

# Membrane-association determinants of the $\omega$ -amino acid monooxygenase PvdA, a pyoverdine biosynthetic enzyme from *Pseudomonas aeruginosa*

Francesco Imperi,<sup>1,2†</sup> Lorenza Putignani,<sup>2‡</sup> Federica Tiburzi,<sup>1,2</sup>  
Cecilia Ambrosi,<sup>1§</sup> Rita Cipollone,<sup>1||</sup> Paolo Ascenzi<sup>1,2</sup> and Paolo Visca<sup>1,2</sup>

<sup>1</sup>Department of Biology, University 'Roma Tre', Viale G. Marconi 446, I-00146 Rome, Italy

<sup>2</sup>National Institute for Infectious Diseases IRCCS, 'Lazzaro Spallanzani', Via Portuense 292, I-00149 Rome, Italy

Correspondence  
Paolo Visca  
visca@uniroma3.it

Received 26 March 2008  
Revised 23 May 2008  
Accepted 28 May 2008

The L-ornithine  $N^{\delta}$ -oxygenase PvdA catalyses the  $N^{\delta}$ -hydroxylation of L-ornithine in many *Pseudomonas* spp., and thus provides an essential enzymic function in the biogenesis of the pyoverdine siderophore. Here, we report a detailed analysis of the membrane topology of the PvdA enzyme from the bacterial pathogen *Pseudomonas aeruginosa*. Membrane topogenic determinants of PvdA were identified by computational analysis, and verified in *Escherichia coli* by constructing a series of translational fusions between PvdA and the PhoA (alkaline phosphatase) reporter enzyme. The inferred topological model resembled a eukaryotic reverse signal-anchor (type III) protein, with a single N-terminal domain anchored to the inner membrane, and the bulk of the protein spanning the cytosol. According to this model, the predicted transmembrane region should overlap the putative FAD-binding site. Cell fractionation and proteinase K accessibility experiments in *P. aeruginosa* confirmed the membrane-bound nature of PvdA, but excluded the transmembrane topology of its N-terminal hydrophobic region. Mutational analysis of PvdA, and complementation assays in a *P. aeruginosa*  $\Delta pvdA$  mutant, demonstrated the dual (structural and functional) role of the PvdA N-terminal domain.

## INTRODUCTION

Iron is one of the most important nutrients for bacteria because of its essential metabolic role. To fulfil their nutritional iron demand, many bacteria synthesize, excrete and ingest high-affinity iron chelators, termed siderophores, which bind environmental iron (generally  $Fe^{3+}$ ), and deliver it to the cell through receptor-mediated active transport (Andrews *et al.*, 2003). Pyoverdine is the major siderophore produced by the opportunistic pathogen

*Pseudomonas aeruginosa*, and it plays an important role in *P. aeruginosa* pathogenicity and ecology (reviewed by Visca *et al.*, 2007).

Structurally, pyoverdine is a chromopeptide consisting of three domains: (i) a fluorescent chromophore, (ii) an acyl side chain, and (iii) a peptide moiety linked via an amide bond to the carboxyl group of the chromophore.  $Fe^{3+}$  is bound by pyoverdine with high affinity to form a very stable octahedral complex (dissociation constant approximately  $10^{-32}$  M). In type 1 pyoverdine from *P. aeruginosa* PAO1, the three bidentate ligands involved in  $Fe^{3+}$  coordination are provided by the catecholic hydroxyl groups of the chromophore and two hydroxamic groups of the formylated  $N^{\delta}$ -hydroxyornithine (OHOrn) residues of the peptide moiety. As a rule, the whole pyoverdine molecule is assembled by non-ribosomal peptide synthases, according to the carrier thiotemplate mechanism (Visca *et al.*, 2007).

In *P. aeruginosa*, L-ornithine (Orn) hydroxylation is catalysed by the Orn  $N^{\delta}$ -oxygenase, which is encoded by the *pvdA* gene (Visca *et al.*, 1994), and belongs to the  $\omega$ -amino acid monooxygenase family (EC 1.14.13.-). Several

†These authors contributed equally to this work.

‡Present address: Children's Hospital and Research Institute 'Bambino Gesù', Piazza S. Onofrio 4, I-00165 Rome, Italy.

§Present address: National Research Council, Istituto di Cristallografia, Sezione di Monterotondo, Monterotondo Stazione, I-00016 Rome, Italy.

||Present address: Department of Experimental Medicine and Biochemical Sciences, University of Rome 'Tor Vergata', Via Montpellier 1, I-00133 Rome, Italy.

Abbreviations: OHOrn,  $N^{\delta}$ -hydroxyornithine; Orn, L-ornithine; TM, transmembrane.

A multiple sequence alignment of PvdA is available with the online version of this paper.

lines of evidence indicate that PvdA plays an essential role in pyoverdine biogenesis by *P. aeruginosa* (Visca *et al.*, 1994, 2007; Putignani *et al.*, 2004). Recently, *P. aeruginosa* PAO1 PvdA has been purified and biochemically characterized as a monomeric enzyme in solution, and it has been shown to require both FAD and NADPH for activity (Meneely & Lamb, 2007).

The  $\omega$ -amino acid monooxygenase family is an elusive group of enzymes that contains two putative dinucleotide-binding motifs (GXGXXG/P and GXGXXG/A for FAD and NAD(P)H, respectively) and a substrate recognition (F/LATGY) domain (Stehr *et al.* 1998; Putignani *et al.*, 2004). Members of this family catalyse the incorporation of one hydroxyl group into the side-chain amino group of  $\omega$ -amino acids by reducing molecular oxygen through concomitant oxidation of both NAD(P)H and FADH<sub>2</sub> (Plattner *et al.*, 1989). Interestingly, the only  $\omega$ -amino acid monooxygenase whose subcellular localization has been investigated to date is the PvdA proximate homologue IucD, which catalyses L-lysine hydroxylation during aerobactin biogenesis in *Escherichia coli* (Herrero *et al.*, 1988). Pioneer topology studies on IucD have suggested that this enzyme is membrane bound by means of at least one transmembrane (TM) domain that encompasses the putative FAD-binding motif at the N-terminal region (Herrero *et al.*, 1988). Accordingly, Viswanatha and coworkers were unable to obtain a soluble form of IucD, unless a recombinant protein with an altered N terminus was constructed (Thariath *et al.*, 1993). However, the group of Diekmann reported that native IucD could be purified to homogeneity in an active form (Plattner *et al.*, 1989), giving rise to a dispute on the assumption that the membrane-associated form of IucD could be an artefact resulting from the moderate hydrophobicity of dinucleotide-binding domains (Dick *et al.*, 1998; Seth *et al.*, 1998; Stehr *et al.* 1998). Therefore, the actual cellular localization of bacterial  $\omega$ -amino acid monooxygenases remains an open issue.

This study was undertaken to explore the membrane topology of the *P. aeruginosa* PAO1 PvdA enzyme, with the aim of providing novel insights into the membrane-association determinants of bacterial  $\omega$ -amino acid monooxygenases. By using *in silico* topology predictions, alkaline phosphatase (PhoA) translational fusions, cell fractionation assays and mutational analysis, we demonstrate that PvdA is anchored to the cytoplasmic membrane with the core of the protein exposed to the cytosol, and that the PvdA N-terminal hydrophobic domain is important for membrane association. Overall, our results provide a valuable background for further investigations on the pyoverdine multienzymic biosynthetic pathway in *P. aeruginosa*, and provide a basis for topological studies on biosynthetic enzymes of other hydroxamate siderophores.

