The Metal Dependence of Pyoverdine Interactions with Its Outer Membrane Receptor FpvA[▽]

Jason Greenwald,^{1*} Gabrielle Zeder-Lutz,² Agnès Hagege,³ Hervé Celia,¹ and Franc Pattus¹

Département Récepteurs et Protéines Membranaires¹ and Département Biotechnologie des Interactions Macromoléculaires,²
Institut Gilbert-Laustrat, UMR 7175-LC1 CNRS, ESBS, Blvd. Sébastien Brant, F-67413 Illkirch, Strasbourg,
France, and Laboratoire de Chimie Analytique et Sciences Séparatives, IPHC-DSA (UMR7178) ECPM,
25 rue Becquerel, 67087 Strasbourg, France³

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To acquire iron, *Pseudomonas aeruginosa* secretes the fluorescent siderophore pyoverdine (Pvd), which chelates iron and shuttles it into the cells via the specific outer membrane transporter FpvA. We studied the role of iron and other metals in the binding and transport of Pvd by FpvA and conclude that there is no significant affinity between FpvA and metal-free Pvd. We found that the fluorescent *in vivo* complex of iron-free FpvA-Pvd is in fact a complex with aluminum (FpvA-Pvd-Al) formed from trace aluminum in the growth medium. When *Pseudomonas aeruginosa* was cultured in a medium that had been treated with a metal affinity resin, the *in vivo* formation of the FpvA-Pvd complex and the recycling of Pvd on FpvA were nearly abolished. The accumulation of Pvd in the periplasm of *Pseudomonas aeruginosa* was also reduced in the treated growth medium, while the addition of 1 μ M AlCl₃ to the treated medium restored the effects of trace metals observed in standard growth medium. Using fluorescent resonance energy transfer and surface plasmon resonance techniques, the *in vitro* interactions between Pvd and detergent-solubilized FpvA were also shown to be metal dependent. We demonstrated that FpvA binds Pvd-Fe but not Pvd and that Pvd did not compete with Pvd-Fe for FpvA binding. In light of our finding that the Pvd-Al complex is transported across the outer membrane of *Pseudomonas aeruginosa*, a model for siderophore recognition based on a metal-induced conformation followed by redox selectivity for iron is discussed.

The poor bioavailability of iron in aerobic environments, which is due to the low solubility of ferric hydroxide, has promoted the evolution of many iron-scavenging and storage molecules in organisms whose requirements for iron exceed the concentration of soluble free iron found under physiological conditions ($\sim 10^{-9}$ M). Mammalian pathogens encounter even lower levels of free iron ($\sim 10^{-18}$ M) due to iron sequestration by the host and have likewise developed mechanisms to efficiently compete for this essential nutrient (23). An indispensable iron acquisition mechanism in gram-negative bacteria involves the secretion of Fe(III)-chelating molecules called siderophores (13) and the expression of their cognate outer membrane transporters (OMTs). A large family of OMTs actively transport ferric siderophores across the outer membrane by coupling the transport to the proton gradient of the inner membrane via the TonB/ExbB/ExbD complex. The TonB-dependent OMTs include the receptors for a wide variety of siderophores as well as for other, non-iron-containing molecules, yet to date, they have all been found to have a conserved structure with a similar binding site for the transported molecule (30). However, one intriguing difference among the siderophore receptors was the reported ability of the pyoverdine (Pvd) receptor from *Pseudomonas aeruginosa* (FpvA) (26) and the ferric citrate receptor from *Escherichia coli* (FecA) (32) to bind their siderophores in the absence of Fe. More recently, iron-free siderophore binding was reported for FptA and

FhuA, the *P. aeruginosa* pyochelin and *E. coli* ferrichrome receptors, respectively (15), and the reported affinities of the iron-free siderophores are always 5- to 20-fold lower than those of the ferric siderophores, which have dissociation constants in the range of 1 nM. These findings raise questions about the mechanism by which the receptors can differentiate the iron-loaded from the empty siderophore. This is particularly intriguing since some siderophores, including Pvd, can regulate their own expression as well as that of several virulence factors via signals that are initiated by binding to their OMTs (19). It is presumably the iron-bound form that signals, since virulence factors should not be expressed until there are enough healthy bacteria, protected by a biofilm, to withstand the immune response. Also, the autoregulation of Pvd expression should depend on the presence of iron, without which the bacteria gain little by synthesizing more Pvd.

It is still not clear how a high-affinity binding to empty siderophores that are normally present in large excess at up to millimolar concentrations would not interfere with ferric siderophore transport. However, our observations on the growth of *Pseudomonas aeruginosa* in metal-deficient media and on the interaction between Pvd and FpvA under controlled metal-free conditions indicate that there is not a high-affinity interaction between metal-free Pvd and FpvA. Our data suggest that the previous reports to the contrary were influenced by trace metal contaminants.

Although it is not evident *a priori* that trace metals will influence the outcome of experiments on a biological system, there are several reasons that the study of siderophores like Pvd can be plagued by artifacts that originate from the low-concentration contaminants that exist in nearly all aqueous

* Corresponding author. Mailing address: Laboratorium für Physikalische Chemie, ETH Zürich, Zürich, Switzerland. Phone: 41(0)446334924. Fax: 41(0)446321021. E-mail: jason.greenwald@phys.chem.ethz.ch.

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buffers and culture media; the most important of these reasons is the broad specificity of siderophores for multivalent metals. Hydroxamate-containing siderophores have high affinity for other metals (2, 31), and although Pvd is principally an iron transporter, it has been previously shown that its complex with Ga(III) (Pvd-Ga) is transported with the same efficiency as iron (12). Another problem can arise from the use of the Pvd fluorescence as a reporter for its metal-loaded state. Because iron quenches the intrinsic fluorescence of Pvd (1), a loss of fluorescence signals the formation of the complex between Pvd and Fe(III) (Pvd-Fe). Conversely, Pvd fluorescence is enhanced by several metals, including gallium and aluminum (8, 11). It is thus not trivial to deconvolute a fluorescence signal in a mixed-metal environment in order to distinguish between Pvd-metal complexes and free Pvd.

MATERIALS AND METHODS

Reagents. Carbenicillin disodium salt was a generous gift from SmithKline Beecham (Welwyn Garden City, Herts, United Kingdom). Pvd, Pvd-Fe, and Pvd-Ga were supplied by Isabelle Schalk and prepared as described previously (1, 9, 11). Sodium *N*-lauroyl sarcosine was purchased from Sigma and *n*-octyl-polyoxyethylene (oPOE) from Bachem. Chemicals for succinate medium (SM) had less than 5 ppm Fe (Merck, Darmstadt), except for the succinic acid, which was ultrapure grade from Sigma. Water with a resistivity of 18.2 M Ω cm was obtained directly from a Milli-Q Plus (Millipore) ultrapure water system.

Bacterial strains and growth media. Two derivatives of the PAO1 strain were used: CDC5(pPVR2), a Pvd-deficient mutant which overproduces FpvA (3), and K691(pPVR2), which overproduces FpvA and produces Pvd (22). The strains were grown in an SM [35 mM K₂HPO₄, 22 mM KH₂PO₄, 1 mM MgSO₄, 35 mM succinic acid, 7.5 mM (NH₄)₂SO₄, 75 mM NaOH] (9) plus trace salts in the presence of 150 μ g/ml carbenicillin. A low-iron-content trace salts solution (PTM4-lo) was prepared as a 1:100 mixture of PTM4 (28) and PTM4(Fe⁻) (PTM4 contains, per liter, 2 g CuSO₄ · 5H₂O, 80 mg NaI, 3 g MnSO₄ · H₂O, 200 mg NaMoO₄ · 2H₂O, 20 mg boric acid, 500 mg CoCl₂, 7 g ZnCl₂, 22 g FeSO₄ · 7H₂O, 200 mg biotin, and 1 ml sulfuric acid). Cultures were routinely grown overnight at 30°C in LB medium to an optical density at 600 nm (OD₆₀₀) of 4 to 5 and then pelleted, washed, and resuspended with SM at a 100-fold dilution. After 7 h at 30°C, the cultures were near an OD₆₀₀ of 1 and overproducing FpvA. The PTM4-lo salts were prepared fresh for each culture from PTM4 and PTM4(Fe⁻) stocks and then diluted 5,000-fold into the medium for a final iron concentration of 160 nM. All metal-deficient cultures were grown in plastic flasks and 50-ml tubes. As precaution against metal contaminants, plastic flasks from the dishwasher were treated with 1 mM EDTA overnight, followed by abundant rinsing with 18.2-M Ω cm water before culture growth.

