Real Time Fluorescent Resonance Energy Transfer Visualization of Ferric Pyoverdine Uptake in Pseudomonas aeruginosa

A ROLE FOR FERROUS IRON

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To acquire iron, Pseudomonas aeruginosa secretes a major fluorescent siderophore, pyoverdine (PvdI), that chelates iron and shuttles it into the cells via the specific outer membrane transporter, FpvAI. We took advantage of the fluorescence properties of PvdI and its metal chelates as well as the efficient FRET between donor tryptophans in FpvAI and PvdI to follow the fate of the siderophore during iron uptake. Our findings with PvdI-Ga and PvdI-Cr uptake indicate that iron reduction is required for the dissociation of PvdI-Fe, that a ligand exchange for iron occurs, and that this dissociation occurs in the periplasm. We also observed a delay between PvdI-Fe dissociation and the rebinding of PvdI to FpvAI, underlining the kinetic independence of metal release and siderophore recycling. Meanwhile, PvdI is not modified but recycled to the medium, still competent for iron chelation and transport. Finally, in vivo fluorescence microscopy revealed patches of PvdI, suggesting that uptake occurs via macromolecular assemblies on the cell surface.

Iron is an essential element for the growth of the vast majority of microorganisms. Under aerobic conditions, the abundance of free iron is limited by the very low solubility of ferric hydroxide. Thus, to maintain the required intracellular levels of iron, bacteria and fungi have developed efficient ferric ion-chelating agents, called siderophores (1), to scavenge iron from the extracellular environment and import it. A major siderophore produced by fluorescent Pseudomonas strains is pyoverdine (PvdI), which has a stability constant for iron of about 10^{32} M^{-1} (2). More than 100 different pyoverdines have been identified, forming a large class of mixed catecholate-hydroxamate siderophores characterized by a conserved dihydroxyquinoline-derived chromophore to which a peptide chain of variable length and composition is attached (3, 4).

In general, the uptake of ferric siderophores into Gram-negative bacteria involves a specific outer membrane transporter (OMT) and an inner membrane ABC transporter (5–7). The energy required for transport across the inner membrane is provided by ATP hydrolysis. The proton motive force of the inner membrane drives OMT-mediated transport across the outer membrane by means of an inner membrane complex comprising TonB, ExbB, and ExbD (8, 9). The PvdI OMT (FpvAI) of Pseudomonas aeruginosa was cloned by Dean and Poole (10) in 1993, and its structure was recently solved (11). FpvAI (11), like FptA (12), FhuA (13, 14), FepA (15), and FecA (16, 17) (the outer membrane transporters of pyochelin in P. aeruginosa and of ferrichrome, ferric enterobactin, and ferric citrate in Escherichia coli, respectively) consists of a C-terminal β-barrel domain and an N-terminal plug domain filling the barrel. The binding site for the ferric siderophore is located above the plug, well outside the membrane, and is composed of residues of the plug and β-barrel domains. The binding site of FpvAI consists mostly of aromatic residues, including six Tyr residues and two Trp residues, and only three hydrophilic residues (11).

The P. aeruginosa FpvAI is the best characterized pyoverdine OMT, whereas only three other transporters of pyoverdines have been cloned: FpvAII and FpvAIII, involved in the uptake of PvdII and PvdIII (18), and FpvB, a second OMT for PvdI in P. aeruginosa (19). Of these known Pvd transporters, only the structure of FpvAI has been solved, and its interactions with its siderophore PvdI have been studied using the fluorescent properties of this siderophore. Iron-free PvdI has spectral properties suitable for fluorescent resonance energy transfer (FRET) with the Trp residues of FpvAI (20). Previous studies using this tech-
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nique have shown that in iron-starved \textit{P. aeruginosa} cells, FpvAI is initially loaded with PvdI and that, in the first round of iron uptake, this bound PvdI dissociates from FpvAI, and PvdI-Fe from the extracellular medium takes its place on the transporter (21, 22). The exchange of PvdI-Fe for metal-free PvdI on FpvAI and the translocation of ferric siderophore are induced by TonB-mediated activation of the transporter (9, 23, 24). In \textit{E. coli}, the ferric siderophore complexes are then transported into the cytoplasm by an ABC transporter (7). Little is known about this step of iron acquisition in Pseudomonads. No ABC transporter has been identified for the PvdL/FpvAI pathway, and the cellular localization of the PvdL-Fe dissociation is unknown.

However, we have previously utilized the efficient FRET between FpvAI and PvdL to show that after iron is released from the siderophore, the iron-free siderophore is recycled via an unknown mechanism to the cell surface, where it again binds to FpvAI (22).

Three mechanisms have been proposed that could explain how bacteria overcome the thermodynamic barrier of iron removal from siderophore ligands: ligand exchange, reduction of the iron, and hydrolysis of the siderophore. A reduction mechanism can be differentiated from ligand exchange by investigating the dissociation while substituting iron with a metal that has a similar ionic radius, the same charge, and the same coordination geometry (25, 26). Cr(III) forms complexes with siderophores that are structurally similar to ferric siderophores but are kinetically inert to ligand substitution, whereas Ga(III) forms complexes with siderophores that are kinetically labile, and exchange with other ligands occurs readily (25, 26). However, gallium has no stable (II) oxidation state, so the metal cannot be removed from the ligand by reduction.