## METHODS

**In silico analyses.** Secondary structure and solvent accessibility were inferred by using Jpred algorithms (<http://barton.ebi.ac.uk/servers/>

jpred.html). Java outputs were manually edited. Hydrophobicity profiles were generated by using Hopp–Woods (Hopp & Woods, 1983) and Kyte–Doolittle (Kyte & Doolittle, 1982) functions over a window size of 19 aa (<http://bip.weizmann.ac.il/index.html>). Prediction of TM domains was accomplished by combining four software programs: TMpred, DAS and TMHMM (all available on the ExPASy server, [www.expasy.org/tools/](http://www.expasy.org/tools/)), and ConPred II (<http://bioinfo.si.hirosaki-u.ac.jp/~ConPred2/>). All user-adjustable parameters were left at their default values.

**Construction of PvdA–PhoA translational fusions.** A series of PvdA–PhoA translational fusions was constructed by PCR amplification, using *P. aeruginosa* PAO1 genomic DNA as a template. Routine genetic manipulations were carried out according to Sambrook *et al.* (1989). The sense primer, which annealed to the start codon of the *pvdA* coding sequence, was paired with antisense primers to generate fusions at appropriate positions. In both sense and antisense primers, the *KpnI* restriction site was included to allow cloning of each amplicon into the compatible restriction site of pBAD*phoA* (Table 1), upstream of the *phoA* gene without a signal sequence, which encodes a PhoA variant lacking the N-terminal signal peptide essential for PhoA export into the periplasm (Melchers *et al.*, 1999). Fusions 12P and 22P were obtained by the oligonucleotide adaptor technique (Invitrogen). Primer and oligonucleotide sequences used in this study are given in Table 2. Correct in-frame cloning and amplicon sequences were verified by automated DNA sequencing. The resultant fusion plasmids were individually introduced into the *phoA*-null mutant *E. coli* strain LMG194 (F<sup>-</sup>  $\Delta$ lacZX74 galE thi rpsL  $\Delta$ phoA  $\Delta$ ara-714 leu::Tn10; Guzman *et al.*, 1995).

**Expression of fusion proteins, PhoA assay, and preparation of *E. coli* subcellular fractions.** *E. coli* LMG194 cells expressing PvdA–PhoA fusion proteins were grown at 37 °C in NZYM medium (Sambrook *et al.*, 1989) supplemented with 100  $\mu$ g ampicillin ml<sup>-1</sup>. PhoA activity was assayed by measuring the rate of *p*-nitrophenyl

**Table 1.** Plasmids and encoded proteins used in this study

Plasmid or construct*	Encoded protein	Reference
pBAD <i>phoA</i>	PhoA†	Melchers <i>et al.</i> (1999)
pBAD <i>phoApvdA</i> 12E	12P	This study
pBAD <i>phoApvdA</i> 22N	22P	This study
pBAD <i>phoApvdA</i> 31E	31P	This study
pBAD <i>phoApvdA</i> 40E	40P	This study
pBAD <i>phoApvdA</i> 210M	21P	This study
pBAD <i>phoApvdA</i> 220A	220P	This study
pBAD <i>phoApvdA</i> 240R	240P	This study
pBAD <i>phoApvdA</i> 443T	443P	This study
pUCP19		Schweizer (1991)
pUCP19 <i>pvdA</i>	PvdA	This study
pUCP19 $\Delta$ 9 <i>pvdA</i>	$\Delta$ 9PvdA	This study
pUCP19 $\Delta$ 9 <i>pvdA</i> (G <sup>15</sup> →D)	$\Delta$ 9PvdA (G <sup>15</sup> →D)	This study
pUCP19 $\Delta$ 30 <i>pvdA</i>	$\Delta$ 30PvdA	This study

\*Construct names report the number of encoded PvdA N-terminal residues and the one-letter symbol of the amino acid at the fusion joint. The amino acid substitutions resulting from the introduction of the *KpnI* site are indicated in bold (D→E, I→M and S→T).

†PhoA enzyme lacking its signal peptide sequence.

**Table 2.** Oligonucleotides used in this study

Oligonucleotide	Sequence (5'→3')*	Restriction site
2-12phoAFW	CCCCGGTACCTACTCAGGCAACTGCAACCGCCGTGGTTCACGAGGTACCGGG	<i>KpnI</i>
2-12phoARV	CCCCGGTACCTCGTGAACACGGCGGTTGCAGTTGCCTGAGTAGGTACCGGG	<i>KpnI</i>
2-22phoAFW	CCCCGGTACCTACTCAGGCAACTGCAACCGCCGTGGTTCACGATCTCATCGGTG TCGGCTTCGGCCCTTCCAAGGTACCGGG	<i>KpnI</i>
2-22phoARV	CCCCGGTACCTTGGAAAGGGCCGAAGCCGACACCGATGAGATCGTGAACCACGG CGGTTGCAGTTGCCTGAGTAGGTACCGGG	<i>KpnI</i>
2-31phoAFW	CCCCGGTACCTACTCAGGCAACTGCA	<i>KpnI</i>
2-31phoARV	GGGGAGGTACCTCCTGGAGGGCAATCGC	<i>KpnI</i>
2-40phoARV	GGGGAGGTACCTCCAGGGCCCTGCGC	<i>KpnI</i>
2-210phoARV	GGGGAGGTACCATCTTCATCGGCTT	<i>KpnI</i>
2-220phoARV	GGGGAGGTACCGCCGCTCTGCC	<i>KpnI</i>
2-240phoARV	GGGGAGGTACCGCAGGATCATGTT	<i>KpnI</i>
2-443phoARV	GGGGAGGTACCGTGCCAGGGCGTGCTC	<i>KpnI</i>
pvdAFW	CCCCGGATCCACTCAGGCAACTGCACC	<i>BamHI</i>
Δ9pvdAFW	CGCGGATCCGTTACGATCTCATCGGTGTCGGC	<i>BamHI</i>
Δ9pvdAG→DFW†	CGCGGATCCGTTACGATCTCATCGATGTCGGC	<i>BamHI</i>
Δ30pvdAFW	CGCGGATCCGAACGGGCCAGGCCGAG	<i>BamHI</i>
pvdARV	CCCCAAGCTTCTGGCCAGGGCGTGCTGG	<i>HindIII</i>
ΔpvdAUPFW	CGAAACAAGGCTTTAAAGCTTG	<i>HindIII</i>
ΔpvdAUPRV	CGCGGATCCGAAGCCGACACCGATGA	<i>BamHI</i>
ΔpvdADOWNFW	CGCGGATCCAGCTGATCGGCCAC	<i>BamHI</i>
ΔpvdADOWNRV	CCCGAATTCAGGGGAAGTGCGGGT	<i>EcoRI</i>

\*Restriction sites are underlined.

†This primer carries a single nucleotide substitution (G→A) that generates the amino acid substitution G→D in the Δ9PvdA(G<sup>15</sup>→D) protein.

phosphate hydrolysis in permeabilized cells (Michaelis *et al.*, 1983), and it was reported in arbitrary units (Melchers *et al.*, 1999). Periplasmic, cytoplasmic and membrane fractions from *E. coli* cells were prepared as described (Neu & Heppel, 1965), using ice-cold 1 mM MgCl<sub>2</sub> instead of water to reduce the amount of shock-released cytoplasmic proteins (Hantash & Earhart, 2000). Immunoblots of PhoA fusion proteins were performed with rabbit anti-PhoA polyclonal antibody (Abcam). Immunodetection was performed with goat anti-rabbit alkaline-phosphatase-conjugated polyclonal antibody (Abcam). Protein concentration was determined using the DC protein assay kit (Bio-Rad). Densitometric measurements were obtained by using Quantity One software and a Gel Doc 2000 CCD camera (Bio-Rad).

