Molecular characteristics of the CupB chaperone-usher pathway and the Tps4 two-partner secretion system in *Pseudomonas aeruginosa*

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This thesis is presented for the degree of Doctor of Philosophy of Imperial College London and Diploma of Imperial College London 2014.
Candidate’s Declaration

I, Daniela Muhl, hereby confirm that this thesis represents my own work and that any external contributions to the research are duly acknowledged.

Daniela Muhl

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Abstract

The opportunistic human pathogen *Pseudomonas aeruginosa* is a threat for immunocompromised individuals, a major cause of nosocomial infections, and is prevalent in patients with cystic fibrosis. The bacterium can form biofilms that help it evade the immune response. It adheres to host cells using molecular adhesins, such as pili assembled by chaperone-usher pathways (Cup). Understanding the adhesion could, therefore, help develop treatments that prevent the establishment of infections.

This thesis considers the CupB system, consisting of an usher (CupB3), two chaperones (CupB2 and CupB4) and two pilin subunits (CupB1 and CupB6). The chaperones target the pilin subunits to the usher assembling a CupB1-containing pilus with a putative CupB6 adhesin at its tip. The *cupB* operon also encodes the TpsA-like protein (two-partner secretion) CupB5, previously suggested to be secreted by CupB3.

The aim of this work was to understand the CupB1-containing pilus assembly and CupB5 secretion mechanism. Genetic and biochemical techniques were used, such as deletion or point mutation, qRT-PCR, pull-down assays, shearing assays, and protein structure prediction or resolution. They led to the following results.

First, each chaperone likely has a cognate substrate: CupB1 interacts with CupB2 and CupB6 with CupB4. Second, the crystal structure solved for CupB6 showed that it has a pilin and a putative adhesin domain, connected by a poly-proline linker. Third, CupB5 secretion was observed to be CupB3-independent and TpsB4-dependent. *tpsB4* is encoded with its substrate *tpsA4*. The expression of the *cupB* and *tpsB4/tpsA4* operons was shown to be controlled by the same regulatory pathway, Roc1, and deletion of the *tpsB4* transporter gene abolished CupB5 secretion. Fourth, a structural analysis indicated that TpsB4 has two POTRA domains, and POTRA-1 interacts with the highly homologue TPS motifs of CupB5 and TpsA4.

Based on these results, the thesis presents a model of CupB pilus assembly and CupB5 secretion.
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1. Introduction

Almost ten out of a hundred people admitted to a European hospital will become infected with pathogenic bacteria during their stay (WHO, 2002). These hospital-acquired (nosocomial) infections lead to high mortality and morbidity, longer treatments, and ultimately, increased cost of medical care. Most infections are caused by the four pathogenic bacteria: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococci* (Bereket et al., 2012). Problematic is that bacteria are easily carried over from medical equipment, people and air. Traditional treatment is becoming increasingly ineffective, as many pathogens have developed resistance to antibiotics. These multidrug-resistant pathogens cause over a half of two million infections acquired in the US hospitals every year (Bereket et al., 2012). About 10% of the infections are due to *P. aeruginosa* (Murray et al., 2005), a Gram-negative bacterium with a strong intrinsic resistance to antibiotics. In 2011–12, it came under the spotlight in the UK when a *P. aeruginosa* outbreak killed three babies in a neonatal intensive care unit at Belfast’s Royal Jubilee Maternity Hospital. *P. aeruginosa* does not only cause these acute infections, but also chronic ones, which are especially problematic in patients suffering cystic fibrosis. Thus understanding the mechanism through which *P. aeruginosa* establishes infections will be crucial to develop anti-bacterial treatments. This Chapter will review data available about *P. aeruginosa* virulence factors, with the focus being on the development and regulation of biofilms and on the release of exoproteins via secretion systems.
1.1 *P. aeruginosa*—an opportunistic pathogen

*P. aeruginosa* usually inhabits soil and water, but can also be isolated from patients with a weak immune system. An opportunistic human pathogen, it is abundant in hospitals and intensive care units (Bergen and Shelhamer, 1996; Foca *et al.*, 2000; Gellatly and Hancock, 2013; Pruitt B. A., 1974). It attaches to medical equipment, such as catheters and tubing, and forms there resilient biofilms that are difficult to remove. Local infections can also disseminate (Costerton, 1999). Acute lung infections caused by *P. aeruginosa* are systematic in patients with a weak immune system: patients treated for cancer and HIV, or those with organ transplants (Williams *et al.*, 2010). The infections often lead to ventilator-associated pneumonia. The patients are intubated with an endotracheal tube, which can damage the tissue. Bacteria on or in the tube can colonise the host via the epithelial breach and progress into the bloodstream. *P. aeruginosa* also induces necrosis and apoptosis of the host cells by injecting toxins via the type III secretion system (T3SS, see Section 1.7.3) (Kenny *et al.*, 2009), ultimately causing sepsis and death of a third of the patients (Williams *et al.*, 2010). The T3SS can also prevent elimination of the bacterium by macrophages (Dacheux *et al.*, 2001; Diaz and Hauser, 2010).

In contrast to acute, chronic infections are a problem in patients suffering cystic fibrosis. Cystic fibrosis (CF) is a genetic disease caused by a mutation in the CF transmembrane regulator, a chloride channel in epithelial cells (Gellatly and Hancock, 2013). In healthy people, the lungs are protected from bacterial infections by a mucus layer. Bacteria embedded within the mucus are removed from the lungs by ciliated epithelial cells, which rhythmically beat their cilia and transport the mucus towards the pharynx. However, malfunction of the CF transmembrane regulator dehydrates the mucus layer of the lungs. The mucus of CF patients becomes thick and viscus and compresses the ciliae. The ciliae can no longer beat properly, and the bacteria trapped in the mucus remain in the lungs. It causes chronic infection. The viscous mucus also prevents an effective immune response by making it difficult for neutrophils (which are phagocytic cells) to
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reach bacteria. Neutrophils usually engulf bacteria and lyse them by releasing reactive oxygen species, lysozymes and defensins. They can also degranulate and, in chronically infected lungs, repeatedly damage the lung tissue and function (Williams and Parkos, 2007). The bacterial infection cannot be cleared, so an inflammatory response persists. It is now the main cause of premature death of CF patients.

When \textit{P. aeruginosa} establishes a chronic infection, it changes the phenotype through pathoadaptive mutations (Mena \textit{et al.}, 2008; Smith \textit{et al.}, 2006). Folkesson \textit{et al.} (2012) have recently analysed the global gene expression profiles of a clinical \textit{P. aeruginosa} strain. They found that the mutations occurred in the three global regulators MucA, LasR and RpoN. \textit{lasR} encodes a transcriptional regulator of quorum sensing (see Section 1.3.3), whereas \textit{rpoN} encodes the $\sigma$-factor $\sigma^{54}$, which activates the expression of many virulence genes. MucA is an anti-$\sigma$-factor that sequesters the $\sigma$-factor $\sigma^{22}$. It releases $\sigma^{22}$ during cell envelope stress and $\sigma^{22}$ triggers, for example, loss of flagellar motility and T3SS, but it also upregulates the production of the exopolysaccharide alginate. \textit{P. aeruginosa} cells that have a mutation in \textit{mucA}, overproduce alginate and have a mucoid phenotype (Martin \textit{et al.}, 1993). The advantage of such a thick layer of alginate is that it protects the bacterium against the innate immune response (Mathee \textit{et al.}, 1999). Moreover, antibiotic treatments also result in a change of the bacterial phenotype: the emergence of small colony variants. These are highly resistant to antibiotics (Häussler \textit{et al.}, 1999), probably due to an overproduction of the Pel and Psl polysaccharides, components of the protective biofilm matrix (see below).

1.2 Formation of biofilms

When \textit{P. aeruginosa} adheres to human host cells and persists there, a person can become chronically infected. The infections are difficult to treat: \textit{P. aeruginosa} is intrinsically highly resistant to antibiotics thanks to multidrug efflux pumps (such as MexAB-OprM) and antimicrobial-modifying enzymes (such as $\beta$-lactamases) (Morita \textit{et al.}, 2014).
Biofilms provide even further resistance. They are bacterial communities (witlingly called “city of microbes” by R. Kolter) attached to abiotic or biotic surfaces. These communities are encased by a protective matrix of extracellular polymeric substance (Watnick and Kolter, 2000). Biofilms protect the bacteria, for example, from the host immune system, antibiotics, pH changes, oxygen radicals and nutrient limitations (Davey and O’Toole, 2000). Bacteria in biofilms slow down their metabolism and are thus not harmed by antibiotics that target growing, metabolically active cells (Pamp et al., 2008; Werner et al., 2004). Biofilms also hamper diffusion of antibiotics. And even having reached their destination, antibiotics might not be effective due to anaerobic conditions in the biofilm (Schobert and Tielen, 2010; Stewart and William Costerton, 2001). Moreover, the biofilm resistance of *P. aeruginosa* is enhanced by high mutation rates (as described above) and horizontal gene transfer (Poole, 2011).

How biofilms develop depends on environmental signals such as nutrient availability (e.g. phosphate and iron limitation, amino acid starvation), host factors (e.g. mucin), and osmolarity (Petrova and Sauer, 2012). Biofilm formation follows distinct and well defined stages (see Figure 1.1): (i) reversible attachment, (ii) irreversible attachment, (iii) formation of microcolonies, (iv) maturation, and (iv) dispersal (Sauer et al., 2002; Tolker-Nielsen et al., 2000; Webb et al., 2003). In *P. aeruginosa* each stage can be described as follows.

(i) **Reversible attachment.** Planktonic and free-swimming *P. aeruginosa* bacteria attach loosely and reversibly to a surface by their flagella and type IV pili (see Section 1.5.2 and 1.5.3). With the help of a flagellum, a bacterium can spin on the surface. It can also assemble its type IV pili and attach them to the surface. The cell then retracts the pili and is pulled towards the attachment point. This form of motility is called twitching (Burrows, 2012a; Petrova and Sauer, 2012). Both spinning and twitching motility allow *P. aeruginosa* to find a surface suitable for attachment.

(ii) **Irreversible attachment.** *P. aeruginosa* cells adhere irreversibly onto the surface by downregulating their flagellum biosynthesis and upregulating the production of adhesive
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Figure 1.1: Stages of biofilm formation by *P. aeruginosa*. The schematic shows the five stages of biofilm formation by *P. aeruginosa*. They can be divided into (i) reversible attachment, (ii) irreversible attachment, (iii) formation of microcolonies, (iv) maturation, and (iv) dispersal. Free-living and motile bacteria (orange) attach first loosely (i) and then irreversibly (ii) to a substrate, replicate and form microcolonies (iii). Microcolonies contain sessile bacteria (grey) and further mature into a mushroom-shaped biofilm (iv). Bacteria in a biofilm are encased by an extracellular polymeric substance matrix (light orange). A subpopulation of the sessile bacteria dies or becomes motile again, causing the biofilm to disperse (v). Motile bacteria can attach somewhere else and start the biofilm cycle again (i).

proteins, such as type IV pili and chaperone-usher pathway pili (see Section 1.10).

(iii) **Microcolony formation.** Bacteria spread across the surface either by clonal replication or by twitching motility. They form microcolonies—small clusters of cells.

(iv) **Maturation.** The microcolonies grow into macrocolonies that can form a mushroom-shaped structure (Klausen *et al.*, 2003b). Type IV piliated bacteria climb the mushroom stalk, which consists of non-motile cells, and form the mushroom cap (Klausen *et al.*, 2003a). Aqueous channels separate the mushroom structures and allow nutrients and oxygen to circulate in different layers of the biofilm as well as waste products to be removed. An extracellular polymeric substance matrix encases the whole community and protects the bacterial population.

(v) **Dispersal.** Nutrient deficiency or accumulation of reactive oxygen species can make life in a biofilm unfavorable for the cells (Barraud *et al.*, 2006; Sauer *et al.*, 2004). These conditions can provoke cell lyses and bacteriophage induction, killing the cells and dispersing the biofilm (Webb *et al.*, 2003). Matrix disruptions destabilise the biofilm and release free-living cells into the environment. The released cells can attach to a new surface and
form a biofilm (McDougald et al., 2012).

A prominent characteristic of a bacterial biofilm is the production of a protective extracellular polymeric substance matrix. The nature and composition of the matrix can vary from one organism to the other, but it is always made of exopolysaccharides, extracellular DNA (eDNA), lipids, proteins and water (Flemming and Wingender, 2010; López et al., 2010). *P. aeruginosa* (although not all strains) produces at least three exopolysaccharides known as Pel, Psl, and alginate (Friedman and Kolter, 2004; Ouellette et al., 2007; Vasseur et al., 2005). Alginate consists of guluronic and manuronic acids and is constitutively produced by mucoid *P. aeruginosa* strains isolated from the lungs of CF patients. As described above, mucoid strains have a mutation in the anti-σ-factor MucA, resulting in the upregulation of alginate biosynthesis (Martin et al., 1993; Pulcrano et al., 2012). Alginate could be important for microcolony formation and biofilm stability, and for protecting the bacterium from the host immune response (Flemming and Wingender, 2010; Mathee et al., 1999).

However, non-mucoid strains, which express only small amounts of alginate, can also form a biofilm. These strains produce Pel and Psl exopolysaccharides. Pel polysaccharides are rich in glucose and are needed to form a pellicle—a biofilm formed at the liquid-air interface when the bacteria grow in static conditions. Moreover, *pel* genes are expressed only after the initial attachment. They are essential for microcolony formation and biofilm structure in *P. aeruginosa* strain PA14. This strain does not produce the Psl exopolysaccharide, as it has a deletion in the *psl* gene cluster (Friedman and Kolter, 2004). The Psl polysaccharide consists of a repeating penta-saccharide made of mannose, glucose, and rhamnose units (Byrd et al., 2009). Psls are constitutively expressed in PAO1, which also produces Pel polysaccharides, and they are required for adherence to biotic and abiotic surfaces and for stabilisation of biofilms (Colvin et al., 2011).

Polysaccharides have long been suspected to be the main component of the biofilm matrix. As is now well known, eDNA is involved as well. eDNA, released from lysed cells, supports intercellular attachment and aggregation, and it maintains the structural in-
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tegrity of the extracellular polymeric substance matrix (Das et al., 2013; Whitchurch et al., 2002). In *P. aeruginosa*, eDNA forms a grid-like structure mainly found in the stalk of the mushroom-like biofilm (Allesen-Holm et al., 2006). As Gloag et al. (2013) have recently postulated, eDNA also coordinates continuous movement of *P. aeruginosa* cells towards the migrating edge of the biofilm. The mushroom-like biofilms do not only contain water channels, but also a network of trails. These trails are layered with eDNA, with which *P. aeruginosa* can spontaneously align. Bacteria easily migrate through the trails by twitching motility towards the migration edge. Furthermore, *P. aeruginosa* can, by secreting an extracellular deoxyribonuclease, utilize eDNA as a nutrient source during starvation (Mulcahy et al., 2010).

Finally, extracellular proteins serve diverse functions ranging from biopolymer-degrading enzymes to virulence factors, lectins, amyloids, type IV and chaperone-usher pili (Flemming and Wingender, 2010). A biopolymer-degrading enzyme released by *P. aeruginosa* is the alginate lyase AlgL (Boyd and Chakrabarty, 1994). AlgL is presumably released when biofilm growth is no longer favorable and helps release bacteria from the biofilm by degradation of alginate. The release or assembly mechanism and functions of virulence factors, lectins, amyloids, type IV and chaperone-usher pathway (Cup) pili will be discussed in Sections 1.7, 1.5 and 1.10.

*P. aeruginosa* can switch between the sessile and planktonic lifestyles depending on the environmental conditions. It switches to the sessile biofilm lifestyle, for example, during a chronic lung infection of CF patients (Hassett et al., 2010; Singh et al., 2000). In the form of a biofilm, *P. aeruginosa* resists the host immune response and can stay in the lungs for the whole patient’s life. In contrast, *P. aeruginosa* planktonic lifestyle is associated with acute infections. The regulatory networks that lead to biofilm formation are complex and involve multiple and intricate signaling pathways. The networks will be discussed in the following chapter.
1.3 Regulation of biofilm formation

Biofilm formation is a subtle process involving a series of successive stages described above. Transition between the stages depends on the environment and involves dedicated regulatory systems. The key regulatory components signaling biofilm formation are (i) the second messenger cyclic di-guanosine monophosphate (c-di-GMP), (ii) two-component systems and (iii) quorum sensing (Petrova and Sauer, 2012). Researchers have just started to unfold the complexity of these signaling networks and the links between them.

1.3.1 Two-component systems

Bacteria sense and adapt to their environment using a central sensory network. The network involves two-component systems (TCS). They usually consist of a sensor histidine kinase and a response regulator (West and Stock, 2001) (see Figure 1.2). The transmembrane or cytoplasmic sensor usually has an input and transmitter domain, and the cytoplasmic response regulator has a receiver and output domain. The kinase senses environmental fluctuations (such as oxygen, temperature, and nutrients) via its input domain. This results in the dimerisation of the sensor kinase. The kinases autophosphorylate in trans a conserved histidine residue in their transmitter domains. They then transfers the phosphoryl group onto a conserved aspartate on the response regulators receiver domain. Subsequently the substrate affinity of the response regulators output domain changes. The output domain can have a helix-turn-helix motif, which allows the response regulator to bind to the DNA and to activate gene transcription. Alternatively, it can carry an enzymatic activity (Galperin, 2010), such as a diguanylate cyclase or a phosphodiesterase involved in the production and degradation of c-di-GMP (see Section 1.3.2). Dephosphorylation of the response regulator by the sensor kinase resets the system. Examples of TCS and their function are summerised in table 1.1.

The phosphotransfer need not be carried out in a single step, but may involve multiple transfers—a process termed phosphorelay (Goulian, 2010) (see Figure 1.2). Because
Figure 1.2: **Types of two-component systems.** TCS usually consists of a sensor kinase that recognizes a certain stimulus via its input domain and a response regulator that when activated carries out a function, such as DNA binding or an enzymatic activity. Having sensed the stimulus, sensor kinases dimerize and autophosphorylate *in trans* a conserved histidine in their transmitter domain. **(classical)** In the classical TCS, the phosphoryl group is then transferred to a conserved aspartate in the receiver domain of a response regulator, activating its output domain. **(hybrid)** The sensor kinases of hybrid TCS are fused to an additional receiver domain. From there the phosphoryl group is transferred to a separate Hpt domain-containing protein and finally to the receiver domain of the response regulator. **(unorthodox)** Unorthodox sensor kinases are fused to a receiver and Hpt domain through which the phosphoryl group is transferred to the response regulator. Figure after Mikkelsen *et al.* (2011).

The phosphoryl group is transferred from histidine to aspartate, the sensor kinases had to evolve to activate their response regulators. An additional module, an Hpt domain (**Histidine phosphotransfer**), is now used for the transfer. Depending on the localisation of the Hpt module, TCS can be classified into hybrid and unorthodox. Hybrid sensors have an additional receiver domain with an aspartate residue and transfer the phosphoryl group onto a separate Hpt domain-containing protein, which then phosphorylates the receiver domain of the response regulator. In contrast, receiver and Hpt domains are a part of the sensor kinase in un-orthodox sensors (Rodrique *et al.*, 2000).

In *P. aeruginosa*, TCS are encoded by 130 genes. This large number allows the bacterium to adapt to a broad range of environments and explains its versatility (Rodrique *et al.*, 2000). TCS are usually insulated to allow a specific response. However, some of the TCS can also cross-talk (Laub and Goulian, 2007) and one sensor kinase can signal to two response regulators. One of the regulators would be the cognate partner, whereas the
second would belong to another TCS. Such cross-talk has been observed, for example, for the Roc1 and Roc2 TCS of *P. aeruginosa* (see Section 1.11.4).

Table 1.1: Two-component systems in *P. aeruginosa*.

<table>
<thead>
<tr>
<th>Sensor</th>
<th>Response</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FleS</td>
<td>FleR</td>
<td>Flagellar motility, adhesin to mucin</td>
<td>Dasgupta et al. (2003); Ritchings et al. (1995)</td>
</tr>
<tr>
<td>PilS</td>
<td>PilR</td>
<td>Type IV pili (T4P)-mediated motility</td>
<td>Hobbs et al. (1993)</td>
</tr>
<tr>
<td>FimS</td>
<td>AlgR</td>
<td>Alginate and cyanide production, quorum sensing, T4P-mediated motility</td>
<td>Deretic et al. (1989); Lizewski et al. (2002, 2004); Morici et al. (2007); Whitchurch et al. (1996); Yu et al. (1997)</td>
</tr>
<tr>
<td>GacS</td>
<td>GacA</td>
<td>Exopolysaccharide production, T4P, T3SS, T6SS, quorum sensing, iron homeostasis, cyanide production</td>
<td>Brencic and Lory (2009); Kay et al. (2006); Parkins et al. (2001); Pessi et al. (2001); Reimmann et al. (1997); Soscia et al. (2007)</td>
</tr>
<tr>
<td>RocS1</td>
<td>RocA1 (SadA), RocR (SadR)</td>
<td>CupB and CupC pili regulation, biofilm formation, T3SS, T1SS, antibiotic resistance</td>
<td>Kuchma et al. (2005); Kulasekara et al. (2005); Sivaneson et al. (2011)</td>
</tr>
<tr>
<td>RocS2</td>
<td>RocA2</td>
<td>CupB and CupC pili regulation, biofilm formation, T3SS, T1SS, antibiotic resistance</td>
<td>Kulasekara et al. (2005); Sivaneson et al. (2011)</td>
</tr>
<tr>
<td>PvrS, RcsC</td>
<td>PvrR, RcsB</td>
<td>CupD pili regulation</td>
<td>Mikkelsen et al. (2013); Nicasstro et al. (2009)</td>
</tr>
<tr>
<td>PprA</td>
<td>PprB</td>
<td>CupE pili and T4b pili regulation, quorum sensing, antibiotic resistance</td>
<td>Bernard et al. (2009); de Bentzmann et al. (2012); Dong et al. (2005); Giraud et al. (2011); Wang et al. (2003)</td>
</tr>
<tr>
<td>BfiS</td>
<td>BfiR</td>
<td>Irreversible attachment during biofilm formation</td>
<td>Petrova and Sauer (2009)</td>
</tr>
<tr>
<td>BfmS</td>
<td>BfmR</td>
<td>Microcolony formation</td>
<td>Petrova and Sauer (2009)</td>
</tr>
<tr>
<td>MifS</td>
<td>MifR</td>
<td>Formation of macrocolonies and mature biofilms</td>
<td>Petrova and Sauer (2009)</td>
</tr>
</tbody>
</table>
1.3. REGULATION OF BIOFILM FORMATION

The GAC signal transduction pathway

The switch between the biofilm and planktonic lifestyle of *P. aeruginosa* is regulated by the Gac signal transduction pathway. It consists of the unorthodox transmembrane sensor kinase GacS and its cognate response regulator GacA (Jimenez et al., 2012) (see Figure 1.3). When stimulated, GacS autophosphorylates and transfers the phosphoryl group to GacA. GacA is a DNA-binding response regulator and binds to the GacA boxes in the promoter region of *rsmY* and *rsmZ* (Brencic and Lory, 2009). It results in the upregulation of the expression of the small non-coding sRNAs RsmY and RsmZ. RsmY and RsmZ then sequester the mRNA-binding protein RsmA and relieve its translational repression. Free RsmA represses the translation of, for example, the type VI secretion system (T6SS) or *pel* and *psl* exopolysaccharides mRNA, which ultimately abolishes biofilm formation. Additionally RsmA promotes *P. aeruginosa* planktonic lifestyle by activating T3SS biosynthesis, rhamnolipid production, and swarming motility (Brencic and Lory, 2009). Thus binding of RsmA by RsmY and RsmZ promotes *P. aeruginosa* biofilm lifestyle.

The activity of the Gac pathway is also fine-tuned by the sensor kinases LadS and RetS, which have an antagonistic impact on the Gac-dependent signaling (Ventre et al., 2006). RetS negatively controls the expression of the sRNAs and promotes a planktonic lifestyle. LadS, in contrast, upregulates *rsmY/rsmZ* expression and activates *P. aeruginosa*’s biofilm lifestyle. Mechanistic insights on how the control may happen have been obtained with RetS, which has been shown to interact directly with GacS by forming a heterodimer (Goodman et al., 2009). Heterodimerisation prevents GacS autophosphorylation and interrupts the phosphotransfer to GacA. RsmA then stays bound to the mRNA of the T6SS and biofilm-related genes and in turn favors expression of genes involved acute infection (for example, the T3SS genes). As an additional level of regulation, RetS and the Gac pathway are also under control of the hybrid sensor kinase PA1611 in *P. aeruginosa*. PA1611 interacts directly with RetS and positively regulates the expression of the sRNAs RsmY and RsmZ, thus favoring *P. aeruginosa*’s biofilm lifestyle (Kong et al., 2013).

Whereas RetS and LadS appear to matter at the early stages of biofilm formation, further de-
1. INTRODUCTION

Figure 1.3: The Gac pathway in *P. aeruginosa*. The main components of this pathway are the sensor kinase GacS and the response regulator GacA. GacS phosphorylates GacA upon stimulation. Gac then activates the transcription of the small sRNAs *rsmY* and *rsmZ*, which sequester and therefore inhibit the translational repressor RsmA. RsmA releases the mRNA, such as mRNA transcribed from *pel* and T6SS genes. GacS function can be fine-tuned by the sensor kinases RetS and LadS. RetS represses the cascade and promotes *P. aeruginosa* motile lifestyle, whereas LadS activates GacS and induces *P. aeruginosa* biofilm lifestyle. Figure adapted from Moscoso et al. (2011).

Development is regulated in a stage-specific manner by additional two-component systems: BfiRS, BfmRS, and MifRS (Petrova and Sauer, 2009). BfiRS is essential for the transition from reversible to irreversible attachment; BfmRS for the transition to microcolony formation; and MifRS for the formation of macrocolonies and mature biofilms. As Petrova and Sauer (2009) suggested, successive activation of the three systems might force a transition to the next biofilm stage. Similarly, interruption of the TCS stops the biofilm cycle at a particular stage. So, for example, mutation in *mifRS* will prevent biofilm maturation.

1.3.2 Second messenger c-di-GMP

Gram-negative bacteria, including *P. aeruginosa*, have a universal signaling network that uses the intracellular concentration of c-di-GMP to switch biofilm lifestyles. High amounts of this second messenger favour a sessile lifestyle. On the other hand, low amounts promote a motile lifestyle (Jenal, 2004; Simm et al., 2004). The concentrations of intracellular c-di-GMP are controlled by the activity of two antagonistic enzymes, GGDEF domain-containing diguanylate
1.3. REGULATION OF BIOFILM FORMATION

cyclases (DGCs), and EAL domain-containing phosphodiesterases (PDEs). DGCs synthesise c-di-GMP from two molecules of GTP. PDEs hydrolyse c-di-GMP to the nucleotide pGpG, which is then converted into GMP (Römling et al., 2013). *P. aeruginosa* encodes 17 DGCs, 5 PDEs and 16 proteins with both GGDEF and EAL domains, although not all influence biofilm formation under static conditions (Kulasakara et al., 2006). Many of the proteins with both EAL and GGDEF domain have a phosphodiesterase activity, which is activated when c-di-GMP binds to the GGDEF side.

A well-studied DGC in *P. aeruginosa* is WspR, part of the Wsp chemosensory signal transduction system. Such systems usually sense the levels of chemical substrates, allowing bacteria to migrate toward a favorable environment (Scott et al., 2012). These substrates are sensed via transmembrane chemoreceptors, which activate a phosphorylation cascade and, subsequently, a response regulator. In *P. aeruginosa*, chemosensory systems also play a role in biofilm formation by influencing the levels of c-di-GMP (Hickman et al., 2005). In the Wsp system, the transmembrane chemoreceptor WspA detects an unknown signal (probably, when the bacterium contacts a surface) and in turn activates the WspE sensor kinase. Upon autophosphorylation, WspE phosphorylates the WspR response regulator and produces high amounts of c-di-GMP.

The synthesised c-di-GMP can bind to PilZ domain-containing proteins, to transcriptional factors, and RNA riboswitches, thus allowing a control on the transcriptional, post-transcriptional, and post-translational level (Ryan, 2013). A transcriptional factor under control of c-di-GMP in *P. aeruginosa* is the master regulator of flagellar biosynthesis, FleQ. At low concentration of c-di-GMP, FleQ positively controls the biosynthesis of the flagellum. It also represses the synthesis of the Pel exopolysaccharides by binding to the *pel* operon promoter (Hickman and Harwood, 2008). At high levels of c-di-GMP, FleQ binds c-di-GMP and relieves its transcriptional repression from the *pel* promoter. It also downregulates the expression of flagellar genes. Upregulation of Pel biosynthesis and downregulation of flagellum biosynthesis thus promotes *P. aeruginosa* biofilm lifestyle (Baraquet and Harwood, 2013; Guttenplan and Kearns, 2013).

As mentioned above, c-di-GMP also binds enzymatically inactive proteins that have a GGDEF and EAL domain and thus activates their function. Such a protein of the Lap system of *Pseudomonas fluorescens* is LapD. It is activated when c-di-GMP binds to its EAL domain. LapD is involved in the secretion and cell-surface binding of the large adhesin protein LapA. LapA
is essential for the irreversible attachment to a surface at the early stages of biofilm formation (Hinsa et al., 2003; Ivanov et al., 2012). It is secreted to the cell surface through a type I secretion system (T1SS, see Section 1.7.1) and anchored in the outer membrane (Ivanov et al., 2012). When the biofilm lifestyle is not favorable, however, LapA is cleaved from the cell surface by the protease LapG (Gjermansen et al., 2010; Newell et al., 2011). LapG’s activity is controlled by LapD depending on the c-di-GMP level. At high concentration of c-di-GMP, LapD interacts with LapG and represses its activity. At low concentrations, LapG is activated by LapD and releases the LapA adhesin from the cell surface. High c-di-GMP concentrations thus promote biofilm formation.

