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Pyochelin-Mediated Iron Transport in *Pseudomonas aeruginosa*: Involvement of a High-Molecular-Mass Outer Membrane Protein

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An iron-regulated outer membrane protein of 75,000 daltons was strongly expressed following iron limitation of strains of *Pseudomonas aeruginosa* which fail to produce pyoverdine. A mutant nonderepressible for this protein (K372) was deficient in pyochelin-mediated iron transport at 150 nM $FeCl_3$, consistent with a role for the 75-kDa protein in ferripyochelin transport. Moreover, ferripyochelin specifically protected the 75-kDa protein against trypsin digestion, supporting an interaction between ferripyochelin and the 75-kDa protein. Previous reports implicated a 14,000-dalton outer membrane protein as the receptor for ferripyochelin (P. A. Sokol and D. E. Woods, Infect. Immun. 40:665–669, 1983) and demonstrated that a mutant (FBP-28) expressing a defective 14-kDa outer membrane protein did not exhibit pyochelin-mediated iron transport (P. A. Sokol, J. Bacteriol. 169:3365–3368, 1987). Nonetheless, we were able to demonstrate (i) that FBP-28 was inducible for the 75-kDa protein under iron-limiting conditions and (ii) that concomitant with the induction of this protein in FBP-28, pyochelin-mediated iron uptake at 150 nM $FeCl_3$ was observed. Interestingly, strain K372 did transport ferripyochelin at higher (750 nM) $FeCl_3$ concentrations, suggesting that a second pyochelin-mediated iron transport system, perhaps involving the 14-kDa outer membrane protein identified previously, operates in *P. aeruginosa*.

The ability of pathogenic bacteria to acquire iron in vivo is essential for growth and, therefore, infection. Not surprisingly, then, bacterial iron transport has been correlated with the ability to cause disease and the severity of disease (10, 12, 15, 23). This need to acquire iron in vivo is, however, complicated by the presence of host iron-binding proteins (e.g., lactoferrin and transferrin) (27), which contribute to, as far as invading microorganisms are concerned, an iron-restricted environment in the host (5, 13, 18). Successful pathogens must, therefore, compete with the host for iron and, in many instances, do so via the production of high-affinity iron chelators called siderophores (22). Siderophores, in conjunction with cell surface receptors, function to transport iron into cells via high-affinity uptake systems (4, 23). The demonstrated abilities of siderophores to remove transferrin-bound iron in vitro (16, 32) and to promote growth in vivo (8, 26, 33) imply an important role for siderophores in the in vivo acquisition of iron.

Pseudomonas aeruginosa, an opportunistic pathogen of humans (3), synthesizes two known siderophores, pyochelin (2, 7) and pyoverdine (9, 11), in response to iron limitation. This organism is also capable of utilizing a number of heterologous siderophores, including enterobactin (25) and ferrioxamine B (6). Pyoverdine efficiently removes transferrin-bound iron in vitro (32), undoubtedly accounting for the ability of pyoverdine-producing strains to grow in vitro in the presence of human transferrin or serum (1). While less efficient at promoting in vitro growth in the presence of human transferrin or serum (1), pyochelin appears to play an important role in the in vivo growth and virulence of *P. aeruginosa* (8). The receptor for ferripyoverdine recently has been identified as an 80,000 (21)- or a 90,000 (24)-dalton outer membrane protein. In contrast, a 14,000-dalton outer membrane protein has been identified as the receptor for ferripyochelin (30). The 14-kDa receptor protein is weakly iron repressible, and its low molecular mass is atypical of iron-siderophore receptor proteins in general. Still, antibodies raised against the purified 14-kDa protein are capable of reducing pyochelin-mediated iron transport by intact cells of *P. aeruginosa* (31), and a mutant deficient in surface expression of this protein is devoid of pyochelin-mediated iron uptake (29). In the present study, we identify a second outer membrane protein which is involved in ferripyochelin uptake and which, in contrast to the 14-kDa protein, is strongly derepressed under iron limitation and has a high molecular mass.

