Recycling of Pyoverdin on the FpvA Receptor after Ferric Pyoverdin Uptake and Dissociation in *Pseudomonas aeruginosa*†

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ABSTRACT: Under iron-limiting conditions, *Pseudomonas aeruginosa* secretes a fluorescent siderophore called pyoverdin (PaA), which, after complexing iron, is transported back into the cells via its outer membrane receptor FpvA. The recent finding that all FpvA receptors on the bacterial cell surface are loaded with iron-free PaA under iron limiting conditions has raised questions about the mechanism by which *P. aeruginosa* transports efficiently iron. We used [^{3}H]PaA′, [^{55}Fe]PaA−Fe, and a kinetically stable chromium−PaA complex to show that iron loading of the receptor occurs through a siderophore displacement mechanism in vivo. Moreover, the fluorescence properties of iron-free PaA revealed that, after PaA−Fe uptake and dissociation, the PaA molecule is recycled into the extracellular medium. We used fluorescence resonance energy transfer (FRET) between the PaA chromophore and the FpvA tryptophans in vivo to monitor the kinetics of PaA displacement by PaA−Fe at the cell surface, the dissociation of iron from the siderophore, and the recycling of PaA back to the receptor on the outer membrane of the bacteria in real time. The loading status of FpvA (PaA versus PaA−Fe) was shown to depend on the relative concentration of the two forms of pyoverdin in the growth medium.

When grown in iron-deficient conditions, many bacteria synthesize and release iron chelators, termed siderophores (*1*, *2*). Siderophores make iron available for use by the cell by solubilizing the ferric ion of insoluble complexes that form under aerobic conditions at physiological pH. In the host, siderophores are thought to sequester iron from iron-containing molecules such as transferrin and lactoferrin and to deliver the iron to the microbial cell.

Iron uptake has been mostly studied in Gram-negative bacteria, particularly in *Escherichia coli* with the ferrichrome siderophore as a prototype (*3*−*5*). The first step of entry of ferrichrome into *E. coli* is mediated by a specific outer membrane receptor. The crystal structures of FhuA and FepA, respectively ferrichrome and ferric enterobactin outer membrane receptors, have been solved (*6*−*8*). Both receptors are composed of a COOH-terminal β-barrel domain and an NH_2-terminal cork domain that fills the barrel interior. The transport into the periplasm via the specific outer membrane receptor requires the protonmotive force (pmf) of the cytoplasmic membrane and an energy transduction complex that includes the cytoplasmic membrane proteins TonB, ExbB, and ExbD (*9*−*13*). The mechanisms by which TonB is coupled to the electrochemical gradient of the cytoplasmic membrane and by which the energy is transferred to the outer membrane receptor are unknown. However, Larsen and collaborators (*14*) have shown that the proton motive force (pmf), ExbB, and the ligand-bound receptor drive conformational changes in TonB, suggesting a dynamic model of energy transduction in which TonB cycles through a set of conformations that differ in potential energy. Once in the periplasm, the ferric siderophore binds to its cognate periplasmic binding protein and is actively transported across the cytoplasmic membrane by an ABC transporter (*15*).

Under conditions of iron limitation, *Pseudomonas aeruginosa*, an opportunistic human pathogen, releases a fluorescent siderophore called pyoverdin (PaA).[1] Until recently, it was commonly believed that iron was transported via the PaA siderophore pathway in *P. aeruginosa*, through binding of the PaA−Fe complex to its receptor called FpvA and by the same kind of mechanism as described for ferrichrome in *E. coli*. However, the recent description that iron-free PaA binds to its outer membrane receptor in *P. aeruginosa* with comparable affinity as the iron-loaded form (*16*, *17*) started to unsettle this belief and to reveal that there may be different iron-uptake mechanisms among Gram-negative bacteria. Alternatively, the binding of iron-free siderophores to their cognate receptors may be a general property, which has not been investigated in detail in other systems. Stintzi and collaborators (*18*) showed that iron-free siderophore binds as its ferric complex to its receptor in *Aeromonas hydrophila*, implying that this property is not restricted to the *P. aeruginosa* PaA uptake system.

In this paper, we used radiolabeled PaA and the unique fluorescence properties of iron-free PaA to clarify more the

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[1] Abbreviations: PaA, iron-free pyoverdine; PaA−Fe, ferric pyoverdine; FRET, fluorescence resonance energy transfer; au, arbitrary unit.

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iron-uptake mechanism in *P. aeruginosa*. First, we showed that PaA−Fe displaces iron-free PaA from FpvA in vivo during iron uptake and that this does not involve an iron exchange step from one siderophore to the other. Furthermore, we demonstrated that, following PaA−Fe uptake, iron is released from the siderophore and iron-free PaA is secreted back into the growth medium and recycled by the cells to re-form a FpvA−PaA complex in the outer membranes.

EXPERIMENTAL PROCEDURES

Chemicals. Carbenicillin disodium salt was a generous gift from SmithKline Beecham (Welwyn Garden City, Herts, U.K.). [^{55}Fe]Cl_3 was purchased from NEN (Boston, MA).

Pyoverdins (PaA, PaA−Fe, [^{3}H]PaA′, and [^{3}H]PaA′−Fe) were prepared as described previously (*17*, *19*−*20*). PaA′, a PaA analogue produced by *P. aeruginosa*, differs from PaA by the presence of an α-keto carboxylic function on the succinamide group (*19*).

Synthesis of Chromium Complexes of Pyoverdins. Chromous chloride (18.3 mg) was added to a vigorously stirred solution of PaA (52.4 mg) in methanol (100 mL) under argon in a glovebox at room temperature, and sodium bicarbonate (20.4 mg) was added immediately. After 10 min, the mixture was removed from the glovebox and bubbled with air for 5 min, and the solvent was evaporated under reduced pressure. The residue was dissolved in 1 mL of 50 mM pyridine/acetic acid buffer, pH 5.0, purified by chromatography on a CM-25 Sephadex column filled with the same buffer (l = 20 cm, ϕ = 1.5 cm), and eluted first with 100 mL of 50 mM pyridine/acetic acid buffer, pH 5.0, and then with a linear gradient of 50 mM to 2 M of the same buffer (2 × 200 mL). Five milliliter fractions were collected. The fractions containing chromic PaA were pooled, evaporated under reduced pressure, lyophilized, and purified by HPLC. This was performed using a Nucleosil ODS 5 μm column (25 × 0.46 cm) maintained at 40 °C and eluted with a solution of 20 mM pyridine/acetic acid buffer, pH 5.0, containing 7% acetonitrile with a flow rate of 0.7 mL/min. The known PaA−Cr(III) complexes have retention times of between 13.0 and 22.0 min.

