The 'P-usher', a novel protein transporter involved in fimbrial assembly and TpsA secretion

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We identified a new bacterial transporter, the *Pseudomonas aeruginosa* CupB3 protein, which is an outer membrane usher involved in pili assembly. In CupB3, the usher domain has fused during evolution with a POTRA (polypeptide-transport-associated)-like domain found in TpsB transporters of two-partner secretion systems. In TpsBs, the POTRA captures the TpsA passenger, which is then transported across the outer membrane through the TpsB β -barrel. We named CupB3 a 'P-usher' for POTRA-like domain-containing usher. We showed that CupB3 assembles CupB1 fimbrial subunits into pili and secretes CupB5, a TpsA-like protein. The CupB3 usher domain has the function of a TpsB β -barrel in CupB5 translocation. We revealed that the POTRA-like domain is neither essential for CupB1 fimbriae assembly nor for cell surface exposition of CupB5, but is crucial to coordinate *bona fide* transport of CupB1 and CupB5 through the usher domain. The P-usher defines a novel transport pathway involving a molecular machine made with old spare parts. *The EMBO Journal* (2008) 27, 2669–2680. doi:10.1038/emboj.2008.197; Published online 2 October 2008

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Introduction

Targeting of proteins to subcellular compartments is a major issue in cell biology. This process involves molecular machines for translocation across biological membranes. In Gram-negative bacteria, proteins are released in the milieu owing to secretion pathways (Kostakioti *et al.*, 2005). Common features are shared between secretion systems

and molecular machines assembling appendages at the bacterial surface. Type II secretion resembles type IV pili assembly (Filloux, 2004), type III secretion resembles flagellar assembly (Cornelis, 2006) and type IV secretion resembles assembly of conjugative F pili (Christie and Cascales, 2005). The type II–IV secretion machines are complex macromolecular systems, whereas the type V secretion system is simple (Henderson *et al.*, 2004). The type Va consists of a unique protein called autotransporter, whereas the type Vb involves two distinct polypeptides, TpsA and TpsB. TpsA is usually an adhesin, such as the filamentous haemagglutinin (FHA) from *Bordetella pertussis* (Coutte *et al.*, 2003), which is transported at the surface. This transport involves the TpsB protein, which is a β -barrel outer membrane protein. Recognition between TpsA and TpsB occurs through an interaction between the secretion signal located at the N terminus of TpsA and the N-terminal periplasmic domain of TpsB (Hodak *et al.*, 2006). The TpsB transporters belong to a superfamily of proteins, including the *Neisseria meningitidis* Omp85 outer membrane protein (Voulhoux *et al.*, 2003) or the eukaryotic Toc75 membrane protein (Schleiff and Soll, 2005), which are required for the insertion of β -barrel membrane proteins into the outer membrane of bacteria or plastids, respectively. These proteins have a C-terminal transmembrane domain forming a β -barrel and an N-terminal region containing 1–5 POTRA (polypeptide-transport-associated) domains, which mediate protein–protein interactions (Gentle *et al.*, 2005).

In many cases, the type Vb secretion system, also known as two-partner secretion (TPS) system, transports bacterial adhesins at the cell surface (Coutte *et al.*, 2003). Bacterial adhesive properties are also conferred by cell surface pili, of which the chaperone–usher-dependent assembly of fimbriae is a remarkable example (Dodson *et al.*, 2001). The assembly process relies on an outer membrane protein called usher, which is a pore-forming β -barrel (Henderson *et al.*, 2004; Li *et al.*, 2004). This channel is used by pilin subunits, which polymerize into fimbriae on the bacterial surface. The pilins are targeted to the usher owing to their association with a periplasmic chaperone (Hultgren *et al.*, 1991; Sauer *et al.*, 2004).

The opportunistic pathogen *Pseudomonas aeruginosa* is equipped with at least five genes encoding TpsA-like proteins and three loci encoding a full set of components belonging to the chaperone–usher pathway (Cup) (Vallet *et al.*, 2001; Filloux *et al.*, 2004a,b). We described the function of two *P. aeruginosa* Cup systems, CupB and CupC (Ruer *et al.*, 2007). Expression of the *cupB* and *cupC* genes is controlled by a two-component regulatory system, namely the RocS1-R-A1 system (Kulasekara *et al.*, 2005). On top of genes encoding Cup components, the *cupB* gene cluster contains the *cupB5* gene, which encodes a protein with similarities to TpsA proteins such as FHA from *B. pertussis*, or HxuA from *Haemophilus influenzae* (Vallet *et al.*, 2001). This is unusual as no obvious molecular mechanistic relation has ever been

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shown between these two systems. Yet a C-terminal motif was found in proteins of the Omp85 family, which is closely related to a motif identified for several usher proteins (Reumann *et al*, 1999).

We have no clue on whether *cupB5* encodes a cell surface protein and if yes, which secretion mechanism is involved in its transport. These are the two main questions that we addressed in this study and which brought us to the characterization of a novel type of bacterial transporter.

Results

The *cupB5* gene encodes a *TpsA*-like protein

The *cupB* gene cluster contains six genes encoding an usher (*cupB3*), a pilin (*cupB1*), an adhesin (*cupB6*) and two chaperones (*cupB2* and *cupB4*) (Figure 1A). The *cupB5* gene encodes a protein of 1018 residues sharing similarities to TpsA proteins (Jacob-Dubuisson *et al*, 2001; Henderson *et al*, 2004). No gene encoding a TpsB transporter was identified in the *cupB* cluster.

The CupB5 protein contains a putative 53-amino acids (aa)-long signal peptide reminiscent of unusually extended signal peptide in TpsAs (type Vb) and autotransporters (type Va) (Desvaux *et al*, 2006), which may exceed 50 aa in length, such as FHA from *B. pertussis* (Lambert-Buisine *et al*, 1998). Five *tpsA-tpsB* gene clusters were identified in the PAO1 genome (Jacob-Dubuisson *et al*, 2001; Filloux *et al*, 2004a).

CupB5 is an orphan TpsA presenting high similarity (44%) with TpsA4 (Figure 1B). CupB5, similar to TpsA4, contains a haemagglutination activity domain (Haemagg_act) at the N terminus (between aa 53 and 167) (Kajava *et al*, 2001) (Figure 1C). This domain is found near the N terminus of several TpsAs designated adhesins, FHAs or haem/haemopexin-binding proteins. In CupB5, 13 repeats of Glug domains (17 for TpsA4) are found between aa positions 585 and 973. The Glug domain is found in many protein-containing Haemagg_act domain (Poulsen *et al*, 1996).