**Generation of the *P. aeruginosa* PAO1ΔpvdA mutant.** Site-specific excision of the entire *pvdA* coding sequence was performed using a *sacB*-based strategy described by Hoang *et al.* (1998). Two regions of approximately 800 bp upstream and downstream of the *pvdA* gene were generated by PCR with specific primer pairs (Table 2). Upstream and downstream fragments were digested with appropriate restriction enzymes, and directionally cloned into the pEX18Tc suicide vector (Hoang *et al.*, 1998). The gentamicin-resistance (Gm<sup>R</sup>)-GFP cassette from pS858 (Hoang *et al.*, 1998) was ligated into the *BamHI* site situated between the two cloned fragments, and the resulting pEXΔpvdA construct was conjugally transferred into *P. aeruginosa* PAO1. Methods for resolution of merodiploids and excision of the Gm<sup>R</sup>-GFP cassette were as described (Hoang *et al.*, 1998). The deletion event was verified by PCR and DNA sequencing, and the resulting mutant was named PAO1ΔpvdA.

**Construction of *pvdA* deletions and point mutations.** The DNA sequences encoding the whole PvdA protein and three N-terminal-deleted PvdA derivatives [Δ9PvdA, Δ9PvdA(G<sup>15</sup>→D) and Δ30PvdA]

were PCR amplified with specific primers containing *BamHI* (sense) and *HindIII* (antisense) restriction sites (Table 2), using *P. aeruginosa* PAO1 genomic DNA as a template. The amplicons were directionally cloned into the same restriction sites of the vector pQE60 (Qiagen), and excised by *XhoI/PvuII* digestion to obtain *pvdA*-deletion derivatives under the control of the T5 promoter provided by pQE60. These DNA fragments were ligated to the compatible *SalI* and *SmaI* sites of the *Escherichia-Pseudomonas* shuttle vector pUCP19 (Schweizer, 1991). Correct frame and sequence were verified by DNA sequencing. The resultant constructs were individually introduced into the PvdA-defective *P. aeruginosa* mutant PAO1ΔpvdA by transformation.

**Complementation and membrane-association assays.** *P. aeruginosa* PAO1, or *P. aeruginosa* PAO1ΔpvdA cells expressing PvdA, Δ9PvdA, Δ9PvdA(G<sup>15</sup>→D) or Δ30PvdA, were grown to late-exponential phase at 37 °C in DCAA medium (Visca *et al.*, 1993). When necessary, the medium was supplemented with 150 μg carbenicillin ml<sup>-1</sup>. Pyoverdine production was assayed by absorbance at 405 nm of culture supernatants (Tiburzi *et al.*, 2008) and by UV fluorescence on cetrimide (Pseudoseal; Acumedia) agar plates. *P. aeruginosa* cells were spheroplasted by the lysozyme/sucrose method (Robles-Price *et al.*, 2004) to release periplasmic proteins. Spheroplasts were then disrupted by sonication, and cell debris was removed by low-speed centrifugation (10 min at 3000 g). Cytosolic and membrane fractions were separated by ultracentrifugation at 55 000 g for 2 h at 4 °C. The specificity of subcellular fractions was verified by measuring relative isocitrate dehydrogenase and lactate dehydrogenase activities, as previously described (Tiburzi *et al.*, 2008). Separation was considered acceptable when subcellular fractions showed less than 2% cross-contamination between enzyme markers of the different fractions. Immunoblot analysis was carried

out using the mouse anti-PvdA monoclonal antibody 3H6D12 raised against the C-terminal region of PvdA (Putignani *et al.*, 2004), a mouse anti-RpoD monoclonal antibody (Neoclone), and a rabbit anti-XcpY polyclonal serum (Michel *et al.*, 1998).

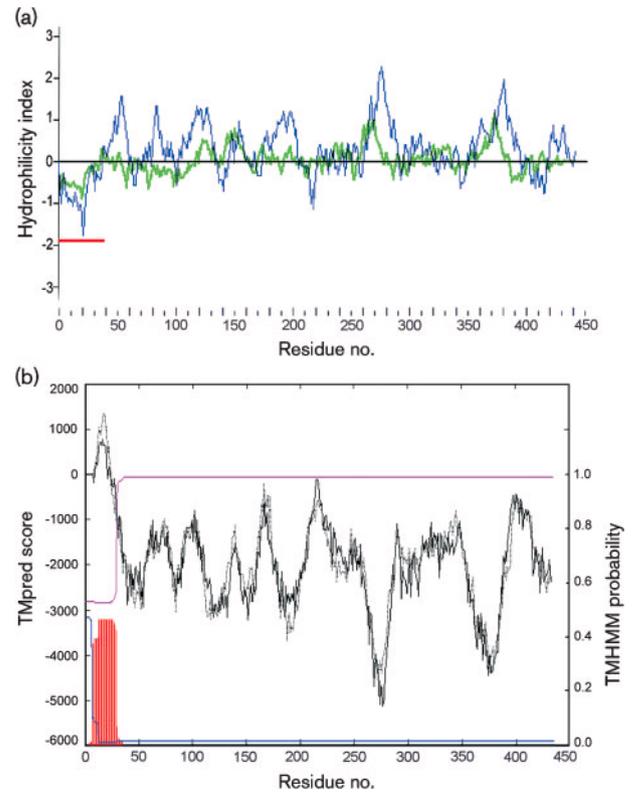
The nature of PvdA association with *P. aeruginosa* PAO1 membranes was assessed by means of different chemical treatments. Membrane samples were treated with 1.0 M NaCl, 0.1 M NaOH, 1.5 M urea, 5.0 M urea, or 2% *N*-lauryl-sarcosine (Sarcosyl), for 30 min at 4 °C, and then centrifuged at 55 000 g for 2 h at 4 °C. Proteins retained in the pellet were directly solubilized in SDS-PAGE loading buffer (Sambrook *et al.*, 1989), while proteins in the supernatants were precipitated with 10% trichloroacetic acid, and suspended in SDS-PAGE loading buffer.

**Proteinase K accessibility assay.** Proteinase K accessibility experiments were performed as described (Arts *et al.*, 2007). Briefly, *P. aeruginosa* PAO1 cells were grown to late-exponential phase at 37 °C in DCAA medium. Cells were spheroplasted by incubation in ice-cold 40% sucrose, 1.5 mM EDTA, 33 mM Tris/HCl (pH 8) and 5 µg lysozyme ml<sup>-1</sup>. After 10 min on ice, and 10 min at 37 °C, spheroplasts were stabilized by the addition of MgCl<sub>2</sub> at 10 mM final concentration. Spheroplasts were then incubated for 1 h on ice in the presence and absence of 50 µg proteinase K ml<sup>-1</sup>. PMSF (2 mM) was added to terminate the reaction, and proteins were precipitated with 10% trichloroacetic acid, and suspended in SDS-PAGE loading buffer.