All of the PaA−Cr(III) complexes possess the same optical characteristics (λ_{max} = 412 nm; ϵ = 21500 M^{-1} L), mass spectrum features (FAB-MS, M^+ = 1383 mu), and electrophoretic features (7 mm; 300 V, pH 5.0 in pyridine/acetic acid buffer, 0.2 M, 30 min). They are conformers in slow equilibrium (*21*).

All of the experiments on PaA−Cr(III) complexes were carried out with each of the conformers, and no significant differences in the results have been observed.

Bacterial Strains and Growth Media. The strains used in this study were the wild-type strain *P. aeruginosa* ATCC 15692 and two mutants: CDC5(pPVR2), which overproduces FpvA and is PaA-deficient (*22*), and K691(pPVR2), which overproduces FpvA and produces PaA (*23*).

All of the strains were grown overnight in a succinate medium (*19*) in the presence of 150 μg/mL carbenicillin for strains CDC5(pPVR2) and K691(pPVR2).

Preparations of Outer Membranes for FRET Experiments. P. aeruginosa CDC5(pPVR2) was grown in 300 mL of liquid medium to an OD_{600} of 0.5−0.7. The cells were harvested by centrifugation, and the pellet was washed twice with 200 mL of fresh medium and then resuspended in 50 mM Tris-HCl (pH 8.0) to an OD_{600} of 0.6. Bacterial suspensions (40 mL) were stirred at 29 °C in the absence or presence of PaA−Fe (25−400 nM). After 1 h at 29 °C, the cell suspensions were harvested by centrifugation and resuspended in 20 mL of 50 mM Tris-HCl buffer (pH 8.0) in the presence of 1 mg/mL lysozyme, 1 mg/mL RNase, and 1 mg/mL DNase. The cells were disrupted by sonication, and outer membranes were prepared as described previously (*16*).

Displacement of [^{3}H]PaA′ by PaA−Fe or PaA−Cr on CDC5(pPVR2) Cells. CDC5(pPVR2) was grown to an OD_{600} of 0.5−0.6. The cells were harvested by centrifugation, and the pellet was washed twice with an equal volume of fresh medium before being resuspended in 50 mM Tris-HCl (pH 8.0) to an OD_{600} of 0.06. To saturate all PaA binding sites on the bacterial cell surfaces, 200 nM [^{3}H]PaA′ was added, and the mixture was incubated for 30 min at 29 °C. Transport was then initiated by adding increasing concentrations of PaA−Fe or PaA−Cr (0.1 nM to 500 μM). After 40 min transport at 29 °C, the cells were filtered (0.45 μM, cellulose nitrate membrane Filters, Whatman), and the radioactivity retained was counted.

Iron Uptake. PaA−[^{55}Fe] (0.25 Ci/mmol) was prepared as described previously (*17*). The iron uptake assays were performed as reported previously (*24*). *P. aeruginosa* ATCC 15692, CDC5(pPVR2), and K691(pPVR2) cells were prepared respectively at an OD_{600} of 0.4, 0.62, and 0.68 in 50 mM Tris-HCl (pH 8.0). The transport assays for *P. aeruginosa* ATCC 15692 were initiated by the addition of 1 μM PaA−[^{55}Fe], or [^{3}H]PaA′−Fe. The transport assays for CDC5(pPVR2) and K691(pPVR2) were initiated by the addition of 100 nM PaA−[^{55}]Fe. Aliquots (100 μL) of the suspension were removed at different times and filtered (0.45 μM, cellulose nitrate membrane filters, Whatman), and the radioactivity retained was counted.

Radioactivity Distribution in P. aeruginosa. P. aeruginosa ATCC 15692 cells were prepared at an OD_{600} of 0.6 in 10 mL of 50 mM Tris-HCl (pH 8.0) buffer. The transport assays were initiated by the addition of 100 nM [^{3}H]PaA′−Fe or [^{3}H]PaA. After 5 min, the transport was stopped by the addition of 10 mL of 1 mM NaN_3 and 50 mM Tris-HCl (pH 8.0) at 4 °C, and the cells were immediately harvested by centrifugation. The cells were then resuspended in 10 mL of Tris-HCl buffer and washed twice, and the remaining radioactivity was counted. Afterward, the cells were broken by sonication in 1 mL of 50 mM Tris-HCl (pH 8.0) in the presence of 1 mg/mL lysozyme, 1 mg/mL RNase, and 1 mg/mL DNase, and the membranes were collected by centrifugation (100000*g*). Radioactivity in the supernatants (cytoplasm and periplasm) was counted. The pellets (outer and inner membranes) were resuspended in 300 μL of 50 mM Tris-HCl (pH 8.0). Inner and outer membranes were separated on a sucrose gradient (*25*). Gradients were fractionated in 1 mL fractions, and the radioactivity in each one was counted.

Fluorescence Spectroscopy. Fluorescence resonance energy transfer (FRET) experiments were performed on membranes or on *P. aeruginosa* cells by use of a SPEX Fluorolog-2 spectrofluorometer (SPEX Industries, Inc., Edison, NJ) as described previously (*16*, *17*).

To show that PaA is recycled into the extracellular media, PaA-deficient cells were resuspended in succinate medium

(*19*) in the presence of 150 μg/mL carbenicillin to a final OD_{600} of 0.6. The cells were then incubated at 29 °C in the presence of 3 μM PaA−Fe. Aliquots of 1 mL were centrifuged, and the supernatant was collected. The fluorescence of the supernatant was measured at 447 nm with the excitation wavelength set at 400 nm.

For the fluorescence energy transfer spectra on outer membranes, the membranes were prepared as described previously (*16*) in 50 mM Tris-HCl (pH 8.0) at a concentration of 160 μg/mL. The excitation wavelength was set at 290 nm.