To investigate the fate of PvdL-Fe in \textit{P. aeruginosa} after transport across the outer membrane by FpvAI, we combined the FRET technique, fluorescence microscopy, and the use of PvdL-Ga and PvdL-Cr complexes. We found that neither PvdL-Cr nor PvdL-Ga could be dissociated by iron-starved \textit{P. aeruginosa}, despite the fact the PvdL-Ga is efficiently transported across that outer membrane, where it accumulates in the periplasm. No recycling of PvdL to the extracellular medium was detected for either PvdL-Cr or PvdL-Ga. This suggests that reduction of Fe(III) to Fe(II) and a ligand exchange are necessary for the complete release of the metal from PvdL. We have also shown that iron release does not involve a permanent chemical modification of PvdL; the siderophore is recycled to the extracellular medium unmodified and fully functional. Finally, fluorescence microscopy showed that there is no accumulation of PvdL-Ga in the cytoplasm, further evidence that the PvdL-metal complex dissociates in the periplasm immediately after its uptake.

\textbf{EXPERIMENTAL PROCEDURES}

\textbf{Chemicals}—Carbenicillin disodium salt was a generous gift from SmithKline Beecham (Welwyn Garden City, Herts, UK). Carbonyl cyanide \textit{p-}((trifluoromethoxy)phenyl)hydrazone (FCCP) and sodium \textit{n}-lauroylsarcosine were purchased from Sigma, and oPOE (\textit{n}-octylpolyoxyethylene) was from Bachem. Pvdls were prepared as described previously for PvdL and PvdL-Fe (2, 27), for PvdL-Ga (28), and for PvdL-Cr (22). Three mutants were used: CDC5(pPVR2), which overproduces FpvAI and is PvdL-deficient (29), K691(pPVR2), which overproduces FpvAI and produces PvdL (30), and the PvdL- and Pch-deficient strain PAD07 (31). The strains were grown overnight in a succinate medium (27) in the presence of 150 \textmu g/ml carbenicillin for CDC5(pPVR2) and K691(pPVR2) and 100 \textmu g/ml streptomycin and 50 \textmu g/ml tetracycline for PAD07.

\textbf{Image Acquisition}—CDC5(pPVR2) cells growing in succinate medium were incubated with or without siderophore, washed once with siderophore-free buffer, and then mounted onto 750-agarose-coated slides, as described previously (32). Images were acquired on an Olympus AX70 microscope with a MicroMax CCD camera using 100-ms exposures. Images were captured using MetaMorph 6.0 (Universal Imaging).

\textbf{Fluorescence Spectroscopy}—Fluorescence experiments were performed on K691(pPVR2) or CDC5(pPVR2) cells with a PTI (Photon Technology International TimeMaster; Bioritech) spectrofluorometer. For all experiments, the sample was stirred at 29 °C in a 1-ml cuvette, the excitation wavelength (\lambda_{em}) was set at 290 nm (for the FRET experiments) or 400 nm (for direct excitation), and the emission of fluorescence (\lambda_{em}) was measured at 447 nm. The cells were washed with 2 volumes of 50 mM Tris·HCl (pH 8.0) and resuspended in the same buffer to a final A_{600} of 2. PvdL-Fe, PvdL-Ga, PvdL-Cr, or PvdL was added, and the fluorescence at 447 nm was measured every 300 ms for the duration of the experiment. As a control, the same experiments were repeated in the absence of the siderophore, and an analysis by SDS-polyacrylamide gels showed the same amount of FpvAI in K691(pPVR2) cells as in CDC5(pPVR2) cells (21).

\textbf{Preparation of Periplasmic, Cytoplasmic, Inner Membrane, and Outer Membrane Fractions}—CDC5(pPVR2) cells were grown in 50 ml of succinate medium to an A_{600} of 1, at which time 600 \textmu M PvdL-Ga or PvdL-Fe was added. After 30 min, the transport was complete based on aliquots that were removed for fluorescence measurements. The cells were pelleted at 6000 \times g, and the pellet was gently rinsed with water and then resuspended in 3 ml of 20% sucrose, 1 mM EDTA, 0.2M Tris, pH 8.0. After 2 min at ambient temperature, with the integrity of the bacterial outer membrane compromised, osmotic shock was achieved by the rapid addition of 4.5 ml of ice-cold water followed by gentle mixing by inversion. After 2 min on ice, the periplasmic fraction was separated from the cells by centrifugation at 6000 \times g for 10 min. The pellet, which was firm and showing no signs of lysis, was gently rinsed with water and then resuspended in 20 ml of 20 mM Tris, pH 8.0. The cells were lysed by sonication, and the cytoplasmic fraction was separated from the insoluble material at 100,000 \times g for 30 min. The inner membrane was extracted from the pellet with 20 ml of 1% sodium \textit{n}-lauroylsarcosine in 20 mM Tris, pH 8.0, followed by a second 100,000 \times g spin. The outer membrane was extracted from the resulting pellet with 2% \textit{n}-octylpolyoxyethylene in 20 mM Tris, pH 8.0, followed by a final high speed centrifugation. The fluorescence intensity (\lambda_{em} = 400 nm, \lambda_{exc} = 450 nm) was measured for each fraction, and the appropriate dilution factors were applied to the measurements so that all reported intensities represent the total fluorescence from the same volume of the initial cultures.
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Characterization of the Recycled PvdI—CDC5(pPVR2) cells at an A_{600} of 2 were incubated in the presence of 300 nm PvdI-Fe at 30 °C in 10 ml of 50 mM Tris-HCl, pH 8.0, buffer. Iron uptake was followed in parallel in 1 ml of the mixture by excitation at 290 nm and monitoring the emission of fluorescence at 447 nm as described above. After 30 min of incubation, the cells were pelleted, and the supernatant containing the recycled PvdI was used for the following experiments.

