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Cloning of the Outer Membrane High-Affinity Fe(III)-Pyochelin Receptor of *Pseudomonas aeruginosa*†

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Pseudomonas aeruginosa **produces the phenolic siderophore pyochelin under iron-limiting conditions. In this study, an Fe(III)-pyochelin transport-negative (Fpt^-) strain, IA613, was isolated and characterized. ^{55}Fe(III)-pyochelin transport assays determined that no Fe(III)-pyochelin associated with the Fpt^- IA613 cells while a significant amount associated with KCN-poisoned Fpt^+ cells. A *P. aeruginosa* genomic library was constructed in the IncP cosmid pLAFR1. The genomic library was mobilized into IA613, and a recombinant cosmid, pCC41, which complemented the Fpt^- phenotype of IA613, was isolated. pCC41 contained a 28-kb insert of *P. aeruginosa* DNA, and the Fpt^--complementing region was localized to a 3.6-kb *Bam*HI-*Eco*RI fragment by deletion and subcloning of the insert. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of IA613 revealed that it lacked a 75-kDa outer membrane protein present in Fpt^+ strains. IA613 strains bearing plasmid pRML303, which carries the 3.6-kb *Bam*HI-*Eco*RI fragment of pCC41, expressed the 75-kDa outer membrane protein and demonstrated a ^{55}Fe(III)-pyochelin transport phenotype identical to that of a wild-type Fpt^+ strain. Minicell analysis demonstrated that the 3.6-kb *Bam*HI-*Eco*RI fragment of pCC41 encoded a protein of ~75 kDa. The results presented here and in a previous report (D. E. Heinrichs, L. Young, and K. Poole, Infect. Immun. 59:3680–3684, 1991) lead to the conclusion that the 75-kDa outer membrane protein is the high-affinity receptor for Fe(III)-pyochelin in *P. aeruginosa*.**

Pseudomonas aeruginosa is a nonfermenting, gram-negative rod found in a wide variety of environments. It is nutritionally versatile and exists as one of the most distinct species within the pseudomonads (43). This bacterium is considered highly pathogenic for individuals with compromised immunity, and a variety of its extracellular products have been assigned as virulence factors (24).

P. aeruginosa produces two siderophores, pyoverdin and pyochelin. Pyoverdin, a hydroxamate siderophore, is a small peptide containing a fluorescent dihydroxyquinoline derivative which is characteristic of the fluorescent siderophores produced by members of *Pseudomonas* rRNA homology group I (11, 45). Pyochelin is a structurally unique phenolate siderophore possessing two sulfur- and nitrogen-containing heterocyclic rings and apparently chelates Fe(III) in a 2:1 [pyochelin:Fe(III)] stoichiometry (3, 8, 12, 13). Two other pseudomonads, *Pseudomonas cepacia* and *Pseudomonas fluorescens*, produce pyochelin (15, 33, 34).

A number of reports support a correlation between pyochelin production and the virulence of *P. aeruginosa*. Pyochelin stimulates bacterial growth in murine infections (9), reverses iron deprivation caused by human serum and transferrin (1), and efficiently removes iron from transferrin (42). Fe(III)-pyochelin is capable of catalyzing hydroxyl radical formation (6) and may play a role in the tissue destruction associated with *Pseudomonas* infections (41). Mutants with defects in pyochelin-mediated Fe(III) transport were observed to be markedly less virulent than wild-type strains of *P. aeruginosa* (35). Pyochelin may also affect the production of other extracellular products (e.g., toxin A, alkaline protease, and elastase) which appear to be under iron regulation (5, 37).

The mechanism of Fe(III)-pyochelin transport is controversial. One group of investigators provided evidence for the involvement of a 14-kDa outer membrane protein, designated ferripyochelin-binding protein (FBP), in pyochelin-mediated Fe(III) transport (33, 36, 38). Another report presented results that a ~90-kDa periplasmic space protein binds Fe(III)-pyochelin (10). Recently, a third group reported the requirement of a 75-kDa outer membrane protein for pyochelin-mediated Fe(III) transport (23).

These conflicting reports instigated this investigation on the nature of the defect in an Fe(III)-pyochelin transport-negative (Fpt^-) mutant of *P. aeruginosa*. A cosmid that complemented the Fpt^- phenotype was isolated, and the region responsible for complementation was subcloned to a 3.6-kb *Bam*HI-*Eco*RI fragment. Recombinant plasmids bearing this fragment encoded a 75-kDa outer membrane protein. The current data on this 75-kDa outer membrane protein indicate that it is the high-affinity receptor for Fe(III)-pyochelin.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. All *P. aeruginosa* strains are strain PAO1 derivatives.

Media and growth conditions. *Escherichia coli* strains were routinely grown in Luria broth (LB) medium with the following antibiotic concentrations (micrograms per milliliter): carbenicillin, 100; kanamycin, 50; and tetracycline, 20. IPTG (isopropyl-β-D-thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were used at 100 and 50 μg/ml, respectively. *P. aeruginosa* strains lacking plasmids were also grown on LB medium. *P. aerug-*

† This paper is dedicated to Jennie K Pierce, Ph.D. (Microbiology) 1986, University of Iowa, Iowa City; 31 October 1958–7 March 1991. "*Every shut eye ain't sleep, every goodbye ain't gone*"—Louis Michaux.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype and/or phenotype	Source or reference
Strains		
E. coli		
DH5α	ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *endA recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*	BRL[a]
HB101	*mcrB mrr hsdS20 recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20 supE44*	27
χ984	*minA minB pdxC purE his rpsL xyl ilv met*	29
JB377	*hsdS leuB6 thr* Δ(*srlR-recA*)*306*::Tn*10*	J. A. Heinemann
P. aeruginosa		
IA602	*pvd-2* Pch$^-$ Sal^{-b}	2
IA613	*pvd-2* Pch$^-$ Sal$^-$ Fpt^{-b}	2
Plasmids		
pBluescriptKS(+)	Extended polylinker pUC derivative	Stratagene[c]
pLAFR1	21.6-kb IncP cosmid	18
pRK2013	IncP *tra*$^+$ ColE1 replicon	17
pRKY55	pTJS133-derived cloning vector containing *lacZ*α complementation fragment of pUC128	This study
pTJS133	11.1-kb IncP replicon	30
pUC128	Extended polylinker pUC derivative	25