To measure the kinetics of fluorescence energy transfer at 447 nm, CDC5(pPVR2) and K691(pPVR2) were grown overnight to an OD_{600} of 0.5−0.7. The cells were then collected by centrifugation, and the pellet was washed twice for CDC5(pPVR2) and four times for K691(pPVR2) with an equal volume of fresh medium. The pellet were resuspended in 50 mM Tris-HCl (pH 8.0) to an OD_{600} of 0.62 for CDC5(pPVR2) and 0.68 or 0.55 for K691(pPVR2). The bacterial suspension (995 μL) was stirred at 29 °C in a 1 mL cuvette. Five microliters of ferric PaA was added to 995 μL of bacterial solution to obtain a final concentration of 100 nM iron-free PaA or PaA−Fe. The fluorescence at 447 nm (excitation wavelength set at 290 nm or at 400 nm) was measured every second for 30 min. To check the stability of the cells at 290 nm, the same experiments were repeated in the absence of the siderophore. For K691(pPVR2) the experiment was repeated in the presence of 25, 40, 50, 100, and 250 nM PaA−Fe or 500 nM PaA−Cr. The number of PaA binding sites or FpvA receptors on the cell surface of CDC5(pPVR2) was determined by binding assays using [^{3}H]PaA′ (*17*). SDS−PAGE gels showed the same FpvA production level in K691(pPVR2) cells as in CDC5(pPVR2) cells.

RESULTS

Mechanism of FpvA−PaA−Fe Complex Formation in Vivo and Fate of FpvA-Bound PaA during Iron Uptake. Previously data suggest that the FpvA receptor is normally bound to PaA in iron-starved *P. aeruginosa* cells in vivo (*16*, *17*). PaA binds to the receptor with high affinity but is not transported into the cell. The presence of FpvA−PaA complexes in the outer membrane raises questions about the mechanisms by which the FpvA−PaA complex is iron loaded and iron is taken up efficiently by the cells. Two possible mechanisms can be figured out: either the FpvA−PaA complex located in *P. aeruginosa* outer membranes captures iron(III) from extracellular PaA−Fe (iron exchange mechanism) or the bound PaA is displaced by the free PaA−Fe complex occurring from the medium (siderophore displacement mechanism). In vitro experiments using a tritiated PaA analogue ([^{3}H]PaA′) as marker have shown that the purified FpvA−PaA receptor is loaded with iron as a result of the displacement mechanism (*17*).

To differentiate between these two mechanisms in vivo and to follow the fate of iron and PaA separately during iron transport, we studied the uptake of PaA−[^{55}Fe] and [^{3}H]PaA′−Fe by *P. aeruginosa* ATCC 15692 cells. [^{3}H]PaA′ was only transported into the cells when it was presented as an iron complex (Figure 1). As described previously (*17*), iron-free

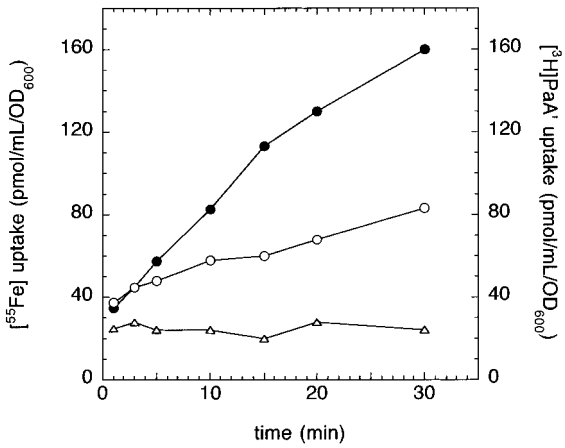

FIGURE 1: Time-dependent uptake of PaA−[^{55}Fe], [^{3}H]PaA′−Fe, and [^{3}H]PaA′ in *P. aeruginosa* ATCC 15692. *P. aeruginosa* ATCC 15692 cells at an OD_{600} of 0.4 were incubated for 15 min in 50 mM Tris-HCl (pH 8.0) before initiation of the transport assays by the addition of 1 μM PaA−[^{55}Fe] (●), [^{3}H]PaA′−Fe (○), or [^{3}H]PaA′ (Δ). Aliquots (100 μL) of the suspension were removed at different times and filtered, and the radioactivity retained was counted.

Table 1: Distribution of [^{3}H]PaA in Different Cellular Compartments of *P. aeruginosa*[a]

	[^{3}H]PaA′	[^{3}H]PaA′−Fe
total radioactivity (%)	100	100
radioactivity lost during washing (%)	99	85
radioactivity bound to outer membrane (%)	0.1	6
radioactivity bound to inner membrane (%)	0.1	6
radioactivity in cytoplasm and periplasm (%)	0.1	3

[a] *P. aeruginosa* ATCC 15692 cells were incubated for 5 min in 50 mM Tris-HCl (pH 8.0) in the presence of 100 nM [^{3}H]PaA′ or [^{3}H]PaA′−Fe, and the total radioactivity was counted. After 5 min incubation, the bacteria were centrifuged, resuspended in 50 mM Tris-HCl (pH 8.0), and washed twice, and the radioactivity lost was counted. For the radioactivity distribution measurements, the cells were broken and the soluble fraction (cytoplasm and periplasm) and outer and inner membranes were fractionated.

[^{3}H]PaA′ binds to the cell surface, but no further increase of [^{3}H]PaA′ uptake was observed with time. However, when ferric PaA was used, [^{3}H]PaA′ seemed to be transported into the cells at a rate similar to that of [^{55}Fe] during the first 3 min of iron uptake, but after 10 min, the transport rate of [^{3}H]PaA′ strongly deviated from that of [^{55}Fe].

This difference in uptake may not be due to iron exchange between unlabeled and radiolabeled PaA within the medium because the excess unlabeled PaA was removed from the medium by centrifugation prior to the addition of the radiolabeled ligands. To solve the uncertainty of whether PaA only accumulates inside the cell when the siderophore is loaded with iron, we directly measured the distribution of radioactivity after washing the cells, breaking them, and separating the outer membrane, inner membrane, and soluble fractions (periplasmic and cytoplasmic). The results presented in Table 1 demonstrate clearly that, after 5 min incubation, 9% of the radioactivity was bound to the inner membrane or in the soluble fraction with [^{3}H]PaA′−Fe, whereas with [^{3}H]PaA′ 99% of the radioactivity had already been lost during the washing step.

