Molecular basis of pyoverdine siderophore recycling in *Pseudomonas aeruginosa*

Francesco Imperia,b, Federica Tiburzi a,b, and Paolo Visca a,b,1

1Department of Biology, University “Roma Tre,” Viale G. Marconi 446, 00146 Roma, Italy; and 2Molecular Microbiology Unit, National Institute for Infectious Disease Istituto Di Ricovero e Cura a Carattere Scientifico “Lazzaro Spallanzani,” Via Portuense 292, 00149 Roma, Italy

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*Pseudomonas aeruginosa* is among the most dreaded Gram-negative pathogens in the hospital setting and the main cause of lung decline and death in patients suffering from cystic fibrosis (CF). In response to iron depletion, *P. aeruginosa* utilizes the endogenous siderophore pyoverdine (PVD) as the primary iron source. In addition to its role in nutrition, PVD is implicated in biofilm control, cell-to-cell communication, and virulence regulation (1). By a mechanism known as surface signaling, PVD-related siderophores are produced by all fluorescent pseudomonads sequenced so far, suggesting that PVD recycling represents a general energy-saving strategy adopted by natural *Pseudomonas* populations.

**Results**

*PA2389, PA2390*, and *opmQ Mutants Accumulate PVD in the Periplasm.*

In a preliminary search for candidate PVD secretion systems, we compared the PVD levels between culture supernatants of wild-type *P. aeruginosa* PAO1 and transposon mutants in genes encoding the MexAB-OprM, PvdE, and PA2389–90-OpmQ transport systems [supporting information (SI) Table S1]. No significant growth differences between wild-type PAO1 and putative PVD secretion mutants were observed under both iron-deplete (iron-deplete Casamino acids or DCAA medium) and -replete (DCAA medium plus 50 μM FeCl3) conditions (Fig. S1). In the iron-deplete medium, PVD production was: (i) marginally affected by mutation in any of the *mexA, mexB, and oprm* genes; (ii) significantly reduced (approximately 50–60% of the wild-type levels) by mutation in any of the *PA2389, PA2390*, and *opmQ* genes; and (iii) completely abolished in the *pvdE* and *pvdA* mutants (Fig. L4).

We took advantage of the fluorescent properties of apo-PVD to determine its amount in cell lysates of wild-type PAO1 and isogenic mutants by fluorimetric measurements (see Fig. L4). The *mexA, mexB*, and *opm* mutants showed intracellular levels of PVD comparable to or even lower than those of the wild type, consistent with their profile of PVD production. PVD was undetectable in cell lysates of the *pvdE* mutant, suggesting that PvdE is not involved in PVD secretion or, alternatively, that it directs the export of a nonfluorescent PVD precursor named ferribactin (1, 7). Conversely, *PA2389, PA2390*, and *opmQ* mutants harboring the *pvdE* gene, which is essential for PVD production (9), in addition, the *pvd* locus contains an operon for a tripartite efflux system of the ABC superfamily (PA2389–90–OpmQ), whose expression mirrors that of PVD synthesis enzymes, being controlled by the PVD-specific sigma factor PvdS. Deletion of this system reduces, but does not abrogate, PVD production (4).

PVD-mediated iron uptake occurs by binding of the ferri-PVD complex to the outer membrane (OM) receptor FpvA (10, 11), while a secondary PVD receptor, called FpvB, provides a minor contribution to PVD uptake (12). Iron release from PVD has been shown to occur in the periplasm, and that apo-PVD is then recycled to the extracellular milieu (13, 14). However, no protein candidates have been proposed for these processes and further studies are required to elucidate the fate of iron and PVD following dissociation in the periplasm (15).

Here, we demonstrate that the tripartite efflux system PA2389–90–OpmQ is implicated in PVD recycling by *P. aeruginosa*, and that PVD recycling and export probably involve alternative pathways.
mutants accumulated five- to sixfold higher amounts of PVD than wild-type PAO1, suggesting an involvement of these genes in PVD export. Therefore, the PA2389 and PA2390 genes were renamed pvdR and pvdT, respectively, to emphasize their role in PVD transport or recycling.