TpsAs have an N-terminal secretion signal, or 'TPS' domain, which is responsible for the interaction with the TpsB transporter, as shown between FHA and the N terminus of the FhaC transporter (Hodak *et al*, 2006). In FHA, the 'TPS' domain structure presents a β -helix fold (Clantin *et al*, 2004; Hodak *et al*, 2006), and secondary structure prediction within the N terminus of CupB5 revealed the presence of a series of β -strands (data not shown). Two conserved motifs, NPNL and NPNGI, have been shown to be important for *Serratia marcescens* TpsA (ShlA) secretion (Schönherr *et al*, 1993). In FHA, the NPNGI motif is critical for FHA-FhaC interaction, whereas the NPNL motif is not required for interaction but needed for FHA secretion (Hodak *et al*, 2006). In HMW1, the NPNL motif is lacking; however, the NPNGI motif and a downstream NTNG motif are required for transport (Grass and St Geme, 2000; Yeo *et al*, 2007). The CupB5 protein possesses the NPNGV motif (aa 138–142), but lacks the

Figure 1 Characteristic of CupB5 and other TpsAs. (A) Genetic organization of the *cupB* gene cluster. The name of the genes is indicated above the arrow and the function is indicated underneath. A code is used for similar functions. (B) A gene cluster encoding a TPS system. The name of the gene is indicated above and the PA number is indicated underneath. TpsB4 is the transporter and TpsA4 is the passenger protein. (C) Domain organization of the CupB5 and TpsA4 proteins. CupB5 and TpsA4 display a haemagglutinin domain (Haemagg_act, bricked boxes) at the N terminus and repeated Glug domains (dotted boxes) at the C terminus. The size in amino acids (aa) of each protein is indicated on the right. (D) Amino-acid sequence alignment of the 'TPS' domains of several TpsAs including FHA (*B. pertussis*), HMW1 and HxuA (*H. influenzae*), TpsA4 and CupB5 (*P. aeruginosa*). The NPNL motif in FHA (black) or NPNGI/V motif in all TpsAs is indicated with bold characters. The downstream NTNG motif in HMW1, together with the motif NVA/GG in HxuA, CupB5 and TpsA4, is also indicated with bold characters. The position of the residue is indicated at the end of each line.

upstream NPNL motif (Figure 1D). This is not unusual for a TpsA protein as HMW1 or HxuA in *H. influenzae* and TpsA4 of *P. aeruginosa* are lacking this motif (Jacob-Dubuisson *et al*, 2001; Yeo *et al*, 2007). Instead, a NVGG motif is found in CupB5 and TpsA4, or a NVAG motif in HxuA, which align with the HMW1 NTNG motif (Figure 1D). In conclusion, based on *in silico* analyses, we suggest that CupB5 possesses the characteristics of a TPS substrate.

CupB5 is a cell surface-exposed protein

We showed that RocS1 overproduction resulted in *cupB* gene expression and CupB1 fimbriae assembly (Kulasekara *et al*, 2005; Ruer *et al*, 2007). The pMMBrocS1 plasmid was thus introduced into the strain PAO1Δ*pilA*Δ*fliC* (PAOΔΔ) or a *cupB5* mutant (PAOΔΔΔ*cupB5*), bacteria were grown on agar plates, scraped and extracts were used for immunoblotting using CupB5 antibodies. The CupB5 protein was readily detected in PAOΔΔ cell extracts (C), but was lacking in the *cupB5* mutant (Figure 2A). By performing shearing of bacterial surface appendages (Ruer *et al*, 2007), large quantities of CupB5 were recovered in the sheared fraction (SF)

Figure 2 Cell surface exposition of CupB5. (A) Cell extracts (C) or material in sheared fractions (SFs) of *P. aeruginosa* strains carrying pMMBrocS1 were prepared. Proteins are separated on an SDS gel, blotted on nitrocellulose and CupB5 is revealed using specific antibodies. CupB5 was detected in C and SF extracts from PAOΔΔ but not in the *cupB5* mutant derivative (PAOΔΔΔ*cupB5*). (B) Material in SFs of *P. aeruginosa* strains carrying pMMBrocS1 was prepared and samples were separated as in (A). Coomassie-stained SDS gel and mass spectrometry reveal the presence of CupB5 and/or TpsA4 in the PAOΔΔ strain or isogenic mutants PAOΔΔΔ*tpsB4* or PAOΔΔΔ*cupB3*. TpsA4 is marked with an asterisk (*), whereas CupB5 is marked with **. (C) Material in sheared fractions (SFs) of *P. aeruginosa* strains carrying pMMBrocS1 was prepared and the presence of CupB5 was verified by immunoblotting (upper panel). CupB5 was not found in SF from the *cupB3* mutant (PAOΔΔΔ*cupB3*). The presence of CupB1 and CupC1 proteins in SF from different *P. aeruginosa* strains was assessed (lower panel). The CupB1 and CupC1 bands were recovered from a Coomassie-stained gel and analysed by mass spectrometry. In all panels, molecular weight standards are indicated on the left (kDa) and proteins of interest are indicated with arrows.

(Figure 2A), indicating that CupB5 is cell surface exposed. The occurrence of CupB5 at the surface is not resulting from a partial cell leakage as intracellular OTCase (ornithine carbamoyltransferase) (cytoplasmic) and DsbA (periplasmic) proteins were not found in the SF (data not shown).

CupB5 export is CupB3 dependent

CupB5 exhibits high similarity to the *P. aeruginosa* TpsA4 protein (Figure 1C and D). We investigated whether the TpsB4 transporter (Figure 1B) could be used for cell surface exposition of CupB5. We engineered a *tpsB4* mutant strain (PAO1ΔΔΔ*tpsB4*) in which we introduced pMMBrocS1. SFs of the *tpsB4* mutant were analysed on Coomassie-stained gels, and mass spectrometry analysis revealed that CupB5 is still exposed, whereas the TpsA4 protein is missing (Figure 2B). We concluded that CupB5 does not use TpsB4 for transport to the cell surface although TpsB4 transports TpsA4.

Furthermore, we found that CupB5 transport is CupB3 usher dependent, as CupB5 was not found in the SF of a *cupB3* mutant (Figure 2C, upper panel). The lack of CupB5 transport is specifically observed in the *cupB3* mutant, as CupB5 is still surface exposed in the parental strain or the mutant lacking the CupC3 usher, PAO1ΔΔΔ*cupC3* (Figure 2C, upper panel). CupB5 secretion was restored when the *cupB3* mutation was *trans*-complemented by introduction of the *cupB3* gene (pBBR*cupB3*) (Figure 2C, upper panel). The presence of CupB1 and CupC1 into *P. aeruginosa* SFs was assessed by mass spectrometry analysis on proteins excised from Coomassie-stained gels (Figure 2C, lower panel). CupB1 was recovered into SF from the parental strain and the *cupC3* mutant but not from the *cupB3* mutant. CupB1 exposition was observed in the *cupB3* mutant on *trans*-complementation with pBBR*cupB3*. CupC1 was present in SF of all tested strains except for the *cupC3* mutant. Finally, CupB3-dependent transport of CupB5 appears specific as the TpsA4 protein is cell surface exposed in the *cupB3* mutant (Figure 2B).

CupB5 is associated with CupB1 fimbriae

Using transmission electron microscopy coupled with immunogold-labelling and CupB5 antibodies, we analysed a series of *P. aeruginosa* strains. We observed that CupB5 was located distal from the PAO1ΔΔ bacterial cell surface (Figure 3A and B) and the gold beads are seen within the CupB1 fimbrial material surrounding the bacteria. In a *cupB3* mutant, CupC1 fimbriae but not CupB1 fimbriae are assembled, and no CupB5 labelling is observed (Figure 3C). This result confirms that CupB5 export is CupB3-dependent. The specific association of CupB5 with CupB1 fimbriae was also observed when no CupC1 fimbriae are assembled as seen by using a *cupC3* mutant (data not shown). Finally, CupB5 labelling was totally absent in a *cupB5* mutant (data not shown). Interestingly, in a *P. aeruginosa* mutant that does not assemble CupC1 and CupB1 fimbriae (PAO1ΔΔΔ*cupC3*Δ*cupB1*), CupB5 is close to the bacterial surface (Figure 3D), a localization also observed in a single *cupB1* mutant (PAO1ΔΔΔ*cupB1*) (data not shown).

