments were performed in triplicate and the results expressed as the mean ± SD.

Fig. 6. Pyoverdin cross-induction and uptake studies in *P. aeruginosa* IA1. Pyoverdin supplementation of iron-deficient succinate growth medium and iron ($^{55}$Fe$^{3+}$) uptake studies were performed as follows: induction with 0:12 and uptake via PAO1 pyoverdins (▲); induction with PAO1 and uptake via PAO1 pyoverdin (■); induction with 0:12 and uptake via 0:12 pyoverdin (●); induction with PAO1 and uptake via 0:12 pyoverdins (□). The uptake mixture contained pyoverdin (80 μg ml$^{-1}$), $^{55}$FeCl$_3$ (115 nM) and 1 ml of cells at an OD$_{470}$ of 1.0. The dashed lines are duplicated from Figs. 3 and 5 for comparison. Experiments were performed in triplicate and the results expressed as the mean ± SD.

Ferri-pyochelin uptake after growth with pyochelin or pyoverdin

Fig. 4 shows the results of ferri-pyochelin uptake studies. Similar to the observations made for ferri-pyoverdin uptake, ferri-pyochelin uptake was increased only after growth with pyochelin. The increase in initial rate of uptake was much greater, rising from 0-3 pmol Fe min$^{-1}$ per 10$^9$ cells to 10-6 pmol Fe min$^{-1}$ per 10$^9$ cells. Pyoverdin pre-treatment had no effect, the rate of iron uptake being 0-3 pmol Fe min$^{-1}$ per 10$^9$ cells.

Pre-treatment with chromatographically distinct pyoverdins

Pyoverdins from eight strains in the laboratory collection were purified and analysed by thin-layer chromatography in methanol/water (70:30, v/v) as described by Bitter *et al.* (1991). The $R_f$ values for PAO1 and 0:12 pyoverdins were 0-38 and 0-22, respectively. Ferri-pyoverdin uptake profiles for *P. aeruginosa* IA1 using pyoverdin from strain 0:12, with and without growth in the presence of this siderophore, are shown in Fig. 5.
Increased uptake was noted with cells grown in the presence of siderophore, but the rate and extent of uptake, even after pre-treatment, were significantly less than that seen with PA01 pyoverdin (Fig. 3). The pretreatment experiments were repeated using either PA01 or 0:12 pyoverdin and uptake experiments performed with 0:12 and PA01 pyoverdins, respectively. Fig. 6 shows the uptake profiles. The increased uptake was the same, irrespective of which pyoverdin had been added to the growth medium.

Discussion

In this work we have shown that P. aeruginosa can respond specifically to the presence of siderophore in the growth medium and not simply to the iron-deficiency imposed by that siderophore. This is based on the finding that growth in the presence of pyoverdin induces ferri-pyoverdin uptake only and growth in the presence of pyochelin increases ferri-pyochelin uptake only. This may go some way to explaining why P. aeruginosa retains two siderophore-mediated iron-uptake systems, despite the observation that the binding constant of iron for pyoverdin (10^12) (Wendenbaum et al., 1983) is significantly greater than that of pyochelin (10^5 at acid pH), which in addition requires two molecules to bind one molecule of iron (Cox & Graham, 1979). We speculate that P. aeruginosa produces low amounts of each siderophore in iron-deficient growth environments and monitors which siderophore is most effective at complexing iron before becoming committed to, and inducing, iron uptake via that route. Although the observations reported in this work were made with the siderophore-deficient strain IA1, we also noted inducible uptake with the siderophore-competent parent strain PA01 (data not shown).

Our finding that the increased uptake of ferri-pyochelin after growth in pyochelin-supplemented medium correlated with increased expression of a 75 kDa IROMP supports the finding of Heinrichs et al. (1991). These workers noted increased expression of a 75 kDa IROMP in PAO 6609, a pyoverdin-biosynthesis-deficient mutant of PA01. They also showed that strain K372, a pirazmonam-resistant mutant of PAO 6609 which lacks this 75 kDa IROMP, failed to transport ferri-pyochelin. We did not observe any changes in expression of the 14 kDa ferri-pyochelin binding protein (Sokol & Woods, 1983), although our studies were carried out using early stationary phase cells when this uptake system is thought to be less important (Heinrichs et al., 1991).

