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Characterization of a novel two-partner secretion system implicated in the virulence of *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is an opportunistic human pathogen implicated in nosocomial infection and infecting people with compromised immune systems such as cystic fibrosis patients. Although multiple genes involved in *P. aeruginosa* pathogenesis have been characterized, the overall mechanism of virulence is not fully understood. In this study, we identified a functional two-partner secretion (TPS) system, composed of the PdtA exoprotein and its cognate pore-forming β-barrel PdtB transporter, which is implicated in the virulence of *P. aeruginosa*. We found that the predicted PdtA exoprotein is related to the HMW-like adhesins subfamily TPS systems. We demonstrate here that limitation of inorganic phosphate (Pi) allows the production of PdtA protein. We show that PdtA is processed during its outer-membrane translocation, with an N-terminal domain released into the extracellular environment and a C-terminal domain associated with the outer membrane of the cell. We also obtained evidence that the transport of PdtA is strictly dependent on the production of PdtB, a result confirming that these proteins constitute a functional TPS system. Furthermore, using the *Caenorhabditis elegans* model of infection, we show that a *pdtA* mutant is less virulent than the wild-type strain.

INTRODUCTION

Secretion of proteins to the surface and outside of cells is ensured by many sophisticated machineries, including two-partner secretion (TPS) systems. These systems are widespread among Gram-negative bacteria and are dedicated to translocate large, predominantly β-helical, exoproteins (TpsA) across the outer membrane of the cell by a β-barrel, channel-forming transporter (TpsB). Both partners usually contain a canonical N-terminal signal peptide that targets them to the Sec pathway for export across the inner membrane (Jacob-Dubuisson *et al.*, 2001). TpsB transporters belong to the Omp85/TpsB superfamily, including the *Neisseria meningitidis* Omp85 outer-membrane protein (Voulhoux *et al.*, 2003) and the eukaryotic Toc75 membrane protein, which insert proteins into the outer membranes of chloroplasts (Schleiff & Soll, 2005). TpsB C-terminal domains form transmembrane β-barrel structures, while the N-terminal regions contain two periplasmic polypeptide-transport associated (POTRA) domains, which are thought to be involved in the recognition and movement of substrates, and initiation of folding. TpsA exoproteins share amino acid similarity within a region called the TPS domain, corresponding to the first 250 residues of the proteins (Mazar & Cotter, 2007). TPS domains are required for translocation and have been shown to interact with POTRA domains of their cognate TpsB transporters (Grass & St Geme, 2000; Renauld-Mongénie *et al.*, 1996). Apart from the well conserved TPS domain, TpsA proteins display low similarity at the amino acid level but all TpsA proteins are predicted to contain numerous β-helical folds (Thanassi *et al.*, 2005). Moreover, sequence comparison of TpsA exoproteins allows the classification into at least four subfamilies with distinct functions: (i) haemolysin/cytolysins such as ShlA of *Serratia marcescens* (Braun *et al.*, 1992), (ii) contact-dependent growth inhibition (Cdi) factors such as CdiA of *Escherichia coli* (Aoki *et al.*, 2005), (iii) adhesins like (iiia) FHA of *Bordetella pertussis* (Relman *et al.*, 1989) or (iiib) HMW1 of *Haemophilus influenzae* (St Geme & Yeo, 2009). With the various functions played by TpsA proteins, TPS systems represent a major virulence determinant for many Gram-negative pathogens (Jacob-Dubuisson *et al.*, 2001).

Two TPS systems have been characterized in the human opportunistic pathogen *Pseudomonas aeruginosa* whose pathogenicity relies on a myriad of secreted proteins (Bleves *et al.*, 2010) and on its growth as biofilms (Flemming & Wingender, 2010). Indeed, the large extracellular protease LepA, secreted by its LepB transporter, modulates host-response through its trypsin-like serine

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Abbreviations: Pi, inorganic phosphate; TPS, two-partner secretion.

The *PA0690* (*pdtA*) and *PA0692* (*pdtB*) gene sequences and corresponding protein sequences are available from the *Pseudomonas* Genome Database (http://www.pseudomonas.com).

protease activity towards human protease-activated receptors (Kida *et al.*, 2008). LepA also contributes to virulence and growth of *P. aeruginosa* in the host in cooperation with the haemolytic phospholipase C (PlcH) (Kida *et al.*, 2011). The second TPS system, composed of CdrA and CdrB proteins, is induced by cyclic-di-GMP, and the extracellular adhesin CdrA is important for localization of the Psl polysaccharide in biofilms, thus maintaining the integrity of these communities (Borlee *et al.*, 2010). On the basis of homology with TPS components in other organisms, two *P. aeruginosa* PA01 genes, *PA0690* and *PA0692*, have been predicted to encode a putative novel TPS system, composed of a large TpsA exoprotein and its TpsB transporter, respectively (Winsor *et al.*, 2011). We have shown recently that *PA0690* and *PA0692* belong to a 25-gene regulon, which is positively regulated by the σ^{VreI} factor under low phosphate conditions through the response regulator PhoB (Faure *et al.*, 2013). σ^{VreI} factor seems to be involved in the control of *P. aeruginosa* virulence (Llamas *et al.*, 2009), but no link has been made with the product of the genes that it controls.

In the present work, we initiated a study on σ^{VreI}-regulon genes by focusing on *PA0690* and *PA0692*. We have designated PA0690 and PA0692 as PdtA (phosphate depletion regulated TPS partner A) and PdtB (phosphate depletion regulated TPS partner B), respectively, based on their regulation by phosphate limitation and sequence homologies with TPS proteins. We used cell fractionation experiments to define localization of PdtA. Herein, we demonstrated that PdtA is proteolytically processed and secreted outside the cell. We further showed that a *pdtB* mutant is unable to secrete PdtA in the supernatant, which strongly suggests that PdtA/PdtB constitutes a TPS system. Finally, we showed that PdtA is implicated in *P. aeruginosa* PAO1 virulence against the *Caenorhabditis elegans* model of infection.

METHODS

Bacterial strains and culture conditions. Strains used in this study are listed in Table 1. Strains were grown at 37 °C in low inorganic phosphate (Pi) medium (Faure *et al.*, 2013). For high Pi medium, 10 mM KH_2PO_4 was added to the low Pi medium. *E. coli* strains were used to propagate plasmids. Plasmids were introduced in *P. aeruginosa* using the conjugative properties of pRK2013 (Figurski & Helinski, 1979) or by electroporation (Choi & Schweizer, 2006). *P. aeruginosa* transconjugants were screened on *Pseudomonas* isolation agar (PIA; Difco) supplemented with appropriate antibiotics. The following antibiotic concentrations were used. For *E. coli*: 50 μg ampicillin (Ap) ml^{-1}; 50 μg streptomycin (Sm) ml^{-1}; 25 μg tetracycline (Tc) ml^{-1}; 25 μg kanamycin (Km) ml^{-1}; 15 μg gentamicin (Gm) ml^{-1}. For *P. aeruginosa*: 300 μg carbenicillin (Cb) ml^{-1}; 2 mg Sm ml^{-1}; 75 μg Gm ml^{-1}.