Our results suggest that the TpsA-like CupB5 and the pilin CupB1 use the CupB3 usher to reach the cell surface. Moreover, the CupB5 protein remains associated with the CupB1 fimbriae at a distal location from the cell surface. In the absence of CupB1 fimbriae, the CupB5 protein is transported to the cell surface in a CupB3-dependent manner, but

Figure 3 Cell surface localization of CupB5, in *P. aeruginosa* strains carrying pMMB*rocS1*, using transmission electron microscopy and immunogold labelling. (A, B) PAO1ΔΔ. (C) PAO1ΔΔΔ*cupB3* lacking the CupB3 'P-usher'. (D) PAO1ΔΔΔ*cupC3ΔcupB1* lacking both CupC1 and CupB1 fimbriae. Magnification in (A, C) is × 50 000; (B, D) is × 140 000.

is not found at a distal location, suggesting that CupB5 and CupB1 transport and assembly might be coordinated through the CupB3 usher.

CupB3 is a POTRA-like-containing usher protein

Because of the unique feature of CupB3, we investigated whether lessons could be learned from its primary sequence or predicted secondary structure. We noticed that the 844-aa-long CupB3 protein, as it is annotated at <http://www.pseudomonas.com>, does not contain a putative signal peptide. We found, 246 bp upstream from the proposed GTG start codon of *cupB3*, an alternative ATG start codon. The newly defined *cupB3* gene encodes a 926-aa-long protein with a predicted signal peptide encompassing residues 1–16 (SignalP). The CupB3 usher is larger than the CupA3 (872 aa) or CupC3 (839 aa) ushers (Figure 4A). The aa sequence comparison between the three ushers revealed that CupB3 has an additional N-terminal domain, which lies between aa 17 and 99 (Figure 4A).

TpsB transporters contain an N-terminal region, called POTRA domain, which may provide a chaperone-like function for the TpsA passenger (Yang and Braun, 2000). As CupB3 serves for the transport of the TpsA-like CupB5 protein, we investigated whether the N-terminal extension of CupB3 has similarity to POTRA domains. Alignments of the *P. aeruginosa* TpsBs (Jacob-Dubuisson *et al*, 2001) with the POTRA domain of ShlB, a canonical *S. marcescens* TpsB, showed that they all have a putative POTRA including two conserved glycine residues (Figure 4B) (Sanchez-Pulido *et al*, 2003). We aligned the N terminus of CupB3 with the POTRA domains and found a match between aa positions 19 and 90. We observed that the putative CupB3 POTRA domain lacks the second conserved glycine, whereas it retained the first

one (Figure 4B). The lack of this residue in POTRA domains is not unique and was reported in HXB1 from *H. influenzae* or FtsQ from *Escherichia coli* (Sanchez-Pulido *et al*, 2003). The 3D structure of the POTRA domain from the *E. coli* YaeT protein shows a tandem α -helices bordered with β -strands (β - α - α - β - β) (Kim *et al*, 2007). The secondary structure of all POTRA domains presented in Figure 4B complied with the β 1- α 1- α 2- β 2- β 3 structure, except for the proposed CupB3 POTRA domain (Figure 4C), which is predicted to be β 1- α 1- α 2- α 3- β 2. However, based on overall similarities, we proposed that CupB3 contains a POTRA-like domain between aa positions 19 and 90, and further assessed its function.

The CupB3 POTRA-like domain coordinates CupB1 and CupB5 transport

We engineered truncated versions of the *cupB3* gene (Figure 5A), which encode proteins lacking the N-terminal (aa 20–80; CupB3ΔPN), the C-terminal (aa 37–97; CupB3ΔPC), the central region (aa 37–80; CupB3ΔPI) or the whole POTRA-like domain (aa 20–97; CupB3ΔP). Each *cupB3*-truncated genes was cloned in the broad host range pBBRMCS-3 plasmid and conjugated into the *P. aeruginosa* *cupB3* mutant containing pMMB*rocS1*. The cell surface localization of CupB5 was investigated in the *cupB3* mutant *trans*-complemented with either the *cupB3*-truncated genes or the full-length *cupB3* gene (Figure 5B). Surprisingly, although CupB5 was not cell surface exposed in a *cupB3* mutant, it could be recovered in the SF on introduction of the *cupB3* gene or any of the *cupB3*-truncated alleles (Figure 5B, upper panel). It suggested that the POTRA-like domain of CupB3 is not required for CupB5 transport to the cell surface. In contrast, we observed that none of the *cupB3*-truncated genes was able to restore CupB1 fimbriae assembly in the

Figure 4 POTRA domain characterization. **(A)** Schematic representation of the CupA3, CupB3 and CupC3 domains. The aa position bordering each domain is indicated. Black bars represent signal peptides and striped bars usher domains. The POTRA-like domain of CupB3 is indicated with a grey box. **(B)** The POTRA domains of ShlB from *S. marcescens*, TpsB1–TpsB5 and CupB3 from *P. aeruginosa*, PFL_1466 from *P. fluorescens* and the fourth POTRA domain of YaeT from *E. coli* have been aligned. Conserved amino acids (Sanchez-Pulido *et al*, 2003) are highlighted in red and blue. The additional residues conserved between CupB3 and TpsB4 are in purple. Amino-acid position within the sequence of each protein has been indicated in green for the first and last residue of each domain. **(C)** The secondary structures predicted by PSIPRED are shown for the POTRA domains of CupB3, TpsB4 and YaeT (fourth). The regions (Pred for secondary structure and AA for amino acids) corresponding to β -strands are in red (E), whereas the region corresponding to α -helices are in blue (H). The conserved motif GY has been indicated with bold characters and is lacking in CupB3.

cupB3 mutant (Figure 5B, lower panel). The recovery of the CupB1 subunits in the SF was observed only when using the parental strain or the *cupB3* mutant *trans*-complemented with the full-length *cupB3* gene (Figure 5B, lower panel). In all cases, recovery of the CupC1 subunit in the SF was not affected (Figure 5B, lower panel). CupC1 was found in variable amounts and mass spectrometry analysis on the corresponding bands did not reveal the presence of CupB1 degradation products or of another protein. These observations unexpectedly suggested that the POTRA-like domain of CupB3 is required for CupB1 assembly but not for transport of the TpsA-like CupB5 protein.

We investigated whether lack of CupB1 assembly through the POTRA-defective CupB3 ushers might be caused by an incomplete translocation of CupB5. We thus engineered a *cupB5* deletion in the *cupB3* mutant strain. Interestingly, *trans*-complementation of the *cupB3cupB5* mutant, with the *cupB3* allele encoding CupB3 lacking its POTRA-like domain

(*cupB3* Δ P), readily restored CupB1 fimbriae assembly (Figure 5B, lower panel). This observation indicates that the POTRA-like domain is not essential for CupB1 transport. In contrast, the POTRA-like domain might be required for a *bona fide* translocation of the TpsA-like CupB5 protein, a crucial event for subsequent CupB1 assembly.