[a] GIBCO Bethesda Research Laboratories, Inc., Gaithersburg, Md.
[b] *P. aeruginosa* phenotypes and genotypes: *pvd*, inability to produce pyoverdin; Pch$^-$, inability to produce pyochelin; Sal$^-$, inability to produce salicylic acid; Fpt$^-$, Fe(III)-pyochelin transport negative.
[c] Stratagene Cloning Systems, La Jolla, Calif.

inosa strains bearing plasmids were selected for and grown on glucose glycerol (GG) agar containing tetracycline. GG medium consisted of the following: 27 mM glucose, 108 mM glycerol, 1× MinA salts (28), and 1 mM $MgCl_2$. When GG medium was prepared as agar, $MgCl_2$ was omitted. When appropriate, freshly prepared tetracycline was added to GG agar at 110 μg/ml. Casamino Acids-phosphate-sulfate (CPS) medium was used as an iron-limiting medium and consisted of 0.8% Casamino Acids (Difco Laboratories, Detroit, Mich.), 5 mM KPO_4 buffer (pH 7.2), and 5 mM K_2SO_4. The above three components were autoclaved together and supplemented with $MgSO_4$ to a final concentration of 1 mM after cooling to 50°C. Freshly prepared tetracycline was added to CPS broth at 75 μg/ml when appropriate. When CPS medium was prepared as agar plates, $MgSO_4$ was omitted. CPS-dipyridyl-salicylic acid (CDS) agar, used for screening pyochelin production and transport, consisted of CPS agar with the following concentrations of supplements: 700 μM 2,2′-dipyridyl, 750 μM sodium salicylate, and 500 μM L-cysteine hydrochloride. These supplements were added as sterile solutions after the cooling of CPS agar to 50°C. Freshly prepared tetracycline was added to CDS agar at 90 μg/ml as needed. Long-term storage of bacterial cultures was at −80°C in 40% glycerol.

^{55}Fe(III) transport assays. *P. aeruginosa* strains were grown in CPS medium for 16 h or until the early stationary phase. Cells were pelleted from 1-liter cultures by centrifugation, washed once with 0.2 volume of 1 mM $MgSO_4$–20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.5), and resuspended in 50 ml of 1 mM $MgSO_4$–1 mM HEPES (pH 7.5). Cells were diluted to an optical density of 0.2 to 0.4 at 600 nm. An optical density of 0.2 at 600 nm corresponded to 6×10^8 bacteria per ml or 0.1 mg (dry weight) per ml. These cells were diluted with an equal volume of 2× transport medium (1% Casamino Acids, 20 mM HEPES [pH 7.5], 2 mM $MgSO_4$, 400 μM 2,2′-dipyridyl) and incubated at 37°C with shaking for 30 min prior to the start of the transport assay in order to promote active respiration of the cells. By incorporating 400 μM 2,2′-dipyridyl into the transport medium, background levels of ^{55}Fe(III) were lowered. The ^{55}Fe(III)-pyochelin complex was prepared in the following manner. Twenty-five micrograms of pyochelin in an ethanolic solution was added to 150 μl of 20 mM HEPES (pH 7.5) and vortexed. Aliquots of a $^{55}FeCl_3$ (Dupont, NEN Research Products, Boston, Mass.; specific activity, 5.5 mCi/μmol) solution were added to the pyochelin solution, and the mixtures were vortexed and allowed to incubate at room temperature for 5 min before addition to 6-ml quantities of assay culture. The amount of $^{55}FeCl_3$ added yielded 0.13 μCi/ml (23.5 nM) or 0.065 μCi/ml (11.7 nM) as indicated herein, and the pyochelin concentration in the assays was ~12 μM. When appropriate, KCN was added to a final concentration of 40 mM in cultures 15 min before the addition of radionuclide.

Samples were removed at various time points and filtered through 0.45-μm-pore-size cellulose acetate filters (catalog no. OE67; Schleicher & Schuell, Inc., Keene, N.H.) in a vacuum-drawn filter manifold (1225 sampling manifold; Millipore Corp., Bedford, Mass.). Filters were dried at 100°C and submerged in scintillation cocktail, and the ^{55}Fe(III) disintegrations per minute of the sample was determined on a Beckman LS 6000LL scintillation spectrometer by using the single-label disintegrations per minute program with an ^{55}Fe quench curve calibration.

Siderophore purification. Pyochelin was isolated and purified as described previously (1). Pyochelin was stored as a dry glass protected from light at −80°C and was dissolved in CH_2Cl_2 to yield concentrated solutions. Dilute solutions in ethyl alcohol were made from concentrated CH_2Cl_2 solutions diluted into ethyl alcohol.

Construction of a genomic library. Chromosomal DNA was prepared from *P. aeruginosa* PAO1 by the method of Goldberg and Ohman (19). Partial *Eco*RI digests of PAO1 DNA were size fractionated on sucrose gradients as de-