For the preparation of metal-depleted medium, Pvd (2 mg) from strain ATCC 27853 (a gift from Valerie Geoffroy) was coupled to 1 ml of *N*-hydroxysuccinimide-activated Sepharose (GE Healthcare, Uppsala) via its lysine side chain according to the manufacturer's recommended protocol. An immobilized Pvd column (IPC) was prepared and used to extract the trace metals from 50 ml SM. The medium was passed over the column with a 1-min contact time. IMAC-extracted medium (SM_{imac}) was prepared by passing 1 liter of SM over a 5-ml HiTrap IMAC Sepharose HP column (GE Healthcare) at 5 ml/min, followed by the addition of 10 ml of the same chelating resin to the flowthrough and mixing with gentle inversion on a rotator for 48 h at 4°C. The resin was allowed to settle and the medium stored at -20°C.

FpvA expression levels in the different medium preparations were monitored by Western blotting of boiled whole-cell lysates. Even loading of the sodium dodecyl sulfate (SDS) gel was ensured by using the equivalent of 200 μ l of each culture at an OD₆₀₀ of 1.0 and then boiling these cells for 5 min in 100 μ l SDS loading buffer. The gel was transferred to a nitrocellulose membrane in 10 mM CAPS (*N*-cyclohexyl-3-aminopropanesulfonic acid)-10% methanol (pH 11) for 1 h at 200 mA. A polyclonal rabbit anti-FpvA antibody was used as a primary antibody (1:2,000 dilution), and the blotting was performed using the Pierce ECL reagents according to the manufacturer's recommendations.

Purification of FpvA. K691(pPVR2) or CDC5(pPVR2) cultures were grown 24 h at 30°C in SM with PTM4-lo salts and the cells harvested and stored frozen at -80°C. The frozen pellets were resuspended in 50 mM Tris (pH 8.0) (20 ml/g of cells) and lysed by sonication followed by a spin at 3,000 $\times$ g for 5 min. The supernatant was mixed with 1% *N*-lauroyl sarcosine, followed by centrifugation

at 100,000 $\times$ g. The pellet was solubilized in 5% oPOE, followed by another centrifugation at 100,000 $\times$ g. The supernatant was loaded onto a 10-ml SourceQ (GE Healthcare) column and eluted with a 30-ml gradient from 0 to 1 M NaCl. The fractions with FpvA also contain FptA in an approximately equal concentration. Lipids were removed by use of a sucrose gradient with equal volumes of 15%, 10%, and 5% sucrose in 20 mM Tris-1% oPOE and the SourceQ elutions layered on top. The samples were centrifuged at 39,000 rpm for 16 h at 12°C in an SW41 rotor (Beckman), and the FpvA-FptA fraction visualized under UV illumination was collected with a syringe needle. The FpvA-FptA mixture was separated by chromatofocusing on a MonoP column (GE Healthcare) equilibrated in 25 mM Na cacodylate (pH 6.3) with elution in PBE74 (pH 3.8). The fractions were adjusted to pH 8 with Tris and then injected on a Superdex 200 gel filtration column pre-equilibrated in 20 mM Tris (pH 8.0)-100 mM NaCl-0.5% oPOE. The eluted sample was concentrated and exchanged into the desired buffer using Millipore Amicon ultracentrifugal filtration devices with a molecular weight cutoff of 50,000.

Fluorescence spectroscopy. Fluorescence experiments were performed on a QuantaMaster UV/visible spectrofluorometer (Photon Technology International, Birmingham, NJ) with a four-cuvette carousel. For *in vivo* experiments, the sample was stirred at 29°C in a 1-ml cuvette. The excitation wavelength (λ_{exc}) was set at 290 nm (for the fluorescent resonance energy transfer [FRET] experiments) or at 400 nm (for direct excitation), and for kinetics experiments, the emission of fluorescence (λ_{em}) was monitored at 450 nm. Recycling of Pvd on FpvA and Pvd-Fe dissociation were monitored simultaneously in the various growth media by using a four-sample carousel and alternating λ_{exc} between 290 nm and 370 nm for each time point. Excitation at 370 nm was used instead of excitation at Pvd's maximum of absorbance of 400 nm in order to minimize the monochromator travel distance between time points.

Preparation of periplasmic, cytoplasmic, inner membrane, and outer membrane fractions. CDC5(pPVR2) cells were grown in 50 ml of SM or SM_{imac} to an OD₆₀₀ of 1, at which time 600 nM Pvd was added. After 30 min, the cells were pelleted at 6,000 $\times$ g and the cellular fractions separated as previously described (12). Briefly, the pellet was resuspended in 3 ml 20% sucrose-1 mM EDTA-0.2 M Tris (pH 8.0), and after 2 min at ambient temperature osmotic shock was applied by the rapid addition of 4.5 ml ice-cold water followed by gentle sample inversion. After 2 min on ice, the periplasmic fraction was separated from the cells by centrifugation at 6,000 $\times$ g for 10 min and the pellet rinsed with water and resuspended in 20 ml of 20 mM Tris (pH 8.0). The cells were lysed by sonication and the cytoplasmic fraction 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 *N*-lauroyl sarcosine in 20 mM Tris (pH 8.0), followed by a second spin at 100,000 $\times$ g. The outer membrane was extracted from the resulting pellet with 2% oPOE in 20 mM Tris (pH 8.0), followed by a final high-speed centrifugation. The fluorescence intensity (λ_{exc} = 400 nm and λ_{em} = 450 nm) was measured for each fraction and the appropriate dilution factors applied to the measurements so that all reported intensities represent the total fluorescence from the same volume of the initial cultures. Minimal cross-contamination of the fractions was verified with Coomassie blue- and silver-stained SDS-polyacrylamide gels (12). Although we consistently found a small amount of FpvA in the periplasmic fraction, it was not significant compared to the amount of Pvd that was recovered there.

Elemental analyses. FpvA-Pvd was purified from K691(pPVR2) as described above, with the addition of 1 mM EDTA in all buffers until the final gel filtration into analysis buffer (10 mM Tris [pH 8.0], 100 mM NaCl, 0.5% oPOE). The elution from the penultimate column (MonoP) was split in two, and half was treated with a 20-fold excess of Pvd-Fe for 1 week before both samples were further purified on a Superdex 200 gel filtration column. The eluted samples (16.7 μ M for FpvA-Pvd and 14.5 μ M for FpvA-Pvd-Fe) were assayed for Fe and Al contents.

ICP-AES (inductively coupled plasma-atomic emission spectroscopy) analyses were performed to determine Fe concentrations in nondiluted samples. Samples were injected via a peristaltic pump (Gilson) equipped with Tygon tubing at a 1-ml/min flow rate. Nebulization of samples was performed by means of a concentric nebulizer (Meinhardt). A JY 38 ICP-AES (Jobin Yvon) was used as the detector. ICP conditions were the following: nebulization gas flow rate, 0.35 liter/min; outer gas flow rate, 12.0 liter/min; and auxiliary gas flow rate, 0.2 liter/min. Detection was performed at 238.204 nm.