The pH of 1 ml of the supernatant (containing the recycled PvdI) was adjusted to 4 with AcOH. 10 μM of 3 μM FeCl₃ was added, the sample was incubated for 5 min, and then the fluorescence emission was monitored (λ_{ex} = 400 nm). The additions of 10 μM of 3 μM FeCl₃ were repeated until there was no more fluorescence at 447 nm.

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For the transport assay, a 1-ml aliquot of this supernatant was incubated with 30 nM 55FeCl₃ for 10 min. The mixture was then added to PADO7 cells prepared at an A_{600} of 1 in 1 ml of 50 mM Tris-HCl, pH 8.0, buffer. After 1, 15, and 30 min of incubation at 37 °C, 100-μl aliquots were removed and filtered. The filters were washed and counted. This experiment was repeated with the incubations at 0 °C.

RESULTS

Dissociation of Iron from PvdI and PvdI Recycling on FpvA

Are Kinetically Independent Steps—The typical fluorescence signal for iron uptake via PvdI in P. aeruginosa, when excited at 290 nm and monitored at 447 nm, displays two steps: a decrease of fluorescence corresponding to the formation of FpvAI-PvdI-Fe upon the addition of PvdI-Fe and a subsequent increase corresponding to the recycling of PvdI on FpvA (formation of FpvAI-PvdI_{recy}) (Fig. 1). During the second step, the fluorescence always returned to exactly the same level as that before the addition of PvdI-Fe, indicating that all of the FpvAI receptors were loaded again with iron-free recycled PvdI. This could be the result of efficient recycling of PvdI to the media and rebinding to FpvAI or a mechanism in which the release of the metal from the siderophore occurs on FpvAI. The second possibility implies that the PvdI would never detach from the FpvAI receptor to enter the cells. If this were case, the increase of fluorescence in Fig. 1 (recycling on FpvA) would correspond to the dissociation of iron from the siderophore while still bound to FpvAI.

We tested the possibility of a PvdI-Fe dissociation on FpvAI. PvdI-deficient CDC5(pPVR2) cells were incubated with PvdI-Fe, and the fluorescence was recorded at 447 nm with excitation wavelengths of 290 nm (FRET) and 400 nm (direct excitation) (Fig. 2A). When PvdI-deficient CDC5(pPVR2) cells are excited at 290 or 400 nm, no FRET occurs, and no fluorescence is observed at 447 nm because no PvdI is produced (22). Moreover, after the addition of PvdI-Fe, the binding of PvdI-Fe to empty FpvAI does not produce any FRET signal. Therefore, with CDC5(pPVR2), only the dissociation of PvdI-Fe (formation of fluorescent PvdI) will produce a fluorescence signal, whereas the recycling of PvdI on FpvAI will be the only source of FRET. In this manner, the dissociation of iron from PvdI was followed with the λ_{ex} = 400 nm, whereas the recycling of PvdI on FpvA was simultaneously followed with λ_{ex} = 290 nm. If PvdI releases the iron while bound to FpvAI, then the fluorescence signal corresponding to PvdI-Fe dissociation should superimpose with that of the PvdI recycling on FpvAI. There was a lag of about 3 min between the signals, suggesting that PvdI-Fe dissociates inside the cells, before recycling of PvdI on FpvAI (formation of FpvAI-PvdI_{recy}). CDC5(pPVR2) cells were also incubated in the presence of metal-free PvdI, and in this case the formation of FpvAI-PvdI started immediately after the addition of the siderophore, with no lag (Fig. 2A) (23).

CDC5(pPVR2) cells were incubated in the presence of various concentrations of PvdI-Fe and the formation of free PvdI (Fig. 2B, λ_{ex} = 400 nm) and of FpvAI-PvdI_{recy} complex (Fig. 2C, λ_{ex} = 290 nm) was followed. The kinetics of PvdI-Fe dissociation and of FpvAI-PvdI_{recy} formation were independent of the initial PvdI-Fe concentration. However, as expected, there was a direct relationship between the PvdI-Fe concentration and the yield of PvdI and of FpvAI-PvdI_{recy}. Also of interest was that the delay before formation of FpvAI-PvdI_{recy} was proportional to the initial concentration of PvdI-Fe; the higher the concentration of PvdI-Fe, the longer the delay before the onset of formation of FpvAI-PvdI_{recy} (Fig. 2C, inset).

PvdI-Cr Is an Antagonist of the FpvA/PvdI System—Chromium forms complexes with siderophores that are structurally similar to siderophore-Fe but are inert to ligand exchange (33). Therefore, if the PvdI-Fe dissociation mechanism involves ligand exchange with another molecule or protein, PvdI-Cr will not be able to act as metal donor in a ligand exchange mechanism, and the transport will be inhibited.

We have previously shown that the PvdI-Cr complex binds to FpvAI with a 10-fold lower affinity than that of PvdI-Fe, and since PvdI-Cr is a nonfluorescent complex, no FRET occurs upon binding of PvdI-Cr to FpvAI (22). When K691(pPVR2) (FpvA'), PvdI') cells were incubated with various concentra-
tions of PvdI-Cr, a TonB-dependent decrease of fluorescence was observed corresponding to displacement of PvdI during the formation of FpvAI-PvdI-Cr; however, there was no subsequent recycling of PvdI on FpvAI (Fig. 3, A and B). Also, no PvdI-Cr dissociation (formation of metal-free PvdI) was detected with \( \lambda_{\text{ex}} = 400 \text{ nm} \) (Fig. 3C). Thus, PvdI-Cr acts as an antagonist of the PvdI iron uptake pathway in \( P. aeruginosa \), implicating ligand exchange for the iron ion in the PvdI-Fe dissociation mechanism.