Molecular cloning and plasmids. Plasmids and primers are listed in Tables 1 and 2, respectively. PCR amplifications from genomic *P. aeruginosa* PAO1 DNA were performed using Expand High Fidelity DNA polymerase (Roche). Oligonucleotides were synthetized by Eurogentec. Plasmid pJN105-pdtB was constructed by cloning the *pdtB* gene with an artificial Shine–Dalgarno sequence (AGGAGGT) as an *Xba*I–*Sac*I PCR fragment into pJN105 vector (Newman & Fuqua, 1999). Plasmid pKNG202-pdtA-FLAG was constructed by first inserting FLAG-epitope-containing annealed oligonucleotides as an *Nhe*I–*Xho*I DNA fragment into pKNG202 (Spagnolo *et al.*, 2011), leading to the pKNG202-FLAG vector. Two *Sal*I–*Nhe*I and *Xho*I–*Bgl*II DNA fragments corresponding to, respectively, upstream and downstream regions of the *pdtA* gene stop codon were then PCR amplified and cloned into pKNG202-FLAG. All constructs were confirmed by DNA sequencing and transferred from *E. coli* to *P. aeruginosa* by triparental mating.

Bacterial strain constructions. The *pdtA* and *pdtB* mutant strains were obtained by first constructing the pKNG101-pdtA and pKNG101-pdtB suicide vectors as follows. Two DNA fragments corresponding to, respectively, upstream and downstream regions of the gene of interest were PCR amplified. The resulting DNA fragments were then used as templates for an overlapping PCR run using the external pair of oligonucleotides. The PCR fragments containing the internal deletion of the gene of interest were digested and cloned into the *Spe*I/*Bam*HI sites of the suicide pKNG101 vector (Kaniga *et al.*, 1991). Once verified by DNA sequencing, pKNG101 recombinant plasmids were transferred from *E. coli* CC118λ*pir* to *P. aeruginosa* PAO1 by triparental mating using the helper plasmid pRK2013 as described by Kaniga *et al.* (1991), thus leading to the Δ*pdtA* and Δ*pdtB* strains. The *vreI* gene placed under control of the P_{LAC} promoter (Faure *et al.*, 2013) was inserted by electroporation into wild-type and PdtA-FLAG strains using a Tn*7*-based integration vector (Choi & Schweizer, 2006). The gentamicin marker was removed by Flp-mediated excision as described by Hoang *et al.* (1998, 2000).

Separation of supernatant and cell fractions. Overnight cultures of each strain were diluted to OD_{600}=0.05 in Pi medium supplemented with 0.1 % L-arabinose or 1 mM IPTG when necessary and grown to OD_{600} ~0.6. Whole-cell lysates and proteins concentrated from supernatants were prepared as follows. Bacterial cells were harvested by centrifugation at 4 °C, 5215 ***g***, for 20 min, and cell pellets were resuspended in loading buffer. The supernatant fraction was then subjected to a second high-speed centrifugation at 20 442 ***g*** for 30 min. Proteins from culture supernatants were then precipitated with 12 % (w/v) TCA, washed with acetone, and resuspended in loading buffer. The protein samples were then heated to 95 °C for 10 min, and separated by SDS-PAGE and immunoblotting.

P. aeruginosa* fractionation.** The bacterial culture equivalent of 1.2 OD_{600} units was resuspended in 10 mM Tris/HCl (pH 8.0), 20 % sucrose and then disrupted by sonication. Unbroken cells were removed by low-speed centrifugation at 1600 ***g at 4 °C for 15 min before separation of the cytoplasm and membrane fractions by ultracentrifugation (45 min at 120 000 ***g***). Cytoplasmic and periplasmic proteins in the supernatant were precipitated with 12 % (w/v) TCA, washed with acetone, and resuspended in loading buffer. Inner- and outer-membrane protein pellets were resuspended in loading buffer.

Isolation and separation of *P. aeruginosa* membranes by sucrose density gradient centrifugation. Inner and outer membranes were separated using discontinuous sucrose gradient sedimentation (Ize *et al.*, 2014). A volume of cells equivalent to 250 OD_{600} units was centrifuged and the pellet was resuspended in 1.5 ml 10 mM Tris/HCl (pH 7.4), 1 × protease inhibitor cocktail (cOmplete EDTA-free; Roche), 10 μg RNase ml^{-1}, 10 μg DNase ml^{-1}, 20 % (w/w) sucrose and lysed by French press treatment. Total membranes were recovered by ultracentrifugation at 120 000 ***g*** at 4 °C for 45 min and resuspended in 0.5 ml 10 mM Tris/HCl (pH 7.4), 5 mM EDTA, 1 × protease inhibitor cocktail, 20 % sucrose. The membrane fraction was then loaded on the top of a discontinuous sucrose gradient composed of 1.5 ml each of 55, 50, 45, 40, 35 and 30 % sucrose solutions (from bottom to the top). Gradients were ultracentrifuged

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics	Source
Strains		
E. coli		
Top10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *ϕ*80*lacZ*ΔM15 Δ*lacX74 nupG recA1 araD139* Δ(*ara-leu*)*7697 galE15 galK16 rpsL*(Sm^R) *endA1* λ^-	Invitrogen
SM10	*thi-1 thr leu tonA acy supE recA*::RP4-2-Tc::Mu (Km^R)	Lab collection
CC118*λpir*	*araD139* Δ(*ara leu*)*7697* Δ*lacX74 phoA20 galE galK thi rpsE rpoB argE*(*Am*) *recA1 λpir* lysogen	Herrero *et al.* (1990)
P. aeruginosa		
PAO1	Wild-type, prototroph	Lab collection
P*pdtA–lacZ*	PAO1 *attB*::miniCTX-P*pdtA-lacZ*	Faure *et al.* (2013)
Δ*pdtB* P*pdtA–lacZ*	Δ*pdtB attB*::miniCTX-P*pdtA-lacZ*	This study
Δ*pdtA*	Chromosomal *pdtA* deletion in PAO1	This study
Δ*pdtB*	Chromosomal *pdtB* deletion in PAO1	This study
PdtA-FLAG	Chromosomal PdtA with a C-terminal FLAG	This study
P_{LAC}-vreI	PAO1 *att*Tn*7*::miniTn*7*-P_{LAC}-vreI	This study
PdtA-FLAG P_{LAC}-vreI	Chromosomal C-terminally FLAG-tagged PdtA *att*Tn*7*::miniTn*7*-P_{LAC}-vreI	This study
PdtA-FLAG Δ*pdtB* P_{LAC}-vreI	Chromosomal C-terminally FLAG-tagged PdtA Δ*pdtB att*Tn*7*::miniTn*7*-P_{LAC}-vreI	This study
Plasmids		
pRK2013	ColE1 *ori tra*$^+$ *mob*$^+$, Km^R	Figurski & Helinski (1979)
pCR2.1	TA cloning vector for PCR products; *lacZα* ColE1 f1 *ori*, Ap^R Km^R	Invitrogen
miniCTX-*lacZ*	Tcr *lacZ*$^+$; self-proficient integration vector with tet, V-*FRT*-*att*PMCS, *ori int* and *oriT*, Tc^R	Lab collection
miniCTX-P*pdtA–lacZ*	*pdtA* promoter fragment cloned upstream of *lacZ* gene in miniCTX-*lacZ*; Tc^R	Faure *et al.* (2013)
pKNG101	Suicide vector in *P. aeruginosa*; *sacB*, Sm^R	Kaniga *et al.* (1991)
pKNG101-Δ*pdtA*	*pdtA* deletion construct; *sacB*, Sm^R	This study
pKNG101-Δ*pdtB*	*pdtB* deletion construct; *sacB*, Sm^R	This study
pKNG202	Suicide vector in *P. aeruginosa*; *sacB*, Sm^R	Spagnolo *et al.* (2011)
pKNG202-FLAG	pKNG202 vector carrying a FLAG in *Nhe*I–*Xho*I; *sacB*, Sm^R	This study
pKNG202-pdtA-FLAG	PdtA with a C-terminal FLAG; *sacB*, Sm^R	This study
pFLP2	Site-specific excision vector, Ap^R	Hoang *et al.* (1998)
pTNS3	Tn*7* insertion helper plasmid, Ap^R	Choi & Schweizer (2006)
pUC18-mini-Tn7T-LAC	pUC18 vector; construction of strains with chromosomal P_{LAC} expression cassette; Ap^R Gm^R	Choi & Schweizer (2006)
pUC18-miniTn7T-LAC-vreI	pUC18-miniTn*7*T-LAC vector carrying in *BamH*I–*Spe*I the 1.3 kb PCR fragment containing the *vreI* gene; Ap^R Gm^R	Faure *et al.* (2013)
pJN105	*araC*-P_{BAD} cassette cloned in pBBR1MCS-5; Gm^R	Newman & Fuqua (1999)
pJN105-pdtB	*pdtB* gene cloned into pJN105; Gm^R	This study

Ap^R, Km^R, Sm^R, Tc^R, Gm^R, resistance to ampicillin, kanamycin, streptomycin, tetracycline and gentamicin, respectively.

at 274 000 ***g*** at 4 °C for 48 h, and 500 µl fractions were collected from the top.