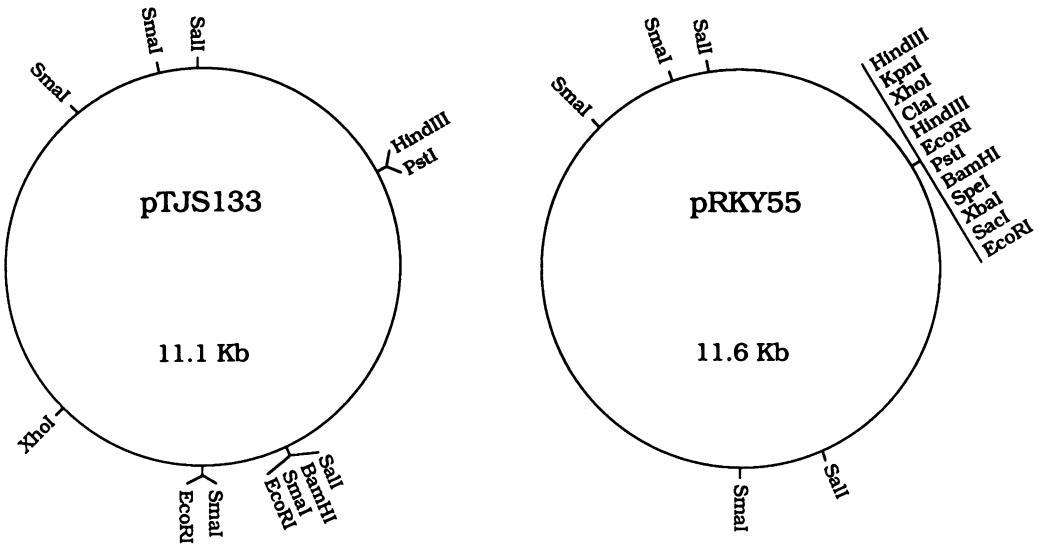

FIG. 1. Maps of pTJS133 and the mobilizable IncP cloning vector pRKY55. The starting replicon was pTJS133, a miniplasmid derivative of RK2 encoding tetracycline resistance (30). The construction of pRKY55 is detailed in Materials and Methods. Blue-white screening for pRKY55 recombinants requires the use of IPTG in the *lacI*$^+$ host DH5α. Sites shown in the polylinker of pRKY55 are not duplicated elsewhere in the vector.

scribed previously (27). Fractions containing DNA fragments of ~20 to 35 kb were ligated into the *Eco*RI site of dephosphorylated cosmid pLAFR1 DNA. The concatamerized DNA was packaged into lambda phage heads by using procedures described by the manufacturer (Gigapack; Stratagene, La Jolla, Calif.). *E. coli* HB101 was infected with packaged cosmids and plated onto LB-tetracycline agar to yield ~3,000 colonies. These colonies were pooled and frozen, and thereafter they served as the *P. aeruginosa* chromosomal DNA library.

Plasmid techniques and DNA manipulations. Cosmids and other plasmids were transferred from *E. coli* to *P. aeruginosa* with triparental matings using pRK2013 as the helper plasmid (16). Plasmid DNA was prepared from *P. aeruginosa* by alkaline lysis (27) and was used to transform *E. coli*. Plasmid minipreps from *E. coli* were prepared by a rapid boiling method described previously (44). Large-scale plasmid purifications from *E. coli* were prepared by alkaline lysis and CsCl density centrifugation. Plasmids were transformed into *E. coli* by standard methods (27).

Use of restriction endonucleases and other DNA-modifying enzymes, agarose gel electrophoresis, cloning methods, and other in vitro DNA techniques were carried out as described previously (27). DNA fragments were electroeluted from agarose gels by previously described methods (32). Specific fragments of cosmid inserts were initially subcloned into pBluescriptKS(+) and pUC128. Desired fragments from the recombinant pUC-type derivatives were subcloned into the mobilizable broad-host-range IncP cloning vector pRKY55 (Fig. 1) in order to allow replication in *P. aeruginosa*. The steps in the construction of pRKY55 are as follows. A single *Eco*RI site (the one nearest to the *Xho*I site in pTJS133) (Fig. 1) was removed by partial digestion of pTJS133 (30) with *Eco*RI, blunt ending with Klenow fragment and deoxynucleoside triphosphates (dNTPs), and religation to yield plasmid pRML105. The remaining *Eco*RI and *Bam*HI sites of pRML105 were removed by simultaneous digestion with *Eco*RI and *Bam*HI, blunt ending with Klenow fragment and dNTPs, and religation to yield plasmid pRML111. The *Xho*I site of pRML111 was similarly removed to yield pRML115. pRML115 was digested with *Hin*dIII and *Pst*I, and the ends were rendered flush by consecutive treatment with Klenow fragment and T4 DNA polymerase. This DNA was ligated to a 517-bp *Hae*II fragment containing the *lacZ*α peptide coding region and polylinker sequences of pUC128 (25) which had been blunt ended with T4 DNA polymerase. This ligation mixture was transformed into *E. coli* DH5α and plated onto LB-tetracycline plates containing IPTG and X-Gal. Blue colonies were screened for the presence of the appropriate plasmid, designated pRKY55. Its structure was confirmed by restriction endonuclease analysis. Use of the blue-white screening properties of pRKY55 in DH5α requires the incorporation of IPTG in agar plates. pRKY55, as an IncP vector, has a low copy number, which has been reported to be between 2 and 5 (30). IncP vectors have not been shown to demonstrate gene dosage effects, probably because of their low copy number, and are therefore good candidates for physiologically accurate complementation by cloned genes.

Outer membrane isolation and analysis. *P. aeruginosa* outer membranes were obtained by the method of Hancock et al. (20, 22) with slight modifications. Lysozyme treatment was omitted after cell disruption in a French pressure cell. Sucrose gradients were prepared in 20 mM HEPES (pH 7.5) in place of Tris. After pelleting of the separated outer membrane fraction, a Triton X-100-insoluble outer membrane fraction was obtained as described previously (31) by using a single incubation in 2% Triton X-100 in 20 mM HEPES (pH 7.5). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of outer membrane proteins was carried out as described previously (20, 26). Outer membrane samples were solubilized for 10 min at 100°C in the sample buffer of Hancock and Carey (20).

