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Mechanism of Ferripyoverdine Uptake by *Pseudomonas aeruginosa* Outer Membrane Transporter FpvA: No Diffusion Channel Formed at Any Time during Ferrisiderophore Uptake

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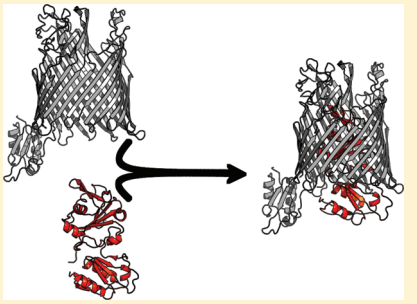
ABSTRACT: To get access to iron, *Pseudomonas aeruginosa* produces the siderophore pyoverdine (PVD), composed of a fluorescent chromophore linked to an octapeptide, and its corresponding outer membrane transporter FpvA. This transporter is composed of three domains: a β-barrel inserted into the membrane, a plug that closes the channel formed by the barrel, and a signaling domain in the periplasm. The plug and the signaling domain are separated by a sequence of five residues called the TonB box, which is necessary for the interaction of FpvA with the inner membrane TonB protein. Genetic deletion of the plug domain resulted in the presence of a β-barrel in the outer membrane unable to bind and transport PVD-Fe. Expression of the soluble plug domain with the TonB box inhibited PVD-^{55}Fe uptake most likely through interaction with TonB in the periplasm. A reconstituted FpvA in the bacterial outer membrane was obtained by the coexpression of separately encoded plug and β-barrel domains, each endowed with a signal sequence and a signaling domain. This resulted in polypeptide complementation after secretion across the cytoplasmic membrane. The reconstituted FpvA bound PVD-Fe with the same affinity as wild-type FpvA, indicating that the resulting transporter is correctly folded and reconstituted in the outer membrane. PVD-Fe uptake was TonB-dependent but 75% less efficient compared to wild-type FpvA. These data are consistent with a gated mechanism in which no open channel with a complete removal of the plug domain for PVD-Fe diffusion is formed in FpvA at any point during the uptake cycle.



Outer membrane transporters are proteins that form transmembrane-gated pores capable of transporting molecules of various sizes,[1] such as siderophores,[2] vitamin B_{12}, carbohydrates,[1] and different metals.[3−5] Siderophores are small molecules synthesized by bacteria as a means of obtaining iron.[6] They have a high affinity for iron, enabling them to capture iron from the environment surrounding the bacterium. The formed ferrisiderophore complexes are then transported across the outer membrane via specific outer membrane transporters, resulting in the uptake of the captured iron into the cell.[7] According to the crystal structure of outer membrane transporters (http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html), these proteins consist of a β-barrel with 11 large extracellular loops (labeled L1 to L11) and periplasmic turns. The lumen of this barrel is occluded by a globular domain called the "plug". Plug domains consist of a four-stranded β-sheet with surrounding loops and helices. This domain is kept in place in the β-barrel by 40−70 hydrogen bonds and two salt bridges involving four highly conserved residues: two Arg residues of the plug domain and two conserved Glu residues in the β-barrel domain.[7−9] In addition to the β-barrel and plug domains, some outer membrane transporters also contain a long periplasmic N-terminal extension that is not involved in the transport of ferrisiderophores but functions in the regulation of transcription.[10]

The binding sites of outer membrane transporters are located in the lumen on the extracellular side of the barrel and are composed of residues from the barrel and the plug domains. These binding sites are highly specific to a single siderophore and with affinities in the nanomolar range.[9] The protonmotive force of the inner membrane drives the ferrisiderophore transport across the outer membrane via the inner membrane protein complex comprising TonB, ExbB, and ExbD.[11,12] The periplasmic C-terminal part of TonB physically interacts with the transporters[13] through the TonB box, a short conserved region located close to the N-terminus.[14,15] This interaction has been demonstrated biochemically by disulfide cross-linking *in vivo* between cysteine residues inserted and by genetic manipulation into the TonB box of BtuB[16] and FecA.[17] Mutations within the TonB box block transporter function.[18−20] The structures of outer membrane transporters in complex with the periplasmic part of TonB have also been solved.[21,22]

The structures of FhuA, BtuB, FecA (outer membrane transporters of *Escherichia coli*), and FpvA (outer membrane transporter of *P. aeruginosa*) have been determined with and without their bound ligands. These ligands are ferrichrome for FhuA, vitamin B_{12} for BtuB, ferricitrate for FecA, and ferripyoverdine for FpvA. In the case of FecA, ligand binding leads to a major change in the conformation of extracellular loops L7 and L8, with those loops closing toward the ferricitrate binding pocket, thereby preventing the escape of the substrate.[23,24] If these loops are

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eliminated by genetic deletion, ferricitrate is no longer bound or transported, confirming the importance of these loops.[25] No such loop closure is seen for FhuA,[26,27] BtuB,[28] and FpvA.[29−31] However, molecular dynamics simulations suggest that L8 of FhuA closes the binding site following the binding of ferrichrome in a manner similar to that observed with FecA;[32] however, deletion of this loop has little effect on ferrichrome transport.[33] Elimination of the L3 and L11 loops of FhuA abolishes ferrichrome binding and transport,[33] consistent with an important role for these loops in transport. Loops L2, L3, and L4 of BtuB are disordered but become ordered following the binding of vitamin B_{12}.[28] Deletion studies have also shown that L7, L8, L9, and L11 are important for the transport of vitamin B_{12}.[34] For FpvA, mutagenesis studies have implicated L4, L7, and L9 in PVD-Fe uptake.[35] Finally, the crystal structures of FpvA bound to noncognate PVDs with high or low affinity have shown that L7 moves, bringing residues Trp599 and Tyr600 closer to the high-affinity PVDs, but not to the low-affinity PVDs, for which little movement of L7 is observed.[36] Thus, the extracellular loops of the barrel are important for uptake.

Ferrisiderophore binding also triggers additional structural changes at the surface of the plug domain which faces the periplasm. Some outer membrane transporters (FhuA and FecA) contain a short helical region, the "switch helix", which unwinds in the presence of bound substrate,[23,24,26,27,37,38] possibly leading to a movement of the TonB box required for interaction with TonB. Multiple conformations of the TonB box have been observed in BtuB: two different ordered conformations in the presence and absence of substrate[39,40] as well as a disordered form, discerned by electron paramagnetic resonance (EPR) spectroscopy [41,42] and biotin labeling.[41] The unwinding of the switch helices in crystal structures of ferrisiderophore-bound FhuA and FecA and the disordering of the TonB box inferred from EPR spectroscopic data on substrate-bound BtuB suggest that TonB may recognize a disordered conformation of the TonB box.

Moreover, in FpvA structures (loaded or not with PVD-Fe) the positioning of the signaling domain in the periplasm depends on the loading status of the transporter, but the fold is not affected by loading.[29,31,43] In unloaded FpvA, the signaling domain interacts with residues of the TonB box of FpvA.[29] This interaction is very similar to those of the TonB boxes of BtuB and FhuA with TonB.[21,22] When loaded with PVD-Fe, the signaling domain moves, most likely activating the signaling cascade, enabling the interaction of the TonB box with TonB.