Proteinase K accessibility. The bacterial culture equivalent of 1.2 OD_{600} units was resuspended in 1 ml 20 mM Tris/HCl (pH 8.0), 10 mM $MgCl_2$ and treated with proteinase K for 5 min on ice, collected by centrifugation at 2375 ***g*** for 5 min, and then analysed by SDS-PAGE and immunoblotting.

Western blot analysis. After transfer onto nitrocellulose membrane, immunoblots were probed with primary antibodies and goat secondary antibodies and revealed with a Super Signal Chemiluminescence system (Thermo Scientific Pierce). To generate anti-PdtA antibody, a peptide (aa 893–908) was synthesized; rabbit polyclonal antibodies against PdtA protein were obtained by immunization of rabbits with this peptide (Eurogentec). Anti-XcpY and anti-LapA polyclonal antibodies were from our laboratory collection while the anti-FLAG monoclonal antibody (clone M2; Sigma-Aldrich) and anti-EF-Tu (Hycult Biotech) were purchased as indicated.

***β*-Galactosidase activity assay.** Strains carrying the P*pdtA–lacZ* transcriptional fusion were grown with agitation at 37 °C in Pi medium and after 300 min 1 ml of culture was harvested. To test activity in the *C. elegans* killing assay, P*pdtA–lacZ* strain was spotted on the centre of nematode growth medium (NGM) (Brenner, 1974) and NGM-Pi plates which were incubated overnight at 25 °C. Bacterial spots were then resuspended in 1 ml LB. The *β*-galactosidase activity was measured and normalized for the cell density as described by Miller (1972). Each assay was run at least in triplicate and the data given are the means.

***C. elegans* killing assay.** Slow killing assays were performed as described previously with modifications as noted below (Sana *et al.*,

Table 2. Oligonucleotides used in this study

Name	Sequence 5′ to 3′	Description
Gene deletion constructs		
SB49	agtactagtTTCGTCAACCCGAACGCAGG	Fw primer *pdtA* mutant with *Spe*I site
SB50	**ggatcagGGCGAAG**GGGTCCTTGAGCA	Rv and overlapped primer *pdtA* mutant
SB51	**cttcgccCTGATCC**TGTAAGGCACCGCC	Fw and overlapped primer *pdtA* mutant
SB52	ttaggatccCGATAGGCGCTGATCAAGTTCC	Rv primer *pdtA* mutant with *Bam*HI site
SB53	AGTACTAGTTCCACGCGCTGCTGCTGCAA	Fw primer *pdtB* mutant with *Spe*I site
SB54	**GTCAGCGACGGTCC**CCCGAAGTTCCAT	Rv and overlapped primer *pdtB* mutant
SB55	**GGACCGTCGCTGAC**CCCGGAGTAATCC	Fw and overlapped primer *pdtB* mutant
SB79	TTAGGATCCGCGCACCGTCGAAGTGATAG	Rv primer *pdtB* mutant with *Bam*HI site
Complementation with pJN105-pdtB		
SB122	AGTTCTAGAAGGAGGTGACCGTGTGAAGCGTTCGCT	Amplification *pdtB* gene with *Xba*I/*Sac*I
SB123	ATTGAGCTCTCAGAAGCTCGCCTGCACGC	
C-terminal FLAG of PdtA		
SB200	ctagcGACTACAAAGACCATGACGGTGATTATAAA-GATCATGATATCGACTACAAAGATGACGACGATAAAc	Fw primer FLAG
SB201	ctcgagTTTATCGTCGTCATCTTTGTAGTCGATATCAT-GATCTTTATAATCACCGTCATGGTCTTTGTAGTCg	Rv primer FLAG
SB207	TTAGTCGACCAACCCGATTCCCGAAGT	3′ end *pdtA* gene amplification
SB208	TGTGCTAGCCAGGATCAGGTTGCCGCGCT	
SB209	ATTCTCGAGTAAGGCACCGCCGCGGA	488 bp amplification downstream *pdtA* gene
SB210	TGTAGATCTTGCGTGCGGCTTCGAC	

Fw, forward; Rv, reverse; restriction sites are underlined; complementary regions are in bold type.

2012). Nematode growth medium minus phosphate (NGM−Pi) plates were made by removing potassium phosphate buffer from the NGM medium and adding 25 mM KCl; the pH of the medium was adjusted with 0.2 M HCl to pH 6.0. L4 to adult stage *C. elegans* were removed from food and placed on unseeded NGM−Pi plates for 24 h at 25 °C. Fifty adult-stage *C. elegans* were picked onto NGM−Pi plates containing overnight growth of each *P. aeruginosa* strain and the number of living versus dead or paralysed worms was scored every 24 h for 12 days. Each strain was tested in triplicate. Animal survival was plotted using PRISM 6.0 for the Macintosh computer program. Survival curves were considered significantly different from the control when P values were <0.05. PRISM calculates survival fractions using the product limit (Kaplan–Meier) method. PRISM compares survival curves by two methods: the log-rank test and the Gehan Breslow Wilcoxon test.

Bioinformatic analysis. The *PA0690* (*pdtA*) and *PA0692* (*pdtB*) gene and corresponding protein sequences are available from the *Pseudomonas* Genome Database (http://www.pseudomonas.com) website (Winsor *et al.*, 2011). The amino acid sequences were analysed with SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP), Interproscan4 (http://www.ebi.ac.uk/Tools/pfa/iprscan), PSIpred (http://bioinf.cs.ucl.ac.uk/psipred), and Phyre (http://www.sbg.bio.ic.ac.uk/phyre2). A phylogenetic tree was generated using the neighbour-joining and JTT matrix-based methods in the MEGA6 software (Tamura *et al.*, 2013).

RESULTS

Bioinformatic analyses suggest that PA0690 and PA0692 belong to a two-partner secretion system

The *Pseudomonas* Genome Database website has predicted PA0690 and PA0692 as hypothetical proteins (Winsor *et al.*, 2011), encoded by two genes that are separated by the *phdA* gene. PhdA is a homologue of the Phd (prevent-host-death) family of proteins implicated in biofilm development (Petrova *et al.*, 2011; Fig. 1a). Eleven putative orthologues of PA0690 are found in different *P. aeruginosa* strains and this protein is produced during infection as antibodies against PA0690 are found in serum of infected *P. aeruginosa* patients (Llamas *et al.*, 2009). To gain insight into the potential function of PA0690 and PA0692, we first performed a bioinformatic analysis. PA0690 has been predicted to encode a 430 kDa exoprotein, with a signal peptide of 38 residues (Fig. 1a). A search for conserved domains revealed the presence of an N terminus haemagglutination activity domain (aa 133 to 248), conserved in the TPS domain of TpsA exoproteins (Kajava *et al.*, 2001; Makhov *et al.*, 1994), and a C terminus conserved sequence domain (aa 3971 to 4081) usually found in association with the haemagglutination activity domain in a number of bacterial filamentous haemagglutinins (Fig. 1a). The remainder of the PA0690 sequence showed no similarity to any database entry. However, the predicted secondary structure showed that PA0690 is rich in β-sheets (data not shown), which suggests that this protein potentially adopts a β-helical folding, a characteristic structure found in other TpsA exoproteins secreted by TPS systems. Phylogenetic analysis indicated that PA0690 belongs to one of the four distinct TpsA exoprotein subfamilies composed of haemolysins with ShlA (*S. marcescens*), contact-dependent growth inhibitors with CdiA (*E. coli*), adhesins with FHA (*B. pertussis*) and HMW1 (*H. influenzae*) (Fig. 1b). The TPS domain of PA0690 is most closely related to the TPS domains of the HMW-like adhesion subfamily.