## RESULTS

### PvdA hydrophobicity, TM domain and secondary structure predictions

Hydropathy plots of the PvdA amino acid sequence, computed by both Kyte–Doolittle (Kyte & Doolittle, 1982) and Hopp–Woods (Hopp & Woods, 1983) algorithms, showed a highly hydrophobic peak encompassing amino acid residues 10–30 (Fig. 1a). Analyses with TMpred, TMHMM, DAS and ConPred II algorithms predicted a single putative TM domain overlapping this hydrophobic region (Fig. 1b, and data not shown), although this showed a moderate probability score with TMHMM. Analysis of the residues flanking the predicted TM region highlighted a net negative charge (−1) on the left site (D<sup>12</sup>) and a zero charge (E<sup>31</sup> plus R<sup>32</sup>) on the right site. This feature could represent an extension of the positive inside rule (von Heijne, 1992), thus resembling protein behaviour in eukaryotes in which cytoplasmic location is conferred by the more positive flanking sequence rather than by a positive charge *per se* (Goder & Spiess, 2001). Indeed, the N<sub>out</sub>/C<sub>in</sub> TM orientation for this presumptive TM domain was strongly suggested by TMpred (Fig. 1b) and ConPred II (the latter probed against eukaryotic databases). The PvdA secondary structure was predicted by the Jpred PSIBLAST algorithm, following multiple alignment with putative or confirmed hydroxylase/monooxygenase homologues (supplementary Fig. S1, available with the online version of this paper). All homologues belong to pyridine nucleotide–disulphide oxidoreductase (Pfam00070) or flavin-binding monooxygenase-like (Pfam00743) families, which are members of the FAD/NAD(P)H-binding Rossmann fold superfamily. The multiple alignment showed the highest amino acid conser-



**Fig. 1.** Hydropathy computations and TM domain predictions for *P. aeruginosa* PAO1 PvdA. (a) Combined hydrophobicity plots generated by the Hopp–Woods (green) and Kyte–Doolittle (blue) methods over a window of 19 aa. The highest hydrophobic peak is indicated by the red segment. (b) TM predictions obtained by TMpred and TMHMM algorithms, and manually superimposed. TMpred scores for out→in (grey dotted line) and in→out (black line) orientation, and TMHMM probabilities for periplasmic (magenta line), cytoplasmic (blue line) and TM (red vertical bars) regions, are shown on the left and right, respectively.

vation over an extended N-terminal region of approximately 110 residues (10–120 aa relative to PvdA) and over two shorter amino acid stretches located in the central and C-terminal regions (210–225 and 345–356 aa relative to PvdA, respectively). Solvent accessibility algorithms predicted the longest stretch of contiguous buried residues at the PvdA N terminus, overlapping the FAD signature (Fig. S1), and corroborated the inferred high hydrophobicity of this region. Finally, PvdA secondary structure predictions highlighted two  $\beta\alpha\beta$  folds (residues 9–46 and 210–240), followed by three consecutive  $\beta$ -strands (Fig. S1); this structure resembles a variant of the typical mononucleotide-binding fold (Dym & Eisenberg, 2001).

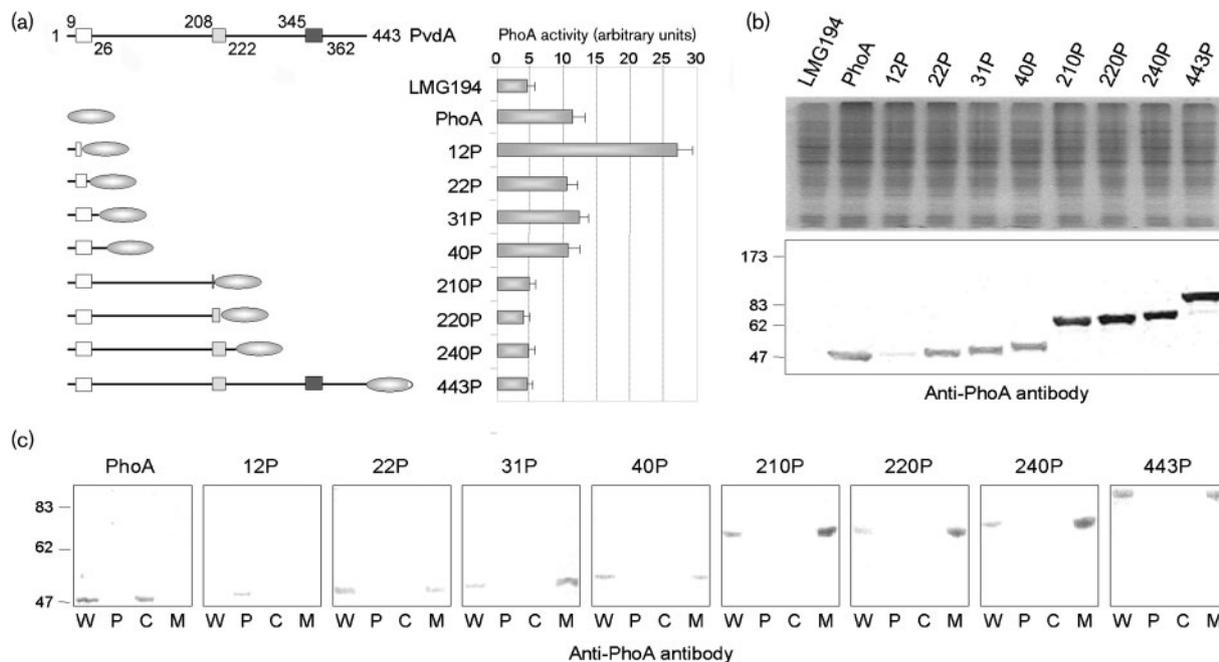
### Topogenic determinants of PvdA

The presence of possible topogenic determinants in the PvdA amino acid sequence was investigated by means of PhoA fusions (Melchers *et al.*, 1999). PvdA coding regions were

fused in-frame with a truncated *phoA* gene encoding a PhoA enzyme lacking the native signal peptide. The eventual presence of topogenic determinant(s) in the fused protein fragment may target PhoA, lacking a signal peptide sequence, across the inner membrane to the periplasm, where PhoA folds and becomes enzymically active (Manoil *et al.*, 1990).

Based on computational analyses, we fused the PhoA reporter enzyme, lacking a signal peptide sequence, with eight different N-terminal fragments of PvdA extending from position 1 to positions 12, 22, 31, 40, 210, 220, 240 and 443 (Fig. 2a). The resulting fusion proteins were named 12P, 22P, 31P, 40P, 210P, 220P, 240P and 443P, respectively (Fig. 2a, Table 1). The enzymic activity of each fusion protein was measured in *E. coli* LMG194, as summarized in Fig. 2(a). The highest PhoA activity value was observed for the 12P fusion, whereas the other fusions showed activities comparable with the PhoA enzyme, lacking a signal peptide sequence (i.e. 22P, 31P and 40P), with the host strain (i.e. 210P, 220P, 240P and 443P) (Fig. 2a). The expression level of each PhoA fusion protein was determined by immunoblot analysis of *E. coli* LMG194 whole-cell lysates with an anti-PhoA antibody. All fusion proteins showed the expected molecular mass, indicating

that none was processed by the heterologous *E. coli* host (Fig. 2b). However, expression levels were variable and extremely low for 12P (Fig. 2b). When PhoA activities were normalized by the protein expression levels, as measured by densitometric analysis, the relative PhoA activity of the 12P fusion increased by at least 40-fold with respect to all the other fusions (data not shown). To further assess the subcellular localization of fusion proteins, cell fractionation experiments were performed. Immunoblot analysis with the anti-PhoA antibody on *E. coli* LMG194 subcellular fractions showed that 22P, 31P, 40P, 210P, 220P, 240P and 443P localized in the membrane fraction, while only 12P was released in the periplasmic fraction (Fig. 2c). As expected, the PhoA control, lacking a signal peptide sequence, was detected in the cytosolic fraction (Fig. 2c). These results indicate that the first 12 residues of PvdA drive the export of PhoA across the inner membrane of *E. coli*, and that a minimum of 22 residues at the PvdA N terminus is able to retain PhoA on the cytosolic leaflet of the membrane. Overall, topological studies in the heterologous host *E. coli* suggested that one single N-terminal domain anchors PvdA to the inner membrane, with the bulk of the protein plausibly exposed to the cytosol.