A conserved glycine residue within the POTRA-like domain is essential for CupB3 function

Amino-acid sequence alignment between POTRA domains revealed the presence of a conserved glycine residue (Sanchez-Pulido *et al*, 2003). In the CupB3 POTRA-like domain, the conserved glycine is at position 28 (G28) (Figure 4B). We examined whether this residue could have a crucial function in CupB3 function. We engineered *cupB3* alleles by substituting the codon corresponding to G28 with an arginine (G28R) or a tryptophan (G28W) codon. These substitutions yielded the *cupB3*_{G28R} and *cupB3*_{G28W} genes,

Figure 5 Functionality of POTRA-truncated CupB3 usher. (A) Schematic representation of CupB3-truncated ushers. The CupB3 usher was truncated of the POTRA-like domain between aa 20–97 (CupB3ΔP) or aa 37–80 (CupB3ΔPI) or aa 20–80 (CupB3ΔPN) or aa 37–97 (CupB3ΔPC). The colour code is similar as in Figure 4A. (B) Analysis of sheared fractions obtained from the PAOΔΔ strain and derivative mutants *cupB3* (PAOΔΔ*cupB3*) or *cupB3cupB5* (PAOΔΔ*cupB3cupB5*) carrying the pMMB*BrocS1* plasmid. Where indicated, the strains also carried a plasmid containing the *cupB3* gene or one of the truncated *cupB3* alleles. Upper panel, immunoblot with antibodies directed against CupB5. Lower panel, Coomassie-stained gel. The positions of CupB5, CupB1 and CupC1 proteins are indicated. The molecular weight standards (kDa) are on the left. (C) Crucial function of the G28 residue in the CupB3 POTRA-like domain. Sheared fractions obtained from the PAOΔΔ strain and derivative *cupB3* mutant (PAOΔΔ*cupB3*) carrying the pMMB*BrocS1* plasmid were analysed. Where indicated, the strains also carried a plasmid containing the *cupB3* gene or one of the *cupB3* alleles encoding the CupB3 derivatives, CupB3_{G28W} or CupB3_{G28R}. Upper panel, immunoblot using antibodies directed against CupB5. Lower panel, Coomassie-stained gel. The positions of CupB5, CupB1 and CupC1 proteins are indicated. The molecular weight standards (kDa) are on the right.

respectively. The resulting CupB3 derivatives were unable to restore CupB1 fimbriae assembly in a *cupB3* mutant but could support surface exposition of CupB5 (Figure 5C). Moreover, in the double *cupB3cupB5* mutant, thus in the absence of CupB5, production of CupB3_{G28W} or CupB3_{G28R} restored CupB1 fimbriae assembly (data not shown). This phenotype is strictly similar to the one observed when *cupB3* or *cupB3cupB5* mutants are complemented with *cupB3* deleted

Figure 6 CupB4- and TpsBPF-dependent transport of CupB5. (A) Comparison between the genetic organization of the *cupB* gene cluster of *P. aeruginosa* (PA) and the *PFL_1462-PFL_1469* gene cluster of *P. fluorescens* (PF). The genes encoding homologous components (function written underneath) are motif-encoded. (B) Analysis of sheared fractions obtained from the PAOΔΔ strain and the isogenic *cupB4* mutant (PAOΔΔ*cupB4*) carrying the pMMB*BrocS1* plasmid. The lower panel is a Coomassie-stained gel, whereas the upper panel is an immunoblot revealed with antibodies against CupB5. The proteins of interest are indicated with arrows and the molecular weight standards (kDa) are on the right. (C) *PFL_1466/TpsB*-dependent transport of *P. aeruginosa* CupB5. Analysis of sheared fractions obtained from the PAOΔΔ strain and the isogenic *cupB3* mutant (PAOΔΔ*cupB3*), which contains, or not, a plasmid encoding the *P. fluorescens* *PFL_1466* gene (pBBR*tpsBPF*) or its derivative encoding a POTRA-truncated TpsB protein (pBBR*tpsBPFΔP*). Coomassie-stained gel (lower panel) and immunoblot using CupB5 antibodies (upper panel). The proteins of interest are indicated with arrows. The molecular weight standards (kDa) are on the right.

for the region corresponding to the POTRA domain (*cupB3ΔP*) (Figure 5B). This result confirms that the G28 residue is likely part of a POTRA-like domain.

CupB1 and CupB5 are not cell surface exposed in the absence of the CupB4 chaperone

The POTRA-like domain of CupB3 does seem to be required for CupB5 translocation but not for its targeting. We investigated whether the CupB4 chaperone could be involved in CupB5 targeting. The CupB4 chaperone is encoded by the *cupB4* gene located upstream from the *cupB5* gene (Figure 6A). A *cupB4* mutant was engineered and shearing experiments were performed. Interestingly, neither CupB1 fimbriae nor the CupB5 protein could be recovered from the cell surface of the *cupB4* mutant as compared with the parental strain (Figure 6B, lower and upper panels, respectively). We concluded that the CupB4 chaperone is required, directly or indirectly, for the cell surface exposition of CupB5 and CupB1.

The *Pseudomonas fluorescens* *cupB*-like cluster encodes a TpsB transporter

In the genome of *Pseudomonas fluorescens*, a *cupB*-like cluster (PFL_1462–PFL_1469) has been identified (Nuccio and Baümler, 2007) (Figure 6A). The gene cluster organization in *P. fluorescens* is different as compared with the *P. aeruginosa* *cupB* cluster, because it contains a gene encoding the TpsB transporter PFL_1466. We cloned the *tpsB* (PFL_1466) gene in pBBR1MCS-3, yielding pBBR*tpsBPF*. We showed that introduction of *tpsBPF* into the *P. aeruginosa* *cupB3* mutant allowed cell surface exposition of CupB5, which is recovered in SF (Figure 6C, upper panel). In contrast, it could not restore CupB1 fimbriae assembly (Figure 6C, lower panel). PFL_1466 does contain a POTRA domain (Figure 4B). We assessed whether the POTRA is required for CupB5 transport, by engineering a gene encoding a POTRA-truncated version of PFL_1466, namely TpsBPFΔP. When the *cupB3* mutant was *trans*-complemented with *tpsBPFΔP*, no CupB5 could be recovered in the SF (Figure 6C, upper panel). Thus, transport of *P. aeruginosa* CupB5 through the *P. fluorescens* TpsB is dependent on the presence of a POTRA domain.

Discussion

The chaperone–usher and TPS pathways

Assembly of pilins into a fibre-like structure at the surface of Gram-negative bacteria often relies on chaperone-usher pathways, in which the central channel of the outer membrane usher accommodates the pilin (Thanassi *et al*, 2002). The TPS systems involve a TpsB protein, which is used for the outer membrane translocation of TpsA proteins. The genes encoding TpsA and TpsB are clustered even though orphan genes exist throughout bacterial genomes (van Ulsen and Tommassen, 2006). TpsB and usher are β -barrel structures, which form pores of equivalent inner diameter (Méli *et al*, 2006).