**PvdI-Ga Is Efficiently Transported and Accumulates in the Periplasm**—A ligand exchange mechanism for PvdI-Fe dissociation does not exclude a requirement for ferric iron reduction to its lower affinity ferrous species. PvdI-Ga was used to investigate this possibility, since Ga(III) has no stable (II) oxidation state and therefore cannot be removed from the siderophore by reduction. Siderophore-Ga complexes are kinetically labile, exchange with other ligands occurs readily (25, 26), and Ga(III) is the closest ion to the ferric ion in size and ligand coordination. Therefore, PvdI-Ga should act as an antagonist of the iron uptake via PvdI only if reduction is involved in the PvdI-metal dissociation process.

We have shown previously that PvdI-Ga has a \( K_i \) of 16 nM for FpvAI and is transported into \( P. aeruginosa \) (28). Since PvdI-Ga is also fluorescent but with about a 2-fold higher quantum yield than PvdI, the removal of gallium from PvdI-Ga would result in a reduction in fluorescence intensity (28). Likewise, there is a large FRET signal when Pvd-Ga binds to FpvAI. We incubated Pvd-deficient CDC5(pPVR2) cells in the presence of 75 nM PvdI-Ga or PvdI-Fe, and dissociation of the metal from the siderophore was monitored by the emission of fluorescence at 447 nm (\( \lambda_{\text{ex}} = 400 \text{ nm} \)) (Fig. 4). The addition of the fluorescent PvdI-Ga complex causes an immediate jump in the fluorescence, followed by a decrease of fluorescence that could correspond to PvdI-Ga dissociation (28) in \( P. aeruginosa \) cells. When compared with the kinetic of PvdI-Fe dissociation (Fig. 4), the plateau for PvdI-Ga fluorescence was reached after 4 min (10 min for PvdI-Fe) and was of a higher fluorescence. This means that either differing amounts of metal-free PvdI were liberated in both experiments or that something else, such as an incomplete dissociation of PvdI-Ga, modulated the PvdI-Ga fluorescence.

To further investigate whether PvdI-Ga can dissociate in \( P. aeruginosa \) cells, we monitored the recycling of PvdI into the extracellular medium during PvdI-Ga uptake. PvdI-deficient CDC5(pPVR2) cells were incubated with 600 nM PvdI-Fe, PvdI-Ga, or PvdI-Cr (Fig. 5A). Aliquots were removed at various times, the cells were pelleted, and the quantity of PvdI or PvdI-Ga was measured in the extracellular medium (\( \lambda_{\text{ex}} =

![FIGURE 2. PvdI-Fe uptake and dissociation kinetics monitored by FRET and by direct excitation of PvdI in CDC5(pPVR2) cells.](http://www.jbc.org/content/282/5/2990/F2.large.jpg)

CDC5(pPVR2) (FpvA⁻, ΔPvdI) cells were washed and resuspended at an \( A_{\text{so}} \) of 2 in 50 mM Tris-HCl (pH 8.0) and incubated at 29 °C. A, the transport assay was initiated by the addition of 300 nM PvdI-Fe to the cells (arrow). Fluorescence at 447 nm was monitored with excitation at 290 nm (black line) and at 400 nm (Δ). CDC5(pPVR2) cells were also incubated with 300 nM PvdI, and fluorescence was monitored at 447 nm with excitation at 290 nm (>). B, the transport assay was initiated by the addition of 50 nM (△), 100 nM (○), 300 nM (◇), or 600 nM (□) PvdI-Fe to the cells (arrow). Fluorescence at 447 nm under excitation at 400 nm was monitored. C, the transport assay was initiated by the addition of 300 nM (○) or 600 nM (□) PvdI-Fe to the cells (arrow). Fluorescence was monitored at 447 nm with excitation at 290 nm. The experiment was repeated also with 50 nM, 100 nM, and 1 μM PvdI-Fe (data not shown). Inset, plot of the time delay for the start of formation of FpvAI-PvdI recy versus the concentration of PvdI-Fe incubated with CDC5(pPVR2) cells.
400 nm and $\lambda_{	ext{em}} = 447$ nm). Efficient recycling of the siderophore to the extracellular medium was observed for PvdI-Fe, as described previously (22), and as expected, no recycling of PvdI-Cr was detected. However, PvdI-Ga was nearly depleted from the media at about the same rate as the recycling of PvdI from PvdI-Fe (Fig. 5A). If PvdI-Fe and PvdI-Ga were transported by $P. aeruginosa$ with the same kinetics and mechanism, then the same amount of fluorescent PvdI would be recycled from both complexes, and a plateau of equivalent fluorescence would be reached. However, PvdI from PvdI-Ga was not recycled as it is from PvdI-Fe, so it must therefore remain associated with the bacteria (Fig. 5A).

