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ORIGINAL ARTICLE

Synthesis of the siderophore pyoverdine in *Pseudomonas aeruginosa* involves a periplasmic maturation

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Abstract Pyoverdines, the main siderophores produced by fluorescent *Pseudomonads*, comprise a fluorescent dihydroxyquinoline chromophore attached to a strain-specific peptide. These molecules are thought to be synthesized as non-fluorescent precursor peptides that are then modified to give functional pyoverdines. Using the fluorescent properties of PVDI, the pyoverdine produced by *Pseudomonas aeruginosa* PAO1, we were able to show that PVDI was not present in the cytoplasm of the bacteria, but large amounts of a fluorescent PVDI precursor PVDIp were stored in the periplasm. Like PVDI, PVDIp is able to transport iron into *P. aeruginosa* cells. Mutation of genes encoding the periplasmic PvdN, PvdO and PvdP proteins prevented accumulation of PVDIp in the periplasm and secretion of PVDI into the growth medium, indicating that these three enzymes are involved in PVDI synthesis. Mutation of the gene encoding PvdQ resulted in the presence of fluorescent PVDI precursor in the periplasm and secretion of a functional fluorescent siderophore that had different isoelectric properties to PVDI, suggesting a role for PvdQ in the periplasmic maturation of PVDI. Mutation of the gene encoding the export ABC transporter PvdE prevented PVDI production and accumulation of PVDIp in the periplasm. These data are consistent with a model in which a PVDI precursor peptide is synthesized in the cytoplasm and exported to the periplasm by PvdE where siderophore maturation, including formation of the chromophore moiety, occurs in a process involving the PvdN, PvdO, PvdP and PvdQ proteins.

Keywords Siderophore biosynthesis · Pyoverdine · Iron uptake · Fluorescence · Fluorescent peptide · *Pseudomonas aeruginosa*

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Introduction

Iron is an essential element for almost all bacteria but under aerobic conditions at neutral pH, iron forms insoluble Fe(III) oxide hydrates and is therefore not readily available. To overcome this problem of iron accessibility many bacteria produce iron chelators called siderophores. These molecules solubilize ferric ions and transport these ions into bacterial cells via specific outer membrane transporters (Braun 2003; Schalk 2008). In Gram negative bacteria, this transport is driven by the proton motive force of the cytoplasmic membrane through a cytoplasmic membrane complex comprising three proteins, TonB, ExbB and ExbD (Postle and Kadner 2003; Koebnik 2005; Wiener 2005). Pyoverdines, a group of structurally related fluorescent siderophores, represent the primary iron uptake system in fluorescent *Pseudomonads*, although many species can also synthesize additional siderophores such as pyochelin and quinolobactin, or can acquire iron bound to a variety of exogenous chelators, including many heterologous siderophores (Cornelis and Matthijs 2002; Poole and McKay 2003; Schalk 2008).

More than 60 different pyoverdines have been identified (Meyer et al. 2008). All pyoverdines are comprised of three distinct structural parts (Abdallah and Pattus 2000; Budzikiewicz 2004), a dihydroxyquinoline chromophore

responsible for their fluorescence, a peptide chain comprising 6–12 amino acids (partially modified) bound to the carboxylic group of the chromophore and a small side chain bound at position C-3 of the chromophore. In most cases, this side chain is a diacid of the Krebs cycle such as succinic acid, malic or α-ketoglutaric acid or one of their amide derivates. The peptide part is unique to each strain and may be linear, or partially or entirely cyclic and its composition and length are specific to the producer strain (Abdallah and Pattus 2000; Budzikiewicz 2004). This peptidic part can have L- and D-amino acids, some of which are unusual, such as N^5-hydroxyornithine, N^5-formyl-N^5-hydroxyornithine and hydroxyaspartate. The catecholate group of the chromophore and the hydroxamate (occasionally β-hydroxy acid) groups of the peptide part provide a high-affinity binding site with association constants as high as 10^{32} M^{-1} for ferric ion (Albrecht-Gary et al. 1994). Pyoverdines produced by *P. aeruginosa* can be of three types (PVDI, II and III) that are distinguished by different peptide chains (Meyer et al. 1997).

Iron uptake by pyoverdines is best understood in *P. aeruginosa* PAO1, which produces PVDI (Fig. 1) [for a review see (Schalk 2008)]. The ferric form of this siderophore is transported across the outer membrane by a specific receptor, FpvAI (Poole et al. 1993b; Cobessi et al. 2005; Schalk 2008). Iron is probably released from the siderophore in the periplasm by a mechanism thought to involve iron reduction, with recycling of the siderophore that can then transport another iron ion (Greenwald et al. 2007). FpvAI is also involved in a signaling cascade, with an anti-sigma factor FpvR and two sigma factors FpvI and PvdS regulating the expression of the genes involved in the iron uptake by this siderophore (*fpvA*), genes encoding the enzymes involved in the biosynthesis of PVDI and genes that encode virulence factors exotoxin A and PrpL protease [for a review see (Visca et al. 2002].

Pyoverdine synthesis is also best understood for *P. aeruginosa* strain PAO1, although available evidence

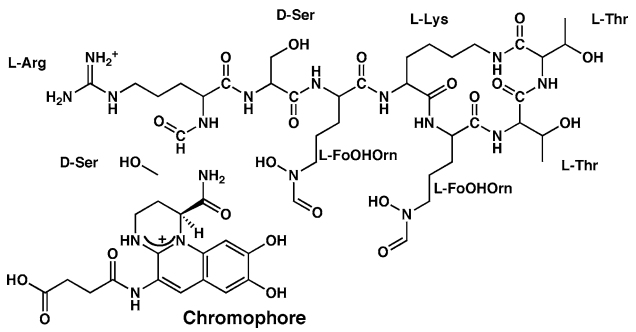

Fig. 1 Chemical structure of PVDI from *P. aeruginosa PAO1*. Standard three-letter codes are used for amino acids, except for *N*-formyl-*N*-hydroxyornithine (FoOHOrn)

indicates that analogous biosynthetic pathways give rise to other PVDs in other strains and species [reviewed in (Visca et al. 2007)]. A precursor molecule is thought to be derived from amino acid precursors that are assembled by non-ribosomal peptide synthetases (NRPSs), with other enzymes giving rise to the substrates for the NRPS enzymes and catalyzing additional reactions that complete the maturation of pyoverdines (Visca et al. 2007). The synthesis of the peptide precursor involves the PvdI, PvdJ, PvdL and PvdD NRPSs (Merriman et al. 1995; Lehoux et al. 2000; Mossialos et al. 2002; Ackerley et al. 2003; Lamont and Martin 2003; Ravel and Cornelis 2003; Visca 2004). The maturation of the chromophore, which is common to all pyoverdines, is likely to involve a series of oxido-reduction steps of a condensation product of D-tyrosine and L-2,4-diaminobutyrate that is part of the peptide precursor (Dorrestein et al. 2003). Consistent with this, PvdL that is proposed to incorporate D-tyrosine and L-2,4-diaminobutyrate into the precursor peptide is the only pyoverdine synthesis NRPS present in the genomes of all fluorescent *Pseudomonads* analyzed so far (Mossialos et al. 2002). Maturation of the chromophore was formerly thought to require the enzymes PvcA–D but it has now been shown that these enzymes are not required for chromophore maturation (Clarke-Pearson and Brady 2008) and the enzymes that carry out this process have not been identified. The PvdH enzyme, which is also common to different fluorescent *pseudomonads*, catalyzes the formation of L-2,4-diaminobutyrate that is thought to be required for chromophore synthesis from aspartate β-semialdehyde (Vandenende et al. 2004). Other pyoverdine synthesis enzymes that have been characterized include PvdA that encodes a membrane-associated ornithine hydrogenase (Visca et al. 1994; Ge and Seah 2006; Imperi et al. 2008), PvdF that encodes a hydroxyornithine-formylating enzyme (McMorran et al. 2001) and PvdYII that encodes a hydroxyornithine acetylase (Lamont et al. 2006). Other enzymes that are required for PVDI synthesis have been implicated through gene mutation studies (Ochsner et al. 2002a; Lamont and Martin 2003), but the precise roles of the corresponding enzymes have not been elucidated. Since some proteins of unknown function that are essential for PVDI synthesis are predicted to be periplasmic (PvdN, PvdO, PvdP and PvdQ) (Ravel and Cornelis 2003; Lewenza et al. 2005), and this was confirmed experimentally for PvdN (Voulhoux et al. 2006), it was proposed that a precursor of PVDI, with an unformed chromophore, is synthesized in the cytoplasm, and matured in the periplasm (Baysse et al. 2002; Visca et al. 2007). The transport of the precursor across the inner membrane may involve PvdE, which is an "export" ABC transporter essential for PVDI production (McMorran et al. 1996; Saurin et al. 1999).