Although c-di-GMP signaling is complex, it overall phenotypically impacts biofilm by influencing expression of one or more determinants involved. As Moscoso et al. (2011) showed, c-di-GMP signaling can also influence the expression and switch of protein secretion systems. As described above, the Gac signal transduction pathway is negatively controlled by the sensor kinase RetS. In the absence of RetS, the T3SS is off but the T6SS is on. The study showed that the levels of c-di-GMP in a retS mutant are increased, resulting in a hyperbiofilm phenotype. Moreover, artificial production and degradation of c-di-GMP can cause a RetS-independent switch between T3SS and T6SS production. Thus, high levels of c-di-GMP also activate the transcription of the T6SS genes, associated with biofilm formation and chronic infections. In contrast, low levels of c-di-GMP repress the T6SS and activate expression of T3SS, whose effectors play a role during acute infections.

### 1.3.3 Quorum sensing

Even before in-depth studies of c-di-GMP and Gac, Davies et al. (1998) suggested that quorum sensing is involved in biofilm formation. Quorum sensing is a regulatory network that cells rely on to control their gene expression when a certain cell density is reached. It synchronises the behavior of bacterial populations. Bacteria monitor the cell density by producing extracellular molecules, called autoinducers (Jimenez et al., 2012).

The autoinducers of Gram-negative bacteria are usually acyl-homoserine lactones (AHLs). Gram-positive bacteria, on the other hand, use small peptides (Li et al., 2012). AHLs, made from fatty
aclyl chains ligated to a lactonized homoserine, are produced by autoinducer synthases, proteins of the LuxI family. The length of the acyl chain can vary, and \textit{P. aeruginosa} produces a 3-oxo-C-12-AHL and a C-4-AHL, which have a C-12 or C-4 acyl chain, respectively. These AHLs are strongly hydrophobic due to the acyl chain. Thus to cross the cell envelope, they might use the MexAB-OprM multi-drug efflux pump (Evans \textit{et al.}, 1998; Pearson \textit{et al.}, 1999). \textit{P. aeruginosa} also produces \textit{Pseudomonas} quinolone signal (PQS), a third autoinducer made from esters. PQS is also hydrophobic and is trafficked among the cells via membrane vesicles (Mashburn and Whiteley, 2005). With increasing cell density, the concentration of extracellular and intracellular AHLs increases and reaches a threshold at which AHLs are bound to a transcriptional regulator of the LuxR family (Fuqua \textit{et al.}, 1996, 1994). Subsequently, the transcriptional regulator binds to the DNA and activates the expression of specific genes.

\textbf{Figure 1.4: The LasI/LasR and RhlI/RhlR quorum sensing system of \textit{P. aeruginosa}.} The LasI synthase produces the autoinducer 3-oxo-C-12-AHL (HSL), which at a certain concentration binds to the LasR transcriptional regulator. LasR upregulates the expression of LasI (autoinduction) and of virulence factors, such as the elastases LasA, LasB and alkaline protease AprA. Simultaneously, LasR activates the expression of the RhlR transcriptional regulator, inducing RhlI synthase production and thus synthesis of the C4-AHL (HSL) autoinducer. C4-AHL is bound by RhlR at a certain threshold, which then upregulates the expression of genes coding for the Rhl synthase, the rhamnolipids RhlAB and the alkaline protease AprA. As can be seen on the left, some of the genes upregulated by both systems overlap. Figure reproduced from Jimenez \textit{et al.} (2012).
The *P. aeruginosa* quorum sensing systems LasI/LasR and RhlI/RhlR produce and recognize a 3-oxo-C-12-AHL and a C-4-AHL, respectively (see Figure 1.4) (Li et al., 2012). The Las system is higher in the expression hierarchy than the Rhl system, as a complex of LasR and 3-oxo-C-12-AHL also activates expression of *rhlI* and *rhlR*. Moreover, both systems are interconnected via the PQS system: LasI/R positively regulates PQS synthesis, whereas RhlI/R inhibits it. The regulons of Las and Rhl partially overlap and regulate the expression of virulence factors, such as elastases LasA and LasB and alkaline protease AprA. These factors play a role during acute infection and host cell damage (Jimenez et al., 2012). That both systems are involved in the later stages of biofilm formation has been shown in a *lasI/rhlI* mutant: the biofilm was 29% thinner than the wild-type biofilm (Davies et al., 1998). Except of forming a biofilm separated by waterchannels, the microcolonies were also densely packed. Moreover Singh et al. (2000) showed that *P. aeruginosa* cells grown in biofilms produce more C-4-AHL than cells grown in broth cultures. In contrast cells in broth cultures synthesise high levels of 3-oxo-C-12-AHL. The analysis of *P. aeruginosa*-containing sputum samples, isolated from the lung of CF patients, showed that the sputum also contained high levels of C-4-AHL. This led to the assumption that *P. aeruginosa* forms biofilm in the CF lung.

The regulatory network of quorum sensing is large, but can branch into other known regulatory pathways. Relevant for biofilm formation, quorum sensing is positively regulated by the response regulator GacA (Pessi et al., 2001; Reimmann et al., 1997). For example, Kay and coworkers observed strongly reduced levels of C-4-AHL in a rsmY and rsmZ double deletion mutant (Kay et al., 2006). Ueda and Wood (2009) found another interesting link between quorum sensing and biofilms. They suggested that quorum sensing and c-di-GMP signaling are linked via the TbpA tyrosine phosphatase in *P. aeruginosa*. First, they identified a *tbpA* mutant that showed increased attachment, biofilm formation, polysaccharide production and aggregation, and had a reduced swimming and swarming motility. Second, they showed that *tbpA* transcription is LasR-dependent. They concluded that a LasR/AHL complex activates expression of the periplasmic tyrosine phosphatase TbpA, which dephosphorylates and deactivates the membrane-bound diguanylate cyclase TbpB. As described above, the reduction in c-di-GMP levels on one hand downregulates the expression of Pel polysaccharides and adhesins and on the other upregulates flagellar motility, subsequently preventing biofilm formation.
Bacterial lifestyles are controlled by complex regulatory networks. As a result, the bacterial cell surface might change and effectors and toxins might be secreted. These changes play a key role in attachment, biofilm formation, motility, and interaction with the host. The transport of proteins on the bacterial surface or the release of exoenzymes and toxins involves a set of multiprotein complexes embedded in the bacterial membranes. These will be described in the following Sections.

1.4 Translocation across the inner membrane

In Gram-negative bacteria, exoproteins are translocated to the cell surface across the cytoplasmic (inner) membrane, the periplasm, and the outer membrane. The inner membrane is made of a phospholipid bilayer with embedded inner membrane proteins and multiprotein complexes. Inner and outer membranes are separated by the periplasm that contains a thin layer of peptidoglycan. The outer membrane consists of a protein- and a lipoprotein-containing asymmetric bilayer. The bilayer is made of lipopolysaccharides on the outer face and phospholipids on the inner face. To transport exoproteins across these membranes, bacteria have developed a multitude of secretion systems. This Section discusses the first step of exporting proteins outside the cytoplasm: the translocation across the inner membrane. This process can use one of two systems: the Sec (including YidC) or TAT translocons.

1.4.1 SecYEG translocon

The SecYEG translocon is an inner membrane (IM) heterotrimeric channel, used to transport proteins and lipoproteins into and across the cytoplasmic membrane (Chatzi et al., 2013; du Plessis et al., 2011). It is the major protein translocon in bacteria. A similar system is also found in archea and in the membrane of the endoplasmic reticulum in eukaryotes (Kudva et al., 2013).

The export of proteins across the inner membrane requires, apart from the inner membrane SecYEG translocon, several additional factors, such as the inner membrane accessory proteins SecDF, the cytoplasmic SecB chaperone and the cytoplasmic SecA ATPase (see Figure 1.5).
Figure 1.5: **Export of unfolded proteins by the SecYEG translocon.** (A) Exoproteins and outer membrane proteins are post-translational translocated by the SecYEG translocon. The preproteins signal peptide is bound by the trigger factor and the SecB chaperone during translation, preventing the misfolding of the preprotein. After translation, SecB targets the preprotein towards the ATPase SecA, in contact with the SecYEG translocon. The preprotein is exported through the SecYEG pore with the help of the SecDF inner membrane protein complex. Lastly, the signal peptide is cleaved off by the SPase I in the periplasm. (B and C) Inner membrane proteins are co-translational inserted into their membrane. The signal recognition particle (SRP) binds to the preproteins N terminus as soon as it exits the ribosome, stops translation and transports the complex toward the membrane-bound FtsY. The preprotein is transferred to the SecYEG translocon (B) or to the YidC insertase (C) and released into the IM. Figure reproduced from du Plessis *et al.* (2011) with permission from Elsevier.

Depending on the destination, even more components are involved: the trigger factor (TF), the signal recognition particle (SRP), and the SRP receptor FtsY. Proteins targeted to the SecYEG translocon are synthesised as preproteins or prelipoproteins with a signal peptide that is cleaved by a signal peptidase (SPase) in the periplasm (Paetzel *et al.*, 2002). The exported protein can either stay in the periplasm, is secreted across the outer membrane via secretion systems (see Section 1.7), or is inserted into the outer membrane via the Bam (β-barrel assembly machinery) or Lol (Lipoprotein outer membrane localization) complex (see Section 1.6).

**Signal peptides**

The signal peptides of preproteins and prelipoproteins differ from each other and are cleaved by two types of SPases (compare Figure 1.6 and 1.12) (Chatzi *et al.*, 2013). The preproteins signal peptide, also known as type I signal peptide, has positively charged residues at the N terminus,
followed by 4-16 hydrophobic core residues and further polar C-terminal residues (von Heijne, 1985). The signal peptide is cleaved at its C terminus by SPase I in the periplasm (Paetzel et al., 2002). Prelipoproteins also have a Sec signal peptide, but it contains an additional conserved lipobox (Okuda and Tokuda, 2011). The processing of prelipoproteins is complex and will be discussed in detail in Section 1.6.2.

Figure 1.6: Signal peptides of Sec and TAT preproteins. The typical signal peptide of SecYEG-exported proteins contains a positive charged N terminus (1-8 amino acids long), a hydrophobic core (4-16 residues) and a polar C terminus (1-11 residues) (Chatzi et al., 2013; von Heijne, 1985). The signal peptide of TAT-exported proteins also has the three domain organisation, however the N terminus contains a conserved S-R-R-X-F-L-K motif with the two conserved arginine residues (orange) that are recognized by the TAT transporter and consists of 3-25 residues (Kudva et al., 2013). Both signal peptides are cleaved by the signal peptidase SPase I (arrow).

Export of preproteins

Preproteins, targeted for export, are translocated post-translationally. To prevent the misfolding and aggregation of the growing polypeptide during translation, its signal peptide is bound by the trigger factor (TF) as soon as its N terminus emerges from the ribosome (Deuerling et al., 1999; Eisner et al., 2003; Hoffmann et al., 2012). After translation, the preprotein is bound by the SecB chaperone (Bechtluft et al., 2007) and targeted to the ATPase SecA, bound to the SecYEG channel (see Figure 1.5A). Upon ATP binding by SecA, SecB is released, and the preprotein is transferred to SecA (Schiebel et al., 1991). At the same time, SecA changes its conformation and pushes the preproteins signal peptide and the following residues into the SecYEG channel. The channel opens and allows the preprotein to emerge step-wise in the periplasm. The step-wise export is enabled by the conformational changes of SecA through the repeated hydrolysis and binding of ATP, which pushes 5 kDa of the preprotein further through the channel (Schiebel et al., 1991; Tomkiewicz et al., 2006). It is still unknown exactly how the conformational change of SecA drives the translocation of the preprotein (Kudva et al., 2013).
The translocation of the preprotein is driven not only by ATP binding and hydrolysis but also by the proton motif force (Driessen, 1992). Depending on the proton motif force, the IM protein complex SecDF pulls the preprotein from the periplasmic side (Tsukazaki et al., 2011). The signal peptidase cleaves off the preprotein’s N-terminal signal peptide (as described above) and releases the protein into the periplasm.

Insertion of proteins into the inner membrane

The Sec complex not only exports preproteins but also inserts proteins into the inner membrane (see Figure 1.5B). The integration of IM proteins is co-translational and requires the signal recognition particle (SRP) and the SRP receptor FtsY. Proteins targeted to the IM have a hydrophobic transmembrane anchor (von Heijne, 1985). The anchor is recognised and bound by the SRP as soon as the protein’s N terminus comes out the ribosome. Binding of the SRP halts the translation, allowing the SRP to target the ribosome:protein complex to membrane-bound FtsY (Ng et al., 1996; Walter and Blobel, 1981). SRP and FtsY form an heteromer that hydrolyses GTP and transfers the ribosome:protein complex to the SecYEG channel (Beckmann et al., 2001). The ribosome then continues with the translation and pushes the protein into the channel. SecY opens laterally and the protein is released into the IM (Becker et al., 2009).

Proteins can also be inserted into the inner membrane by the YidC insertase (see Figure 1.5C), working either independently of or together with the SecYEG translocon (Kedrov et al., 2013; Kol et al., 2009). Although it is unclear how YidC integrates proteins into the inner membrane, Kedrov et al. (2013) showed that a YidC monomer interacts with a translating ribosome.

1.4.2 TAT translocon

Proteins that are exported by the Sec system have to remain unfolded. Instead, the twin-arginine-transporter (TAT) translocates folded proteins, oligomerised or in complex with cofactors, across the inner membrane. Many of these proteins are cofactor-containing redox proteins (Kudva et al., 2013). The components of the TAT translocon are the three inner membrane proteins TatC, TatA and TatB (see Figure 1.7). TatC is the central component of the pathway and spans the membrane with six transmembrane domains (Behrendt et al., 2004). TatB and
1.4. TRANSLOCATION ACROSS THE INNER MEMBRANE

TatA have a single domain (Hu et al., 2010; Kudva et al., 2013). It is yet unknown whether cytoplasmic chaperones, specifically dedicated to the TAT complex, are required to target the preprotein. However, TAT substrates have been observed in complex with general chaperones, such as TF, DnaK and SlyD (Graubner et al., 2007; Jong et al., 2004).

Figure 1.7: Export of folded proteins by the TAT translocon. (1) Translocation starts with the recognition of the preproteins signal peptide via TatC, which forms an oligomeric complex with TatA in the inner membrane. (2) The signal peptide is bound in the TatBC binding pocket in the form of a hairpin-like structure. TatA polymerises, interacts with the TatBC:preprotein complex and (3) forms the translocation pore through which the preprotein is translocated in a proton motive force (PMF)-dependent manner. Lastly, the signal peptidase SPase I cleaves off the signal peptide at the periplasmic interface of the inner membrane and releases the now mature protein. (4) The TatABC complex depolymerises for another round of translocation. Figure reproduced from Palmer and Berks (2012) with permission from Macmillan Publishers Ltd.

Signal peptides

Signal peptides recognised by the TAT system, have the same organisation as those recognised by the Sec pathway: positive N-terminal residues, a hydrophobic core, and polar C-terminal residues (see Figure 1.6). However, TAT signal peptides are less hydrophobic (Cristóbal et al., 1999), are usually longer, and have the characteristically conserved S-R-R-X-F-L-K motif (Berks, 1996), with the two arginines responsible for the name “Twin Arginine Translocation”. The preproteins are processed by the signal peptidase SPase I, as described above for the Sec pathway (Lüke et al., 2009; Yahr and Wickner, 2001).

Export of preproteins

Translocation starts with heterodimerisation of TatC and TatB (de Leeuw et al., 2002). The arriving substrate is first recognised by TatC and then bound by both TatC and TatB (see
Figure 1.7). TatC functions as a signal peptide insertase and imbeds the preproteins signal peptide into a TatBC-binding pocket in the IM, where it adopts a hairpin-like structure (Fröbel et al., 2012). It is not yet fully understood how the TAT substrate is translocated, but Lee et al. (2006) suggested that several TatC:TatB dimers form an oligomer with TatB in the middle. Then, a TatA tetramer associates with the TatC:TatB oligomer. TatA has been proposed to either form the translocation pore (see Figure 1.7) (Gohlke et al., 2005; Walther et al., 2013) or to destabilise the IM, allowing direct passage of substrates through the lipid bilayer (Brüser and Sanders, 2003). In the latter case, TatC could pull the substrate across the membrane. Independent of the translocation model, the whole process is driven by the proton motif force, which appears to be coupled to the transfer of the TAT substrate through the inner membrane (Theg et al., 2005). When translocation is completed, the signal peptidase SPase I cleaves off the signal peptide, releasing the protein into the periplasm (Kudva et al., 2013). Like for the SecYEG translocon, the protein stays in the periplasm, is secreted across the outer membrane via secretion systems (see Section 1.7), or is inserted into the outer membrane via the Bam or Lol complex (see Section 1.6).

1.5 Extracellular Appendages

Extracellular appendages are proteinaceous filaments that protrude from the bacterial cell surface. They can be involved in attachment, biofilm formation and/or motility. Sometimes, the subunits forming the filament are first transported by the Sec pathway, but alternative and specific routes are also embedded in the biogenesis of the filament. One type of extracellular appendages is known as type I pilus in *E. coli* and is assembled by the chaperone-usher pathway. The chaperone-usher pathway is the main topic of this thesis and is covered in detail in Section 1.10. The other most known and characterised appendages are the unipolar flagellum, the type IV pili (T4P) and the curli fibers. Curli biogenesis depends on the extracellular nucleation-precipitation pathway. Flagellum and T4P are assembled by specific machineries whose mechanism is similar to that of the secretion systems. The assembly of these extracellular appendages will be reviewed in the following Section.
1.5.1 Curli fiber biogenesis

Curli fibers are functional amyloids, essential for adhesion to different surfaces and for structuring the biofilm scaffold during biofilm maturation as observed for *E. coli* strain K-12 (Kikuchi *et al.*, 2005). They are also the major component in the extracellular biofilm matrix of uropathogenic *E. coli* (Hung *et al.*, 2013) and have been shown to promote internalisation of *E. coli* into eukaryotic cells (Gophna *et al.*, 2001), probably by binding to host proteins such as fibronectin (Olsén *et al.*, 1989) and plasminogen (Sjöbring *et al.*, 1994).

Csg curli fiber assembly in *E. coli*

Biogenesis of curli fibers is well studied in *E. coli* (Olsén *et al.*, 1989) and *Salmonella enteritidis* (Collinson *et al.*, 1991). It involves the extracellular nucleation-precipitation pathway as well as the SecYEG translocon that first exports the curli proteins, required for curli biogenesis, into the periplasm. In *E. coli*, curli are transcribed by two divergent operons: *csgBAC* and *csgDEFG* (see Figure 1.8A) (Hammar *et al.*, 1995). CsgA is the major fiber subunit, whose polymerisation is induced by the nucleator CsgB (Bian and Normark, 1997). CsgB can also form short polymers at the cell surface in the absence of CsgA. CsgC is a small periplasmic protein with an immunoglobuline-like fold and a CXC motif. The role of CsgC is unknown. However, because the CXC motif is structurally similar to the N terminus of DsbD (a membrane protein essential for the correct formation of disulfide bonds in the periplasm), CsgC could be involved in folding of CsgA (Taylor *et al.*, 2011). CsgE and CsgF have a chaperone-like function and have been suggested to support secretion and cell surface attachment of CsgA and CsgB (Nenninger *et al.*, 2011, 2009). Finally, CsgG forms an octameric pore in the outer membrane and is required for the secretion of Csg proteins (Robinson *et al.*, 2006).

Evans and Chapman (2013) proposed a model of curli assembly where, having been exported to the periplasm, CsgG forms the outer membrane pore (see Figure 1.8B). Outside on the cell surface, CsgF binds to the CsgG pore and interacts with the CsgB nucleator, causing CsgB to attach to the surface and form an amyloid fiber template. Meanwhile, CsgE chaperones bind CsgA fiber subunits in the periplasm and target them to the pore. As soon as CsgA exits the pore, it interacts with the CsgB template and assembles/precipitates into a curli fiber that folds
Figure 1.8: *E. coli* and *P. aeruginosa* curli operons and curli biogenesis in *E. coli*. (A) Curli fibers in *E. coli* are encoded by two divergent *csg* operons. *csgA* encodes the major fiber subunit, *csgB* the nucleator, *csgC* a periplasmic protein, *csgD* a transcriptional activator, *csgE* and *csgF* chaperones and *csgG* an outer membrane pore. (B) Assembly of curli fibers in *E. coli*. Proteins are colour-coded as in (A). All proteins are displayed as cartoons, except for GsgG’s periplasmic portion for which β-strands and loops are shown (purple). CsgG forms a pore in the OM through which the fiber subunits are translocated. On the extracellular side, CsgF interacts with CsgG and CsgB, causing CsgB to nucleate at the surface. In the periplasm, the CsgA major fiber is targeted to CsgG by CsgE. Upon reaching the cell surface via CsgG, CsgA interacts with the CsgB template and assembles into a curli fiber. Figure B reproduced from Evans and Chapman (2013) with permission from Elsevier. (C) The *fap* operon (PA1951-PA1956) encodes curli fibers in *P. aeruginosa*. Genes are coloured as in (A). In contrast to *E. coli* curli fibers in *P. aeruginosa* are only encoded in one operon.

into β-sheets and assembles into a cross-β structure (Collinson et al., 1991; Nelson et al., 2005). The process is called extracellular nucleation-precipitation. The formed curlis are thin and non-branching. They are made from strongly aggregated proteins, are highly stable, and can only be depolymerised by extreme chemical treatments (Chapman et al., 2002).

**Fap curli fibers in *P. aeruginosa***

Curli fibers, known as functional bacterial amyloids (FuBa), have also been identified in *P. aeruginosa* PAO1, *P. fluorescens* Pf-5 and UK4, and in *Pseudomonas putida* F1 (Dueholm et al., 2010, 2013). Although endogenous FuBa are not detectable under standard laboratory conditions in these strains, they can be detected when expressed from a plasmid. Recombinant expressed FuBa cause strong cell aggregation and pellicle formation even in liquid cultures, indicating their roles in attachment to abiotic surfaces and in stabilisation of the biofilm scaffold (Dueholm et al., 2013).

In contrast to *E. coli*, curli in *P. aeruginosa* strain PAO1 are encoded by the single *fapF-A*
(functional amyloids in *Pseudomonas*, see Figure 1.8C) operon (Dueholm *et al.*, 2010, 2013). Overexpression and purification of recombinant curlis and their subsequent analysis by SDS-PAGE and mass spectrometry showed that they consist of mostly FapC (PA1954) and some FapB (PA1955) and FapA (PA1956) proteins. Moreover, a *fapA* mutant produced short curli-like structures that contained mainly FapB and little FapC, and had a strong biofilm phenotype. Thus, FapA might have chaperone-like functions and be essential for the proportion of FapB and FapC proteins in the assembled curli. A bioinformatic analysis predicted that FapF (PA1951) is the outer membrane pore and FapD (PA1953) is a protease.

Using this data, Dueholm *et al.* (2013) developed a model of the extracellular nucleation-precipitation of Fap curli. In this model, the periplasmic chaperone, FapA, targets the nucleator FapB and major fiber FapC towards the outer membrane β-barrel pore, FapF. FapB and FapC are secreted via FapF and proteolytically processed by the protease FapD. On the cell surface, FapB nucleates and initiates FapC polymerisation into the cross-β structure of amyloid fibers. The role of FapE (PA1952) is still unclear, but it was shown to associate with the extracellular amyloid fiber.

### 1.5.2 Type IV pili biogenesis

Type IV pili (T4P), discovered in *P. aeruginosa*, are long helical fibers that bacteria use to adhere to host cells or abiotic surfaces (Kang *et al.*, 1997; Saiman *et al.*, 1990), to glide and twitch across a substrate (Burrows, 2005; Mattick, 2002), to take up DNA (Aas *et al.*, 2002), and to form microcolonies and mushroom-like structures during biofilm formation (O’Toole and Kolter, 1998). Three classes of T4P, distinguished by characteristics in the pilin subunit, have been so far discovered. *P. aeruginosa* is the only bacterium that assembles all three T4P systems (Burrows, 2012a): T4aP (PA4525 - PA4528, PA4550 - PA4556, PA0395 - PA0396, PA3805, PA5044 - PA5048), T4bP (PA14,59240 - PA14,59360) and Tad (tight adherence pili, PA4297 - PA4306). T4a pilins are only 150 - 175 residues long, whereas T4b pilins are 180 - 200 residues long. Tad pilins, despite being a subclass of T4b pili, are the smallest among T4P, with only an average size of 50 - 80 amino acids (Giltner *et al.*, 2012).
Signal peptides

In contrast to preproteins and prelipoproteins that have a type I and type II signal peptide, T4P prepilins and T2SS prepseudopilins (see Section 1.7.2) have a type III signal peptide. It is found at the preproteins hydrophobic N terminus and contains a leader peptide. The leader peptide differs in length and composition among T4P (see Figure 1.9A): T4aP have the shortest leader peptide, consisting of only 5-6 residues. T4bP in contrast have a 15-30 residue long leader peptide and the one of TadP is 10-26 aa long. Tad pili can be further differentiated by their Flp motif consisting of the conserved Gly (−1), Gln (+5) and Tyr (+6) residues.

The prepilins and prepseudopilins are inserted into the inner membrane via the signal recognition pathway of the SecYEG translocation machinery (see Section 1.4.1) (Arts et al., 2007a). Once inserted into the inner membrane, the type III signal peptide is cleaved off just behind a conserved glycine residue by cytoplasmic prepilin peptidases (see Figure 1.9A). In *P. aeruginosa*, the PilD (also known as XcpA) prepilin peptidase cleaves T4a and T4b prepilins as well as T2SS prepseudopilins (Carter et al., 2010; Korotkov et al., 2012). Tad prepilins are cleaved by their own peptidase FppA (de Bentzmann et al., 2006). The first residue of mature pilins, a phenylalanine in T4aP and non-conserved residues in T4bP, is further methylated by PilD (Strom et al., 1993).

Components of the T4aP assembly machinery

Although not every step in the biogenesis of T4aP is understood yet, their assembly machine in *P. aeruginosa* is well characterised. The T4aP assembly machine consists of four subcomplexes: the outer membrane, (inner membrane) motor, alignment and pilus subcomplex (Burrows, 2012a) (see Figure 1.9B). The outer membrane subcomplex is made of the PilQ (PA5046) secretin, the channel through which the pilus is assembled (Berry et al., 2012), and the lipoprotein pilotin PilF (PA3805), required for the oligomerisation of PilQ and its insertion into the outer membrane (Koo et al., 2008). PilQ is a secretin protein. Such proteins are also found in the T2SS and T3SS (see Section 1.7) (Filloux, 2004; Koster et al., 1997). Energy for the pilus assembly and retraction is provided by the inner membrane motor proteins PilB (PA4526), PilT (PA0395) and PilU (PA0396), associated with the platform protein PilC (PA4527). The PilB
Figure 1.9: **Signal peptides of type IV pili and P. aeruginosa’s T4aP assembly system.** (A) Signal peptide characteristics of major and minor pilins of T4aP, T4bP and Tad pili. Shown are full-length proteins with an N-terminal leader peptide followed by the mature region consisting of hydrophobic and hydrophilic domains. + represents positively charged, orange conserved and X non-conserved amino acids. The arrow shows the cleavage side of the prepilin peptidase. **(B)** Assembly system of T4aP in *P. aeruginosa*. Shown are the pilus subcomplex: the major pilin PilA and the minor pilins FimU, PilVWXE (here referred to as mP); motor subcomplex: the ATPases PilB, PilT and PilU; alignment subcomplex PilP, PilO, PilN and PilM; outer membrane subcomplex: the PilQ secretin, the PilF lipoprotein; and the PilD prepilin peptidase and the PilC platform protein. OM is the outer membrane, PP the periplasm and IM the inner membrane. Figure adapted after Burrows (2012b).

ATPase is required for pilus assembly, whereas PilT and PilU ATPases are essential for pilus retraction (Whitchurch and Mattick, 1994; Wolfgang et al., 1998). Associated with the motor subcomplex is the PilD (PA4528) prepilin peptidase. The alignment complex, connecting the inner and outer membrane, consists of PilM (PA5044), PilN (PA5045), PilO (PA5046), PilP (PA5047) and FimV (PA3115) and coordinates pilus assembly through the secretin pore (Ayers
et al., 2009; Tammam et al., 2013). Finally, the pilus subcomplex is formed by the major subunit PilA (PA4525) and the minor subunits FimU (PA4550), PilV (PA4551), PilW (PA4552), PilX (PA4553) and PilE (PA4556). All these subunits are cleaved and methylated by PilD and the minor subunits are thought to initiate pilus biogenesis (Douzi et al., 2009).

Assembly and disassembly of the T4aP

The major and minor prepilins are inserted into the inner membrane via the signal recognition pathway (see Section 1.4.1) and are further processed by the PilD prepilin peptidase. The mature pilins are positioned with their hydrophobic N terminus embedded into the inner membrane and their hydrophilic C terminus pointed into the periplasm (Burrows, 2012a). The assembled minor pilins form an initiation complex for pilus biogenesis. The pilus grows by addition of PilA subunits at the base of the initiation complex, powered by PilB. Moreover, it has been speculated that PilB changes its conformation upon ATP binding and causes PilC to push the pilus out of the inner membrane. Subsequently, a new pilin subunit can be inserted into the inner membrane (Misic et al., 2010; Satyshur et al., 2007). In the pilus, the subunits interact through a salt bridge between the conserved +5 residues (Glu, see Figure 1.9A) and through the methylated N terminuses of the neighbouring pilins, causing the pilus to fold into a right-handed helix (Craig et al., 2006). The pilus then binds to a surface and is retracted through depolymerisation catalysed by the retraction ATPase PilT. Pilin subunits are recycled and used to assemble a new T4a pilus (Burrows, 2012a).