MATERIALS AND METHODS

Bacterial strains and growth media. *P. aeruginosa* PAO 6609 (*met-9011 amiE200 rpsL pvd-9*) (14), PAO1 (wild type) (25), and FBP-28 (a Tn*5* insertion mutant of PAO1 deficient in surface expression of a 14-kDa pyochelin-binding protein) (29) have been described previously. *P. aeruginosa* K372 is a spontaneous pirazmonam-resistant derivative of PAO6609 which fails to express a 75-kDa outer membrane protein under iron-limiting conditions (this study). Iron-deficient medium consisted of BM2 minimal medium supplemented with 0.5 mM $MgSO_4$ and 20 mM potassium succinate (25). This medium was made iron sufficient by the addition of $FeSO_4$ (50 μM). Methionine (1 mM) was added to the above-described media as required. For the culturing of strain FBP-28, kanamycin (500 μg/ml) was included in all growth media. Solid media were obtained by the addition of 1.5% (wt/vol) Bacto Agar (Difco).

Purification of siderophores. Pyochelin was prepared from 500-ml cultures of PAO6609 grown in iron-deficient minimal medium by extraction of acidified (pH 2.0) culture supernatants with ethyl acetate (7) and purification on Sephadex LH-20 (LKB-Pharmacia) as described previously (20). Pyoverdine was prepared as described previously (24).

Outer membrane preparation and SDS-polyacrylamide gel electrophoresis. Outer membranes were prepared by differ-

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ential Triton X-100 extraction of cell envelopes as described previously (28). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out as described previously (19) with 7% (wt/vol) acrylamide in the running gel but with 2-mercaptoethanol being omitted from the sample buffer.

Transport assays. *P. aeruginosa* cells used in ^{55}Fe uptake assays were grown in iron-deficient minimal medium to an A_{600} of 1.0. Five milliliters of the cell culture was harvested by filtration on 0.45-μm-pore-size membrane filters, washed twice with an equal volume of iron-deficient minimal medium previously treated with Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.) to remove contaminating iron (25), and resuspended in an equal volume of the same medium. Cells were shaken (150 rpm) for 5 min at 37°C prior to assay. Pyochelin (60 μM) was mixed with various concentrations of $^{55}FeCl_3$ in the presence of nitrilotriacetate (1 μM) at least 15 min before the start of the assay. Twenty microliters of this mixture was added to 1 ml of cells in a 10-ml disposable culture tube to initiate uptake. Cells were vortexed gently, and aliquots (200 μl) were removed at intervals, filtered on 0.45-μm-pore-size membrane filters (GN-6; Gelman Sciences Inc., Ann Arbor, Mich.), and washed twice with 10 ml of H_2O. Membranes were dried and counted in scintillation fluid by use of the tritium channel of an LKB model 1215 Rackbeta liquid scintillation counter (Pharmacia-LKB Biotechnology AB, Uppsala, Sweden). In some experiments, an equal volume of H_2O replaced pyochelin in uptake assays. For assays that included KCN-treated cells, KCN (10 mM) was added to cells 15 min prior to harvesting.

Preparation of detergent-soluble 75-kDa protein. Outer membranes prepared from 4 liters of iron-limited PAO6609 were resuspended in 40 ml of 20 mM Tris-HCl (pH 8.0)–2% (wt/vol) Triton X-100–1 M NaCl. The insoluble fraction was collected by centrifugation (180,000 × *g*, 45 min). The extraction was repeated, and the pellet was resuspended in 40 ml of 1% (wt/vol) Zwittergent Z3-14 (Calbiochem)–20 mM Tris-HCl (pH 8.0). Following centrifugation (180,000 × *g*, 45 min), the soluble fraction (containing the 75-kDa protein) was recovered and loaded onto a 5-ml DEAE–Sepharose CL-6B (Pharmacia) column equilibrated with 1% (wt/vol) Zwittergent Z3-14–20 mM Tris-HCl (pH 8.0) (column buffer). The column was washed with 10 ml of column buffer and then with 5 ml of column buffer containing 0.1 M NaCl. Protein was eluted from the column with a 0.1 to 0.35 M NaCl gradient in column buffer. Fractions were analyzed on SDS-polyacrylamide gels, and 75-kDa protein-containing fractions were recovered and stored at −20°C.