In the present work, the fluorescent properties of PVDI were used to investigate this siderophore synthesis pathway and the roles of the ABC transporter PvdE and the PvdN, PvdO, PvdP and PvdQ proteins in PVDI synthesis.

Materials and methods

Chemicals

Chemicals were purchased from Sigma unless otherwise stated. PVDI and PVDI-Fe were prepared as described previously (Albrecht-Gary et al. 1994).

Bacterial strains and growth

The strains used in this study are described in Table 1. Strains were grown at 37°C in succinate medium (composition in g/L is K_2HPO_4, 6.0; KH_2PO_4, 3.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4.7H_2O$, 0.2; sodium succinate, 4.0 and the pH was adjusted to 7.0 by addition of NaOH). For *P. aeruginosa* PAO1 OT11 and the corresponding mutants the medium was supplemented with 1 mM each of leucine and proline.

Table 1 Strains used in this study

Name	Genotype/phenotype	References
PAO1	Wild type	Royle et al. (1981)
PAD06	*pvdA*::ΩSm/Sp	Takase et al. (2000)
PAD06(pPVR2)	*pvdA*::ΩSm/Sp, FpvA$^+$	Brillet et al. (2007)
PAD07	*pvdA*::ΩSm/Sp, Δ*pchD*::ΩTc	Takase et al. (2000)
PAO1*fpvA*	Δ*fpvA*	Shirley and Lamont (2009)
PAO1*fpvA*, *fpv*R	Δ*fpvA*, Δ*fpvR*	This study
PAO1*pvdN*	*pvdN::Km*	Lamont and Martin (2003)
PAO1*pvdO*	*pvdO*::Km	Lamont and Martin (2003)
PAO1*pvdP*	*pvdP*::Km	Lamont and Martin (2003)
PAO1*pvdQ*	*pvdQ*::pEXGm	Kovach et al. (1995)
PAO1 OT11	*leu pro*	McMorran et al. (1996)
PAO1 OT11*pvdE*	*pvdE*::Km	Mascarenhas et al. (2002)
PAO1 OT11*pvdE* (pBBR4)	*pvdE*::Km, Cb^R	This study
PAO1 OT11*pvdE* (pBBR4::*pvdE*)	Pvd$^+$, Cb^R	This study

Genetic manipulations

Enzymes were purchased from Roche and used under the conditions recommended by the manufacturer. Plasmid and chromosomal DNA were isolated using the Roche high pure plasmid isolation kit and the Ultra Clean Microbial DNA isolation kit (Mo Bio), respectively, according to the manufacturers' instructions. Treatment of DNA with enzymes, subcloning of DNA, and transformation of plasmid constructs into *E. coli* and *P. aeruginosa* were carried out using standard methods (Sambrook and Russel 2001; Chuanchuen et al. 2002). DNA fragments were amplified from *P. aeruginosa* PAO1 genomic DNA by PCR using PCR SuperMix High Fidelity obtained from Invitrogen. All products and plasmid constructs were verified by DNA sequencing. Deletion of the entire *fpvA* gene was carried out using plasmid pJSS2 as described previously (Shen et al. 2002). A deletion of *fpvR* extending from just after the 5′ end of the gene to just before the 3′ end was engineered using pEX18Tc (Hoang et al. 1998). Flanking DNA fragments were amplified by PCR with primers 5′-CGC**GAATTC**AGTCGTTGAACTCCATCCGG-3′ and 5′-GGC**GGTACC**CGAGCGAAACAAGGCTTTAA-3′ 5′- (upstream) and 5′-CGC**GGTACC**GTTAAATTTAGCC GCCCTGG-3′ GCC**TCTAGA**GGCGTTTTCCATCAGCT GTC-3′ (downstream), with introduced restriction sites shown in bold. The PCR products were digested with *Eco*RI and *KpnI*, and *KpnI* and *XbaI*, and cloned sequentially into pEX18Tc. The resulting plasmid was transformed into *E. coli* strain S17-1 (Simon et al. 1986) and transferred into *P. aeruginosa* by conjugation, resulting in homologous recombination between the chromosomal target and one of the deletion-flanking fragments located on the plasmid. Bacteria were then spread onto L-agar containing 5% sucrose to select for a second recombination event leaving a deletion of *fpvR*. Bacteria in which *fpvR* had been deleted were identified by PCR.

To construct plasmid pBBR4::*pvdE*, an *Eco*RI–*Pst*I restriction fragment spanning the *pvdE* gene was excised from pSOT4 (Rombel et al. 1995) and cloned into pBBR4 (Kovach et al. 1995) that had been digested with the same enzymes.

Image acquisition

Cells grown overnight in succinate medium were incubated with or without PVDI, washed once with siderophore-free buffer, and then mounted onto S750-agarose coated slides, as described previously (Mascarenhas et al. 2002). Images were acquired on a Leica DM RXA2 (objective: Leica HCX PL APO 100 × 1.40–0.7 OIL CS) microscope with a photometric coolSNAP HQ camera. Images were captured using MetaMorph 6.0 (Universal Imaging).

Cell fractionation

For periplasm and cytoplasm preparations, 2.5×10^{10} bacteria (25 mL of OD_{600} of 1) were collected by centrifugation after overnight growth in iron-limited succinate medium, washed twice with 50 mM Tris–HCl pH 8.0 and re-suspended in 1 mL of buffer A (200 mM Tris–HCl, pH 8.0, 20% sucrose). To obtain spheroplasts, 10 µL of 100 mg/mL Lysozym (Euromedex) was slowly added to the cells and the mixtures were slowly shaken at room temperature for 40 min. After preparation of the spheroplasts, the periplasmic fractions were isolated by centrifugation (15 min at 8,000*g*). The pellets containing the spheroplasts were washed twice with buffer A. The spheroplasts were re-suspended in 750 µL cold water and the mixtures incubated for 1 h at room temperature in the presence of benzonase (1 µL, from Sigma). The cytoplasmic fractions were isolated by ultracentrifugation (40 min at 120,000*g*). The volume of the cytoplasmic and periplasmic fractions was adjusted to 1 mL with 50 mM Tris–HCl pH 8.0 buffer and the fluorescence in each fraction was monitored. The proteins were separated on SDS PAGE (12%). For western blotting, proteins were transferred from the gel onto nitrocellulose membranes. The membranes were blocked by incubation overnight in phosphate-buffered saline [PBS (pH 7.6), 5% dried milk] and then incubated in blocking buffer with primary antibody against β-lactamase (Chemicon, dilution 1/2,500), followed by a second incubation in blocking buffer with horse-radish peroxidase-conjugated goat anti-rabbit IgG antibody (Sigma, dilution 1/10,000). Blots were developed using the enhanced chemiluminescence protocol (SuperSignal West Pico Chemiluminescent Subtrat, Pierce).