Minicell analysis of plasmid-encoded proteins. Plasmids to be analyzed for plasmid-encoded proteins were introduced into *E. coli* χ984 by electroporation. Minicell isolation and labeling were carried out as described previously (29). Briefly, minicells were isolated by serial passages through

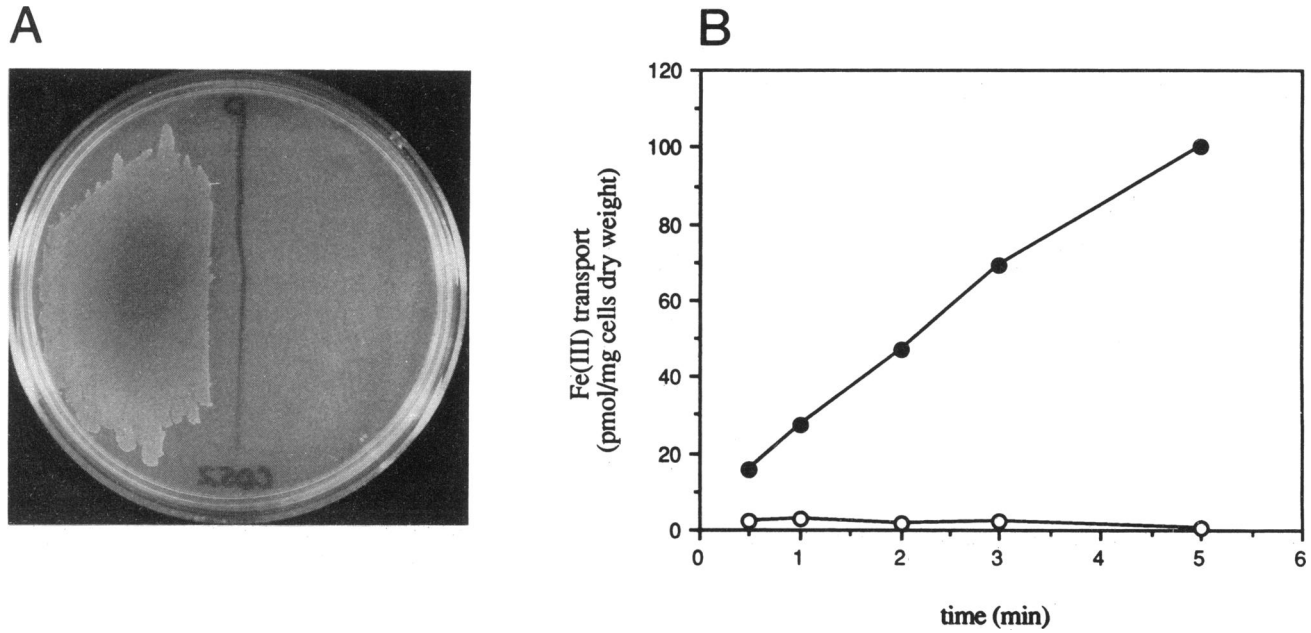

FIG. 2. Growth and Fe(III)-pyochelin transport phenotypes of *P. aeruginosa* IA602 (Fpt^+) and IA613 (Fpt^-). (A) Growth of IA602 and IA613 on CDS plates. Single colonies of IA602 and IA613 from GG agar plates were picked and smeared onto separate halves of a CDS plate and incubated at 37°C for 30 h. Left side of the plate, IA602; right side of the plate, IA613. (B) $^{55}Fe(III)$-pyochelin transport by IA602 and IA613. IA602 (●) and IA613 (○) cells were diluted to an A_{600} of 0.1 in 1× transport medium, and $^{55}Fe(III)$-pyochelin was added to give concentrations of 0.13 μCi/ml and 23.5 nM with respect to $^{55}Fe(III)$. $^{55}Fe(III)$ transport assay protocols are given in Materials and Methods. Curves are representative of three experiments.

sucrose step gradients. Minicells were starved for 1 h at 37°C in M9 medium (28) containing adenine, histidine, isoleucine, and valine each at a concentration of 50 μg/ml and 2,2′-dipyridyl at a concentration of 150 μM. [^{35}S]methionine (Dupont, NEN) was added to yield a concentration of 0.5 mCi/ml, and the minicells were incubated for an additional hour. Minicells were removed from the suspension by centrifugation and were washed with H_2O to remove unincorporated label. The minicells were solubilized in sample buffer and electrophoresed on SDS-PAGE gels as described above. Gels were fixed and treated with Entensify (Dupont, NEN) prior to fluorography.

RESULTS

Characterization of a mutant defective in pyochelin-mediated transport of Fe(III). The ability of pyochelin-mediated transport of Fe(III) to reverse iron starvation caused by nonutilizable Fe(III) chelators (i.e., 2,2′-dipyridyl) was investigated. The Pch^- Sal^- *P. aeruginosa* IA602 mutant (2) was used because of its siderophore phenotype. IA602 is defective in both pyoverdin and pyochelin biosynthesis but is capable of producing pyochelin when provided with exogenous salicylic acid. When grown on CPS agar (which contains no 2,2′-dipyridyl), IA602 grew as well as Pch^+ strains. A concentration of 700 μM 2,2′-dipyridyl in CPS agar was found to strongly inhibit the growth of IA602. The addition of salicylic acid to a final concentration of 750 μM (CDS agar) restored the ability of IA602 to grow in the presence of 2,2′-dipyridyl. The incorporation of exogenously supplied salicylic acid into pyochelin by IA602 promoted the removal of iron from the nonutilizable 2,2′-dipyridyl complexes and thus the growth of IA602 via pyochelin-mediated transport of Fe(III). The growth promotion afforded by the presence of salicylic acid in CDS agar was dependent upon the Pch^- Sal^- phenotype of mutants; no Pch^- Sal^+ mutants were capable of growing on CDS agar. These observations indicate that pyochelin biosynthesis and pyochelin-mediated Fe(III) transport, not salicylic acid-mediated transport of Fe(III), were responsible for growth on CDS.

When the remaining Pch^- Sal^- mutants isolated by Ankenbauer and Cox (2) were inoculated onto CDS agar, one strain, IA613, was unable to grow (Fig. 2A). Although fully capable of synthesizing pyochelin when provided with salicylic acid, pyochelin was not able to reverse iron starvation elicited by 2,2′-dipyridyl, suggesting that this strain had a defect in pyochelin-mediated iron transport or some subsequent step in iron utilization. $^{55}Fe(III)$ transport assays revealed that the defect occurred at the level of iron transport (Fig. 2B). IA613 showed no accumulation of $^{55}Fe(III)$, while IA602 demonstrated rapid $^{55}Fe(III)$ accumulation during these assays. This phenotype in IA613 was designated Fpt^-.