After the uptake of the PvdI-Fe and PvdI-Ga complexes, we measured the amount of fluorescent PvdI or PvdI-Ga in the periplasm, cytoplasm, and inner and outer membranes to determine where the PvdI-Ga was localized (Fig. 5B). The majority of PvdI-Ga remained in the periplasm, apparently trapped and unable to be recycled to the extracellular medium. After PvdI-Fe uptake, there was less PvdI in the periplasm than in the case of PvdI-Ga due to the efficient recycling of PvdI. The remaining fluorescence in the extracellular medium and outer membrane (Fig. 5B) may reflect an equilibrium at these unnaturally high levels of Pvd-metal complexes or similarly an overloading of the PvdI transport pathway as would be consistent with the previously observed saturation of FpvAI at 450 nM PvdI-Fe (22). In order to confirm that the observed partitioning of Pvd-Ga into the periplasmic fraction was not due to an inefficient fractionation procedure, samples from all four cell fractions were analyzed on Tricine-SDS-PAGE and visualized by

![FIGURE 3. Pvd-Cr binding kinetics monitored by fluorescence in K691(pPVR2) cells.](image)

K691(pPVR2) ($FpvAI^{+}$, PvdI$^{+}$) cells were washed and resuspended at an $A_{600}$ of 2 in 50 mM Tris-HCl (pH 8.0) and incubated at 29 °C. A, after the addition of 3 μM PvdI-Cr (arrow), fluorescence at 447 nm was monitored with excitation at 290 nm (Δ). The experiment was repeated with K691(pPVR2) cells in the absence of siderophore (○) and with cells preincubated with 200 μM FCCP (□). B, after the addition of 300 nM (△), 1.2 μM (○), or 3 μM PvdI-Cr (□) (arrow), fluorescence at 447 nm was monitored with excitation at 290 nm. The experiment was repeated in the absence of siderophore (○) and in the presence of 300 nM PvdI-Fe (black line). C, after the addition of 3 μM PvdI-Cr, fluorescence was monitored at 447 nm with excitation at 290 nm (△) and at 400 nm (□).
both silver and Coomassie staining (Fig. 5C). The Tricine buffer system allowed the clear visualization of peptides down to 10 kDa, revealing a unique staining pattern for each fraction with no visible contamination of small peptides from the cytoplasm in the periplasm. Furthermore, the large difference in Pvd-Ga concentration observed in each fraction is not likely to originate from a minor contamination during the cell fractionation.

*P. aeruginosa* imported PvdI-Ga with a similar kinetic to PvdI-Fe (Fig. 5A); however, the PvdI-Ga accumulated in the periplasm (Fig. 5B) instead of being recycled to the media. Our findings are consistent with an incomplete dissociation of PvdI-Ga (kinetic in Fig. 4), such that the siderophore is unable to be recycled in the extracellular medium. A reduction of Fe(III), thereby lowering PvdI affinity for iron, appears to be required for a complete and efficient release of the metal from PvdI in vivo.

**Recycled PvdI Is Able to Chelate Iron and to Start a New Iron Uptake Cycle**—Siderophore-iron dissociation in Gram-negative bacteria could also involve hydrolysis of the siderophore to overcome the high affinity for iron. Recycled PvdI has the same spectral properties as PvdI extracted from *P. aeruginosa* cultures (22), suggesting that the chromophore is not modified by the bacteria. To test whether the peptide moiety is modified, particularly the two formylhydroxy-ornithines involved in the metal coordination, the ability of PvdIrecy to chelate iron was investigated. PvdIrecy was incubated with stoichiometric amounts of FeCl₃, and formation of PvdIrecy-Fe was observed by the decrease in fluorescence ($\lambda_{\text{ex}} = 400$ nm, $\lambda_{\text{em}} = 447$ nm). After confirming that PvdIrecy was able to chelate iron efficiently (Fig. 6), we then loaded PvdIrecy with $^{55}$Fe and demonstrated that it was able to transport iron into PvdI- and Pch-deficient *P. aeruginosa* cells (Fig. 7). Thus, the PvdI recycled after iron uptake and release is a fully functional siderophore, able to chelate iron and to start a new iron uptake cycle.

**PvdI Bound to FpvAI Is Located in Patches at the Cell Surface**—Because of its fluorescent properties, Pvd is suitable for imaging in living cells by fluorescence microscopy. We immobilized growing Pvd- and Pch-deficient *P. aeruginosa* (PAD07) cells on agarose, and as expected, only low levels of background fluorescence were detectable in the CFP filter set (Fig. 8A). However, when 300 nM PvdI was added to the cells, there was strong fluorescence at the periphery of washed cells (Fig. 8B). Interestingly, most cells (85%, 180 cells counted) contained one region of higher fluorescence intensity, most often located close to one cell pole, but also occurring at the lateral edges (see enlargements in Fig. 8B). These experiments clearly demonstrate that PvdI binding sites are nonuniformly distrib-

![FIGURE 5. A, recycling of PvdI to the extracellular medium. The appearance of fluorescence ($\lambda_{\text{ex}} = 400$ nm, $\lambda_{\text{em}} = 447$ nm) in the culture media of CDC5(pPVR2) (PvdI⁺) upon incubation in the absence (●) or in the presence of 600 nM PvdI-Fe (○), PvdI-Ga (△), or PvdI-Cr (□). B, cellular distribution of PvdI after PvdI-metal uptake. Growing cultures of CDC5(pPVR2) at an $A_{600}$ of 0.8 were incubated for 30 min in the presence of 600 nM PvdI-Fe (black) or PvdI-Ga (white), after which the amount of PvdI and PvdI-Ga was quantified in the media and each cellular location (periplasm, cytoplasm, inner membrane, and outer membrane). Values are expressed as a percentage of the total fluorescence recovered from each culture. C, Tricine SDS-PAGE of cell fractions. Equal volumes of each cell fraction were run on a 11% Tricine SDS-polyacrylamide gel and visualized by both silver and Coomassie staining. The molecular weight standards (M) are in lane 1, and lanes 2–5 show periplasm (P), cytoplasm (C), inner membrane (I), and outer membrane (O). The unique band pattern for each fraction demonstrates that there is no significant contamination of the periplasm by other cell fractions. The protein standard masses, noted on the left, are in kilodaltons.](http://www.jbc.org/content/282/5/2992)
uted in the cell outer membrane. However, the significance of this nonuniform staining pattern is not immediately evident. This result also supports the findings that PvdI binds to the FpvAI OMT in the absence of iron and is retained at the receptor for an extended period of time (at least 10 min). In agreement with in vitro data, the addition of PvdI-Fe to the cells does not give rise to a fluorescent signal (Fig. 8C), even after incubation for 30 min. Thus, there was no change in intracellular fluorescence that might have indicated the dissociation of PvdI-Fe. We previously estimated that only 10% of the receptors are activated by TonB and able to transport PvdI-Fe (23), so the amount of PvdI-Fe transported and dissociated inside the cells may not be sufficient for detection of PvdIrecy by fluorescence microscopy. Further experiments are necessary before conclusions can be drawn. The addition of PvdI-Ga resulted in a fluorescent pattern that was indistinguishable from that of free PvdI (Fig. 8, compare D with A). Even after 20 min, PvdI-Ga fluorescence was only observed at the periphery of cells and not inside the cell, further evidence that PvdI-Ga does not accumulate in the cytoplasm.