Electrophoretic analysis of PVDs

PVDI and its precursors were purified on a column of octadecylsilane (Lichroprep RP 18, 40–63 µm, Merck). The column was first washed with an aqueous solution of acetic acid at pH 4.0, to remove the bulk of inorganic salts and then the siderophores were eluted with a 1:1 mixture of acetonitrile/50 mM pyridine–acetate buffer pH 5.0. After evaporation, the siderophores were dissolved in 50 mM pyridine–acetate buffer pH 5.0. Electrophoretic analysis of pyoverdines on cellulose acetate membranes (Midifilm, Biomidi) was carried out using a horizontal electrophoresis tank as described (Albrecht-Gary et al. 1994). Electrophoresis was performed in 100 mM pyridine–acetic acid pH 5.0 at a constant voltage (300 V) for 30 min.

Fluorescence spectroscopy

Fluorescence experiments were carried out using a PTI (Photon Technology International TimeMaster, Bioritech) spectrofluorometer. For all experiments, the sample was stirred at 29°C in a 1 mL cuvette, the excitation wavelength (λ_{exc}) was set at 400 nm (for direct excitation), and the emission of fluorescence (λ_{em}) was measured at 450 nm. The cells, after overnight growth in iron-limited medium, were washed with two volumes of 50 mM Tris–HCl (pH 8.0) and re-suspended in the same buffer to a final OD_{600} of 0.75. To follow PVDI production, the bacterial suspension (900 µL) was stirred in a 1 mL cuvette and the fluorescence monitored. The fluorescent emission spectra of PVDI and its precursors were performed with purified siderophores in solution in 50 mM Tris–HCl pH 8.0. The siderophores were purified as described above for electrophoretic analysis.

^{55}Fe uptake assay

The iron uptake assays were carried up as described previously (Schalk et al. 2001). PAD07 cells at an OD_{600} of 1, preincubated as required with 200 µM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), were incubated in the presence of 200 nM siderophore–^{55}Fe complexes. At different times aliquots were removed, filtered and the radioactivity retained by the bacteria counted.

Iron chelation by PVDI and PVDI precursors

After overnight growth in succinate medium, *P. aeruginosa* PAO1 and PAO1*pvdQ* cells were collected by centrifugation and the siderophore was prepared from the supernatants. PVDI and PVDI precursors were purified as described above for electrophoretic analysis, diluted to 500 nM in 50 mM pyridine–acetate buffer (pH 5.0). Aliquots of $FeCl_3$ (stock solution at 100 µM, 1 mM and 10 mM) were added and the fluorescence monitored at 450 nm (excitation wavelength 400 nm) after 5 min incubation.

Mass spectrometry analyses of PVDI precursors

Samples were analyzed using LC–MS at the Service de Spectrométrie de Masse de la Faculté de Pharmacie de l'Université de Strasbourg. Samples were dissolved in DMSO, injected on a Thermo HypersilGold C18 column ($1 \times 3 \times 1.9$ cm) and analyzed in electrospray (ES-TOF) experiments performed on a Bruker Daltonic MicroTOF mass spectrometer.

Results

Presence of important amounts of PVDI in the periplasm of *P. aeruginosa* PAO1

We first investigated whether chromophore formation occurs in the periplasm. PVDI is characterized by a typical

fluorescence at 450 nm (excitation wavelength 400 nm), due to the presence of its chromophore moiety. Only PVDI and any PVDI precursors with a formed chromophore are fluorescent. To identify the cellular compartment of chromophore formation, we used the fluorescent properties of the chromophore to look for its presence in the cytoplasm and/or in the periplasm of *P. aeruginosa* cells. Two approaches were used, cellular fractionation (Fig. 2) and fluorescent microscopy (Fig. 3). When the periplasm and the cytoplasm of *P. aeruginosa* PAO1 cells were fractionated, a large amount of fluorescence was detected in the periplasmic fraction and almost no fluorescence in the cytoplasmic fraction (Fig. 2a). This result is consistent with the hypothesis that maturation of the PVDI chromophore takes place in the periplasm although it could also indicated that the chromophore is formed in the cytoplasm, exported immediately after its formation across the inner membrane and stored in the periplasm. To check that fractionation occurred with minimal contamination of one cell compartment by the other, the bacteria were transformed with plasmid pUCP18 (West et al. 1994) that carries a gene encoding a periplasmic β-lactamase. Analysis of the cytoplasmic and periplasmic fractions of these cells showed that the fractionation procedure was very efficient with only trace amounts of β-lactamase in the cytoplasmic fractions (Fig. 2b).

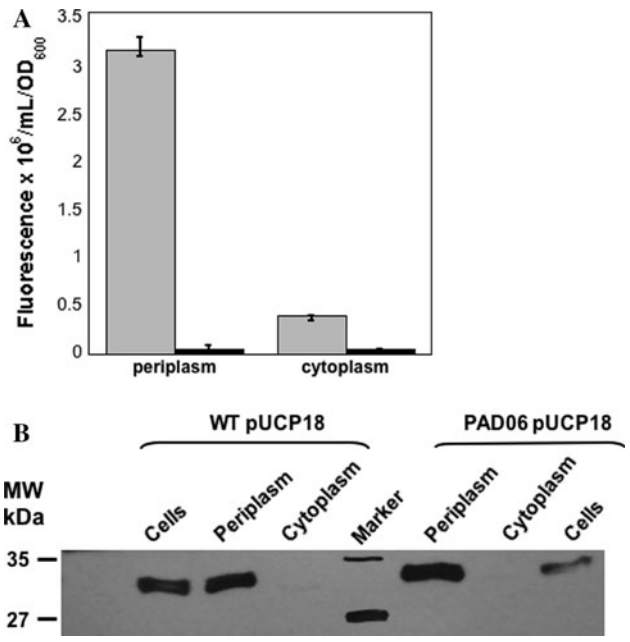

Fig. 2 **a** Fluorescence in the cytoplasmic and periplasmic fractions of *P. aeruginosa* PAOI (*gray bars*) and PAD06 (unable to produce PVDI) (*black bars*). Spheroplasts were prepared for separation of the periplasmic and cytoplasmic fractions. Before extraction of the cytoplasm, the spheroplasts were washed twice. The samples (periplasm and cytoplasm), each in a volume of 1 mL, were excited at 400 nm and the fluorescence was monitored at 450 nm. The observed fluorescence is proportional to the amount of PVDI present in each sub-cellular fraction. The data are means of three independent experiments. **b** Western blot analyzes of the cytoplasmic and periplasmic fractions. The equivalent of 0.1 OD_{600} units of PAO1 and PAD06, transformed with plasmid pUCP18 that carries a gene encoding a periplasmic β-lactamase, were separated by SDS PAGE. Proteins were blotted onto nitrocellulose and β lactamase detected using an antibody. Molecular weight markers (in kDa) are shown on the *left*