It has been suggested that ferrisiderophore uptake across the outer membrane by the outer membrane transporter involves the following mechanisms: (i) the formation of a channel, through a major change in the conformation of the plug domain, (ii) the movement of this domain out of the β-barrel, or (iii) a combination of the two.[44−46] However, it is not possible to determine which of these possibilities is correct on the basis of existing experimental data. Recent single-molecule unfolding experiments in the context of outer membrane transporters have suggested that TonB may induce the total or partial unfolding of the plug domain by exerting a very modest mechanical force on the transporter.[47]

We investigated the mechanism of ferrisiderophore translocation across outer membrane transporters by coexpressing, in *Pseudomonas aeruginosa*, an FpvA derivative lacking the plug domain and an FpvA derivative lacking the β-barrel domain, each endowed with a signal sequence and a signaling domain to get proper expression levels of both forms of the transporter. Such an approach has previously been used for FhuA in *E. coli*[48] and for the heme outer membrane transporter HasR in *Serratia marscescens*.[49] Both reconstituted transporters were located in the outer membrane, but a TonB-dependent uptake was observed only for FhuA. In addition, the fluorescent properties of PVD[50−53] were used to monitor transport by this reconstituted FpvA transporter, the subsequent iron release from the siderophore in the periplasm, and the apo PVD recycling in the extracellular medium. We began by cloning the sequences encoding the various domains of FpvA, expressing them separately in *P. aeruginosa* and studying their biological properties. We then produced a reconstituted FpvA transporter in an FpvA mutant and investigated its ability to transport PVD-Fe using ^{55}Fe and spectroscopic approaches.

MATERIALS AND METHODS

Chemicals and Siderophores. $[^{55}Fe]Cl_3$ was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). The protonophore CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) was purchased from Sigma. PVD was purified as previously described.[54] Anti-FpvA and anti-TonB polyclonal antisera were prepared from purified FpvA and $TonB_{pp}$. New Zealand rabbits were immunized with 150 μg of receptor in 1 mL of PBS and Freund's complete adjuvant V.

Bacterial Strains, Plasmids, and Growth Conditions. The *P. aeruginosa* strains, the *E. coli* strains, and the plasmids used in this study are listed in Table 1. Bacteria were usually grown in LB broth (Difco) medium at 37 °C. The *P. aeruginosa* strains were grown overnight at 30 °C in an iron-deficient succinate medium (composition: 6 g/L K_2HPO_4, 3 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4 \cdot 7H_2O$, and 4 g/L sodium succinate, with the pH adjusted to 7.0 by adding NaOH). Antibiotics (50 $\mu g \cdot mL^{-1}$ gentamycin, 150 $\mu g \cdot mL^{-1}$ carbenicillin, 100 $\mu g \cdot mL^{-1}$ ampicillin, and 100 $g \cdot mL^{-1}$ tetracyclin) were added, when required.

Plasmid Construction. All enzymes for DNA manipulation were purchased from Fermentas and used according to the manufacturer's instructions. *E. coli* strain TOP10 (Invitrogen) was used as a host strain for all plasmids. The *fpvA* gene was amplified by PCR, using *Pfu* polymerase (Fermentas), from the pPVR2 plasmid,[55] which carries the *fpvA* gene on a 4.6 kb *Sph*I fragment. The primers used were FpvA4 (5′ GATCGGATCCCATCAGCGAAACCGCGAAC 3′) and FpvA6 (5′ GCACTCTAGACCGCTGTTCATGTTCCATCCG 3′), which contain *Bam*HI and *Xba*I restriction sites, respectively. Primer FpvA4 binds 1091 bp upstream from the ATG start codon, and primer FpvA6 binds 1042 bp downstream from the TAA stop codon. The resulting PCR product was inserted into pUC18 (Invitrogen) cut with *Bam*HI and *Xba*I to generate pUC_{FpvA}. The fragment encoding the signaling domain, the TonB box, and the plug domain (residues 44−276) of *fpvA* was amplified from pPVR2 with oligonucleotides FpvA4 and FpvA5 (5′ GCACAAGCTTTTATTCATGGGTAGGTTTCTTGCGG 3′). These oligonucleotides introduce *Bam*HI and *Hin*dIII restriction sites, respectively. The amplified fragment was digested and ligated into pMMB190 and pUCP18, giving $pMMB_{SD\text{-}TB\text{-}Plug}$ and $pUCP_{SD\text{-}TB\text{-}Plug}$, respectively. Oligonucleotides FpvA4 and FpvA9 (5′ GATCAAGCTTTTAGGGTGCCCAACTGGTTGGA 3′) were used to insert the fragment encoding the signaling domain and the TonB box (residues 44−139) into pUCP18, giving $pUCP_{SD\text{-}TB}$. We generated $pUCP_{SD}$ by inserting the

Table 1. *E. coli* and *P. aeruginosa* Strains and Plasmids Used[a]

strain or plasmid	relevant characteristics	source or ref
P. aeruginosa strains		
PAO1	wild-type strain	68
PAO1*fpvA*	derivative of PAO1; *fpvA*::Genta	69
PAO6382	derivative of PAO1; *pvdF*::kana	70
PAO1*pvdFfpvA*	derivative of PAO1Δ*fpvA*; *pvdF*::kana	69
PAS022	derivative of PAO1; *fpvA*Δ*139*−*276* encoding *fpvA* barrel domain, chromosomally integrated	this study
PAS033	derivative of PAO6382; *fpvA*Δ*139*−*276* encoding *fpvA* barrel domain, chromosomally integrated	this study
E. coli strains		
TOP10	*supE44 DlacU169 (ϕ80lacZDM15) hsdR17 recA1 endA1 gyrA96 thi-1relA1*	Invitrogen
S17-1	*pro thi hsdR recA*; chromosomal RP4 (Tra+ Tcs Kms Aps); Tpr Smr	71
BL21 (DE3)	F^- *ompT* $hsdS_B(r_{B^-}m_{B^-})$ *gal dcm* (λ DE3)	Novagen
plasmids		
pUC18	Ap^R, ColE1 *lacI ϕ*80d*lacZ*	72
pUCP18	Ap^R, *pMMB66EH, tac promoter, LacZ*α	73
pMMB190	Ap^R, *pMMB66EH, tac promoter, LacZ*α	74
pJET1/blunt vector	rep (pMB1), *bla* (Ap^R), *eco47IR*, PlacUV5	Fermentas
$pMMB_{SD\text{-}TB\text{-}Plug}$	pMMB190 $fpvA_{1-276}$ (encoding FpvA signaling domain, TonB box and plug)	this study
pUC_{FpvA}	pUC18 *fpvA* (encoding *fpvA*)	this study
$pUC_{\Delta Plug}$	pUC18 $fpvA_{\Delta 139-276}$ (encoding *fpvA* barrel and signaling domains)	this study
$pUCP_{SD\text{-}TB}$	pUCP18 $fpvA_{1-139}$ (encoding *fpvA* signaling domain and TonB box)	this study
$pUCP_{SD\text{-}TB\text{-}Plug}$	pUCP18 $fpvA_{1-276}$ (encoding *fpvA* signaling domain, TonB box and plug)	this study
$pUCP_{\Delta Plug}$	pUCP18 $fpvA_{\Delta 139-276}$ (encoding the *fpvA* barrel and signaling domains)	this study
$pUCP_{SD}$	pUCP18 $fpvA_{1-128}$ (encoding *fpvA* signaling domain)	this study
pET_{SD}	pET20(b+) $fpvA_{44-128}$	this study
$pET_{SD\text{-}TB}$	pET20(b+) $fpvA_{44-139}$	this study
$pET_{SD\text{-}TB\text{-}Plug}$	pET20(b+) $fpvA_{44-276}$	this study
suicide vector and mutator		
pME3088	suicide vector; Tc^R ; ColE1 replicon ; *Eco*RI *Kpn*I *Dra*II *Xho*I *Hin*dIII polylinker	56
$pME_{\Delta Plug}$	pME3088 $fpvA_{\Delta 139-276}$ with 1000pb flanking regions	this study