Targeting of a TpsA substrate to its cognate TpsB transporter is mediated through interaction between the TpsA secretion domain and the periplasmic domain of the TpsB transporter. The periplasmic region of FhaC (TpsB) contains two POTRA domains, which are required for FHA binding and secretion (Clantin *et al*, 2007). Other POTRA domain-containing proteins from the Omp85 superfamily, such as YaeT from *E. coli*, bind polypeptides by β -strand augmentation and assist folding and insertion of β -barrel proteins into the outer membrane of bacteria (Kim *et al*, 2007; Tommassen, 2007). The structure of the YaeT periplasmic fragment shows four POTRA domains that share a common fold (β - α - α - β - β) (Kim *et al*, 2007), but are not functionally equivalent (Bos *et al*, 2007). Overall, POTRA domains are potentially responsible for nucleating folding in the proteins they are interacting with.

How is CupB5 transported to the cell surface?

In our study, we characterized an orphan *tpsA*-like gene, *cupB5*. CupB5 displays several features of TpsA passenger proteins. We revealed by using different approaches that CupB5 is transported at the bacterial surface and seems intertwined with CupB1 fimbriae.

We investigated whether a TpsB transporter could be involved in CupB5 secretion. There are five different

TPS systems encoded on the *P. aeruginosa* genome (Jacob-Dubuisson *et al*, 2001; Filloux *et al*, 2004a). We focused on the TpsB transporter, the substrate of which presented the highest homology to CupB5, that is, TpsA4. We found that a *tpsB4* mutant is not affected for CupB5 translocation. Instead, we showed that CupB5 uses the CupB3 protein to reach the bacterial cell surface. Our results demonstrate that the TpsA-like protein CupB5 is transported to the cell surface through an usher protein rather than classically through a TpsB transporter. This is consistent with the presence of the *cupB5* gene within the *cupB* gene cluster.

Is CupB3 an unusual usher protein?

Outer membrane usher proteins have a three-domain organization in which a central β -barrel domain is flanked by distinct N- and C-terminal domains (Thanassi *et al*, 2002). The periplasmic N-terminal domain is required for specific binding of the chaperone–subunit complexes (Dodson *et al*, 1993; Ng *et al*, 2004). This is reminiscent of the TpsB domain organization with the N terminus-containing POTRA domain required for specificity and substrate recognition.

We compared the aa sequence of the *P. aeruginosa* ushers, CupA3, CupB3 and CupC3, and found that CupB3 displayed an extended N-terminal region. This extended region might be coupled to the gain of a new function, that is, secretion of the TpsA-like protein CupB5 in addition to CupB1 fimbriae assembly. Sequence alignment of this N-terminal region with POTRA domains found in TpsB or Omp85 (YaeT) proteins showed significant similarities. The predicted secondary structure of this region is β - α - α - β , which is only slightly different from the more canonical β - α - α - β - β structure found in POTRA domains (Clantin *et al*, 2007; Yeo *et al*, 2007). It should be noted that among the conserved glycine residues, which are usually found in POTRA domains, only the first glycine is conserved in the CupB3 POTRA-like domain. As with other POTRA, this glycine is located within a loop connecting the first strand with the first helix. The second glycine is lacking, which introduces a small gap in the alignment. This small gap might be responsible for the difference in secondary structure predictions. Nevertheless, bioinformatics comparison of the CupB3 N terminus with known POTRA domains led to the conclusion that the usher CupB3 contains a POTRA-like domain, which might be involved in interaction with and folding of the TpsA-like protein CupB5.

CupB3 has a POTRA-like domain connected to an usher

CupB3 combines two functions within one single polypeptide. We showed that it has the classical function of ushers assembling fimbriae (Ruer *et al*, 2007), but during evolution CupB3 has acquired a feature of TpsB transporters, the POTRA domain, which likely recognizes and folds the CupB5 protein. We assessed this possibility by deleting parts of the POTRA-like region and tested whether the truncated CupB3 protein could support CupB5 assembly. Surprisingly, not only we observed that CupB5 could be found at the cell surface when the *cupB3* mutant was complemented with any of the truncated *cupB3* alleles but instead the assembly of CupB1 fimbriae was abolished. One explanation could be that CupB5 is transported to the bacterial surface but is misfolded and not properly released from the CupB3 channel, thus preventing access to the usher for CupB1 fimbrial subunits. This hypothesis is attractive

because we observed using transmission electron microscopy and immunogold-labelling that CupB5 is not found proximal to the surface, but distal, and probably is integrated within or associated with the CupB1 fimbriae. It is thus a possibility that CupB5 comes first into the usher and is displaced from the surface by the growing CupB1 fimbriae. To assess the possibility of CupB5 blocking access to the usher, we introduced a *cupB5* deletion in the *cupB3* mutant and assessed the restoration of CupB1 fimbriae assembly when the strain is complemented by *cupB3* alleles. Strikingly, in this background, CupB1 fimbriae were readily assembled at the cell surface, showing that this process is POTRA independent. In other words, our data demonstrate that the POTRA-like domain of CupB3 is not required for CupB5 targeting, but likely it is involved in proper folding of CupB5 during its translocation through CupB3. Even though CupB5 is cell surface exposed, it might not be properly secreted/translocated and may remain connected to the usher. The POTRA-like domain might have a crucial function to complete the secretion/translocation process, which finally results in the release of CupB5 from the usher. Finally, our observation suggests that assembly of CupB5 and CupB1 fimbriae is coordinated and kinetically ordered.

If the POTRA-like domain of CupB3 is not essential for targeting CupB5, alternative pathways might be considered. One possibility is that CupB5 is targeted to the usher in a chaperone-dependent manner, similar to fimbrial subunits. We tested the role of the CupB4 chaperone in the process and found that a *cupB4* mutant was unable to expose either CupB5 or CupB1 onto the cell surface (Figure 6B). Nevertheless, we could not conclude that the CupB4 chaperone is directly involved in CupB5 targeting. Indeed, the 3D structure of the PapC usher revealed the presence of a plug within its central channel (Remaut *et al*, 2008). It is proposed that the adhesin-chaperone complex, such as FimH-FimC (So and Thanassi, 2006; Munera *et al*, 2007; Remaut *et al*, 2008), relays a conformational change to the plug domain resulting in the opening of the usher channel. It is thus a possibility that the CupB4 chaperone is required for targeting of CupB6, the putative adhesin in the CupB system (Figure 6A), which could be needed to open the CupB3 channel. The activated channel will then allow CupB1 and CupB5 transport and assembly.

