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

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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 μM 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 FpvA-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 (~10⁻¹⁰ M). Mammalian pathogens encounter even lower levels of free iron (~10⁻¹⁰ 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 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

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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 Schall 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 MΩ cm was ultrapure grade from Sigma. Water with a resistivity of 18.2 MΩ 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 K91(pPVR2), which overproduces FpvA and produces Pvd (22). The strains were grown in an SM [35 mM K2HPO4, 2 mM KH2PO4, 1 mM MgSO4, 35 mM K6391(pPVR2), which overproduces FpvA and produces Pvd (22). The strains were grown in an SM [35 mM K2HPO4, 2 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 μg/ml carbenicillin. A low-iron-content trace salts solution (PTM4-lo) was prepared as a 1:100 mixture of PTM4 (28) and PTM4(Feþ) (PTM4 contains, per liter, 2 g CuSO4·5 H2O, 200 g FeSO4·7 H2O, 200 mg NaMoO4·2 H2O, 20 mg boric acid, 500 mg CoCl2· 7 g MnCl2·22 g FeCl3·4 H2O, 200 mg biotin, and 1 ml sulfurlc 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 3 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(Feþ) stocks and then diluted 5,000-fold into the medium for a final iron concentration of 160 mM. 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-MΩ water before culture growth.

Preparation of periplasmic, cytoplasmic, inner membrane, and outer membrane fractions. CDC5(pPVR2) cells were grown in 50 ml of SM or SMimac to an OD600 of 1, at which time 600 nm Pvd was added. After 30 min, the cells were pelleted at 6,000 × g and the cellular fractions separated as previously described (12). Briefly, the pellet was resuspended in 5 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 sample inversion. After 2 min on ice, the periplasmic fraction was separated from the cells by centrifugation at 6,000 × 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 × 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 × 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 (λex = 400 nm and λem = 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 K91(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) 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 (λem) 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 λem 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 nonspecific phototransfer of the Pvd-Fe complex with distilled water.

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. Determinations of samples were 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-milliliter/minute 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 diaminopolylethyleneglycol (PEG) [2·(2′-ethylendioxy)bis(ethyl-amine)] (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 (DMSO)/H₂O (7:1, vol/vol), to which was added 3 mg 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (ABC-RK, Karlsruhe). After 1.5 h at room temperature, 2 μl of the diamin-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 Resource S column equilibrated in the same buffer. Elution was carried out at 3 ml/min with a 30-mM 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 threelfold in 0.5% AcOH, reinfected 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 M⁻¹ 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 mm². 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-Fe 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 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.
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-hydroxysuccinimide-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 affinity 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 depletions 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 immobilized metal from GE Healthcare.)

**FIG. 1.** Fluorescence of Pvd in SM and Tris buffer with EDTA and aluminum. Emission spectra of Pvd in various solutions (λ_{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).

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

<table>
<thead>
<tr>
<th>Pvd concn</th>
<th>Medium</th>
<th>Normalized fluorescence intensity* at:</th>
<th>λ_{em} (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 μM</td>
<td>SM</td>
<td>1.00  1.04</td>
<td>458</td>
</tr>
<tr>
<td></td>
<td>SM_{imac}</td>
<td>0.51  0.55</td>
<td>453</td>
</tr>
<tr>
<td></td>
<td>SM-EDTA</td>
<td>0.54  0.60</td>
<td>453</td>
</tr>
<tr>
<td>100 nm</td>
<td>SM</td>
<td>1.00  (19.6) 1.03 (19.0)</td>
<td>458</td>
</tr>
<tr>
<td></td>
<td>SM_{imac}</td>
<td>0.83  (12.1) 0.91 (11.1)</td>
<td>458</td>
</tr>
<tr>
<td></td>
<td>SM-EDTA</td>
<td>0.55  (19.2) 0.65 (16.2)</td>
<td>453</td>
</tr>
</tbody>
</table>

* The fluorescence intensity (λ_{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.

* EDTA (100 μM) was added to the SM 10 min before the addition of Pvd.
lized Pvd. Therefore, in all subsequent experiments, the media were supplemented with a modified PTM4 trace salts solution (see Materials and Methods), and then the cells were centrifuged, washed with 50 mM Tris (pH 8.0), and resuspended at an OD_{600} 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 μl of the cells at an OD_{600} of 2 were pelleted, resuspended in 100 μl SDS sample loading buffer, and incubated for 5 min at 95°C, and then 20 μl 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}. 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 solution, and then the cells were centrifuged, washed with 50 mM Tris (pH 8.0), and resuspended at an OD_{600} 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 μl of the cells at an OD_{600} of 2 were pelleted, resuspended in 100 μl SDS sample loading buffer, and incubated for 5 min at 95°C, and then 20 μl 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}. FIG. 3. Pvd binding to FpvA in vivo monitored by FRET. CDC5(pPVR2) was grown to an OD_{600} of 1, then incubated with 1 μM Pvd (A) or 1 μM 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 μM FeSO_{4} (3).
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 SMimac were able to bind Pvd.

The recycling of Pvd onto FpvA is diminished in SMimac. 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(pPV2) cells that were grown in either SM or SMimac. 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 the direct excitation of Pvd fluorescence (Fig. 4, middle). The dissociation was faster in the SMimac-cultured cells, which can be explained by a lack of competition with other metals (see Discussion). The lower overall fluorescence intensity in SMimac 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 SMimac-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(pPV2) cells grown in SM and SMimac. The cell fractions were separated as previously described (12) and the Pvd concentration reported as the percentage of recovered fluorescence from an equivalent culture volume (Fig. 5). In order to directly compare the intensities from the two cultures, 1 μM AlCl$_3$ 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 binding of Pvd to FpvA in the absence of metals. The experiment was also performed with cells grown in SMimac supplemented with 1 μM AlCl$_3$. 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 SMimac 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.
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 μM) 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 Kᵣ (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 μM Pvd was mixed with 20 μM 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 μM 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 μM FpvA sample gave a response of 90 RU for binding to the immobilized Pvd-Fe surface, while a 5 μM sample gave 5 RU on the iron-free surface. Upon subsequent reloading of the surface with iron, it was possible to recover 90% of 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 kₐ = (7 ± 3) × 10⁴ M⁻¹ s⁻¹ and kₐf = (1.2 ± 0.13) × 10⁻² s⁻¹, yielding an equilibrium constant (Kᵣ = kₐf/kₐ) of (1.9 ± 0.8) × 10⁻⁷ M. The maximum binding (R₉) values were less than 10% of the theoretical R₉ 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 μM (a 10-fold molar excess over FpvA), did not inhibit FpvA binding to the immobilized Pvd-Fe surface. The range of Kᵣ values that can be evaluated with this method depends on the measurable range
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...
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 nM 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 MΩcm 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$_{limac}$ suggests that much of the trace metals have been removed: based on the 100 nM versus 2 μM fluorescence intensity, ~100 nM fluorescence-enhancing trace metal remains (Table 2). The relatively low rate of inner-sphere H$_2$O substitution for Al(III) could account for the remaining metal in SM$_{limac}$, 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$_{limac}$ 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 outcompeting 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
we found that binding of the metal-free Pvd to FpvA could not complex with FpvA. Consistent with the observations above, measurement of its interaction with FpvA. In this manner, a can be loaded with iron or easily stripped of metal before measurement of its interaction with FpvA. This difference can be explained by the relative trans-

FpvA purification, then it would be difficult to explain how 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 fluores-

We have shown that the FpvA-Pvd complex that is formed 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 μM) 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.
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 dissocation 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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