To visualize PVD accumulation by pvdR-, pvdT-, and opmQ mutants, intracellular PVD was detected in living cells by confocal laser-scanning microscopy (Fig. 1B). The fluorescence emission levels of pvdR-, pvdT-, and opmQ-mutant cells largely exceeded that of wild-type cells. No fluorescence was detected in the PVD-defective pvdA mutant or in iron-replete wild-type cells (see Fig. 1B and Fig. S2), validating the identification of the

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Next, we sought to investigate the involvement of the PvdRT-OpmQ efflux system in recycling PVD after FpvA-mediated ferri-PVD uptake. To this aim, iron-deplete cells of pvdA, pvdApvdR, and fpvApvdA mutants were incubated with the nonfluorescent ferri-PVD complex (1 μM). Recycling of apo-PVD was monitored fluorimetrically as the appearance of PVD-specific fluorescence in culture supernatants (14). The pvdA strain efficiently recycled apo-PVD in the culture medium, while both the pvdApvdR and fpvApvdA double mutants did not (Fig. 3D). Nevertheless, pvdApvdR cells accumulated apo-PVD (see Fig. 3D), indicating that they are able to remove iron from ferri-PVD in the periplasm but are unable to recycle apo-PVD. Moreover, pvdA and pvdApvdR cells showed similar ability to use PVD as an iron source, as determined by a feeding assay in which exogenously added PVD was used to sustain bacterial growth under conditions of extreme iron restriction (DCAA plus 600 μM dipirydil) (Fig. 3E). Taken together, our results substantiate the hypothesis of Greenwald et al. (14), according to which PVD recycling and PVD-iron dissociation in the periplasm are independent processes.

The PvdRT-OpmQ System Is Not Involved in Export of Endogenous PVD. To assess the involvement of the PvdRT-OpmQ efflux system in secretion of de novo synthesized PVD, we investigated the effect of PvdRT-OpmQ inactivation in an FpvA-deficient mutant, which retains the ability to produce PVD (2) but is severely impaired in its uptake (see Fig. 3). Remarkably, the amount of PVD stored in the periplasm did not differ significantly between fpvA and fpvApvdR mutants, while it was much lower than that of PAO1 and pvdR mutant (Fig. 4A). Extracellular PVD levels were also comparable between fpvA and fpvApvdR (see Fig. 4A), denoting no defect in secretion of de novo produced PVD by the fpvApvdR mutant. Accordingly, confocal microscopy failed to reveal significant intracellular accumulation of PVD by both fpvA and fpvApvdR mutants (Fig. 4B). The barely detectable fluorescence shown by fpvApvdR cells was comparable to that of a fpvApvdRfpvA triple mutant fed with exogenous PVD (see Fig. 4B), suggesting that the weak fluorescence detectable in the fpvApvdR background originates from impaired recycling of some exogenous PVD acquired via an FpvA-independent route, likely through the secondary receptor FpvB. Indeed, no residual fluorescence was detected in a tonB1fpvA double mutant (see Fig. S2), in which receptor-dependent siderophore uptake is completely abrogated (16).

In conclusion, these results indicate that periplasmic PVD accumulation by PvdRT-OpmQ-deficient P. aeruginosa cells originates from impaired recycling of exogenous PVD, rather than from a defect in secretion of endogenous PVD.

**Discussion**

Prerequisites for a system to be implicated in any step of PVD secretion are the accumulation of intracellular PVD by the corresponding mutants, together with reduced or impaired PVD secretion. We therefore addressed the role of MexAB-OpmM, PvdE, and PvdRT-OpmQ systems in PVD secretion by measuring the amount of secreted PVD and the level of PVD-related fluorescence retained by P. aeruginosa cells. Our results rule out any significant contribution of the MexAB-OpmM pump to PVD secretion (see Fig. 1), while highlighting an essential role of PvdE in PVD biogenesis, as shown by the PVD-deficient phenotype of the pvdE mutant. In fact, PVD-related fluorescence was undetectable in pvdE mutant cells (see Fig. 1), and no hydroxamate-containing PVD precursors could be detected in pvdE culture supernatants (SI Text). We therefore suggest that PvdE could export the nonfluorescent PVD precursor ferribactin from the cytosol to the periplasm without being involved in the secretion of mature PVD into the extracellular milieu. Accordingly,
maturation of the PVD chromophore has been proposed to occur in the periplasm (7).

Only mutation of the PvdRT-OpmQ exporter resulted in both reduction of extracellular PVD levels and remarkable intracellular accumulation of PVD (see Fig. 1). PvdRT-OpmQ displays the typical architecture of Gram-negative tripartite ABC-type exporters, consisting of an IM component with both ATPase and permease domains, a periplasmic membrane fusion protein, and a TolC-like OM protein (see SI Text for details). The pvdRT-opmQ gene cluster was found to be iron-regulated and under the tight control of the PVD-specific PvdS sigma factor (Table S2), in line with previous transcriptomics data (4).