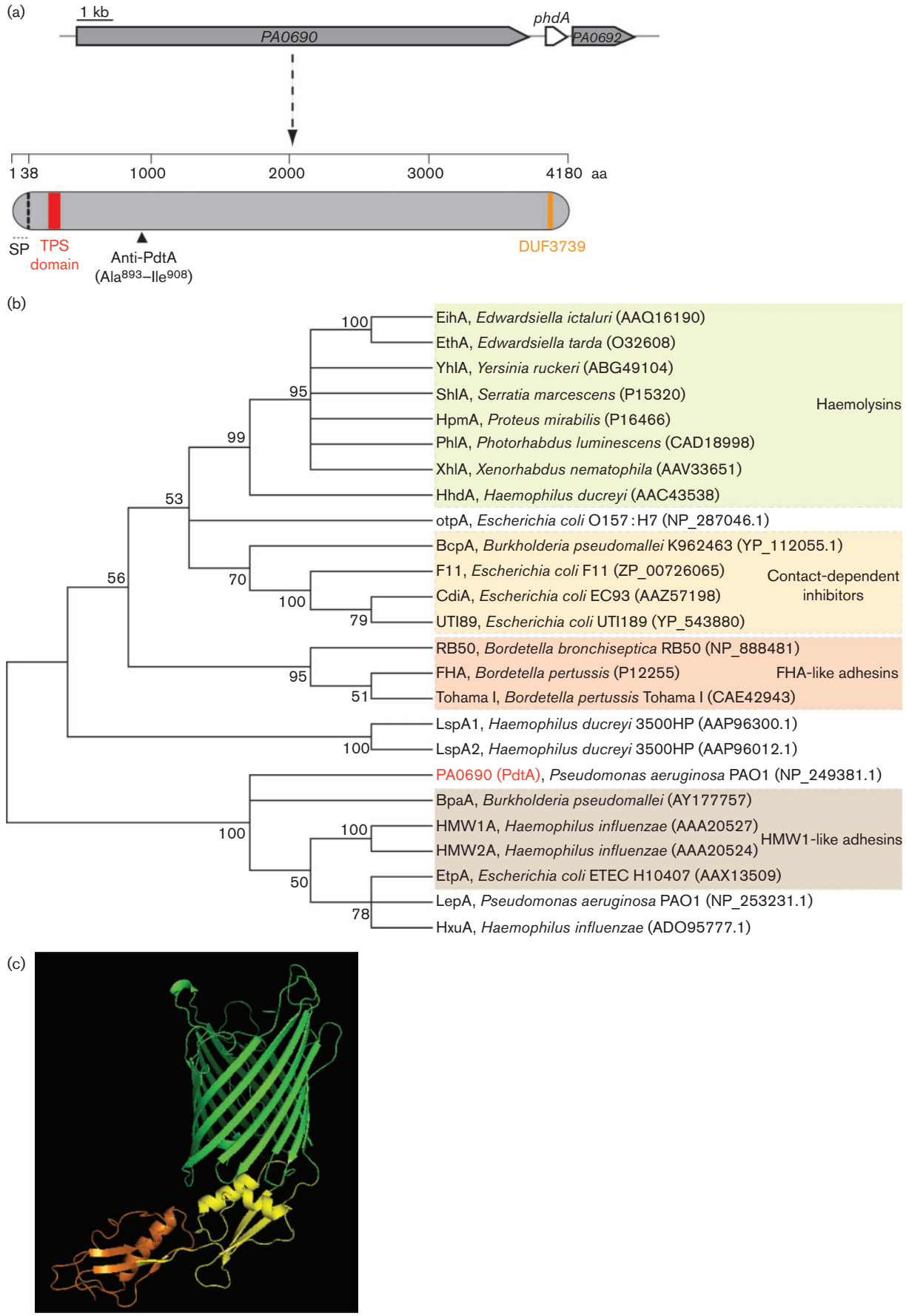

Fig. 1. Computational analyses of PA0690 and PA0692 proteins. (a) Chromosomal locus of *PA0690*/*PA0692* genes and schematic representation of PA0690 (PdtA) protein. The conserved TPS domain is shown in red and represents the PF05860 haemagglutination activity domain. DUF3739, in orange, is found in bacteria, and is approximately 110 aa in length. The DUF3739 family is found in association with PF05860. The region corresponding to the predicted signal peptide (SP) and the location of the peptide (residues Ala893–Ile908) used to generate anti-PdtA polyclonal antibodies are indicated. (b) Phylogenetic tree showing the relationship between TPS domains of PA0690 and representative TpsA exoproteins. Accession numbers are shown in parentheses. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree, number of amino acid substitutions per site. Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. All positions containing gaps and missing data were eliminated. There were a total of 110 positions in the final dataset. (c) Tertiary structure prediction of PA0692 protein using Phyre software. β-Barrel structure is represented in green and the two POTRA domains are coloured in yellow and orange.

The *PA0692* gene is predicted to encode a 59 kDa outer-membrane protein, belonging to the Omp85/TpsB transporter family, with a signal peptide of 31 residues. The PA0692 3D model, based on the structure of FhaC (TpsB from *B. pertussis*; Clantin *et al.*, 2007), revealed a clear two-domain organization (Fig. 1c). The C-terminal domain is predicted to form a β-barrel structure similar to FhaC, which forms a channel in the outer membrane (Jacob-Dubuisson *et al.*, 1999). The N-terminal domain contains two predicted POTRA domains, which are thought to be involved in the recognition of its TpsA partner in the periplasm (Delattre *et al.*, 2011). On the basis of homologies with components of TPS systems and the fact that PA0690/PA0692 gene expression is positively regulated by Pi limitation (Faure *et al.*, 2013), we renamed PA0690 as PdtA (phosphate depletion regulated TPS partner A) and PA0692 as PdtB (phosphate depletion regulated TPS partner B).

After processing, PdtA is associated with cells and secreted in the supernatant

PdtA homologies with TPS systems indicated that the protein is secreted and/or cell-surface associated. To investigate the localization of PdtA, we generated polyclonal antibodies against the N-terminal part of PdtA (anti-PdtA; Fig. 1a) and, additionally, we replaced the *pdtA* gene at its locus on the chromosome by a copy encoding a C-terminally FLAG-tagged PdtA protein. Thus the N- and C-terminal regions could be followed simultaneously using the appropriate antibodies. As expression of the *pdtA* gene is induced under low Pi growth conditions (Faure *et al.*, 2013), we determined the expression pattern along the growth curve. We observed that *pdtA* expression occurs in the exponential phase (Fig. 2a), and localization experiments were then conducted with cultures grown to an OD_{600} ~0.6, when *pdtA* is highly expressed (Fig. 2a). Using anti-PdtA antibody, a polypeptide with a considerably slower mobility than the 250 kDa molecular mass marker was detected in the whole cell fraction, probably corresponding to the full-length form of PdtA (PdtA-FL) after cleavage of the signal peptide (~426 kDa), since no band appeared with the Δ*pdtA* mutant (Fig. 2b). In addition, this polypeptide was not detected after addition of Pi to the growth medium (Fig. 2b). As a control, the inner-membrane XcpY protein was found under all conditions tested. These results show that the production of PdtA is regulated by the concentration of phosphate.