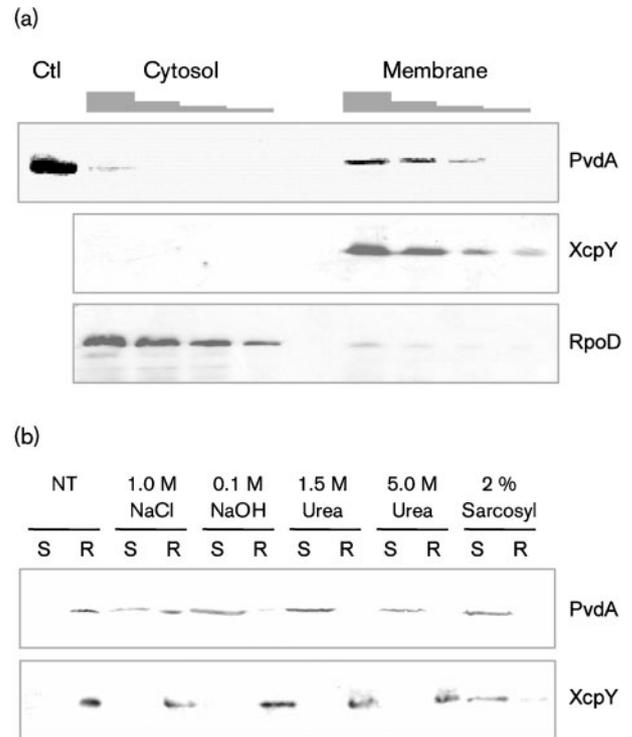
**Fig. 2.** Enzymic activity and expression profile of PvdA–PhoA fusion proteins. (a) Linear representation (not to scale) of PvdA and PvdA–PhoA fusions. FAD, NADPH and LATGY domains are represented as white, light grey and dark grey boxes, respectively, and their amino acid positions are indicated in full-length PvdA. The PhoA, lacking a signal peptide sequence (grey ellipse), and the *E. coli* LMG194 host strain were included as negative controls. The histogram shows mean PhoA activity values ( $\pm$  SD) of three independent experiments. (b) Coomassie Blue staining (upper panel) and immunoblot analysis (lower panel) of cell extracts (5 and 10 µg protein, respectively) from *E. coli* LMG194 cells expressing different PvdA–PhoA fusions with the anti-PhoA antibody. (c) Cell fractionation immunoblot analysis from *E. coli* LMG194 cells as reported in (b). A 3 µg quantity of protein for whole-cell extract (W), periplasmic (P), cytosolic (C) and membrane-bound (M) fractions was loaded onto gels. Molecular mass standards (kDa) are indicated on the left. The immunoblots are representative of three experiments showing similar results.

### Membrane association of PvdA

To verify the ability of PvdA to associate with the membranes *in vivo*, we performed experiments of cell fractionation in the homologous *P. aeruginosa* host. Bacteria were grown in iron-depleted DCAA medium to induce PvdA expression. Cells were spheroplasted to release the periplasmic protein fraction, and the resulting spheroplasts were lysed by sonication. Soluble (cytosolic) and membrane proteins were separated by ultracentrifugation. Isovolumes of each fraction (or appropriate dilutions) were resolved by SDS-PAGE to determine the distribution of PvdA between the cytosolic and membrane compartments of *P. aeruginosa* cells. Immunoblot analysis with an anti-PvdA monoclonal antibody showed that almost the whole cellular pool of PvdA co-sedimented with *P. aeruginosa* membranes, corroborating the membrane localization of PvdA in *P. aeruginosa* PAO1 cells (Fig. 3a). Detection of the vegetative sigma factor RpoD and the inner-membrane protein XcpY almost entirely in the cytosolic and membrane fractions, respectively, confirmed the selectivity of the fractionation procedure (Fig. 3a).

To investigate the biochemical nature of the membrane association of PvdA, membranes from *P. aeruginosa* PAO1 were treated with chemical agents that disrupt different types of protein bonding with membranes (Fig. 3b). PvdA was released from membranes by alkali (0.1 M NaOH) and denaturant (1.5 M and 5.0 M urea), both of which solubilize peripheral membrane proteins, while allowing integral membrane proteins to remain anchored to the lipid bilayer (Rosenberg, 2005 and references therein). High salt (1.0 M NaCl) treatment, which is known to affect protein–protein and protein–phospholipid electrostatic interactions (Rosenberg, 2005), caused only partial solubilization of PvdA from membranes. In contrast, the integral inner-membrane protein XcpY was not solubilized by any of these treatments, confirming that membrane integrity was preserved upon alkali, urea and salt treatments. Finally, both PvdA and XcpY were retrieved in the soluble fraction after treatment of membranes with Sarcosyl (2%), which is a detergent that selectively disrupts the inner membrane without affecting the stability of the outer membrane (Filip *et al.*, 1973), and this suggests that PvdA is localized at the inner membrane level. Overall, these results indicate that PvdA behaves as a peripheral protein, and rule out any unspecific co-sedimentation of PvdA with the *P. aeruginosa* membranes.

While *in silico* topology and PhoA fusion assays suggested that the PvdA N-terminal hydrophobic region (amino acid residues 10–30) could be responsible for PvdA association with membranes, solubilization assays argued against the existence of a canonical TM domain by which PvdA can actually cross the lipid bilayer. To verify whether PvdA is entirely exposed to the cytosolic leaflet of the inner membrane, a proteinase K accessibility assay was performed on spheroplasts obtained from iron-starved *P. aeruginosa* cells. As expected, treatment of the spheroplasts with proteinase K resulted in substantial signal reduction for the type II bitopic inner-membrane protein

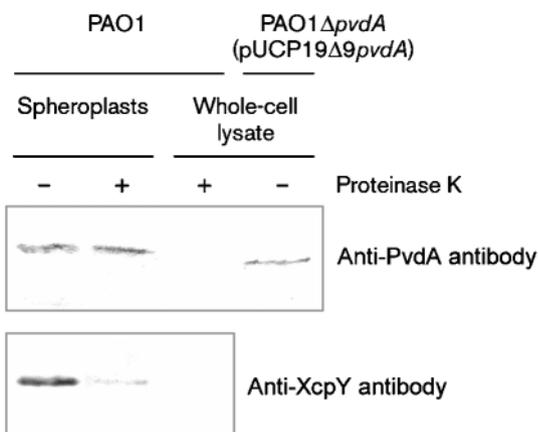


**Fig. 3.** Subcellular localization of PvdA, RpoD, and XcpY in *P. aeruginosa* PAO1. (a) Partition of PvdA, RpoD and XcpY between the cytosolic and membrane protein fractions from iron-depleted *P. aeruginosa* PAO1 cells. Twofold serial dilutions were loaded onto the gel starting from equal volumes of each protein fraction (equivalent to 5  $\mu$ g cytosolic protein), resolved by SDS-PAGE, and electroblotted. Purified full-length PvdA (30 ng) was used as positive control (Ctl). (b) Membrane samples from *P. aeruginosa* PAO1 were untreated (NT), or treated with 1.0 M NaCl, 0.1 M NaOH, 1.5 M urea, 5.0 M urea or 2% sarcosyl, as indicated. After sedimentation of the membranes, equal volumes of the supernatant (S) and membrane-retained proteins (R) were resolved by SDS-PAGE and electroblotted. Proteins were probed with antibodies directed against PvdA and XcpY, as indicated. The immunoblots are representative of three experiments showing similar results.