ICP-mass spectrometry (ICP-MS) analyses were performed to determine Al and Mn concentrations in samples diluted 10-fold. Samples were injected via a peristaltic pump (Gilson) equipped with Tygon tubing at a 1-ml/min flow rate. Nebulization of samples was performed by means of a concentric nebulizer (Meinhardt type C; flow rate, 1 ml/min). A PlasmaQuad 3 ICP-MS (thermo-elemental) was used as the elemental detector. ICP conditions were the following: nebulization gas flow rate, 0.75 liter/min; outer gas flow rate, 13.5 liter/min;

auxiliary gas flow rate, 1.8 liter/min. The plasma power was set to 1,350 W, and ion lens voltages were adjusted to maximize the signals detected at $m/z = 27$ and $m/z = 55$ for Al and Mn, respectively.

Synthesis of NP-Pvd. An amine-containing derivative of Pvd was synthesized by coupling a diamino-polyethylene glycol (PEG) [2,2'-(ethylenedioxy)bis(ethylamine)] (Aldrich) to the succinate moiety on Pvd using standard carbodiimide chemistry. Pvd-Fe (0.78 mg, 0.56 μmol) was dissolved in 0.7 ml dimethyl sulfoxide-H₂O (7:1, vol/vol), to which was added 3 mg 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (ABCR Karlsruhe). After 1.5 h at room temperature, 2 μl of the diamino-PEG was added to give approximately 20 mM, or a 25-fold excess over Pvd-Fe. After 3 h, the reaction mixture was diluted 20-fold into 0.5% acetic acid (AcOH)–1 mM EDTA and injected on a 6-ml ResourceS column equilibrated in the same buffer. Elution was carried out at 3 ml/min with a 30-ml gradient from 0 to 0.5 M NaCl. The first major peak was the metal-free starting material, and the second was the desired product based on matrix-assisted laser desorption ionization–time-of-flight MS. The product was diluted threefold in 0.5% AcOH, reinjected on the same column, and eluted with a 30 ml gradient to 1.25 M ammonium acetate in 1% AcOH. The eluted peak (1 ml) was frozen at -80°C , lyophilized, and resuspended in 50 μl H₂O, giving a concentration of 1 mM amino-PEG-Pvd (NP-Pvd) based on the extinction coefficient of Pvd at pH 5 of $16,500 \text{ M}^{-1}\text{cm}^{-1}$. The NP-Pvd was reloaded with iron by the addition of 20 mM sodium citrate (pH 5) with 1 mM FeCl₃ to give a twofold excess of iron to Pvd.

SPR. Surface plasmon resonance (SPR) measurements were performed at 25°C using a Biacore 2000 instrument. The response measured in Biacore experiments is related to the accumulation of mass at the sensor surface and is recorded as a function of time using arbitrary resonance units (RU), where a signal of 1 RU corresponds to the binding of approximately 1 pg of material per mm^2 . The NP-Pvd-Fe was immobilized (48 RU) on a CM5 sensor surface from a 10 μM solution in 0.5 mM sodium citrate (pH 5.0) via standard amine-coupling chemistry (16). A surface treated with the same chemistry but omitting the NP-Pvd-Fe injection was used as reference surface. The running buffer for the NP-Pvd-Fe immobilization was 10 mM HEPES–150 mM NaCl (pH 7.5). For the FpvA interaction and siderophore competition analysis, the running buffer was 10 mM Tris–150 mM NaCl–0.8% oPOE (pH 8.0) (TNO). EDTA (0.4 mM) was added to this running buffer for experiments involving iron stripping from immobilized Pvd surfaces and reloading. To remove iron from the immobilized NP-Pvd-Fe surface, the surface was treated by three pulses (2 min) of formic acid 1%–8 mM EDTA. The NP-Pvd surface was reloaded with iron by the injection (twice for 2 min each) of 10 mM citrate–0.5 mM FeCl₃ (pH 5.0).

Interaction assays and data processing and analysis. Solubilized and purified FpvA (7.5 nM to 2 μM) was injected for 120 s on the NP-Pvd-Fe surfaces, followed by a 600-s buffer injection. All sensorgrams were processed by double referencing, and the binding profiles were analyzed by global fitting using the simple 1:1 Langmuir model from the BIAevaluation 4.1 software (21).

For competition experiments, increasing amounts of Pvd-Fe or Pvd were incubated (>60 min, room temperature) with a constant concentration of FpvA (250 nM or 1,000 nM, respectively). The FpvA-siderophore mixture was then allowed to flow over the NP-Pvd-Fe surface to measure the free FpvA that can still bind to the surface. The free protein concentration was deduced from the initial slope recorded between 12 and 20 s after the injection start, using a calibration curve established by injecting known FpvA concentrations on the same surface. The “solution affinity” model from the BIAevaluation 4.1 software was used to evaluate the equilibrium constant from the plot of free concentrations of FpvA against the total concentration of siderophore.

RESULTS

The purified in vivo-formed FpvA-Pvd complex contains aluminum. The purification of an iron-free FpvA-Pvd complex from the Pvd-producing and FpvA-overexpressing strain K691(pPVR2) has been previously reported (26) and has been used to solve the crystal structure of the FpvA-Pvd complex (6). Although those authors noted in their description of the structure that there was a significant density in the Pvd molecule where an iron would normally be bound, it was not attributed to a metal and thus was not modeled. Therefore, we analyzed the purified protein by ICP-AES and ICP-MS in order to see what metals could be associated with Pvd in the complex. As a control, the same protein was incubated in a

TABLE 1. Elemental analysis of FpvA-Pvd complexes

Buffer or protein (concn, μM^a)	Concn (μM), mean $\pm$ SD		
	Fe ^b	Al ^c	Mn ^c
Buffer	<1	0.4 ± 0.1	<0.2
FpvA-Pvd (16.7)	2.5 ± 0.1	4.8 ± 0.4	1.1 ± 0.1
FpvA-Pvd-Fe (14.5)	13.7 ± 0.2	0.3 ± 0.1	ND ^d

^a Protein concentration calculated using $\epsilon_{280} = 161,300 \text{ cm}^{-1} \text{ M}^{-1}$.

^b Analyses were performed by ICP-AES.

^c Analyses were performed by ICP-MS.

^d ND, not detected.

20-fold excess of Pvd-Fe for 1 week to completely exchange the bound Pvd for Pvd-Fe. Both protein samples were passed over a Superdex 200 column before being subjected to elemental analysis. The buffer from the gel filtration (10 mM Tris, 100 mM NaCl, 0.5% oPOE) was also analyzed as a blank. The analyses showed that there was more aluminum in the FpvA-Pvd sample than in either the FpvA-Pvd-Fe or blank sample (Table 1) and that it was present in the same concentration as the Pvd in the FpvA-Pvd complex. In our hands there is a batch-to-batch variability in FpvA-Pvd preparations, in that they have different amounts of the copurified contaminants apo-FpvA and FpvA-Pvd-Fe. To assess whether Pvd in the preformed in vivo FpvA-Pvd complex might be picking up iron or aluminum from the buffers during purification, we performed the lysis and membrane extraction steps of the purification in 50 μM Fe(II)SO₄ and 90 μM Fe(III)Cl₃ and found that this excess of iron did not reduce the FRET in the detergent-solubilized outer membrane compared to the control (data not shown). Therefore, aluminum must be already bound to Pvd in the FpvA-Pvd complex in vivo, which explains why iron is not picked up by the complex during or after purification. The small contaminant of FpvA-Pvd-Fe could form from apo-FpvA and metal-free Pvd, which is always present in excess during the cell lysis step of the purification.