Evolutionary comparison between the *P. aeruginosa* and *P. fluorescens* cupB clusters

The transformation of an usher into a POTRA-like containing usher, or P-ushe, is exciting from an evolution point of view. The most closely related chaperone-usher gene cluster to the *P. aeruginosa* cupB cluster is the *P. fluorescens* PFL_1462–PFL_1469 cluster (Nuccio and Baümler, 2007). The main difference is that the *P. fluorescens* cupB5-like gene (PFL_1467) is associated with a gene encoding a TpsB-like transporter (PFL_1466). In *B. pertussis*, it was earlier reported that chaperone usher genes (*fimABCD*) and *tps* genes (*fhaBC*) could be linked (Willems *et al*, 1994). However, it was shown that FhaC is essential for FHA production but not for fimbriae biogenesis. In *Pseudomonas*, the vicinity between these two types of assembly pathway may have favoured genetic recombination and the fusion of an usher and a TpsB transporter into a P-ushe. We tested the functionality of the TpsB protein from *P. fluorescens* towards the *P. aeruginosa* CupB5

protein. Interestingly, the TpsB transporter (PFL_1466) is able to restore CupB5 transport in the *P. aeruginosa* strain deleted for the gene encoding the CupB3 P-ushe. However, the TpsB protein is not able to fully replace the function of the P-ushe as no CupB1 fimbriae were assembled at the cell surface. Furthermore, we showed that CupB5 transport is dependent on the presence of the POTRA domain in TpsB (PFL_1466), which confirms that CupB5 transport requires a POTRA either in CupB3 or in PFL_1466. This original observation strengthens the possibility that the *P. aeruginosa* CupB3 hybrid P-ushe has emerged from the fusion between a gene encoding an usher and a gene encoding a TpsB protein.

Conclusion

The CupB3 protein is an usher that has evolved into a novel class of bacterial transporter, that we called the P-ushe. The P-ushe CupB3 is a composite of two transporters previously described as involved in separate transport mechanisms known as type Vb secretion and chaperone-usher pathways. The usher domain may have fused during evolution to the POTRA domain of a TpsB transporter. The P-ushe CupB3 has a central function in monitoring both transport processes, assembly of CupB1 fimbriae and transport of CupB5 (TpsA), through one single outer membrane pore. The detailed mechanistic of the process, which probably involves sequential events, remains to be elucidated. We may propose that in the absence of the POTRA in CupB3, CupB5 translocation is partially abortive, probably due to CupB5 misfolding. Misfolded CupB5 jams the CupB3 P-ushe, locks the CupB3 channel and prevents access for CupB1 fimbriae.

Materials and methods

Bacterial strains, plasmids and growth conditions

The strains and plasmids used are listed in Table I. Strains were grown at 30 or 37°C on solid agar M63 medium supplemented with 0.4% L-arginine and 1 mM MgSO₄, in the presence of 1 mM IPTG. Recombinant plasmids were introduced into *P. aeruginosa* using conjugative properties of pRK2013. Transconjugants were selected on *Pseudomonas* isolation agar supplemented with appropriate antibiotic concentrations. For *E. coli*: ampicillin (Ap) 50 µg ml⁻¹, gentamycin (Gm) 25 µg ml⁻¹, kanamycin (Km) 25 µg ml⁻¹, tetracycline (Tc) 15 µg ml⁻¹ and streptomycin (Sm) 50 µg ml⁻¹. For *P. aeruginosa*: carbenicillin (Cb) 300 µg ml⁻¹, gentamycin (Gm) 80 µg ml⁻¹, tetracycline (Tc) 200 µg ml⁻¹ and streptomycin (Sm) 2 mg ml⁻¹.

P. aeruginosa mutant construction

PCR was used to generate 550-bp DNA fragments located upstream from the *cupB1*, *cupB4*, *cupB5* and *tpsB4* genes using the DelB1Up5/DelB1Up3, DelB4Up5/DelB4Up3, DelB5Up5/DelB5Up3 and DelB4Up5/DelB4Up3 oligonucleotide pairs, respectively, whereas 550 bp downstream DNA fragments were obtained with the DelB1Dn5/DelB1Dn3, DelB4Dn5/DelB4Dn3, DelB5Dn5/DelB5Dn3 and DelB4Dn5/DelB4Dn3 oligonucleotide pairs, respectively (Table II). We used appropriate DNA fragments as matrix and performed overlapping PCR with external pair of oligonucleotides DelB1Up5/DelB1Dn3, DelB4Up5/DelB4Dn3, DelB5Up5/DelB5Dn3 and DelB4Up5/DelB4Dn3 (Table II), to generate the *cupB1*, *cupB4*, *cupB5* and *tpsB4* mutagenic DNA fragments, respectively. The mutagenic DNA fragments were cloned into pCR2.1 and re-cloned into the pKNG101 or pEX18 suicide vectors, as BamHI/EcoRV, SpeI/NsiI, EcoRI/XbaI or SmaI fragments for *cupB1*, *cupB4*, *cupB5* or *tpsB4*, respectively. The recombinant plasmids were transferred into *P. aeruginosa* and the mutants were selected on LB plates containing 5% sucrose supplemented with Irgazan at 25 µg ml⁻¹ and appropriate antibiotics (Kaniga *et al*, 1991).