XcpY (Fig. 4), which exposes a large region to the periplasmic space (Bleves *et al.*, 1996). In contrast, PvdA was protected from the protease in spheroplasts, while it was completely digested upon treatment of the *P. aeruginosa* whole-cell lysate with proteinase K (Fig. 4). The SDS-PAGE mobility of PvdA from proteinase-K-treated spheroplasts was identical to that of the native protein from untreated spheroplasts, while it differed from that of the  $\Delta$ 9PvdA deletion derivative, which lacks the first 9 aa at the PvdA N terminus (see below); the  $\Delta$ 9PvdA deletion derivative was used as size control in case of N-terminal proteolysis (Fig. 4).

### A dual role for the PvdA N-terminal region

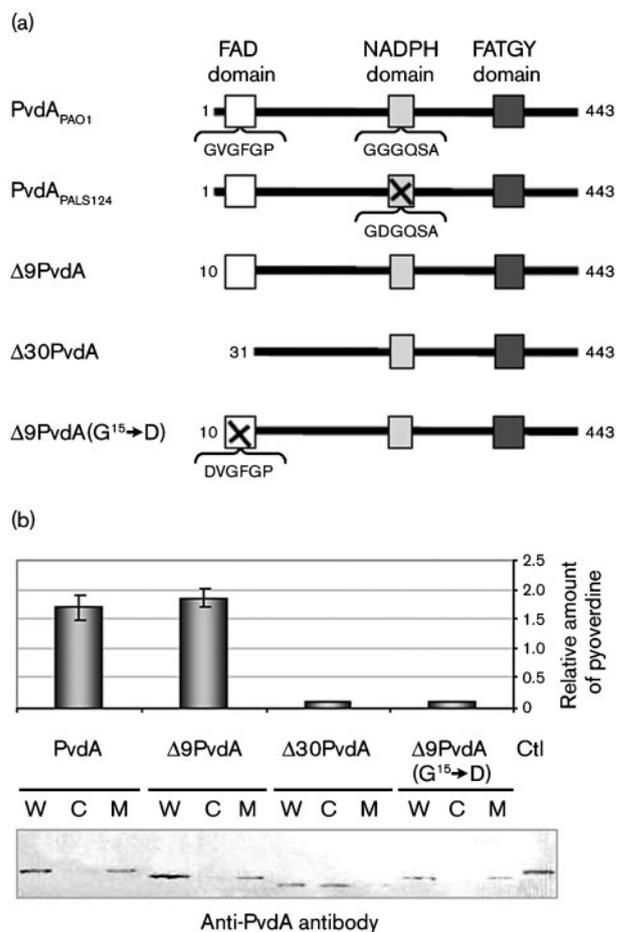
We have previously generated by chemical mutagenesis the pyoverdine defective PALS124 *P. aeruginosa* mutant, and



**Fig. 4.** Proteinase K accessibility of PvdA and XcpY in spheroplasts obtained from iron-starved *P. aeruginosa* cells. *P. aeruginosa* PAO1 spheroplasts, and whole-cell lysates of PAO1 and PAO1 $\Delta pvdA$ (pUCP19 $\Delta 9pvdA$ ) strains were treated (+) or not (-) with 50  $\mu\text{g}$  proteinase K  $\text{ml}^{-1}$ , as indicated. A 10  $\mu\text{g}$  quantity of proteins from each sample was resolved by SDS-PAGE, immunoblotted, and probed with anti-PvdA and anti-XcpY antibodies.

shown that it is unable to produce OHOrn (Visca *et al.*, 1992). We observed by immunoblotting that this mutant expressed a full-length (apparent mass approx. 49 kDa) PvdA under conditions of iron starvation (data not shown), suggesting that PvdA<sub>PALS124</sub> could carry an amino acid replacement that compromises its enzymic activity. Sequencing of the whole *pvdA* gene revealed that PALS124 carries a single mutation at codon 215 (GGC $\rightarrow$ GAC) of *pvdA*, resulting in a single amino acid substitution (G<sup>215</sup> $\rightarrow$ D) in the NADPH-binding motif (G<sup>214</sup>GGQSA<sup>219</sup> in wild-type PvdA). This finding corroborates the importance of the G residues in the dinucleotide-binding motif, in line with their role in the formation of the tight turn necessary for protein binding to the pyrophosphate group of FAD and NAD(P)H (Dym & Eisenberg, 2001).

Since the N-terminal region of PvdA appears to be endowed with both structural (membrane-interaction) and functional (FAD-binding) roles, three PvdA deletion derivatives [ $\Delta 9$ PvdA,  $\Delta 9$ PvdA(G<sup>15</sup> $\rightarrow$ D) and  $\Delta 30$ PvdA] were expressed in the PvdA-defective *P. aeruginosa* strain PAO1 $\Delta pvdA$ . The  $\Delta 9$ PvdA and  $\Delta 30$ PvdA proteins were PvdA derivatives lacking the first 9 and 30 aa at the N terminus, respectively, while the  $\Delta 9$ PvdA(G<sup>15</sup> $\rightarrow$ D) protein originated from  $\Delta 9$ PvdA by a single amino acid substitution (G $\rightarrow$ D at position 15) in the FAD-binding motif (Fig. 5a). The full-length PvdA protein was used as positive control (Fig. 5a). While all proteins were efficiently expressed in PAO1 $\Delta pvdA$  cells, only PvdA and  $\Delta 9$ PvdA were able to complement the  $\Delta pvdA$  mutation by restoring pyoverdine production. Conversely, PvdA derivatives deleted of, or mutated in, the FAD signature did not restore pyoverdine production (Fig. 5b). The subcellular



**Fig. 5.** Activity and membrane association of PvdA derivatives carrying mutations in the N-terminal region expressed in the PAO1 $\Delta pvdA$  mutant. (a) Schematic representation of PvdA derivatives used. The amino acid sequence of the FAD signature is indicated for the full-length PvdA and for  $\Delta 9$ PvdA(G<sup>15</sup> $\rightarrow$ D). The black cross represents the single amino acid mutation (G $\rightarrow$ D) in the FAD-binding motif of  $\Delta 9$ PvdA(G<sup>15</sup> $\rightarrow$ D) and in the NADPH-binding motif of PvdA<sub>PALS124</sub>. (b) A 5  $\mu\text{g}$  quantity of whole *P. aeruginosa* PAO1 $\Delta pvdA$  proteins (W), and equal volumes of cytosolic (C) and membrane (M) protein fractions (equivalent to 5  $\mu\text{g}$  of cytosolic proteins), were probed with the 3H6D12 anti-PvdA antibody. Purified full-length PvdA (10 ng) was used as positive control (Ctl). The immunoblot is representative of three experiments showing similar results. The histogram shows the amount of pyoverdine produced by each strain normalized by the number of cells ( $A_{405}/A_{600}$ ). Values are means ( $\pm$ SD) of three independent experiments.

localization of these PvdA derivatives was also investigated by means of cell fractionation experiments, and by immunoblot analysis with an anti-PvdA antibody. As shown in Fig. 5(b), both  $\Delta 9$ PvdA and  $\Delta 9$ PvdA(G<sup>15</sup> $\rightarrow$ D) sedimented with cell membranes as their native counterpart. On the other hand, a large amount of  $\Delta 30$ PvdA was detected in the soluble cytosolic fraction (Fig. 5b), indicating that deletion of the PvdA N-terminal

hydrophobic region results in a redistribution of the enzyme from the membrane to the cytosolic compartment. Taken together, these results indicate that, although PvdA retains its activity upon deletion of the nine N-terminal residues, it requires a functional FAD-binding motif for the catalytic activity. In addition, the hydrophobic region encompassing amino acid residues 10–30 is essential for membrane association of PvdA, and this confirms the dual role of the PvdA N-terminal domain.