[a] For plasmids $pMMB_{SD\text{-}TB\text{-}Plug}$, pUC_{FpvA}, $pUCP_{\Delta Plug}$, $pUCP_{SD\text{-}TB}$, $pUCP_{SD\text{-}TB\text{-}Plug}$, $pMC_{\Delta Plug}$, and $pUCP_{SD}$, *fpvA* was cloned with its promotor.

sequence encoding the signaling domain lacking the TonB box into pUCP18, using oligonucleotides FpvA4 and FpvA10 (3′ GCACAAGCTTTTAGCCGAGATCGACGCTGCTGTC 5′). For all FpvA derivatives, the oligonucleotides used were designed to amplify the 1091 bp immediately upstream from the *fpvA* ATG start codon for the expression of these genes under the control of the *fpvA* promoter.

All FpvA derivatives were also inserted into the pET20(b+) vector for expression and purification in *E. coli*. All oligonucleotides were designed to allow insertion into the pET20(b+) vector in frame with the pelB signal sequence and the C-terminal 6-histidine. The fragment encoding the signaling domain, the plug, and the TonB box of FpvA (residues 44−276) was amplified from pPVR2 by PCR with the *Pfu* DNA polymerase (Fermentas) and the oligonucleotides FpvA1 (5′ GATCCCATGGCCCAGGAAGTCGAGTTC 3′) and FpvA3 (5′ GCATCTCGAGTTCATGGGTAGGTTTCTTGC 3′) introducing *Nco*I and *Xho*I sites, respectively. The PCR fragment was inserted into pET-20b(+) to give $pET_{SD\text{-}TB\text{-}Plug}$. The signaling domain (residues 44−128) was cloned using oligonucleotides FpvA1 and FpvA18 (5′ GATACTCGAGGAGATCGACGCTGCTGTCCGC 3′) giving plasmid pET_{SD}. The sequences encoding the signaling domain and the TonB box (residues 44−139) were inserted into pET-20b(+) in the same way, using oligonucleotides FpvA1 and FpvA2 (5′ GTCACTCGAGGGTGCCCAACTGGTTG 3′) to generate $pET_{SD\text{-}TB}$. The resulting plasmids were introduced into the BL21(DE3) strain by transformation. The introduced genes are expressed under the control of the T7 promoter with expression induced by adding 1 mM isopropyl β-D-thiogalactoside (IPTG).

Mutant Construction. The plug domain (residues 139−276) was deleted by inverse PCR using pUC_{FpvA} as a template and phosphorylated oligonucleotides FpvA7 (GGTGCCCAACTG GTTGGAGGT) and FpvA8 (TTCAAGGGCCATGTCGA ACTG). The remaining fragment was self-ligated, giving $pUC_{\Delta Plug}$ (pUC18::$fpvA_{\Delta Plug}$). This DNA fragment, encoding the barrel domain, was subcloned into pUCP18 to give $pUCP_{\Delta Plug}$ for expression of the barrel domain in *P. aeruginosa* strains. An FpvA transporter lacking the plug domain was obtained by amplifying the *fpvA* fragment encoding the barrel domain by PCR from $pUC_{\Delta Plug}$ with oligonucleotides FpvA*Hin*dIII (5′ GCACAAGCTTCCGCTGTTCATGTTCCATCCG 3′ and FpvA*Eco*RI (5′ GATCGAATTCCATCAGCGAAACCGCGAAC 3′). The PCR product was digested with *Eco*RI and *Hin*dIII and ligated into the suicide plasmid pME3088,[56] linearized by digestion with the same enzymes, to create $pME_{\Delta Plug}$. Mutations in the chromosomal *fpvA* gene of *P. aeruginosa* were generated by transferring $pME_{\Delta Plug}$ from *E. coli* S17-1 to the

PAO1 and PAO6382 strains and integration of the plasmid into the chromosome with selection for tetracycline resistance. A second crossing-over event excising the vector was achieved by enrichment for tetracycline-sensitive cells[57] to generate the corresponding mutants PAS022 and PAS033, respectively. All gene replacement mutants were checked by PCR and Western blot analysis (data not shown).

Immunoblot Analysis. We subjected 0.2 OD_{600} unit of cells (corresponding to 1.2×10^8 cells) to SDS−PAGE. Proteins were transferred onto nitrocellulose membranes by electroblotting (Bio-Rad). The nitrocellulose membranes were then blocked by incubation for 20 min in phosphate-buffered saline (PBS, pH 7.6, 10% dried milk powder) followed by incubation in blocking buffer supplemented with a primary anti-FpvA antibody.[58] They were then incubated in blocking buffer supplemented with a peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Sigma, dilution 1/10000). Antibody binding was detected by enhanced chemiluminescence (SuperSignal West Pico chemiluminescent subtrate; Pierce).

Cell Fractionation. For periplasm and cytoplasm preparations, overnight bacterial cultures in iron-limited succinate medium were centrifuged to obtain a cell pellet, which was washed twice with 50 mM Tris-HCl, pH 8.0, and resuspended in 1 mL of buffer A (200 mM Tris-HCl, pH 8.0, 20% sucrose). Spheroplasts were obtained by adding 10 μL of 100 mg/mL lysozyme (Euromedex) to the suspension and shaking the mixture gently at 4 °C for 15 min. The suspension was then centrifuged (15 min at 6700*g*) to obtain the spheroplast pellet and the periplasmic fraction (the supernatant). Spheroplast pellets were washed with 500 μL of buffer A. Spheroplasts were resuspended in 750 μL of cold water by vortexing, and the resulting suspension was incubated for 1 h at room temperature with benzonase (1 μL of a 50-fold dilution of benzonase ≥250 units/μL from Sigma). The cytoplasmic fractions were isolated by ultracentrifugation (40 min at 120000*g*). Pellets were resuspended in about 3.5 mL of buffer A with a Potter-Elvehjem homogenizer. The resulting suspension was applied to the top of a sucrose gradient containing, from bottom to top, Tris−70% sucrose (4 mL), Tris−50% sucrose (4 mL), and Tris−20% sucrose (4 mL). The sucrose gradient was centrifuged in a Beckman SW40 rotor at 35000 rpm for 15 h. We then collected 1 mL fractions from the sucrose gradient. Protein concentration was determined by the Bradford method.[59] Fractions containing inner membranes and outer membranes were collected and analyzed by SDS−PAGE (12% polyacrylamide gel) followed by Western blotting.