Table I Strains and plasmids used in this study

Strain or plasmid	Description or phenotype ^a	Reference/source
<i>E. coli</i>		
TG1	<i>supE</i> $\Delta(lac-proAB)$ <i>thi</i> <i>hsdR</i> $\Delta 5$ (<i>F'</i> <i>traD</i> 36 <i>rpoA</i> ⁺ <i>B</i> ⁺ <i>lacI</i> ^q $\Delta\Delta$ M15)	Lab collection
BL21 (DE3)	<i>F</i> [−] <i>ompT</i> <i>gal</i> [<i>dcm</i>] [<i>lon</i>] <i>hsdS</i> _B (<i>r</i> _K <i>m</i> _B [−]) <i>E. coli</i> B strain with DE3, a prophage carrying the T7 RNA polymerase gene	Lab collection
TOP10F'	<i>F'</i> (<i>lacI</i> ^q Tn10 (Tet ^R)) <i>mrcA</i> $\Delta(mrr-hsdRMS-mcrBC)$	Invitrogen
CC118 (λ pir)	Φ 80 <i>lacZ</i> Δ M15 Δ <i>lacX</i> 74 <i>recA1</i>	Lab collection
SM10	Host strain for pKNG101 replication, $\Delta(ara-leu)$ <i>araD</i> Δ <i>lacX</i> 74 <i>galE</i> <i>galK</i> <i>phoA</i> 20 <i>thi-1</i> <i>rpsE</i> <i>rpoB</i> <i>argE</i> (Am) <i>recA1</i> R ^R (λ pir) <i>thi-1</i> <i>thr</i> <i>leu</i> <i>tonA</i> <i>lacY</i> <i>supE</i> <i>recA</i> ::RP4-2-Tc::Mu (Km ^R)	Lab collection
<i>P. aeruginosa</i>		
PAO1	Wild-type strain	Lab collection
PAO1 Δ <i>pilA</i> Δ <i>fliC</i>	PAO1 deletion mutant for the <i>pilA</i> and <i>fliC</i> genes	Lab collection
PAO1 Δ <i>pilA</i> Δ <i>fliC</i> Δ <i>cupB3</i>	PAO1 deletion mutant for the <i>pilA</i> , <i>fliC</i> and <i>cupB3</i> genes	Ruer <i>et al</i> (2007)
PAO1 Δ <i>pilA</i> Δ <i>fliC</i> Δ <i>cupC3</i>	PAO1 deletion mutant for the <i>pilA</i> , <i>fliC</i> and <i>cupC3</i> genes	Ruer <i>et al</i> (2007)
PAO1 Δ <i>pilA</i> Δ <i>fliC</i> Δ <i>cupB1</i>	PAO1 deletion mutant for the <i>pilA</i> , <i>fliC</i> and <i>cupB1</i> genes	This study
PAO1 Δ <i>pilA</i> Δ <i>fliC</i> Δ <i>cupB4</i>	PAO1 deletion mutant for the <i>pilA</i> , <i>fliC</i> and <i>cupB4</i> genes	This study
PAO1 Δ <i>pilA</i> Δ <i>fliC</i> Δ <i>cupB5</i>	PAO1 deletion mutant for the <i>pilA</i> , <i>fliC</i> and <i>cupB5</i> genes	This study
PAO1 Δ <i>pilA</i> Δ <i>fliC</i> Δ <i>tpsB4</i>	PAO1 deletion mutant for the <i>pilA</i> , <i>fliC</i> and <i>tpsB4</i> genes	This study
PAO1 Δ <i>pilA</i> Δ <i>fliC</i> Δ <i>cupB3</i> Δ <i>cupB5</i>	PAO1 deletion mutant for the <i>pilA</i> , <i>fliC</i> , <i>cupB3</i> and <i>cupB5</i> genes	This study
PAO1 Δ <i>pilA</i> Δ <i>fliC</i> Δ <i>cupC3</i> Δ <i>cupB1</i>	PAO1 deletion mutant for the <i>pilA</i> , <i>fliC</i> , <i>cupC3</i> and <i>cupB1</i> genes	This study
<i>P. fluorescens</i>		
Pf-5	Wild-type strain	Paulsen <i>et al</i> (2005)
<i>Cloning plasmids</i>		
pCR2.1	TA cloning vector for PCR products, <i>lacZ</i> α ColE1 f1 <i>ori</i> , Ap ^R , Km ^R	Invitrogen
pMMB67EH-GW	Broad host range vector, IncQ, <i>ptac</i> , <i>lacZ</i> α , Gm ^R	Lab collection
pBBR1MCS-3	Broad host range vector, Tc ^R	Lab collection
pEX18	Ap ^R ; <i>oriT</i> ⁺ <i>sacB</i> ⁺ , gene replacement vector with MCS from pUC18	Lab collection
pKNG101	Suicide vector in <i>P. aeruginosa</i> , <i>sacB</i> , Sm ^R	Lab collection
pET-Dest42	Destination vector for GATEWAY technology, T7 promoter, Ap ^R	Invitrogen
<i>Plasmids</i>		
pBBR <i>cupB3</i>	<i>cupB3</i> gene cloned in pBBR1MCS-3, Tc ^R	Ruer <i>et al</i> (2007)
pBBR <i>cupB3</i> Δ P	<i>cupB3</i> gene deleted for the POTRA domain, in pBBR1MCS-3, Tc ^R	This study
pBBR <i>cupB3</i> Δ PI	<i>cupB3</i> gene deleted for nucleotides encoding aa 37–80 of POTRA domain, in pBBR1MCS-3, Tc ^R	This study
pBBR <i>cupB3</i> Δ PN	<i>cupB3</i> gene deleted for nucleotides encoding aa 20–80 of POTRA domain, in pBBR1MCS-3, Tc ^R	This study
pBBR <i>cupB3</i> Δ PC	<i>cupB3</i> gene deleted for nucleotides encoding aa 37–97 of POTRA domain, in pBBR1MCS-3, Tc ^R	This study
pBBR <i>cupB3</i> _{G28R}	<i>cupB3</i> gene with glycine 28 substituted in arginine cloned in pBBR1MCS-3, Tc ^R	This study
pBBR <i>cupB3</i> _{G28W}	<i>cupB3</i> gene with glycine 28 substituted in tryptophan cloned in pBBR1MCS-3, Tc ^R	This study
pBBR <i>tpsBPF</i>	<i>tpsB</i> (PFL_1466) gene of <i>P. fluorescens</i> cloned in pBBR1MCS-3, Tc ^R	This study
pBBR <i>tpsBPF</i> Δ P	<i>tpsB</i> (PFL_1466) gene of <i>P. fluorescens</i> deleted for the POTRA domain in pBBR1MCS-3, Tc ^R	This study
pMMB <i>rocS1</i>	<i>rocS1</i> gene cloned in pMMB67EH-GW, Gm ^R	Kulasekara <i>et al</i> (2005)
pET-Dest42 <i>cupB5</i>	<i>cupB5</i> gene cloned in pET-Dest42, Ap ^R	This study
pRK2013	ColE1 <i>ori</i> , <i>tra</i> ⁺ , <i>mob</i> ⁺ , Km ^R	Lab collection

^aSm^R, streptomycin resistance; Ap^R, ampicillin resistance; Km^R, kanamycin resistance; Gm^R, gentamycin resistance; Tc^R, tetracycline resistance.

Construction of *cupB3* alleles

See Supplementary data.

Cloning of *P. fluorescens* *tpsB*/PFL_1466 genes and derivatives

The PFL_1466 gene from *P. fluorescens* was PCR amplified from Pf-5 genomic DNA using the oligonucleotide pair PFTpsBUP/PFTpsBDn (Table II), thus leading to an amplicon of 1715 bp. The resulting DNA fragment was cloned into pCR2.1 and subcloned into the pBBR1MCS-3 using *KpnI*/*XbaI* restriction sites.

PCR amplification of DNA fragments corresponding to the N-terminal and C-terminal parts of *P. fluorescens* *TpsB* protein lacking the POTRA domain was performed using the PFTpsBAPUP/PFTpsBAPOTRADn and the PFTpsBAPOTRAUP/PFTpsBAPDn oligonucleotide pairs, thus leading to DNA fragments of 221 and

1160 bp, respectively. The resulting pairs of DNA fragments were used as templates for an overlapping PCR using the external pair of oligonucleotides PFTpsBAPUP/PFTpsBAPDn (Table II). The resulting DNA fragment had a size of 1381 bp, thus yielding the *tpsBPFAP* allele. The *tpsBPFAP* gene was cloned into pCR2.1 and subcloned into pBBR1MCS-3 digested with *XbaI*/*SpeI* restriction sites (Table I).

Production of antibodies directed against *CupB5*

Two peptides selected within the aa sequence of CupB5, AEFNGNSSRYRFTGPS and AEATGKVS GGSNSRVG, were synthesized (Eurogentec). Two rabbits were inoculated with the peptides at a concentration of 200 μ g ml^{−1}. The immunization included an injection followed by three boosters at 15-day, 1- and 2-month intervals. The rabbits were bled, the sera were collected and purified against the peptides. Sera specificity was tested on whole