DISCUSSION

Two mechanisms for the release of iron from a siderophore-iron(III) complex have been reported in the literature. One involves hydrolysis of the siderophore-Fe complex, as in the case of ferric enterobactin in *E. coli*, in which the cyclic triester is hydrolyzed by esterases (34). The second mechanism involves the reduction of Fe(III) to Fe(II), the ferrous ion having a much lower affinity for the siderophore (35). Fe(II) is then easily released to a ligand with a higher affinity for Fe(II) than that of the siderophore (36). We demonstrated that PvdI, after having released iron inside the bacteria, is recycled to the extracellular medium in a form that is still able to bind and transport iron (Figs. 6 and 7). Clearly, the PvdI-Fe dissociation does not involve hydrolysis or chemical modifications of the siderophore that permanently lower its affinity for Fe(III). Despite the huge amount of PvdI produced by the cells in iron-limited planktonic cultures (200 mg/liter), PvdI is not consumed during the iron uptake process but is efficiently recycled to the extracellular medium. This efficient use of PvdI might be because the synthesis of PvdI is complex (37), involving many enzymes and consuming substantial energy and substrates.

Fluorescence measurements during PvdI-Cr uptake showed that there was no dissociation of the kinetically stable metal-siderophore complex (Fig. 3C), evidence that there is a ligand...
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exchange for iron during its uptake. Although no candidates have been identified, this exchange mechanism might involve a periplasmic binding protein of an ABC transporter, since the dissociation appears to occur in the periplasm.

Unlike the PvdI-Cr complex, which is substitution-inert, the PvdI-Ga complex is kinetically labile, and exchange with other ligands occurs readily. Since gallium has no stable (II) oxidation state, the bacteria cannot remove the metal from the ligand by reduction. Based on the decrease in fluorescence during its uptake, PvdI-Ga seemed to dissociate in P. aeruginosa cells (Fig. 4). However, the rate of decrease was more than 2 times faster than PvdI-Fe dissociation, suggesting that we might actually be observing a rapid step that occurs just before the complete dissociation of PvdI-Ga. Subsequently, we found that PvdI from PvdI-Ga was not recycled to the extracellular medium but accumulates in the periplasm (Fig. 5), as if the siderophore was still associated with the metal in this cellular compartment. Presumably, gallium does not completely dissociate from PvdI despite the decrease in fluorescence observed during uptake (Fig. 4). Formation of a ternary complex between PvdI-Ga and another protein, like a periplasmic binding protein or a reductase, could be an explanation for the decrease of fluorescence observed in Fig. 4. Such a mechanism would be similar to the one described for Fe(III) release from ferrozine, [36], in which a ternary complex forms between ferrozine B and another iron-chelating molecule, sulfonated bathophenanthroline, in a rapid step followed by a rate-limiting reduction of the ternary complex by glutathione or ascorbate. Another explanation for the decrease of fluorescence observed in Fig. 4 could be a protonation of the catecholate coordination in the PvdI-Ga complex. It would be expected to lower the fluorescence (28) while at the same time decreasing the affinity of the siderophore for the metal. The kinetic parameters for the formation and the dissociation of PvdI-Fe in acidic conditions have been reported (2). The in vitro dissociation mechanism occurs by an unfolding of PvdI via successive protonations of the coordination sites starting with the catecholate coordination site (2). Thus, it is reasonable to speculate that an in vivo protonation would occur on the catechol, explaining the decrease of fluorescence observed in Fig. 4. Following such a protonation, although the chromophore would no longer be involved in the chelation of the metal, there would be significant residual affinity from the two remaining hydroxamates. However, for a complete dissociation of the metal from the siderophore, a reduction step and capture of the iron by another ligand must be necessary. Further investigations are needed to identify the source of the protons for the putative first step of the PvdI-Fe dissociation, the source of electrons for the reduction, and the periplasmic iron binding molecule for the ligand exchange. Ferrisiderophore reductase activities have been reported in P. aeruginosa, so the reduction of iron in PvdI-Fe is plausible; however, the corresponding enzymes have not been identified, and the activities have been located in the cytoplasm (38, 39). Also it has been shown in vitro that ferric siderophore from Gram-negative bacteria could be reduced by a simple chemical process rather than by an enzymatic process (40). In this case, flavins in the oxidized form (FMN, FAD, and riboflavin) are enzymatically reduced by electron transfer from NADH or NADPH, and the reduced flavins in turn transfer their electrons to the ferric siderophores. This reduction reaction is also greatly stimulated by Fe(II) acceptors, such as ferrozine.