Because of its fluorescent properties, PVDI is suitable for imaging in living cells by fluorescence microscopy. We immobilized growing *P. aeruginosa* PAO1 and different mutants on agarose. A strong fluorescence at wavelengths corresponding to the emission of fluorescence of PVDI was only detected on the circumference of PAO1 cells (Fig. 3a). This fluorescence was absent in a PVDI-deficient mutant (PAD06, Fig. 3b), demonstrating that the fluorescence is due to the presence of PVDI. The PVDI-Fe outer membrane transporter, FpvA, is able to bind PVDI at the cell surface when it is complexed with non-ferric ions such as Ga^{3+} or Al^{3+} (Schalk et al. 1999, 2001; Braud et al. 2009). These metals, unlike Fe^{3+} ions, do not quench fluorescence of PVDI (Yoder and Kisaalita 2006; Braud et al. 2009). In order to discriminate between fluorescence due to PVDI bound to FpvA at the cell surface and fluorescence due to the presence of PVDI in the periplasm, PVDI-deficient PAD06 and PAD06(pPVR2) cells carrying a plasmid that overexpresses FpvA were incubated in the presence of metal-free PVDI (Fig. 3c). Fluorescence was observed at the cell periphery for the strain overexpressing FpvA (PAD06(pPVR2)) and preincubated with PVDI, but with a markedly lower intensity compared to *P. aeruginosa* PAO1 cells (Fig. 3a). These observations indicate that the fluorescence seen at the cell circumference of PAO1 cells is mostly due to accumulation of PVDI in the periplasm although overexpression of FpvA can give observable binding of PVDI to FpvA at the outer membrane. For the same purpose, PAO1*fpvA* and PAO1*fpvAfpvR* strains were observed by fluorescent microscopy (Fig. 3f, g). In these strains, FpvA is not expressed and so PVDI cannot bind to FpvA at the cell surface although it can be synthesized. Mutations in *fpvA* result in reduced PVDI synthesis relative to wild-type bacteria and this reduction is overcome when *fpvR* is also mutated (Lamont et al. 2002). In these strains the fluorescence at the cell periphery was higher than for PAD06 expressing FpvA (Fig. 3g) and was similar to the fluorescence seen in strain PAO1. All these data demonstrate that the fluorescence seen in PAO1 cells is mostly due to the presence of PVDI in the periplasm. The data shown in Figs. 2 and 3 indicate that PVDI or a fluorescent precursor accumulates in the periplasm before its secretion into the extracellular medium. These data suggest also that the final steps of chromophore biogenesis may take place in the periplasm.

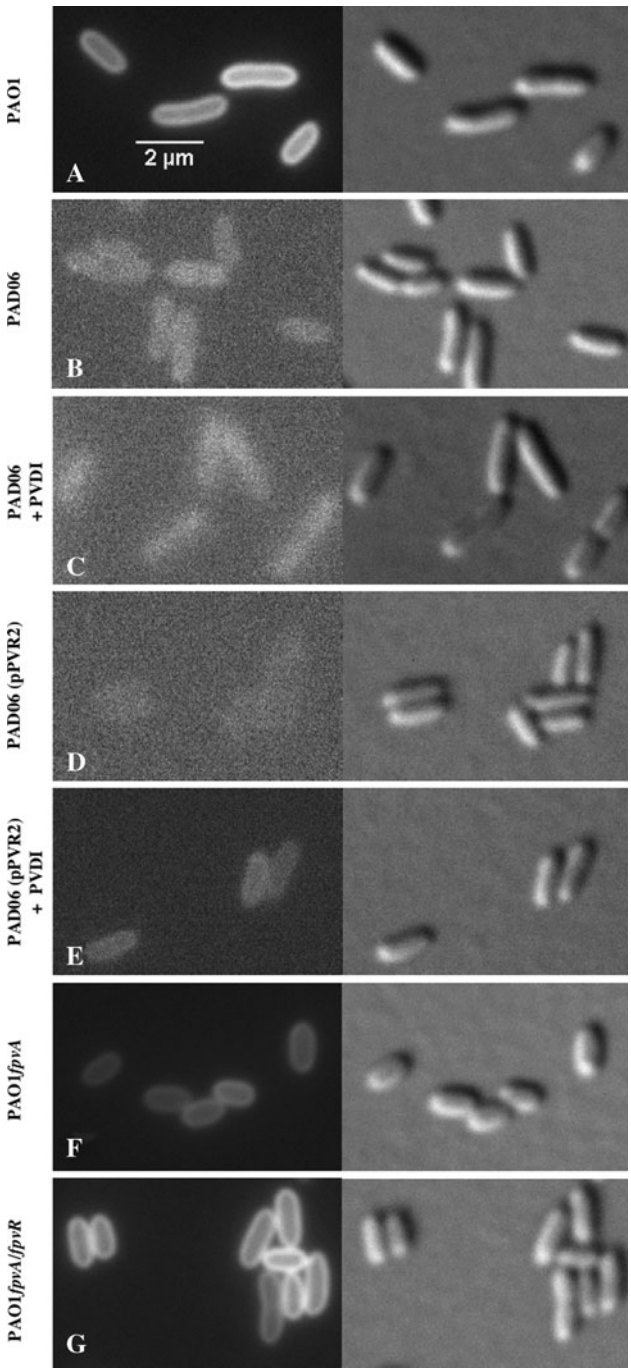

Fig. 3 Fluorescence microscopy of *P. aeruginosa* PAO1 and different mutants. For all the strains the left picture was taken with Ex 425-445HQ, Em 460-510HQ, Dc 450 (filter specification) and the right with DIC (Differential Interference Contrast) filter (*white bar* 2 µm). The experiment has been carried out with *P. aeruginosa* wild type PAO1 cells (**a**), PAD06, a strain unable to produce PVDI (**b**), PAD06 cells incubated with PVDI (**c**), PAD06(pPVR2), a strain unable to produce PVDI and overexpressing FpvA (**d**), PAD06(pPVR2) incubated with PVDI (**e**), PAO1*fpvA*, a *fpvA* mutated strain (**f**) and PAO1*fpvA*/*fpvR*, a *fpvA* and *fpvR* mutated strain (**g**). When the cells were incubated with PVDI (strains PAD06 and PAD06(pPVR2)) siderophore excess was removed by washing prior to microscopy

Chemical and biochemical characteristics of the periplasmic PVDI (PVDIp)

The data above show that PVDI or a fluorescent precursor accumulates in the periplasm of *P. aeruginosa* PAO1. Spheroplasts of *P. aeruginosa* PAO1 were prepared to extract the fluorescent material from the periplasmic fraction. Further purification was carried out on a column of octadecylsilane used previously to purify PVDs (Albrecht-Gary et al. 1994). The eluted fluorescent material (named PVDIp) gave *m/z* 1,301.40 [PVDI found in the extracellular medium being *m/z* 1,334.60, (correspond to PVDI + H^+)] (Demange et al. 1990). The migration pattern of PVDIp on cellulose acetate membrane was different from that of PVDI harvested from the extracellular medium, indicating a different pI (Fig. 4a). In both cases a major spot was observed with a minor spot. However, the migration distances of the major spots were slightly different for both molecules indicating a small difference in the structures and the minor spots had quite different mobilities. These data indicate that a single major fluorescent compound, PVDIp, accumulates in the periplasm. We extracted an average of 320 µg/OD_{600} of PVDIp from 1 L culture of *P. aeruginosa* PAO1. This represents 0.16% of the amount of PVDI (200 mg/OD_{600}) purified from the extracellular medium. To evaluate its ability to chelate iron and transport iron, PVDIp and PVDI harvested in the extracellular medium from the same culture were loaded with ^{55}Fe and incubated with cells of the PVDI and pyochelin-deficient mutant PAD07. PVDIp was able to transport iron into PAD07 with the same efficiency as PVDI from the extracellular medium (Fig. 4b). This transport was abolished when the cells were treated with the protonophore CCCP, which inhibits any TonB-dependent transport (Clément et al. 2004). Fluorescence of PVDI from the extracellular medium was quenched by an excess of Fe^{3+}, as expected (Folschweiller et al. 2002), but the fluorescence of PVDIp was not (Fig. 4c). Collectively, these data indicate that PVDIp is the only major fluorescent PVDI precursor that accumulates in the periplasm and that PVDIp is able to chelate $Fe^{3+}$and transport it into *P. aeruginosa*.