The repeated cycles of pilus assembly, tethering and retraction, allow the bacterium to twitch across a surface (Wall and Kaiser, 1999). Twitching is independent of the flagellar movement (see below) and is required for biofilm formation. For example, a P. aeruginosa pilA mutant, which does not assemble T4aP, is not able to form microcolonies and differentiate into biofilms (O’Toole and Kolter, 1998). It indicates that T4aP are essential for the initial attachment, when bacteria search for a nutrient-rich substrate to colonise and develop into microcolonies. Klausen et al. (2003b) even showed that the maturation of microcolonies to a mushroom-shaped biofilm requires T4aP, as only piliated bacteria can climb a biofilm stalk to form a cap.
1.5.3 Unipolar flagellum

*P. aeruginosa* has a single polar flagellum that it uses to swim in a liquid environment or to swarm on a slippery surface (Köhler et al., 2000). The flagellum is a long helical filament made of thousands of FliC flagellin subunits (PA1092) that polymerise at the flagellum tip (see Figure 1.10). To reach the tip, flagellin subunits travel from the cytoplasm through an inner membrane basal body, a periplasm- and outer membrane-spanning rod, an axle-like hook and, finally, the already assembled flagellum (Macnab, 2003). The flagellum assembly machine is evolutionary related to the T3SS that directly injects effectors into the cytosol of eukaryotic host cells (see Section 1.7.3).

The flagellum can be rotated clock- and counterclock-wise through an ion-powered motor complex connected to the basal body (see Figure 1.10). The motor consists of interacting rotor and stator protein complexes (Berg and Anderson, 1973; Macnab, 1977; Silverman and Simon, 1974). The FliG, FliM and FliN rotor subunits form a ring attached to the basal body and the stator complex. The stator is made from MotA and MotB subunits (Berg, 2003; Tang et al., 1996). MotA and MotB form the ion channel. The ion motive force induces a conformational change in MotA (Blair, 2003; Braun and Blair, 2001) and, since MotA is connected to the rotor via FliG, this change creates a torque rotating the flagellum and moving the bacterium. *P. aeruginosa* encodes two pairs of stator proteins: MotA/B (PA4954/PA4953) and MotC/D (PA1460/PA1461) (Doyle et al., 2004; Toutain et al., 2005). A single stator pair alone is sufficient to drive the bacterium’s swimming motility, but both are needed for swarming. However it is still unknown if the flagellar motor contains a mix of these stators (Toutain et al., 2007).

Over thirty proteins are required in total for the assembly and anchoring of the filament in *P. aeruginosa*. These proteins are encoded in five regions spread across the genome: region I: PA1077-1105, region II: PA1441-1464, region III: PA3348-3352, region IV: PA1461/1462 and region V: PA3526 (Canals et al., 2006; Winsor et al., 2011). Among these genes are also two chemotactic clusters, cluster I (PA1457-1464) and cluster V (PA3348/3349), which are involved in flagellar motility (Ferrández et al., 2002; Kato et al., 1999; Masduki et al., 1995). The flagellum does not only allow *P. aeruginosa* to swim towards nutrient sources, but is also essential for its ability to form biofilms: A non-flagellated mutant was not able to attach to PVC and to form
biofilms, indicating that the flagellum is required for the initial attachment (O’Toole and Kolter, 1998; Vallet et al., 2001). Moreover, in combination with rhamnolipids, which are surface-wetting surfactants produced by the bacterium (Caiazza et al., 2005), the flagellum lets *P. aeruginosa* swarm across a surface. Lastly, sessile bacteria in a biofilm lose their flagella, because motility would destabilise the biofilm scaffold (Guttenplan and Kearns, 2013).

### 1.6 Insertion into the outer membrane

The outer membrane of Gram-negative bacteria contains up to 50% integral membrane proteins and lipoproteins. These proteins participate in such functions as secretion of exoproteins, uptake of nutrients, and sensing of environmental cues. They have to be inserted in or anchored to the outer membrane (OM) in such a way that the membrane remains undamaged and still protects the bacterium from the environment. Two assembly machineries orchestrate these steps in Gram-negative bacteria: the Bam complex that inserts OM proteins into the OM and the Lol
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complex that anchors lipoproteins to it.

1.6.1 Bam complex: Assembly of OM proteins

Inner membrane proteins have hydrophobic α-helical transmembrane domains and are inserted into the inner membrane via the SRP pathway of the Sec translocon. On the other hand, outer membrane proteins (OMP) fold into large β-barrels that consist of amphipathic antiparallel β-sheets (Koebnik et al., 2000). The β-barrel assembly machinery (Bam) complex and additional periplasmic chaperones are needed to prevent their misfolding and to assist their assembly in the OM (see Figure 1.11A).

The Bam complex is located in the OM and consists of the BamA:BamB and BamC:BamD:BamE modules (Ricci and Silhavy, 2012; Wu et al., 2005). BamA is an outer membrane protein that inserts OMPs into the membrane with the help of the BamB and BamC:D:E lipoproteins. BamA, a member of the Omp85 superfamily of protein transporters, consists of a C-terminal OM β-barrel pore. The pore is connected to five periplasmic polypeptide-transport associated (POTRA) domains, located at the N terminus of the protein (see Figure 1.11B) (Grijpstra et al., 2013; Sanchez-Pulido et al., 2003; Voulhoux et al., 2003). The POTRA domains fold into a three-stranded β-sheet and into two α-helices (Kim et al., 2007; Zhang et al., 2011). They are essential for the interaction with the Bam lipoproteins (Kim et al., 2007) and the OMP substrate (Robert et al., 2006; Struyvé et al., 1991). Several studies showed that BamA interacts directly with BamB and BamD (Malinverni et al., 2006; Sklar et al., 2007; Wu et al., 2005) and the assembly of BamA has even been reconstructed in proteoliposomes. Hagan et al. (2013) showed that BamB and BamD bind unfolded substrates, thus retaining them at the OM, and then trigger the assembly of BamA.

OMPs reach the periplasm via the SecYEG translocation machinery (see Figure 1.11A) (Solov’eva et al., 2012). Once in the periplasm, chaperones interact with the linear OMPs to prevent their misfolding, help with their proper folding, and target them to the Bam complex. In E. coli, the chaperones Skp and SurA are involved directly with the Bam complex. Skp binds the unfolded OMPs still translocated by SecYEG, prevents their degradation, and passes them on to BamA (Chen and Henning, 1996; de Cock et al., 1999; Harms et al., 2001). SurA helps to fold the OMPs
into their native conformation and coordinates the recognition of the proteins by BamA (Lazar and Kolter, 1996; Rouvière and Gross, 1996). To avoid accumulation of misfolded proteins in the periplasm, the protease DegP degrades and sequesters the non-native OMPs (CastilloKeller and Misra, 2003).

Figure 1.11: Assembly of outer membrane proteins by the Bam complex. (A) Preproteins are either inserted into the IM or are exported into the periplasm by the Sec translocon. Proteins destined for the OM, are bound by periplasmic chaperones (SurA, Skp, DegP) that transfer them through the periplasm towards the Bam complex. BamA recognises the preprotein and together with BamB and BamCDE supports the folding of the protein into a \( \beta \)-barrel and its insertion into the OM. Figure reproduced from Okuda and Tokuda (2011) with permission from Annual Reviews. (B) Crystal structure of BamA from Neisseria gonorrhoeae (PDB: 4K3B). BamA forms a 16-stranded \( \beta \)-barrel. The barrel is connected to five periplasmic POTRA domains, which form the N terminus of the protein.

### Assembly process

BamAs POTRA domains recognise a C-terminal signature motif (Kim et al., 2007; Robert et al., 2006; Struyvè et al., 1991). The signature motif mainly consists of alternating hydrophobic residues (HR): C terminus-Phe-X2-Tyr/HR-X4-HR-X6-HR-X8-HR. These last ten residues are proposed to fold into \( \beta \)-strand which might interact with the POTRA domains by \( \beta \)-augmentation. The assembly of the OMP is supported by BamB, which gives protection needed by OMP to fold into an amphipathic \( \beta \)-barrel (see Figure 1.11A) (Selkrig et al., 2013). Moreover, an interaction of the BamC:D:E lipoproteins with BamA causes BamA to change its conformation and to assemble OMPs into the asymmetric lipid bilayer (Ricci and Silhavy, 2012; Rigel et al., 2013). Little is known about the assembly process. However, Noinaj et al.
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(2013) suggested that BamA destabilises the outer membrane and inserts the OMP either independently or dependently of its $\beta$-barrel domain. Simple OMPs, which have been suggested to self-assemble in the periplasm, could be assembled independently of BamAs $\beta$-barrel. In this case, BamA might localise the OMP close to the OM with the help of its POTRA domains and facilitate the OMPs insertion by destabilising the OM. More complex OMP might also interact with BamA’s POTRA domains. But in contrast to simple OMP, the unfolded polypeptide would be threaded through BamA’s $\beta$-barrel pore. Then the barrel opens laterally and releases the now folding protein into the destabilised membrane.

As the periplasm has no energy source, the energy for the OMP biogenesis could come from the protein folding itself (Selkrig et al., 2013): Proteins are driven to fold into their native conformation, the most stable and lowest energy state. The role of the Bam complex would be to assist with assembly by lowering the activation energy for protein folding.

1.6.2 Lol complex: Anchoring of lipoproteins

Lipoproteins, such as BamBCDE, contain hydrophobic fatty acids, which anchor the lipoproteins to either the periplasmic side of the inner membrane (IM) or to the periplasmic side of the outer membrane. The lipoproteins targeted for the outer membrane cannot cross the hydrophilic periplasm because of their hydrophobic moiety. To allow their passage, OM lipoproteins require help from the Lol (Localisation of lipoproteins) complex that shields their hydrophobic residues and anchors them to the outer membrane (Okuda and Tokuda, 2011).

Biogenesis of lipoproteins

Like preproteins, prelipoproteins are transported across the inner membrane by the Sec machinery. To be recognised by the Sec machinery, prelipoproteins have a type II signal peptide, consisting of the typical Sec signal peptide (see Section 1.4.1) with an additional lipoprotein-specific lipobox (see Figure 1.12A). The lipobox contains a conserved Leu-Ala/Ser-Gly/Ala-Cys motif, with cysteine as the first residue in the mature protein (Hayashi and Wu, 1990; Tokuda and Matsuyama, 2004). In contrast to the cleavage of preproteins, the processing of prelipoproteins is complex and involves three periplasmic enzymes (Okuda and Tokuda, 2011; Sankaran
First, a prolipoprotein diacylglycerol transferase links a diacylglycerol to the cysteine in the lipobox. Then, the signal peptidase SPase II cleaves the signal peptide upstream of the Cys residues. Finally, an apolipoprotein transacylase transfers an acetyl chain to the same Cys. The addition of the hydrophobic fatty acids anchors the mature lipoprotein in the inner membrane with its proteinous moiety pointing into the periplasm. Whether lipoproteins are targeted to the OM via the Lol complex or are retained in the IM, depends on the nature of the residue at position +2 of its N terminus (see Figure 1.12B). Whereas OM lipoproteins usually have a serine at position +2, IM lipoproteins have an aspartate (Terada et al., 2001; Yamaguchi et al., 1988).

Transfer of OM lipoproteins via the Lol complex

Components of the Lol complex are the inner membrane ATP-binding cassette (ABC) transporter LolCDE, the periplasmic chaperone LolA, and the outer membrane lipoprotein acceptor LolB (see Figure 1.12B) (Okuda and Tokuda, 2011). ABC transporters typically consist of four domains: two membrane-associated domains and two ATPase domains. They are either separate proteins or a fused combination of proteins (Higgins, 2001; Holland and A. Blight, 1999). The LolCDE ABC transporter consists of two cytoplasmic and nucleotide-binding LolD proteins and the two transmembrane proteins LolC and LolE (Yakushi et al., 2000). LolD powers the release of the lipoprotein from the IM, via LolCE, by binding and hydrolyses of ATP. The lipoprotein is then transferred from LolC to LolA. LolA forms an incomplete β-barrel, made from 11 antiparallel β-strands, with a lid consisting of three α-helices on top (Takeda et al., 2003). The β-barrel forms a hydrophobic cavity, which is thought to take up the hydrophobic acyl chains of the lipoprotein emerging from the ABC transporter. The now shielded acyl-chains can be easily transported through the periplasm towards LolB, which is anchored to the periplasmic side of the outer membrane. LolB has a structure similar to LolA, and it has been suggested that the lipoprotein is transferred between both proteins in a mouth-to-mouth fashion (Nakada et al., 2009; Okuda and Tokuda, 2009). Only when both cavities, one from LolA and one from LolB, oppose each other, is the acyl-chain of the lipoprotein moved. Next, LolB releases the lipoprotein into the outer membrane (via a yet unknown process).

The Lol system is well studied in *E. coli*, but little is known about it in *P. aeruginosa*. 
P. aeruginosa has five Lol homologues, which show a high sequence similarity to E. coli’s Lol system: LolA (PA2614), LolB (PA4668), LolC (PA2988), LolD (PA2987) and LolE (PA2968). In contrast to E. coli, where the sorting of lipoproteins depends on the +2 residues, the +3 and +4 residues can also function as a sorting signal in P. aeruginosa. Thus, Lys in position three and Ser in position four can also retain lipoproteins in the inner membrane (Tanaka et al., 2007).

Figure 1.12: Lipoprotein processing by the Lol complex. (A) Schematic of a full-length prelipoprotein. Prelipoproteins are inserted into the IM via the SEC machinery and thus have the typical Sec signal peptide (see Section 1.4.1). Additionally they have a lipobox, which is cleaved by the SPase II. Cys is the first residue in the mature lipoprotein and is further processed by the addition of diacylglycerol and acetyl-chains, thus anchoring the lipoprotein in the membranes. (B) Lipoproteins targeted for the OM (X) are released from the IM by the LolCDE ABC transporter, which transfers the lipoprotein to the periplasmic LolA chaperone through binding and hydrolysis of ATP. LolA targets it to the OM-bound LolB receptor and LolB anchors the lipoprotein to the OM by an unknown mechanism. Inner membrane lipoproteins have an Asp at position +2 of the N terminus and thus stay anchored to the IM. Figure B reproduced from Okuda and Tokuda (2011) with permission from Annual Reviews.
1.7 Translocation across the outer membrane

The outer membrane proteins and lipoproteins assembled by the Lol and Bam machines can be components of various secretion systems. These systems deliver a vast array of proteins across the cell envelope and can even inject them into the host. These proteins, for example, degrade macromolecules and are required for the subsequent uptake of nutrients. Or, like in pathogenic bacteria, exoproteins mediate the infection of the host. The secretion systems span the cell envelope and thus keep the hydrophilic exoproteins protected during their secretion across the hydrophobic membranes (see Figure 1.13).

![Secretion systems diagram](image_url)

Figure 1.13: *P. aeruginosa* secretion systems. The T1SS, T3SS and T6SS directly secrete their substrates from the cytoplasm into the extracellular medium or eukaryotic host cell. In contrast, the T2SS and the T5SS rely on the Sec and Tat machines to export substrates from the cytoplasm into the periplasm. From there, the T2SS and T5SS translocate their substrates across the outer membrane. For the T5SS only the T5aSS (classical autotransporters) and T5bSS (two-partner secretion) are shown. Not depicted is the T5dSS (hybrid autotransporter) also present in *P. aeruginosa*. Figure reproduced from Bleves et al. (2010) with permission from Elsevier.

Secretion systems can span either both IM and OM or only the OM, whereas those spanning IM and OM usually translocate their substrates in a one-step mechanism. Those spanning the OM work together with the Sec and TAT machines (see Section 1.4). Seven secretion systems have been classified so far: type I (T1SS), type II (T2SS), type III (T3SS), type IV (T4SS), type V (T4SS), type VI (T6SS) and type VII (T7SS). Other secretion systems not included
in the numerical classification are the chaperone-usher (CU) pathway and the Por secretion system (PorSS). So far, the T7SS has been only identified in *Mycobacteria*, where it secretes virulence factors involved in the manipulation of the host immune response (Houben *et al.*, 2013; Weerdenburg *et al.*, 2012). The PorSS, assembled in *Porphyromonas gingivalis*, delivers the virulence factor gingipain, a cysteine proteinase, across the cell surface (Sato *et al.*, 2013).

As an opportunistic human pathogen, *P. aeruginosa* assembles the T1SS, T2SS, T3SS, T5SS, T6SS and the CU pathway (see Figure 1.13). However, it does not assemble the T4SS (Bleves *et al.*, 2010), which either delivers DNA by conjugation or exports effector proteins (Christie *et al.*, 2014). This Section reviews the T1SS, T2SS, T3SS and T6SS, whereas the T5SS and the CU pathway are reviewed in Sections 1.10 and 1.8.

### 1.7.1 Type I secretion system

The T1SS transports exoproteins across both membranes in one step. The system is made of an OMP, a membrane fusion protein (MFP) and an ABC transporter (Bleves *et al.*, 2010).

**Recognition of T1SS substrates**

The sizes of T1SS substrates vary between 20 and 900 kDa. The substrates usually have a C-terminal secretion signal (Filloux, 2010; Thomas *et al.*, 2013). Although no universal secretion signal could be identified, several typical features have been proposed: five essential residues located in the secretion signal, an amphipathic helix or a secondary structural motif. The presence of a C-terminal secretion signal indicates that the substrate is post-translationally recognised. Thus, to keep it in a secretion-competent state, other motifs could be recognised and bound by the transporter as observed for hemolysin A of uropathogenic *E. coli* (UPEC, see below) (Lecher *et al.*, 2012) and the hemophore HasA of *Serratia marcescens* (Masi and Wandersman, 2010). That T1SS substrates are also stabilised by chaperones, has been shown for HasA, kept unfolded by the SecB chaperone (Sapriel *et al.*, 2003).
Translocation via the T1SS

One of the best characterised T1SS is involved in the secretion of hemolysin A (HlyA) by UPEC (Goebel and Hedgpeth, 1982; Welch et al., 1981). It consists of the MFP HlyD, the ABC transporter HlyB and the OMP TolC (see Figure 1.14). Whereas the HlyB and HlyD proteins are specific for HlyA secretion, TolC is not only associated with this T1SS, but is also a component of drug efflux pumps. In *P. aeruginosa* strain PAO1, for example, the TolC homologue OprM (PA0427) interacts with MexAB, one of four major efflux systems (Mex) (Hancock and Brinkman, 2002). The MexAB-OprM efflux pump is one of the elements that causes *P. aeruginosa* multidrug resistance, because it exports various antibiotics, such as fluoroquinolones and β-lactams (Li et al., 1995; Srikumar et al., 1999).

Figure 1.14: Secretion of HlyA by the T1SS. The T1SS secretes substrates, such as HlyA, in one step across inner (IM) and outer membranes (OM). The HlyB ABC transporter is connected to the OM protein TolC by the membrane fusion protein HlyD. The secretion of the unfolded HlyA is energized through the hydrolysis of ATP by HlyB. As soon as HlyA exits the TolC pore, it folds and becomes active. The crystal structure of TolC is shown: TolC is a homotrimer and the protomers are colored in red, blue and green. The homotrimer forms a 12-stranded β-barrel in the outer membrane. The periplasmic part of the homotrimer folds into 12 helices that span the periplasm and interact with HlyD. Figure reproduced from Koronakis et al. (2004) with permission from Annual Reviews.

In UPEC, the Hly T1SS assembles as soon as HlyA interacts with HlyB and HlyD at the IM (Thomas et al., 2013). The following conformational change of HlyD recruits TolC to the machinery. TolC forms a bridge that extends from the OM towards the IM, where it interacts with the MFP protein HlyD (see Figure 1.14, center) (Lee et al., 2012). The energy for translocation is provided by the ABC transporter HlyB that binds and hydrolyses ATP (Zaitseva et al.,...
The substrates transport through the HlyD:TolC channel shields it from the hydrophobic membranes and protects the bacterium from its toxic effects.

Substrate activation depends on the intracellular Ca\(^{2+}\) concentration if the substrates have a RTX motif (Kuhnert et al., 1997). RTX motifs are rich in glycine and aspartate residues and bind Ca\(^{2+}\). The bound Ca\(^{2+}\) can activate protein folding. Because the Ca\(^{2+}\) concentration in the cytoplasm is too low to trigger protein folding, proteins with an RTX motif are secreted in an unfolded conformation (Thomas et al., 2013). In contrast, the Ca\(^{2+}\) concentration on the cell surface is higher and causes the emerging protein to fold.

**T1SS effectors in *P. aeruginosa***

The function of T1SS effectors ranges from adhesins, lipases, proteases, RTX-toxins, to heme-binding and S-layer proteins (Thomas et al., 2013). *P. aeruginosa*, for example, secretes three T1SS effectors: AprA (PA1249), AprX (PA1245) and HasAp (PA3403) (Filloux, 2011). AprA is an alkaline protease that modulates the host immune response by degrading cytokines and complement proteins (Laarman et al., 2012). It also cleaves free flagellin, the major component of the flagellum, and thus prevents the bacterium’s detection through Toll-like receptor 5 present on monocytes, immature dendritic cells, and epithelial cells (Bardoel et al., 2011). AprA has a Ca\(^{2+}\)-binding RTX motif and is activated upon binding of Ca\(^{2+}\) at the cell surface (Zhang et al., 2012). Translocation of AprA requires the ABC transporter AprD (PA1246), the MFP AprE (PA1247) and the OMP AprF (PA1248) (Guzzo et al., 1990). This system secretes not only AprA but also a protein of an unknown function called AprX (Duong et al., 2001). HasAp (heme acquisition system) is a hemophore. Hemophores bind heme released from hemoglobin and traffic it to an OM receptor (HasR, PA3408) (Ochsner et al., 2000). The heme is then taken up into the cytoplasm, where it is degraded and the released iron used for the cellular metabolism (Idei et al., 1999; Létoffé et al., 1998). HasAp is secreted by the HasDEF T1SS: HasD (PA3406) is the ABC transporter, HasF (PA3404) the OMP and HasE (PA3405) the MFP.
1.7.2 Type II secretion system

The T2SS transports substrates by a two-step mechanism across the inner- and outer membranes. These substrates include toxins or enzymes such as proteases and lipases (Douzi et al., 2012). The first step, the export of unfolded or folded/oligomerised substrates into the periplasm, is carried out by the Sec and TAT machineries (Pugsley, 1993; Voulhoux et al., 2001). The second step, the secretion of substrates into the extracellular space, requires the T2SS machinery. The T2SS has been discovered in *Klebsiella oxytoca* and *P. aeruginosa* and was named Pul or Xcp system, respectively. The names have been subsequently unified to Gsp (for general secretion pathway) but remained Xcp for all *Pseudomonas* species. Both names will be used here in the following manner: Gsp/Xcp.

The T2SS is usually made of 12–15 proteins. They assemble into three subcomplexes located in the IM, periplasm and OM (see Figure 1.15A). The IM platform consists of the IMPs GspC/XcpP, GspF/XcpS, GspL/XcpY, GspM/XcpZ; and the cytoplasmic ATPase GspE/XcpR (Arts et al., 2007b; Bleves et al., 1996; Michel et al., 1998). The ATPase is associated with the inner side of the IM via GspL/XcpY. The OM subcomplex harbors a secretion pore formed by 12-15 subunits of the homomultimer GspD/XcpQ (Collins et al., 2001; Koster et al., 1997). Targeting to and assembly of the GspD/XcpQ secretin in the OM is assisted by the pilotin GspS (Collin et al., 2007). GspS is an OM lipoprotein with a chaperone function that also protects the secretin from degradation (Daefler et al., 1997; Hardie et al., 1996). Not all T2SS have a GspS homologue, for example the Xcp and Hxc system in *P. aeruginosa* (Viarre et al., 2009). HxcQ is a liposecretin, and lipoproteins are targeted to the OM via the Lol complex (see Section 1.6.2). Alternatively, some secretins may use other yet unknown chaperones (Douzi et al., 2012). The third subcomplex is the pseudopilus consisting of the major GspG/XcpT pseudopilin and the minor GspH/XcpU, GspI/XcpV, XcpJ and XcpK pseudopilins (Bally et al., 1992; Bleves et al., 1998).

The pseudopilus is a pilus-like structure similar to the type IV pilus (see Section 1.5.2). Like the type IV pilus, it forms a right-handed helix, but without visible extrusion at the cell surface (Craig et al., 2006). Instead, it could push the T2SS substrates across the secretin towards the extracellular space (see below). The pseudopilus consists of major and minor pseudopilins that,
like T4aP, have a 5–6 amino acid long and positively charged leader sequence at their hydrophobic N terminus (see Section 1.5.2). The N terminus targets them for a co-translational insertion into the IM by the Sec/SRP pathway (see Figure 1.15A). Once in the IM, the leader peptide is cleaved by the prepilin peptidase GspO/XcpA/PilD that also cleaves the leader peptide of T4P (Nunn and Lory, 1992, 1993). The hydrophobic residues fold into a transmembrane domain in the IM, whereas the periplasmic C terminus folds into a globular structure (Bleves et al., 1998; Nunn and Lory, 1993).

The minor pilins possibly initiate pseudopilus assembly by forming a quaternary complex in the IM (Douzi et al., 2009; Durand et al., 2005; Sauvonnet et al., 2000). Subsequently, GspG/XcpT major pseudopilin subunits polymerise at the initiation side into the pseudopilus. The pseudopilus grows from the tip to the base and consequently has the minor pilins on its tip. Energy for the pseudopilus assembly is provided by the ATPase GspE/XcpR (Craig et al., 2006).

**Recognition of T2SS substrates**

Although the T2SS is specific for its cognate substrate (Lindeberg et al., 1996), the secretion motif recognised by the T2SS machinery has not yet been identified. The substrates might display several motifs spread across the primary sequence of the exoprotein (Sandkvist, 2001). These motifs, coming together upon the protein’s folding, might provide a conformational signal recognised by the IMP GspC/XcpP. Direct interaction between GspC/XcpP and substrate has recently been shown by surface plasmon resonance: GspC/XcpP bound LasB, one of the major T2SS substrates of *P. aeruginosa* (Douzi et al., 2009). It suggests that GspC/XcpP locates the substrates close to the T2SS machinery after they have been exported into the periplasm via the Sec or TAT machineries. The interaction between GspC/XcpP and the substrate changes the conformation of GspL/XcpY and activates the ATPase GspE/XcpR. These events drive the assembly of the pseudopilus and the translocation of the substrate: The substrate, now in the T2SS cage, interacts with the pseudopilins and is expelled from the cell across the secretin channel by the growing pseudopilus (see Figure 1.15B).
Figure 1.15: **Assembly of the pseudopilus and secretion of T2SS substrates.** (A) Assembly of the pseudopilus. Major (GspG) and minor prepilins (GspK, I, J, H) are inserted into the IM via the SRP pathway of the Sec machine. Their signal peptide is cleaved by a prepilin peptidase (PPP). The minor pilins initiate pseudopilus assembly by forming a complex in the T2SS. The T2SS consists of an IM platform (green), a cytoplasmic hexameric ATPase (GspE, orange) and an OM secretin (GspD, blue) that is anchored to the OM via the pilotin GspS. Subsequent addition of major pilins results in the growth of a pseudopilus. (B) Secretion of substrates by the T2SS. The components of the T2SS are colored as in A, except the minor pilins are in silver, major pilins in pink, the OM secretin is blue, cyan and purple. A potential substrate (AB) is shown in yellow. (1) The T2SS has started to assemble the pseudopilus (silver, pink) and is ready for translocation. (2) The substrate, already exported into the periplasm by the Sec or TAT machines, is loaded into the cavity atop the pseudopilus. (3) The pseudopilus grows through the polymerisation of the major pilin (GspG) at its base, powered by the ATPase GspE. (4) The growing pseudopilus pushes the substrate (AB) out of the cell and into the extracellular space. Reprinted by permission from Macmillan Publishers Ltd from Korotkov et al. (2012).

**Model of T2SS**

Unlike for the type IV pili, whose retraction is powered by an ATPase (Skerker and Berg, 2001; Wolfgang et al., 1998), no evidence has been found that pseudopilus retraction involves an alternative ATPase. Two models, the piston model and the Archimedes screw model have been developed to explain the secretion mechanism (Nunn, 1999; Wandersman et al., 2013). In the former model, the pseudopilus acts like a piston that pushes the tip-bound substrate out of the
cell (see Figure 1.15). The pseudopilus’s length has to be controlled, probably by GspK/XcpX (Durand et al., 2005). GspK/XcpX is present at the pseudopilus tip and is pushed towards the secretin through the growing pseudopilus. At contact with the secretin, GspK/XcpX might change its conformation and destabilise the pseudopilus. Repeated cycles of disassembly and ATP-dependent assembly of the pseudopilus would then allow the secretion of several substrates.

The Archimedes screw model proposes that the substrates bind to the major pseudopilin GspG/XcpT and that, during pseudopilus biogenesis, the pilus rotates like a screw turned into a hole. The rotation would then allow the passage of the pilus-bound substrates through the secretin. However, an argument against this model is that the pseudopilus does not protrude from the cell surface.