The internalization of both PaA and iron by the bacteria strongly suggests that in vivo the receptor is loaded with iron in a manner similar to that in vitro, through a siderophore

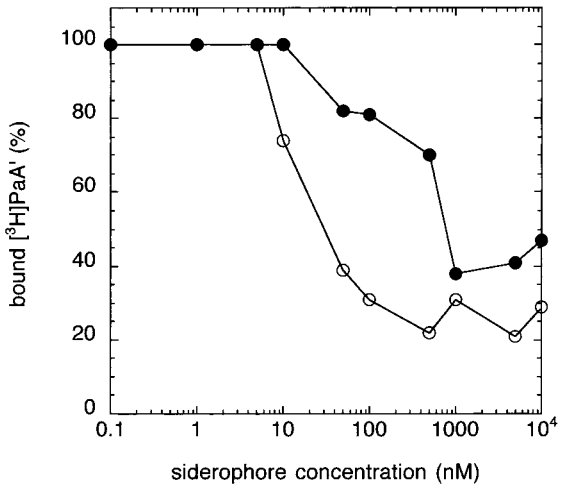

FIGURE 2: Displacement of [^{3}H]PaA′ by PaA−Fe or PaA−Cr on CDC5(pPVR2) cells. CDC5(pPVR2) (*fpvA*$^+$, ΔPaA) cells at an OD_{600} of 0.06 were incubated for 30 min at 29 °C in 50 mM Tris-HCl (pH 8.0) in the presence of 200 nM [^{3}H]PaA′ before initiation of the transport assays by the addition of increasing concentrations of PaA−Fe (○) or PaA−Cr (●) (0.1−10000 nM). After 40 min transport at 29 °C, the cell suspensions were filtered, and the radioactivity retained was counted.

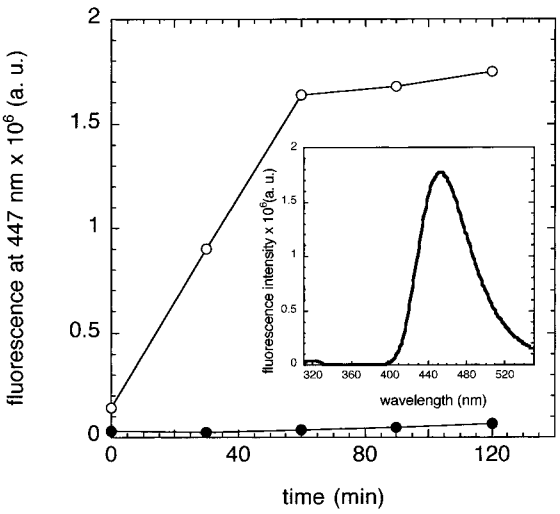

FIGURE 3: Appearance of fluorescence (λ_{exc} = 400 nm, λ_{em} = 447 nm) in the culture media of CDC5(pPVR2) (*fpvA*$^+$, ΔPaA) upon incubation in the absence (●) or in the presence (○) of 3 μM PaA−Fe. The bacteria were removed by centrifugation before fluorescence was measured. Insert: Fluorescence emission spectrum (excitation wavelength 400 nm) for the last measurement of the time course in the presence of PaA−Fe.

displacement mechanism rather than through an iron exchange mechanism. With the latter mechanism [^{55}Fe] uptake only is expected to occur. The fact that the transport kinetics of [^{3}H]PaA′−Fe were slower than those of PaA−[^{55}Fe] when ferric PaA was used is not in contradiction with this displacement mechanism because the results with [^{3}H]PaA′−Fe could be interpreted as being a [^{3}H]PaA′ recycling mechanism, where iron is released from the siderophore into the cells and the [^{3}H]PaA′ is excreted back into the extracellular medium. The recycling of the siderophore should decrease the [^{3}H]PaA′ accumulation rate, whereas the true uptake rate is identical to the iron uptake rate but, in contrast to iron, coupled with a secretion step.

Another experiment was performed to confirm this displacement mechanism. PaA-deficient CDC5(pPVR2) (*fpvA*$^+$, ΔPaA) cells, which produce siderophore-free FpvA at the cell surface, were first incubated in the presence of [^{3}H]PaA′ to saturate all binding sites with iron-free labeled siderophore (FpvA-[^{3}H]PaA′). The cells were then incubated in increasing concentrations of unlabeled PaA−Fe or PaA−Cr for 40 min in a competition experiment (Figure 2). Ferric siderophore complexes are kinetically labile (*26*), but if the ferric ion is replaced by chromic ion, this should induce kinetic inertness (*26*−*28*). Thereby, this stable PaA−Cr complex will not be able to act as a metal donor for the FpvA−PaA complexes and will only be able to displace PaA on FpvA. PaA−Fe and PaA−Cr were both able to displace [^{3}H]PaA′ from FpvA in vivo (Figure 2). Thus, during iron uptake, PaA−Fe does not act as an iron donor but displaces iron-free [^{3}H]PaA′ bound to FpvA; otherwise, no decrease of bound [^{3}H]PaA′ should be observed in the presence of PaA−Fe or PaA−Cr (Figure 2). The Cr complex is 1 order of magnitude less efficient than the Fe complex at displacing the PaA from its receptor. This is consistent with the difference in affinities between PaA−Fe (8 nM; *16*) and PaA−Cr (88 nM; data not shown) for FpvA. Twenty percent of the bound [^{3}H]PaA′ was not displaced by PaA−Fe or PaA−Cr and may be attributed to nonspecific binding of PaA.

Recycling of PaA after Iron Uptake. Our data suggested that, after iron uptake and dissociation from the siderophore, PaA is recycled into the extracellular medium (Figure 1). To confirm this hypothesis, PaA-deficient CDC5(pPVR2) cells were incubated in the presence of PaA−Fe, and the fluorescence emission at 447 nm (direct excitation of the chromophore, excitation wavelength set at 400 nm) in the extracellular medium was measured. Fluorescence appeared in the culture growth media after the removal of the bacteria by centrifugation. The fluorescence emission spectrum (insert in Figure 3) of the culture medium after the removal of the cells (t = 120 min; excitation wavelength, 400 nm) shows a typical fluorescence emission spectrum of iron-free PaA (*29*). As these cells are unable to synthesize PaA de novo, these results further demonstrate that PaA delivers iron(III) to the bacterium via a PaA-specific iron(III) uptake system, that iron dissociates from PaA, and that the unloaded siderophore is released back to the extracellular medium.

Different States of the FpvA Receptor during Iron Uptake. The above results confirm that, in iron-starved *P. aeruginosa* cells, the receptor is initially loaded with PaA and that, in the first round of iron uptake, PaA−Fe from the extracellular medium displaces the bound PaA. They also show that the PaA molecule taken up with the iron is released back to the medium. This raises questions about what happens next and which form the FpvA receptor is in after iron uptake (FpvA, FpvA−PaA, or FpvA−PaA−Fe).