Trypsin digests. Samples of Zwittergent Z3-14-soluble 75-kDa protein (50 μg) were incubated with trypsin (2.5 mg/ml) in 100 μl of 50 mM Tris-HCl (pH 8.0) at 37°C in the presence or absence of ferripyochelin (0.2 mM $FeCl_3$–20 mM pyochelin) or ferripyoverdine (0.2 mM $FeCl_3$–20 mM pyoverdine). Ten-microliter samples were removed before and after 2 h of incubation, heated for 5 min in sample loading buffer (19) at 95°C, and subjected to SDS-polyacrylamide gel electrophoresis.

RESULTS

Identification of a 75-kDa iron-regulated outer membrane protein involved in ferripyochelin transport. Growth of wild-type *P. aeruginosa* PAO1 under iron-limiting conditions at 37°C revealed the presence of several high-molecular-mass outer membrane proteins which were absent in cells cultured under iron-sufficient conditions (Fig. 1, compare lanes 1 and 2). A number of these iron-regulated outer membrane proteins, including one of 75 kDa, were present in noticeably increased levels in PAO1 cells grown at 42°C (Fig. 1, lane 4) as compared with 37°C (Fig. 1, lane 2). Notably, *P. aeruginosa* fails to synthesize pyoverdine at the elevated temperature (24), although pyochelin production is unaffected (23a). A mutant defective in pyoverdine biosynthesis, PAO6609 (14), showed even stronger derepression of the 75-kDa protein under iron-limiting conditions (Fig. 1, lane 6). The high molecular mass of the 75-kDa protein and its inducibility by iron limitation are typical features of iron-siderophore receptor proteins. Still, *P. aeruginosa* produces only two known siderophores, pyochelin and pyoverdine, for which receptors have already been identified. Moreover, screening of culture supernatants for novel iron-chelating compounds which might utilize the 75-kDa protein as a receptor were unsuccessful. Nonetheless, evidence for multiple transport systems for both pyoverdine (24) and enterobactin (25) in *P. aeruginosa* has been reported, such that the 75-kDa protein may play a role in iron transport mediated by a known pseudomonal siderophore.

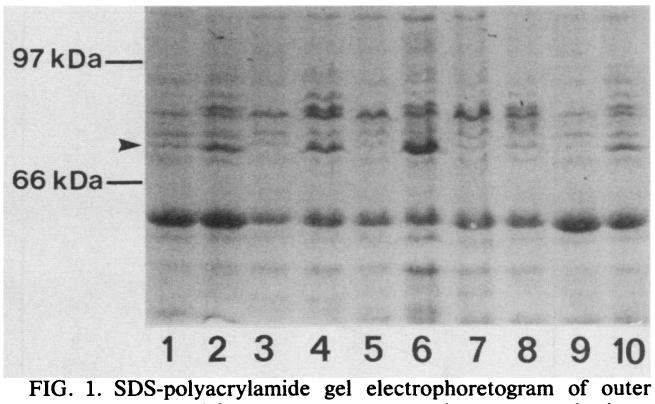

FIG. 1. SDS-polyacrylamide gel electrophoretogram of outer membranes prepared from *P. aeruginosa* strains grown under iron-sufficient (odd-numbered lanes) or iron-limiting (even-numbered lanes) conditions. Lanes: 1 to 4, PAO1; 5 and 6, PAO6609; 7 and 8, K372; 9 and 10, FBP-28. Outer membranes were prepared from cells cultured at 37°C, except for those shown in lanes 3 and 4, which were prepared from cells cultured at 42°C. Outer membranes were heated for 5 min at 95°C prior to electrophoresis. The arrowhead indicates the 75-kDa protein.