**T2SS effectors in *P. aeruginosa***

In *P. aeruginosa*, two T2SS have been well characterised: Xcp (PA3095-PA3105) and Hxc (PA0677-PA0688) (Filloux, 2011). For the Xcp machinery, 14 substrates, whose genes are scattered across the genome, have been identified so far. The Xcp machine secretes, for example, the lipase LipA (PA2862), the elastase LasB (PA324), the hemolytic phospholipase C PlcH (PA0844), and the alkaline phosphatase PhoA (PA3296). The Hxc machinery is expressed mostly at low levels of phosphate and has been shown to translocate the low-molecular weight alkaline phosphatases LapA (PA0688) and LapB (PA0689) (Ball et al., 2002). These proteins are important *P. aeruginosa* virulence factors letting the bacterium modulate and damage the host immune response. The elastase LasB, for example, has been implicated in chronic lung infections, where it degrades lung surfactant proteins A and D (Mariencheck et al., 2003). These proteins usually coat *P. aeruginosa* cells and label them for phagocytosis. Moreover, LasB degrades the tight-junctions between epithelial cells that results in a damage of the protective respiratory epithelium and allows *P. aeruginosa* to travel across the epithelial monolayer (Azghani, 1996). In contrast, the hemolytic phospholipase C PlcH is linked to acute infections and inflammations. It has been observed to degrade phospholipids of eukaryotic cell membranes and surfactant proteins (König et al., 1997; Wiener-Kronish et al., 1993).
1.7.3 Type III secretion system

The T3SS translocates its effectors in a single step from the cytoplasm into the cytosol of eukaryotic host cells. To do so, effectors are injected directly into the host cell with a needle-like structure called injectisome. The T3SS is evolutionarily related to the flagellum assembly machinery (see Section 1.5.3) (Saier, 2004), which explains their structural and functional similarities. Flagellum and injectisome, for example, are both anchored in the bacterial membranes via a basal body. Around twenty of the proteins that form the basal body have homologues in the flagellar machine and T3SS. The flagellum and injectisome are both hollow structures and substrates are secreted through these hollow tunnels with high velocity (Büttner, 2012; Erhardt et al., 2010). However, their substrates are different: whereas the flagellar machine secretes flagellar and hook proteins, the T3SS transports needle proteins and effectors.

The best studied T3SSs are the Yop system assembled by *Yersinia spp* and the SPI-I/II systems of *Salmonella spp* (Büttner, 2012). Insight has also been obtained into the Psc T3SS of *P. aeruginosa*, although it was discovered only in 1996 (Azghani, 1996). The T3SS is a complex machine that involves a translocation apparatus, chaperones, and effector proteins. The translocation apparatus of the T3SS consists of extracellular, membrane-spanning, and cytoplasmic components (Büttner, 2012). The membrane-spanning components are the OM secretin, the basal body, and the export apparatus. To assemble the secretin, 12-15 PscC (PA1716) protomers are targeted to the OM and oligomerise with the help of the PscW pilotin (Bleves et al., 2010; Burghout et al., 2004). Moreover, the basal body engulfs an inner and outer ring. The inner ring has been suggested to contain the IM lipoprotein PscJ (PA1723) (Burns et al., 2008).

Connected to the basal body is the extracellular component formed by a needle-like structure (Pastor et al., 2005). The needle, made from polymerised PscF (PA1719) subunits, is hollow and has a helical fold. It is the channel through which effector proteins are secreted from the bacterial cytoplasm into the host’s cytosol (Büttner, 2012). However, the needle does not actually pierce the host plasma membrane. Piercing requires the translocon, a heteromeric complex of PopD (PA1709), PopB (PA1708) and PcrV (PA1706) proteins. The translocon forms a pore in the host plasma membrane through which the effectors are released (Mueller et al., 2008; Sarker et al., 1998). Pore formation requires the insertion of PopB and PopD’s hydrophobic domains.
into the host membrane (Hauser, 2009; Schoehn et al., 2003). In contrast, the hydrophilic PcrV is not a part of the actual pore, although it is essential for the pore construction (Goure et al., 2004).

Not much is known about the composition of the cytoplasmic component of the T3SS. It is predicted to have an IM C ring with an associated hexameric ATPase, PscN (PA1697). Furthermore, proteins essential for the regulation of the ATPase activity and the docking of T3SS substrate have also been predicted to be a part of the cytoplasmic component. The ATPase is not required for the secretion of the substrates, but drives the unfolding and docking of the substrates to the basal body (Akeda and Galán, 2005). The secretion of the partially folded or unfolded substrates relies on the proton motive force (Wilharm et al., 2004).

**Targeting of T3SS substrates**

The T3SS also involves cytoplasmic chaperones essential for the stabilisation and targeting of the translocators and effectors. Depending on the structure, the chaperones can be put into one
of two classes (Parsot et al., 2003). Class I chaperones support the export of the effectors and are made of α-helices and β-sheets. On the other hand, class II chaperones target translocators and mainly contain α-helical tetratricopeptide repeats (Job et al., 2010). PopD and PopB, for example, are stabilised by the PcrH (PA1707) chaperone (Bröms et al., 2003; Schoehn et al., 2003), whereas the PscF needle subunits are kept from premature polymerisation by the PscE (PA1718) and PscG (PA1720) chaperones (Quinaud et al., 2005). The effector toxins also have cognate chaperones that prevent their secretion and toxic effects on the cell but also target them to the T3SS. *P. aeruginosa* has four effector toxins: ExoS, ExoT, ExoU and ExoY (Bleves et al., 2010). Although the cognate chaperone for ExoY is yet unknown, it has been established that ExoS and ExoT interact with SpcS (Shen et al., 2008) and ExoU with SpcU (Finck-Barbançon et al., 1998; Halavaty et al., 2012). The chaperones recognise and bind to the chaperone-binding domains present at the substrate’s N terminus (Hauser, 2009). Moreover, all T3SS substrates have an N-terminal secretion signal, prior to the chaperone-binding side, that targets them for secretion via the T3SS (Arnold et al., 2009; Lloyd et al., 2002; Samudrala et al., 2009).

The secretion of the T3SS has to be orderly orchestrated. The needle and the translocators have to be secreted and assembled before the effectors are targeted for T3SS. The hierarchy between the effector and translocators not only depends on their N-terminal secretion signal and chaperone but might also involve additional motifs (Büttner, 2012). This is the case for the PopD translocator of *P. aeruginosa* (Tomalka et al., 2012). PopD requires its N-terminal secretion signal, its cognate PcrH chaperone and two additional N- and C-terminal translocator export signals (TES) for translocation. Whereas the first two features enable PopD’s secretion, the TES allow for PopDs export prior to effector secretion.

**Model of T3SS**

The T3SS is activated upon contact with a host cell, resulting in the biogenesis of the needle and translocation of the effectors (Galle et al., 2012). Because the export apparatus is present at a basal level, it can immediately export the PscF needle subunits, which polymerise into the needle-like structure. As has been observed for the flagellum, these proteins might polymerise at the needle tip (Poyraz et al., 2010). That the length of the needle is regulated by a molecular
ruler has been observed in *Yersinia enterocolitica*: deletions or insertions in the molecular ruler YcsP produced shorter and longer needles, respectively (Journet et al., 2003). It has thus been proposed that the C terminus of the ruler remains attached to the base of the T3SS, and the N terminus is connected to the needle tip (Büttner, 2012). Growth of the needle would stretch the ruler. A fully elongated ruler would then signal the bacterium to stop needle assembly and to start transporting the translocators to form the pore in the host membrane. In *P. aeruginosa*, the needle length could depend on PscP (PA1695) (Agrain et al., 2005).

The signaled switch from needle assembly to pore formation also activates the PopB and PopD translocators, which are released from their cytoplasmic chaperone Perc (Bröms et al., 2003; Schoehn et al., 2003). Perc inhibits the toxic effect these translocators might have on the bacterial cell (Neyt and Cornelis, 1999). Once released, they travel through the needle to the tip, oligomerise and bind to lipid rafts, regions rich in cholesterol and glycosphingolipids, on the hosts plasma membrane (Schoehn et al., 2003). PopD:PopB then assemble into a channel-like structure, punctuating the host membrane. Effector toxins are now secreted through the needle and released into the host cell cytosol.

**T3SS effectors in *P. aeruginosa***

As mentioned above, *P. aeruginosa* has four T3SS effector toxins: ExoS, ExoT, ExoU and ExoY. Usually, not all four effectors are present in a single *Pseudomonas* strain (Feltman et al., 2001). All strains have ExoT and ExoY, however, ExoS or ExoU are mutually exclusive. For example, ExoU is present in *P. aeruginosa* strain PA14 on a pathogenicity island and is absent from strain PAO1. Moreover, these effectors are inactive in the bacterium not only because they are bound to their chaperone but also because they require an host co-factor for activation (Galle et al., 2012). Having reached the host cell cytosol, ExoS, ExoU and ExoT are targeted to the plasma membrane because of their membrane-localisation domain (Rabin et al., 2006). At the plasma membrane, the toxins damage the host cells as described below.

ExoS and ExoT have two functional domains: an N-terminal GTPase activating (GAP) domain and a C-terminal adenosine diphosphate ribosyltransferase (ADPRT) domain (Radke et al., 1999). The GAP domain inactivates Rho GTPases (Kazmierczak and Engel, 2002; Pederson
et al., 1999), essential for the organisation of the host cytoskeleton (Ridley, 2012). When the GTPases are inactive, the actin skeleton collapses; cells start to round and detach from each other (Garrity-Ryan et al., 2000). Furthermore, fewer bacteria are taken up and killed by phagocytic cells causing the pathogen to spread. Moreover, the ADPRT activity of ExoT arrests wound healing, allowing bacteria to enter the body via an epithelial breach (Garrity-Ryan et al., 2004). It is also involved in inducing apoptosis. ExoS and ExoT have similar, but also specific, targets. In case of ExoS, the activity associated with the ADPRT domain disrupts the actin cytoskeleton, induces cell death (Kaufman et al., 2000), and prevents DNA synthesis and endocytosis (Galle et al., 2012).

ExoU has a patatin-like domain (see Section 1.8.5), which functions as a phospholipase after ExoU has been activated by the host co-factor superoxide dismutase (Rabin and Hauser, 2005; Sato et al., 2006, 2005). ExoU is a very potent exotoxin that induces cytotoxicity levels hundred times higher then ExoS. It causes cell lysis, especially of epithelial cells and macrophages, by degrading phospholipids of the host plasma membrane (Finck-Barbançon et al., 1997). Lysis of epithelial cells causes strong tissue damage, giving \textit{P. aeruginosa} a way to penetrate the host. Neutrophils are also killed by ExoUs activity, weakening the immune response and leaving the host more susceptible to secondary infections (Diaz et al., 2008).

ExoY is an adenylate cyclase that degrades cAMP (Yahr et al., 1998), which is also important for the organisation of the cytoskeleton. Although the exact role of ExoY is yet unclear, it can kill yeast cells (Arnoldo et al., 2008) and cause rounding of immune cells (Cowell et al., 2005).

### 1.7.4 Type VI secretion system

The T6SS secretes its effectors in a single step across the inner and outer membranes. Effectors can be injected in either eukaryotic or bacterial cells with the help of a puncturing device. T6SS have been identified in several bacteria, such as \textit{Agrobacterium tumefaciens}, \textit{S. enterica}, \textit{Vibrio cholerae}, \textit{Rhizobium leguminosarum} and \textit{P. aeruginosa}. \textit{P. aeruginosa} encodes three T6SS scattered across its genome: H1-T6SS, H2-T6SS and H3-T6SS (Filloux et al., 2008). The system studied best is the H1-T6SS, discussed in the next Section.
Assembly of the T6SS

Usually, T6SS are assembled from 12 core and various accessory proteins (see Figure 1.17). The core components include membrane-associated proteins (IcmF, DotU and Lip), bacteriophage-like proteins (Hcp, VgrG, HsiB, HsiC and HsiF), and proteins with unknown function (HsiG, HsiH, HsiA, HsiE)(Silverman et al., 2012). The membrane-associated protein IcmF is predicted to interact with the IM protein DotU (Silverman et al., 2012) and the OM lipoprotein Lip (Felisberto-Rodrigues et al., 2011). Furthermore, Silverman et al. (2012) suggested that these three proteins form a channel that spans the IM and OM and envelopes the tube components of the T6SS. The tube is hollow and is made from several oligomerised Hcp hexamers (Mougous et al., 2006; Osipiuk et al., 2011). Effector toxins might be secreted through this tube, although it is not known if they travel through the assembled tube or are preloaded during assembly. The tube is surrounded by a tubular sheath made from HsiB and HsiC cogwheels stacked on top of each other (Lossi et al., 2013). The sheath can extend and contract, and it so pushes the Hcp tube out of the bacterial cell (Basler et al., 2012). At the tip of the tube sits the puncturing device made from a VgrG trimer. The trimer forms a sharp needle structure that allows the Hcp tube to perforate the cell envelope (Cascales and Cambillau, 2012).

The HsiF baseplate connects the sheath with the IM, probably by interacting with IcmF, and is used to assemble the secretion apparatus. Associated with the secretion apparatus is the cytoplasmic ATPase ClpV. ClpV disassembles the HsiB:HsiC sheath, letting it contract (Bönemann et al., 2009). The repeated cycles of assembly, disassembly, and recycling that occur in the cytoplasm allow the sheath to extend and retract and push the tube through the cell envelope (Basler et al., 2012).

Finally, some T6SS use additional accessory proteins. An example is the Sci-1 system assembled by enteroaggregative E. coli. This system uses the TagL protein to anchor the T6SS to the cell envelope (Aschtgen et al., 2012). TagL has a periplasmic domain with a peptidoglycan binding domain. In the absence of such accessory proteins, the peptidoglycan binding domain is usually found in the DotU protein.
**1. INTRODUCTION**

Figure 1.17: **Comparison of bacteriophage and *P. aeruginosa*'s H1-T6SS.** Phage and T6SS proteins that have structural similarities are presented in the same color. (Left) The bacteriophage injects its DNA into the cytoplasm of target cells with the help of a tail tube (gp19, yellow) that has a spike (gp27/gp5, red) at its tip. The tail tube is encompassed by a gp18 sheath (blue). (Center and right) The H1-T6SS of *P. aeruginosa* resembles an inverted phage tail and spike. In *P. aeruginosa*'s T6SS, the sheath is assembled by the HsiB and HsiC proteins. Whereas the tail tube is made from Hcp proteins and has VgrGs (similar to the phages spike) on its tip. The VgrGs pierce the membranes of target cells, into which the T6SS effectors (Tse toxins) are released. Tube and sheath are enveloped by a protein complex of IcmF, DotU (green) and Lip (pink). In the cytoplasm, the ClpV ATPase associates with the HsiB/HsiC sheath. ClpV catalyses the disassembly of the sheath and thus allows it to retract. Figure reproduced from Filloux (2013).

**T6SS: an inverted bacteriophage**

The Hcp and VgrG proteins have a strong structural similarity to the bacteriophage tail tube and spike, respectively (Leiman *et al.*, 2009). Bacteriophages are bacterial viruses that infect target cells by injecting their DNA. Infected bacteria replicate the phage DNA and produce structural phage components, which assemble into new bacteriophages. These are released through lysis of the infected bacterium. To inject the DNA, bacteriophages use a long tail tube and a puncturing device - the spike - that pushes through the bacterial cell envelope (see Figure 1.17) (Leiman *et al.*, 2010; Rossmann *et al.*, 2004). But contrary to the phage, *P. aeruginosa* uses the T6SS tube and puncturing device to break through its own cell envelope and, probably, through the cell envelope of its target cells (Filloux *et al.*, 2008; Kanamaru, 2009). VgrG proteins have a two domain structure, with the N terminus resembling the bacteriophage protein gp27 and the
C terminus gp5 (Filloux, 2013). In the bacteriophage, these two proteins form a heterotrimeric complex localised at the tip of the gp19 (Hcp) tail tube. This tail tube is encased by a sheath consisting of gp18 (HsiB and HsiC) subunits. Another protein complex found in the T6SS and bacteriophage is a baseplate made from HsiF or gp25 (Lossi et al., 2011).

**T6SS effectors**

T6SSs are expressed only when bacteria come in contact with a target cell (either another bacterium (Hood et al., 2010) or an eukaryotic cell (Ma et al., 2009)) and then deliver effectors into these cells. The effectors can induce cell death resulting in a growth/survival advantage for the bacterial population with the T6SS (Schwarz et al., 2010). So far, two types of effectors released by the T6SS have been discovered: evolved VgrGs and toxins. In contrast to canonical VgrGs that form the puncturing device of the needle, evolved VgrGs do not only pierce the cell envelope but also have a special function due to a C-terminal extension. VgrG-1 from *V. cholerae* has an actin crosslinking domain at its C terminus (Ma and Mekalanos, 2010). This domain acts on the cytoskeleton and can induce cell death. Thus, *V. cholerae* is protected from phagocytosis by macrophages or amoeba that have been injected with VgrG-1 (Ma et al., 2009). Another evolved VgrG has been discovered in *Aeromonas hydrophila*. Here, the C-terminal extension is an actin ADP-ribosyltransferase, whose injection results in damaging of the cytoskeleton and apoptosis of HeLa cells (Suarez et al., 2010). Therefore, the evolved VgrGs identified so far seem to be involved in eukaryotic host cell interactions.

In contrast, the effect of the discovered toxins might be essential for competition between bacteria. In *P. aeruginosa*, the H1-T6SS secretes the three effector toxins Tse-1, Tse-2 and Tse-3 (Hood et al., 2010; Russell et al., 2011). These proteins are encoded together with an immunity protein. When expressed, it interacts with the toxin and prevents its toxic effect on the cell (Benz et al., 2012; Li et al., 2012). Tse-1 and Tse-3 are released into the periplasm of Gram-negative target cells, where they hydrolyse peptidoglycan (Russell et al., 2011). Peptidoglycan is a part of the cell envelope and stabilises the cell shape. Its degradation causes cell rounding and eventually death of bursting cells. Although the mode of action of Tse-2 is still unclear, it is targeted to the cytoplasm, where it can arrest cell growth (Hood et al., 2010).
Lastly, the T6SS might be an important tool for *P. aeruginosa* to invade the host (by weakening the host immune response) and defend its niche against competitive bacteria with the help of toxins. Bacterial competitions might be found during chronic lung infections, especially in the lung of cystic fibrosis patients. The sera of such patients contains high titers of antibodies against Hcp1 - the tube structure of the T6SS (Mougous *et al.*, 2006). Hence, the T6SS has been proposed to be a trait of chronic infections.

1.8 Type V secretion systems

The T5SS is probably the simplest of all known secretion systems. It relies on the insertion of a pore in the OM through which the passenger proteins are translocated (see Figure 1.18). The passenger protein and the pore are either fused together in a single polypeptide (autotransporters) or are encoded as two separate proteins (two-partner secretion system) (Henderson *et al.*, 2000). Autotransporters can be subdivided into T5a—classical, T5c—trimeric, T5d—hybrid, and T5e—inverted autotransporters.

The preproteins of all subtypes of autotransporters have a classical N-terminal Sec signal sequence, a translocator domain (TD), and a passenger domain (PD) (Grijpstra *et al.*, 2013). Around 10% of T5SS proteins have an extended signal peptide that can be over 50 amino acids long. The N-terminal extension of these signal peptides has additional positively charged and hydrophobic residues (Dautin and Bernstein, 2007; Henderson *et al.*, 1998). The extension is highly conserved among T5SS, suggesting that it has a function during secretion. Its exact role is unknown, but it might prevent aggregation and misfolding of preproteins in the periplasm during SecYEG translocation (Szabady *et al.*, 2005). Once in the periplasm, the TD is inserted into the OM by the Bam complex, where it forms a \(\beta\)-barrel (Ieva and Bernstein, 2009; Rossiter *et al.*, 2011). The passenger is exposed to the surface or secreted and exerts its role in, for example, biofilm formation, adhesion (Klemm *et al.*, 2006), toxicity (Kimura *et al.*, 1999), and immunomodulation (Lin *et al.*, 1997) (see Table 1.2). This Section will review the different subtypes of autotransporters and the two-partner secretion system, which is classified as T5b.
1.8. TYPE V SECRETION SYSTEMS

Figure 1.18: Overview of the T5SS. All T5SS proteins have an N-terminal signal peptide, a passenger and translocator domain. The signal peptide targets them for export across the inner membrane by the Sec machinery. Once in the periplasm, proteins are stabilised and/or guided to the outer membrane (OM) by the Skp, SurA and DegP chaperones. DegP also has protease activity and degrades misfolded proteins. The Bam complex assists with the assembly of the translocator domain to a β-barrel in the OM. The passenger domain is then translocated into the extracellular space (E), where it is either released or stays attached to the cell surface. Type 5a is the classical autotransporter, 5b the two-component system, 5c the trimeric autotransporter, 5d the hybrid autotransporter and 5e the inverted autotransporter. Figure adapted after Grijpstra et al. (2013).

Table 1.2: Examples of T5SS and their function.

<table>
<thead>
<tr>
<th>T5SS</th>
<th>Bacterium</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>T5aSS</td>
<td>IgA, N. meningitidis</td>
<td>cleaves human secretory and serum immunoglobulin A1, initiation of biofilm formation</td>
<td>Almogren et al. (2003); Arenas et al. (2013); Pohlner et al. (1987)</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>NalP</td>
<td>N. meningitidis</td>
<td>cleaves human complement C3 and IgA protease, initiation of biofilm formation</td>
<td>Arenas et al. (2013); Del Tordello et al. (2014)</td>
</tr>
</tbody>
</table>
### 1. INTRODUCTION

continued: Table 1.2

<table>
<thead>
<tr>
<th>T5SS</th>
<th>Bacterium</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hap</td>
<td><em>H. influenzae</em></td>
<td>microcolony formation, adherence to epithelial cells and extracellular matrix</td>
<td>Fink <em>et al.</em> (2003)</td>
</tr>
<tr>
<td>App</td>
<td><em>N. meningitidis</em></td>
<td>adhesion to host cells</td>
<td>Hadi <em>et al.</em> (2001); Serruto <em>et al.</em> (2003)</td>
</tr>
<tr>
<td>OmpB</td>
<td><em>Rickettsia conorii</em></td>
<td>binds serum factor H</td>
<td>Riley <em>et al.</em> (2012)</td>
</tr>
<tr>
<td>EspP</td>
<td><em>E. coli</em> O157:H7</td>
<td>degrades proteins of the complement cascade, biofilm formation, proteolytic activity</td>
<td>Weiss and Brockmeyer (2013)</td>
</tr>
<tr>
<td>IcsA</td>
<td><em>Shigella flexneri</em></td>
<td>actin tail assembly protein, spread between host cells</td>
<td>Bernardini <em>et al.</em> (1989); Goldberg and Theriot (1995)</td>
</tr>
<tr>
<td>Pet</td>
<td><em>E. coli</em></td>
<td>serine protease, degradation of the actin-binding protein foddrin</td>
<td>Navarro-García <em>et al.</em> (2007)</td>
</tr>
<tr>
<td>BrkA</td>
<td><em>Bordetella pertussis</em></td>
<td>inhibits human complement cascade</td>
<td>Barnes and Weiss (2001)</td>
</tr>
<tr>
<td>EstA</td>
<td><em>P. aeruginosa</em></td>
<td>lypolitic esterase</td>
<td>Wilhelm <em>et al.</em> (1999)</td>
</tr>
<tr>
<td>AaaA</td>
<td><em>P. aeruginosa</em></td>
<td>arginine-specific aminopeptidase</td>
<td>Luckett <em>et al.</em> (2012)</td>
</tr>
<tr>
<td>EprS</td>
<td><em>P. aeruginosa</em></td>
<td>cleaves the human specific receptors PAR-1, PAR-2 and PAR-4</td>
<td>Kida <em>et al.</em> (2013)</td>
</tr>
<tr>
<td>SphB1</td>
<td><em>B. pertussis</em></td>
<td>cleavage of the haemagglutinin FHA</td>
<td>Coutte <em>et al.</em> (2001)</td>
</tr>
</tbody>
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<tr>
<th>T5cSS</th>
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<tbody>
<tr>
<td>DsrA</td>
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<tr>
<td>AtaA</td>
</tr>
<tr>
<td>SadA</td>
</tr>
<tr>
<td>ApaA</td>
</tr>
<tr>
<td>YadA</td>
</tr>
</tbody>
</table>
1.8. TYPE V SECRETION SYSTEMS

continued: Table 1.2

<table>
<thead>
<tr>
<th>T5SS</th>
<th>Bacterium</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NhhA</td>
<td><em>N. meningitidis</em></td>
<td>bacterial adhesion to host cells and extracellular matrix components</td>
<td>Scarselli <em>et al.</em> (2006)</td>
</tr>
<tr>
<td>Hia</td>
<td><em>H. influenzae</em></td>
<td>attachment to human epithelial cells</td>
<td>Barenkamp and St Geme (1996)</td>
</tr>
</tbody>
</table>

**T5eSS**

| Int    | *E. coli*         | attachment to host cells through Tir receptor binding                    | Hartland *et al.* (1999); Luo *et al.* (2000) |
| Inv    | *Yersinia* spec.  | colonisation, internalisation into the host cell                          | Mikula *et al.* (2012b)           |

**T5bSS**

(TpsA/TpsB)

| FHA/FhaC   | *B. pertussis*    | filamentous haemagglutinin, binding to macrophages, epithelial cells and cilia, biofilm formation | Locht *et al.* (1993); Serra *et al.* (2011) |
| HMW1/      | *H. influenzae*   | adherence to human epithelial cells                                       | St Geme (1994)                    |
| HMW1B      |                   |                                                                         |                                  |
| ShlA/ShlB  | *S. marcescens*   | hemolysin, cell lysis                                                    | Hertle *et al.* (1999)           |
| CdiA/CdiB  | *E. coli* EC93    | contact-dependent growth inhibition of bacteria                           | Hayes *et al.* (2014)            |
| BcpA/BcpB  | *Burkholderia* thailandensis | autoaggregation, adherence to abiotic surfaces                      | Anderson *et al.* (2012)         |
| XacFhaB/XacFhaC | *Xanthomonas axonopodis pv. citri* | attachment to a surface, biofilm formation                        | Gottig *et al.* (2009)           |
| TpsA4/TpsB4 (LepA/LepB) | *P. aeruginosa* | cleavage of the human specific receptors PAR-1, PAR-2 and PAR-4, degradation of hemoglobin | Kida *et al.* (2008, 2011)       |
| CdrA/CdrB  | *P. aeruginosa*   | crosslinks Psl polysaccharides, biofilm formation                        | Borlee *et al.* (2010)           |
| HecA/HecB  | *Erwinia chrysanthemi* | attachment, aggregation, epidermal cell killing                           | Rojas *et al.* (2002)            |
| EtpA/EtpB  | *E. coli*         | adherence to intestinal cells                                             | Roy *et al.* (2009)              |
| HrpA/HrpB  | *N. meningitidis* | intracellular survival                                                    | Talà *et al.* (2008)             |
1.8.1 **T5a—classical autotransporters**

T5a proteins have the typical two-domain structure of autotransporters, with the passenger at the N terminus and the TD at the C terminus (see Figure 1.18). In contrast to T5bSS (see 1.8.4), the TD forms in the OM a 12-stranded β-barrel closed by an α-helix (see Figure 1.19) (Barnard *et al.*, 2007; Oomen *et al.*, 2004; van den Berg, 2010). Moreover, most passengers have been predicted to fold into a β-helical stalk with globular surface domains (Kajava and Steven, 2006). Whereas the majority of the passengers stays connected to the TD pore, some are cleaved and released into the extracellular space. For example, the IgA protease of *Neisseria meningitidis* is released from the cell envelope by the serine protease NalP (van Ulsen *et al.*, 2003). NalP is an outer membrane protein that cleaves IgA on the extracellular side of the OM. It does not only processes IgA but also cleaves the App adhesin and the AusI protein (whose function is unknown) (van Ulsen *et al.*, 2006, 2003). Other IgA proteases, such as Hap of *H. influenzae*, have an autocatalytic cleavage activity and can, when close to a second IgA protease, intermolecularly cleave and release the passenger (Fink *et al.*, 2001). The TD remains in the OM after the release. Little is known about the function of a TD pore left behind, but in *Rickettsia conorii*, for example, the OmpB TD has been observed to bind serum factor H, which makes the bacterium immune to serum killing (Riley *et al.*, 2012).

<table>
<thead>
<tr>
<th>T5SS</th>
<th>Bacterium</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HxuA/HxB</td>
<td><em>H. influenzae</em></td>
<td>utilization of heme</td>
<td>Baelen <em>et al.</em> (2013); Cope <em>et al.</em> (1995)</td>
</tr>
<tr>
<td>T5dSS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PlpD</td>
<td><em>P. aeruginosa</em></td>
<td>Patatin-like protein</td>
<td>Salacha <em>et al.</em> (2010)</td>
</tr>
</tbody>
</table>
1.8. TYPE V SECRETION SYSTEMS

Figure 1.19: **Crystal structures of T5SS.** Shown are the structures of the passenger and translocator domains in cartoon representation, either as single molecules or as one polypeptide. Translocator domains are shown in purple, except for YadA, where each protomer is highlighted. Passenger domains are rainbow colored. The structure of EibD only shows one protomer. The structures were obtained from the protein data bank and the PDB codes are given next to the proteins name. For the T5dSS no structures are available.

**Mechanism of T5a secretion**

Depending on the autotransporter, T5as might require periplasmic chaperones to stabilise and guide them. Chaperones involved in T5a protein interactions are DegP, Skp or SurA (Grijpstra et al., 2013). They are not specific to the T5SS but also guide OMP to the OM (see Section 1.6.1). DegP has protease activity and degrades misfolded proteins (see Section 1.10). However, it also has a chaperone function (Spiess et al., 1999). It is, for example, required for the secretion of the serine protease EspP, secreted by the *E. coli* strain O157:H7 (Ruiz-Perez et al., 2009). Although deletion of *degP* in the experiment of Ruiz-Perez et al. (2009) did not prevent the insertion of the TD into the OM, less EspP was secreted. They suggested it was due to the misfolding of the passengers in the periplasm. A T5a that has been shown to not require
DegP is IcsA, which is secreted by *Shigella flexneri* (Purdy *et al.*, 2007). Other T5as require specific chaperones, such as in enteroaggregative *E. coli*, where the VirK chaperone helps with the secretion of the Pet toxin by stabilising the passenger domain and aiding the TD assembly in the OM (Tapia-Pastrana *et al.*, 2012).