Processing during or after translocation of TpsA exoproteins is frequent. No band smaller than the PdtA-FL, corresponding to an N-terminal processed form of PdtA, was detected in whole cells using the anti-PdtA antibody (Fig. 2b). However, this antibody could not detect a C-terminal processed version of PdtA. In order to determine the potential processing of PdtA, we examined the localization of the C-terminally FLAG-tagged PdtA protein (Fig. 2c). Interestingly, two forms of PdtA were detected within whole cells expressing PdtA-FLAG using an anti-FLAG antibody, the unprocessed full-length form (PdtA-FL) and a smaller processed version of PdtA (~115 kDa; Fig. 2c, PdtA-C) corresponding to the C-terminal part of the protein. As this band was also observed in the wild-type strain with an anti-PdtA antibody raised against the C-terminal domain of PdtA (gift from M. Llamas, Spanish Council for Scientific Research, data not shown), we ruled out the possibility of an unspecific processing due to the presence of the FLAG epitope. Notably, both forms appeared cell-associated and no protein was detected in the secreted fraction (data not shown). We hypothesize that the untagged N-terminal processed form of PdtA could be rapidly degraded in cells or released into the culture supernatant in an insufficient amount to be detected. As the detection level of PdtA showed variability among experiments, probably due to its high molecular mass, we favoured the second hypothesis. To overcome this potential detection problem, we increased the quantity of σ^{VreI} factor to overexpress *pdtA* gene expression (Faure *et al.*, 2013). In order to do this, we introduced the *vreI* gene under the control of the P_{LAC} promoter at the *glmS* locus of PAO1 strain using a Tn*7*-based vector (Choi & Schweizer, 2006). Under conditions of PdtA overproduction, a smaller form than the full-length PdtA (PdtA-sf) was detected in culture supernatant using the N-terminal anti-PdtA but not with an anti-FLAG antibody (Fig. 2d and data not shown). This secretion is specific and not simply due to cell lysis as the cytoplasmic EF-Tu protein is only found in the whole cell fraction and LapA, the main substrate of the type II Hxc secretion system, is detected within the secreted fraction.

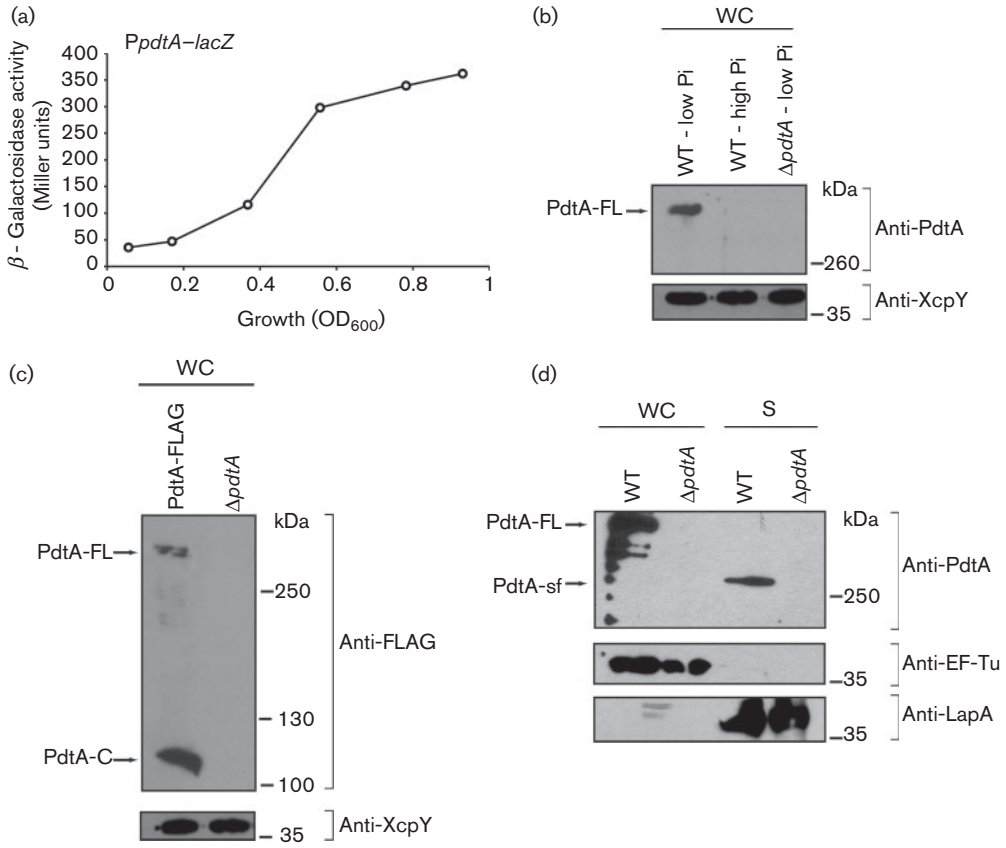

Fig. 2. Localization of PdtA protein in whole-cell and supernatant fractions. (a) P*pdtA–lacZ* strain, in which the *pdtA* promoter is fused to *lacZ*, was grown in low Pi medium and analysed for β-galactosidase activity at different growth times. (b) PdtA is produced under low Pi growth conditions. Whole cells from *P. aeruginosa* PAO1 and *pdtA* mutant strains grown to OD_{600} ~0.6 in low or high Pi medium were analysed. Samples equivalent to 0.25 OD_{600} units were loaded on 6 % acrylamide SDS-PAGE gels and subjected to immunodetection with anti-PdtA directed against the N terminus of PdtA. As control, samples of 0.1 OD_{600} units were loaded on 12 % acrylamide SDS-PAGE gels for immunoblotting with anti-XcpY-specific antiserum (lower panel). (c) PdtA is processed. PdtA-FLAG PAO1 strain, with a C-terminally FLAG-tagged PdtA, was grown in low Pi to OD_{600} ~0.6 and 0.25 OD units or 0.1 OD_{600} units of whole cells were loaded on 6 or 12 % acrylamide SDS-PAGE gels for immunoblotting with anti-FLAG (upper panel) and anti-XcpY (lower panel), respectively. (d) PdtA is secreted. Whole cells and supernatant were separated from P_{LAC}-vrel PAO1, with an IPTG-inducible *vrel* gene at the *glmS* locus, and its isogenic *pdtA* mutant grown in low Pi supplemented with 1 mM IPTG to OD_{600} ~0.6. Samples from whole cells (0.25 OD_{600} units) and supernatant (5 OD_{600} units) were loaded on 6 % acrylamide SDS-PAGE gel and subjected to immunodetection with anti-PdtA (upper panel). As controls, samples were loaded on 12 % acrylamide SDS-PAGE gel for immunoblotting with anti-EF-Tu (middle panel) and anti-LapA (lower panel). Molecular markers are indicated to the right. WC, whole cell; S, supernatant; PdtA-FL, full-length; PdtA-C, C-terminal; PdtA-sf, secreted form.

The sum of the PdtA-C and PdtA-sf molecular masses could correspond to the size of the PdtA-FL despite the difficulty in estimating precisely the size of such high molecular mass proteins by acrylamide SDS-PAGE.