Trace metals present in minimal media modulate Pvd fluorescence. We use SM for iron-limited *Pseudomonas* cultures, and since metal binding modulates Pvd fluorescence, we used this fluorescence as a probe of trace metals in SM. In Fig. 1A the fluorescence of 2 μM Pvd in SM is compared to that in SM plus 100 μM EDTA. The fluorescence intensity of Pvd in SM was nearly twofold higher, and remarkably, it was not immediately affected by the subsequent addition of EDTA. Thus, this modulation in Pvd fluorescence appears to be the result of a competition between EDTA and Pvd for a metal that displays slow kinetics of ligand exchange. A similar fluorescence modulation can be replicated with aluminum and EDTA in 10 mM Tris. As demonstrated in Fig. 1B, EDTA had no direct effect on Pvd fluorescence, but instead it was through the sequestration of a metal that the fluorescence was modulated. When Al(III) was added to 20 mM Tris with Pvd, there was an increase in fluorescence (trace 6), which was not reduced by subsequent addition of EDTA (trace 5); however, when EDTA was added to the Al(III) before the Pvd, then the fluorescence (trace 4) remained the same as Pvd alone (trace 1). Also, in both SM and aluminum-spiked Tris buffer, there was a concomitant red shift of the emission spectra by 5 nm upon a metal-induced fluorescence increase.

FIG. 1. Fluorescence of Pvd in SM and Tris buffer with EDTA and aluminum. Emission spectra of Pvd in various solutions ($\lambda_{\text{exc}} = 400$ nm) are shown. When EDTA was present, the additions of EDTA and Pvd were always separated by 5 min to allow a more complete metal chelation. (A) Two micromolar Pvd in SM (1), SM plus 100 μM EDTA (2), and SM with addition 5 min later of 100 μM EDTA (3). (B) One micromolar Pvd, 40 μM EDTA, and/or 20 μM AlCl_3 in 20 mM Tris (pH 8.0) listed in the order of addition: Pvd alone (1); Pvd and EDTA; (2) EDTA and Pvd (3); EDTA, Al, and Pvd (4); Al, Pvd, and EDTA (5); and Pvd and Al (6).

Immobilized Pvd and IMAC Sepharose extract a Pvd fluorescence-enhancing metal. In order to study the role of trace metals in the Pvd-Fe uptake pathway, a method was developed for making a defined minimal medium in which the majority of trace multivalent metals that are present in standard medium preparations have been removed. Without a prior knowledge of the metals which might influence ferric Pvd transport, a metal extraction system that mimics the potentially broad spectrum of Pvd-metal affinities was initially chosen. Pvd from *P. aeruginosa* strain ATCC 27853 was immobilized on *N*-hydroxy-succinimide-activated Sepharose via its lysine side chain, thus creating a metal affinity resin. SM was passed over the immobilized Pvd to yield SM_{ip} . Addition of 160 nM Pvd to SM and SM_{ip} confirmed via a 30% drop in fluorescence that a fluorescence-enhancing species had been removed by the immobilized Pvd column (data not shown).

The cost of a large-scale immobilized Pvd column, which would be needed to prepare several liters of SM_{ip} , inspired the development of an alternative approach. Although we found that iminodiacetate-based metal chelating resins did not remove the fluorescence-enhancing metals in SM, the more efficient chelation by IMAC Sepharose (a proprietary metal af-

finity resin from GE Healthcare) proved to be more effective. To overcome the slow kinetics of ligand exchange for some metals, the metal removal was performed in two steps: on a column (5-min contact time) and then for 48 h in batch with fresh resin. For these manipulations, there was ~ 1 mM MgSO_4 in the medium, and thus the only trace metals effectively removed were those that are chelated by IMAC Sepharose with a much higher affinity than Mg(II) . The fluorescence emission spectra of 2 μM and 100 nM Pvd in SM and SM_{imac} showed that the IMAC resin removes a large amount of the fluorescence-enhancing metal (Table 2). However, the smaller difference in fluorescence with the lower Pvd concentration suggests that there are still some metals left in solution. This is supported by the fact that preaddition of EDTA can further decrease the fluorescence of 100 nM Pvd but has little effect on 2 μM Pvd. Once again, the red shift in the emission is correlated with trace metals in solution. Fluorescence measurements at 24 h after sample preparation showed an increased intensity, suggesting that the kinetics of ligand exchange for some metals are slow and that, for the metals concerned, Pvd has a higher affinity than EDTA. Because there is a competition between Pvd and EDTA for metals that can both enhance and quench fluorescence, the observed time-dependent fluorescence modulation is a function of the concentrations and binding kinetics of all the metals in solution. Therefore, the fluorescence modulation is too complex to interpret apart from the conclusions that a trace metal that enhances Pvd fluorescence exists in SM at a concentration on the order of 2 μM and that it is largely removed in SM_{ip} and SM_{imac} .

Reduction of in vivo FRET in *P. aeruginosa* grown in metal-poor media. Previous studies have demonstrated that Pvd is bound to FpvA in vivo when *P. aeruginosa* is cultured in iron-deficient media by taking advantage of a fortuitous signal of the interaction between FpvA and Pvd: the FRET which occurs between the tryptophans of FpvA and the bound Pvd (25, 26). To investigate the effect of metal depletion on in vivo FRET, SM_{ip} and SM_{imac} were compared with SM as media for the growth of *P. aeruginosa*. However, the metal-depleted media did not support bacterial growth above a cell density (A_{600}) of 0.3 OD unit, compared to 1.2 OD units in SM, likely due to a lack of some essential nutrients such as iron and other trace metals that were extracted by IMAC Sepharose and immobi-

TABLE 2. Fluorescence intensity of Pvd in SM, SM_{imac} , and SM plus EDTA

Pvd concn	Medium	Normalized fluorescence intensity ^a at:		$\lambda_{\text{em max}}$
		<1 h	24 h	
2 μM	SM	1.00	1.04	458
	SM_{imac}	0.51	0.55	453
	SM-EDTA ^b	0.54	0.60	453
100 nM	SM	1.00 (19.6)	1.03 (19.0)	458
	SM_{imac}	0.83 (12.1)	0.91 (11.1)	458
	SM-EDTA	0.55 (19.2)	0.65 (16.2)	453

^a The fluorescence intensity ($\lambda_{\text{exc}} = 400$ nm) was integrated between 410 and 550 nm and normalized to the value for the fresh SM sample. The ratio of the fluorescence at 2 μM to that at 100 nM (20-fold difference in Pvd concentration) is in parentheses.

^b EDTA (100 μM) was added to the SM 10 min before the addition of Pvd.

FIG. 2. FRET and FpvA expression from bacteria cultured in metal-deficient medium. K691(pPVR2) was grown overnight in SM, SM_{ip}, and SM_{imac} with PTM4-lo trace salts (see Materials and Methods), and then the cells were centrifuged, washed with 50 mM Tris (pH 8.0), and resuspended at an OD₆₀₀ of 2. (A) FRET (λ_{exc} = 290 nm), expressed as the emission intensity normalized to the tryptophan fluorescence in order account for small differences in cell density between samples. Dashed line, SM; thin line, SM_{ip}; thick line, SM_{imac}. (B) Western blot with polyclonal anti-FpvA antibodies of whole-cell lysates. For each lane, 500 μ l of the cells at an OD₆₀₀ of 2 were pelleted, resuspended in 100 μ l SDS sample loading buffer, and incubated for 5 min at 95°C, and then 20 μ l was run on a 13.5% SDS-acrylamide gel. Lanes: 1, 250 ng purified FpvA; 2, K691(pPVR2) in SM_{imac}; 3, K691(pPVR2) in SM; 4, K691(pPVR2) in SM_{ip}; 5, CDC5(pPVR2) in SM; 6, CDC5(pPVR2) in SM_{imac}.

lized Pvd. Therefore, in all subsequent experiments, the media were supplemented with a modified PTM4 trace salts solution which included 160 nM Fe₂SO₄ (see Materials and Methods). When the FpvA-overexpressing strain K691(pPVR2) was grown in the trace salts-supplemented SM, SM_{ip}, and SM_{imac}, we observed a large difference in the amount of in vivo FRET despite similar levels of FpvA expression and Pvd production (Fig. 2A). FpvA expression was qualitatively compared by Western blotting of the whole-cell lysates, and the Pvd concentration in the medium was calculated from its extinction coefficient at 405 nm. The Pvd concentrations varied from 50 to 150 μ M depending on the age of the culture, and FpvA expression levels were similar in all the media (Fig. 2B). Although both metal removal protocols cause a reduction in the in vivo FRET, the 48-h batch method used in the SM_{imac} preparation is more efficient in reducing the FRET than the short exposure used in the SM_{ip} preparation.