The mechanism leading to the secretion of the T5a passenger domain or its exposition at the cell surface is still under debate. Three models have been proposed: the threading model, the hairpin model and the Bam complex-assisted model. In all three, the Bam complex inserts the TD into the OM, where it forms the 12-stranded β-barrel pore. In the threading model (Barnard *et al.*, 2007), the N terminus of the passenger is threaded through the pore. Thus, the N terminus is secreted first and the rest of the protein follows with an N to C terminus directionality. In the hairpin model (Oliver *et al.*, 2003), the passenger domain forms a hairpin in the β-barrel allowing the protein to be secreted from the C to N terminus. However, these two models do not consider that the channel within the barrel is narrow and closed by an alpha helix. Taking these features into account makes it more difficult to imagine how a polypeptide can be threaded through (Grijpstra *et al.*, 2013).

In the Bam complex-assisted model (see Figure 1.20), BamA - the OM β-barrel protein of the Bam complex - plays a major role not only in the insertion of the TD but also in the secretion of the passenger (Ieva and Bernstein, 2009; Saurí *et al.*, 2011). However, the exact function of BamA is unclear. BamA has been proposed to either secrete the passenger through its own pore (Oomen *et al.*, 2004) or help it through at the BamA-TD interface (Ieva *et al.*, 2011). The hypothesis that BamA secretes the passenger has strong parallels to the secretion mechanism of two-partner secretion systems (see Section 1.8.4). In these systems, an Omp85 family protein forms a β-barrel pore in the OM through which it secretes its cargo (Wandersman *et al.*, 2013). The secreted protein usually forms a β-helix on the cell surface. Similarly, BamA belongs to the Omp85 family, and the passenger domains usually have a β-helical fold. In contrast, in the model proposed by Ieva *et al.* (2011), the passenger is secreted through the BamA-TD interface. Ieva *et al.* (2011) suggest that the TD assembles already in the periplasm into a half-folded β-barrel. The passenger would form a hairpin in the barrel stabilised by the Skp chaperone. The Bam complex would then insert the TD into the OM and assist its folding. At the same time, the passenger would be secreted through the BamA-TD interface. This could be possible, because
the Bam complex is thought to increase the permeability of the OM during the biogenesis of OMP. Thus, the membrane at the BamA-TD interface would have an increased permeability. It would allow BamA to help the passenger hairpin across the OM.

Figure 1.20: Bam complex-assisted model of T5aSS assembly. (I) Upon export of the T5a into the periplasm via the Sec machine (not depicted), the translocator (green) domain partially folds into a β-barrel and the passenger (purple) forms a hairpin the barrel lumen. (II) The Skp and SurA chaperones stabilise the T5a protein and guide it to the Bam complex (BamA in red and BamBD in orange) in the outer membrane. The Bam complex inserts the translocator domain into the OM and the passenger is secreted (from C to N terminus) at the BamA:translocator interface. (III) Secretion is driven by the folding of the autochaperone domain, located at the passengers C terminus. (IV and V) The passenger is released and the translocator finally assembles into a fully folded β-barrel. Figure reproduced from Ieva et al. (2011).

The final question is how the transport of the passenger domain is energised in the absence of ATP in the periplasm. A study by Oliver et al. (2003), using the BrkA autotransporter from B. pertussis, suggested that the secretion is driven by the folding of a conserved region, called autochaperone, on the cell surface. The autochaperone is located at the C terminus of the passenger domain, and its deletion rendered the BrkA passenger unstable and more vulnerable for degradation. Thus, Oliver et al. (2003) suggested that the autochaperone starts to fold upon reaching the extracellular space and thereby drives the translocation of the residual passenger. It, however, would only work if the passenger is secreted from the C to N terminus. Finally, most passengers are predicted to adopt a β-helical fold at the cell surface and the folding would thus prevent the protein from backsliding into the cell (Grijpstra et al., 2013).
1. INTRODUCTION

**T5aSS in *P. aeruginosa***

*P. aeruginosa* has been predicted to produce three T5a proteins whose TDs fold into the 12-stranded \(\beta\)-barrel: PA5112 (EstA), PA0328 (AaaA) and PA3535 (EprS) (Stover *et al.*, 2000). The structure of EstA has been solved, indeed revealing a 12-stranded OM \(\beta\)-barrel (see Figure 1.19). In contrast to the \(\beta\)-helical structure of classical passenger domains, the EstA passenger has a globular \(\alpha\)-\(\beta\)-\(\alpha\)-fold with a central \(\beta\)-sheet and several loops (van den Berg, 2010). Moreover, EstA is not cleaved from the TD after translocation and has lypolitic esterase activity (Wilhelm *et al.*, 1999).

The passenger of the AaaA (arginine-specific aminopeptidase of *P. aeruginosa*) T5aSS also remains attached to the TD and cleaves N-terminal arginines from peptides (Luckett *et al.*, 2012). The released arginine could be used by the bacterium and provide a growth advantage in the absence of other nutrients.

In the third *P. aeruginosa* T5aSS, the EprS passenger domain is released into the extracellular medium and functions as a serine protease (Kida *et al.*, 2013). EprS does not carry out autoproteolysis but activates the human immune response by cleaving human specific receptors (PAR-1, PAR-2 and PAR-4), present on the surface of various cells.

### 1.8.2 T5c—trimeric autotransporters

Another secretion mode for autotransporters, known as T5c, involves the formation of trimeric autotransporters. In contrast to the classical T5a, the T5c consists of three protomers that form a 12-stranded \(\beta\)-barrel linked to three passenger domains (see Figures 1.18 and 1.19) (Leo *et al.*, 2012). To assemble the barrel, each protomer donates four \(\beta\)-strands. Moreover, the passengers form a lollipop-like structure with a globular head connected by a neck to a stalk. The stalk consists of three tangled \(\alpha\)-helices that stretch from the channel toward the extracellular space. The helix core contains several hydrophilic residues with the most frequent one being asparagine (Hartmann *et al.*, 2009). These motifs could keep the autotransporters in the periplasm in an unfolded and soluble state. The region N-terminal to the \(\beta\)-barrel can have right-handed or left-handed coiled-coils (Alvarez *et al.*, 2010). The right-handed coiled-coils usually contain a YxD sequence motif, whereas the left-handed coiled-coils have a RxD motif. These motifs have
been implicated to activate the folding of the passengers as soon as it reaches the cell surface (Leo et al., 2012). The length of the stalk of the passenger varies not only between bacteria but also in the same organism, as has been shown for H. influenzae, which secretes two T5c: Hia and Hsf (Spahich and St Geme, 2011). Both are essential for adherence to epithelial cells. But depending on the thickness or presence of the capsule, the bacterium expresses either the shorter Hia or the longer Hsf. This allows the adhesin to emerge from the capsule, and the bacterium can adhere properly to its host.

**Mechanism of T5c secretion**

Little is known about the assembly mechanism of the trimeric T5cs (Cotter et al., 2005). No evidence exists that periplasmic chaperones are involved. However, T5cs are, like T5a proteins, inserted into the OM by the Bam complex. Lehr et al. (2010) showed that YadA, a trimeric T5c expressed by Y. enterocolitica, co-localises and directly interacts with BamA. Moreover, the trimeric barrel could assemble already in the periplasm before interacting with the Bam complex. A study by Grosskinsky et al. (2007) indicated that the passengers are translocated through the pore of their translocator domain. The barrel lumen of YadA is lined with small residues, e.g. glycine and alanine. Mutation of a conserved glycine to bulky histidine or asparagine destabilises the YadA trimer, resulting in YadA degradation by DegP.

The current model of trimeric autotransporter assembly (see Figure 1.21) states that the assembly processes of T5a and T5c are identical except for the presence of three polypeptides in the T5cSS (Leo et al., 2012). The translocator domain of the T5c is inserted into the OM by the Bam complex. Then, like in the hairpin model, the region N-terminal of the TD forms a hairpin through the pore lumen. In contrast to T5a, three hairpins would be simultaneously threaded through the pore. The coiled-coil region would be the first to arrive at the cell surface and induce the folding of the three passengers. The passengers trimerise during or after the folding and form the lollipop-like structure.
1. INTRODUCTION

**Figure 1.21: Hairpin model of T5cSS assembly.** The preproteins (green) are translocated across the inner membrane via the Sec machine (pink) and their Sec signal peptide (yellow) is cleaved. The proteins might be stabilised and kept in an unfolded state by unknown chaperones (orange) in the periplasm. The Bam complex (purple) recognises the C-terminal signature motif (see Section 1.6.1) of the three polypeptides with its POTRA domains and helps with the assembly of the translocator domain (brown) in the outer membrane. Each protomer donates four \(\beta\)-strands and also inserts one hairpin into the barrel. Secretion is carried out from the C to N terminus and driven by the folding of the coiled-coils at the passengers C terminus. Once at the cell surface the passengers trimerise into the lollipop-like structure. Figure reproduced with permission from Leo et al. (2012).

**Examples of T5cSS**

Most trimeric ATs are adhesins not released from their pore, and they have a role in biofilm formation and host cell attachment (see Table 1.2). Both the globular head and the stalk can have adhesive properties. The sticky head of the YadA adhesin binds e.g. collagen from host cells and tissues, and the stalk binds human factor H (Biedzka-Sarek et al., 2008; El Tahir and Skurnik, 2001; Nummelin et al., 2004). No trimeric AT has been identified so far in *P. aeruginosa*.

### 1.8.3 T5e—inverted autotransporters

There are ATs where the TD is located at the N terminus and the passenger at the C terminus of the protein. They are called inverted ATs or T5e (see Figure 1.18). T5e proteins belong to the invasin/intimin (see below) family of adhesins that mediate adhesion to and invasion into host cells (Tsai et al., 2010). The passenger domains of T5eSS consist of a C-terminal lectin-like domain, several Big (Bacterial immunoglobulin-like) domains, and an external protease resistant
domain (see Figure 1.22) (Fairman et al., 2012; Hamburger et al., 1999; Luo et al., 2000). Both lectin-like and Big domains are essential for the attachment of bacteria to a surface. The TD forms a 12-stranded $\beta$-barrel in the OM and is closed by a periplasmic $\alpha$-helix connected by an extended pore-spanning linker with the protease resistant domain (Fairman et al., 2012).

Figure 1.22: Domains of intimin. Shown are the crystal structures of the intimin translocator (PDB: 4E1S) and passenger (PDB: 1F00) in cartoon representation. The structure of the first big domain (green) and the protease resistant domain (pink) have not been solved yet.

**Mechanism of T5e secretion**

The assembly of the T5eSS supposedly is similar to that of the classical T5aSS. As previously described, SurA and Skp chaperones stabilise the preprotein, and the Bam complex inserts the TD into the OM (Bodelón et al., 2009; Oberhettinger et al., 2012). The TD pore is narrow and might allow secretion of an unfolded hairpin of the preprotein (Fairman et al., 2012). However, the lectin-like domain contains a disulphide bond, formed by DsbA in the periplasm (Hamburger et al., 1999). A folded protein would not fit through the TD pore. Therefore, secretion of the passenger might require the Bam complex, with translocation occurring at the BamA:TD interface. The secretion might be driven by the folding of the protease resistant domain, which reaches the cell surface initially in the shape of a hairpin and might function as an autochaperone (Grijpstra et al., 2013).

**Examples of T5eSS**

Inverted AT are mostly found in $\gamma$-proteobacteria but also in cyanobacteria, $\alpha$- and $\beta$- proteobacteria and the best studied members are intimin and invasin (Tsai et al., 2010). The Int intimin produced by enteropathogenic *E. coli* (Jerse et al., 1990) binds, via its lectin-like domain,
the Tir receptor on the surface of epithelial cells (Hartland et al., 1999; Luo et al., 2000). The Tir receptor is not produced by the host itself, but is injected into the host in a T3SS-dependent manner (Kenny et al., 1997). The interaction between intimin and Tir receptor allows the bacterium to attach to the host cell. The interaction between these two proteins induces actin polymerisation and the subsequent formation of a cytoskeleton pedestal where the bacterium resides during infection (Goosney et al., 1999).

The Inv invasin (see Figure 1.19), an adhesin produced by Yersinia species, is essential for the initial colonisation and then internalisation of bacteria into the host cell (Mikula et al., 2012a). The lectin-like and Big domains of invasin bind receptors of the β-1 integrin family on the surface of epithelial cells, allowing the bacteria to attach tightly (Hamburger et al., 1999; Isberg and Leong, 1990). The host cell then rearranges its cytoskeleton, which results in formation of pseudopod structures engulfing the bacteria. The bacteria are then internalised and can travel further to the lymphoid tissue, where they multiply and subsequently cause plague (Aepfelbacher et al., 2007; Deuretzbacher et al., 2009).

1.8.4 T5b—two-partner secretion systems

Usually, the two-partner secretion (TPS) system consists of two independent polypeptides: a TpsB protein transporter and its cognate passenger, TpsA (see Figure 1.18). TpsAs are large proteins that have a conserved domain at their N terminus, called TPS domain (Clantin et al., 2004; Jacob-Dubuisson et al., 2001). TpsBs are around 60 kDa proteins that have periplasmic polypeptide-transport associated (POTRA) domains and a C-terminal outer membrane β-barrel (see Figure 1.19) (Clantin et al., 2007; Sanchez-Pulido et al., 2003). Both proteins are synthesized as precursors and are transported across the IM by the SecYEG pathway (Chevalier et al., 2004; Grass and St. Geme, 2000). Once in the periplasm, TpsB transporters are inserted into the OM by the Bam complex (Jacob-Dubuisson et al., 2013). Unfolded TpsA substrates transit through the periplasm and are secreted by the TpsB transporter. It is yet unknown if TpsA and TpsB proteins are stabilised by chaperones during their transfer through the periplasm.

One of the best studied is the FHA/FhaC (TpsA/TpsB) TPS system of B. pertussis. FHA is a filamentous haemagglutinin that has adhesive properties and binds to macrophages, epithe-
lial cells, and cilia (Locht et al., 1993). Moreover, FhaC is the only TpsB transporter whose full-length structure has been solved (Clantin et al., 2007). As the secretion of FHA by FhaC has been well studied, the characteristics of TPS systems and their secretion mechanism will be explained in the following Sections with the help of this model system.

Characterisation of TpsB transporters

TpsB proteins assemble to 16-stranded $\beta$-barrels in the OM with the help of the Bam complex (see Figure 1.23A). It is in contrast to the translocator domains of T5aSS, which form 12-stranded $\beta$-barrels (Jacob-Dubuisson et al., 2013). The lumen of the TpsB barrel is closed by an $\alpha$-helix, called H1, and a hairpin loop, called L6. The sequence of TpsBs is usually not conserved, however, the L6 loop contains a conserved VRGY/F motif (Delattre et al., 2010; Leonard-Rivera and Misra, 2012). The motif seems to be essential for the secretion of FHA by FhaC, as mutation of the arginine to alanine reduced secretion of FHA by 90% (Delattre et al., 2010). Furthermore, Jacob-Dubuisson et al. (2013) suggested that the F/GxDxG motif (x are hydrophobic residues, see Figure 1.23), located in the $\beta$13-strand of the $\beta$-barrel close to the L6 loop, is also essential for secretion.

Another feature of TpsB transporters are their periplasmic and N-terminal POTRA domains. TpsB proteins can have up to seven POTRAs, and FhaC, for example, has two, POTRA-1 and POTRA-2 (see Figure 1.23A) (Clantin et al., 2007). Whereas POTRA-2 is connected to the outer membrane $\beta$-barrel part of FhaC, the N-terminal POTRA-1 is connected to the H1 $\alpha$-helix via a proline-rich linker (Jacob-Dubuisson et al., 2013). POTRAs usually have a $\beta$-$\alpha$-$\alpha$-$\beta$-$\beta$ fold (see Figure 1.23B) (Sanchez-Pulido et al., 2003), although there are some variations: in FhaC, the first helix is substituted by a loop. Only the core of POTRA domains is conserved; it contains two glycines and hydrophobic residues (see Figure 1.23C). In contrast, surface residues are variable. POTRA domains are essential for the recognition of and interaction with the cognate TpsA substrate. The variability of the surface residues would thus allow the various TpsB transporters to recognise their cognate substrate. Delattre et al. (2011) showed that both POTRA domains of FhaC are essential for the interaction with FHA. They introduced point mutations into the POTRA domains of FhaC and identified the binding efficiency between FHA
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Figure 1.23: Characteristics of a TpsB transporter. (A) FhaC (PDB: 2QDZ), shown in a cut-away view, forms a 16-stranded \( \beta \)-barrel (purple) in the outer membrane. The pore lumen is plugged by an \( \alpha \)-helix (H1, blue) and a loop (L6, orange and red). The N terminus of FhaC contains two POTRA domains, POTRA-1 (pink) and POTRA-2 (light yellow). POTRA-1 is connected to H1 via a linker (dashed pink line), whereas POTRA-2 is connected to the \( \beta \)-barrel. FhaC has two conserved motifs: F/GxDxG (cyan) in \( \beta \)13-strand of the barrel and VRGY/F (orange) in the L6 loop. (B) Cartoon presentation of POTRA-4 from BamA (PDB: 3O65). The POTRA domain has the typical \( \beta \)-\( \alpha \)-\( \alpha \)-\( \beta \)-\( \beta \)-\( \beta \) fold. (C) POTRA domains have two conserved glycines. The POTRA domains of TpsBs (PA2463, PA0040, TpsB4, CdrB and PA0692) are aligned with the POTRAs of FHA (B. pertussis) and ShlB (S. marcescens). Conserved residues are shown in rose. The first two glycines are the ones conserved in POTRA domains. The alignment was created with Clustal Omega.

and FhaC using surface plasmon resonance spectroscopy. They showed that the edge between \( \alpha2 \) and \( \beta2 \) of POTRA-1 and the hydrophobic groove between \( \alpha4 \) and \( \beta5 \) of POTRA-2 are essential for the interaction with FHA.

The structure of TpsB transporters varies, as observed for HMW1B - a TpsB transporter that secretes the high molecular weight proteins 1 and 2 (HMW1 and HMW2) in *Haemophilus influenzae* (St Gene and Yeo, 2009). HMW1B lacks the H1 helix and the proline-rich linker.
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Moreover, the TpsB proteins belonging to the HMWB subfamily have a conserved phenylalanine as the first residue in the F/GXDXG motif instead of glycine (Jacob-Dubuisson et al., 2013).

Characterisation of the TPS domain

TpsA proteins have a 250 residue long TPS domain at their N terminus (Clantin et al., 2004; Khan et al., 2011; Weaver et al., 2009; Yeo et al., 2007). According to Clantin et al. (2004), the TPS domain is organised into four alternating regions that are either less conserved (LC) or conserved (C): LC1-C1-LC2-C2 (see Figure 1.24). Region C1 contains conserved motifs, which determine two subtypes of TpsA proteins (Yeo et al., 2007). Both subtypes have a conserved NPNGI motif, whereas only subtype 1 has an additional NPNL motif.

![Characterisation of the TPS domain](Figure 1.24: Characteristics of the TPS domain. Shown is the TPS domain of FHA from *B. pertussis* according to Clantin et al. (2004). The conserved (C1 and C2) and less conserved regions (LC1 and LC2) are indicated. The conserved residues are shown in bold and the conserved TPS motifs, NPNGI and NPNL, are underlined. Amino acid numbers are indicated. The alignment was created with Clustal Omega.)

The TPS motif serves as a secretion signal that targets the TpsA towards its cognate TpsB transporter and is recognised by the POTRA domains of TpsB. POTRA and TPS domains probably interact by β-augmentation (Kim et al., 2007; Koenig et al., 2010) - an intermolecular process in which the β-sheet of the POTRA interacts with short β-strands of TpsA (Harrison, 1996). The specificity of the recognition relies on the conservation of residues within the TPS domain. Mutation of conserved residues strongly affects secretion. For example, mutation of the first asparagine of the NPNL and NPNGI motifs impairs TpsA translocation (Jacob-Dubuisson et al., 1997; Schönherr et al., 1993). Moreover, Hodak et al. (2006) introduced additional point mutations into the C1 and C2 regions and identified residues that abolished (S₃₂, N₄₉, N₆₆, S₈₆,
E93, N118, M225) and strongly decreased (N34, N105) FHA secretion. Surprisingly, only a few of these variants (E93, N118, M225, N105) also had decreased binding efficiency towards FhaC, as shown by pull-down assays. It indicates that the TPS domain has an additional role other than recognition of and interaction with the POTRA domains. Furthermore, only the unfolded protein is recognised by the POTRA domains and is subsequently translocated (Delattre et al., 2011).

Another hallmark of TpsA proteins is that they fold into right-handed parallel β-helices as soon as they reach the cell surface (see Figure 1.19) (Jacob-Dubuisson et al., 2013). Each coil of the helix is made from three parallel β-sheets, with aliphatic hydrophobic amino acids in the center of the folded protein. Moreover, the N terminus and the side of the helix display anti-parallel β-strands. The number of coils and, therefore, the protein length differs among TpsAs.

**Translocation of TpsA via TpsB**

Because the structure of the TpsB transporter in complex with its substrate remains unsolved, one can only speculate that TpsAs are secreted through the TpsB channel (Méli et al., 2006). As described above, the pore lumen of FhaC is plugged by the H1 helix and the L6 loop. Thus, to allow the passage of FHA, FhaC has to undergo a conformational change. It has been shown that the H1 helix can move from the channel into the periplasm and that the L6 loop is a dynamic one (Guédin et al., 2000; Guérin et al., 2014). Guérin et al. (2014) observed that even in a resting FhaC, the H1 helix periodically moves into the periplasm. The H1 helix is connected to POTRA-1 via a linker and the linker has been suggested to interact with the POTRA domains. The POTRA domains are not able to interact with the TPS domain of the TpsA when occupied by the linker. Thus movement of the H1 helix into the periplasm also repositions the linker. The POTRA would then be able to interact with the TPS domain.

The secretion of TpsA is an active process and thus requires energy. However, free energy is available neither in the periplasm nor across the OM. Thus, it has been speculated that the energy comes from the folding of the TpsA on the cell surface (Jacob-Dubuisson et al., 2013). As it is not exactly known how TpsA is translocated by TpsB, two models have been proposed: the "TPS first model" and the "hairpin model". Their basis is that TpsA, either fully translocated
or still in the process of translocation across the inner membrane via the Sec machine, interacts with the POTRA domains of TpsB via its TPS motif (Delattre et al., 2011; Hodak et al., 2006). In the "TPS first model" the TpsA's N-terminal TPS domain interacts with TpsB's POTRA domains and then progresses into the TpsB channel (see Figure 1.25A). Consequently, the TPS domain emerges first from the TpsB (Jacob-Dubuisson et al., 2013). It nucleates immediately and forms a template for the folding of the $\beta$-helix, thereby driving the secretion of the rest of the protein. 

In vitro evidence for this model has been given using S. marcescens ShlA hemolysin (TpsA). An addition of a secreted and folded ShlA TPS domain to an inactive full-length ShlA activated the folding of the full-length protein (Schiebel et al., 1989; Walker et al., 2004). It shows that the folded TPS domain can indeed serve as a template and drive folding.

Figure 1.25: **TPS first and hairpin model of TPS secretion.** TpsA and TpsB are first exported into the periplasm by the Sec machine and TpsB (blue) is inserted into the OM by the Bam complex (not shown). The TpsA protein (red), which moves in an unfolded conformation through the periplasm, has a N-terminal TPS domain (yellow), and a C-terminal pro-domain (green). (A) TPS first model. The unfolded TPS domain of TpsA interacts with the POTRA domains of TpsB. The TPS domain is transferred into the pore and TpsA is secreted from the N to C terminus. Secretion is driven by the folding of the emerging TPS domain. (B) Hairpin model. The unfolded TPS domain is also recognised by the POTRA domains of TpsB. But, now the portion C-terminal to the TPS domain forms a hairpin in the TpsB pore. The hairpin starts to fold at the cell surface and drives translocation of the TpsA. (A and B) The pro-domain is cleaved off by the SphB1 protease and intracellular degraded. In the TPS first model, TpsA is released from the cell surface, whereas in the hairpin model TpsA either stays anchored to the transporter or is released by an unknown mechanism. Figure reproduced Mazar and Cotter (2007) with permission from Elsevier.

In contrast, in the “hairpin model” the TPS domain remains connected to the POTRA domains during the whole secretion process (Leo et al., 2012; Mazar and Cotter, 2006) (see Figure 1.25B). Again, the TPS domain of TpsA interacts with the POTRA domains of TpsB. But in contrast to the “TPS first model”, residues downstream of the TPS domain form a hairpin in the TpsB channel. The folding of these residues at the cell surface drives the secretion of TpsA’s C terminus. The
N-terminal TPS motif of TpsA would remain attached to the POTRA domains during secretion and the fully exposed C terminus would carry out TpsA’s function. It is likely to be so for the Cdi TPS system of *E. coli* (see below): the C terminus of the secreted CdiA (TpsA) stretches to and interacts with a neighboring target cell, while the N terminus remains anchored to the cell surface. Mazur and Cotter (2006) also suggested that FHA is secreted via the hairpin model. The C terminus of FHA contains a pro-domain, with an intramolecular chaperone function (Renauld-Mongénie *et al.*, 1996). FHA is secreted until only the TPS domain and pro-domain remain in the periplasm. The pro-domain halts secretion, allowing the mature C-terminal domain of FHA to fold. Next, the SphB1 protease (which is a T5aSS) cleaves the pro-domain, which is degraded intracellularly. FHA stays N-terminally anchored to the cell surface or is released by an unknown mechanism.

**Diversity of TPS systems**

TPS systems have diverse functions, amino acid sequences, and genomic organisations. They are involved in, for example, adherence (Roy *et al.*, 2009), biofilm formation (Serra *et al.*, 2011), proteolysis (Kida *et al.*, 2008), contact-dependent growth inhibition (CDI) (Aoki *et al.*, 2005), and iron acquisition (Cope *et al.*, 1995; Kida *et al.*, 2011) (see Table 1.2). Moreover, accumulating data shows that TPS systems do not necessarily consist of two partners only. Some involve accessory proteins or several substrates, or may require more than one transporter. For example, the FhaC transporter of *B. bronchiseptica* secretes two substrates, FhaB and FhaS (Julio and Cotter, 2005). Moreover, the secretion of the HMW1 and HMW2 adhesin by *H. influenzae* requires the accessory enzymes HMW1C and HMW2C, respectively (St Géme and Grass, 1998). Both enzymes are glycosyltransferases, and HMW1C has been shown to transfer hexose residues onto HMW1 in the cytoplasm, thereby protecting HMW1 from proteolytic degradation and promoting its binding to the cell surface (Grass and St. Géme, 2000). HMW1 and HMW2 are secreted across the OM via their cognate TpsB transporters, HMW1B or HMW2B. HMW1 has been shown to stay anchored to HMW1B by disulfide bond formation, whereas the N terminus is proteolytically processed (Buscher *et al.*, 2006).

Many TPS systems are encoded in an operon with the *tpsB* gene upstream of the *tpsA* gene (Jacob-Dubuisson *et al.*, 2013). However, loci have been identified that contain one *tpsB* to-
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together with two tpsA s: an example is the *Burkholderia ambifaria* strain AMMD (Bamb.5443, Bamb.5444, Bamb.5446). When a TpsB transports two independent TpsAs, the corresponding genes do not have to be located in the same operon and the second tpsA gene can be located elsewhere on the genome. It happens, for example, in *N. meningitidis*. This bacterium has three TPS systems (van Ulsen *et al.*, 2008), and although the genes encoding these systems are scattered across the genome, all three systems are co-expressed. Tps1 comprises the TpsA1a and TpsA1b proteins and the TpsB1 transporter; Tps2 contains the TpsA2a and TpsA2b proteins and the TpsB2 transporter; whereas Tps3 only comprises one TpsA3 protein. A Clustal Omega alignment of the TPS domains showed that TpsA1a and TpsA1b have 100% identity, whereas the identity between TpsA2a and TpsA2b is 93% (see Figure 1.26). TpsA3 has an identity of 63% towards the TpsA2 proteins and 45% towards the TpsA1 proteins. Moreover, the TpsA1 and TpsA2 proteins are 42% identical. ur Rahman and van Ulsen (2013) showed that the TpsB2 transporter indeed secretes all five TpsA s, whereas TpsB1 can only secrete TpsA1a and TpsA1b.

It indicates that the TpsB1 transporter has a higher specificity than the TpsB2 transporter.

| TpsA1a          | NTGAPLNIQPSTNGRLSHNYQFDVNGKAGVLNDRNH---NPVVKGSASA49 |
| TpsA1b          | NTGAPLNIQPSTNGRLSHNYQFDVNGKAGVLNDRNH---NPVVKGSASA49 |
| TpsA3           | ANGLFQVIQPSTSGSVPNFKQDFDEKGVILNNRSCNQTLQGWINQPMELGAREA60 |
| TpsA2a          | GNGTPQVNIQPSTSAVSNQYAQFDVGRGQAIILNNRSCNQTLQGWINQPMELGAREA60 |
| TpsA2b          | GNGTPQVNIQPSTSAVSNQYAQFDVGRGQAIILNNRSCNQTLQGWINQPMELGAREA60 |

Figure 1.26: TPS domain alignment. The TPS domains of TpsA1a, TpsA1b, TpsA2a, TpsA2b and TpsA3 from *N. meningitidis* were aligned using Clustal Omega. Conserved residues are labeled with an asterisk. The TPS motifs, NPNL and NPNGI, are highlighted in purple.