Several groups have reported IROMPs in the range 80-90 kDa that are associated with ferri-pyoverdin uptake in different strains (Cornelis et al., 1989; Meyer et al., 1990; Poole et al., 1991). In this work, ferri-pyoverdin uptake correlated with expression of an 85 kDa IROMP, although we were unable to inhibit uptake with the anti-85 kDa IROMP mAb. However, in previous studies we have shown only poor interaction with whole cells, possibly as a result of being shielded by lipopolysaccharide or because the mAb was raised against an epitope which is not surface-exposed (Smith et al., 1991). It was interesting to note that uptake by IA1 of ferri-pyoverdins from other strains occurred at different rates, suggesting that either the receptor for its own pyoverdin had a broader substrate specificity, with not all ferri-pyoverdin complexes being taken up at the same rate, or that a second ferri-pyoverdin receptor exists. The induction studies with different pyoverdins, from PA01 and strain 0:12, suggest that the same receptor is induced (Fig. 1, lanes 1, 2 and 3), and this was supported further by the increased reactivity with the anti-85 kDa IROMP mAb (Fig. 2, lanes 1, 2 and 3). Pretreatment with either pyoverdin resulted in the same rate of uptake; pre-treatment with 0:12 pyoverdin resulted in the same induction of PA01 ferri-pyoverdin uptake as pretreatment with PA01 pyoverdin itself. The reverse was also true, with PA01 pyoverdin pre-treatment resulting in the same induction of 0:12 ferri-pyoverdin uptake as with 0:12 pyoverdin itself. Competition studies with 55Fe-0:12 pyoverdin and non-radioactive Fe-PA01 complex proved inconclusive (data not shown). It was expected that the rate of uptake of 55Fe-0:12 complex would be reduced in the presence of Fe-PA01 pyoverdin complex due to competition at the receptor. However, the opposite was observed. This was attributed to transport of 55Fe as the 55Fe-PA01 complex, which resulted from equilibration between the two pyoverdins. These data suggest therefore that with the pyoverdins and strains used in this study, the uptake and induction phenomena require just one receptor. However, it cannot be ruled out that there is a second uptake system, and the functions of other high molecular mass IROMPs, notably the 81 and 83 kDa proteins, remain to be determined. Poole et al. (1991) have noted residual uptake of ferri-pyoverdin complex in a mutant deficient in a 90 kDa IROMP and suggested that this is due to a second transport system. Interestingly, these workers were not able to demonstrate an induction phenomenon in this strain. Similarly, we were unable to demonstrate induction in a strain rendered unable to transport ferri-pyoverdin through resistance to P. aeruginosa pyocin Sa (Smith et al., 1992). Both these strains lacked a major IROMP associated with ferri-pyoverdin uptake, which suggests that the IROMP must be present for the induction phenomenon reported in this work to be seen.

Poole et al. (1990) have noted an induction pheno-
menon in *P. aeruginosa*, but with the enterobacterial siderophore enterobactin. Growth inhibition of a pyoverdin-deficient strain imposed by the non-metabolizable chelator ethylene diamine-di(o-hydroxyphenol acetic acid) could be reversed by addition of enterobactin. A novel 80 kDa IROMP was detected and enterobactin-grown cells transported ferri-enterobactin in an energy-dependent manner at low iron concentrations. Leong *et al.* (1991) have noted an induction response to exogenous siderophore in the related fluorescent pseudomonad *P. putida*. In their study, the gene coding for a second pseudobactin uptake system (*pupB*), located on plasmid pMK15, was identified by its ability to render transformed strains no longer susceptible to starvation by pseudobactin BN7, the native siderophore of strain BN7. However, an additional OM protein was expressed in strain A124/pMK15 only after growth in the presence of pseudobactin BN7. Moreover, these workers noted new OM proteins when strains were grown in the presence of pseudobactin B10 and pyoverdin, but not deferriferriochrome A. Our studies extend these observations to *P. aeruginosa* and to siderophores which are not structurally related, i.e. pyoverdin and pyochelin. At the present stage, it is unclear how the signal of siderophore present in the growth medium is detected by the cell, but evidence is now emerging for positive and negative iron regulators in *P. aeruginosa*. Prince *et al.* (1991) have identified a Fur homologue in *P. aeruginosa* and have also noted that the *E. coli fur* gene will regulate exotoxin A expression. Several workers have identified positive regulators of iron-regulated gene expression, notably the *regA* gene in exotoxin A expression (Wick *et al.*, 1990) and LasR regulation of elastase expression (Gambello & Igleswki, 1991) in *P. aeruginosa*, and a transcriptional activator associated with a gene from the siderophore biosynthesis/uptake cluster of a fluorescent plant pseudomonad (O’Sullivan & O’Gara, 1991).

In summary we have shown that *P. aeruginosa* can respond specifically to the presence either of pyoverdin or pyochelin in the growth medium. We are currently cloning siderophore biosynthesis/uptake genes to determine how this response is regulated.

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**References**