Loss of Pvd binding in *P. aeruginosa* grown in SM_{imac}. The in vivo binding kinetics and affinity of Pvd were reported for CDC5(pPVR2) cells grown in standard SM preparations. In

FIG. 3. Pvd binding to FpvA in vivo monitored by FRET. CDC5(pPVR2) was grown to an OD₆₀₀ of 1, then incubated with 1 μ M Pvd (A) or 1 μ M Pvd-Ga (B) for 5 min on ice, and then washed in and resuspended in 50 mM Tris (pH 8.0). The emission spectrum for each sample was normalized to the tryptophan fluorescence, and then the control without Pvd or Pvd-Ga was subtracted. The three traces are for cells grown in SM (1), SM_{imac} (2), and SM plus 80 μ M FeSO₄ (3).

order to test whether this reported binding is metal dependent, we grew CDC5(pPVR2), a Pvd-deficient strain that overexpresses FpvA, in SM and SM_{imac}. Growing cultures of CDC5(pPVR2) at an OD of 0.8 were put on ice, followed by the addition of 1 μ M Pvd or Pvd-Ga. After 10 min, the cells were centrifuged, washed, and resuspended in Tris buffer for FRET measurements. The addition of metal-free Pvd gave rise to FRET only in SM (Fig. 3A). A similar expression level of FpvA in the different media was confirmed by Western blotting (Fig. 2B) as well as by monitoring the FRET between FpvA and the fluorescent complex of Ga(III) with Pvd (Pvd-Ga) (Fig. 3B). The binding assay was performed on ice in order to slow the transport of Pvd to better observe the binding of Pvd without the internalization of Pvd-metal complexes. However, the same result was obtained at room temperature (data not shown). This experiment was repeated for bacteria grown in SM supplemented with 80 μ M FeSO₄, in an effort to suppress FpvA expression, as a control for nonspecific or FpvA-independent interactions of Pvd-Ga or Pvd with the bacteria. Nei-

ther Pvd nor Pvd-Ga incubation produced FRET for the cells in the iron-supplemented medium, confirming that the FRET induced by Pvd-Ga incubation arises from FpvA-Pvd-Ga complexes in the outer membrane. Thus, despite similar FpvA expression levels, only the cells grown in the standard SM and not those grown in SM_{imac} were able to bind Pvd.

The recycling of Pvd onto FpvA is diminished in SM_{imac}. It has been previously shown that after the transport and dissociation of Pvd-Fe by *P. aeruginosa*, the Pvd is recycled (Pvd_{recy}) to the extracellular medium as well as onto FpvA (24). In wild-type bacteria, the recycling is observed as a decrease in FRET following the addition of Pvd-Fe, followed by a rise back to the original level. This corresponds to a displacement of Pvd by Pvd-Fe, followed by a rebinding of Pvd to FpvA. Although the mechanism of recycling is unknown, the binding of Pvd_{recy} to FpvA suggested that the receptor might be involved in the Pvd-Fe dissociation and/or recycling. In order to test the metal dependence of this process, we compared the recycling of Pvd on FpvA in CDC5(pPVR2) cells that were grown in either SM or SM_{imac}. Since CDC5 cells do not produce Pvd, the recycling is observed as a drop in FRET only upon successive additions of Pvd-Fe. This recycling was much more pronounced in the SM-cultured cells, which is consistent with a metal dependence (Fig. 4, top). The kinetics of Pvd-Fe dissociation were simultaneously followed by direct excitation of Pvd fluorescence (Fig. 4, middle). The dissociation was faster in the SM_{imac}-cultured cells, which can be explained by a lack of competition with other metals (see Discussion). The lower overall fluorescence intensity in SM_{imac} is due to the fluorescence-enhancing Pvd-metal complexes that occur in SM. The ratio between the direct excitation and FRET fluorescence is a sensitive signal for the amount of Pvd that is free in solution versus bound to FpvA. Free Pvd fluorescence at 450 nm with excitation at 290 nm was about 1/12th as much as with excitation at 370 nm. The FRET between Pvd and FpvA makes this ratio close to 1 in purified FpvA-Pvd complexes. Consequently, the bottom panel of Fig. 4, which plots this ratio during Pvd-Fe uptake, depicts the kinetics and extent of FpvA-Pvd complex formation in vivo. For the SM_{imac}-cultured cells, this ratio remained higher and more stable during the three additions of Pvd-Fe, while for the bacteria grown in SM, the addition of Pvd-Fe displaced Pvd from the receptor (rise in ratio), followed by a rebinding of the Pvd to FpvA (drop in ratio).

Periplasmic accumulation of Pvd is metal dependent. The accumulation of Pvd-Ga complexes in the periplasm of *P. aeruginosa* was recently demonstrated and presented as evidence for the periplasmic dissociation of Pvd-Fe and involvement of a ferrous intermediate during iron transport by FpvA (12). In these experiments, most of the Pvd fluorescence after transport of Pvd-Fe was recovered in the extracellular medium; however, it also accumulated, although to a much lesser extent than for Pvd-Ga, in the periplasm. The accumulation in the periplasm could be due to inefficient recycling of metal-free Pvd or perhaps due to another Pvd-metal complex that cannot be dissociated by the bacteria. To further investigate these possibilities, the cellular localization of Pvd fluorescence was monitored after Pvd treatment of CDC5(pPVR2) cells grown in SM and SM_{imac}. The cell fractions were separated as previously described (12) and the Pvd concentration reported as the percentage of recovered fluorescence from an equivalent cul-

FIG. 4. Kinetics of Pvd-Fe dissociation and recycling in bacteria cultured in metal-deficient medium. CDC5(pPVR2) was grown to an OD₆₀₀ of 1 and then washed with 20 mM Tris (pH 8.0) and resuspended at an OD₆₀₀ of 2. The kinetics of Pvd recycling (top panel; $\lambda_{\text{exc}} = 290$ nm) and Pvd-Fe dissociation (middle panel; $\lambda_{\text{exc}} = 370$ nm) were monitored at 450 nm following three additions of 300 nM Pvd-Fe (vertical dashed lines) and plotted as the change in fluorescence intensity from $t = 0$. The ratio between the two signals (direct excitation and FRET) is plotted in the lower panel.