Role of PvdE in PVDI production

The peptide backbone of PVDI is assembled by NRPS that are thought to be in the cytoplasm giving rise to a PVDI precursor with a lacking a chromophore (Merriman et al. 1995; Lehoux et al. 2000; Mossialos et al. 2002; Ackerley et al. 2003; Lamont and Martin 2003; Ravel and Cornelis 2003; Visca 2004). The data presented in Figs. 2 and 3 indicate that this precursor is then transported across the inner membrane into the periplasm for maturation, as

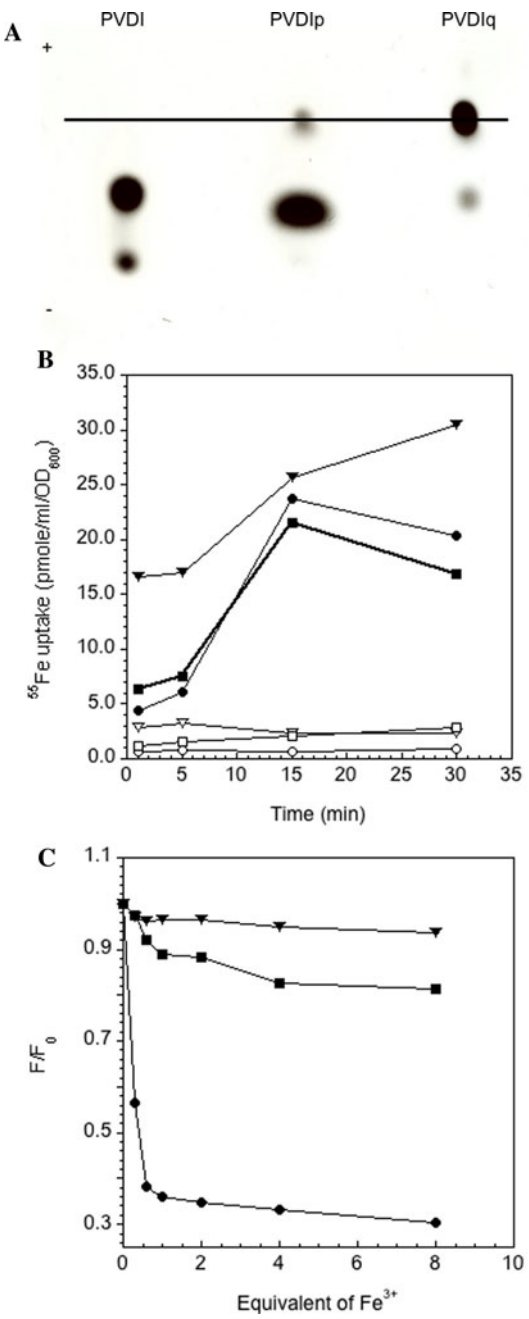

Fig. 4 Characteristics of PVDI, PVDIp and PVDIq. **a** Isoelectrofocusing migration of pyoverdine on cellulose acetate membranes. The *black line* indicates where the samples were loaded onto the membrane. PVDIp was isolated from the periplasm of *P. aeruginosa* PAO1 and PVDI and PVDIq were purified from the extracellular medium of strains PAO1 and PAOI*pvdQ*, respectively, **b** transport of ^{55}Fe into *P. aeruginosa* PAD07. Suspensions of PAD07 at an OD_{600} of 1 were incubated with 200 nM PVDI-^{55}Fe (*filled circle*), PVDIp-^{55}Fe (*filled square*) and PVDIq-^{55}Fe (*filled inverted triangle*) and aliquots were removed at different times and filtered. The amounts of radioactivity retained by bacteria collected on the filters were determined. The experiment was repeated with cells pretreated with 200 µM CCCP (PVDI-^{55}Fe (*open circle*), PVDIp-^{55}Fe (*open square*) and PVDIq-^{55}Fe (*open inverted triangle*). PVDIp, pyoverdine prepared from the periplasm of *P. aeruginosa* PAO1; PVDIq, pyoverdine produced by PAO1*pvdQ* **c** fluorescence of the different forms of PVDI in the presence of Fe^{3+}. PVDI (*filled circle*), PVDIp (*filled square*) and PVDIq (*filled inverted triangle*) were diluted to 500 nM in 50 mM pyridine AcOH buffer (pH 5.0). Aliquots of $FeCl_3$ were added and the fluorescence was monitored at 450 nm (excitation at 400 nm). F_0 Fluorescence at time 0, F Fluorescence at time t

revealed by the presence of PVDIp in this cell compartment. A likely candidate for the transport of a PVDI non-fluorescent precursor across the inner membrane into the periplasm is PvdE, an "export" ABC transporter with a fused ATPase and permease component. Mutation of *pvdE* affects PVDI production (McMorran et al. 1996; Saurin et al. 1999) but its precise role in PVDI synthesis and/or transport has not been elucidated. The kinetics of formation of the chromophore in the periplasm can be followed by monitoring fluorescence in living cells. *P. aeruginosa* PAO1 OT11 (Pvd^+), the corresponding *pvdE* mutant and the complemented strain were grown in iron-limited medium, collected, re-suspended in buffer and the fluorescence monitored (Fig. 5). Fluorescence increased and no plateau was reached for strain PAO1 OT11, showing that the synthesis and maturation of PVDI is continuous. Mutation of *pvdE* almost completely prevented PVDI production, and PVDI production was restored when the mutation was complemented. Consistent with these kinetic data, fluorescence microscopy showed no fluorescence in the periplasm for the PAO1 OT11*pvdE* mutant (Fig. 6a). When the mutant was complemented with a plasmid carrying *pvdE*, fluorescence was detected in the periplasm as for the wild-type strain (Fig. 6a). Periplasmic and cytoplasmic fractions as well as inner and outer membrane preparations were isolated from wild-type bacteria, the *pvdE* mutant and the complemented mutant. The fluorescence at 450 nm was monitored in each fractions (Fig. 6b). A fivefold higher amount of fluorescence was observed for the periplasmic fraction from the Pvd^+ PAO1 OT11 strain compared to the *pvdE* mutant. Fluorescence was mostly restored when the mutation was complemented with the plasmid-borne *pvdE* gene. No fluorescence was seen in the cytoplasm (Fig. 6b) or detected in the inner and outer membrane preparations (data not shown) for either the wild-type or *pvdE* mutant bacteria. Collectively, these data and the fact that PvdE belongs to the family of ABC transporters suggest that PvdE enables transport of a PVDI precursor across the inner membrane. When this transport cannot occur through mutation of *pvdE* no fluorescence appears in the periplasm and no PVDI is released into the extracellular medium (McMorran et al. 1996; Saurin et al. 1999).