We have previously shown that the fluorescence spectroscopy properties of iron-free PaA allow the transfer of fluorescence resonance energy (FRET) between tryptophans of the FpvA receptor and iron-free PaA when excited at 290 nm (*16*, *17*, *29*). After complexation with iron(III), the metal quenches the fluorescence of PaA (emission at 447 nm): the ferric PaA complex is no longer fluorescent and no FRET can be observed for the FpvA−PaA−Fe complex. For these reasons, FRET can be a very powerful tool for distinguishing between the different forms of FpvA: FpvA−PaA (FRET) and FpvA/FpvA−PaA−Fe/FpvA−PaA−Cr (no FRET).

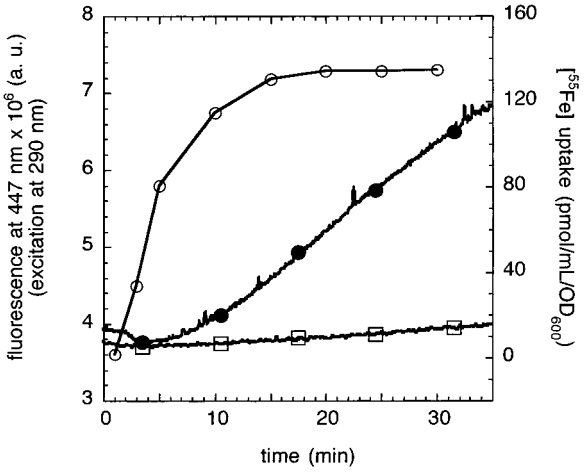

FIGURE 4: Iron uptake monitored by FRET in CDC5(pPVR2) cells in the presence of low concentrations of PaA−Fe. CDC5(pPVR2) (*fpvA*$^+$, ΔPaA) cells at an OD_{600} of 0.62 were incubated for 15 min in 50 mM Tris-HCl (pH 8.0) at 29 °C. The transport assay was initiated by the addition of 100 nM PaA−[^{55}Fe] to the cells. Aliquots (100 μL) were removed at different times, filtered, and counted (○). Simultaneously, the increase in fluorescence at 447 nm was monitored by measuring the emission of fluorescence at 447 nm (excitation set at 290 nm) every second for 30 min (●). The same fluorescence measurements were repeated with CDC5(pPVR2) cells in the absence of siderophore (□).

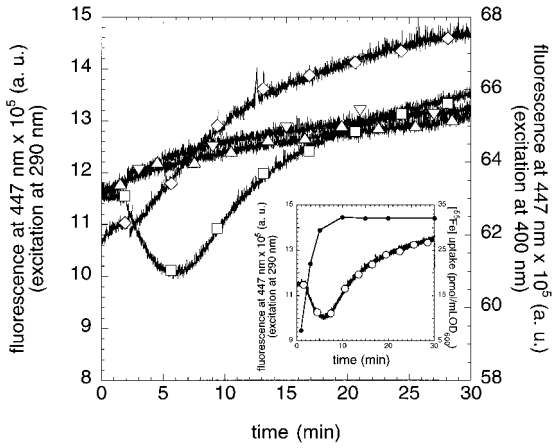

FIGURE 5: Iron uptake monitored by FRET in K691(pPVR2) cells in the presence of low concentrations of PaA−Fe. The PaA-producing, FpvA-overproducing K691(pPVR2) cells at an OD_{600} of 0.68 were incubated for 15 min in 50 mM Tris-HCl (pH 8.0) at 29 °C. After the addition of 100 nM PaA−Fe, the variation in fluorescence at 447 nm was monitored by measuring the emission of fluorescence at 447 nm every second for 30 min, with the excitation set at 290 nm (□) or at 400 nm (◇). The same fluorescence measurements (excitation set at 290 nm) were repeated with K691(pPVR2) cells in the absence of siderophore (△), in the presence of 100 nM iron-free PaA (▽), or in the presence of 100 nM PaA−[^{55}Fe] (insert, ○). For this last experiment, aliquots (100 μL) were removed at different times, filtered, and counted (insert, ●).

To determine in which form the FpvA receptor is after iron uptake, PaA-deficient CDC5(pPVR2) cells were incubated in the presence of 100 nM PaA−Fe, such that PaA−Fe was not in large excess compared to FpvA (22 nM). The number of PaA binding sites or FpvA receptors on the cell surfaces was estimated by a binding assay using [^{3}H]PaA′ (*17*). When PaA-deficient CDC5(pPVR2) cells are excited at 290 nm, no FRET occurred, and no emission of fluorescence was observed at 447 nm because no PaA is produced (*17*). Therefore, any FpvA−PaA complexes formed upon incubation of the cells with PaA−Fe can be followed by monitoring the increase in fluorescence energy transfer at 447 nm (FRET, excitation wavelength set at 290 nm) (Figure 4). The binding of ferric PaA to free binding sites on FpvA does not produce any FRET signal. Simultaneously, the same cell preparation was used to monitor [^{55}Fe] transport to assess the relationship between the kinetics of FpvA−PaA formation and [^{55}Fe] uptake.

Iron uptake kinetics reached a plateau after 15 min (900 s) (Figure 4). This iron uptake led to a simultaneous increase in fluorescence energy transfer, suggesting that the FpvA−PaA complex was formed at a much slower rate than iron uptake (after 30 min, no plateau was reached).

To confirm that the FRET signal was indeed due to the formation of FpvA−PaA complexes during or after iron uptake and not to a FRET signal due to another component within the bacteria, outer membranes were prepared from CDC5(pPVR2) (*fpvA*$^+$, ΔPaA) cells that had been incubated in a low concentration of PaA−Fe. During this incubation, iron uptake was allowed to occur in the cells. An emission peak at 447 nm (excitation 290 nm) was observed with these outer membranes (data not shown). The difference between the spectra in the presence and in the absence of PaA−Fe gave a typical fluorescent emission spectrum for PaA. Binding assays using [^{3}H]PaA′ on outer membranes prepared from CDC5(pPVR2) cells revealed a single class of binding sites with an affinity of 16 nM for iron-free PaA. This implies that, the only protein present in the outer membranes of *P. aeruginosa* that can bind iron-free PaA is FpvA. Moreover, CDC5(pPVR2) cells are PaA-deficient, which means that the only source of PaA in the above experiment was the PaA−Fe complex added at the beginning of the experiment. These data suggest that, after PaA−Fe uptake by *P. aeruginosa*, iron is released into the bacteria and iron-free PaA is recycled to re-form a FpvA−PaA complex in the outer membrane.