ture volume (Fig. 5). In order to directly compare the intensities from the two cultures, 1 μM AlCl₃ was added to all the samples prior to fluorescence measurements. In this manner, all of the Pvd is in a metal complex and the differences in quantum yield between Pvd and Pvd-metal complexes are minimized. Figure 5 shows that for cells grown in the metal-poor medium, there was much less Pvd accumulation in the periplasm. Also, there was less Pvd in the outer membrane fraction, in agreement with the findings in Fig. 2 that show reduced in vivo binding to FpvA in the absence of metals. The experiment was also performed with cells grown in SM_{imac} supplemented with 1 μM AlCl₃. In this case the results were like those for bacteria cultured in SM. Thus, the accumulation of Pvd in the periplasm is metal dependent, and 1 μM Al(III) is sufficient to reproduce this accumulation in metal-poor medium. Consistent with this metal dependence is the fact the addition of Al(III) to the fractionated samples had no effect on the SM or SM-Al samples but caused a dramatic increase in the fluorescence of the SM_{imac} samples, putting their total fluorescence (sum of individual cell fractions) on par with the fluorescence from the SM samples. It is worth noting that the volume of the periplasm (as well as that of the other cell fractions) is many hundreds if not thousands of times smaller

FIG. 5. Cellular distribution of fluorescence after exposure to exogenous Pvd. CDC5(pPVR2) was cultured in SM, SM_{imac}, and SM_{imac} plus 1 μ M AlCl₃ to an OD₆₀₀ of 0.8, at which point it was incubated for 30 min in the presence of 600 nM Pvd. The cell fractions (periplasm, cytoplasm, inner membrane, and outer membrane) were separated (see Materials and Methods), and the fluorescence in the media and in four cellular locations was quantified. Values are expressed as a percentage of the total fluorescence recovered from each culture.

than the culture volume that it occupies, so the concentration of Pvd in the periplasm is significantly higher than that in the extracellular medium despite the lower fluorescence measurement. We also tested the growth of *P. aeruginosa* in SM with a range of AlCl₃ concentrations (10 to 800 μ M) to see if it could be a growth inhibitor. A twofold reduction in doubling time was observed for all samples with aluminum, but with a similar final cell density.

Purified FpvA binding to Pvd is metal dependent in vitro.

The reported values for the apparent association kinetics between FpvA and Pvd in vitro were derived from FRET measurements with purified and detergent-solubilized FpvA that had been expressed in the Pvd-deficient strain CDC5 (5). An unusual feature of this association was that its rate was independent of the concentration of Pvd. Although this can be explained by a fast initial binding step that does not give rise to FRET, the possibility that trace metals in the buffers were the cause of the concentration independence needs to be investigated. This is particularly relevant since the measurements were performed only in the concentration range near the reported K_D (equilibrium dissociation constant) (~ 10 nM), a range that is below the limits of detection for trace metals such as iron and aluminum. If the binding of Pvd to FpvA in vitro is dependent on the trace metals in the buffers, then when the buffers are treated with the IMAC resin, a loss of binding should be observed. Also, if the trace metal concentration in protein solubilization buffer (20 mM Tris [pH 8], 1% oPOE) is not higher than that in the SM recipe, there will be a loss of Pvd binding when the concentrations of Pvd and FpvA are relatively high if the binding is metal dependent. When 30 μ M Pvd was mixed with 20 μ M FpvA, a small amount of complex

quickly formed, but based on the degree of FRET, there was no further complex formation after 2 days (Fig. 6A). However, following a 200-fold dilution of this mixture (100 nM FpvA and 150 nM Pvd), the complex continued to form, reaching completion within 24 h. Our experience with the purification of FpvA-Pvd complexes from a Pvd-producing strain indicates that upon excitation at 290 nm, the ratio of Trp fluorescence to Pvd fluorescence (FRET) falls in the range of 1:3 to 1:3.5 when all FpvA molecules are bound to a Pvd. By this measure, the FpvA molecules in the dilute samples are 100% bound to Pvd, whereas the higher-concentration samples reach only 10 to 20% binding. This inverse dependence on the concentration could be explained by a self-association of FpvA or Pvd that inhibits their interaction with each other. This possibility can be excluded in a number of ways but most simply by adding Fe(III) or Al(III) at the same concentration as Pvd, which quickly leads to complete complex formation even at 20 μ M FpvA (data not shown). Furthermore, the exposure of buffers to IMAC Sepharose or the addition of metal chelators such as EDTA or ferrichrome all led to a reduction of complex formation even at low concentrations of FpvA and Pvd (Fig. 6C and D). Only a trace metal contaminant in the buffers or protein stocks can explain the observed inverse concentration dependence seen in Fig. 6A.

SPR confirms an iron requirement for FpvA-Pvd interactions. We introduced a primary amine to Pvd via a PEG linker on the succinate moiety in order to be able to immobilize Pvd to a Biacore sensor chip by primary amine coupling. The succinate moiety was chosen as the attachment site because it has been previously demonstrated that the attachment of bulky groups to this part of Pvd had no effect on iron chelation and transport (27). Using the immobilized Pvd surface, we were able to compare the affinities of FpvA for Pvd and Pvd-Fe in a controlled and quantitative manner. Figure 7A shows that the binding of FpvA to the immobilized Pvd is iron dependent. A 0.5 μ M FpvA sample gave a response of 90 RU for binding to the immobilized Pvd-Fe surface, while a 5 μ M sample gave 5 RU on the iron-free surface. Upon subsequent reloading of the surface with iron, it was possible to recover 90% the initial FpvA binding activity.

The FpvA binding parameters for immobilized Pvd-Fe were evaluated from a global fit of the binding profiles to a simple 1:1 Langmuir model (Fig. 7B). The kinetic association and dissociation constants calculated from three independent experiments were $k_{on} = (7 \pm 3) \times 10^4$ M⁻¹ s⁻¹ and $k_{off} = (1.2 \pm 0.13) \times 10^{-2}$ s⁻¹, yielding an equilibrium constant ($K_D = k_{off}/k_{on}$) of $(1.9 \pm 0.8) \times 10^{-7}$ M. The maximum binding (R_{max}) values were less than 10% of the theoretical R_{max} in all three experiments, suggesting limited access of the binding site of the immobilized NP-Pvd to the FpvA. The immobilization of ligands can in some cases markedly affect the access and the binding site (18), and solution equilibrium measurements can provide an alternative method for measuring binding activity without these effects. Figure 7C demonstrates FpvA binding to Pvd in solution, observed as the inhibition of FpvA binding to the immobilized Pvd-Fe surface. Pvd-Fe and NP-Pvd-Fe showed similar binding activities, while the metal-free Pvd, even at 10 μ M (a 10-fold molar excess over FpvA), did not inhibit FpvA binding to the immobilized Pvd-Fe surface. The range of K_D values that can be evaluated with this method depends on the measurable range

FIG. 6. Concentration and metal dependence of the FpvA-Pvd complex in vitro monitored by FRET. FpvA in pure or crude (initial ion-exchange column elution contaminated with FptA, the pyochelin receptor) form was mixed with pure Pvd in 20 mM Tris–1% oPOE at different concentrations and with different metal chelators. The FRET ($\lambda_{\text{exc}} = 290$ nm) was measured 48 h after the initial mixture of FpvA and Pvd. (A) Mixtures of 20 μM pure FpvA plus 30 μM Pvd were diluted 200-fold (to 100 nM FpvA) for fluorescence measurements. Black line, 48 h of incubation and then measurement immediately after dilution; gray line, 50-fold dilution (to 400 nM FpvA) for 48 h and then 4-fold dilution for immediate measurement; dotted line, 24 h of incubation, 200-fold dilution, and then measurement 24 h after dilution. (B) Pure FpvA at 150 nM mixed with 300 nM Pvd in standard (dashed line) or IMAC-treated (solid line) buffer. (C) Crude ~ 3 μM FpvA mixed with 5 μM Pvd in standard buffer (dashed line) or buffer pretreated for 5 min with 100 μM EDTA (solid line). (D) Pure FpvA at 150 nM in standard buffer (dashed line) or in buffer with 700 nM ferrichrome (solid line) and 24 h later mixed with 300 nM Pvd.

of free FpvA. The lowest free FpvA concentration that can be accurately measured using a calibration curve constructed with known concentrations of FpvA is approximately 5 nM (slope, <0.18 RU/s) (data not shown).