An example of the functions of TPS systems is the CDI activity of the CdiA/CdiB systems. These systems were first discovered in *E. coli* EC93, but are also present in, for example, *N. meningitides* MC58 and *Burkholderia pseudomallei* (Hayes *et al.*, 2014). The CdiA/CdiB system is encoded in the cdiBAI gene cluster (Aoki *et al.*, 2005). CdiA is the equivalent of a TpsA, whereas CdiB is the TpsB transporter. In most cases, the C-terminal part of CdiA is a bacterial toxin, also called CdiA-CT. To protect itself from the toxic activity of CdiA-CT, the
bacterium also produces an immunity protein, CdiI (Aoki et al., 2005; Ruhe et al., 2013). CdiA is translocated via CdiB across the OM and then stretches, due to its β-helical structure, from CdiB towards a neighbouring cell. Interestingly, the receptor for CdiA-CT on the neighbouring cell is the OMP BamA (Aoki et al., 2008). Upon BamA binding, CdiA-CT is released from its N terminus and is translocated into the target cell via an unknown mechanism (Webb et al., 2013). Depending on the CdiA-CT, it can either degrade nucleic acids or disrupt the inner membrane of bacterial target cells, resulting in a growth defect (Hayes et al., 2014).

1.8.5 T5d—hybrid autotransporter

The class of hybrid autotransporters is called so because they contain features of classical T5a and TpsB. The only T5d transporter characterised so far is PlpD (patatin-like protein, PA3339) of *P. aeruginosa* (Salacha et al., 2010). PlpD, like classical T5a, has an N-terminal signal peptide, a central passenger and a C-terminal translocator domain (see Figure 1.18). However, in contrast to T5as, the TD is predicted to form a 16-stranded β-barrel and to have a periplasmic POTRA domain, as found in the TpsB transporter of TPS systems. This POTRA domain is predicted to fold into a typical β-α-α-β-β structure. Yet, the role of the POTRA is unclear, since the TD is fused to the passenger domain and, therefore, no targeting might be required. One possibility is that the POTRA domain helps keep the passenger in a translocation-efficient state and direct its N- to C-terminal secretion (Grijpstra et al., 2013). The passenger domain of PlpD is released into the extracellular space by an unknown mechanism (Salacha et al., 2010). The PlpD passenger is a patatin-like protein (PLP), a lipolytic enzyme first characterised in potato tubers where it serves as a storage protein (Banerji and Flieger, 2004). PLPs from potatoes also play a role in the defence against pathogens by acting as lipid acylhydrolases (Banerji and Flieger, 2004; Shewry, 2003). Moreover, orthologs are found in several plant and animal pathogens. The cytotoxin ExoU of *P. aeruginosa* is also a PLP. ExoU is injected into host cells via the T3SS (see Section 1.7.3). There, it induces a rapid cell death by degrading phospholipids in the host cell membrane (Finck-Barbançon et al., 1997; Sato et al., 2005).
1.9 TPS systems in *P. aeruginosa*

The genome of *P. aeruginosa* strain PAO1 encodes six TPS systems (see Figure 1.27): Tps1: PA2462-PA2463; Tps2: PA0040-PA0041; Tps3: PA4624-PA4625; Tps4: PA4540-PA4541, Tps5: PA0690-PA0692 and Tps6: PA2542-PA2543 together with the orphan TpsA PA4082 (Filloux, 2004, 2011). An amino acid alignment of the TPS domains shows that PA0041 and PA2462 TPS domain is similar to the one of filamentous haemaglutinin FHA of *B. pertussis* (see Figure 1.28). In contrast, the TPS domains of PA0690, PA4082, PA4541 and PA4625 resemble the TPS domains of the adhesin HMW1 of *H. influenzae*. Moreover, the TpsB transporters are predicted to have the typical POTRA domain with the two conserved glycine residues (see Figure 1.23C). Although several studies have tried to shed light on the function of these systems, hardly anything is known, and no structural data are available.

**Figure 1.27:** Genomic organisation of the *tpsA/tpsB* operons in *P. aeruginosa* PAO1. Except for the PA0690-PA0692 gene cluster, all operons encode only one *tpsA* and one *tpsB* gene.

**Tps1 PA2462-PA2463**

Attila et al. (2008) identified PA2462 and PA2463 to be virulence genes required for the infection of the plant model organisms poplar and barley. They observed that a *P. aeruginosa* strain PAO1 PA2462 deletion mutant had a decreased haemolytic activity towards red blood cells compared to the wild type.

**Tps2 PA0040-PA0041**

PA0040 has been predicted to have a POTRA domain and PA0041, a haemagglutination activity domain. Nouwens (2003) showed that the secretion of PA0041 is negatively regulated by the
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Figure 1.28: Protein sequence alignment of the TPS domains of *P. aeruginosa* TpsAs. The TPS domains of the *P. aeruginosa* TpsAs (PA2462, PA0041, CdrA, TpsA4, PA0690 and CupB5) were aligned with the TpsAs of *B. pertussis* (FHA), *S. marcescens* (ShlA) and *H. influenzae* (HMW1). The conserved NPNL and NPNGI motifs are shown in purple, whereas conserved residues are shown in rose. The first and last amino acid residue of the full-length proteins is written at the start and stop of the alignment.

Las and Rhl quorum sensing systems. A quorum sensing mutant released high levels of PA0041 in the supernatant at the stationary growth phase. In contrast, the supernatant of the wild type culture contained less PA0041.

**Tps3 PA4624-PA4625 (CdrB/CdrA)**

Borlee et al. (2010) showed that the expression of PA4624 and PA4625 is regulated by c-di-GMP and therefore the proteins were called c-di-GMP-regulated (Cdr) TPS partners ((Starkey et al., 2009). CdrB is the putative TpsB transporter, and CdrA is predicted to form, like other TpsA proteins, an extended β-helix. In addition, CdrA has a RGD integrin binding motif and a carbohydrate binding domain. The integrin binding motif might be essential for the interaction with host cells, and the carbohydrate binding domain might play a role in binding Psl.
carbohydrates, components of the EPS matrix in biofilms (see Section 1.2). Mature CdrA has indeed been shown to crosslink Psl polysaccharides and thus to stabilise the biofilm structure. The expression of cdrBA is regulated not only by c-di-GMP but also by the transcriptional regulator FleQ (Borlee et al., 2010). FleQ binds to the promoter region upstream of cdrBA and prevents transcription. At high levels of c-di-GMP, as observed in biofilms, c-di-GMP binds to FleQ, and FleQ drops off the promoter (Hickman and Harwood, 2008), allowing the transcription of cdrBA.

After or during secretion by CdrB, CdrA is processed by a yet unknown protease. A Western Blot revealed the presence of a 150 kDa protein instead of the predicted 220 kDa form (Borlee et al., 2010). N-terminal and C-terminal cleavage of CdrA supposedly generated the mature and functional CdrA.

**Tps4 PA4540-PA4541**

PA4540 and PA4541 are also known as LepB/TpsB4 and LepA/TpsA4. Using a zymogram, Kida et al. (2008) observed that clinical *Pseudomonas* strains, but not the PAO1 strain, secreted a 100 kDa large protein, which could degrade casein. This large exoprotease (Lep) was identified as PA4541/TpsA4. The tpsA4 gene is clustered with tpsB4 in an operon, where tpsB4 is located upstream of tpsA4. A sequence analysis revealed that TpsA4 has an N-terminal NPNG motif, a trypsin-like serine protease motif, and a RGD cell attachment motif. TpsB4, possibly the transporter for TpsA4, is predicted to have one POTRA domain and a 18–22 β-strands TD domain forming the β-barrel.

TpsA4 modulates host immune and inflammatory responses by cleaving human specific receptors (PAR-1, PAR-2 and PAR-4), which then activate the transcription factor NFκB. Moreover, TpsA4 degrades haemoglobin, probably to release peptides and iron that *Pseudomonas* can use to grow. Kida et al. (2011) also showed that *Pseudomonas* can use erythrocytes as a sole nutrient source when it secretes TpsA4 together with the hemolytic phospholipase C (PlcH).

**Tps5 PA0690-PA0692**

This Tps system has not been yet studied. The tpsA gene PA0690 is separated from the tpsB
gene PA0692 by another gene PA0691, encoding a putative transposase.

**Tps6 PA2542-PA2543**

This Tps has also not been studied. PA2542 has some similarity to PA2462 and to filamentous haemagglutinins in Xanthomonas species (Filloux, 2004).

**Orphan TpsA PA4082**

PA4082, also known as CupB5, is encoded among the $cupB$ gene cluster that encodes components of a chaperone-usher pathway (see Section 1.11). It will be the major focus of this thesis and will be discussed in Chapter 4.

### 1.10 Chaperone-usher pathways

Bacteria have developed many strategies to bind and attach to biotic and abiotic surfaces. One of them is to produce adhesive pilus protruding from the bacterial cell surface. Such pilus are assembled by the chaperone-usher (CU) pathway. The main components of the CU pathway are an outer membrane usher pore, periplasmic chaperones, and pilin subunits. These components are exported across the inner membrane via the SecYEG translocon prior to assembly of the pilin subunits into a pilus. Best studied are CU pathways assembling type 1 pili and P pili on the surface of UPEC (Geibel et al., 2013; Sauer et al., 2004; Waksman and Hultgren, 2009). Therefore, this chapter will discuss the molecular assembly mechanism of P and type I pili as an example for CU pili biogenesis. It will also review the genetic diversity of CU operons and the role of the CU pili.

P pili and their assembly machine are encoded in the $pap$ operon (see Figure 1.29A): $papA$ (pilin subunit), $papH$ (terminator), $papC$ (usher), $papD$ (chaperone), $papK$ (adapter), $papE$ (tip component), $papF$ (adaptor), and $papG$ (adhesin) (Hung et al., 2009). These components assemble a right-handed superhelical pilus rod (consisting of PapA) with a thin fibrillum at the distal tip (see Figure 1.29B) (Kuehn et al., 1992). The rod is a long and rigid structure consisting of up to 1000 PapA pilin subunits. It is connected by the PapK adapter to the highly flexible
tip fibrillum, made of one PapG adhesin, one PapF adapter subunit, and 5-10 PapE subunits. Pilus synthesis is terminated in the OM by the PapH terminator (Verger et al., 2006).

Figure 1.29: Components of the P pili and type I pili in *E. coli*. (A) P pili are encoded by the *pap* operon and type I pili by the *fim* operon. The operon does not only encode structural components, but also regulatory proteins (white). The gene products of *papJ* and *fimI* are essential for the proper assembly of the P and type I pili (Tennent et al., 1990; Valenski et al., 2003) (B and C) Schematic illustration of the type I pilus (B) and the P pilus (C). Colors are as in A. Pap and Fim components are first exported into the periplasm (P) by the Sec machine. The PapC and FimD ushers form a pore in the outer membrane (OM). Up to 1000 copies of PapA or FimA are assembled into a rigid pilus rod by the usher. At the tip of the rod sits the flexible fibrillum. The PapG and FimH adhesins confer adhesive properties. Figures B and C are reproduced from Geibel and Waksman (2013) with permission from Elsevier.

Type 1 pili also form a helical pilus rod with a flexible tip fibrillum (Busch and Waksman, 2012). The pili and their components are encoded in the *fim* operon (see Figure 1.29A): *fimA* (pilin subunit), *fimC* (chaperone), *fimD* (usher), *fimFG* (linker subunits) and *fimH* (adhesin) (Hung et al., 2009). The fibrillum consists of the FimH adhesin and the FimF and FimG linker.
subunits (Hanson and Brinton, 1988). Because each subunit is present only once, the type I pili fibrillum is shorter than that of the P pilus (Hahn et al., 2002). FimG links the fibrillum to the pilus rod generated by approximately 1000 copies of the pilin FimA (see Figure 1.29C). No termination subunit has been identified so far.

1.10.1 Molecular mechanisms of CU pilus biogenesis

The first step of pilus biogenesis is the transport of pilus subunits across the inner membrane by the Sec translocon (see Section 1.4.1) (Jacob-Dubuisson et al., 2004). Once in the periplasm, the DsbA oxidoreductase catalyses the formation of disulfide bonds in the unfolded subunits (Crespo et al., 2012). Subsequently, the CU chaperones interact with their cognate subunits and accelerate their folding into a stable intermediate (Di Yu et al., 2012; Vetsch et al., 2004, 2002). For example, FimC accelerates the folding of FimG by 100 times (Vetsch et al., 2004). The interaction between chaperones and subunits also prevents the subunits from misfolding and protects them from degradation by DegP (Jones et al., 1997; MacIntyre et al., 2001; Vetsch et al., 2002). DegP is a periplasmic protease, whose expression is upregulated by the Cpx-envelope stress system (Danese et al., 1995). This two-component system (see Section 1.3.1) consists of the IM sensor kinase CpxA, the cytoplasmic response regulator CpxR and the periplasmic inhibitor CpxP (Hunke et al., 2012). The Cpx system is activated by numerous signals, such as misfolded proteins, salt, and pH. In the absence of a stimulus, CpxP interacts with the periplasmic portion of CpxA and inhibits CpxAs activity. However, upon stimulus sensing, CpxP is released from CpxA. The latter then activates the transcriptional activator CpxR that upregulates the expression of target genes, for example degP (Price and Raivio, 2009). Another function of the chaperones is to target their subunits to the outer membrane usher that forms the platform for pilus assembly (Thanassi et al., 2002).

Donor strand complementation

Unfolded subunits have a high energy state and are unstable. The interaction with the chaperone lowers a subunit’s energy state and keeps it in a stable and folded conformation. Subunits are highly unstable because of their incomplete immunoglobulin-like (Ig-like) fold. An Ig-like
fold typically is made from seven antiparallel β-strands; however, subunits are missing the seventh C-terminal β-strand (see Figure 1.30A) (Choudhury et al., 1999; Sauer et al., 1999). The missing strand creates a deep hydrophobic groove on the surface of the subunit and its absence consequently destabilises the subunit. Chaperones have two Ig-like domains that form a boomerang-like structure (see Figure 1.30B) (Holmgren and Bränden, 1989; Kuehn et al., 1993). But in contrast to the subunits, each chaperone’s Ig-like domain consists of seven β-strands. The seventh strand, also called G1 strand, of the N-terminal domain is essential to stabilise the subunits fold.

Figure 1.30: Donor-strand complementation and exchange and crystal structure of the CupB2 chaperone. (A) The pilin (pink) has an incomplete Ig-like fold consisting of only six (instead of seven) antiparallel β-strands. The missing strand creates a hydrophobic groove (between the A2 and F strand) on the surface of the subunit and makes the subunit unstable by itself. Thus a chaperone donates its seventh β-strand (yellow, also termed G1) and inserts it in a parallel fashion between the A2 and F strand. This process is called donor strand complementation. (B) Crystal structure of the CupB2 chaperone of P. aeruginosa (PDB: 3Q48). The chaperone has two Ig-like domains that fold into a boomerang-like shape. Each Ig-like domain consists of seven β-strands. CupB2 has a short F1G1 loop (dashed lines) and is therefore classified as a FGS chaperone (see below). (C) Donor strand exchange at the usher. The G1 strand of the chaperone is exchanged against the Nte peptide (green) of an incoming chaperone-subunit complex. The Nte peptide is inserted antiparallel to the F strand, which stabilises the subunits even more. Figures A and C were adapted after Busch and Waksman (2012).
The chaperone’s G1 strand contains four alternating hydrophobic residues, P1, P2, P3 and P4. These residues have counterparts in the hydrophobic groove of the subunits: the P1, P2, P3 and P4 pockets (see Figure 1.31A). The subunits, except the terminator PapH, also have a P5 pocket (see Figure 1.31A and 1.31B). The pocket is required for subunit polymerisation at the usher (see below) (Verger et al., 2006). In a process called donor strand complementation (DSC) the chaperone donates in trans its G1 strand to fill the P1–P4 pockets, leaving the P5 pocket empty (see Figure 1.31A and 1.30A). Importantly, the chaperone inserts its G1 strand parallel to the subunits F strand, which results in an atypical Ig-like fold (in a typical Ig-like structure, the G1 strand runs antiparallel to the F strand). Because of the atypical fold, the subunit has not reached its lowest energy state yet and is still able to assemble into a pilus. Subunits in the pilus have the lowest energy state when compared with the unfolded subunits and the one in complex with a chaperone.

Figure 1.31: Accessibility of the P5 pocket. (A) The PapD chaperone is shown in complex with the PapK adapter. The G1 strand of PapD contains the P1-P4 residues. These are inserted into the hydrophobic groove of PapK, where they fill the P1-P4 pockets of the subunit. Importantly the P5 pocket is left free, so that PapK can undergo DSE with the following PapA subunit. (B) The PapD chaperone is in complex with the PapH terminator. PapH does not have a P5 pocket and cannot undergo DSE. Thus being the last subunit that is incorporated into the pilus, it terminates pilus biogenesis. (C and D) The fibrillum subunits PapE and PapK polymerise at the usher. PapK inserts its Nte peptide into the hydrophobic groove, thereby displacing the chaperones G1 strand. PapK starts to insert its Nte at the P5 pocket up to the P2 pocket, thus pushing the chaperone out. The Nte also contains hydrophobic residues, termed P2-P4 residue. The proper positioning of the Nte in the groove depends from a conserved glycine in position P4. Only a small Gly is able to fill the bulky P4 pocket in the groove. Figure reproduced from Geibel and Waksman (2013) with permission from Elsevier.
Donor strand exchange

The chaperones target the subunits to the usher, where the subunits polymerise to form a pilus by a process called donor strand exchange (DSE, see Figure 1.30C). During DSE, the G1 strand of the chaperone is swapped against an N-terminal extension (Nte) peptide that protrudes from the subunit in the subunit:chaperone complex. The Nte peptide is 10–20 residues long and is not part of the Ig-like fold. Moreover, adhesin subunits, such as PapG and FimH, usually do not have an Nte peptide (Geibel and Waksman, 2013). The Nte sequences are relatively conserved and contain a conserved glycine and cysteine, as well as four alternating hydrophobic residues called P2–P5 residues (see Figure 1.32) (Lee et al., 2004). During DSE, the G1 strand of the chaperone:subunit complex at the usher is exchanged against the Nte peptide of an incoming pilin subunit. Hereby, the Nte peptide is inserted in an antiparallel fashion: it gets in at the P5 pocket and fills up the groove towards the P2 pocket (see Figure 1.31D). Once the P5 residue of the Nte peptide enters the P5 pocket, the DSE relies on a zip-in-zip-out mechanism: the G1 strand of the chaperone gradually zips out, and at the same time the Nte of the incoming subunit zips in (Remaut et al., 2006).

![Amino acid alignment of subunits Nte peptides](image-url)

Figure 1.32: Amino acid alignment of subunits Nte peptides. The Nte peptides of *P. aeruginosa* CupB1 pilin and *E. coli* PapA pilin, PapF and PapK fibrillum subunits were aligned. The P2 to P5 residues are indicated. Strictly conserved residues are shown in purple and hydrophobic residues in pink. Figure adapted after Lee et al. (2004).

The correct positioning of a subunit’s Nte peptide relies on its P4 residue (see Figure 1.31D and Figure 1.32). The P4 residue is a highly conserved small glycine (Rose et al., 2008; Verger et al., 2007). In contrast, amino acids in the P4 pocket are usually bulky aromatic residues. Consequently, the P4 pocket can only be accessed by a small residue, such as glycine. Large P4 residues, in contrast, would cause constraints and destabilise the subunits, preventing pilus biogenesis (Busch and Waksman, 2012).
Structure and function of the usher

The DSE takes place at the usher - an OM pore - that catalyses the assembly of the pilus. The usher consists of five domains: a periplasmic N-terminal domain (NTD), two periplasmic C-terminal domains (CTD1 and CTD2), an internal plug domain, and an outer membrane pore (see Figure 1.33A) (Nishiyama et al., 2003; Thanassi et al., 2002). The structure of the PapC usher has revealed a large $\beta$-barrel made up of 24 $\beta$-strands (Remaut et al., 2008). In a resting/inactivated state, the usher forms a dimer and each monomer is closed by a $\beta$-sandwich plug in its pore lumen (Li et al., 2004; Remaut et al., 2008).

![Figure 1.33: Crystal structure of the activated FimD usher (PDB: 3RFZ) and the FimH adhesin (PDB: 1QUN). (A) The usher has a periplasmic N-terminal domain (NTD, red) and two periplasmic C-terminal domains (CTD1, yellow and CTD2, orange). It also comprises an outer membrane pore (blue) that folds into a 24-stranded $\beta$-barrel. In the resting usher the pore is closed by a plug (green). But when activated, the plug swings out into the periplasm as can be seen here. (B) FimH has an N-terminal lectin domain and a C-terminal pilin domain. The pilin domain undergoes DSE with FimG in the growing pilus, whereas the lectin domain binds to D-mannosylated receptors on the surface of eukaryotic cells.](image)

The usher has to be activated prior to pilus assembly. The activation relies on an interaction of the usher with the adhesin:chaperone complex. The adhesin has a two domain structure, usually an N-terminal lectin domain (FimH$_L$) and a C-terminal pilin domain (FimH$_P$, see Figure 1.33B and below) (Choudhury et al., 1999; Hultgren, 1989). Structural data for a complex consisting of FimD:FimC:FimH (usher:chaperone:adhesin) shows FimH$_L$ trapped in the pore lumen and the plug in the periplasm, next to the NTD domain (Phan et al., 2011). It is not understood how the lectin domain of FimH gets into the pore lumen despite the presence of the plug.
However, electrophysiology experiments indicate that the plug is not constantly closing the pore (Mapingire et al., 2009). It swings frequently into the periplasm and so allows FimH to access the pore lumen. To accommodate FimH and the growing pilus in the pore lumen, the usher pore changes from a kidney shape to a circular one (Huang et al., 2009; Nishiyama et al., 2008). This transition also influences the oligomerisation state of the usher, which now dissociates into two monomers. Recent structural data support the idea that the activated usher is indeed a monomer (Allen et al., 2013; Phan et al., 2011).

The chaperone:subunit complexes are targeted to the ushers NTD (Nishiyama et al., 2005). The targeting is controlled by an allosteric mechanism (see Figure 1.34A) (Di Yu et al., 2012). It has been shown for the Caf1M:Caf1 (chaperone:subunit) complex required for the assembly of the F capsule in Yersinia pestis. Caf1M has an usher-binding surface, which recognises and binds the N terminus of the usher NTD. Moreover, Caf1M has a proline-containing structural lock in its N-terminal domain. In an unoccupied chaperone (without subunit), the usher-binding surface is inaccessible due to the lock and prevents the interaction between the chaperone and the usher. But as soon as the Caf1 subunit binds to Caf1M, the lock opens and, through a conformational change, twists away from the usher-binding surface. Thereby it exposes a binding site for a highly conserved Phe present at the extreme N terminus of the ushers NTD. The affinity of the Caf1M:Caf1 complex for the usher increases, and the complex binds the usher NTD to undergo DSE. According to Di Yu et al. (2012) a similar allosteric mechanism might be involved in the traffic control of other CU pathways.

Model of type I pilus biogenesis

A model for type I pilus assembly at the usher (see Figure 1.34B and 1.35) can be deduced from the crystal structures of FimD bound to FimC:FimH (Phan et al., 2011) and to FimC:FimF:FimG:FimH (Geibel et al., 2013). Phan et al. (2011) showed that the FimC:FimH complex is bound to the CTDs of the usher. It is the starting point of the model. The next incoming subunit would be FimG in complex with the FimC chaperone. FimC:FimG are recruited by the usher NTD:plug complex, which positions FimC:FimG so that the Nte of FimG is very close to the groove of the CTD-bound FimH (Geibel and Waksman, 2013; Volkan et al.,
1. INTRODUCTION

Figure 1.34: **Interactions at the usher.** (A) Schematic representation of the allosteric mechanism that enables the interaction between usher and chaperone:subunit complex. The free chaperone Caf1M, cannot bind to the usher because its binding interface is obscured by a lock (yellow). But, after binding the pilin subunit Caf1, a structural change leads to the opening of the lock and makes the usher binding interface accessible for the usher Caf1A. Thus the chaperone can only bind the N terminus of the usher (Caf1A_N), when it is in complex with a subunit. Figure adapted after Di Yu et al. (2012). (B) Model of the transfer of the subunits from the FimD usher NTD to the CTD. The FimD usher has been activated by an interaction with the FimH:FimC complex that is bound to its CTD2. Consequently, the plug moved into the periplasm, interacted with the NTD and the lectin domain of FimH entered the pore. (1) The next subunit, FimG, is targeted to the ushers NTD by the FimC chaperone. FimC interacts with the NTD and positions FimG so that it can undergo DSE with FimH. (2) As FimH is now stabilised by the Nte peptide of FimG, the FimC chaperone dissociates from the usher. (3) The FimH:FimG complex moves upward into the pore (see Figure 1.35) and (4) is transferred onto the CTD. The NTD can now accept another chaperone:subunit complex. Figure adapted after Geibel et al. (2013).

2012). Because FimH and FimG are now close to each other, they can undergo DSE. Subsequently, the FimC chaperone dissociates from the CTD. As the CTD is now unoccupied, the FimH:FimG:FimC complex is transferred to it, and FimH is pulled into the pore. Such transfer of the pilin:chaperone complex from NTD to CTD has been confirmed by solving the structure of the FimD:FimC:FimF:FimG:FimH complex (see Figure 1.35) (Geibel et al., 2013). Examination shows that the complex interacts with the CTD only. The now unoccupied NTD is ready to receive the next subunit:chaperone complex. For the type I pili, it is one FimF adapter subunit, followed by up to a thousand FimA pilin subunits. Repeated cycles will result in the biogenesis of a pilus.

Only little is known about how the subunit:chaperone complex is transferred to the CTD. However, Volkan et al. (2012) and Geibel et al. (2013) have given first insights into this mechanistic
Subunit transfer at the usher. To be transferred from the NTD (blue) to the CTD (purple) subunits have to move upwards by 50 Å and rotate counterclockwise by 110°. The proteins and the usher domains are colored as in Figure 1.34B. The FimC chaperone and FimD usher are shown as ribbons, whereas FimH, FimG and FimF subunits are displayed as spheres. For FimH the lectin (FimH$_L$) and pilin domain (FimH$_P$) are labeled. The right figure shows the crystal structure of the FimD:FimC:FimF:FimG:FimH complex, solved by Geibel et al. (2013). Figure reproduced from Geibel and Waksman (2013) with permission from Elsevier.

Volkan et al. (2012) showed that the CTD2 can catalyse the dissociation of the subunit:chaperone complex from the NTD. It is possible only when two conditions are fulfilled: (i) the pilus has to move upwards by 53 Å and (ii) it has to rotate by 110° counterclockwise (see Figure 1.35) (Geibel et al., 2013). A simulation of the subunit transport by the usher has revealed that the subunits follow a helical low energy path when entering and leaving the usher.

To follow this path, the subunits have to lift and rotate counterclockwise when entering the pore. Ultimately, the upward motion and rotation facilitate the transfer of the pilus from the NTD to the CTD.

Another unsolved question is how the subunits are pulled or pushed through the usher pore. The low energy stream is probably not essential, because it does not create an energy gradient along which the subunits could migrate (Geibel and Waksman, 2013). However, Geibel and Waksman
(2013) speculated that the subunits are pulled through the usher. They can only be pulled when
the back-sliding of the growing pilus into the cell is blocked. The pilus back-sliding is prevented
by the folding of FimH and FimA on the cell surface: the angle between the FimH$_L$ and FimH$_P$
domains differs before and after its translocation (Geibel et al., 2013). Before translocation
(FimH in complex with FimD:FimC), the FimH$_L$ and FimH$_P$ domains are in a straight line.
But after translocation (FimH in complex with FimD:FimC:FimF:FimG), both domains are
angled towards each other. This angling of the domains might prevent the back-sliding of the
pilus into the cell. Polymerisation of FimA also seems to prevent the back-sliding. Once at the
cell surface, FimA folds into a right-handed and super-helical pilus made of 3.3 subunits per
turn (Hahn et al., 2002). As only one subunit can pass through the usher, the super-helical pilus
cannot slide back.

Termination of pilus biogenesis

P pilus biogenesis is terminated by integration of the terminator subunit PapH (Verger et al.,
2006). This is in contrast to type I pili for which no terminator has been identified so far. PapD
targets PapH to the ushers NTD, which is in complex with the plug domain. PapH provides
its Nte peptide to the accepting subunit already at the ushers CTDs (Volkan et al., 2012).
Importantly, PapH has no P5 pocket. Thus, it is not able to undergo DSE with subsequent
subunits and terminates pilus polymerisation (see Figure 1.29C and 1.31B) (Remaut et al.,
2006). Volkan et al. (2013) showed that PapH anchors the pilus to the plug domain: A $papH$
deletion mutant released P pili and had an increased sensitivity to the antibiotic erythromycin.
Erythromycin diffused into the cell through the open usher, which was kept open due to the
interaction between the plug and the NTD.

Subunit ordering

Various pilin subunits polymerise in a specific order at the usher. The first subunit to arrive is
always the adhesin, thus activating the usher for pilus assembly. Compared to other subunits,
the adhesin has the highest affinity for binding the usher. It is because both the pilin and lectin
domains bind to the usher’s NTD, as has been shown for PapG of the P pilus. In contrast,
the other subunits have only one domain - the pilin domain - that interacts with the NTD. Consequently, the adhesin interacts with the usher NTD prior the fibrillum and pilin subunits (Morrissey et al., 2012; Nishiyama et al., 2003; Saulino et al., 1998; Volkan et al., 2012).