Roles of PvdN, PvdO, PvdP and PvdQ in PVDI production

The data described above suggest that a PVDI precursor is synthesized in the cytoplasm of *P. aeruginosa* PAO1 and

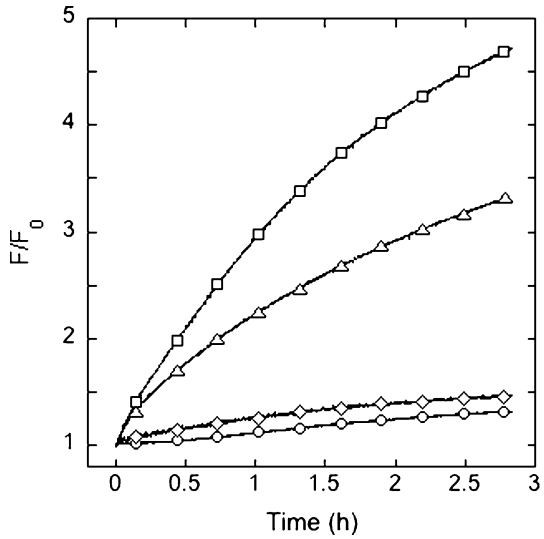

Fig. 5 Effect of *pvdE* mutation on PVDI synthesis. *P. aeruginosa* PAO1 OT11 (*open square*), OT11*pvdE* (*open circle*), PAO1 OT11*pvdE*(pBBR4) (*open diamond*) and PAO1 OT11*pvdE*(pBBR4::*pvdE*) (*open triangle*) cells at an OD_{600} of 1 were incubated at 37°C in 50 mM Tris–HCl pH 8.0 and the total fluorescence monitored at 450 nm (excitation at 400 nm). F_0 fluorescence at time 0, F fluorescence at time t

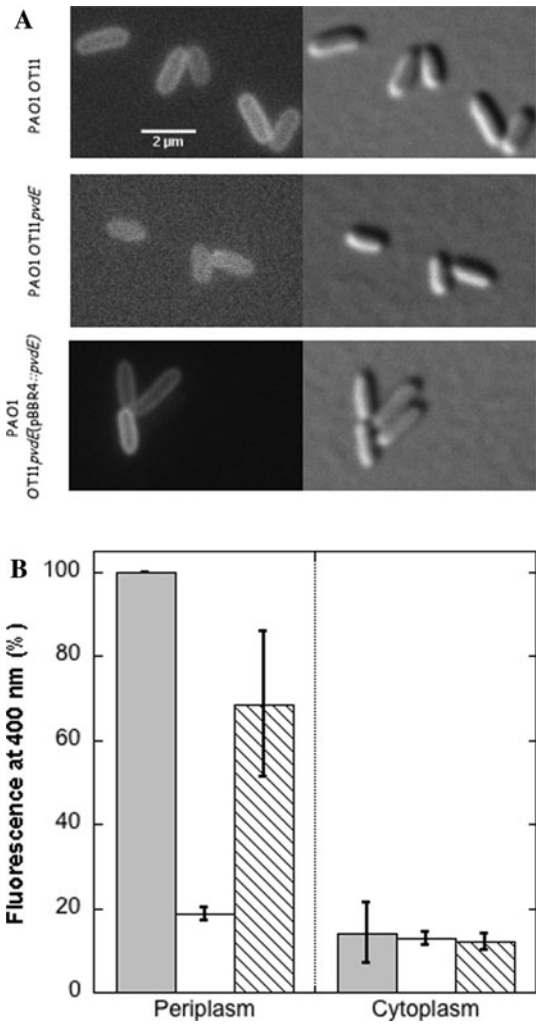

Fig. 6 **a** Fluorescence microscopy of *P. aeruginosa* PAO1 OT11, PAO1 OT11*pvdE* and PAO1 OT11*pvdE*(pBBR4::*pvdE*). For all the strains the left picture was taken with Ex 425-445HQ, Em 460-510HQ, Dc 450 (filter specification) and the right with DCI (Differential Interference Contrast) filter (*White bar* 2 µm). **b** Fluorescence in the cytoplasmic and periplasmic fractions of *P. aeruginosa* PAO1 OT11 (*gray bars*), PAO1 OT11*pvdE* (*white bars*) and PAO1 OT11*pvdE*(pBBR4::*pvdE*) (*striped bars*). The spheroplasts were prepared as in Fig. 2 for separation of the periplasmic and cytoplasmic fractions and fluorescence was determined (excited at 400 nm and emission monitored at 450 nm). The data are mean values of three independent experiments

transported across the inner membrane by PvdE, with further steps of PVDI synthesis taking place in the periplasm before release of PVDI into the extracellular medium. The *pvdN*, *pvdO*, *pvdP* and *pvdQ* genes are required for pyoverdine synthesis (Lamont and Martin 2003) and PvdN has been shown to be located in the periplasm (Voulhoux et al. 2006); *pvdO*, *pvdP* and *pvdQ* are also predicted to encode periplasmic enzymes (Lewenza et al. 2005). The kinetics of PVDI chromophore formation was followed for *pvdN*, *pvdO*, *pvdP* and *pvdQ* mutants. No increase of fluorescence was observed for the *pvdN*, *pvdO* and *pvdP* mutants (Fig. 7a), indicating that these three enzymes must be involved either in chromophore formation or in a step preceding it. Consistent with this observation, no PVDI was found in the extracellular medium of an overnight culture of these three mutants (Fig. 7b). When *pvdQ* was mutated, chromophore formation was only slightly affected (a 17% decrease of fluorescence compared to PAO1, Fig. 7a) but the production of PVDI after overnight culture was more dramatically decreased (70%; Fig. 7b). PvdQ, which is predicted to be an acylase, must be involved in PVDI maturation, in a step following chromophore formation. Fluorescence microscopy studies showed that this mutant accumulates fluorescent siderophore in the periplasm in the same way as strain PAO1 (data not shown).

The fluorescence spectrum of PVDIq, the pyoverdine released into the extracellular medium by the *pvdQ* mutant, showed a small shift of the maximum of emission of fluorescence (456 nm in pyridine acetate buffer) compared to PVDI secreted by PAO1 (452 nm in the same buffer). Electrophoretic analysis of PVDIq on cellulose acetate membranes showed no migration, showing a different pI from PVDI (Fig. 4a). Mass spectrum analyses of PVDIq gave *m/z* 1,560.85 [PVDI found in the extracellular medium being *m/z* 1,334.60 (corresponding to PVDI + H^+)] a mass slightly higher than PVDIp (1,334.60), indicating that PVDI maturation involves at least one precursor with a molecular mass larger than the final product. PVDIq was able to transport ^{55}Fe into PAD07 cells (Δ*pvd* Δ*pch*) with

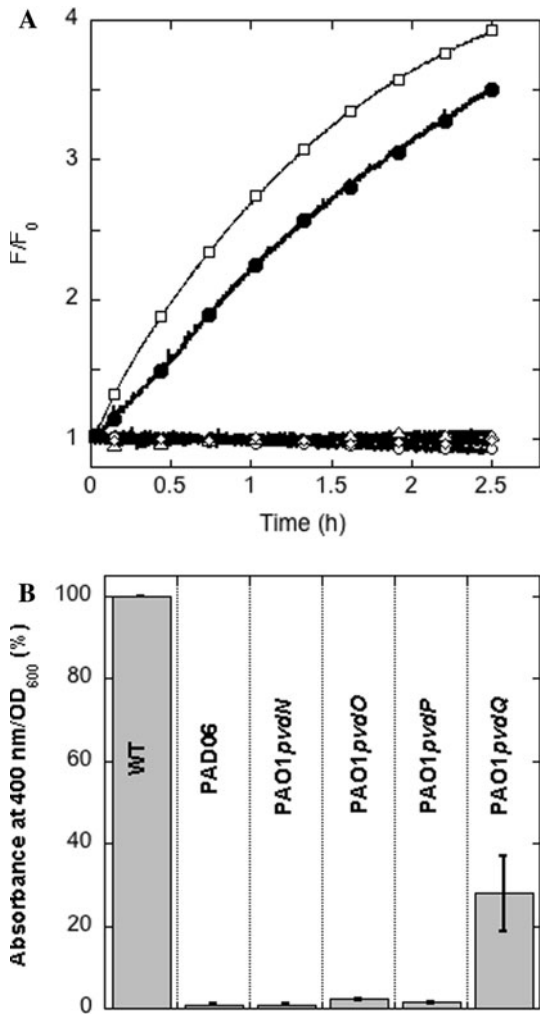

Fig. 7 **a** Kinetics of PVDI production. Cells of the strains (PAO1, *open square*; PAO1*pvdN*, *open circle*; PAO1*pvdO*, *open triangle*; PAO1*pvdP*, *open diamond* and PAO1*pvdQ*, *filled circle*) at an OD_{600} of 1 in 50 mM Tris–HCl were incubated at 37°C and fluorescence at 450 nm (excitation at 400 nm) was monitored for 3 h. F_0 fluorescence at time 0, F fluorescence at time t. **b** Effects of mutations on PVD production after overnight culture. The amounts of PVDI produced were estimated as previously, for an OD_{600} of 1, by pelleting the cells and monitoring the absorbance at 400 nm in the supernatant (Nader et al. 2007). The data are mean values of three independent experiments