Theoretical considerations for equilibrium affinity measurements indicate that a high affinity (K_D in the range of a few nM or less) cannot be determined with good accuracy because the free protein concentrations are too low to be measured under

FIG. 7. SPR analysis of FpvA-Pvd interactions with and without iron. The binding of FpvA to immobilized NP-Pvd was monitored by SPR and was shown to be iron dependent. (A) The profiles in black correspond to the binding of 500 nM FpvA to iron-loaded, immobilized NP-Pvd-Fe (solid line) and again after the surface was stripped of iron and subsequently reloaded with iron (dashed line). In red is the binding profile of FpvA (5 μM) for the iron-free NP-Pvd surface. (B) Kinetic analysis of FpvA binding to immobilized NP-Pvd-Fe. The binding curves (red) corresponding to the injection of various concentrations FpvA (from 7.5 to 250 nM) on the NP-Pvd-Fe surface are overlaid with the global fit for a simple one-to-one interaction (black). (C) The binding activities of NP-Pvd-Fe and Pvd-Fe are similar in solution. Iron-free Pvd (empty circles), Pvd-Fe (black circles), and NP-Pvd-Fe (gray circles) were mixed in a molar ratio of 0.1 to 10 with a fixed concentration of FpvA (250 nM for Pvd-Fe and NP-Pvd-Fe or 1,000 nM for Pvd without iron). The solution binding is observed as a decrease in the free FpvA that can bind to the surface and is expressed as a relative response compared to the response for FpvA alone.

these experimental conditions (33). An equilibrium dissociation constant in the nanomolar range (0.5 ± 3.6 nM) was evaluated for the NP-Pvd-Fe interaction with FpvA, and since Pvd-Fe and the amine derivative have very similar binding profiles (Fig. 7C), it is expected that they have similar affinities in solution. This is in relatively good agreement with the literature values of 0.5 to 1.5 nM for the *in vivo* Pvd-Fe binding to FpvA (5, 15, 25).

DISCUSSION

Pvds, like the other hydroxamate and catecholate siderophores, have extraordinarily high affinity for Fe(III) ($\log K_a$, >30) (1), but as they have a structurally flexible scaffold which can accommodate many ionic radii and coordination geometries, they also have significant affinity for a number of multivalent metal ions (2). Trace metals in buffers and salts are normally overlooked in bacterial medium preparations; however, in some experiments these low-concentration species can have unwanted and unexpected effects. In this study, elemental MS of the FpvA-Pvd complex that is copurified from *P. aeruginosa* showed that aluminum is present at the same molar concentration as the siderophore and that this aluminum is replaced by iron when the complex is incubated with excess Pvd-Fe. Thus, the original misinterpretation of the Pvd metal-loaded state was likely due to the unexpected formation of a fluorescent Pvd-Al complex that was derived from the aluminum found at trace levels in the media and buffers used in the experiments. Of the metal ions that are efficiently chelated by Pvd, aluminum is a plausible source of interference in experiments that involve the measurement of Pvd-FpvA interactions due to its ubiquitous nature and the thermodynamic stability of aluminum-hydroxamate siderophore complexes ($\log K_a = 21.5$) (2, 8).

In this study, the fluorescence of Pvd was an indispensable biophysical probe because of its sensitivity to its environment and in particular to the metal-loading state of the siderophore. The Pvd complex with iron (Pvd-Fe) is not fluorescent, while aluminum and gallium complexes (Pvd-Al and Pvd-Ga) are nearly twice as fluorescent as metal-free Pvd. The trace iron is in fact the only source of this essential nutrient in the SM used for iron-limited cell cultures. When special care is taken to remove this iron, the bacteria do not grow above 0.3 OD unit. Since the standard reagents used in biochemistry labs do not guarantee less than 0.0005% of most trace elements, the levels of a trace metal such as aluminum can be as much as 300 nM in a 20 mM Tris buffer. Considering the many other sources of metal contamination, such as glassware that has not been acid washed, metal spatulas, or aluminum foil, it is clear that precautions need to be taken when working with siderophores such as Pvd.

Despite the steps that we took in preparing metal-free media and buffers as well as rinsing plastic ware with 18.2 M Ω cm water and avoiding the use of metal and glass in the preparation of samples, there was still evidence of metal contamination, albeit at a much lower level. The levels are near the limits for detection using even the most sensitive technique, ICP-MS, which can detect a few parts per billion, which for aluminum is around 100 nM. The Pvd fluorescence in SM_{imac} suggests that much of the trace metals have been removed: based on the 100

nM versus 2 μ M fluorescence intensity, $\sim$ 100 nM fluorescence-enhancing trace metal remains (Table 2). The relatively low rate of inner-sphere H₂O substitution for Al(III) could account for the remaining metal in SM_{imac}, and a longer contact with the chelating resin may help (14). However, the remaining aluminum might be insoluble inorganic salts that are not chelated by the IMAC resin but which during bacterial growth can be chelated by Pvd. Also, there are clearly significant trace metals in the *in vitro* samples used for Fig. 6, owing partially to the protein sample itself, which was not treated with IMAC resin. The best metal removal according to Fig. 6 was achieved, not surprisingly, by ferrichrome (Fig. 6D), which has a much higher affinity for Al(III) and Fe(III) than EDTA (Fig. 6C) and can better compete with Pvd for these metals. The 24-h incubation of the samples before FRET measurement allowed an approach toward the equilibrium that favors Pvd-Fe and Pvd-Al complexes over the EDTA complexes but not over the ferrichrome-metal complexes. The *in vivo* kinetics of Pvd-Fe dissociation and recycling are also consistent with a small amount of trace metal in the SM_{imac}, as evidenced by the small decrease in the FRET signal upon subsequent rounds of Pvd-Fe uptake (Fig. 4). However this decrease is smaller and recovers faster than with the cells grown in the standard SM.

The previous findings that Pvd and other siderophores can bind to their receptors in the absence of iron are therefore not surprising considering the fact that the reported equilibrium constants of the metal-free siderophores are lower than the concentration of trace iron and aluminum likely to be present in the media and buffers used (15, 25). The hydroxamate siderophores have an affinity many orders of magnitude higher than their receptor binding constants, and so under normal trace metal conditions, all of the metal-free siderophores subjected to the assay are rapidly metal loaded. For FecA, the only report of metal-free citrate binding is the crystal structure that was solved from a crystal grown from empty FecA in the presence of 5 mM citrate. Despite a potentially high physiological concentration of citrate, the affinity of FecA for citrate has not been reported for this system. It is possible then that ferric citrate has a much higher affinity for FecA, simply out-competing the more abundant citrate. The data on FptA binding to pyochelin are more ambiguous, because while the crystal structure of FptA-pyochelin clearly has iron bound, it appears to have picked up the iron during purification (7). To date, there is no report of other pyochelin-metal chelates which can bind to FptA; however, several other metals do regulate the expression of FptA, which suggests that it can evolve or has in fact evolved to transport other metals (29). It still remains to test FptA-pyochelin interactions under metal-depleted conditions. Thus, we can conclude only that for the hydroxamate- and catecholate-based siderophores (Pvd, ferrichrome, and enterobactin), the binding and transport of other metals interfere with affinity measurements for the metal-free siderophores and their receptors. The main implication of our conclusion in light of the previous reports of a special mechanism for the metal-free binding of siderophores (25) is that the actual mechanism is simpler. The results of studies in which a metal-free environment was not ensured before carrying out metal-free siderophore experiments need to be reinterpreted. Thus, the implications are wide reaching but are also easy to include in preexisting models. For example, the siderophore signaling

through OMTs via an anti-sigma factor (19) can be reinterpreted as requiring the presence of iron or at least a metal. Whether or not the receptors distinguish the type of metal that is present in the bound siderophore during this type of signaling remains to be determined.