Table II Oligonucleotides used in this study

	Oligonucleotides (5'–3') ^a
<i>Mutation</i>	
<i>cupB1</i>	
DelB1Up5	GACCGGATGGTTGATAGGTCC
DelB1Up3	CGCCCCGGGTGATTTCTTTGGAG TTGTGG
DelB1Dn5	CAGCCCGGGGCGGCGGTGACGACGAA
DelB1Dn3	TGGGCGGAACCTCCAGGATATT
<i>cupB4</i>	
DelB4Up5	CACGCGCCAAATGCCAGGGGCGCGGC
DelB4Up3	CGAACTCCTAACGCGGTACAGCGTCCGC
DelB4Dn5	GTGACCGGCGTTAGGAGTTCGGCA GGCCCCG
DelB4Dn3	GGATATCGTGGCCTTGGTGCCGATCAC
<i>cupB5</i>	
DelB5Up5	AAAGAAATTCGTGAGCTACCGCTGC
DelB5Up3	GGACCCGGGTGCTTCTTCTCCAGTGAA
DelB5Dn5	GCACCCGGGTCCCTGGCGGACCTCCCTT
DelB5Dn3	GCTCTAGAACCCGCTTGACGCCGTCTCT
<i>tpsB4</i>	
DeltpsB4Up5	AGAACGGCATTTCTAGCGTGCGTCTCTG
DeltpsB4Up3	AGCTTGCTCATCCCATGAGGCCGTACGGA
DeltpsB4Dn5	CCTCATGGGATGACCAAGCTCCCGCCCGC
DeltpsB4Dn3	CGAGGAAGTCCCTGTGCGCCA
<i>Geneengineering/cloning</i>	
<i>cupB3</i>	
CupB3Up	CGCGGTACCCGCGCCATTCTCGACGA
CupB3Dn	GCTCTAGACGGTACAGCGTGCG
CupB3EVSup	GTTTGCACTGCGACCCT
CupB3EVSdn	TTCGAAGAATCCAGGCTCA
<i>cupB3ΔP</i>	
ΔPOTRAUp	TGGGCAGGTCATCGTCTTCGACGCGC
ΔPOTRADn	CGAAGACGATGACCTGCCCACCGTAA
<i>cupB3ΔPI</i>	
ΔPOTRA37–80Up	TTCCCTGCGACCCGCGGTGGCCGTT
ΔPOTRA37–80Dn	CCACCGCGGTGCGCAGGAACGAACG
<i>cupB3ΔPN</i>	
ΔPOTRA20–80Up	TGGGCAGGTCCCGCGGTGGCCGTT
ΔPOTRA20–80Dn	CCACCGCGGGACCTGCCCACCGTAA
<i>cupB3ΔPC</i>	
ΔPOTRA37–97Up	TTCCCTGCGAATCGTCTTCGACGCGC
ΔPOTRA37–97Dn	CGAAGACGATTGCGCAGGAACGAACGG
<i>cupB3_{G28R}</i>	
CupB3G28RUp	GATCCCTCGCTGTATCGCCA
CupB3G28RDn	TGGCGATACAGCGAGGGATC
<i>cupB3_{G28W}</i>	
CupB3G28WUp	GATCCCCCACTGTATCGCCATT
CupB3G28WDn	GAATGGCGATACAGTGGGGGATC
<i>tpsBPF PFL₁₄₆₆</i>	
PFTpsBUp	GGGGTACCGAACGGCGCTGGCAGCGATT
PFTpsBDn	GCTCTAGACGGCACCACTCCGAAGCAC
<i>tpsBPFΔP PFL₁₄₆₆</i>	
PFTpsBΔPUp	GGACTAGTTGAACGCGCTGGCAGCGA
PFTpsBΔPDn	GCTCTAGAAATTAACGCCCTGCAACCTAT
PFTpsBΔPOTRAUp	CGGCATCCGCGACGACCTTCCCGG GGTCCG
PFTpsBΔPOTRADn	GAAGGTGCTGCGGATGCCGGA GGTGTCGG

^aRestriction sites have been underlined in sequence. *EcoRI* in DelB5Up5; *XbaI* in DelB5Dn3, CupB3Dn, PFTpsBDn or PFTpsBΔPOTRADn; *KpnI* in CupB3Up and PFTpsBUp and *SpeI* in PFTpsBΔPOTRAUp.

cell extracts of *E. coli* TG1 strains expressing the *cupB5* gene (pET-Dest42cupB5).

Shearing the bacterial cell surface

Bacteria were scraped from M63 agar plates. The equivalent of 5 U of OD₆₀₀ was resuspended in L broth. Shearing of cell surface

proteins or appendages was performed by gentle agitation at 4°C overnight. Bacteria were harvested by centrifugation and supernatant-containing sheared material was centrifuged at 17 600 g for 10 min at 4°C. Supernatant was subjected to ammonium sulphate precipitations as described earlier (Ruer *et al*, 2007). The pellets were resuspended in 500 μl of Tris 10 mM, pH 8. A protease inhibitor cocktail (Boehringer) was added to the sample to minimize proteolytic degradation.

SDS–PAGE electrophoresis and western blot analysis

The samples containing whole cell extract or sheared material were adjusted to 0.025 OD₆₀₀ equivalent unit per microlitre in SDS–PAGE loading buffer. The samples were boiled for 10 min and proteins were separated by electrophoresis on a 12% polyacrylamide gel. The proteins were either stained with Coomassie blue or blotted onto nitrocellulose membranes. The CupB5 protein was immunodetected using the CupB5 polyclonal antibodies at a dilution of 1:1000. A peroxidase-conjugated goat anti-rabbit IgG at a dilution of 1:5000 in TBS containing 10% milk and 0.1% Tween was further applied and the presence of the protein was further revealed using a chemoluminescence revelation kit (Pierce). To check for cellular leakage, immunoblotting was performed to detect the cytoplasmic OTCase using antibodies at a dilution of 1:500 and the periplasmic DsbA protein using antibodies at a dilution of 1:10 000.

Transmission electron microscopy

Bacterial cells obtained in conditions described for the shearing experiments were processed for transmission electron microscopy. They were collected in a tube containing 50 μl of 10 mM PBS. A drop of the bacterial suspension was placed on Formvar- and carbon-coated copper grids and left for 5 min. Grids were fixed with 1% paraformaldehyde for 5 min and rinsed two times for 5 min with 10 mM PBS. For CupB5 protein detection, grids were further incubated with a 5% BSA solution in 10 mM PBS for 10 min and incubated with the CupB5 antibodies at a 1:100 dilution in 0.5% BSA and 10 mM PBS for 45 min. Grids were finally incubated with 5 nm colloidal gold protein A solution (Tebu) for 30 min in 0.5% BSA and 10 mM PBS. After repetitive washes in 10 mM PBS and in water, grids were immersed in a drop of 1% uranyl acetate for 1 min and were examined in a JEOL 1200EX transmission electron microscope operating at 80.0 kV.

Mass spectrometry

Proteins were excised from Coomassie blue-stained gels and subjected to procedures routinely performed by the IBSM proteomic platform (<http://www.ibsm.cnrs-mrs.fr/ifrc/servtech/seq/>). Briefly, samples were treated through reduction and alkylation processes, further digested with trypsin, desalted and subjected to MALDI-TOF analysis. Internal scale up was done with ions coming from trypsin autolysis and from keratin. Mass values were compared with theoretical values from peptides referenced in databases.

Bioinformatic analysis

The genome sequence of *P. aeruginosa* and *P. fluorescens* and their annotations are available at <http://www.pseudomonas.com>. The aa sequences of the *cupB3* and *cupB5* gene products were analysed with software such as Signal3P, LipoP, PsiPRED, SMART, ClustalW and T-COFFEE, available at <http://www.embl-heidelberg.de/predictprotein/predictprotein.html>.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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