To gain insight into the processing of PdtA, we determined the localization of the different forms of PdtA over cell growth. We first observed that the production of PdtA correlates with its gene expression (Figs 2b and 3). Western analysis further showed that PdtA is processed soon after its production and a total maturation occurs in stationary phase as no full-length PdtA can be detected any more (Fig. 3). These results suggest that PdtA contains a large N-terminal fragment that is cleaved and secreted during translocation and a smaller C-terminal domain, which stays associated with the cells.

PdtA fractionates with the outer membrane

Based on bioinformatic analyses, PdtA contains a 38 aa signal peptide that targets the protein to the general Sec secretory machinery (Fig. 1a). To determine the cellular localization of PdtA, we performed a whole-cell fractionation with cells overproducing PdtA, using the cytoplasmic

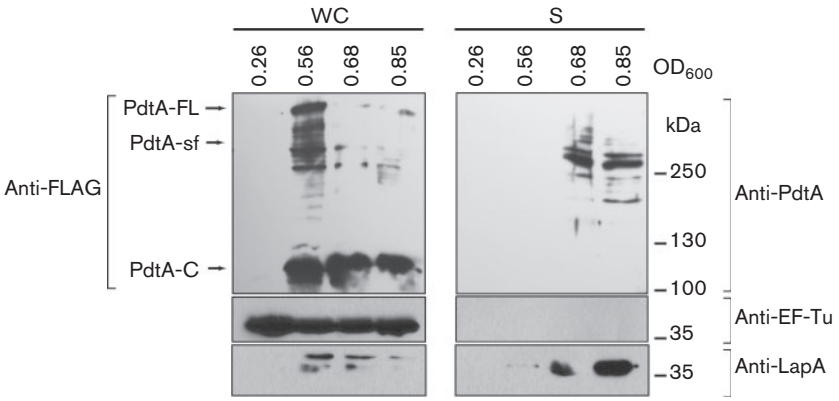

Fig. 3. PdtA is rapidly processed. PdtA-FLAG P_{LAC}-vreI PAO1 strain, grown in low Pi supplemented with 1 mM IPTG, was divided into whole cell (WC) and secreted (S) fractions at different OD_{600} growth. Samples were analysed by 6 % acrylamide SDS-PAGE and immunoblotting with anti-FLAG (WC, upper panel) and anti-PdtA antibodies (S, upper panel) or by 12 % acrylamide SDS-PAGE and immunoblotting with anti-EF-Tu (WC, S; middle panel) and anti-LapA antibodies (WC, S; lower panel). Anti-FLAG immunoreactive proteins of the size of PdtA and its major proteolytic products were present in whole cells. Molecular markers are indicated to the right. PdtA-FL, full-length; PdtA-C, C-terminal; PdtA-sf, secreted form.

EF-Tu and the inner-membrane XcpY proteins as controls. As shown in Fig. 4(a), full-length PdtA, as well as the XcpY protein, were localized in the total-membrane fraction. The same result was obtained using the anti-FLAG antibody, showing that the cleaved C-terminal part of PdtA is also extracted with the membrane fraction (data not shown). To test whether both forms of PdtA associate with the inner membrane or outer membrane, we used sedimentation density-gradient centrifugation to separate membrane fractions, using the outer-membrane porins and inner-membrane XcpY protein as controls (Fig. 4b). Our results clearly demonstrate that full-length PdtA as well as the C-terminal domain of PdtA associate with the outer-membrane fractions. However, a portion of the C-terminal domain of PdtA is retained in the inner-membrane fraction or in fractions with intermediate densities (see Discussion).

PdtA is exposed to the cell surface

To test whether the outer-membrane PdtA protein is exposed to the cell surface, we performed proteinase K accessibility experiments. Full-length PdtA was degraded after proteinase K treatment in whole cells (Fig. 5a, b). By contrast, the outer-membrane C-terminal was not degraded upon treatment, similar to the controls XcpY and EF-Tu (Fig. 5b). These results suggest that PdtA is anchored to the outer membrane via its C terminus, most likely with an N-terminal domain exposed to the surface

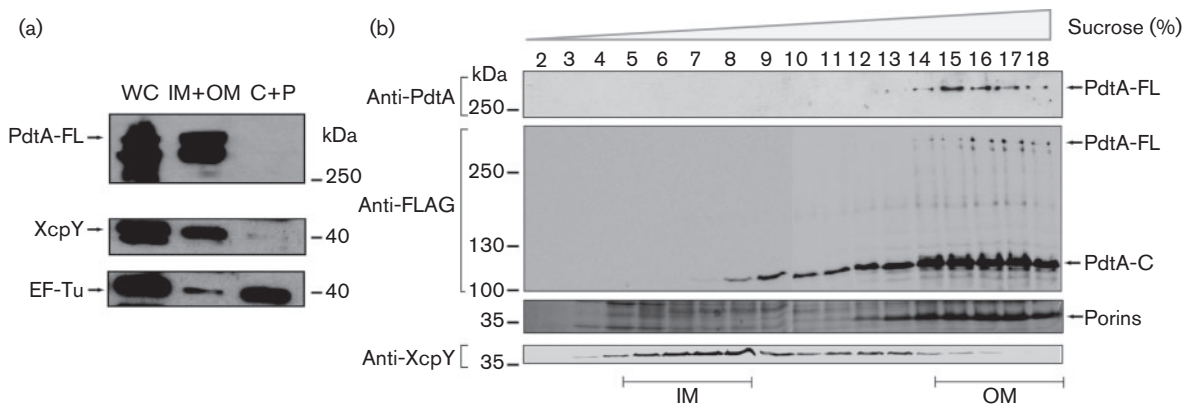

Fig. 4. PdtA is an outer-membrane protein. (a) PdtA localizes with the membranes. Whole cells (WC), total membranes (IM + OM) and cytoplasmic/periplasmic fractions (C + P) from P_{LAC}-vreI PAO1 strain were analysed by Western blot using anti-PdtA, anti-Xcp and anti-EF-Tu antibodies (from top to bottom panel). (b) PdtA co-fractionates with the outer membranes. Total membranes from PdtA-FLAG P_{LAC}-vreI PAO1 strain were separated by sedimentation on a discontinuous sucrose gradient. Collected fractions were analysed for contents using anti-PdtA, anti-FLAG, Coomassie blue coloration and anti-XcpY antibodies (from top to bottom panel). Fraction numbers are indicated above. Molecular markers are indicated on the left. PdtA-FL, full-length; PdtA-C, C-terminal; IM, inner membrane; OM, outer membrane.

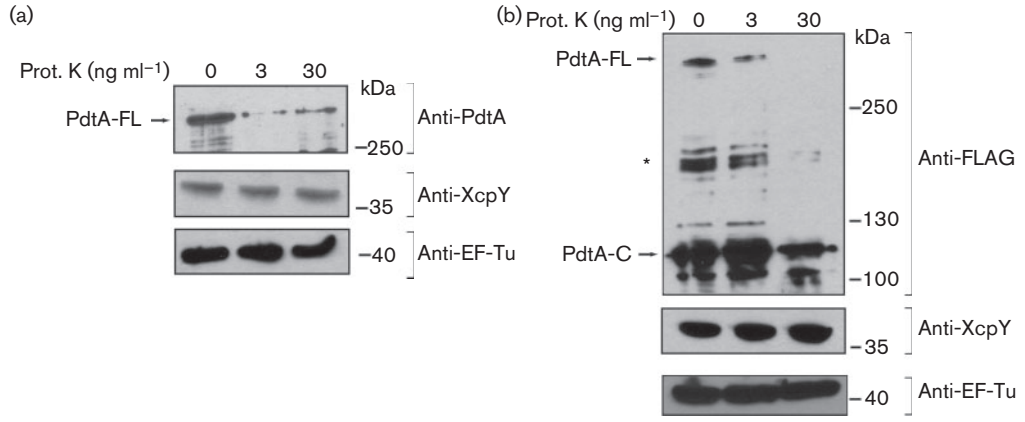

Fig. 5. PdtA is exposed to the cell surface. P_{LAC}-vrel whole cells producing wild-type or FLAG-tagged PdtA were treated with different concentrations of proteinase K (Prot. K). Samples were loaded on 6 % acrylamide SDS-PAGE gels for immunoblotting with anti-PdtA (a) or anti-FLAG antibodies (b). Controls, using anti-XcpY and EF-Tu antibodies (a, b), were carried out by loading samples on 12 % acrylamide SDS-PAGE gels. An asterisk indicates degradation products of PdtA. Molecular markers are indicated to the right. PdtA-FL, full-length; PdtA-C, C-terminal.

and a C-terminal domain exposed at the periplasmic side and/or embedded inside the outer membrane.