In the above experiments, conditions were chosen to avoid de novo synthesis of PaA. In the following experiments, the conditions were much closer to those encountered by the wild-type strains, as K691(pPVR2) cells (*fpvA*$^+$, PaA) produce both PaA and its receptor FpvA.

The above experiments were repeated using *P. aeruginosa* K691(pPVR2) cells (*fpvA*$^+$, PaA) (Figure 5). It is assumed that under our experimental conditions all of the FpvA receptor binding sites on the K691(pPVR2) cell surfaces were occupied by iron-free PaA prior to the addition of ferric PaA (*17*). When these cells were excited at 290 nm, FRET occurred, and a fluorescence emission peak was observed at 447 nm. As above, the K691(pPVR2) cells (*fpvA*$^+$, PaA) were incubated in the presence of 100 nM ferric PaA, such that it was not in large excess compared to FpvA (about 27 nM). The formation of the FpvA−PaA−Fe complexes was followed by monitoring the loss of fluorescence energy transfer at 447 nm (*17*). Simultaneously, the same cell preparations were used to monitor [^{55}Fe] transport to assess the relationship between the kinetics of FpvA−PaA−Fe formation and [^{55}Fe] uptake.

Iron uptake reached a plateau after 8 min (500 s) (Figure 5, insert). The variation of fluorescence emission at 447 nm was more complex (Figure 5). After the addition of

PaA−Fe, the fluorescence emission decreased during the first 400 s (6.5 min) and reached a minimum corresponding to the beginning of the plateau in the [^{55}Fe] uptake plot. This decrease in the emission of fluorescence indicated the formation of FpvA−PaA−Fe complexes, which is, therefore, the first step of iron uptake. The fluorescence at 447 nm subsequently increased and reached the same level as observed for the K691(pPVR2) cells incubated in the absence of siderophore (△, Figure 5) or in the presence of PaA (▽, Figure 5). This phenomenon indicated the gradual reloading of all FpvA sites on the cell surfaces with iron-free PaA. The addition of 40 nM iron-free PaA to the K691(pPVR2) cells (▽, Figure 5) did not change the FRET signal compared to the control (△, Figure 5), indicating that the PaA fluorophore is not directly excited at 290 nm and that unbound PaA does not contribute to the signal. This clearly shows that, when excited at 290 nm, the variation of emission of fluorescence at 447 nm is only due to the formation of FpvA−PaA−Fe or FpvA−PaA complexes. When the bacteria were excited directly at 400 nm (no FRET but direct excitation of the PaA chromophore), only an increase in fluorescence at 447 nm was observed, corresponding to the production of iron-free PaA following Fe^{3+} uptake, Fe^{3+} release, and de novo synthesis of PaA by the cell (◇, Figure 5). The small increase in the emission of fluorescence at 447 nm in the absence of ferric PaA or in the presence of iron-free PaA (▽ and △, respectively, Figure 5) is probably due to bacterial growth and de novo synthesis of the FpvA receptor and its loading with PaA. These experiments on K691(pPVR2) cells clearly show that the first step during iron uptake is the formation of FpvA−PaA−Fe complexes on the cell surfaces. After iron uptake and probably iron release, the FpvA receptor is loaded with another PaA molecule, thus forming FpvA−PaA complexes at the cell surfaces as observed above in CDC5(pPVR2) (*fpvA*$^+$, ΔPaA) cells (Figures 4 and 5).

Effect of the Concentration of PaA−Fe on the Different States of the FpvA Receptor in K691(pPVR2) (fpvA$^+$*, PaA) Cells.* To investigate the effect of increasing concentrations of PaA−Fe on the distribution of FpvA states during iron uptake, K691(pPVR2) cells (*fpvA*$^+$, PaA) were incubated in the presence of increasing concentrations of PaA−Fe, and the FRET signal was monitored at 447 nm (excitation set at 290 nm). For all PaA−Fe concentrations the first step was a concentration-dependent decrease in the fluorescence at 447 nm (Figure 6A), indicating the formation of FpvA−PaA−Fe complexes. The kinetics were biphasic in the presence of up to 100 nM PaA−Fe. Fluorescence reached a minimum and then increased to almost the same level as before the addition of PaA−Fe, indicating that the receptors were reloaded with iron-free PaA. The time course was monophasic at the highest PaA−Fe concentrations (250 nM), indicating that all of the receptors remained loaded with PaA−Fe and that recycled or newly synthesized PaA molecules were unable to bind to the receptor.

To confirm these results, PaA-deficient CDC5(pPVR2) cells at an OD_{600nm} of 0.6 were incubated in the presence of increasing concentrations of PaA−Fe. After 1 h of incubation during which transport occurs, the cells were broken, the outer membranes were isolated, and the emission of fluorescence at 447 nm was measured (excitation wavelength set at 290 nm) (Figure 6B). Under these conditions, the only

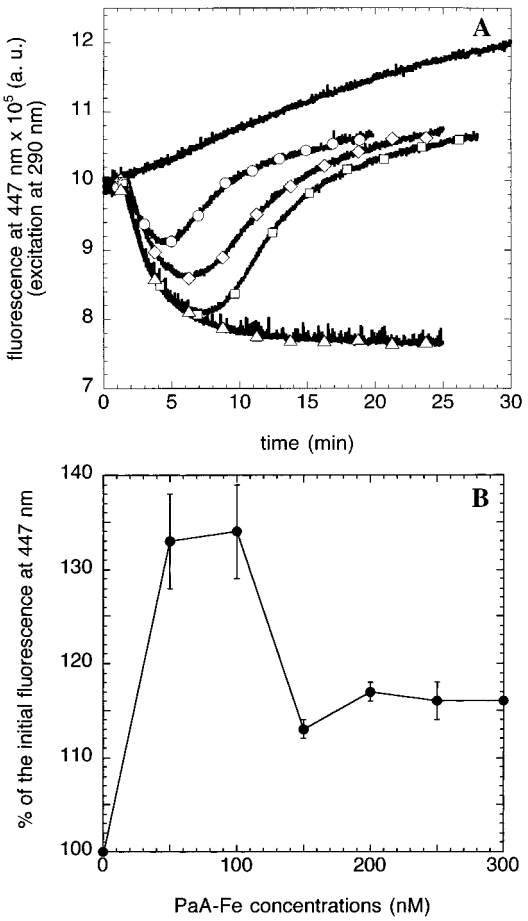

FIGURE 6: (A) Iron uptake monitored by FRET in K691(pPVR2) cells in the presence of increasing concentrations of PaA−Fe. The PaA-producing, FpvA-overproducing K691(pPVR2) cells at an OD_{600} of 0.55 were incubated for 15 min in 50 mM Tris-HCl (pH 8.0) at 29 °C. After the addition of 0 nM (black line), 25 nM (○), 50 nM (◇), 100 nM (□), and 250 nM (△) PaA−Fe, the variation in fluorescence at 447 nm (excitation set at 290 nm) was monitored by measuring the emission of fluorescence at 447 nm every second for 30 min. (B) FRET at 447 nm was monitored on outer membranes prepared from CDC5(pPVR2) (*fpvA*$^+$, ΔPaA) cells incubated in the presence of increasing concentrations of PaA−Fe (0−300 nM) for 1 h as described in Experimental Procedures.