In light of the strong induction of the 75-kDa protein in strains deficient in pyoverdine synthesis and the absence of a high-molecular-mass receptor for ferripyochelin, a role for the 75-kDa protein in ferripyochelin transport was assessed. A derivative of PAO6609 defective in the expression of the 75-kDa protein under iron-limiting conditions (Fig. 1, compare lanes 7 and 8 and lanes 5 and 6) was recovered following screening of mutants capable of growth on iron-deficient minimal medium plates containing 0.0155 μg of pirazmonam (E. R. Squibb & Sons, Princeton, N.J.), an iron-binding monobactam which appears to gain entry into cells via iron uptake systems (5a), per ml. The mutant, strain K372, in contrast to its parent strain, PAO6609, was almost completely devoid of pyochelin-mediated iron uptake at 150 nM $^{55}FeCl_3$ (in the presence of a molar excess of pyochelin) (Fig. 2). Pyoverdine-mediated iron uptake and citrate-mediated iron uptake were not, however, altered in K372 (data not shown).

Ferripyochelin protects the 75-kDa protein from trypsin digestion. To confirm an interaction between the 75-kDa

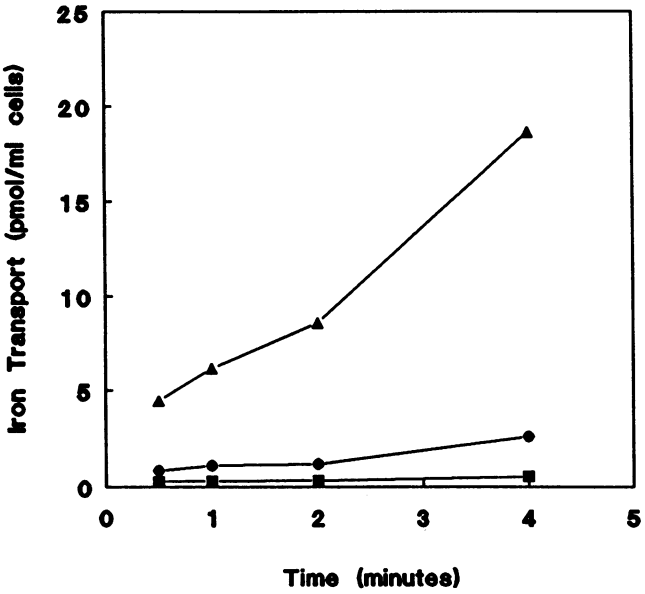

FIG. 2. Pyochelin-mediated iron ($^{55}Fe^{3+}$) transport by *P. aeruginosa* PAO6609 (▲) and K372 (●) cultured in iron-deficient medium. The uptake mixture contained pyochelin (60 μM), nitrilotriacetate (1 μM), $^{55}FeCl_3$ (150 nM), and 1 ml of cells at an A_{600} of 1.0. ■, Transport in the presence of 10 mM KCN or in the absence of the siderophore. Data are representative of four experiments.