Cell fractionation assays showed that PVD is accumulated in the periplasm of pvdRT-opmQ mutants, independent of the deleted component of the tripartite system (see Fig. 2), thus indicating that the PvdRT-OpmQ system is involved in secretion of PVD from the periplasmic space to the extracellular milieu. Previous studies localized the substrate binding site of some resistance-nodulation division-type (RND) efflux pumps in the periplasmic compartment, and suggested that substrate extrusion from the periplasm could represent a strategy to protect the cytoplasm from potential hazards (17–19). This work provides substantial evidence that ABC exporters can also extrude substrates from the periplasm, in line with a previous report showing that the macrolide efflux pump MacAB-TolC of Escherichia coli is also implicated in secretion of periplasmic enterotoxin II (20). Although PvdRT-OpmQ and MacAB-TolC share remarkable structural similarity (SI Text), the PvdRT-OpmQ efflux system does not provide any contribution to P. aeruginosa resistance to antibiotics belonging to the classes of tetracyclines.
aminoglycosides, β-lactams and macrolides, and chloramphenicol (Table S3).

Periplasmic accumulation of fluorescent PVD in P. aeruginosa cells deficient in PvdRT-OpmQ could be explained by two nonmutually exclusive mechanisms. According to the “de novo pathway,” PVD production could require PvdE-dependent transport of the ferribactin precursor through the IM. Ferribactin would undergo maturation in the periplasm before extracellular release through a secretion device whose impairment would cause periplasmic accumulation of PVD. In the “recycling pathway,” mature PVD would enter the periplasm by FpvA-mediated uptake, where it would be retained in the absence of a recycling system.

To verify the involvement of PvdRT-OpmQ in either of these processes, accumulation of PVD by PvdRT-OpmQ-deficient cells was assessed in PVD synthesis- or uptake-defective backgrounds. With respect to the biosynthetic (pvdA) mutant, the pvdApvdR double mutant accumulated higher amounts of exogenously-provided PVD in the periplasm (see Fig. 3 A–C), and was impaired in recycling of periplasmic PVD (see Fig. 3D).

On the other hand, comparable levels of extracellular PVD and no periplasmic PVD retention were observed in the PVD-producing uptake-mutant fpvA, irrespective of the pvdR mutation (see Fig. 4). These results lead us to propose that the PvdRT-OpmQ system is responsible for exogenous PVD recycling, while excluding its involvement in secretion of de novo synthesized PVD. Coherently, P. aeruginosa mutants with an impaired PvdRT-OpmQ system secrete up to 180 μM PVD in DCAA medium (see Fig. 1 and ref. 4), and our preliminary results suggest that the lower PVD levels in their culture supernatants are a result of reduced PVD synthesis rather than of impaired secretion of de novo synthesized PVD. A schematic of PVD trafficking in P. aeruginosa is shown in Fig. 5.

What is the benefit for P. aeruginosa of having a system for PVD recycling? The energetic cost of PVD synthesis is prohibitive, and this is confirmed by the fact that PVD producers are outcompeted by nonproducers in growth competition assays (21). Although PVD recycling does not provide a great advantage to iron-deplete laboratory cultures, where the PVD concentration attains up to 350 μM, circumstances may differ in natural environments, such as the CF lung, where PVD is approximately 1 μM (22). Moreover, P. aeruginosa adopts a biofilm mode of growth in the CF lung, which could further reduce the local availability of PVD. Notably, PVD-deficient mutants have been isolated from sputa of CF patients (23, 24), indicating that P. aeruginosa occurs in the CF lung as a mixed community of PVD producers and PVD-deficient cheaters. In vitro and in vivo studies demonstrated that both growth and virulence of these mixed populations are negatively affected by increasing proportions of PVD cheaters over PVD producers (reviewed in ref. 25). The ability to recycle PVD and, thus, to maintain PVD levels at biologically meaningful concentrations with minor costs for the whole population. The biological relevance of an energy-saving device for PVD recycling is testified by the presence of pvdRT-opmQ orthologs in the genome of all fluorescent pseudomonads sequenced so far (26) (Table S4).

Materials and Methods

Growth Conditions. Bacterial strains and plasmids used in this study are listed in Table S1. P. aeruginosa transposon insertion mutants were purchased from a P. aeruginosa Transposon Mutant Library (www.genome.washington.edu/}

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**Fig. 4.** Effect of impaired PVD uptake on PVD accumulation in the periplasm. P. aeruginosa mutants were grown for 14 h at 37 °C in DCAA. (A) Quantification of extracellular (black bars, Left y axis), periplasmic and spheroplasts-associated PVD (gray and white bars, respectively; Right y axis). Values are mean (± SD) of three independent assays. (B) Confocal microscopy images of cells. For each strain, the left and right panels show the visible and the corresponding 405-nm laser line confocal microscopy pictures, respectively.