Expression and Purification of the Signaling and Plug Domains of FpvA and $TonB_{pp}$. *E. coli* BL21(DE3) cells were transformed with pET_{SD}, $pET_{SD\text{-}TB}$, and $pET_{SD\text{-}TB\text{-}Plug}$. A single colony from freshly transformed cells was used to inoculate 5 mL of LB broth supplemented with 100 μg/mL ampicillin. The culture was incubated overnight at 37 °C under shaking at 220 rpm. This preculture was then used to inoculate 2 L of LB broth supplemented with 100 μg/mL ampicillin. At an OD_{600} of approximately 0.6, 1 mM isopropyl β-D-thiogalactoside (IPTG) was added for induction. The culture was incubated at 37 °C, with shaking, for a further 2 h, and the cells were pelleted. The periplasmic fraction was prepared as described above. Complete protease inhibitor tablets (Roche) were added to this fraction, and the proteins present were purified by nickel affinity chromatography using a (Ni-NTA) column (Amersham) equilibrated with 20 mM NaP_i, pH 7.5, buffer supplemented with 10 mM imidazole, pH 7.5. Proteins specifically bound to the column were eluted with 20 mM NaP_i, pH 7.5, buffer supplemented with 500 mM imidazole. The fractions were collected and analyzed by SDS−PAGE (12% polyacrylamide gel). The His-tagged $TonB_{pp}$ protein was produced and purified as previously described.[60]

Coimmunoprecipitation. Twenty microliters of protein A−Sepahrose beads (Amersham Biosciences) at 20 mg/mL were washed with 500 μL of 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl and resuspended in 30 μL of this buffer in the presence of anti-FpvA or anti-TonB antibodies. After 30 min under stirring, the beads were washed three times with 250 μL of PBS containing 0.5% of Tween 20 and afterward resuspended in 30 μL of this same buffer. Purified $TonB_{pp}$ and $FpvA_{DS}$, $FpvA_{SD\text{-}TB}$, or $FpvA_{SD\text{-}TB\text{-}Plug}$ at 5 μM were added to these beads in 1:1 ratio. After 2 h incubation at 4 °C under stirring, the beads were pelleted and washed with 750 μL of PBS containing 0.05% BSA and 0.5% Tween 20 to eliminate unbound proteins. Twenty microliters of this washing (Sup) and the beads (Pel) were resuspended in SDS−PAGE loading buffer and heated at 100 °C during 10 min. Coprecipitated proteins were identified by Western blot analysis using anti-$TonB_{pp}$ and anti-FpvA antibodies.

Iron Uptake Assays. PVD-^{55}Fe (0.25 Ci/mmol) was prepared as previously described[53] with a 4-fold excess of PVD over iron. The uptake assays were carried out as described previously.[53] An overnight culture in iron-limited medium was harvested, and the bacteria were prepared at an OD_{600} of 1 in 50 mM Tris-HCl (pH 8.0) and incubated at 37 °C. Transport assays were initiated by adding 100 nM PVD-^{55}Fe. Aliquots (100 μL) of the suspension were removed at different times, filtered, and washed with 2 mL of 50 mM Tris-HCl (pH 8.0), and the retained radioactivity was counted. The experiment was repeated with cells that had previously been treated with 200 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a protonophore that inhibits iron uptake.[61]

Ligand-Binding Assays. For *in vivo* determination of the apparent dissociation constant for the binding of PVD-Fe to FpvA, we used a filtration assay described previously.[53] After overnight growth in succinate medium, cells were washed in 50 mM Tris-HCl (pH 8.0) buffer, and the density of the suspension was adjusted to an OD_{600} of 0.15. The cells were then incubated at 0 °C in a final volume of 500 μL in the presence of 200 μM protonophore CCCP and various concentrations of PVD-^{55}Fe (0.1−500 μM, 2.1 Ci/mmol) for 1 h. We evaluated the nonspecific binding of the ^{55}Fe-loaded siderophore by repeating the binding experiment, in parallel, with PAO1*pvdFfpvA*.

Fluorescence Spectroscopy. Fluorescence experiments were performed with a PTI (Photon Technology International TimeMaster; Bioritech) spectrofluorometer. The cells were washed with 2 volumes of 50 mM Tris-HCl, pH 8.0, and resuspended in the same buffer to a final OD_{600} of 1. For all experiments, the sample was stirred at 29 °C in a 1 mL cuvette, the excitation wavelength was set at 400 nm, and fluorescence emission was measured at 447 nm, every 300 ms, for the duration of the experiment. PVD recycling assays: PVD recycling was monitored as described previously.[52]

RESULTS

The FpvA β-Barrel Lacking the Plug Domain ($FpvA_{\Delta plug}$) Cannot Bind and Transport PVD-Fe. We investigated the role

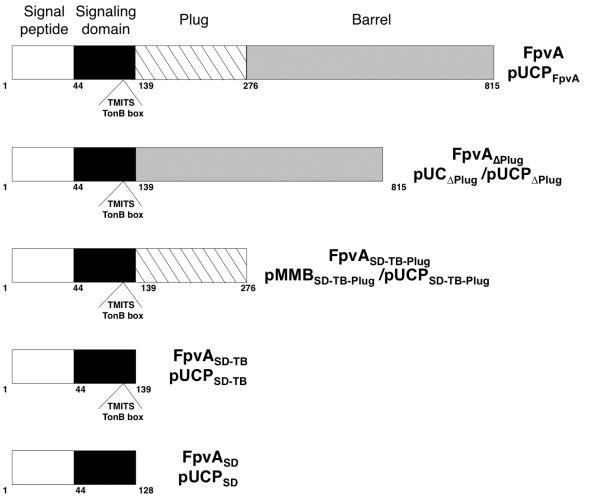

Figure 1. The various domains of FpvA cloned, with the names of the corresponding plasmids, TMITS being the TonB box sequence.

of the β-barrel domain of FpvA in PVD-Fe translocation across the outer membrane by evaluating the extent to which FpvA$_{\Delta Plug}$ bound and interacted with ferrisiderophore in *P. aeruginosa* outer membranes. We used plasmid pUCP$_{\Delta Plug}$, containing the *fpvA*$_{\Delta Plug}$ coding sequence (Figure 1), to produce this transporter under the control of the *fpvA* promoter in PAO1*fpvA* and PAO1*pvdFfpvA* (PAO1*fpvA* deleted for the *fpvA* gene and PAO1*pvdFfpvA* for *fpvA* and *pvdF*, PVD synthetase F). Western blot analyses of the outer membranes of the PAO1*fpvA* and PAO1*fpvA*(pUCP$_{\Delta Plug}$) (PAO1*fpvA* carrying pUCP$_{\Delta Plug}$) strains indicated the presence of a 72 kDa band, corresponding to the expected molecular mass of FpvA$_{\Delta Plug}$ (Figure 2A). In the absence of the plug domain, the β-barrel domain was incorporated into the outer membrane but in lower amounts to those for wild-type FpvA.

Although the binding site for PVD-Fe consists of residues from both the plug and barrel domains, no binding of PVD-Fe was observed in PAO1*pvdFfpvA*(pUCP$_{\Delta Plug}$) cells, even with concentrations of PVD-^{55}Fe as high as 500 μM (data not shown). Thus, even if binding does occur, it must have a K_d above the millimolar range. This lack of binding of the siderophore suggests that the conformation of the outer membrane of the FpvA β-barrel without the plug domain may be different from that of the β-barrel in wild-type FpvA, preventing PVD-Fe binding. We investigated the ability of the channel without the plug domain to transport PVD-Fe using ^{55}Fe. FpvA$_{\Delta Plug}$ in *P. aeruginosa* cells was clearly unable to transport iron across the outer membrane (Figure 2B): no ^{55}Fe was incorporated into PAO1*fpvA*-(pUCP$_{\Delta Plug}$) (Figure 2B) and PAO1*fpvA*.