The initial stages of Fe(III)-pyochelin transport were analyzed with short-term transport assays. By keeping the sampling times less than 1 min, it was hoped that two rates of Fe(III)-pyochelin transport could be observed: (i) the rate representing the substrate binding at the Fe(III)-pyochelin receptor and (ii) the rate of active transport of Fe(III) into the cytoplasm following the initial binding at the receptor.

As observed in Fig. 3, there was no measurable Fe(III) associated with cells of the Fpt^- strain, IA613. The only Fe(III) transport rate seen with the Fpt^+ strain, IA602, was that of active transport. An initial rate of ligand binding via a high-affinity receptor expected to be much faster than the active transport rate was not observed. However, the calculated extrapolation of the IA602 $^{55}Fe(III)$ uptake curve to 0 s does not intersect the *y* axis at 0 pmol/mg of cells but instead

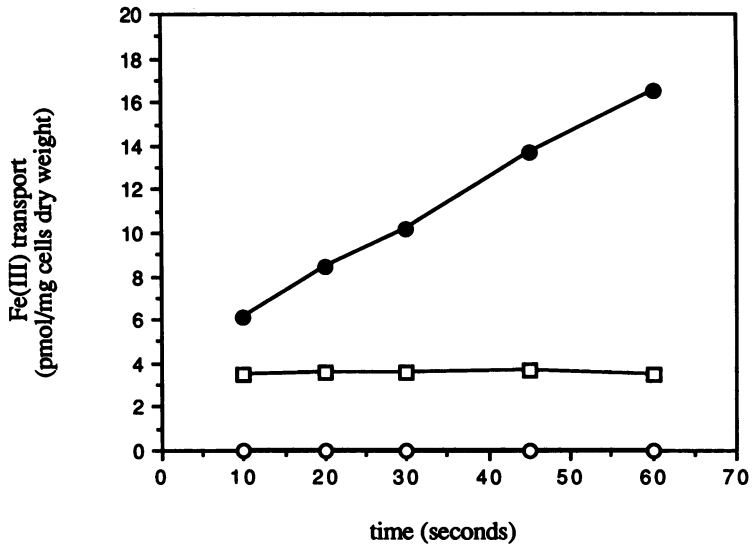

FIG. 3. Short-term analysis of ^{55}Fe(III)-pyochelin transport by Fpt$^+$ and Fpt$^-$ strains of *P. aeruginosa*. Cells were diluted to an A_{600} of 0.2 in 1× transport medium, and ^{55}Fe(III)-pyochelin was added to give concentrations of 0.065 μCi/ml and 11.7 nM with respect to ^{55}Fe(III). Curves represent IA602 (●), IA613 (○), and IA602 supplemented with 40 mM KCN (□). KCN was added 15 min prior to initiation of the assay. ^{55}Fe(III) transport assay protocols are given in Materials and Methods. Note that the time scale in this figure is in seconds rather than minutes, as in Fig. 2B and 4B. Results are the average of two separate assays.

at ~4.1 pmol/mg of cells. If no cell association mechanism other than active transport existed, then the IA602 ^{55}Fe(III) uptake curve would at best be colinear with the origin. As this is not observed, ligand-receptor binding must account for this discrepancy. Since the first technically feasible time point is at ~10 s, the initial binding of Fe(III)-pyochelin by its receptor occurs between 0 and 10 s and is extremely rapid.

The Fpt$^-$ strain, IA613, demonstrated no Fe(III)-pyochelin transport in these short-term transport assays (Fig. 3); IA613 assay values were identical to those of the controls lacking bacteria. The total absence of measurable Fe(III) associated with IA613 cells shows that IA613 lacks the ability to bind the Fe(III)-pyochelin substrate, suggesting that its defect is in the Fe(III)-pyochelin receptor.

In order to determine the level of Fe(III)-pyochelin binding to the Fe(III)-pyochelin receptor, IA602 cells were treated with KCN prior to ^{55}Fe(III) transport assays. KCN inhibits the energy-dependent active transport of Fe(III)-pyochelin, and therefore results obtained from assays done in the presence of KCN represent the Fe(III)-pyochelin ligand-receptor binding interaction. A constant 3.6 pmol of ^{55}Fe(III) per mg (dry weight) of cells is observed over the 60-s assay (Fig. 3). This value corresponds well with the extrapolated 0-s value of ~4.1 pmol/mg of cells from the IA602 ^{55}Fe(III) uptake curve in the absence of KCN. The similarity of these two values indicates that the ligand-receptor binding is nearly instantaneous. By comparison with the Fe(III) transport demonstrated by the Fpt$^-$ strain, IA613, it can be concluded that IA602 possesses a high-affinity Fe(III)-pyochelin receptor while IA613 does not.

Complementation of Fpt$^-$ phenotype by *P. aeruginosa* genomic library. A genomic library of *P. aeruginosa* was constructed by using the IncP cosmid pLAFR1. The genomic library was mated en masse into IA613 by using pRK2013 as a helper plasmid. The mating mixtures were plated on GG-tetracycline agar. IA613 cosmid-bearing transconjugants were screened for complementation of the Fpt$^-$ phenotype by picking colonies onto CDS-tetracycline agar. The rationale was based on the fact that although IA613 was capable of producing pyochelin on CDS agar, it would be unable to grow unless provided with a cosmid specifically complementing the Fpt$^-$ phenotype; tetracycline was added to maintain the cosmids during growth on screening agar. Approximately 1,500 individual transconjugants were picked onto CDS-tetracycline agar and incubated at 37°C. After 5 days, one transconjugant showed good growth on CDS-tetracycline agar. The complementing cosmid was reintroduced into *E. coli*, and its complementation of the Fpt$^-$ phenotype was reconfirmed after conjugation again into IA613. This cosmid, designated pCC41, has an insert of ~28 kb, and a restriction map using *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Hin*dIII, *Xba*I, and *Xho*I was constructed (Fig. 4).