PdtA is exported in a PdtB-dependent manner

Bioinformatics analyses suggested that PdtA and PdtB constitute a TPS system. To test whether PdtB is required for the secretion of PdtA, we constructed a *pdtB* deletion mutant and a plasmid allowing the arabinose-inducible production of the PdtB protein. Western blot analyses using the anti-FLAG or anti-PdtA antibodies showed that PdtA was no longer detected in the whole cells or supernatant fraction of a Δ*pdtB* strain, but the PdtA localization was restored when PdtB was supplied *in trans* (Fig. 6a). We verified that the absence of PdtB does not affect the expression of the *pdtA* gene using a P*pdtA–lacZ* transcriptional fusion and showed that the promoter activity was identical in the wild-type and *pdtB* mutant strains (Fig. 6b). We supposed that the absence of PdtA is presumably due to the instability of the protein that is trapped in the periplasm, which is consistent with previous reports on FHA undergoing a rapid proteolytic degradation in the periplasm in the absence of its transporter FhaC (Guédin *et al.*, 1998; Jacob-Dubuisson *et al.*, 1997). These results strongly suggest that PdtB is the transporter required for the translocation of PdtA and that PdtA and PdtB proteins function as a TPS system.

PdtA does not display adhesive or proteolytic activities

Because a variety of TpsA exoproteins have shown adhesive activity and PdtA is part of a larger HMW-like adhesins clade (Fig. 1b), we tested biofilm formation (i.e. bacterial aggregation) on polyethylene plastic wells and glass tubes. Crystal violet staining showed no biofilm formation differences between wild-type and *pdtA* mutant strains (data not shown). We also tested bacterial adherence to human bronchial epithelial cell line 16HBE14o- using a standard adherence assay and microscopic visualization. Deletion of *pdtA* did not change the number of cell-associated bacteria (data not shown). These results suggest that PdtA does not function as an adhesin under our experimental conditions. The possibility that PdtA possesses proteolytic activities was also tested by casein and gelatin zymography. However, none of the standard conditions tested could suggest that PdtA contains protease activities (data not shown).

A *pdtA* mutant is attenuated for *C. elegans* killing

TPS systems play important roles in virulence (Jacob-Dubuisson *et al.*, 2001). Moreover, the low Pi condition was shown to activate expression of virulence factors (Zaborin *et al.*, 2009). We therefore tested whether PdtA protein could have a role in the virulence of *P. aeruginosa* PAO1 strain using the *C. elegans* slow killing assay (Garvis *et al.*, 2009; Tan *et al.*, 1999), although we could not determine the functions carried by PdtA. Killing kinetics are much faster for bacteria grown in phosphate-limiting medium due to induction of expression of virulence factors by phosphate limitation (Zaborin *et al.*, 2009 and data not shown). We first showed that the *pdtA* gene is expressed in a wild-type strain grown on NGM−Pi (Fig. 7a). This expression is in concordance with previous microarray data from experiments performed on NGM versus NGM−Pi plates (GEO database GSE30967; Zaborin *et al.*, 2009). Killing assays performed in phosphate-limiting medium showed that wild-type cells killed the worms at a 50 % lethal dose in 2 days (Fig. 7b). In contrast to the wild-type, the *pdtA*

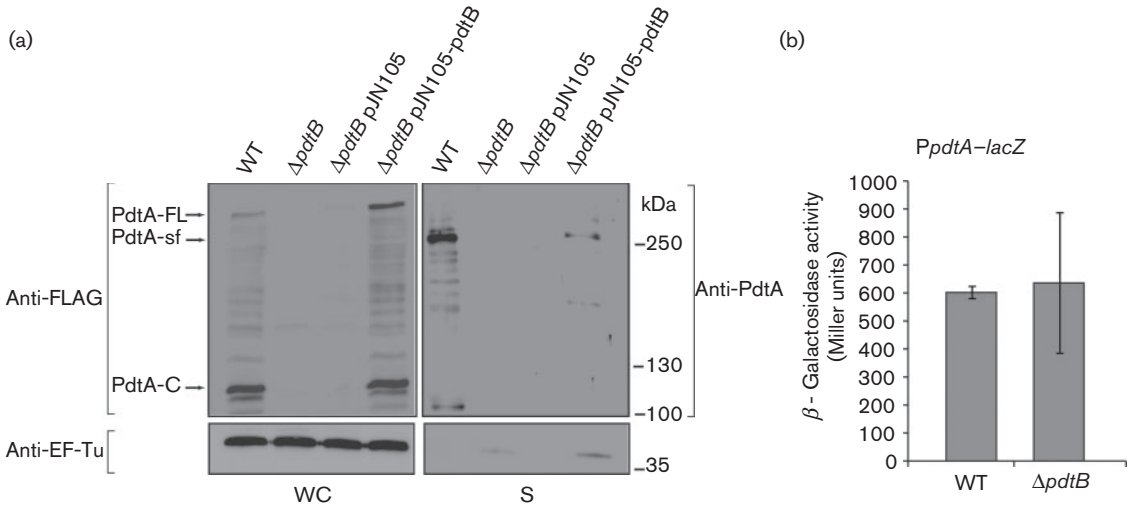

Fig. 6. PdtA is exported via PdtB. (a) P_{LAC}-vrel cells producing FLAG-tagged PdtA and its isogenic *pdtB* P_{LAC}-vrel mutant carrying pJN105 plasmid encoding or not the PdtB protein were grown in low Pi with 1 mM IPTG. After 220 min of growth, 0.1 % L-arabinose was added. Whole cell (WC) and secreted (S) sample fractions were loaded on 6 % acrylamide SDS-PAGE gels and subjected to immunodetection with anti-FLAG (left panel) and anti-PdtA antibodies (right panel). As control, anti-EF-Tu antibody was used to immunodetect samples loaded on a 12 % acrylamide SDS-PAGE gel (lower panel). Molecular markers are indicated to the right. WT, wild-type PdtA-FLAG; PdtA-FL, full-length; PdtA-C, C-terminal; PdtA-sf, secreted form. (b) *pdtA* is expressed in the absence of PdtB. PAO1 and *pdtB* mutant strains carrying the P*pdtA–lacZ* transcriptional fusion were grown in low Pi to OD_{600} ~0.6 and β-galactosidase activities were measured. Bars represent mean ± SD of β-galactosidase activity for at least three independent experiments.

mutant was significantly attenuated in lethality against *C. elegans*, killing the worms at a 50 % lethal dose in 4 days (Fig. 7b). This decreased virulence cannot be explained by a growth defect of the *pdtA* mutant as both strains tested have similar growth curves (data not shown). This result showed that PdtA plays a role in *P. aeruginosa* PAO1 virulence against *C. elegans*.