protein and ferripyochelin, which would be expected given the apparent role of this protein in ferripyochelin uptake, we made attempts to demonstrate ferripyochelin binding to detergent-solubilized 75-kDa protein and outer membranes containing the 75-kDa protein. Unfortunately, a high level of apparently nonspecific binding was observed in these experiments, independent of the presence of the 75-kDa protein. Previous reports have demonstrated, however, that substrate-binding or receptor proteins may be protected from proteolytic digestion in the presence of the appropriate substrate or ligand (e.g., 17), presumably as a result of substrate binding-dependent changes in protein conformation. To demonstrate an interaction between the 75-kDa protein and ferripyochelin, then, we made attempts to demonstrate that ferripyochelin could protect the 75-kDa protein from proteolytic digestion. Trypsin was initially tested for its ability to degrade the 75-kDa protein, which it did quite readily (Fig. 3, compare lanes 2 and 1). When digestions were carried out in the presence of ferripyochelin, however, the 75-kDa protein was largely protected (Fig. 3, lane 4). Moreover, the protection afforded by ferripyochelin was specific for the 75-kDa protein: additional proteins contaminating the 75-kDa protein preparations were completely degraded by trypsin in the presence of ferripyochelin (Fig. 3, lane 4). As expected, the protection was also siderophore specific: ferripyoverdine was unable to prevent trypsin digestion of the 75-kDa protein (Fig. 3, lane 6). Finally, exposure of the 75-kDa protein to 1% (wt/vol) SDS at 95°C rendered it susceptible to digestion by trypsin in the presence of ferripyochelin (data not shown), ruling out any effect of ferripyochelin on trypsin itself and, more importantly, indicating that the protective action of ferripyochelin required a native conformation on the part of the 75-kDa protein. These data support a specific interaction between the 75-kDa protein and ferripyochelin.

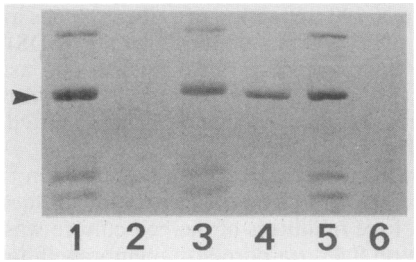

FIG. 3. SDS-polyacrylamide gel electrophoretogram of trypsin digests of detergent-solubilized 75-kDa protein. Zwittergent Z3-14-soluble fractions of the 75-kDa protein (50 μg of total protein) were incubated at 37°C in the presence of trypsin (2.5 mg/ml) with no additions (lanes 1 and 2), with ferripyochelin (lanes 3 and 4), and with ferripyoverdine (lanes 5 and 6) for 0 min (odd-numbered lanes) and 120 min (even-numbered lanes). Samples were heated at 95°C in sample loading buffer for 5 min prior to electrophoresis. The 75-kDa protein is identified by an arrowhead.

Identification of the 75-kDa protein in strain FBP-28. The demonstration that a 75-kDa outer membrane protein is involved in ferripyochelin transport in *P. aeruginosa* contrasts with earlier data showing that a defect in a 14-kDa outer membrane protein was sufficient to abolish ferripyochelin transport in this organism (29). Further examination of *P. aeruginosa* FBP-28, one of the mutants reportedly defective in ferripyochelin transport as a result of a defect in the 14-kDa ferripyochelin receptor (25), revealed, however, that the 75-kDa protein was, indeed, inducible in this strain under conditions of iron limitation (Fig. 1, compare lanes 10 and 9). Furthermore, the protein was induced to levels comparable to those in PAO1 (Fig. 1, lane 2), the parent strain of FBP-28. Induction of the protein correlated with the ability of iron-limited FBP-28 cells to transport ferripyochelin (Fig. 4), although at a level below that in PAO1 cells.

Identification of a second uptake system for ferripyochelin.