## DISCUSSION

In past years, pyoverdine biosynthesis has been extensively studied in light of the role of this siderophore in *P. aeruginosa* virulence, initiation of infection and pathogenesis (reviewed by Visca *et al.*, 2007). It is now well established that pyoverdine synthesis relies on four non-ribosomal peptide synthetases that direct the synthesis of the peptide backbone, and on at least three accessory enzymes responsible for synthesis of the non-proteinogenic amino acid precursors. Nevertheless, the subcellular localization of pyoverdine biosynthetic enzymes remains uncertain.

Herein, we provide evidence for the membrane-bound nature of PvdA, which is a key enzyme of the pyoverdine biosynthetic pathway (Visca *et al.*, 1994, 2007). Similar to its close homologue IucD (Herrero *et al.*, 1988), PvdA possesses a major hydrophobic region overlapping the putative FAD-binding motif, and *in silico* analyses predicted a single TM domain at the N-terminal region (Fig. 1b) flanked by a charge bias rather than a cytosolic net positive charge typical of bacterial TM domains (von Heijne, 1992). On this basis, the membrane topology of PvdA was experimentally tested by means of a series of PvdA–PhoA translational fusions in the heterologous host *E. coli*. The results indicate that the 12 N-terminal residues of PvdA are able to target the reporter PhoA protein through the inner membrane into the periplasm, while the following hydrophobic residues seem to be responsible for anchoring the enzyme to the inner membrane (Fig. 2). Given the lack of other topogenic determinants, we expect the bulk of the protein to be exposed to the cytoplasm. In view of this, PvdA could have a membrane topology reminiscent of a eukaryotic reverse signal-anchor (type III) membrane protein that translocates its N-terminal end across the membrane in an  $N_{out}/C_{in}$  orientation (Goder & Spiess, 2001). According to this model, PvdA co-sediments with *P. aeruginosa* membranes in cell fractionation experiments, and its N-terminal hydrophobic region appears to be essential for membrane association (Figs 3a and 5b). However, we demonstrated that native PvdA can be released from membranes by chemical treatments that do not affect membrane stability (Fig. 3b), thus excluding the existence of a canonical TM domain in the PvdA N-terminal region. Accordingly, PvdA was resistant to treatment of *P. aeruginosa* spheroplasts with proteinase K (Fig. 4), and this suggests that PvdA lacks a protease-accessible periplasmic domain. Overall, these observations are compatible with the finding that a

recombinant soluble form of PvdA can be obtained by hexahistidine tagging the N terminus, but not the C terminus (Ge & Seah, 2006; Meneely & Lamb, 2007), and this suggests that the hexahistidine tag could provide the N-terminal domain with a hydrophilic character capable of counteracting its intrinsic hydrophobicity.

Most membrane proteins span the bilayer with long  $\alpha$ -helical stretches. However, the predicted secondary structure of the PvdA N-terminal region consists of a short  $\beta$ -sheet (from H<sup>11</sup> to V<sup>16</sup>) and an  $\alpha$ -helix (from S<sup>21</sup> to Q<sup>30</sup>) separated by two residues generally involved in  $\alpha$ -helical breaking (G<sup>19</sup> and P<sup>20</sup>). P and G residues are widely distributed in TM domains of many integral membrane proteins (Williams & Deber, 1991; Landolt-Marticorena *et al.*, 1993), and their helical propensity is greatly enhanced in lipid bilayers (Li *et al.*, 1996; Deber *et al.*, 1999). Nevertheless, the N-terminal hydrophobic region of PvdA overlaps the putative FAD-binding motif. We showed that this motif is essential for PvdA activity, since a single amino acid substitution in the FAD-binding motif of PvdA abrogated the genetic complementation (i.e. restoration of pyoverdine synthesis) in the PvdA-defective *P. aeruginosa* mutant (Fig. 5b). This confirms the crucial role of the FAD signature in PvdA activity and function.

It is difficult to reconcile the inclusion of a functional FAD signature within a structural membrane-anchoring domain. We can only speculate that the PvdA N-terminal hydrophobic domain interacts with the lipid bilayer by forming a U-shaped or re-entrant loop aided by contiguous G<sup>19</sup>–P<sup>20</sup> residues without actually crossing the membrane. Then, part of the FAD-binding domain may interact with the membrane, concomitantly leaving exposed residues to the intracellular milieu. Hence, the observed export of the 12P translational fusion into the periplasm of *E. coli* cells should plausibly be an artefact due to the lack of constraints otherwise imposed by the downstream structure(s). Notably, a similar cryptic signal peptide unmasked upon fusion to the PhoA reporter enzyme has been detected in the HMWP2 protein of *Yersinia* spp. (Guilvout *et al.*, 1995). In view of this, it is worth noting that the first 9 aa at the N terminus of PvdA are not required for either membrane association or enzymic activity (Fig. 5). The dual role of the PvdA N-terminal hydrophobic domain is very intriguing, and deserves further studies to refine residues responsible for membrane anchoring, and to elucidate the actual secondary structure of this domain.

Overall, the membrane localization for PvdA is biologically meaningful. Since PvdA is involved in iron metabolism, and requires oxygen for oxygenase activity, the membrane location would facilitate oxygen recruitment from the outer environment, and thereby contribute to the maintenance of a reducing intracellular milieu. On the other hand, the putative substrate-binding pocket (LATGY) would be localized on the cytoplasmic side, and as result it would be accessible to the substrate (Orn). To the best of our knowledge, in *P. aeruginosa*, OHOrn is exclusively

utilized for pyoverdine biosynthesis. Moreover, because of its high instability at neutral pH (Akers & Neilands, 1973), cytoplasmic OHOrn should promptly be formylated and/or sequestered into the nascent pyoverdine backbone by the pyoverdine biosynthesis machinery. In this scenario, our work provides the basis for future studies aimed at investigating the subcellular localization of the whole pyoverdine biosynthesis machinery.

## ACKNOWLEDGEMENTS

We wish to thank Dr M. Ehrmann (University of Cardiff) for critical contribution to experimental design, and for providing us with the *E. coli* strain LMG194 and the pBAD*phoA* plasmid, Dr I. J. Schalk (Université Strasbourg 1) for providing us with the anti-XcpY antibody, and E. Nebuloso (National Institute for Infectious Disease 'L. Spallanzani') for DNA sequencing. This work was supported by grants to P.V. from the Ministry for Health of Italy ('Ricerca Corrente 2006' to the National Institute for Infectious Diseases 'L. Spallanzani'), the Ministry of University and Research of Italy (PRIN-2006), and the Italian Cystic Fibrosis Research Foundation (grant FFC#10/2007), with the contribution of 'Gruppo Aziende Ferraresi', 'Delegazione FFC di Ferrara' and 'Gruppo di Sostegno FFC di Comacchio'.