the same efficiency as PVDI from PAO1 (Fig. 4b) and this transport was abolished when the cells were treated with the protonophore CCCP. However, almost no quenching of the PVDIq fluorescence was observed in the presence of an excess of Fe^{3+} (Fig. 4c) indicating a chelation that does not affect fluorescence.

Discussion

The spectral properties of PVDI have been used previously by our group to investigate the interaction between PVDI and its outer membrane transporter FpvAI (Schalk et al. 2001, 2002; Clément et al. 2004; Greenwald et al. 2007). Here, we used this approach to investigate PVDI synthesis. The biochemical functions of a number of enzymes involved in PVDI synthesis are well established [reviewed in (Visca et al. 2007)] but those of other enzymes that are required for this process are not. However, the predicted periplasmic locations of PvdN, PvdO, PvdP and PvdQ (Lewenza et al. 2005), which has been verified experimentally for PvdN (Voulhoux et al. 2006), suggested that synthesis of PVDI starts in the cytoplasm and is continued in the periplasm. Fluorescence detection of the PVDI chromophore showed that in *P. aeruginosa* PAO1 this is located in the periplasm with only a small amount of fluorescence detected in the cytoplasm (Figs. 2, 3). Fluorescence in the cytoplasmic fraction following cellular fractionation is most likely due to slight contamination of the cytoplasmic fractions by the periplasmic fractions. PVDIp, the periplasmic form of PVDI, showed a different migration pattern on cellulose acetate membranes to PVDI secreted into the extracellular medium by PAO1 (Fig. 4b), has slightly different fluorescence properties from PVDI, and its fluorescence is not quenched by Fe^{3+} in the same way as PVDI (Fig. 4c). Mass spectrum analyses of PVDIp revealed a mass of 1,301.40 compared with 1,334.60 for PVDI. When PVDIp was loaded with ^{55}Fe, the formed complex was transported into *P. aeruginosa* (Fig. 4a), indicating that the specific outer membrane transporter FpvA is able to recognize this PVDI precursor as the alternative pyoverdine receptor FpvB does not transport ^{55}Fe in our experimental conditions (unpublished data). Siderophore outer membrane transporters are highly siderophore specific. Among the 70 different pyoverdines presently identified (Meyer et al. 2008), *P. aeruginosa* PAO1 is only able to use pyoverdines produced by *Pseudomonas fluorescens* ATCC 13525 or 18.1 in addition to PVDI (Meyer et al. 1999; Fuchs et al. 2001). Structural and biochemical studies of FpvA have shown that the siderophore binding to this transporter is influence by the structure of the first three to four amino acids of the PVDI peptide moiety, while the C-terminus of the peptide does not seem to be involved extensively in recognition by FpvA (Greenwald et al. 2009). All these findings indicate that PVDIp has a structure that is sufficiently similar to PVDI to enable it to be recognized and transported by FpvA, although it is different from PVDI in its molecular weight and pI. These observations suggest that PVDIp is a PVDI fluorescent precursor. The fluorescent properties of PVDI are conferred by the chromophore. The PVDI chromophore is thought to be derived from a multistep oxidative cascade of a peptide precursor as part of the process that results in the presence of the catechol moiety of PVDI, with formation of the catechol group being the

last step in this process (Dorrestein et al. 2003; Dorrestein and Begley 2005). Since the fluorescence of PVDIp is not quenched by iron chelation (Fig. 4c), PVDIp may be a precursor of PVDI in which the chromophore has been formed but the catechol has not. Ferribactins, PVDI precursors in which the chromophore and catecholate moiety have not been formed, are able to chelate Fe^{3+} ions via hydroxamate ligands [reviewed in (Budzikiewicz 2004)] and the two hydroxamate groups of PVDI are able to efficiently chelate iron in the absence of the catecholate group and are involved in the initial step of the formation of PVDI-Fe (Albrecht-Gary et al. 1994). The two hydroxamate groups would be sufficient for PVDIp to chelate iron with the formed complex being able to bind to FpvAI and be transported into the bacteria. Cell fractionation (Fig. 2) and fluorescent microscopy (Fig. 3) indicated that PVDIp is concentrated in the periplasm. However, the amount of PVDIp extracted from this small cell compartment is extremely low (320 μg/L/OD_{600}; 0.16% of the amount of PVDI purified from the extracellular medium) and this has so far prevented elucidation of the structure of this PVDI precursor.

It is surprising that cells of *P. aeruginosa* store siderophore precursor in the periplasm. PVDI is able to chelate a number of metal ions in addition to iron (Baysse et al. 2000; Braud et al. 2009) so that these molecules are potentially able to chelate any free metal present in this cell compartment and perhaps also metal bound to metalloproteins. The differences between PVDIp and PVDI may indicate different mechanisms of chelation for the two molecules as discussed above resulting in a lower affinity of PVDIp for metal ions compared to PVDI and this may be important for maintaining metal ion homeostasis in the periplasm. Here again, further studies are necessary to clarify this point.

As discussed above, fluorescence corresponding to PVDI chromophore was detected in the periplasm, with almost no fluorescence in the cytoplasm of *P. aeruginosa* PAO1 cells (Figs. 2, 3). This strongly suggests that either a PVDI precursor with an unformed chromophore is synthesized in the cytoplasm and transported across the inner membrane with chromophore maturation occurring in the periplasm, or chromophore cyclization occurs in the cytoplasm with an immediate export of the formed molecule into the periplasm. The transport across the inner membrane apparently involves PvdE, an "export" ABC transporter, with a fused ATPase and permease. When *pvdE* was mutated, production of secreted PVDI was dramatically decreased (Fig. 5), PVDIp could not be detected in the periplasm and fluorescence did not accumulate in the cytoplasm (Fig. 6). PvdE is apparently involved in the transport of a PVDI precursor across the inner membrane. This transported molecule is most likely to be a non-fluorescent PVDI precursor that is transported into the periplasm via PvdE, with maturation of the PVDI chromophore occurring only in this second cell compartment. A second possibility is that the chromophore is formed in the cytoplasm and PVDI biosynthesis is directly coupled to transport across the inner membrane by PvdE. However, synthesis of PVDI requires PvdN, PvdO and PvdP that are likely to be involved in the formation of the chromophore or in a step preceding it. In their absence no fluorescence was detected in the bacterial cells (Fig. 7a) and PVDI was not released into the extracellular medium (Fig. 7b). The periplasmic localization of PvdN has been shown experimentally (Voulhoux et al. 2006) and the presence of signal sequences for the other proteins makes it likely that they are also localized in the periplasm (Fig. 8). The cellular localization of these enzymes and the fact that their mutation prevents the presence of PVDIp in the periplasm as well as PVDI production indicates that PVDI chromophore formation occurs in the periplasm and the molecule transported by PvdE is a non-fluorescent precursor of PVDI. A reaction pathway has been proposed for the formation of the PVDI chromophore from a synthetic chromophore precursor (Dorrestein et al. 2003). Cell extracts from wild-type *P. aeruginosa*, but not a mutant strain that does not express PVDI synthesis genes, catalyzed reactions that were consistent with this scheme (Dorrestein et al. 2003). It remains to be determined whether PvdN, PvdO and PvdP catalyze specific steps in the proposed pathway.