Deletion analysis and subcloning of cosmid pCC41. Deletions of pCC41 generated by restriction endonuclease digestion and religation resulted in plasmids pCC43, pCC44, pCC45, and pCC46 (Fig. 4), which were mobilized into IA613 and screened for complementation of the Fpt$^-$ phenotype via growth on CDS agar. Each of these plasmids was defective for complementation of the Fpt$^-$ phenotype, and the limited deletion in pCC44 indicates that the Fpt$^-$-complementing region resides near the *Eco*RI site at 9 kb on the pCC41 map (Fig. 4).

In order to localize the complementing DNA further, fragments of pCC41 were subcloned into pBluescriptKS(+) and pUC128 and these fragments were similarly subcloned into the mobilizable IncP cloning vector pRKY55 (Fig. 1). The resulting recombinant plasmids were mobilized into IA613 and screened for the Fpt$^+$ phenotype by growth on CDS agar. As shown in Fig. 4 and 5A, strains carrying plasmids pRML300, pRML301, pRML302, and pRML303 all possessed the Fpt$^+$ phenotype as determined by growth on CDS agar. The plasmid pRML303 localized the Fpt$^-$-complementing region of pCC41 to a 3.6-kb *Bam*HI-*Eco*RI fragment.

pRML303 complements the ^{55}Fe(III)-pyochelin transport

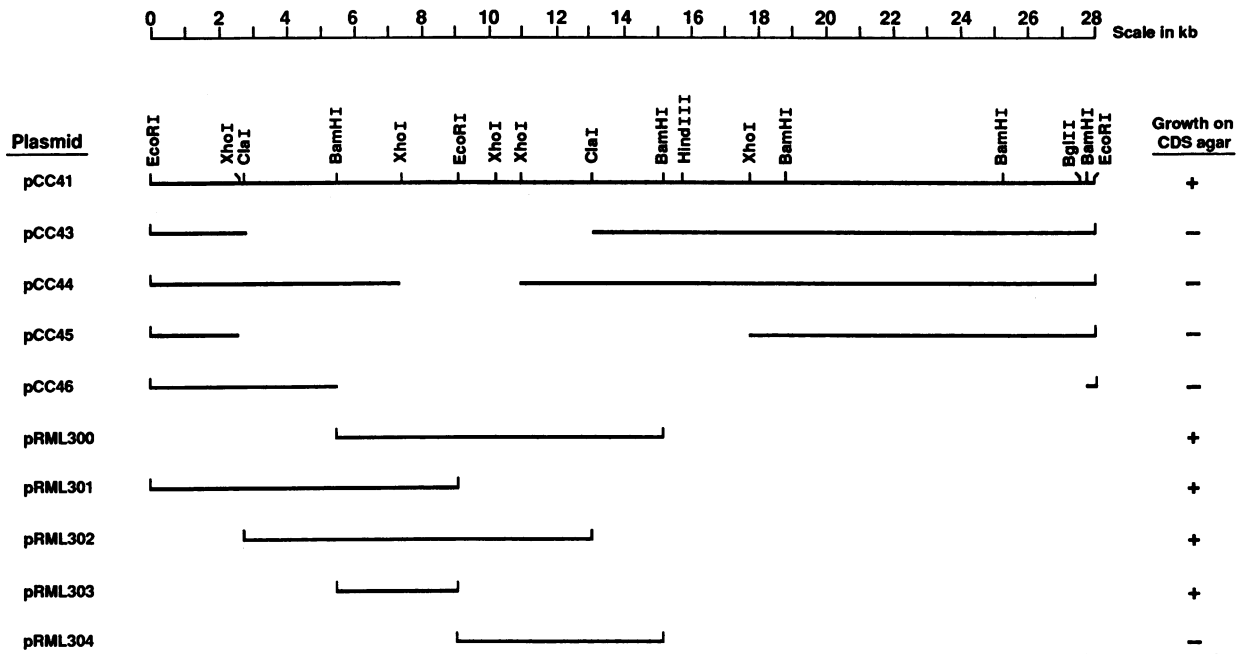

FIG. 4. Localization of Fpt$^-$-complementing sequences of recombinant cosmid pCC41. The plasmid pCC41 was isolated as complementing the Fpt$^-$ defect in IA613. A restriction map of the insert in pCC41, using the indicated enzymes and *Xba*I, was generated; no *Xba*I sites were found in the insert. Below the pCC41 map are plasmid derivatives containing the indicated sequences of the pCC41 insert. Plasmids pCC43, pCC44, pCC45, and pCC46 are pCC41 derivatives obtained by in vitro deletions with *Cla*I, *Xho*I, *Xho*I, and *Bam*HI, respectively. pRML300, pRML301, pRML302, pRML303, and pRML304 are pRKY55 derivatives carrying the indicated subcloned regions of pCC41. Each of the plasmids was mobilized into IA613, and the transconjugants were patched onto CDS plates. Growth was determined at 30 h. Growth was scored as follows: +, confluent growth; −, no growth.

defect in IA613. The ability of pRML303 to complement the Fpt$^-$ phenotype of IA613 was determined quantitatively in ^{55}Fe(III)-pyochelin transport assays. As observed above, IA602 transported ^{55}Fe(III)-pyochelin very rapidly, while IA613 accumulated no ^{55}Fe(III) over 5 min (Fig. 5B). IA613 cells bearing plasmid pRKY55 had ^{55}Fe(III) transport activity identical to that of IA613 alone. Cells with the recombinant plasmid pRML303 transported ^{55}Fe(III)-pyochelin at levels nearly identical to those demonstrated by IA602. These results, along with the CDS agar growth phenotypes, confirm that the Fpt$^-$ phenotype is complemented by pRML303.