Presence in the Periplasm of Soluble Domains of FpvA Carrying the TonB Box Inhibits PVD-Fe Uptake. Soluble FpvA fragments of various lengths (Figure 1) were produced in the periplasm of *P. aeruginosa* PAO1 cells to study their ability to modulate ^{55}Fe uptake. The pUCP$_{SD}$ plasmid encodes the signaling domain alone (*fpvA*$_{SD}$); pUCP$_{SD\text{-}TB}$ encodes the FpvA signaling domain with the TonB box (*fpvA*$_{SD\text{-}TB}$) and pUCP$_{SD\text{-}TB\text{-}Plug}$ encodes a protein composed of the FpvA signaling domain, the TonB box, and the plug domain (*fpvA*$_{SD\text{-}TB\text{-}Plug}$). These soluble forms of FpvA are produced under the control of the *fpvA* promoter. They also include a signal peptide and must therefore be exported through the bacterial inner membrane.

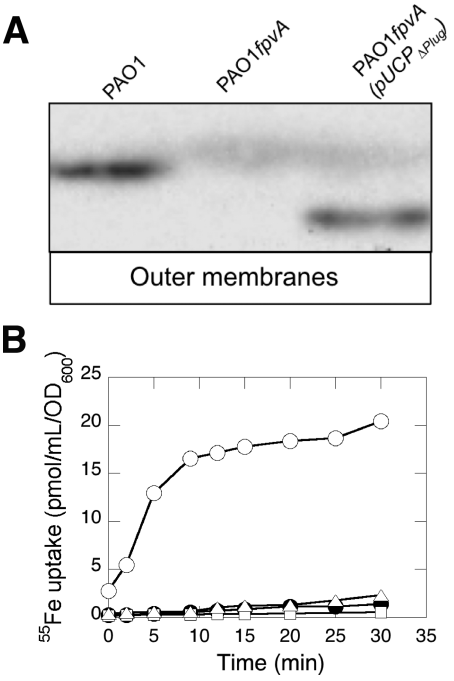

Figure 2. (A) Immunoblot of outer membrane preparations of PAO1, PAO1*fpvA*, and PAO1*fpvA*(pUCP$_{\Delta Plug}$) cells with polyclonal anti-FpvA antibodies. Outer membranes, prepared from cells grown to an OD$_{600}$ of 1 in succinate medium at 37 °C, were subjected to SDS−PAGE in a 12% polyacrylamide gel and transferred to nitrocellulose membranes. FpvA and FpvA$_{\Delta Plug}$ were detected with polyclonal anti-FpvA antibodies. For lanes 1, 2, and 3 the same amount of protein was loaded. (B) Time-dependent uptake of PVD-^{55}Fe in *P. aeruginosa* PAO1 (○), PAO1*fpvA* (□), and PAO1*fpvA*(pUCP$_{\Delta Plug}$) (△) strains. Cells at an OD$_{600}$ of 1 were incubated for 15 min at 37 °C in 50 mM Tris-HCl (pH 8.0) before the initiation of transport assays by the addition of 100 nM PVD-^{55}Fe. Samples (100 μL) of the suspension were removed at various times and filtered, and the radioactivity retained was counted. The results are expressed as picomoles of PVD-^{55}Fe transported per milliliter of cells at an OD$_{600}$ of 1. The experiment was repeated with the protonophore CCCP at a concentration of 200 μM (PAO1 (●); data not shown for the other two strains).

The strains expressing domains including the TonB box (PAO1(pUCP$_{SD\text{-}TB\text{-}Plug}$) and PAO1(pUCP$_{SD\text{-}TB}$)) incorporated smaller amounts of ^{55}Fe than PAO1 cells, and this incorporation also occurred at a slower rate for PAO1-(pUCP$_{SD\text{-}TB\text{-}Plug}$) (Figure 3A). In the absence of the TonB box (PAO1(pUCP$_{SD}$)), no inhibition of ^{55}Fe uptake was observed (Figure 3A). Thus, the inhibition observed when FpvA$_{SD\text{-}TB}$ and FpvA$_{SD\text{-}TB\text{-}Plug}$ are expressed may result from partial trapping of TonB of FpvA by the FpvA polypeptides present in these bacteria.

We tested both hypotheses by inserting sequences encoding the truncated forms of FpvA into pET20(b+) vector, expressing them in *E. coli*, and purifying the proteins produced for investigation of their interaction with purified truncated TonB protein. The soluble domains of FpvA were purified by spheroplast preparation from the periplasm of BL21(DE3) cells transformed with pET$_{SD\text{-}TB}$, pET$_{SD\text{-}TB\text{-}Plug}$, or pET$_{SD}$, demonstrating that these fragments are able to be exported through the bacterial inner membrane and are present in the periplasm. The cloning and purification of a soluble periplasmic form of TonB lacking

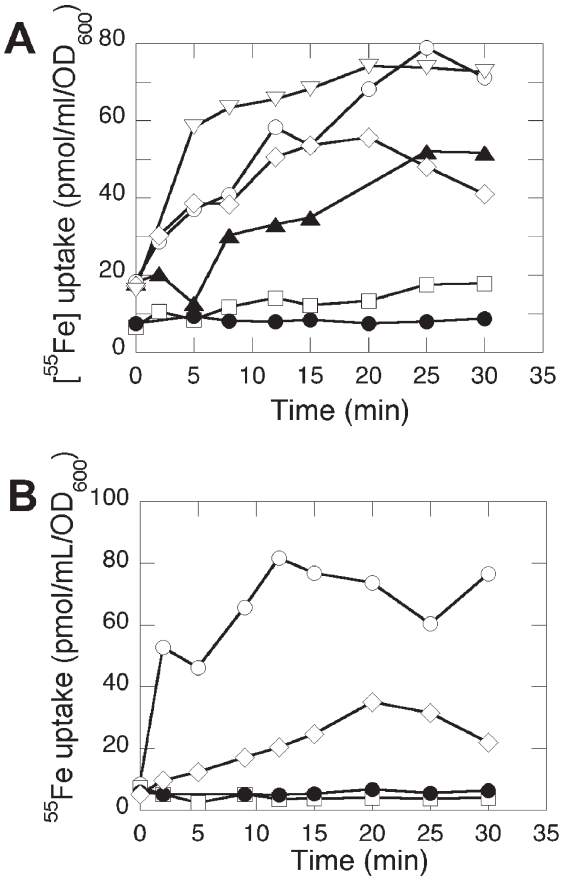

Figure 3. (A) Time-dependent uptake of PVD-^{55}Fe in *P. aeruginosa* PAO1 (○), PAO1*fpvA* (□), PAO1(pUCP$_{SD-TB}$) (◇), PAO1 (pUCP$_{SD-TB-Plug}$) (▲), and PAO1(pUCP$_{SD}$) (▽) strains. Cells at an OD_{600} of 1 were incubated for 15 min in 50 mM Tris-HCl (pH 8.0) at 37 °C before the initiation of transport assays by the addition of 100 nM PVD-^{55}Fe. Samples (100 μL) of the suspension were removed at various times and filtered, and the radioactivity retained was counted. The experiment was repeated with the protonophore CCCP at a concentration of 200 μM (PAO1 (●); data are not shown for the other two strains). (B) Time-dependent uptake of PVD-^{55}Fe in *P. aeruginosa* PAO1 (○), PAS022 (□), and PAS022(pMMB$_{SD-TB-Plug}$) (◇) strains. The experiment was carried out as in (A), and the experiment was repeated with the protonophore CCCP at a concentration of 200 μM (PAO1 (●); data not shown for the other two strains).