When *pvdQ* was mutated, the rate of chromophore formation was only slightly decreased (Fig. 7a) but production of fluorescent siderophore in an overnight culture was 70% decreased. Since this mutant was able to form a fluorescent siderophore, PvdQ must be involved in PVDI synthesis either in a step following chromophore maturation or in a step converting an immature (but fluorescent) chromophore into the final form. The pyoverdine produced by PAO1 *pvdQ*, PVDIq, has a higher molecular weight than PVDI (1,560.85 Da instead of 1,334.60 Da) and migrated differently to PVDI on cellulose acetate (Fig. 4), indicating that the molecules have different pIs. PvdQ was shown to be an acylase for quorum sensing signal molecules (Huang et al. 2006) and synthesis of the PVDI precursor peptide is predicted to begin with incorporation of an acyl group (Mossialos et al. 2002) so that a possibility is that PvdQ is responsible for removing this group during PVDI synthesis. Moreover, iron chelation studies with PVDIq did not show the quenching of fluorescence characteristic of the formation of a ferrichelate complex although PVDIq efficiently transported ^{55}Fe into *P. aeruginosa* cells. It may be that PvdIq is a PVDI precursor with an immature chromophore lacking catechol function as proposed above for PVDIp. If this was the case, iron would only be chelated by the two hydroxamate functions in the

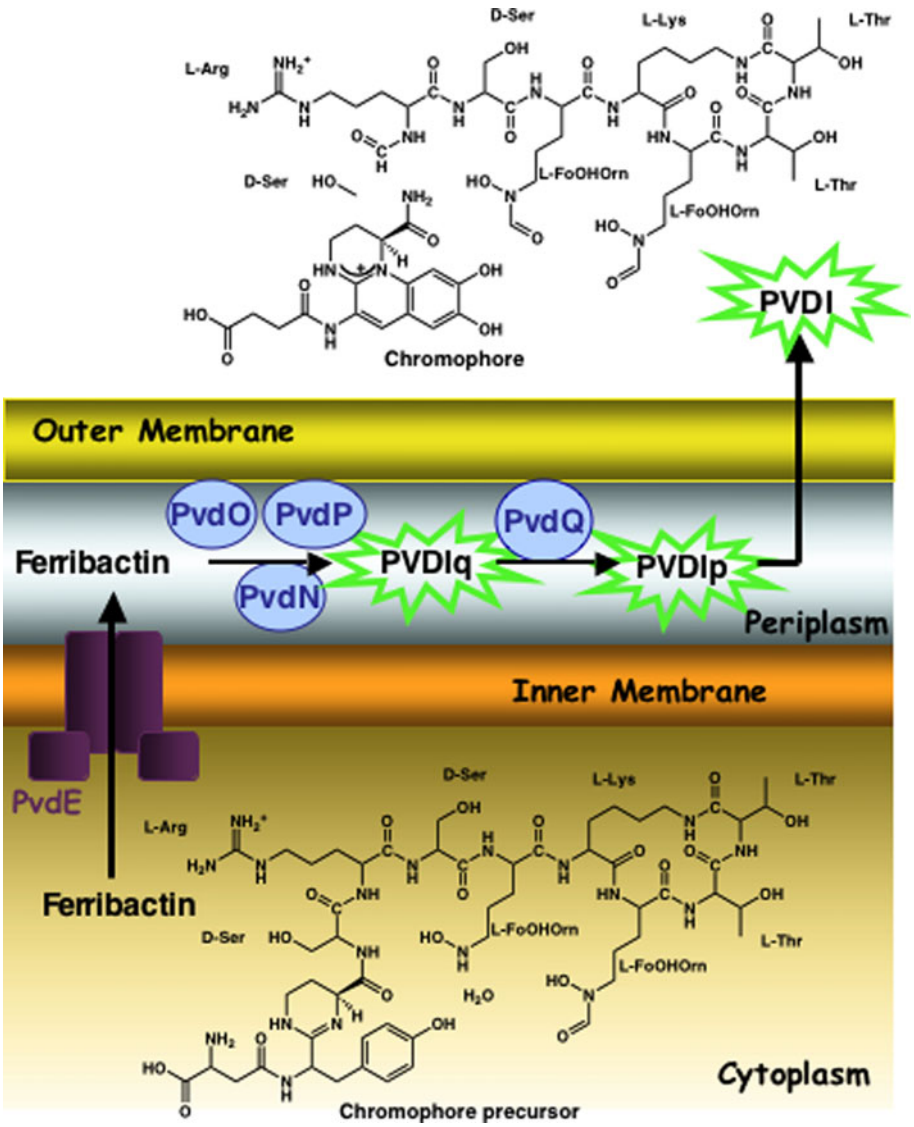

Fig. 8 Model for Ferribactin maturation in *P. aeruginosa*. Ferribactin, a non-fluorescent PVDI precursor with an unformed chromophore, is synthesised in the cytoplasm. This molecule is transported across the inner membrane by the ABC transporter PvdE. The chromophore is formed in the periplasm with PvdN, PvdO and PvdP catalyzing steps in this process to form PVDIq that has a fluorescent chromophore. PvdQ is involved in conversion of PVDIq into PVDIp a pyoverdine precursor stored in the periplasm before being converted into PVDI and secreted by an unknown transporter. Only PVDI, PVDIp and PVDIq are fluorescent

PVDIq complex, which would be sufficient to enable transport into *P. aeruginosa* but not to efficiently quench fluorescence.

Collectively, our data show that a PVDI precursor with a non-cyclisized chromophore is synthesized in the cytoplasm and transported across the inner membrane by the "export" ABC transporter PvdE. Once in the periplasm the chromophore is formed from the precursor and PvdN, PvdO and PvdP are likely to catalyze steps in this process. PvdQ is also involved in the maturation of the siderophore in the periplasm, probably in one of the last steps of the maturation of the chromophore. In this cell compartment the siderophore accumulates in a form (PVDIp) different from PVDI, but that is nonetheless able to chelate iron and to transport it into *P. aeruginosa*, by interacting with the specific outer membrane transporter FpvA. The mechanism of PVDI secretion across the outer membrane remains to be determined. In the case of catecholate siderophores, it has been suggested that an efflux pump is involved in this step (Page et al. 2003). The *PVDI* locus contains three genes potentially encoding an efflux pump but previous studies have shown that mutations in these genes do not prevent secretion of PVDI (Ochsner et al. 2002b; Lamont and Martin 2003). Another efflux pump has also been proposed to be involved in PVDI secretion (MexABOprM) but its role has not been further investigated (Poole et al. 1993a).

In conclusion, our data show that the final steps of pyoverdine synthesis take place in the periplasm and that a siderophore precursor accumulates there. Periplasmic accumulation or storage of a siderophore precursor has not been shown for any other bacteria. It is possible that other siderophores are stored by bacteria in the periplasm but since they are not fluorescent, their accumulation has not been observed because of lack of appropriate tools.

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