The fibrillum subunits are also assembled in an ordered fashion (Dodson et al., 1993). They prefer to interact with their neighbour in the polymerised pilus. This preference relies on the rate of the DSE between subunits. In the type I pilus, the DSE rate between FimF:FimG is 3 per minute, whereas the DSE rate between FimA:FimF is slower, only 0.03 per minute (Geibel and Waksman, 2013). The DSE rate depends on (i) the steric fit of hydrophobic groove and Nte peptide of accepting and incoming subunit, (ii) the fit between the P5 pocket and the P5 residue, and (iii) the accessibility of the P5 pocket (Ford et al., 2012; Verger et al., 2008). After the fibrillum is assembled, the polymerisation seems to pause, as the DSE rate of FimF with FimA is very slow. The subsequent polymerisation of FimA subunits is much faster (960 per minute), supporting rapid pilus biogenesis (Geibel et al., 2013).

1.10.2 Classification of CU pathways

CU pathways can be classified according to the protein sequence of the usher (Nuccio and Bäumler, 2007) or according to the structure of the chaperone (Hung et al., 1996; Zav’yalov et al., 1995). The usher belongs to the fimbrial usher protein (FUP) family found among the Proteobacteria, Cyanobacteria and Deinococcus-Thermus phyla. Nuccio and Bäumler (2007) divide the FUP family into six clades: α, β, γ, κ, π and σ. They originate from a common ancestor. The γ clade is further subdivided into four subclasses: γ₁, γ₂, γ₃ and γ₄. Clade names (except for β and γ, which have been assigned alphabetically) refer to a prominent member of this clade: (α) alternate CU family, (κ) K88 fimbriae, (π) pyelonephritis associated fimbriae, and (σ) spore coat protein U from Myxococcus xanthus. Components of the alternate CU family have little or no sequence homology with components of the classical CU pathway (Nuccio and Bäumler, 2007). However, they do have a strong structural similarity to the components of the classical CU pathway (Li et al., 2009). Additionally, the pilus morphology and the pilus assembly mechanism of both CU pathways are highly similar (Froehlich et al., 1994). Some members of the σ-family assemble non-fimbrial structures: M. xanthus secretes the protein U, required for
spore coat formation at the late stages of the bacterium’s development (Gollop et al., 1991).

Hung et al. (1996) and Zav’yalov et al. (1995) based their classification on the structure of the chaperones. Depending on the length of the loop between the F1 and G1 β-strands, they distinguish FGL (long loop) and FGS (short loop, see Figure 1.30B) chaperones. Because of the loop, FGL and FGS chaperones behave differently during the DSC and DSE. Examples for FGS chaperones are the discussed PapD and FimC chaperones required for P and type I pili biogenesis, respectively. FGL chaperones are Caf1M from Y. pestis (Chapman et al., 1999; Zavialov et al., 2003b) and SafB from S. enterica (Salih et al., 2008). Caf1M is required for the biogenesis of the F capsule, whereas SafB participates in Saf pilus assembly. As described above, during the DSC, chaperones insert their G1 strand into the subunit’s hydrophobic groove, containing the P1–P5 pockets. Because of the short loop, FGS chaperones fill the groove only up to the P4 pocket (Waksman and Hultgren, 2009). The P5 pocket is left empty and therefore readily available for the DSE. FGL chaperones, in contrast, have an additional P5 residue on their G1 strand. The P5 residue is essential for the formation of a stable chaperone:subunit complex and the assembly of the fiber (MacIntyre et al., 2001). It binds periodically to the P5 pocket in the subunits groove during DSC. However, the accessibility of the P5 pocket is crucial for the DSE, where a ternary complex (consisting of the chaperone:subunit complex and the incoming subunit) is formed. A study by Yu et al. (2012) showed that the the steric fit of the chaperones P5 residue in the subunits P5 pocket is not optimal and thus the chaperones P5 residue can be easily removed during DSE. Interestingly, mutation of the subunits or chaperones P5 residues to smaller or larger P5 residues, respectively, also inhibits fiber assembly. Remaut et al. (2006) also suggested that a dynamic equilibrium exists between a locally bound and unbound state of the P5 pocket. The study showed that a P5 pocket residue mutated to a residue with a better steric fit moves the equilibrium towards the closed/occupied state and slows down the DSE. Finally, FGL chaperones belong to the γ3 clade of FUPs, whereas FGS chaperones are members of the α, β, κ, π, σ, γ1, γ2 and γ4 clades.
1.10.3 Role and function

Many diseases are caused by pathogens that assemble CU pili on their cell surface (see Table 1.3) (Nuccio and Bäumler, 2007; Soto and Hultgren, 1999). The CU pili are, for example, involved in the attachment to and invasion into host cells as well as the formation of biofilms on biotic and abiotic surfaces. Rod-like CU pili usually confer their adhesive properties through the tip adhesin. Two of the best studied CU adhesins are the ones at the tip of type 1 pili and P pili.

Table 1.3: Fimbrial and non-fimbrial CU structures and their association with disease or function. UTI = urinary tract infection, UPEC = uropathogenic E. coli., ETEC = enterotoxigenic E. coli

<table>
<thead>
<tr>
<th>Pili/Structure</th>
<th>Bacterium</th>
<th>Disease/Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S fimbriae</td>
<td>UPEC</td>
<td>UTI</td>
<td>Johnson and Stell (2000)</td>
</tr>
<tr>
<td>type 1C fimbriae</td>
<td>UPEC</td>
<td>UTI</td>
<td>Pere et al. (1985)</td>
</tr>
<tr>
<td>987P fimbriae</td>
<td>ETEC</td>
<td>diarrhea in piglets</td>
<td>Dean (1990)</td>
</tr>
<tr>
<td>CS3 pili</td>
<td>ETEC</td>
<td>diarrhea, UTI</td>
<td>Servin (2005)</td>
</tr>
<tr>
<td>CS6 polyadhesin</td>
<td>ETEC</td>
<td>diarrhea, intestine colonisation</td>
<td>Roy et al. (2012); Svennerholm et al. (1988)</td>
</tr>
<tr>
<td>F1 capsule-like antigen</td>
<td>Y. pestis</td>
<td>bulbonic plague, anti-phagocytic properties</td>
<td>Du et al. (2002); Liu et al. (2006)</td>
</tr>
<tr>
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<td>Klebsiella pneumonia</td>
<td>biofilm formation on biotic and abiotic surfaces</td>
<td>Murphy and Clegg (2012)</td>
</tr>
<tr>
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<td>Proteus mirabilis</td>
<td>pyelonephritis in mouse model</td>
<td>Bahrani et al. (1994)</td>
</tr>
<tr>
<td>spore coat protein U</td>
<td>M. xanthus</td>
<td>spore coat formation</td>
<td>Gollop et al. (1991)</td>
</tr>
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<td>UPEC</td>
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<td>Lane and Mobley (2007)</td>
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<td>type I pili</td>
<td>UPEC</td>
<td>UTI</td>
<td>Reisner et al. (2014)</td>
</tr>
<tr>
<td>Cup pili</td>
<td>P. aeruginosa</td>
<td>biofilm formation</td>
<td>Giraud et al. (2009)</td>
</tr>
</tbody>
</table>

Type 1 pili, which belong to the $\gamma_1$ clade, carry at their tip a mannose-specific adhesin that recognises D-mannosylated receptors on the surface of epithelial bladder cells (Korea et al., 2011).
Having attached to the cells, bacteria can be internalised. Internalised bacteria are taken up into exocytic vesicles or are released into the cytosol. Cytosolic bacteria rapidly replicate and form intracellular bacterial communities, so-called pods (Anderson et al., 2003). Like biofilms, these communities are protected from the host immune system and antibiotic treatments. Bacteria can disperse from pods, be exocytosed and infect neighboring cells, causing acute and recurrent urinary tract infections (Wright et al., 2007).

P pili, members of the π clade, bind Galα-1,4-Galβ moieties of glycolipids in epithelial kidney cells (Kuehn et al., 1992; Roberts et al., 1994). Moreover, their expression is associated with kidney infections. Type 1 and P pili are suggested to work together to colonise the host. Type 1 pili would be expressed early in infection to colonise and spread in the urinary tract, followed by P pili expression as soon as bacteria reach the kidneys (Snyder et al., 2005).

Fimbriae of the γ2 clade recognise mainly intestinal epithelial cells, whereas γ3 fimbriae bind, for example, neutrophils and erythrocytes. The receptor binding domain of the γ4 fimbriae is still unknown, and although the binding domains of α fimbriae are well studied, the receptors on epithelial intestinal cells have not been identified yet (Korea et al., 2011).

### 1.10.4 CU pathway regulation

Genes encoding CU pathway proteins are usually clustered on the genome and most frequently organised in an operon. The gene clusters encode at least one chaperone and one pilin subunit, and always a single usher. Some clusters encode additional genes such as adhesins. Moreover, the order of the genes in the operons differs between the FUP clades (Nuccio and Bäumler, 2007).

Regulation of CU protein expression is complex and involves, for example, invertible DNA elements, methylation, regulatory proteins, and c-di-GMP (Clegg et al., 2011). Moreover, many bacteria produce several CU pili. *E. coli* K-12, for example, encodes 11 CU operons (Korea et al., 2010) and *P. aeruginosa* encodes five (Giraud and de Bentzmann, 2012). These CU operons are cryptic under laboratory conditions, and most are under control of the global regulator H-NS (histone-like nucleoid structuring protein). H-NS downregulates gene expression according to environmental stimuli (Dorman, 2009). The cryptic CU operons are, however, expressed and
functional *in vivo* (Clayton *et al.*, 2008; van Diemen *et al.*, 2005). Sequentially or synergistically expressed CU pili would allow bacteria to colonise different niches.

An example of interplay between regulatory proteins and invertible DNA elements to control CU pili expression is the regulation of type I pili biogenesis. *fim* genes are phase variably expressed due to an invertible DNA element - the *fim* switch - just upstream of the first gene in the operon, *fimA*. The *fim* switch harbors the *fimA* promoter and is surrounded by two inverted DNA repeats (Corcoran and Dorman, 2009; Holden *et al.*, 2007). Whether the *fim* genes are expressed depends on the orientation of the *fim* switch and, therefore, of the promoter. The inversion of the DNA element requires the recombinases FimB and FimE, which work independently. Whereas FimB promotes the inversion of the DNA element towards the off phase, FimE mediates the inversion towards both off and on phases (Adicipitaningrum *et al.*, 2009; Clegg *et al.*, 2011). The orientation of the *fim* switch also depends on the DNA supercoiling and additional proteins. Binding of the regulatory protein Lrp and integration host factor IHF to the *fim* switch, relaxes the supercoiled DNA and activates *fim* gene expression (Blomfield *et al.*, 1993; Corcoran and Dorman, 2009). However, in the absence of Lrp and IHF, the *fim* switch is inverted by the recombinases. This restores a binding site for H-NS, otherwise interrupted in the on phase orientation.

### 1.11 CU pathways in *P. aeruginosa*

Five different CU pathways have been identified in *P. aeruginosa* (see Figure 1.36). The *cupA* (*chaperone-usher pathway*), *cupB* (Vallet *et al.*, 2001), *cupC* (Vallet *et al.*, 2001), and *cupE* (Giraud *et al.*, 2011) operons are present in the genome of several *P. aeruginosa* strains sequenced so far, for example PAK, PA14, M18, PA7 and PAO1 (www.pseudomonas.com). Moreover, the *cupB*, *cupC* and *cupE* clusters can also be found in the genome of *Pseudomonas protegens* and *P. fluorescens*, and *cupC* and *cupE* also in several *Pseudomonas putida* strains and in *Pseudomonas resinovorans NBRC 106553*. In contrast, *cupD* is only present on a pathogenicity island in *P. aeruginosa* strain PA14 (Mikkelsen *et al.*, 2009). Moreover, the CupA, CupB, CupC and CupD pathways belong to the γ4 clade of FUPs, whereas the CupE pathway is a member of the σ clade (Nuccio and Bäumler, 2007).
This Section discusses recent findings about genomic organisation, structure, and assembly mechanism of the \textit{P. aeruginosa} CU components. Moreover, it describes the complex regulation of the CU pathways. Only little is known about the physiological role of CU pili in \textit{P. aeruginosa}, but there have been indications that they are involved at various stages during biofilm formation.

![Genetic organisation of the cup clusters in \textit{P. aeruginosa}.](image)

The \textit{cup} cluster is the simplest cluster and encodes one pilin subunit, one chaperone and one usher. The \textit{cupA}, \textit{cupB} and \textit{cupD} clusters encode additional proteins: a second chaperone and an adhesin. The \textit{cupB} cluster also encodes a TpsA-like protein and the \textit{cupE} operon three fimbrial-like proteins.

### 1.11.1 Classical CU pathways - CupA, CupC and CupD

**The CupA pathway**

The \textit{cupA} operon encodes the components of a classical CU pathway: the CupA1 (PA2128) pilin subunit, the CupA2 (PA2129) and CupA5 (PA2132) chaperones, the CupA3 (PA2130) usher, and the CupA4 (PA2131) adhesin. CupA2 and CupA5 are both FGS chaperones (see Section 1.10.2). CupA4 is a large protein of 453 amino acids, and it is predicted not to have the typical two-domain structure of the CU adhesins. Nonetheless, CupA4 shares similarity with some putative fimbrial CU subunits in \textit{S. enterica} and \textit{Salmonella typhimurium} (Filloux \textit{et al.}, 2004).

CupA pili have been first discovered by a transposon library screen of a \textit{P. aeruginosa} (strain PAK) mutant lacking type IV pili (\textit{\Delta pilA}). Disruptions of the \textit{cupA2} or \textit{cupA3} genes resulted in
mutants forming 70% less biofilm than the parental ΔpilA mutant. Moreover, a cupAβ mutant, introduced in the PAK wild type strain, formed even less biofilms than a type IV-piliated wild type. This indicated that CupA pili support the stable attachment of bacteria to abiotic surfaces during the early stages of biofilm formation (Vallet et al., 2001). Also, the CupA pilus seems to be important for host colonisation: a cupAβ deletion mutant, isolated from a CF patient, is required in the chronic infection of rat lungs (Winstanley et al., 2009).

The CupC pathway

The cupC operon was identified by analysing the genome sequence of P. aeruginosa (Vallet et al., 2001). The CupC pathway seems to be the simplest CU pathway in P. aeruginosa: it consists only of the CupC1 (PA0992) pilin subunit, the CupC2 (PA0993) chaperone, and the CupC3 (PA0994) usher. The P. aeruginosa genome also encodes an orphan subunit (PA5284) with the typical two-domain structure of the FimH adhesin. Although a Pfam server search could identify the pilin domain (Pfam00419), the presence of an adhesin domain is only speculative. That the CupC3 usher assembles the CupC1 pilus has been shown by shearing assays, where sheared fractions of a cupC3 deletion mutant did not contain CupC1 pili (Ruer et al., 2007). The CupC1 pilin, however, did accumulate in the cells. Moreover, the assembled CupC1 pilus could be visualised by immune electron microscopy with antibodies directed against CupC1.

The CupC1 pilus has been shown to participate in biofilm formation, especially at the early stages of microcolony formation and cell clustering. Contradictory results have been published on how strongly CupB1 (see below) and CupC1 pili influence biofilm formation (Kulasekara et al., 2005; Ruer et al., 2007). However, CupB1 and CupC1 pili might work together to improve cell-cell interactions and, therefore, the establishment of the bacterial community (Kulasekara et al., 2005).

The CupD pathway

The cupD operon encodes the CupD1 (PA14_59710) pilin subunit, the CupD2 (PA14_59720) and CupD5 (PA14_59760) chaperones, the CupD3 (PA14_59735) usher, and the CupD4 (PA14_59750) adhesin (Mikkelsen et al., 2009). The CupD4 putative adhesin has also been predicted to have a pilin domain (Pfam00419) and the two-domain structure of CU adhesins. The operon is located
on the PAPI-1 genomic island of the P. aeruginosa strain PA14 (Mikkelsen et al., 2009). PA14 is more virulent than the P. aeruginosa strain PAO1 (Rahme et al., 1995). Expression of CupD fimbriae results in the development of small colony variants and in a decrease of P. aeruginosa’s swimming motility. Moreover, CupD pili promote bacterial attachment and biofilm formation in vitro (Mikkelsen et al., 2009).

1.11.2 Hybrid CU pathways - CupB

The CupB pathway is a hybrid CU pathway, because it encodes not only the classical CU components but also a TpsA-like protein, called CupB5 (PA4082) (Ruer et al., 2008). The CU components are the CupB1 (PA4086) pilin subunit, the CupB2 (PA4085) and CupB4 (PA4083) chaperones, the CupB3 (PA4084) usher and the CupB6 (PA4081) adhesin (Vallet et al., 2001). It is not unusual for clusters of the $\gamma_4$ clade to contain genes that encode proteins of TPS systems (Jacob-Dubuisson et al., 2013). In B. pertussis, for example, the fim gene cluster is flanked up- and downstream by the TpsA protein FhaB and the TpsB transporter FhaC, respectively (Nuccio and Bäumler, 2007). In P. fluorescens, a whole tpsB/tpsA operon (PF1466/PF1467) is integrated in the CU cluster (Ruer et al., 2008).

CupB1 is predicted to be a typical CU pilin with a Pfam00419 domain. It can be detected on the cell surface of P. aeruginosa by shearing assays (Ruer et al., 2008). That CupB1 assembly is dependent on the CupB3 usher was also shown by Ruer et al. (2008) using Coomassie-staining of sheared fractions of a cupB3 deletion mutant. In their experiments, CupB1 was not detectable in the sheared fractions. CupB2 and CupB4 are both FGS chaperones, and the structure of CupB2 has been solved by Cai et al. (2011). CupB2 is a typical CU chaperone with two Ig-like domains that have a boomerang-like shape (see Figure 1.30B). Moreover, Cai et al. (2011) showed, by limited trypsin digest and strep-Tactin pull-down assays, that the CupB1 pilin and the CupB2 chaperone interact with each other. CupB6 is predicted to be a putative adhesin because it has the two-domain structure of CU adhesin. Although the C-terminal pilin domain (Pfam00419) was identified by the Pfam server, the presence of an adhesin domain remains speculative. The TpsA-like protein CupB5 has an N-terminal TPS motif including the conserved NPNG box, present in TpsA proteins. Moreover CupB5 has an N-terminal haemagglutinin activity domain
and C-terminal Glug domain repeats (Ruer et al., 2008). These domains are usually found in adhesins secreted by TPS systems, such as FHA of *B. pertussis*.

Interestingly, the *cupB* operon does not encode a TpsB transporter that could be dedicated to the secretion of CupB5. However, Ruer et al. (2008) suggested the presence of a POTRA-like domain upstream of the N-terminal domain of CupB3 (see Figure 1.37A and B). POTRA domains usually have two conserved glycine residues and fold into $\beta$-$\alpha$-$\alpha$-$\beta$-$\beta$ structure (Sanchez-Pulido et al., 2003). Although CupB3 has only the first conserved glycine, and its POTRA is predicted to resemble a $\beta$-$\alpha$-$\alpha$-$\beta$ fold, it was called P-usher which stands for POTRA-containing usher.

Ruer et al. (2008) reported that the CupB3 P-usher has a dual function: (i) secretion of CupB5 and (ii) assembly of the CupB1 pilus. To confirm the importance of the POTRA-like domain of CupB3 for CupB5 secretion, Ruer et al. (2008) generated POTRA truncations and also engineered a point-mutation in the conserved glycine. Yet, whereas CupB5 secretion was abolished in a *cupB3* deletion mutant, CupB5 was still present on the cell surface in all CupB3 variants with alteration in the POTRA-like domain. However, the truncations of the POTRA-like domain influenced the extracellular localisation of CupB5. Whereas CupB5 was located away from the cell in a wild type strain, it was close to the cell in a POTRA-truncated strain. It indicated that CupB5 was still targeted to the P-usher and that the secretion started, but then CupB5 might have got stuck in the usher pore, thus blocking subsequent assembly of the CupB1 pilus. This hypothesis is supported by the observation that POTRA-truncated variants could indeed

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**Figure 1.37:** The POTRA-like domain of the CupB3 P-usher. (A) Domain structure of CupB3 as predicted by the Pfam server. Numbers represent amino acids. (B) Amino acid sequence of the POTRA-like domain as proposed by Ruer et al. (2008). The conserved glycine is highlighted in red and residues conserved among POTRA domains are shown in green. The first and last amino acid residue numbers are indicated.
not assemble the CupB1 pilus. Consequently, Ruer et al. (2008) proposed that CupB5 is secreted prior to CupB1. Another observation to support this model was that the CupB1 pilus is readily assembled in this genetic background if the cupB5 gene is deleted, thus making the CupB3 channel free. It confirms that the POTRA-like domain is not needed for CupB1 assembly, but a POTRA-defective CupB3 disturbs CupB1 pilus biogenesis when CupB5 is present. Although the CupB pathway is the best studied CU pathway in P. aeruginosa, the data available is inconclusive. Thus, an aim of this work was to investigate the molecular assembly of the CupB1 pilus by the CupB3 P-usher and the translocation of the orphan CupB5.

The CupB pilus has been suggested to play a role during microcolony formation at the early stages of biofilm formation. Expression of the cupB operon in the P. aeruginosa strain PAK enhanced the pellicle formation in comparison to the wild type strain (Kulasekara et al., 2005). Moreover, a cupB3 mutant, which does not assemble CupB1 pili, formed less dense biofilms than the wild type did (Ruer et al., 2007).

1.11.3 Archaic CU pathways - CupE

The cupE operon is an archaic CU pathway and belongs to the σ clade of FUPs, whose most prominent member is spore coat protein U of M. xanthus. The components of the CupE system are the CupE1 (PA4648), CupE2 (PA4649), and CupE3 (PA4650) pilin subunits, the CupE4 (PA4651) chaperone, the CupE5 (PA4652) usher, and the CupE6 (PA4653) adhesin (Giraud et al., 2011). All three pilin subunits are predicted to fold into an antiparallel β-strand scaffold that differs from the typical incomplete Ig-like fold of CU pilin subunits. Instead, the pilins have a COG5430 domain with a spore coat protein U subdomain (Pf05229). Giraud et al. (2011) suggested that the COG5430 domain is a new conserved domain among archaic CU systems. Although the pilin subunits have an Nte peptide, they do not have the four alternating hydrophobic residues (P2-P5 residues) of classical CU subunits, indicating that the subunits polymerise in a process different from the DSE. CupE1 seems to form the core of the pilus, as it was still detectable in sheared fractions of a cupE2 and cupE3 mutant. The CupE4 chaperone is a member of the FGS family. The CupE6 adhesin has a two-domain structure, whereby the C-terminal domain contains the conserved COG5430 domain (Giraud et al., 2011; Nuccio and
Bäumler, 2007). Additionally, both domains contain a spore coat protein U subdomain. Lastly, CupE pili seem to be involved in biofilm formation by promoting cell clustering and formation of the typical mushroom shape.

1.11.4 Regulation of CU pathways in *P. aeruginosa*

The regulation of the *cup* operons in *P. aeruginosa* is complex and involves (depending on the *cup* cluster) an H-NS family protein, two-component systems, c-di-GMP and other regulatory systems. The following Section discusses these control elements for each *cup* operon (Giraud and de Bentzmann, 2012).

**MvaT, anaerobiosis and c-di-GMP: *cupA***

The *cupA* operon is expressed in a phase-variable manner under anaerobic conditions, e.g. in the CF lung (see Figure 1.38) (Vallet-Gely *et al.*, 2005). Under anaerobiosis, the *cupA* gene expression is positively regulated by the regulatory proteins CgrABC (*cupA* gene regulator, PA2127, PA2126.1 and PA2126) and the global regulator of anaerobic gene expression Anr (PA1544) (Vallet-Gely *et al.*, 2007). Anr senses oxygen deprivation and activates directly the expression of the *cgrABC* genes, probably by binding to a putative binding site upstream of *cgrA*. Subsequently, the CgrABC proteins upregulate *cupA* gene expression. The exact activity of the Cgr proteins is unknown. However, bioinformatic studies predict that CgrA belongs to the adenine nucleotide α-hydrolase superfamily (McManus and Dove, 2011). CgrB is a putative member of the GNAT family of acetyltransferases, whereas CgrC is a member of the ParB family of DNA-binding proteins. McManus and Dove (2011) showed that CrgB and CrgC interact with each other, which is essential for the phase-variable expression of the *cupA* genes. Moreover, chromatin immunoprecipitation revealed that CgrC binds to the *cupA* promoter (McManus and Dove, 2011). Speculations are that CgrB acetylates CgrA or CgrC to activate them (Giraud and de Bentzmann, 2012).

The *cupA* genes are only phase-variably expressed in the absence of the negative regulator MvaT (PA4315) (Vallet-Gely *et al.*, 2005). MvaT belongs to the H-NS protein family that regulates the expression of CU pili in *E. coli* (Korea *et al.*, 2010; Olsen and Klemm, 1994). MvaT represses
the \textit{cupA} gene expression under standard laboratory conditions (Vallet \textit{et al.}, 2004; Vallet-Gely \textit{et al.}, 2005, 2007). It has been suggested that MvaT either binds directly to the \textit{cupA} promoter (Castang \textit{et al.}, 2008) or represses the transcription of the \textit{cgrABC} genes (Vallet-Gely \textit{et al.}, 2007). \textit{P. aeruginosa} encodes another H-NS protein family protein, MvaU (PA2667), which can form homodimers or heterodimers with MvaT and represses \textit{cupA} gene expression (Vallet-Gely \textit{et al.}, 2005).

![Diagram of cupA gene expression regulation](image)

**Figure 1.38:** Regulation of \textit{cupA} gene expression by anaerobiosis, MvaT and levels of c-di-GMP. The transcriptional regulators MvaT and MvaU repress \textit{cupA} gene expression either directly by binding to the \textit{cupA} promoter or indirectly by repressing transcription of the \textit{cgr} operon, whose gene products positively regulate \textit{cupA} expression. In the absence of MvaT and MvaU, \textit{cupA} genes are phase-variable expressed: anaerobiosis is sensed by Anr. Anr activates expression of the \textit{cgr} genes, which activate \textit{cupA} expression by binding to its promoter region. High levels of c-di-GMP also induce \textit{cupA} synthesis. PA1120, MorA and WspR have GGDEF domains and thus produce c-di-GMP. In contrast, PvrR and PA2133 contain EAL domains and degrade c-di-GMP. Moreover, SDS stress also induces \textit{cupA} expression. Sensing of SDS stress involves the transmembrane protein SiaA, two proteins of unknown function (SiaB and SiaC) and the putative diguanylate cyclase SiaD (Klebensberger \textit{et al.}, 2009). Figure reproduced from Giraud and de Bentzmann (2012).

Important for biofilm formation, the \textit{cupA} gene expression is also controlled by the levels of intracellular c-di-GMP (see Figure 1.38) (Meissner \textit{et al.}, 2007). As discussed in Section 1.3.2,
c-di-GMP regulates the switch between the free-living and sessile lifestyle of *P. aeruginosa*. High levels of c-di-GMP upregulate fimbrial gene expression, such as *cupA*, and activate the biofilm lifestyle of the bacterium. The diguanylate cyclases MorA (PA4601) and PA1120 synthesise c-di-GMP and thus upregulate the *cupA* gene expression. In contrast, the phosphodiesterase PvrR (PA14_59790) degrades c-di-GMP, resulting in the downregulation of the *cupA* expression (Meissner *et al.*, 2007). The *cupA* gene expression is also controlled by the chemosensory Wsp pathway (D’Argenio *et al.*, 2002). Overexpression of the WspR diguanylate cyclase increases the c-di-GMP levels and CupA protein production. C-di-GMP levels can also be decreased by PA2133. PA2133 is the last gene in the *cupA* operon (www.pseudomonas.org). Despite being encoded with the *cupA* proteins, the effect of PA2133 on *cupA* expression has not yet been verified.

**Roc1/Roc2 TCS, MvaT and paerucumarin: *cupB* and *cupC***

In contrast to the *cupA* operon, the *cupB* and *cupC* operon are not phase variably expressed. However, both operons are also under control of the transcriptional repressor MvaT (PA4315) that represses their transcription (Vallet *et al.*, 2004). MvaT does not repress the *cupB* and *cupC* gene expression as strongly as it does the *cupA* gene expression. It has been shown by Northern blot hybridisation in a PAO1 wild type strain, where *cupB* and *cupC* mRNA was still detectable. But a *mvaT* mutant increased *cup* mRNA levels, showing that MvaT regulates negatively *cupB* and *cupC* gene expression.

The expression of the *cupB* and *cupC* operons is also regulated by the Roc1 and Roc2 (regulator of *cup* genes, see Figure 1.39) two-component systems (Kulasekara *et al.*, 2005). The Roc1 system consists of the sensor kinase RocS1 (PA3946) and two response regulators RocA1 (PA3948) and RocR (PA3947). RocS1R1A1 are also known as SadSRA (Kuchma *et al.*, 2005). The sensor kinase RocS2 (PA3044) and the response regulator RocA2 (PA3045) are part of the Roc2 system. RocS1 and RocS2 are unorthodox sensor kinases (see Section 1.3.1) (Kulasekara *et al.*, 2005). Moreover, RocS1 has two tandem Sbp3 (solute binding proteins) domains at its periplasmic N terminus and a PAS domain at its cytoplasmic C terminus. Kulasekara *et al.* (2005) speculate that the Sbp3 domain could recognise and bind a ligand, whereas the PAS domain senses oxygen levels in the cell. Stimulation of both Sbp3 and PAS domain could then
activate the phosphorelay. A study by Sivaneson et al. (2011) showed that both sensor kinases can signal to the RocA1 and RocA2 response regulators. RocA and RocA2 are typical response regulators with a helix-turn-helix DNA binding motif in their output domain (Kulasara et al., 2005), and thus might bind directly to the DNA to activate gene expression.