After uptake through the outer membrane by FpvAI, dissociation of PvdI-Fe is rapid (Fig. 2A), and there is no intracellular accumulation of the metal form of this siderophore. Cells osmotically shocked after incubating with [55Fe]ferrir[b]Pvd (41) and Mössbauer spectroscopy studies (42) showed no accumulation of PvdI-Fe inside the cells. Furthermore, we found that after overloading the PvdI uptake pathway with PvdI-Fe, less than 0.5% of the total fluorescence was in the cytoplasm (Fig. 5B), a level that is barely above the background level in untreated cells (data not shown). In the case of PvdI-Ga overloading, ~2% of the fluorescence was in the cytoplasmic fraction, and ~70% was in the periplasm. The small amount in the cytoplasmic fraction is probably a contamination from incomplete extraction of the periplasm during osmotic shock. In contrast to PvdI-Fe uptake, cytoplasmic accumulation of ferric enterobactin in E. coli and ferricoprogens in Neurospora crassa (43) before the ligand is released has been observed by Mössbauer spectroscopy.

To further confirm the cellular location of PvdI-Ga during transport and the likely location of PvdI-Fe dissociation, we used fluorescence microscopy. PvdI-deficient CDC5(pVR2) cells were incubated in the presence of PvdI-Ga, after which the complex was not detectable in the cytoplasm (Fig. 8D). The absence of PvdI-Ga in the cytoplasm points to the periplasm as the likely location of PvdI-Fe dissociation. Moreover, the genome of P. aeruginosa contains the genes of only a few ABC transporters, whereas there are numerous outer membrane transporter genes (44, 45). Therefore, it would be logical that the ferric siderophore complexes used by P. aeruginosa dissociate in the periplasm and that only the iron is transported into the cytoplasm by an ABC transporter. In addition, we have recently reported that at least one enzyme involved in the synthesis of Pvd is localized in the periplasm and that the final Pvd is probably only produced in this cellular compartment (46). It is therefore possible that no mechanism exists for the export of Pvd from the cytosol in P. aeruginosa, although perhaps one exists for a synthetic precursor of Pvd. The reason that the dissociation of Pvd-Fe and the final step of Pvd synthesis occur in the periplasm may in fact be that it obviates the risk of accumulating such a potent iron chelator in the cytoplasm, where it would compete with the cytoplasmic iron-dependent enzymes.

The novel use of fluorescence microscopy to investigate the PvdI iron uptake pathway in living P. aeruginosa cells allowed us to visualize PvdI and PvdI-Ga bound to their OMT, FpvAI, at the cell surface. Although the data confirmed that under iron-limited conditions the FpvAI receptors at the cell surface are loaded with iron-free PvdI (Fig. 6B) (6, 20, 21), we also found that about 85% of the cells showed a region of higher fluorescence (Fig. 6A) (6, 20, 21). The fate of ferric pyoverdine in P. aeruginosa, therefore, is probably only produced in this cellular compartment (46). It is therefore possible that no mechanism exists for the export of Pvd from the cytosol in P. aeruginosa, although perhaps one exists for a synthetic precursor of Pvd. The reason that the dissociation of Pvd-Fe and the final step of Pvd synthesis occur in the periplasm may in fact be that it obviates the risk of accumulating such a potent iron chelator in the cytoplasm, where it would compete with the cytoplasmic iron-dependent enzymes.

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The delay in PvdI recycling after iron release (Fig. 2) is sufficiently long for the PvdI-Fe complex to be transported into the periplasm, dissociated, and re-exported to the outer mem-
brane. However, the FRET after the full iron uptake cycle returns to the same level as before the addition of PvdI-Fe (Fig. 1), which is also consistent with a mechanism in which PvdI brings the iron to the cells without dissociating from the OMT. The delay between PvdI-Fe dissociation and PvdI recycling on FpvAI can also be explained by a mechanism in which the plug domain of FpvA is pulled into the periplasm. In this scenario, PvdI-Fe could remain bound to this partially or totally unfolded plug domain, where iron release occurs by a reduction and ligand exchange, possibly involving an apoperiplasmic protein.

With the plug domain dislodged from the barrel, none of the Trp residues in FpvAI would be close to the metal-free fluorescent siderophore, and no FRET would occur. FpvAI contains 17 Trps, but they are all located in the β-barrel domain. After release of iron by the plug-bound siderophore, the plug with fluorescent PvdI refolds into the β-barrel domain such that FRET is restored. Regardless of the actual mechanism of recycling, the delay before the observed onset of PvdI recycling in Fig. 2 can be due to another phenomenon. The macroscopic FRET only begins to appear after the PvdI-Fe uptake process has reduced the concentration of PvdI-Fe in the extracellular medium to a threshold that allows PvdI to begin to accumulate on FpvAI. Before this threshold is reached, the thermodynamics favor a displacement of PvdI by the surplus of Pvd-Fe in the extracellular medium.

This study provides the first evidence for the role of iron reduction in the in vivo dissociation of PvdI-Fe, improving our understanding of the mechanism of PvdI-Fe uptake in P. aeruginosa and iron uptake by pyoverdines into fluorescent Pseudomonads in general. We conclude that metal-siderophore dissociation via the FpvAI/PvdI pathway occurs without chemical modification of PvdI and rather by a reduction of Fe(III) followed by ligand exchange. Our data indicate that PvdI dissociates from iron in the periplasm, the location of PvdI-Ga accumulation, and that free PvdI is then recycled to the extracellular medium, where it can undergo a new cycle of iron uptake. Thus, the mechanism reported here for PvdI and relevant for more than 100 currently known pyoverdines is different from that described previously for ferric enterobactin and ferrichrome. Further studies are necessary to determine whether the PvdI-Fe dissociation occurs on FpvAI, to identify what catalyzes the reduction of Fe(III), and to identify the apoperiplasmic binding protein involved in the capture of the iron released from PvdI.