## REFERENCES

- Akers, H. A. & Neilands, J. B. (1973). A hydroxamic acid present in *Rhodotorula pilimanae* cultures grown at low pH and its metabolic relation to rhodotorulic acid. *Biochemistry* **12**, 1006–1010.
- Andrews, S. C., Robinson, A. K. & Rodriguez-Quinones, F. (2003). Bacterial iron homeostasis. *FEMS Microbiol Rev* **27**, 215–237.
- Arts, J., van Boxtel, R., Filloux, A., Tommassen, J. & Koster, M. (2007). Export of the pseudopilin XcpT of the *Pseudomonas aeruginosa* type II secretion system via the signal recognition particle–Sec pathway. *J Bacteriol* **189**, 2069–2076.
- Bleves, S., Lazdunski, A. & Filloux, A. (1996). Membrane topology of three Xcp proteins involved in exoprotein transport by *Pseudomonas aeruginosa*. *J Bacteriol* **178**, 4297–4300.
- Deber, C. M., Liu, L. P. & Wang, C. (1999). Perspective: peptides as mimics of transmembrane segments in proteins. *J Pept Res* **54**, 200–205.
- Dick, S., Marrone, L., Thariath, A., Valvano, M. A. & Viswanatha, T. (1998). Cofactor- and substrate-binding domains in flavin-dependent N-hydroxylating enzymes. *Trends Biochem Sci* **23**, 414–415.
- Dym, O. & Eisenberg, D. (2001). Sequence-structure analysis of FAD-containing proteins. *Protein Sci* **10**, 1712–1728.
- Filip, C., Fletcher, G., Wulff, J. L. & Earhart, C. F. (1973). Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. *J Bacteriol* **115**, 717–722.
- Ge, L. & Seah, S. Y. (2006). Heterologous expression, purification, and characterization of an L-ornithine N<sup>5</sup>-hydroxylase involved in pyoverdine siderophore biosynthesis in *Pseudomonas aeruginosa*. *J Bacteriol* **188**, 7205–7210.
- Goder, V. & Spiess, M. (2001). Topogenesis of membrane proteins: determinants and dynamics. *FEBS Lett* **504**, 87–93.
- Guilvout, I., Carniel, E. & Pugsley, A. P. (1995). *Yersinia* spp. HMWP2, a cytosolic protein with a cryptic internal signal sequence which can promote alkaline phosphatase export. *J Bacteriol* **177**, 1780–1787.
- Guzman, L. M., Belin, D., Carson, M. J. & Beckwith, J. (1995). Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* **177**, 4121–4130.
- Hantash, F. M. & Earhart, C. F. (2000). Membrane association of the *Escherichia coli* enterobactin synthase proteins EntB/G, EntE, and EntF. *J Bacteriol* **182**, 1768–1773.
- Herrero, M., de Lorenzo, V. & Neilands, J. B. (1988). Nucleotide sequence of the *iucD* gene of the pColV-K30 aerobactin operon and topology of its product studied with *phoA* and *lacZ* gene fusions. *J Bacteriol* **170**, 56–64.
- Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J. & Schweizer, H. P. (1998). A broad-host-range FLP-FRT recombination system for site-specific excision of chromosomally located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* **212**, 77–86.
- Hopp, T. P. & Woods, K. R. (1983). A computer program for predicting protein antigenic determinants. *Mol Immunol* **20**, 483–489.
- Kyte, J. & Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J Mol Biol* **157**, 105–132.
- Landolt-Marticorena, C., Williams, K. A., Deber, C. M. & Reithmeier, R. A. (1993). Non-random distribution of amino acids in the transmembrane segments of human type I single span membrane proteins. *J Mol Biol* **229**, 602–608.
- Li, S. C., Goto, N. K., Williams, K. A. & Deber, C. M. (1996).  $\alpha$ -Helical, but not  $\beta$ -sheet, propensity of proline is determined by peptide environment. *Proc Natl Acad Sci U S A* **93**, 6676–6681.
- Manoil, C., Mekalanos, J. J. & Beckwith, J. (1990). Alkaline phosphatase fusions: sensors of subcellular location. *J Bacteriol* **172**, 515–518.
- Melchers, K., Schuhmacher, A., Buhmann, A., Weitzenegger, T., Belin, D., Grau, S. & Ehrmann, M. (1999). Membrane topology of CadA homologous P-type ATPase of *Helicobacter pylori* as determined by expression of *phoA* fusions in *Escherichia coli* and the positive inside rule. *Res Microbiol* **150**, 507–520.
- Meneely, K. M. & Lamb, A. L. (2007). Biochemical characterization of a flavin adenine dinucleotide-dependent monooxygenase, ornithine hydroxylase from *Pseudomonas aeruginosa*, suggests a novel reaction mechanism. *Biochemistry* **46**, 11930–11937.
- Michaelis, S., Inouye, H., Oliver, D. & Beckwith, J. (1983). Mutations that alter the signal sequence of alkaline phosphatase in *Escherichia coli*. *J Bacteriol* **154**, 366–374.
- Michel, G., Bleves, S., Ball, G., Lazdunski, A. & Filloux, A. (1998). Mutual stabilization of the XcpZ and XcpY components of the secretory apparatus in *Pseudomonas aeruginosa*. *Microbiology* **144**, 3379–3386.
- Neu, H. C. & Heppel, L. A. (1965). The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. *J Biol Chem* **240**, 3685–3692.
- Plattner, H. J., Pfefferle, P., Romaguera, A., Waschutza, S. & Diekmann, H. (1989). Isolation and some properties of lysine N<sup>6</sup>-hydroxylase from *Escherichia coli* strain EN222. *Biol Met* **2**, 1–5.
- Putignani, L., Ambrosi, C., Ascenzi, P. & Visca, P. (2004). Expression of L-ornithine Ndelta-oxygenase (PvdA) in fluorescent *Pseudomonas* species: an immunochemical and *in silico* study. *Biochem Biophys Res Commun* **313**, 245–257.
- Robles-Price, A., Wong, T. Y., Sletta, H., Valla, S. & Schiller, N. L. (2004). AlgX is a periplasmic protein required for alginate biosynthesis in *Pseudomonas aeruginosa*. *J Bacteriol* **186**, 7369–7377.
- Rosenberg, I. M. (2005). *Protein Analysis and Purification: Benchtop Techniques*, 2nd edn. Cambridge, MA: Birkhauser Boston.

- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schweizer, H. P. (1991).** *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. *Gene* **97**, 109–121.
- Seth, O., Smau, L., Welte, W., Ghisla, S., Stehr, M., Diekmann, H. & Macheroux, P. (1998).** A reply to Dick *et al.* *Trends Biochem Sci* **23**, 414–415.
- Stehr, M., Diekmann, H., Smau, L., Seth, O., Ghisla, S., Singh, M. & Macheroux, P. (1998).** A hydrophobic sequence motif common to *N*-hydroxylating enzymes. *Trends Biochem Sci* **23**, 56–57.
- Thariath, A., Socha, D., Valvano, M. A. & Viswanatha, T. (1993).** Construction and biochemical characterization of recombinant cytoplasmic forms of the IucD protein (lysine:N6-hydroxylase) encoded by the pColV-K30 aerobactin gene cluster. *J Bacteriol* **175**, 589–596.
- Tiburzi, F., Imperi, F. & Visca, P. (2008).** Intracellular levels and activity of PvdS, the major iron starvation sigma factor of *Pseudomonas aeruginosa*. *Mol Microbiol* **67**, 213–227.
- Visca, P., Serino, L. & Orsi, N. (1992).** Isolation and characterization of *Pseudomonas aeruginosa* mutants blocked in the synthesis of pyoverdine. *J Bacteriol* **174**, 5727–5731.
- Visca, P., Ciervo, A., Sanfilippo, V. & Orsi, N. (1993).** Iron-regulated salicylate synthesis by *Pseudomonas* spp. *J Gen Microbiol* **139**, 1995–2001.
- Visca, P., Ciervo, A. & Orsi, N. (1994).** Cloning and nucleotide sequence of the *pvdA* gene encoding the pyoverdine biosynthetic enzyme L-ornithine  $N^{\beta}$ -oxygenase in *Pseudomonas aeruginosa*. *J Bacteriol* **176**, 1128–1140.
- Visca, P., Imperi, F. & Lamont, I. L. (2007).** Pyoverdine siderophores: from biogenesis to biosignificance. *Trends Microbiol* **15**, 22–30.
- von Heijne, G. (1992).** Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. *J Mol Biol* **225**, 487–494.
- Williams, K. A. & Deber, C. M. (1991).** Proline residues in transmembrane helices: structural or dynamic role? *Biochemistry* **30**, 8919–8923.

---

Edited by: P. Cornelis