A 75-kDa outer membrane protein confers the Fpt$^+$ phenotype. Since the Fpt$^-$ defect in IA613 totally abolishes any ^{55}Fe(III)-pyochelin binding or internalization, it was expected that the genetic lesion affected an early stage in Fe(III)-pyochelin transport, most likely at the outer membrane. Cytoplasmic and outer membranes of IA602, IA613, IA613(pRKY55), and IA613(pRML303) were prepared from CPS-grown cells as described in Materials and Methods. Outer membranes were further extracted with Triton X-100 to remove any cytoplasmic membrane contamination. These membrane fractions were analyzed by SDS-PAGE. Comparison of the outer membrane profiles of IA602 and IA613 revealed the absence of a major 75-kDa protein in IA613 (Fig. 6). Analysis of the outer membranes of IA613 (pRKY55) and IA613(pRML303) demonstrated that the presence of pRML303 in IA613 restored the expression of the 75-kDa outer membrane protein such that its profile is identical to that of IA602 (Fig. 6). Both Fpt$^+$ and Fpt$^-$ strains expressed the 14-kDa outer membrane protein corresponding to FBP (38) as determined by SDS-PAGE, and no other differences in outer or cytoplasmic membrane protein profiles were observed between any of these four strains.

Identification of the gene product by minicell analysis. In order to confirm that the 75-kDa outer membrane protein was encoded by the 3.6-kb *Bam*HI-*Eco*RI fragment of pCC41, this fragment was cloned into pBluescriptKS(+) to yield pRML207. This pUC-based plasmid was introduced into the minicell-producing strain χ984, and minicell isolation and labeling were carried out as previously described (29). The autoradiographs show that a ~75-kDa protein was produced in minicells bearing pRML207 (Fig. 7, lane 2), while minicells bearing plasmid pBluescriptKS(+) did not produce this polypeptide (lane 1). Therefore, it is concluded that a 75-kDa protein is encoded by the 3.6-kb *Bam*HI-*Eco*RI fragment of pCC41 and that this protein is responsible for the Fpt$^+$ phenotype conferred by plasmids bearing this fragment.

DISCUSSION

The binding and subsequent transport of Fe(III)-pyochelin may involve at least three proteins: a 14-kDa outer membrane protein (38), a ~90-kDa periplasmic space protein (10), and a 75-kDa outer membrane protein (23). This study reports the cloning of a 75-kDa outer membrane protein which is required for Fe(III)-pyochelin transport. The Fpt$^-$ strain IA613 was unable to transport Fe(III) via pyochelin; this defect was complemented to wild-type levels by the plasmid pRML303. It should be noted that the Fpt$^-$ phenotype is distinct from the FBP-deficient phenotype (36) in two ways. The Fpt$^-$ phenotype results in a total inability to transport Fe(III)-pyochelin, whereas cells with the FBP-

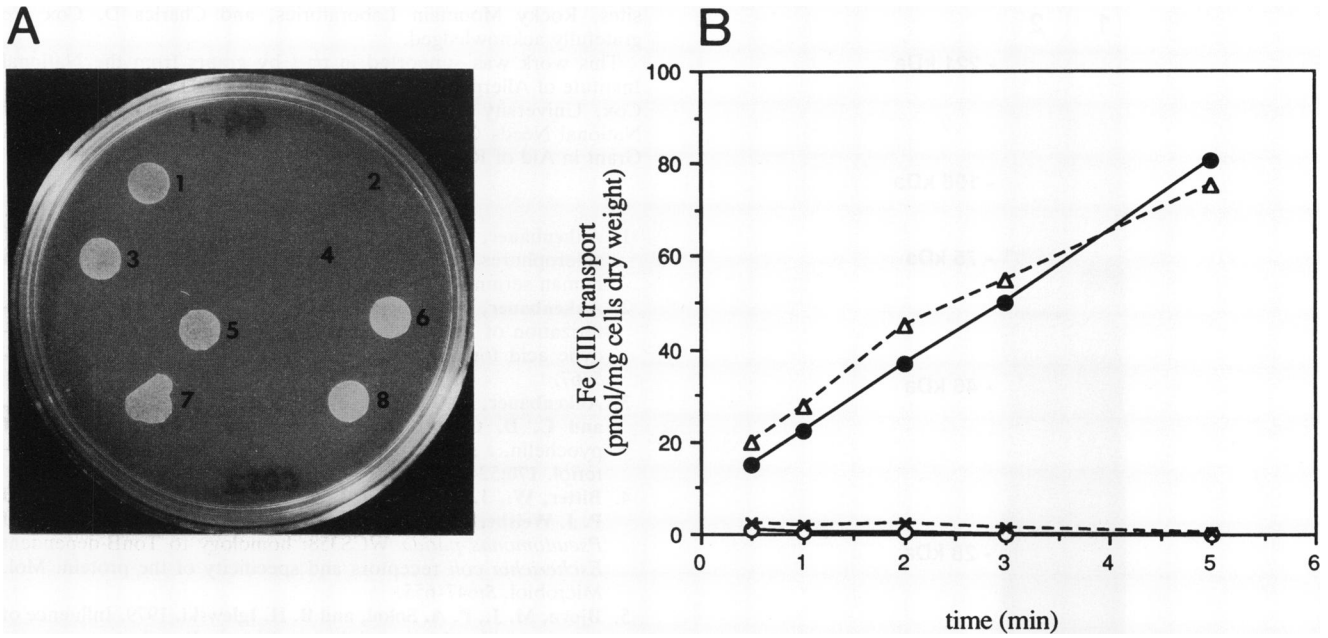

FIG. 5. Growth and Fe(III)-pyochelin transport phenotypes resulting from recombinant plasmids in IA613 (Fpt^-). (A) Growth of recombinant-plasmid-bearing cultures on CDS plates. Various strains grown in GG medium (containing tetracycline for plasmid maintenance when appropriate) were diluted, and 10 μl (5×10^5 CFU) of cell suspension was spotted onto the surface of CDS agar and incubated at 37°C for 30 h. Different strains are indicated numerically: 1, IA602; 2, IA613; 3, IA613(pCC41); 4, IA613(pRKY55); 5, IA613(pRML300); 6, IA613(pRML301); 7, IA613(pRML302); 8, IA613(pRML303). (B) ^{55}Fe(III)-pyochelin transport by recombinant-plasmid-bearing cultures. IA602 (●), IA613 (○), IA613(pRKY55) (×), and IA613(pRML303) (△) cells were diluted to an A_{600} of 0.1 in 1× transport medium, and ^{55}Fe(III)-pyochelin was added to give concentrations of 0.13 μCi/ml and 23.5 nM with respect to ^{55}Fe(III). ^{55}Fe(III) transport assay protocols are given in Materials and Methods. Curves are representative of three experiments.

deficient phenotype are attenuated, but not totally abolished, in their ability to transport Fe(III)-pyochelin (23). Furthermore, Fpt^- cells lack a 75-kDa outer membrane protein which FBP-deficient cells possess. These results corroborate and extend the findings of Heinrichs et al. (23) that a 75-kDa outer membrane protein is involved in Fe(III)-pyochelin transport.