source of iron-free PaA was from the dissociation of the PaA−Fe complex. In the presence of low PaA−Fe concentrations (below 150 nM), the FpvA receptors at the cell surfaces were reloaded with recycled PaA, and reloading was inhibited at higher PaA−Fe concentrations (Figure 6B).

Inhibition of PaA Recycling in CDC5(pPVR2) (fpvA$^+$*, ΔPaA) Cells upon Incubation with PaA−Cr.* The chromium complex of PaA, PaA−Cr, is a nonfluorescent complex, and no FRET should occur upon binding of PaA−Cr to FpvA. Moreover, PaA−Cr is a kinetically stable complex (*26*, *27*). Therefore, PaA−Cr cannot act as a metal donor for the FpvA−PaA complexes. In fact, PaA−Cr can only displace PaA from FpvA in the outer membranes. K691(pPVR2) (*fpvA*$^+$, PaA) cells were incubated in the presence of a nonsaturable concentration of PaA−Fe or PaA−Cr, and the formation of FpvA−PaA−Fe or FpvA−PaA−Cr complexes was followed by monitoring the decrease of fluorescence energy transfer at 447 nm (Figure 7). Due to the difference in the affinities between PaA−Fe (8 nM; *16*) and PaA−Cr (88 nM) to FpvA, 10 times more PaA−Cr was used than PaA−Fe.

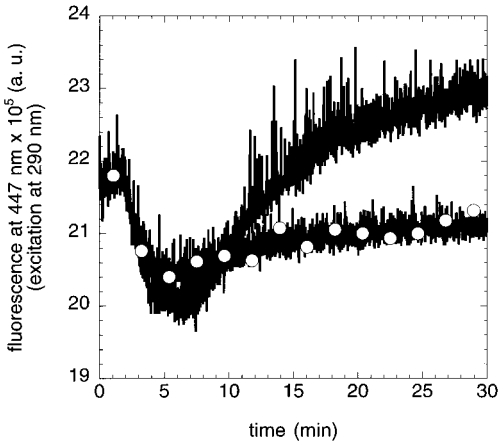

FIGURE 7: PaA exchange monitored by FRET in K691(pPVR2) cells in the presence of nonsaturating concentrations of PaA−Fe or PaA−Cr. CDC5(pPVR2) (*fpvA*$^+$, ΔPaA) cells at an OD_{600} of 0.62 were incubated for 15 min in 50 mM Tris-HCl (pH 8.0) at 29 °C. The transport assay was initiated by the addition of 40 nM PaA−Fe (black line) or 500 nM PaA−Cr (○) to the cells. The variation in fluorescence at 447 nm was monitored by measuring the emission of fluorescence at 447 nm (excitation set at 290 nm) every second for 30 min.

In the presence of PaA−Cr or PaA−Fe, a decrease of fluorescence energy transfer was observed during the first 500 s, corresponding to the formation of FpvA−PaA−Cr or FpvA−PaA−Fe complexes (Figure 7). PaA−Cr displaced PaA from FpvA with the same kinetics as PaA−Fe, but with the chromium complex no biphasic behavior and no increase in fluorescence due to recycling were observed. The formation of an FpvA−PaA−Cr complex clearly showed that the mechanism of FpvA−PaA−Fe formation during iron uptake in *P. aeruginosa* is a displacement mechanism. There are two potential reasons why the fluorescence did not increase with the chromium complex and the resulting lack of recycling of PaA: (i) PaA−Cr is not taken up by the cells in the same way as PaA (*17*), and instead it acts as an antagonist of the FpvA receptor; (ii) PaA−Cr is transported into the cells, but Cr is not released due to the stability of the complex. So far, we have no data showing whether PaA−Cr is or is not transported inside the cells such as PaA−Fe, and therefore, we are not able at present to answer the question above.

DISCUSSION

The recent findings (*16*, *17*) that under iron-limiting conditions the pyoverdin (PaA) binding site of the FpvA receptor from *P. aeruginosa* is fully occupied by iron-free PaA has raised several questions about the mechanism by which the receptor is loaded with iron at the cell surface and the efficiency of iron uptake in the presence of large excess of iron-free siderophore in the extracellular medium.

We have demonstrated that the first step of the iron-uptake pathway is the displacement of the bound PaA from the FpvA receptor by extracellular PaA−Fe to form the iron-loaded receptor rather than an exchange of the metal ion between PaA and PaA−Fe. Moreover, the uptake of [^{3}H]PaA′ when *P. aeruginosa* cells are incubated in the presence of [^{3}H]PaA′−Fe (Figure 1 and Table 1) and the ability of PaA−Fe and PaA−Cr (this latter cannot release the chromic ion) to displace preloaded [^{3}H]PaA′ from *P. aeruginosa* cells (Figure 2) are consistent with the siderophore displacement mechanism but not with the iron exchange mechanism. The rapid loss of fluorescence resonance energy transfer (FRET) observed on intact cells upon incubation with PaA−Fe that accompanies [^{55}Fe] uptake (Figure 5) clearly shows that the loading of FpvA with iron at the cell surface is an early step in the iron-uptake pathway. In addition, the formation of this FpvA−PaA−Fe complex is dependent on the concentration of PaA−Fe (Figure 6A).