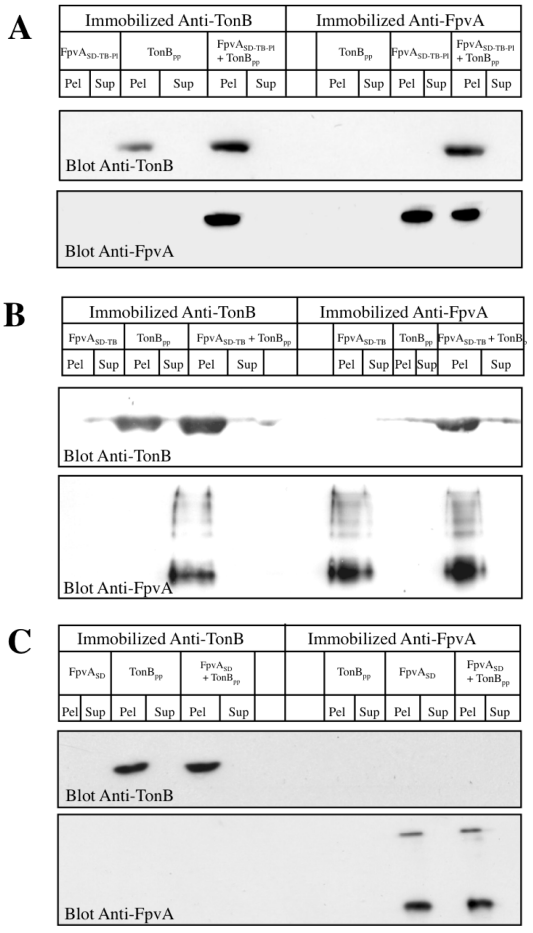

Figure 4. *In vitro* interaction between TonB$_{PP}$ and FpvA$_{SD-TB-Plug}$ (A), FpvA$_{SD-TB}$ (B), and FpvA$_{SD}$ (C). For each panel, the different purified proteins were incubated in the presence of either A-Sepharose bead bound anti-TonB or anti-FpvA as follows: soluble forms of FpvA alone (FpvA$_{SD-TB-Plug}$ in panel A, FpvA$_{SD-TB}$ in panel B, and FpvA$_{SD}$ in panel C), TonB$_{PP}$ alone, and soluble form of FpvA with 1 equiv of TonB$_{PP}$. After 2 h incubation at 4 °C, the beads were pelleted and washed. Twenty microliters of the last washing step (Sup) and the pelleted beads (Pel) were analyzed on 12% acrylamide gel, followed by electrotransfer onto nitrocellulose membranes. Proteins were detected with polyclonal anti-FpvA and anti-TonB antibodies. TonB$_{PP}$ is a recombinant TonB1 protein lacking the N-terminal inner membrane anchor (TonB$_{PP}$ corresponds to residues 109−342).

the N-terminal inner membrane anchor (TonB$_{PP}$) have been described before.[60] TonB$_{PP}$ was incubated in a 1:1 ratio with each of the three FpvA fragments separately and immunoprecipitated using polyclonal anti-TonB or anti-FpvA antibodies. The mixtures were then analyzed by SDS−PAGE and Western blotting using again anti-FpvA and anti-TonB antibodies. A coimmunoprecipitation was observed only for the FpvA fragments carrying the TonB box, FpvA$_{SD-TB-Plug}$, and FpvA$_{SD-TB}$ (Figure 4A,B). No coimmunoprecipitation was observed for the signaling domain alone (Figure 4C), demonstrating the requirement of the TonB box for interaction between TonB and the soluble FpvA fragments studied here. The interaction between FpvA and His-tagged truncated forms of FpvA was investigated using Ni-NTA agarose beads, and no interaction was seen (data not shown). In conclusion, the results of these coimmunoprecipitation experiments are consistent with an *in vivo* trapping of TonB by FpvA$_{SD-TB}$ and FpvA$_{SD-TB-Plug}$, inhibiting iron uptake.

Coexpression of Sequences Encoding FpvA$_{SD-TB-Plug}$ and the β-Barrel (FpvA$_{\Delta Plug}$): Cellular Distribution of the Reconstituted FpvA in *P. aeruginosa* Cells and Ability To Bind PVD-Fe. We obtained a reconstituted FpvA transporter for investigation of the mechanism of transport via FpvA by producing the FpvA$_{SD-TB-Plug}$ and FpvA$_{\Delta Plug}$ polypeptides together in the same strain. The PAS022 strain, expressing only FpvA$_{\Delta Plug}$, was transformed with pMMB$_{SD-TB-Plug}$, which encodes *fpvA$_{SD-TB-Plug}$*. Both genes were endowed with the *fpvA* signal sequence, directing the secretion of the fragments across the cytoplasmic membrane. PAO1, PAS022, and PAS022(pMMB$_{SD-TB-Plug}$) cells were lysed, and the outer membrane, inner membrane, periplasm, and cytoplasmic fractions were analyzed by SDS−PAGE, followed by Western blotting with anti-FpvA polyclonal antibodies. Both domains, FpvA$_{\Delta Plug}$ and FpvA$_{SD-TB-Plug}$, were present in both the outer and inner membrane fractions

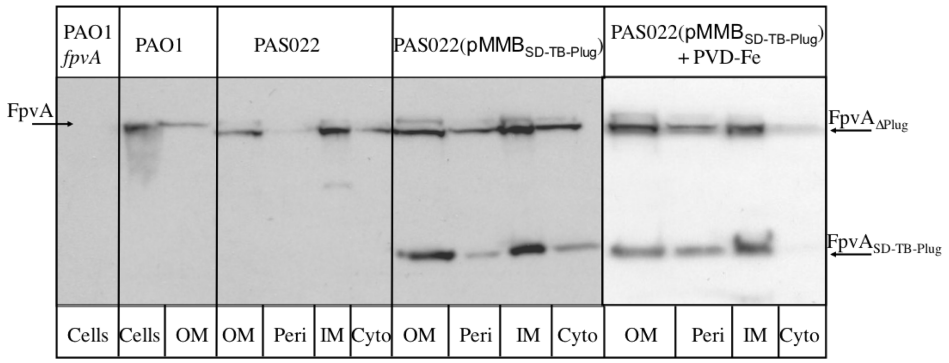

Figure 5. Production and cellular distribution of wild-type FpvA, $FpvA_{\Delta Plug}$, and reconstituted FpvA. The cells were grown in succinate medium, at 37 °C, to an OD_{600} of 1. Outer and inner membranes and soluble fractions were isolated and subjected to SDS−PAGE in a 12% polyacrylamide gel. FpvA and FpvA mutants were detected with polyclonal anti-FpvA antibodies. For PAS022($pMMB_{SD\text{-}TB\text{-}Plug}$) the experiment was repeated with incubation of the cells in the presence of 300 nM PVD-Fe before cellular fractionation.