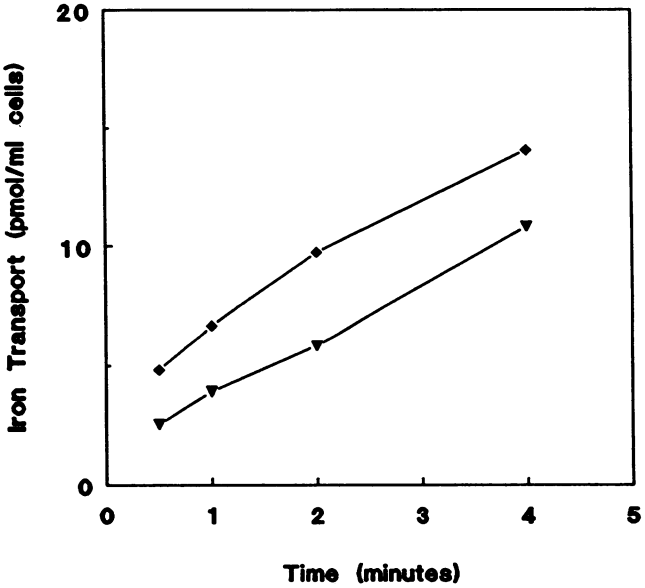

FIG. 4. Pyochelin-mediated iron ($^{55}Fe^{3+}$) transport by *P. aeruginosa* PAO1 (◆) and FBP-28 (▼) cultured in iron-deficient medium. The composition of the uptake mixture is described in the legend to Fig. 2. Data are representative of three experiments.

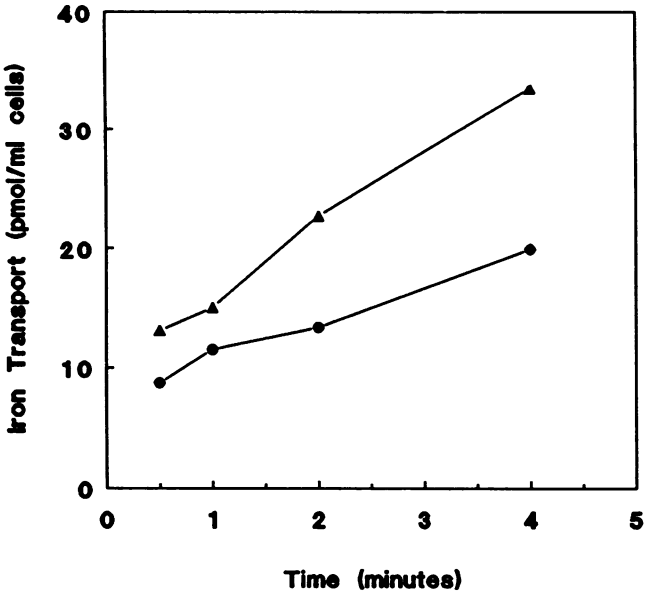

FIG. 5. Pyochelin-mediated iron ($^{55}Fe^{3+}$) transport by *P. aeruginosa* PAO6609 (▲) and K372 (●) cultured in iron-deficient medium. The composition of the uptake mixture is described in the legend to Fig. 2, except that the concentration of $^{55}FeCl_3$ was 750 nM. Data are representative of three experiments.

Given the bulk of previously published data implicating the 14-kDa protein in ferripyochelin uptake, the current study supports the conclusion that at least two uptake systems for ferripyochelin exist in *P. aeruginosa*. Indeed, although mutant K372 showed drastically reduced ferripyochelin uptake compared with that in PAO6609, uptake above that seen in the absence of the siderophore or in the presence of KCN was observed. This result suggested that the mutant, while deficient in uptake, was still capable of ferripyochelin transport. When transport assays were conducted with fivefold-higher concentrations of iron (i.e., 750 nM) (in the presence of a molar excess of pyochelin), pyochelin-mediated iron transport was readily measured in K372 (Fig. 5). These data are consistent with the existence of a second uptake system for ferripyochelin in this strain. While the identity of the 75-kDa protein present in the outer membranes of iron-limited K372 (Fig. 1, lane 8) is unknown, its presence in cells grown in iron-sufficient medium (Fig. 1), which do not demonstrate pyochelin-mediated iron uptake (data not shown), suggests that this protein is not involved in ferripyochelin transport and therefore does not share identity with the iron-repressible 75-kDa protein.