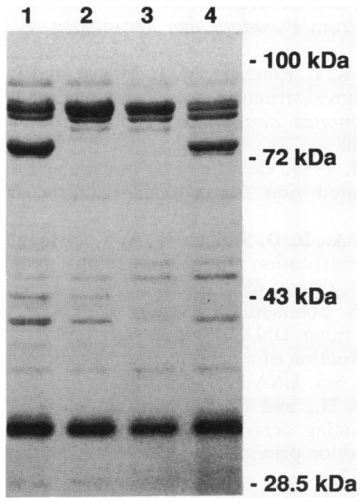

FIG. 6. SDS-polyacrylamide gel of outer membranes proteins of Fpt^+ and Fpt^- *P. aeruginosa* strains. Triton X-100-insoluble outer membranes were prepared from CPS-grown cells as described in Materials and Methods. Lanes: 1, IA602; 2, IA613; 3, IA613 (pRKY55); 4, IA613(pRML303). The PAGE gel shown here is a 9% polyacrylamide gel; SDS-PAGE and sample preparation protocols are described in Materials and Methods.

Several lines of evidence lead to the conclusion that the 75-kDa outer membrane protein is the high-affinity Fe(III)-pyochelin receptor: (i) cells lacking the 75-kDa outer membrane protein are totally defective in Fe(III)-pyochelin transport; (ii) cells lacking the 75-kDa outer membrane protein do not bind Fe(III)-pyochelin, whereas cyanide-poisoned cells expressing the 75-kDa outer membrane protein demonstrate significant binding of the substrate; (iii) the rapid association of low concentrations of Fe(III)-pyochelin with cells expressing the 75-kDa outer membrane protein demonstrates the high-affinity uptake mediated by this protein; (iv) the purified 75-kDa outer membrane protein binds Fe(III)-pyochelin as determined by trypsin protection assays (23); and (v) the molecular mass of the 75-kDa outer membrane protein is similar to the molecular masses of other known outer membrane receptors for Fe(III)-siderophore chelates, such as the 81-kDa FepA protein of *E. coli* (21), the 76-kDa IutA protein of *E. coli* (46), the 86-kDa OM2 protein of *Vibrio anguillarum* (14), and the 86-kDa PupA protein of *Pseudomonas putida* (4). Although the 75-kDa outer membrane protein was not observed to bind radiolabeled Fe(III)-pyochelin in SDS-PAGE gels in the in vitro studies identifying the 14-kDa FBP (38) and the ~90-kDa periplasmic space FBP (10), these results are not surprising for denatured polypeptides. The results of the in vivo studies presented here and the data from the in vitro assays reported by Heinrichs et al. (23) establish the 75-kDa outer membrane protein as the high-affinity Fe(III)-pyochelin receptor.

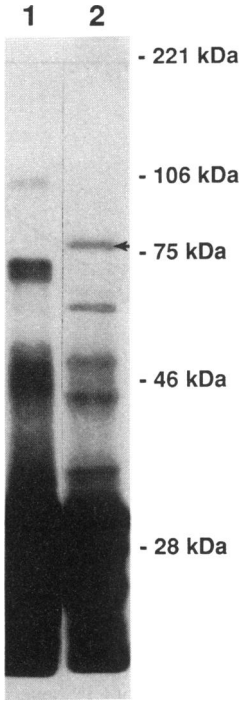

FIG. 7. Autoradiograph of ^{35}S-labeled proteins expressed from plasmid-containing minicells. χ984 minicells containing pBluescript KS(+) and pRML207 were prepared and labeled as described in Materials and Methods. Samples were loaded onto SDS-PAGE (10% polyacrylamide) gels, and the resulting gels were treated with Entensify prior to exposure to Kodak XAR-2 film. pRML207 is pBluescriptKS(+) containing the 3.6-kb *Bam*HI-*Eco*RI fragment of pCC41. Lanes: 1, minicells containing pBluescriptKS(+); 2, minicells containing pRML207. Migration positions of molecular mass standards are shown in the right margin. The ~75-kDa protein expressed from pRML207 is indicated by the arrow. The autoradiograph for lane 1 was slightly overexposed in order to show that the ~75-kDa protein was not present in minicells containing the control vector.

Although the 75-kDa outer membrane protein is the Fe(III)-pyochelin receptor, the roles of the 14-kDa outer membrane FBP (38) and the ~90-kDa periplasmic space FBP (10) remain unclear. The reports of Sokol et al. demonstrated that the 14-kDa FBP has a role in Fe(III)-pyochelin transport (36, 38, 40). However, mutants lacking FBP are only slightly diminished in their ability to transport Fe(III)-pyochelin (23), and even high concentrations of antibodies to FBP do not abolish Fe(III)-pyochelin transport (39, 40). Since the 14-kDa FBP is an outer membrane protein, it may form a complex with the 75-kDa outer membrane protein to help facilitate or stabilize high-affinity binding of the Fe(III)-pyochelin ligand to its receptor. Careful analysis and comparison of the ^{55}Fe(III)-pyochelin transport kinetics of Fpt$^-$ and FBP-negative mutants would likely resolve these questions concerning the respective functions of FBP and the 75-kDa outer membrane proteins. Less is known about the ~90-kDa periplasmic FBP (10). Although no function has yet been assigned to this protein, it may be the periplasmic ferripyochelin iron reductase described by Cox (7).

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