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**Fig. 5.** PVD trafficking across the P. aeruginosa cell envelope. Ferribactin is synthesized in the cytoplasm and secreted by PvdE into the periplasm, where maturation to PVD is suggested to take place (1, 7). Export of de novo synthesized PVD occurs by a still uncharacterized mechanism. Ferri-PVD is translocated by FpvA into the periplasm, where Fe(III) is dissociated, plausibly by reduction to Fe(II) (14). Internalized PVD is recycled by the PvdRT-OpmQ system. It is hypothesized that Fe(II) enters the cytoplasm by a classic periplasmic binding protein (PBP)/IM ABC importer (15). Experimentally confirmed and hypothetical steps are in yellow and gray, respectively.
P. aeruginosa was grown at 37 °C in DCAA medium (27), supplemented or not with 50 μM FeCl₃ as iron-deplete and -replete conditions, respectively. Exogenous PVD was added as PVD-conditioned medium (28), corresponding to a culture filtrate of the pyoverdine-defective P. aeruginosa PA6331 grown in DCAA medium. As a control, the medium conditioned by the Pvd- and pyoverdine-defective PA6331pvdA was used.

Construction of Deletion Mutants and Complementing Plasmids. E. coli was routinely used for recombinant DNA manipulations. Site-specific excision of pvdR and pvdA genes was performed as previously described (29). For complementation of pvdR, pvdT, and opmQ genes, a 4.7-kb fragment encompassing the pvdR-T-opmQ operon with its putative promoter (region 2642032–2646727 of the P. aeruginosa PA01 chromosome, www.pseudomonas.com) was amplified by PCR and cloned in pUCP19, yielding plasmid pUCPvdR-­pvdT-opmQ (see Table 5).

Subcellular Fractions. Intracellular PVD determinations were performed on P. aeruginosa cells grown for 14 h in DCAA, and washed three times with 30 mM Tris-HCl (pH 7), 150 mM NaCl. Bacterial pellets (5 × 10⁹ cells) were resuspended in 1 ml of 10 mM Tris-HCl (pH 8), 100 mM NaCl, and lysed by sonication. Cell debris were removed by low-speed centrifugation (7,000 × g, 10 min, 4 °C), and PVD concentration was determined using appropriate dilutions of cell lysates in 100 mM Tris-HCl (pH 8).

Periplasmic fractions were obtained by spheroplasting P. aeruginosa cells with lysyozyme and MgCl₂ as described (30). Briefly, bacterial pellets (5 × 10⁹ cells) were resuspended in 1 ml of 10 mM Tris-HCl (pH 8), 200 mM MgCl₂, 0.5 mM MgCl₂, and incubated for 30 min at room temperature with gentle shaking. Suspensions were centrifuged (11,000 × g, 15 min, 4 °C) to collect the periplasm-containing supernatants. The resulting pellets were suspended in 1 ml of 10 mM Tris-HCl (pH 8), 100 mM NaCl, and lysed by sonication. Cell debris were removed to obtain the spheroplasts fractions. The specificity and purity of cell fractions were verified by using specific subcellular protein markers as described (30).

PVD Measurements. PVD from culture supernatants was detected in 100 mM Tris-HCl (pH 8), and measured as OD₅₆₅ normalized by the cell density of bacterial cultures (OD₅₆₅). Intracellular PVD was quantified fluorometrically by recording the emission at 455 nm upon excitation at 398 nm in a LS50B Luminescence spectrometer (Perkin-Elmer), using a standard curve generated with known PVD concentrations (0–3 μM in 100 mM Tris-HCl, pH 8).

Kinetics of PVD Uptake and Recycling. Kinetic studies of PVD uptake were performed by incubating 3 × 10⁸ cells/ml in the presence of 50 μM PVD in DCAA medium supplemented with 300 μg/ml kanamycin (Km) and 100 μg/ml chloramphenicol (Cm) to halt bacterial growth. At given time points, cells from 1-ml samples were washed, lysed by sonication, and cell debris removed by centrifugation for fluorometric PVD measurements.

PVD recycling studies were performed by incubating 2 × 10⁹ cells/ml with 1 μM ferri-PVD in DCAA medium supplemented with 300 μg/ml Km and 100 μg/ml Cm. At given time points, 1-ml samples were centrifuged, and the appearance of apo-PVD was monitored fluorometrically in both supernatants and intact cells.

Confocal Microscopy. Cells (5 × 10⁹) from iron-deplete or-replete cultures were washed and mounted onto agarose-coated slides as described (31). Images were electronically acquired through a Leica SP5 confocal laser scanning microscope (Leica Microsystems) using a violet laser diode emitting at 405 nm. Laser intensity was kept constant at 11% for all images, without adjustments of contrast and brightness.

Note Added in Proof. Since the work described in this paper was completed and submitted for publication, the involvement of PvdE in ferribactin secretion from the cytoplasm has been proposed by Yeterian E, et al. (32).

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