In an effort to quantify the relative affinities of Pvd and its metal complexes for FpvA in a metal-controlled environment, we designed an SPR-based assay in which an immobilized Pvd can be loaded with iron or easily stripped of metal before measurement of its interaction with FpvA. In this manner, a single surface could be used for the assay of any Pvd-metal complex with FpvA. Consistent with the observations above, we found that binding of the metal-free Pvd to FpvA could not be observed with up to 5 μ M FpvA (Fig. 7A) and that it did not inhibit FpvA-Pvd-Fe complex formation even with a 10-fold molar excess at a concentration of 10 μ M (Fig. 7C). The lower affinity of FpvA for the immobilized NP-Pvd-Fe measured in this assay ($\sim$ 200 nM) compared to values reported for Pvd-Fe binding to FpvA *in vivo* ($\sim$ 1 nM) is likely due to the immobilization of the Pvd via the succinate moiety of the chromophore (5, 15, 25). The crystal structure of FpvA-Pvd-Fe shows that this end of the chromophore is oriented toward FpvA, suggesting that in the case of the immobilized Pvd, the PEG linker might restrain the Pvd too close to the surface matrix for unimpeded access by FpvA. This explanation is also consistent with the lower-than-expected maximum response on the sensor surface that occurs when some fraction of the immobilized Pvd is completely inaccessible for binding by FpvA. Since the solution affinity of the modified NP-Pvd-Fe is similar to that of Pvd-Fe, as demonstrated in the competition analysis (Fig. 7C), a steric hindrance very likely causes the lower-than-expected affinity and $R_{\max}$. There may be more optimal, although synthetically very challenging, sites on the Pvd peptide for the immobilization site, and perhaps a longer PEG linker would reduce the steric hindrance. However, the facts that the SPR signal arises only for the iron-loaded surface and that it is abolished by competition with Pvd-Fe but not Pvd confirm that it represents the formation of the natural FpvA-Pvd-Fe complex.

We have shown that the FpvA-Pvd complex that is purified from Pvd-producing bacteria is primarily a complex with aluminum with a small amount of iron (Table 1). The possibility that a metal is picked up by the FpvA-Pvd complex during the purification is not likely considering the fact that adding a large excess of Fe(II) and Fe(III) (40 and 90 μ M) to the buffers for the bacterial lysis and membrane extraction does not lead to a loss of FRET in the detergent-solubilized outer membrane fractions. Furthermore, we purified the FpvA samples for ICP-MS or AES in the presence of 1 mM EDTA until the final gel filtration step. If Pvd picked up trace aluminum during the FpvA purification, then it would be difficult to explain how a large excess of iron did not interfere with formation of FpvA-Pvd-Al complexes. The FRET observed in Pvd-producing strains is strong evidence that an iron-free FpvA-Pvd complex forms *in vivo*; however, the simplest explanation considering the above data is that it is actually an FpvA-Pvd-Al complex. It is not clear why a large percentage of FpvA remains loaded with Pvd-Al, unlike what occurs with Pvd-Fe and Pvd-Ga complexes. This difference can be explained by the relative transport efficiencies of the metal complexes as well as the fact the

noniron complexes cannot be dissociated, remaining at very high concentrations in the periplasm. We show that Pvd-Al complexes accumulate in the periplasm (Fig. 5) yet that the majority of the Pvd-Al remains in the medium, unlike the case for Pvd-Ga transport, where nearly all of the fluorescence accumulates in the periplasm (12). Despite the lower Pvd fluorescence recovered from the periplasm, the concentration of Pvd-Al in the periplasm is still much higher than that in the medium, considering the relatively small volume occupied by the periplasm. As previously reported, the efficiency of Pvd-Al transport is lower than that of Pvd-Ga transport (11), and thus a small amount of Pvd-Al recycling or diffusion through the outer membrane would lead to an equilibrium where FpvA is partially loaded with Pvd-Al. While it is difficult to quantify the degree of Pvd loading of FpvA *in vivo*, purification of FpvA from Pvd-producing strains yields samples with various degrees of FRET. This can be due to a mixture of FpvA-Pvd-Al with unloaded FpvA or with FpvA-Pvd-Fe. Our data indicate that iron is present in the FpvA-Pvd sample (Table 1); however, we have also found that preparations of FpvA-Pvd with low FRET were able to bind to Pvd-Ga (shown by an increase in FRET and a decrease in Trp fluorescence upon addition of Pvd-Ga). It should be noted that upon cell lysis, any FpvA that is not already loaded with Pvd-Al *in vivo* would likely encounter a pool of Pvd-Al from the periplasm. Some Pvd-Fe may also exist inside the cells, as is seen with bacteria that have been artificially overloaded with exogenous Pvd-Fe, or it can arise from Pvd chelation of iron from buffers during cell lysis. Thus, there may be an *in vitro* competition between various Pvd-metal chelates for binding to FpvA during cell lysis. However, as demonstrated previously, the purified FpvA-Pvd-Al complex does not pick up free iron (26), so any iron in the *in vitro* complex should arise from Pvd-Fe binding to FpvA and not from a metal substitution with an FpvA-Pvd-Al complex.

The biological significance of aluminum transport is not clear, but since aluminum and gallium bioavailability in an infected host is low, a pathogenic bacterium is unlikely to encounter interference from these metals during infection. However, the fluorescent pseudomonads live in soil and water and can survive in a variety of niches where they can encounter higher levels of toxic metals. Considering the fact that the affinity of siderophores for iron compared to other common metals (2) is sufficient to ensure the formation of siderophore-Fe complexes in a mixed-metal environment, bacteria gain little by designing a more specific siderophore transport system. We found that AlCl_3 did not significantly inhibit growth in SM, which is consistent with a similar report on the ferrichrome-producing fungus *Ustilago sphaerogena* in iron-deficient medium (10). The fact that higher levels of Al(III) are not more toxic may be due to its low solubility at neutral and basic pHs. However, siderophores may also act to sequester toxic metals so that they do not interfere in metabolic processes. This is consistent with the protective effect of Pvd against the antimicrobial and antibiofilm activities of gallium (17) and the higher sensitivity to vanadium in Pvd-deficient mutants (4). The finding that bacteria do not remove Ga(III) and Al(III) from siderophore complexes is also consistent with a metal reduction-based mechanism of Fe(III) removal that would serve two purposes: to make a thermodynamically unfavorable dissociation possible and to differentiate between an essential nutrient and toxic metals.

Taken together our data support a model of siderophore-receptor interactions that echoes the observations made over 35 years ago by Emery on ferrichrome-mediated metal transport in *U. sphaerogena*: "The specificity data indicate that conformation of the chelate, rather than charge or solubility, is the basis for recognition by the transporter system" (10). We have demonstrated both *in vivo* and *in vitro* that there is a metal dependence for the interaction of Pvd with its receptor and that Al(III), in addition to Ga(III) and Fe(III), is transported by FpvA. The hydroxamate chelates of iron form an octahedral geometry, and both Fe(III) and Al(III) have been shown to form a similar geometry with ferrichrome (20). Based on the ability of Al(III) and Ga(III), and to some extent Ti(III), to be transported by Fe(III) transporters, it appears that the trivalent group III elements are unique in forming an iron-like coordination with siderophores, thereby restricting the conformation of their siderophore chelates to a similar geometry. Thus, a model of siderophore recognition that depends on a metal-induced conformation is consistent with our findings and with previously reported data on the specificity of siderophore transporters (10). The specificity of iron transport can be further refined by the siderophore-metal dissociation mechanism, which selects for iron based on a reduction to the ferrous ion, which has a lower affinity for the siderophore and can thus be extracted by a periplasmic iron binding molecule.

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