(Figure 5) and in lower amounts in the cytoplasm and periplasm. Cellular fractionation was followed in parallel by monitoring the fluorescence of PVD. About 89% and 11% of the fluorescence were found in the periplasmic and cytoplasmic fractions, respectively, indicating that cytoplasm and periplasm were properly separated. In *P. aeruginosa* cells, fluorescent PVD is only present in the periplasm and not in the cytoplasm.[62] Thus, FpvA was reconstituted in the outer membrane. The presence of $FpvA_{\Delta Plug}$ and $FpvA_{SD\text{-}TB\text{-}Plug}$ at the inner membrane probably results from the secretion of these proteins across this membrane, with $FpvA_{SD\text{-}TB\text{-}Plug}$ also interacting with TonB, as shown above. Coexpression of $FpvA_{\Delta Plug}$ and $FpvA_{SD\text{-}TB\text{-}Plug}$ led to plug localization in the outer membrane, demonstrating interaction between these two FpvA domains *in vivo*.

Binding of PVD-^{55}Fe to cells expressing the reconstituted transporter ($PAS033(pMMB_{SD\text{-}TB\text{-}Plug})$) showed an equivalent affinity as the binding to cells expressing the wild-type transporter (FpvA, $K_d = 0.5 \pm 1.1$ nM;[61] reconstituted FpvA receptor, $K_d = 0.42 \pm 0.15$ nM). Thus, the reconstituted transporter is incorporated into the outer membrane, exposed on the cell surface, and correctly folded, like wild-type FpvA, with all the residues of the PVD-Fe binding site present and in the correct conformation for interaction with PVD-Fe.

Ability of the Reconstituted FpvA Receptor to Incorporate PVD-^{55}Fe in *P. aeruginosa*. We investigated the ability of the separately synthesized barrel and plug domains to form an active TonB-dependent receptor for PVD-Fe uptake by carrying out uptake experiments with ^{55}Fe (Figure 3B). The level of ^{55}Fe incorporation was 75% lower in $PAS022(pMMB_{SD\text{-}TB\text{-}Plug})$ cells expressing reconstituted FpvA than in PAO1 cells expressing wild-type FpvA. The experiment was repeated in the presence of the protonophore CCCP, which inhibits TonB-dependent transport in bacteria. In the presence of this protonophore, no ^{55}Fe was incorporated into the cells, indicating that the iron uptake observed was TonB-dependent and not due to PVD-^{55}Fe diffusion across the outer membrane. Thus, at no point in the PVD-Fe uptake process is the reconstituted FpvA transformed into an open channel by the complete release of $FpvA_{SD\text{-}TB\text{-}Plug}$ into the periplasm. Moreover, cells expressing reconstituted FpvA incubated in the presence and absence of a large excess of PVD-Fe apparently did not significantly differ in terms of the amount of $FpvA_{SD\text{-}TB\text{-}Plug}$ present in the different cell compartments after cell fractionation (Figure 5). The same amount of soluble $FpvA_{SD\text{-}TB\text{-}Plug}$ was still present in the outer membranes, indicating that this part of FpvA may not completely dissociate from $FpvA_{\Delta Plug}$ during PVD-Fe uptake.

PVD-Fe Dissociation and PVD Recycling in Cells Expressing Reconstituted FpvA. We previously showed that, after PVD-Fe uptake across the outer membrane, iron is released from PVD in the periplasm and the siderophore recycled in the extracellular medium by the PvdRT-OpmQ efflux pump.[51,52,63] The kinetics of iron release from the siderophore can be followed *in vivo* in real time in cells unable to produce PVD.[51] Cells are incubated in the presence of PVD-Fe and excited at 400 nm, the excitation wavelength of metal-free PVD, and fluorescence emission is monitored at 447 nm. PVD-Fe is not fluorescent because iron quenches the fluorescence of the molecule, but the release of the metal generates apo-PVD, which is fluorescent at 447 nm.[50] We studied the kinetics of iron release from PVD after transport across the outer membrane by reconstituted FpvA receptor in a strain unable to produce PVD ($PAS033(pMMB_{SD\text{-}TB\text{-}Plug})$). An increase in fluorescence corresponding to the formation of fluorescent metal-free PVD was observed in PAO6382 and $PAS033(pMMB_{SD\text{-}TB\text{-}Plug})$ cells (Figure 6A) due to iron release from the siderophore. No such increase in fluorescence was observed in cells expressing only $FpvA_{\Delta Plug}$ (PAS033 cells). However, the increase in fluorescence at 447 nm was slower in $PAS033(pMMB_{SD\text{-}TB\text{-}Plug})$ cells expressing the reconstituted transporter than in cells expressing wild-type FpvA and reached a lower plateau. This observation is consistent with the transport rate shown in Figure 3B: smaller amounts of PVD-Fe are transported into the periplasm by reconstituted FpvA than by the wild-type protein.

The fluorescent properties of PVD can also be used to follow the recycling of metal-free PVD in the extracellular medium after the transport of iron into the bacteria.[52] PAS033, $PAS033(pMMB_{SD\text{-}TB\text{-}Plug})$, and PAO6382 cells unable to produce PVD were incubated in the presence of PVD-Fe, which is not fluorescent. After various periods of time, aliquots were removed, the cells were harvested by centrifugation, and fluorescence at 447 nm was monitored in the extracellular medium (excitation at 400 nm). An increase in the fluorescence of the extracellular medium was observed for $PAS033(pMMB_{SD\text{-}TB\text{-}Plug})$ cells, corresponding to the recycling of metal-free PVD outside the bacteria (Figure 6B). However, this recycling occurred at a rate about 70% slower than that in PAO6382 cells. This difference in

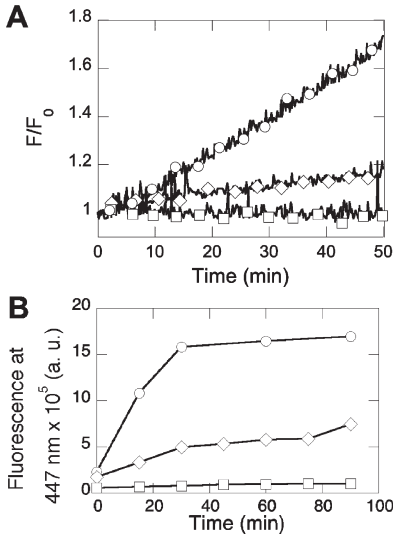

Figure 6. (A) PVD-Fe dissociation kinetics monitored by direct excitation of PVD in PAO6382 (○), PAS033 (□), and PAS033-(pMMB$_{SD\text{-}TB\text{-}Plug}$) (◇) cells. Cells were washed and resuspended at an OD_{600} of 2 in 50 mM Tris-HCl (pH 8.0) and incubated at 29 °C. After the addition of 300 nM PVD-Fe, the change in fluorescence (excitation set at 400 nm) was monitored by measuring the emission of fluorescence at 447 nm, every second, for 50 min. As a control, the experiment was repeated for the three strains in the absence of PVD-Fe, and no increase in fluorescence was observed (kinetics not shown). (B) Recycling of PVD in the extracellular medium after the release of iron in PAO6382 (○), PAS033 (□), and PAS033-(pMMB$_{SD\text{-}TB\text{-}Plug}$) (◇) cells. The cells, at an OD_{600} of 2 in 50 mM Tris-HCl (pH 8.0), were incubated at 29 °C in the presence of 2 μM PVD-Fe. At various times, 1 mL of the culture was centrifuged, and the emission of fluorescence of the supernatant was monitored at 447 nm (excitation set at 400 nm). No increase in fluorescence was observed when the experiment was repeated for each strain in the absence of PVD-Fe (data not shown).

kinetics was probably due to the smaller amount of PVD-Fe transported in PAS033(pMMB$_{SD\text{-}TB\text{-}Plug}$) cells than in PAO6382 cells. No recycling was observed in the PAS033 strain expressing only FpvA$_{\Delta Plug}$.