DISCUSSION

In this study, we provided evidence that previously uncharacterized *pdtA* and *pdtB* genes encode a TPS system. We found that in the absence of PdtB no translocation of PdtA across the outer membrane is observed. Indeed, PdtA was not detected any more in the Δ*pdtB* cells, suggesting a

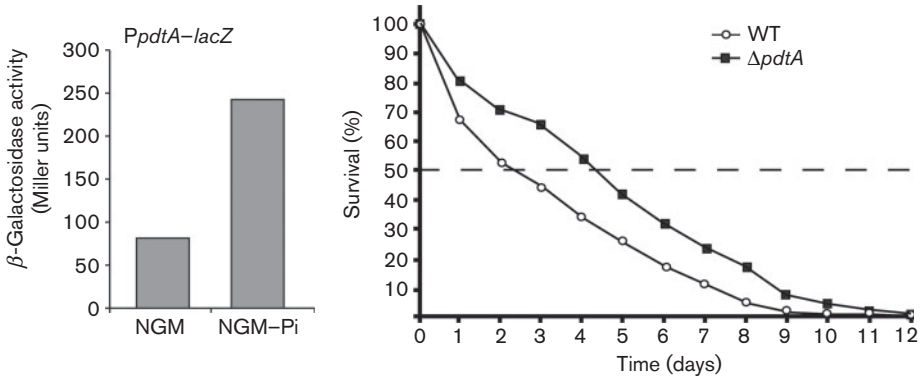

Fig. 7. PdtA is implicated in *P. aeruginosa* virulence on *C. elegans*. (a) *pdtA* is expressed on NGM–Pi plates. PAO1 strain carrying the P*pdtA–lacZ* transcriptional fusion was spotted on NGM or NGM–Pi plates and β-galactosidase activity was measured after 24 h of growth at 37 °C. Data represent one experiment. (b) A Δ*pdtA* mutant is attenuated in *C. elegans* killing. Comparison of slow killing assay between *C. elegans* fed with wild-type PAO1 (open circles) and Δ*pdtA* mutant (filled squares) on NGM–Pi (n=25, P<0.0001). Percentage nematode survival (y-axis) is shown with respect to number of days post-infection (x-axis).

potential degradation in the periplasm as observed for FHA in the absence of its transporter (Jacob-Dubuisson *et al.*, 1997). Taken together, our data strongly suggest that PdtB functions as the PdtA transporter. Alternatively, it is possible that an unidentified transporter uses PdtB as a co-factor to translocate PdtA. The genetic organization of the PdtA/PdtB system differs from most TPS systems, in which the two *tps* genes are located in a single operon. We demonstrated previously that *pdtA* and *pdtB* have their own promoter and despite the fact they are arranged in different transcriptional units, they are co-activated by the response regulator PhoB and σ^{VreI} factor under low phosphate conditions (Faure *et al.*, 2013). This unusual genetic organization has been also reported for the *fhaB* and *fhaC*, and *tpsA* and *tpsB* genes of *B. pertussis*, respectively. While *fhaC* forms an operon with the *fimBDC* locus encoding fimbriae, *fhaB*, located upstream, has a separate promoter, but both TPS partners are co-regulated by the BvgA/S/R two-component system (Willems *et al.*, 1992, 1994).

A correlation exists between TpsA biogenesis and the classification into exoprotein subfamilies. Proteins from the contact-dependent growth inhibitor and haemolysin subfamilies do not appear to be processed and are surface-associated by their N terminus (Hertle, 2005; Webb *et al.*, 2013). In the case of the adhesin subfamilies, the N terminus of mature FHA remains associated with the outer membrane, while the C-terminal prodomain is removed (Mazar & Cotter, 2006). By contrast, HMW1 adhesin is synthesized with an N-terminal prodomain that is removed during outer-membrane translocation and mature HMW1 is associated with the cell surface by its C terminus (Buscher *et al.*, 2006). In this study, we showed that PdtA protein is processed over time, and is found associated with cells as well as in cell culture supernatants. Indeed, PdtA contains an N-terminal domain (about two-thirds of the entire protein) that is secreted in the extracellular medium while about one-third of PdtA (PdtA-C) remains in the outer-membrane fraction via the C terminus. Using sedimentation sucrose gradients and proteinase K accessibility tests, we demonstrated that the PdtA-C domain is not exposed to the cell surface and a portion of this domain co-localized with inner-membrane fractions or fractions with intermediate densities. All the TpsA exoproteins that have been characterized to date would be long enough to span the periplasm. Indeed, intermediate localization has been observed during FHA biogenesis, as the C-terminal prodomain remains intracellular while the mature FHA domain is extracellular (Noël *et al.*, 2012). It is then conceivable that PdtA-C co-localizes with inner-membrane or intermediate fractions because translocation and processing through the outer membrane occurred while the PdtA exoprotein was still engaged in the Sec machinery. While it was demonstrated that the processing of FHA involves the autotransporter SphB1 (Coutte *et al.*, 2001), its nature and the cellular compartment in which the proteolytic cleavage of PdtA occurs are still unknown. We observed that three forms of PdtA appeared during the proteolytic maturation of this protein. However, we do not know which forms carry the protein activity and this question remains to be determined.

Our results suggest that PdtA/PdtB constitute a real TPS system, but they also point out that PdtA has unique properties. PdtA does not present other conserved motifs than those of the TPS system. It lacks the signature CXXC motif found in TPS haemolysin and no C-terminal nuclease domain was revealed, as is the case for contact-dependent growth inhibitor exoproteins. Based on our phylogenetic analysis, PdtA is related to the HMW-like adhesins subfamilies. The position of PdtA in this tree seems accurate because PdtB has an identical position in a phylogenetic analysis performed with the TpsB transporters (data not shown). This result shows the shared evolutionary histories of the transporters and their substrates. We tested adhesive properties of PdtA, but we observed that this protein did not display adhesive activity under the conditions tested. This result was not surprising given the weak homologies between PdtA and the known adhesins. Furthermore, two TpsA proteins, the protease LepA and HxuA, involved in iron acquisition, are also related to the HMW-like adhesins subfamily (Fig. 1b). This larger HMW-like clade shows functional diversity and should be considered as an evolutionary structure. Evolution of these proteins seems mysterious and it is likely that their role in environmental interactions has driven rapid adaptive selection and, therefore, sequence divergence.

We have shown previously that *pdtAB* genes are expressed under low Pi growth conditions (Faure *et al.*, 2013). Many of the Pi-regulated genes encode virulence determinants. For example, the phosphate-binding protein PstS, produced in low Pi, oligomerizes in appendages on the cell surface of multi-drug-resistant *P. aeruginosa* strains and contributes to altering barrier function of intestinal epithelial cells (Zaborina *et al.*, 2008). Similarly, the haemolytic phospholipase C, a substrate of the Xcp type II secretion system, contributes to virulence in a mouse infection model (Ostroff *et al.*, 1989). *C. elegans* lethality is higher in Pi-depleted media, thus making the worm a good model to study the contribution of virulence factors specifically produced at low Pi concentration (Zaborin *et al.*, 2009). Using this infection model, we demonstrated that PdtA is implicated in the virulence of *P. aeruginosa*. Because homologues of PdtA are only found in pathogenic *Pseudomonas*, it may suggest that this protein plays an important role in the pathogenesis of these bacteria. However, the function played by PdtA during *C. elegans* infection is still unknown. On the basis of our data, we suspect that the PdtA/PdtB system forms a new subfamily distinct from the major subfamilies of TPS systems. Future studies of the exact biogenesis of PdtA and its exact role should provide important information to fully understand the mechanism of secretion via the TPS pathways. Comprehension of this mechanism will be more apparent as additional members of the TPS superfamily are characterized.

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