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REFERENCES
1. Boukhalfa, H., and Crumbliss, A. L. (2002) Biometals 15, 325–339
2. Albrecht-Gary, A. M., Blanc, S., Rochel, N., Ocactkan, A. Z., and Abdallah, M. A. (1994) Inorg. Chem. 33, 6391–6402
3. Meyer, J. M., Geoffroy, V. A., Baida, N., Gardan, L., Izard, D., Lemanceau, P., Achouak, W., and Palleroni, N. J. (2002) Appl. Environ. Microbiol. 68, 2745–2753
4. Budzikiwcz, H. (1997) Z. Naturforsch. C 52, 713–720
5. Braun, V. (2003) Front. Biosci. 8, 1409–1421
6. Schalk, I. J., Yue, W. W., and Buchanan, S. K. (2004) Mol. Microbiol. 54, 14–22
7. Koster, W. (2001) Res. Microbiol. 152, 291–301
8. Moeck, G. S., and Coulton, J. W. (1998) Mol. Microbiol. 28, 675–681
9. Postle, K., and Kadner, R. J. (2003) Mol. Microbiol. 49, 869–882
10. Dean, C. R., and Poole, K. (1993) Mol. Microbiol. 8, 1095–1103
11. Conti, C., Celia, H., Foschini, N., Schalk, I. J., Abdallah, M. A., and Pattus, F. (2005) J. Mol. Biol. 34, 121–134
12. Conti, C., Celia, H., and Pattus, F. (2005) J. Mol. Biol. 352, 893–904
13. Ferguson, A. D., Breid, J., Diederichs, K., Welte, W., and Coulton, J. W. (1998) Protein Sci. 7, 1636–1638
14. Schalk, I. J., Yue, W. W., and Buchanan, S. K. (2003) J. Mol. Biol. 332, 353–368
15. Schalk, I. J., Abdallah, M. A., and Pattus, F. (2002) Biochimie 84, 351–360
16. Schalk, I. J., Abdallah, M. A., and Pattus, F. (2002) Biochemistry 41, 1663–1671
17. Clément, E., Mesini, P. J., Pattus, F., Abdallah, M. A., and Schalk, I. J. (2004) Biochemistry 43, 7954–7965
18. Postle, K., Zhao, Q., Neshat, S., Heinrichs, D. E., and Dean, C. R. (1996) Microbiology 142, 1449–1458
19. Emery, T., and Hoffer, P. B. (1980) J. Nucl. Med. 21, 935–939
20. Ecker, D. J., and Emery, T. (1983) J. Bacteriol. 155, 616–622
21. Demange, P., Wendenbaum, S., Linet, C., Mertz, C., Cung, M. T., and Dell, A. Abdallah, M. A. (1990) Biol. Metals 3, 155–170
22. Fonschewer, N., Gallay, J., Vincent, M., Abdallah, M. A., Pattus, F., and Schalk, I. J. (2002) Biochemistry 41, 14691–14694
23. Hert, J., Hennard, C., Dugave, C., Poole, K., Abdallah, M. A., and Pattus, F. (2001) Mol. Microbiol. 39, 351–360
24. Takase, H., Nitanai, H., Hoshino, K., and Otani, T. (2000) Infect. Immun. 68, 1834–1839
25. Mascal, J., Soppa, J., Sturikov, A. V., and Graumann, P. L. (2002) EMBO J. 21, 3108–3118
26. Clevenger, A., and Neilsen, G. (1976) J. Bacteriol. 126, 823–830
27. Langman, L., Young, I. G., Frost, G. E., Rosenberg, H., and Gibson, F. (1972) J. Bacteriol. 112, 1142–1149
28. Matzkan, B. F., Anemuller, S., Schunemann, V., Trautwein, A. X., and Hantke, K. (2004) Biochemistry 43, 1386–1392
29. Mies, K. A., Wirgau, J. I., and Crumbliss, A. L. (2006) Biometals 19, 115–126
30. Ravel, J., and Cornelis, P. (2003) Trends Microbiol. 11, 195–200
31. Halle, F., and Meyer, J. M. (1992) Eur. J. Biochem. 209, 621–627
32. Halle, F., and Meyer, J. M. (1992) Eur. J. Biochem. 209, 613–620
33. Coves, J., and Fontecave, M. (1993) Eur. J. Biochem. 211, 635–641
34. Royt, P. W. (1990) Biol. Met. 3, 28–33
35. Mielczarek, E. V., Royt, P. W., and Toth-Allen, J. (1990) Biol. Met. 3, 34–38
36. Matzkan, B. F. (1997) in Iron Transport in Microbes, Plants, and Animals (Winkelmann, G. v. d. H. D.; Neilnads, J. B., ed) VCH Verlagsgesellschaft, Weinheim, Germany
37. Poole, K., and McKay, G. A. (2003) Front. Biosci. 8, d661–d686
38. Schalk, I. (2006) Pseudomonas: Molecular Biology of Emerging Issues (Ramos, J. L., and Levesque, R., eds) Vol. 4, Kluwer Publishers, Dordrecht, The Netherlands
39. Voulhoux, R., Filloux, A., and Schalk, I. J. (2006) J. Bacteriol. 188, 3317–3323
Real Time Fluorescent Resonance Energy Transfer Visualization of Ferric Pyoverdine Uptake in *Pseudomonas aeruginosa*: A ROLE FOR FERROUS IRON

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