■ DISCUSSION

The structures of four TonB-dependent receptors (FhuA, FepA, FecA, and BtuB) of *E. coli* and two (FpvA and FptA) of *P. aeruginosa* and one (FauA) of *B. pertussis* have been solved, with medium to high resolution (http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html). All of these membrane proteins are involved in transport of ferrisiderophores except BtuB, which is the vitamin B_{12} transporter in *E. coli*. Despite all of these structures, the mechanism of ferrisiderophore translocation across the outer membrane remains unsolved. To further investigate in the understanding of this mechanism, we expressed separately or coexpressed in *P. aeruginosa* the different domains of FpvA and studied their biological properties.

Expression of the soluble domains FpvA$_{SD\text{-}TB\text{-}Plug}$ and FpvA$_{SD\text{-}TB}$ in PAO1 resulted in an inhibition of PVD-Fe uptake compared to PAO1 (Figure 3A), most likely due to *in vivo* trapping of TonB by these proteins. The TonB boxes of FpvA$_{SD\text{-}TB}$ and FpvA$_{SD\text{-}TB\text{-}Plug}$ produced in the absence of the barrel in the periplasm adopt a conformation suitable for interaction with TonB, thereby blocking transport. For FpvA$_{SD}$, no interaction with TonB is possible due to the absence of the TonB box. Also, no inhibition of iron uptake was therefore observed. Previous studies have shown that the overproduction of TonB$_{pp}$ in *P. aeruginosa* PAO1 also inhibits ^{55}Fe uptake, most likely through the trapping of the FpvA TonB box.[60]

In the absence of the plug domain, the β-barrel domain (FpvA$_{\Delta Plug}$) is inserted into the outer membrane but can neither bind PVD-Fe nor transport this substrate by either a TonB-dependent mechanism or diffusion (Figure 2). Consequently, the protein does not behave as a channel, either because the extracellular loops close the channel or because the barrel collapses under the pressure of the lipids in the membrane. The expression of FhuA without its plug domain in *E. coli* also results in the incorporation of this protein into the outer membrane but induces an increase in permeability for ferrichrome and antibiotics, consistent with its folding into a β-barrel that forms a channel allowing the diffusion of these molecules.[48] The HasR β-barrel of the heme outer membrane transporter is also incorporated into the outer membrane and binds the hemophore HasA, but binding in this system is based purely on interactions with the extracellular loops and does not involve interactions with the plug domain. However, the HasR β-barrel has no intrinsic active heme transport properties.[49] FpvA$_{\Delta Plug}$, like the HasR β-barrel, does not form a large open channel in the absence of the plug, because no PVD-Fe diffusion was observed. The channel may be closed by the folding down of the external loops of the β-barrel, or the β-barrel may itself adopt a different conformation or collapse under lipid pressure when present in the outer membrane in the absence of the plug. Moreover, based on the crystal structure of FpvA-PVD-Fe,[31] PVD-Fe binding to the transporter requires both the plug and β-barrel domains, which is not the case for binding of the hemophore to HasR.[64] All of these data confirm that the binding and transport of PVD-Fe via FpvA requires the presence of both the barrel and the plug domain.

The reconstituted FpvA receptor, obtained by the coproduction of separately encoded plug and β-barrel domains, each endowed with a signal sequence and a signaling domain, was present in the outer membranes (Figure 5) and bound PVD-Fe with the same affinity as the wild-type FpvA. This implies that the plug was correctly and functionally inserted into the barrel despite the production of these proteins separately *in vivo*. The similar binding affinity for PVD-Fe of the reconstituted and wild-type FpvA transporters indicates that the barrel and the plug domains function independently and are able to associate in exactly the same conformation even when they are not part of the same polypeptide chain. It is unknown whether this reconstitution occurs after β-barrel incorporation into the outer membrane or in the periplasm, with a periplasmic, partially folded β-barrel intermediate. Such intermediates have been described for several outer membrane porins.[65,66]

Reconstituted FpvA transported PVD-Fe into the periplasm but with a lower efficiency than wild-type FpvA (Figure 3B). This uptake was TonB-dependent, with no diffusion due to the complete release of FpvA$_{SD\text{-}TB\text{-}Plug}$ into the periplasm, consistent with no diffusion channel formation at any time during PVD-Fe uptake. The 75% lower ^{55}Fe incorporation in cells expressing reconstituted FpvA compared to PAO1 cells expressing wild-type FpvA may be due to several of the following reasons: (i) reconstituted FpvA may be less expressed than wild-type FpvA; (ii) some TonB may be trapped, as described above, by

$FpvA_{SD\text{-}TB\text{-}Plug}$ present in the periplasm, preventing its interaction with reconstituted FpvA in the outer membrane; (iii) the presence of two signaling domains in the reconstituted FpvA transporter may affect transport ($FpvA_{SD\text{-}TB\text{-}Plug}$ and $FpvA_{\Delta Plug}$ were each expressed with a signaling domain to ensure a proper production of the two polypeptides); or (iv) the reconstituted FpvA transporter may be unable to adopt some of the plug and barrel domain conformations required for efficient uptake. The total absence of PVD-^{55}Fe diffusion in PAS022($pMMB_{SD\text{-}TB\text{-}Plug}$) indicates that the mechanism of uptake must involve a gated mechanism, with closing of the binding site by some extracellular loops as shown for FecA,[67] before formation of the channel allowing PVD-Fe transport.

The kinetics of PVD-Fe dissociation in the periplasm and apo PVD recycling with cells expressing the reconstituted FpvA transporter (Figure 6) indicate that PVD-Fe does not get stuck in this reconstituted transporter in the outer membrane but is instead transported into the periplasm, where iron is released from the siderophore, leading to the recycling of PVD in the extracellular medium mediated by PvdRT-OpmQ. Reconstituted FpvA, despite being composed of two polypeptides, each with a signaling domain, can carry out complete iron uptake cycles but with a 75% lower efficiency than the wild-type protein. In conclusion, all of these data are consistent with a gated mechanism without a complete opened channel formation for PVD-Fe diffusion.

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ABBREVIATIONS

PVD and PVD-Fe, iron-free and ferric pyoverdine, respectively, produced by *Pseudomonas aeruginosa* 15692; FpvA, PVD outer membrane receptor.

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