This displacement mechanism is also consistent with the known strain specificity of the pyoverdin-mediated iron uptake in fluorescent *Pseudomonas* (*30*−*32*). Although the pyoverdins produced by different strains are very similar in their overall structure, they differ in the nature and location of their peptide chain and of their amino acids. The strict strain specificity of most pyoverdin-mediated iron transport systems is thought to be due to the highly specific recognition of the cognate siderophore by the outer membrane ferric pyoverdin receptor (*2*). A mechanism involving iron exchange between the receptor-bound siderophore and the extracellular ferric siderophore has been suggested by Stintzi and collaborators (*18*) for the nonspecific iron uptake by *A. hydrophila*. However, this mechanism would not be consistent with the strict strain specificity of the pyoverdin-mediated iron uptake in *Pseudomonas*.

The second major finding of this study is that the release of iron from PaA can be followed by monitoring fluorescence recovery by direct excitation of the PaA chromophore and that the loading status of the FpvA receptor can be monitored by FRET. It is important to emphasize that all of these in vivo FRET experiments have to be done with cells overexpressing the FpvA receptor [CDC5(pPVR2) and K691(pPVR2)]. When the receptor is not overexpressed as in ATCC 15 692 cells, the variation of fluorescence at 447 nm during iron uptake can hardly be measured in intact cells. Although FRET probably occurs between PaA and properly located tryptophans from other components of the iron uptake machinery (periplasmic binding protein, cytoplasmic membrane transporter), this FRET contribution is probably negligible in cells overexpressing FpvA. In addition, we have previously shown (*17*) that FRET was observed only in the presence of overexpressed FpvA and PaA but not when one of the components is missing (PaA-deficient strain overexpressing FpvA or FpvA-deficient strain producing PaA).

The difference between the incorporation kinetics of the two radiolabeled PaA−Fe (PaA−[^{55}Fe] and [^{3}H]PaA′−Fe) by *P. aeruginosa* cells (Figure 1) suggests that [^{55}Fe] dissociates from the siderophore within the cell and that [^{3}H]PaA′ is excreted back into the extracellular medium. Similar results have been described previously on *P. aeruginosa* with ^{14}C-labeled PaA (*33*). But here we directly measured the PaA release into the medium by monitoring the appearance of fluorescence in the growth medium of PaA-deficient CDC5(pPVR2) cells following incubation with nonfluorescent PaA−Fe (Figure 3). As CDC5 cells do not synthesize PaA, ferric PaA must deliver iron(III) to the microorganism via a PaA-specific iron(III) uptake system, and then after the iron is released, the iron-free PaA returns to the extracellular medium. A similar conclusion was reached by Weizman and collaborators (*34*) for the ferrichrome uptake pathway in *Pseudomonas putida* using a fluorescently labeled ferrichrome.

Moreover, monitoring the FRET signal with the same PaA-deficient strain (Figure 4) showed that PaA is not only secreted into the medium but also rebinds to the FpvA receptor on the outer membrane. There was a clear delay between [^{55}Fe] uptake and the increase of fluorescence energy transfer (Figure 4), indicating that the formation of the FpvA−PaA complex is an event following iron uptake which requires a dissociation step within the cell. With the PaA−Cr complex this dissociation step, and therefore recycling, does not occur. It is not clear whether iron-free PaA is recycled first through a step involving the formation of a FpvA−PaA complex and subsequent release into the medium or whether the PaA is first released into the medium and then binds to FpvA in the outer membranes. Previous binding studies with [^{3}H]PaA′ or PaA−[^{55}Fe] on purified FpvA receptors or on *P. aeruginosa* outer membranes showed only the occurrence of one single binding site for PaA or PaA−Fe which is absent in the outer membranes of FpvA-deficient strains. A release mechanism involving FpvA implies that PaA should be able to bind to this unique binding site on FpvA either from the extracellular side or from the periplasmic side of the outer membranes.

The FRET data (Figure 6) for PaA-producing K691-(pPVR2) cells incubated in the presence of increasing concentrations of PaA−Fe illustrate the dynamics of interconversion of the FpvA receptor between two possible states during iron uptake. In an iron-deficient medium, cells synthesize and excrete large amounts of PaA, and the FpvA receptor remains PaA-loaded (FpvA−PaA) (*17*). The presence of nonsaturating concentrations of PaA−Fe in the medium promotes iron loading of the receptor through siderophore and ferric siderophore exchange (decrease of the FRET signal) and triggers the uptake of the PaA−Fe complex. The PaA−Fe complex dissociates within the cells, iron-free PaA is recycled, and the PaA-loaded state of the receptor is restored (increase of the FRET signal). Conversely, when the cells are incubated in the presence of excess PaA−Fe, FpvA adopts its iron-loaded form (FpvA−PaA−Fe). This is because the excess PaA−Fe in the extracellular medium probably displaces the FpvA-bound PaA after PaA recycling.

It is not known how this cycle is regulated by the TonB machinery and the proton motive force (pmf), but the methods (double labeling, FRET) described here provide the necessary tools to tackle this question.

In conclusion, our study on PaA-mediated iron transport in *P. aeruginosa* revealed a different iron transport mechanism than the one usually proposed for ferrichrome in *E. coli* (*3*−*5*) or suggested by Stintzi and collaborators (*18*) for *A. hydrophila*. Although these iron transport mechanisms involve TonB-regulated outer membrane receptors, several differences exist. First, ferrichrome is not synthesized by *E. coli* whereas its receptor FhuA is. However, *P. aeruginosa* synthesizes both the PaA siderophore and its highly specific outer membrane receptor FpvA. Second, in *A. hydrophila*, the outer membrane receptor described by Stintzi and collaborators (*18*) is not specific for a single class of siderophores such as FpvA. Finally, the PaA receptor FpvA belongs to a subset of siderophore receptors that are also involved in signal transduction. For example, the binding of ferric citrate and pseudobactin BN7/8 to their cognate receptors in the outer membrane, respectively FecA in *E. coli* and PupB in *P. putida*, induces the expression of the genes responsible for siderophore uptake (reviewed in ref *15*), a process that can be separated from the transport of the siderophore across the outer membrane. There is some evidence that PaA positively regulates the expression of the *fpva* gene (*35*). As the loading status of FpvA (free PaA versus Fe−PaA) depends on the relative concentration of the two forms of PaA in the medium, this specific property may be linked to this regulatory role in *Pseudomonas*. FpvA may sense iron availability and then either interacts or not with the signal transduction machinery depending on its loading status with PaA or PaA−Fe.

A large number of questions remain about the precise mechanism by which iron is transported through two membranes and how this mechanism is strictly regulated to avoid the deleterious side effects of iron. It is now of paramount importance to locate the compartment in which iron dissociates from its chelator, to locate the recycling pathway, and to study the mechanism of iron transport through the inner membrane.

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