DISCUSSION

Outer membrane receptors for Fe(III)-siderophore complexes are typically iron-repressible proteins with high molecular masses (23). Interestingly, however, the ferripyochelin receptor of *P. aeruginosa* has been identified as a low-molecular-mass (14-kDa) outer membrane protein (30). In the present report, we describe a second outer membrane protein, strongly derepressed under conditions of iron limitation and with a high molecular mass (75 kDa), that is involved in pyochelin-mediated iron transport. Furthermore, we demonstrate the presence of this 75-kDa protein and the corresponding transport system in *P. aeruginosa* FBP-28, a strain synthesizing a defective 14-kDa ferripyochelin receptor and reportedly unable to transport ferripyochelin (29). The earlier failure to observe pyochelin-mediated iron uptake in FBP-28 is probably attributable to the growth of the strain to the early log phase in preparation for transport assays in the earlier study. In our experience, induction of the 75-kDa protein required growth to the late log or early stationary phase (A_{600}, ≥1.0) (13a). Early-log-phase FBP-28 cells presumably would have lacked the 75-kDa protein and thus have failed to exhibit pyochelin-mediated iron uptake. Still, FBP-28 exhibits a reduced ability to transport ferripyochelin relative to PAO1, presumably reflecting the loss of 14-kDa protein-dependent ferripyochelin uptake in this mutant.

In addition to the defect in ferripyochelin uptake, the loss of the iron-regulated 75-kDa protein in K372 is also associated with the derepression of a number of iron-regulated outer membrane proteins under what are normally iron-sufficient conditions (Fig. 1). Although the 50 μM $FeSO_4$ present in the iron-sufficient medium is sufficient to repress the synthesis of these proteins in parent strain PAO6609, apparently the added defect in iron uptake resulting from the loss of the 75-kDa protein renders K372 unable to transport enough iron at 50 μM $FeSO_4$ to repress the synthesis of these proteins.

The fact that 14- and 75-kDa outer membrane proteins have been implicated in pyochelin-mediated iron transport supports the existence of two uptake systems for ferripyochelin in *P. aeruginosa*. Consistent with this idea, strain K372 still exhibited pyochelin-mediated iron uptake despite the loss of the 75-kDa protein. Furthermore, Western blot analysis with a 14-kDa protein-specific antiserum confirmed the presence of the 14-kDa protein in K372 (28a), suggesting that it may be responsible for the ferripyochelin uptake observed in K372. Apparently, then, multiple uptake systems for a particular Fe(III)-siderophore complex, while absent in the much-studied *Escherichia coli* (3), may be a general phenomenon in *P. aeruginosa*. Indeed, we have also provided evidence for at least two uptake systems for ferripyoverdine (24) and ferrienterobactin (25) in this organism. For a particular siderophore, however, the transport systems identified operate at different substrate concentrations, suggesting differences in the affinities of the two systems. Indeed, uptake dependent on the high-molecular-mass iron-regulated outer membrane proteins appears to be of high affinity, operating at iron concentrations 5- to 10-fold lower than those required for uptake via the second uptake system.

While the presence of multiple uptake systems for a given Fe(III)-siderophore complex may be advantageous in the event of the loss of one system via mutation, one wonders if *P. aeruginosa* evolved multiple uptake systems merely as insurance against a mutational loss of one of them. The apparent differences in affinity suggest, instead, that the putative low-affinity systems function during marginal iron limitation while the high-affinity systems function during more severe iron limitation. In support of this idea, ferripyochelin uptake dependent on the 75-kDa protein is only observed in late-log- or early-stationary-phase cells which, in batch cultures, are expected to be more iron limited than early-log-phase cells. The latter appear to transport ferripyochelin exclusively via the 14-kDa protein. Alternatively, the low-affinity system may be less specific than its high-affinity counterpart, capable of recognizing complexes of Fe(III) and closely related siderophores. Thus, additional, heterologous siderophores could be used by *P. aeruginosa*

to promote iron uptake and assist the organism in its competition for available iron in vivo. Other than ferrioxamine B and enterobactin (21), however, no other siderophores have been examined in detail for their ability to mediate iron uptake in *P. aeruginosa*.

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