The RocA2 regulon is known, and RocA2 has been shown, in a microarray, to upregulate the expression of biofilm-related genes, such as cupE4 (PA4651), and downregulate the expression of genes of the T3SS (pcrI, popB, pvrV, exoT, see Section 1.7.3), flagella (PA1087, see Section 1.5.3), multi-drug efflux pumps (mexAB, oprM), and T1SS (hasD, see Section 1.7.1) (Sivaneson et al., 2011). Moreover, RocA1 activates the expression of the cupC gene cluster. Additionally, RocS1 and RocS2 upregulate the expression of the cupB operon via an unknown regulator. Thus the Roc1 and Roc2 TCS together promote the sessile lifestyle of P. aeruginosa and the associated chronic infections. In contrast, they repress P. aeruginosa motile lifestyle and associated acute infections. It is unknown why P. aeruginosa also downregulates its MexAB-OprM (PA0425-PA0427) efflux pumps, as it seems counterintuitive to lower the multi-drug resistance in biofilms.

However, that the MexAB-OprM efflux pump is not essential for the biofilm lifestyle has also been shown by de Kievit et al. (2001) and Vettoretti et al. (2009). de Kievit et al. (2001) followed the mexAB and oprM expression levels during the biofilm development cycle by transcriptional fusion to gfp. They observed that the mexAB and oprM expression was indeed repressed. Furthermore, the P. aeruginosa cells isolated from the sputum of CF patients either lost or inactivated their MexAB-OprM efflux pumps (Vettoretti et al., 2009). P. aeruginosa has been suggested to form biofilms in the CF lung (Bjarnsholt et al., 2009), confirming the link between the sessile lifestyle and the downregulation of the multi-drug efflux pump.

Another protein stimulated by the Roc1 and Roc2 TCS is the RocR response regulator (Sivaneson et al., 2011). RocR does have an EAL domain in its output domain (Kulasara et al., 2005) and is a c-di-GMP-degrading phosphodiesterase (Rao et al., 2008). Chen et al. (2012) solved the structure of a RocR mutant that crystallised as a RocR tetramer. It has been suggested that the tetramer undergoes a conformational change upon phosphorylation by the sensor kinase. This change would modulate its affinity towards c-di-GMP, which is now bound and degraded. Degradation of c-di-GMP favours the motile lifestyle of P. aeruginosa. Thus RocR indirectly downregulates the expression of the cupB and cupC gene clusters, as these are
expressed during the biofilm lifestyle (Kulasekara et al., 2005). The antagonistic regulation of \textit{cupB} and \textit{cupC} gene expression via RocA1 and RocR might allow bacteria to disperse from a biofilm to find a new niche.

Figure 1.39: Overview of the TCS that control expression of the \textit{cupB}, \textit{cupC}, \textit{cupD} and \textit{cupE} gene clusters in \textit{P. aeruginosa}. The protein domains are labeled as follows: sensory domains = Spb3, PAS, PAC, GAF; transmitter domain = H1; receiver domain = D1; Hpt domain = H2; second receiver domain = D2; output domains either have a helix-turn-helix (HTH) motif for DNA-binding or a phosphodiesterase (EAL) activity. OM is outer membrane, P is periplasm, IM is inner membrane and C is cytoplasm. (Left) \textit{cupB} and \textit{cupC} gene expression are under control of the Roc1 and Roc2 TCS. The unorthodox sensor kinases RocS1 and RocS2 can both signal to the response regulators RocA1, RocA2, RocR and the unknown protein X. Phosphorylation of RocA1 activates \textit{cupC} gene expression, whereas phosphorylation of RocA2 represses the \textit{mexAB-oprM} cluster. Activated protein X upregulates \textit{cupB} expression and phosphorylated RocR degrades c-di-GMP and thus indirectly (dashes lines) downregulates \textit{cupB} and \textit{cupC} expression. Figure adapted after Sivaneson et al. (2011). (Centre) \textit{cupD} gene expression is controlled by the regulatory RcsC/B and PvrS/R networks. The hybrid sensor kinase PvrS likely phosphorylates the unorthodox kinase RcsC, which then activates the response regulator RcsB. RcsB binds to the \textit{cupD} promoter region and activates \textit{cupD} gene expression. RcsC negatively regulates \textit{cupD} transcription, as it also dephosphorylates RcsB. \textit{cupD} expression is also under negative control of the phosphodiesterase PvrR. PvrR degrades c-di-GMP and thus indirectly downregulates production of CupD proteins. Figure adapted after Mikkelsen et al. (2013). (Right) The expression of the \textit{cupE} genes is controlled by the PprA/B TCS. The classical sensor kinase PprA phosphorylates the response regulator PprB. Activated PprB then induces expression of the \textit{cupE} genes. Figure adapted after Giraud and de Bentzmann (2012).
Qaisar *et al.* (2013) also showed that the expression of the *cupB* and *cupC* operons is regulated by the gene products of the *pvc* gene cluster. The *pvc* gene cluster is under positive control of the transcriptional regulator PtxR (PA2258). The *pvcABCD* genes (PA2254-PA2257) encode enzymes that produce the metabolite paerucumarin (Clarke-Pearson and Brady, 2008). The specific function of paerucumarin is yet unknown, but Qaisar *et al.* (2013) showed that it upregulates the expression of the Roc1 and Roc2 TCS. Moreover, the authors suggested two possibilities for paerucumarin to activate *roc1* and *roc2* expression: either paerucumarin binds to their promoter region in complex with an unknown regulator protein or it is sensed by the sensors in the periplasm. Subsequently, the Roc1 and Roc2 TCS activate *cupC* and *cupB* gene expression and promote biofilm formation.

**PvrS/R and RcsC/B regulatory networks: *cupD***

In contrast to the *cupA, cupB* and *cupC* operons, the expression of the *cupD* genes is not under control of MvaT (Nicastro *et al.*, 2009). Mikkelsen *et al.* (2009) and Nicastro *et al.* (2009) showed that the *cupD* operon is under control of the regulatory PvrS/R (PA14_59800/PA14_59790) and RcsC/B (PA14_59780 /PA14_59770) networks. The RcsC/B network resembles the Roc1 TCS, because it consists of an unorthodox RcsC sensor kinase, having, like RocS1, a cytoplasmic PAS domain. The response regulator RcsB has a DNA binding domain and binds to a conserved RcsB box (G−78A−77A−76) in the promoter region of the *cupD* operon (Mikkelsen *et al.*, 2009).

The PvrS/R network consists of the hybrid sensor kinase PvrS and the response regulator PvrR. PvrR has, like RocR, an EAL domain and is a phosphodiesterase. It was discovered by a cosmid library screen (obtained from the chromosome of *P. aeruginosa* strain PA14) in rough SCV of *P. aeruginosa* (Drenkard and Ausubel, 2002). Overexpression of PvrR reverted the rough SCV to the wild type phenotype. The revertants did not form biofilms and were susceptible to antibiotics.

Activation of the phosphorelay depends on the sensor kinase PvrS, which indirectly activates the response regulator RcsB - likely by phosphorylating RcsC (Mikkelsen *et al.*, 2013). RcsB in turn activates the expression of the *cupD* genes and the biofilm lifestyle. In contrast, the sensor kinase RcsC, which also has a phosphatase activity, negatively regulates the *cupD* expression,
probably by dephosphorylating the RcsB response regulator. It is unknown what protein signals to PvrR for activation, but upon activation, PvrR decreases the intracellular levels of c-di-GMP and induces the switch towards the motile lifestyle. Thus PvrR, like RocR, indirectly down-regulates the cupD expression. The RcsB response regulator also upregulates the expression of the rcsC/B and pvrS/R genes. Consequently, the genes enhancing the cupD expression and, therefore, biofilm formation are expressed at the same time as the genes promoting the biofilm dispersal. This antagonistic regulation might make it possible for individual bacteria to fine-tune the dispersal from a biofilm (Mikkelsen et al., 2013). In the CF lung, for example, these bacteria would be under selective pressure caused by antibiotic treatments. It can result in an emergence of new resistant phenotypes, allowing the bacterial population to evolve further (Drenkard and Ausubel, 2002).

PprA/PprB TCS: cupE

The regulation of the cupE expression is under control of the PprA/PprB (PA4293/PA4296) two-component system (Giraud et al., 2011). PprA, a classical sensor kinase, activates the response regulator PprB by phosphorylation (Wang et al., 2003). PprB has a DNA-binding domain and controls directly the transcription of the cupE gene cluster by binding to the cupE1 promoter region. The PprB regulon has been identified by transcriptomic analysis: PprB upregulates the expression of, for example, bapABCD (biofilm-associated protein, PA1874-PA1877) and T4b pili (see Section 1.5.2) (de Bentzmann et al., 2012). BapABCD encodes a T1SS that secretes the adhesin BapA. BapA, CupE pili, T4b pili, and eDNA have been shown to be a part of a hyperbiofilm, produced in cells expressing PprA and PprB. Bacteria with a hyperbiofilm phenotype were hyper-susceptible to drugs and displayed decreased virulence in a chronic and acute infection model of Drosophila melanogaster (de Bentzmann et al., 2012). The decreased virulence is caused by the downregulation of the T3SS, whereas the drug hyper-susceptibility might be associated with a increase in the membrane permeability, influenced by PprA/PprB (Wang et al., 2003). Moreover, recent DNA microarray experiments showed that pprA gene expression, as well as T4b pili, CupE pili and T6SSs genes expression, are upregulated by the GacA regulon (Wei et al., 2013). The Gac TCS is involved in the lifestyle switch of P. aeruginosa from motile to sessile (see Section 1.3.1).
2. Aims of this study

Although a lot is known about the CU pathway and the TPS system, the molecular mechanism of CupB pili biogenesis and CupB5 secretion, as well as their role in *P. aeruginosa* pathogenesis, are still not well understood. Because of the genetic organisation of the cupB cluster, the intricate association of CU and TPS pathways, and the previous report that TpsA transport and pili assembly could be mixed (Ruer *et al.*, 2008), this study further investigated the assembly mechanism. The overall aim was to gain insight into the role of CupB fimbriae in attachment, biofilm, and host colonisation, suggesting that CupB is a target for the development of antibacterial treatments restraining *P. aeruginosa* infections. More specifically, the aims were:

1. **To understand the molecular mechanism involved in CupB pilus assembly (Chapter 3).** In contrast to classical CU pathways, the CupB pathway has two periplasmic chaperones, CupB2 and CupB4. It is unknown if each chaperone has a cognate subunit substrate. The possible role of CupB6, a putative adhesin, in opening the usher CupB3 was also considered. Lastly, Cup pili have been implicated to promote biofilm formation. Thus their role in biofilm formation was also investigated.

2. **To investigate CupB5 secretion (Chapter 4).** CupB5 could be secreted by the CupB3 P-usher (Ruer *et al.*, 2008). The aim was to confirm the CupB3-dependent transport and further investigate whether another transporter of the TpsB family could be involved. Additional insight should be gained into the role of the TPS domain of CupB5 during the secretion process. It should be studied whether this domain is required for an interaction with the POTRA-like domain of CupB3 or is involved in an interaction with a cognate TpsB in a more classical manner.
3. **To solve the structures of various CupB components (Chapter 5).** The primary target was the CupB3 P-usher for its unique characteristics, but also other ushers from *P. aeruginosa*. The CupB6 adhesin is a key component and obtaining the structure would result in further understanding the role of CupB fimbriae. Finally, the crystal structure of POTRA domains from a possible TpsB and its interaction with the CupB5 TPS domain were investigated. The study of the structure was done in collaboration with Prof S. Matthews (Imperial College London) and Prof G. Waksman (Birkbeck College, London).
3. Molecular mechanism of CupB pilus assembly

3.1 Results

The pili assembled by the CU pathway have been well studied in *E. coli*, but little is known about the assembly mechanism in *P. aeruginosa*. Furthermore, the knowledge about the role of *P. aeruginosa*’s Cup pili is scarce: some *cup* mutants show defects in attachment or biofilm formation, e.g. *cupA*, *cupD* or *cupE* (Giraud et al., 2011; Mikkelsen et al., 2009; Vallet et al., 2001). The CupB pili, the focus of this study, are not expressed under laboratory conditions, but production/assembly can be induced by the sensor kinase RocS1 (Kulasekara et al., 2005). Whereas RocS1 can activate the RocA1 or RocA2 response regulators to control the expression of the *cupC* genes, it impacts the expression of *cupB* genes via an unknown response regulator (see Chapter 1.11.4).

In this study, two approaches have been developed to control the expression of the *cupB* gene cluster: (i) an IPTG-dependent expression of RocS1 from the broad host range vector pMMB67EH-GW (called pRocS1 here, see Figure 3.1A), as previously shown by Ruer et al. (2008), and (ii) a chromosomal insertion of the arabinose-inducible *pBAD* promoter, together with the regulatory *araC* gene, upstream of the first gene in the *cupB* operon, *cupB1* (see Figure 3.1B). The advantage of the latter approach is that the *pBAD* promoter tightly and exclusively controls the expression of the *cupB* genes. Thus, no other RocS1-regulated genes are expressed. It is particularly important since it will allow (i) to study the CupB-dependent phenotypes independently of other genes belonging to the RocS1 regulon and (ii) to assess directly the relationship between CupB5 and the other CupB components. The strain PAO1Δ*pilAΔfliC*
3. MOLECULAR MECHANISM OF CUPB PILUS ASSEMBLY

(PAO1ΔΔ), defective for type IV pili and flagella, was used as a parental strain for all genetic manipulations. Type IV pili and flagella are the main players in surface attachment and biofilm formation (O’Toole and Kolter, 1998) and their presence is likely to mask any influence of CupB pili (Ruer et al., 2008, 2007; Vallet et al., 2001).

![Figure 3.1: Induction of cupB gene expression. (A) cupB gene induction via the sensor kinase RocS1. (Top) Overexpression of plasmid-encoded RocS1 upregulates cupB and cupC gene expression, downregulates mexAB-oprM transcription and influences the expression of additional genes as well. (Bottom) PAO1ΔΔ, conjugated with pMMB-rocS1 or pMMB67EH-GW, was grown on M63 agar plates supplemented with IPTG. Cells were scraped from the plates, resuspended in LB, harvested by centrifugation and analysed by Western Blot using CupB1, CupB6 and CupB5 antibodies. The results shown have been validated by at least three independent experiments. (B) cupB gene expression via the arabinose-inducible pBAD promoter. (Top) Schematic showing the insertion of the pBAD promoter and the regulatory araC gene. (Bottom) PAO1ΔΔ::pBAD was grown on M63 agar plates supplemented with 0–1% arabinose or 0.4% glucose (Glu). Cells were scraped from the plates, resuspended in LB, harvested by centrifugation and cell fractions analysed by Western Blot using CupB1, CupB6, CupB5 and RNA-polymerase (RNA-P) antibodies. The assay has been carried out once.

A series of antibodies were produced directed against individual CupB components, notably CupB1, CupB5 and CupB6. Western blot analysis was then used to evaluate whether CupB proteins are produced in either of the two inducible contexts described above. Figure 3.1A shows that overexpression of RocS1 in the parental strain PAO1ΔΔ led to the production of various, but readily detectable, levels of CupB1, CupB5 and CupB6. The induction of the pBAD promoter upon addition of arabinose also resulted in the production of all CupB components tested (see Figure 3.1B). Increasing concentrations of arabinose upregulated the expression of
cupB1, cupB5 and cupB6 in a dose-dependent manner, starting at 0.1–0.2% arabinose and peaking at 1%. In the presence of glucose, which represses expression from the $p_{BAD}$ promoter, the cupB gene products are not detectable, indicating a tight control via the $p_{BAD}$ system. The amount of RNA polymerase detected is constant at all conditions.

Attempts were made to design another induction method by inserting the $p_{BAD}$ promoter upstream of the rocS1 gene in PAO1ΔΔ, creating PAO1ΔΔ::$p_{BAD}$-rocS1 (see Figure 3.2A). Note that rocS1 is clustered with the gene encoding the response regulator RocR1, located upstream of rocS1. To test if rocS1 is expressed in this context, increasing concentration of arabinose were added to the bacterial culture, cells harvested, and analysed by SDS-PAGE and Western Blot. PAO1ΔΔ/pRocS1 was a positive control, and as can be seen in the Coomassie-stained SDS-PAGE gel of Figure 3.2B, a band of 126 kDa was detected in the cell fractions. This band was absent in the non-induced strain, indicating that it corresponds to RocS1. However, induced PAO1ΔΔ::$p_{BAD}$-rocS1 did not express RocS1, as no band was present at the correct size. Moreover, a Western Blot using antibodies against CupB1, CupB6 and CupB5 showed that none of the CupB proteins was expressed (data not shown).

To dissect the role of every single CupB component in the assembly of CupB pili, clean deletion mutants were created for every cupB gene, except cupB4 (PAO1ΔΔΔcupB4) and cupB5 (PAO1ΔΔΔcupB5), already available in the strain collection. The deletions were introduced into the parental strain PAO1ΔΔ.

### 3.1.1 CupB3-dependent secretion of CupB1 and CupB6

The CupB3 usher is essential for the assembly of the CupB1 pilus when *P. aeruginosa* strains PAO1 and PAK, conjugated with pRocS1, are grown on semi-solid M63 agar plates (Ruer et al., 2008, 2007). Briefly, Ruer and collaborators grew bacteria on M63 agar plates for up to 4 days at 30°C, before scraping them from the plate with a spatula. Bacteria were then resuspended in LB medium and appendages sheared off by gentle rocking over night. Cell and sheared fractions were separated by centrifugation and sheared proteins precipitated with ammonium sulphate. Several attempts were made to reproduce this exact technique (results not shown). However, sheared fractions without a significant cytoplasmic lysis and periplasmic leakage were
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Figure 3.2: Construction and expression profile of PAO1ΔΔ::pBAD-rocS1. (A) The rocR gene is located upstream of rocS1 and both genes are separated by 101 base pairs. TGA (bold) is the rocR stop codon and ATG (bold) is the rocS1 start codon. The pBAD promoter and araC gene were inserted 26 bp upstream of rocS1, thus making sure that the ribosome-binding site of rocS1 is not disturbed. (B) Coomassie-stained SDS-PAGE gel of PAO1ΔΔ/pRocS1, PAO1ΔΔ/pM MB67EH-GW (empty vector control, EV) and PAO1ΔΔ::pBAD-rocS1 cell fractions. Bacteria were grown in liquid cultures for 24 h at 30°C in M63 medium under static conditions. Cultures were supplemented with 0.1 mM IPTG, 0.1% to 1% arabinose (Ara) or 0.4% glucose (Glu). Cell samples were harvested by centrifugation. RocS1 is indicated at 126 kDa (asterisk). M is the marker, sizes are shown in kDa on the left. The experiment has been done once.

not obtained. Problems were that bacteria were tightly anchored to the agar on the plates after incubation and were difficult to scrape. It was also difficult to resuspend the bacteria in LB medium because cells adhered to the spatula (used for scraping) and formed cell lumps. Resuspension by pipette was impossible, as this would have broken the cell envelope and released cellular proteins.

Another problem was that even after shearing by gently moving the bacterial resuspension on a rocker overnight at 4°C, the cell lumps were still present. Because of the lumps, the shearing
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Figure 3.3: Shearing assays of \textit{P. aeruginosa} grown on M63 agar plates. (A) 24 hours growth on M63 agar plates. PAO1\(\Delta\)/pRocS1 (\(\Delta\)) and PAO1\(\Delta\Delta\Delta\)/pRocS1 (\(\Delta\B3\)) were grown on M63 agar plates, supplemented with 0.1 mM IPTG, for 24 h at 30\(\degree\)C. Cells were scraped off the agar plates with plastic loops and resuspended in LB. Appendages were sheared off overnight at 4\(\degree\)C and cell and sheared fractions were separated by centrifugation. Sheared fractions were precipitated with ammonium sulphate and proteins harvested by centrifugation. Samples were analysed by SDS-PAGE and Western Blot using antibodies directed against CupB5, CupB1, DsbA and RNA polymerase. The results shown have been obtained once. (B) Four days growth on M63 agar plates. PAO1\(\Delta\)/pRocS1 (\(\Delta\)) and PAO1\(\Delta\Delta\Delta\)/pRocS1 (\(\Delta\B3\)) were grown on M63 agar plates, supplemented 1 mM IPTG, for four days at 30\(\degree\)C. Cells were scraped off the agar plates with a glass spatula and resuspended in LB. Appendages were sheared off overnight at 4\(\degree\)C and cell and sheared fractions were separated by centrifugation. Sheared fractions were precipitated with ammonium sulphate and harvested by ultracentrifugation (exact protocol after Ruer \textit{et al.} (2008)). Samples were analysed by SDS-PAGE and Western Blot using antibodies directed against CupB5, CupB6, CupB1, DsbA and RNA-polymerase (RNA-P). The experiment was done once.

was not optimal and the cell density could not be estimated properly. Nonetheless, the cell and sheared fractions were separated by centrifugation. The sheared fractions were subjected to ammonium sulphate precipitation and pellets harvested by centrifugation. The cell and sheared fractions were analysed by SDS-PAGE and Western Blot. Figure 3.3A shows an example Western Blot of the cell and sheared fractions of PAO1\(\Delta\)/pRocS1 and PAO1\(\Delta\Delta\Delta\)/pRocS1 that were grown on agar plates for 24 hours. Both DsbA and RNA-polymerase were detected in the sheared fractions, indicating that cellular lysis and periplasmic leakage occurred during sample preparation. Moreover, Figure 3.3B shows sheared and cell fractions harvested from the same strains, but grown on agar plates for four days. Although the sheared fractions were not contaminated with periplasmic proteins, no CupB proteins were detectable in the cell fractions. It is possible that the CupB pili break off more easily from the cell surface after four days of incubation (due to differences in "age" and therefore stability) and thus only appendages were isolated from the agar plates. The cell bodies could stay attached to the agar and were thus...
not obtained during scraping. However, when the four day assay was carried out before, cellular CupB proteins could be detected (results not shown). As cell and sheared fractions are present on one single membrane, it excludes the possibility of malfunctioning CupB antibodies. This experiment was abandoned.

As an alternative to the shearing assay from agar plates, bacteria were grown under static conditions in M63 liquid medium. Bacteria, prepared from liquid cultures, were less prone to cytoplasmic lysis and periplasmic leakage. Other than using the liquid medium and shortening the shearing time to 3 hours, the preparation of the sheared fractions followed the protocol described above. Briefly, bacteria were incubated at 30°C for 24 h, and appendages were sheared off with a magnetic stirrer. The OD$_{600}$ of the bacterial cultures was measured. Notably, the cupB3 mutant (containing either pRocS1 or the p$_{BAD}$ promoter) had a growth defect when compared to the parental strain (data not shown). We excluded that the defect was due to CupB1 production by constructing a cupB1 and cupB3 double mutant, which still had a growth defect. To obtain comparable protein levels, the OD$_{600}$ of all strains was set to one. The cell and sheared fractions were separated by centrifugation, sheared proteins precipitated with ammonium sulphate and harvested by centrifugation. Fractions were analysed by Western Blot.

As Figure 3.4A shows, overexpression of RocS1 in the parental strain PAO1∆∆ resulted in the production and extracellular assembly of CupB1 and CupB6. In contrast, in the absence of the CupB3 usher, CupB1 and CupB6 were not assembled on the cell surface. It confirms the data of Ruer et al. (2008) and supports the hypothesis that both subunits are transported in a classical manner through the usher pore. The results were similar when monitoring cupB gene expression strains with the arabinose inducible p$_{BAD}$ promoter (see Figure 3.4B). In contrast to CupB1, CupB6 was detectable in the cell fractions of the cupB3 mutant (see Figure 3.4A and B). Probably, accumulation of CupB1, the most abundant subunit, induces a stress response. In E. coli for example, misfolded proteins induce the Cpx response. It activates the DegP protease, which degrades misfolded pilins in the periplasm (DiGiuseppe and Silhavy, 2003).

CupB1 and CupB6 translocation to the cell surface could be restored upon complementation by introducing the pBBR1-Mcs4-cupB3 plasmid in the cupB3 mutant, thus confirming the CupB3-dependent assembly of the CupB pilus (see Figure 3.5). Notably, the complementation is not
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Figure 3.4: Translocation of CupB1 pilins, CupB6 adhesins and CupB5 TpsA-like proteins. Western blot analysis of the cell and sheared fraction obtained from PAO1ΔΔ (ΔΔ), PAO1ΔΔΔcupB1 (ΔB1), PAO1ΔΔΔcupB2 (ΔB2), PAO1ΔΔΔcupB3 (ΔB3), PAO1ΔΔΔcupB4 (ΔB4), PAO1ΔΔΔcupB5 (ΔB5) and PAO1ΔΔΔcupB6 (ΔB6) using antibodies derived against CupB5, CupB6, CupB1, RNA-Polymerase and DsbA. (A) Induction of cupB gene expression via RocS1. The strains, all conjugated with the RocS1 expressing vector pMMB-rocS1, were grown in liquid culture under static conditions for 24 h at 30°C in the presence of 0.01 mM IPTG. (B) Induction of cupB gene expression via the pBAD promoter. All strains have a chromosomal insertion of the pBAD promoter upstream of cupB1 and were grown in liquid culture under static conditions for 24 h at 30°C in the presence of 0.4% arabinose. The results have been validated by at least three independent experiments.

very efficient. This might be because the assay was not carried out with fresh transconjugants, but transconjugants streaked from glycerol stocks. Furthermore, CupB5 is not required for the assembly of the CupB pilus. Its lack did not affect the secretion of CupB1 or CupB6: both are still detected in the sheared fraction of the cupB5 mutant (see Figure 3.4A and B).

The activation of the usher relies on an initial interaction with an adhesin:chaperone complex (Munera et al., 2007, 2008; Phan et al., 2011). As CupB6 is predicted to be the adhesin of the CupB pathway, CupB6 in complex with either one of the two chaperones could activate
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Figure 3.5: CupB3 dependent secretion of CupB1 and CupB6 in the complemented PAO1ΔΔΔcupB3::pBAD mutant. Western Blot analysis of the cell and sheared fractions obtained from PAO1ΔΔ::pBAD (ΔΔ), PAO1ΔΔΔcupB6::pBAD (ΔB6), PAO1ΔΔΔcupB5::pBAD (ΔB5) and PAO1ΔΔΔcupB3::pBAD (ΔB3) using antibodies derived against CupB5, CupB6, CupB1, RNA-polymerase and DsbA. PAO1ΔΔΔcupB3::pBAD was conjugated with pBBR1-Mcs4-cupB3 (pB3) for complementation or with pBBR1-Mcs4 (pEV) as a control. cupB gene expression was induced by adding 0.4% arabinose to liquid cultures grown under static conditions at 30°C for 24 h. M is the marker and corresponding protein sizes are given in kDa. The results have been validated by three independent experiments.

the CupB3 usher and subsequent assembly of the CupB1 fimbriae. Here, the deletion of the cupB6 gene prevented CupB1 transport to the cell surface. It also resulted in the degradation of CupB1 in the cell fractions as observed in the cupB3 mutant (see Figure 3.4A and B). However, the deletion of the cupB1 gene also resulted in CupB6 being absent from the cell and sheared fractions. It is, thus, unclear whether the phenotypes observed are directly linked to the mutation or whether polar effects on downstream genes, such as in the cupB1 mutant, play a role. Although polar effects on downstream genes are unlikely (because CupB5 is expressed or secreted in all mutants), they should be excluded by either complementation or quantitative RT-PCR.

3.1.2 Targeting of CupB1 and CupB6 to the CupB3 usher

The cupB gene cluster encodes, in contrast to classical CU clusters, two periplasmic chaperones, CupB2 and CupB4. To date, it is still unclear whether each chaperone has a cognate substrate or whether they can both interact with the CupB1 pilin and the CupB6 adhesin. However, some indications about the specificity of the two chaperones haven been given by Ruer et al.
Ruer et al. (2008) reported that the CupB1 pilus is not assembled in a \textit{cupB4} deletion mutant, and speculated that CupB4 interacts with the CupB6 adhesin as a prerequisite to open the CupB3 usher. The experiments presented in this thesis confirmed that the cell surface exposure of CupB1 is lost in a \textit{cupB4} mutant. Moreover, they also showed that CupB6 translocation is lost in the absence of the CupB4 chaperone (see Figure 3.4A and B). Interestingly, CupB6 was totally degraded in the cell fraction of PAO1\textDelta\textDelta\textDelta_{cupB4::pBAD}, whereas traces of CupB1 are still visible. This observation suggests that CupB4 is more likely to be the specific chaperone for CupB6.

The \textit{cupB2} mutant translocated neither CupB1 nor CupB6 (see Figure 3.4A and B). However, and in contrast to the \textit{cupB4} mutant, CupB6 was still detected in the cell fractions, but CupB1 was not. Since the lack of the CupB2 chaperone had such a severe effect on CupB1s stability, it suggests that CupB2 interacts with CupB1 rather than with CupB6. This conclusion is also supported by the data of Cai et al. (2011), who showed, by limited trypsin digest and streptactin pull-down assays, that CupB1 and CupB2 interact with each other. Although Cai et al. (2011) tried to establish the role of CupB4 towards CupB1 and CupB6, they could not see any interaction.

Pull-down assays

Overall, the data suggest that two chaperone:subunit couples exist: CupB2:CupB1 and CupB4:CupB6. To further assess this hypothesis, pull-down assays were carried out by producing recombinant proteins in \textit{E. coli}. \textit{cupB1} or \textit{cupB6}, both with their natural stop codons, were cloned into the expression vector pET28b. Pilin and adhesin are expected to be unstable in the absence of the chaperone, resulting in their periplasmic aggregation or degradation (Jones \textit{et al.}, 1997; MacIntyre \textit{et al.}, 2001). Thus, the untagged CupB1 and untagged CupB6 were also produced from pET28b derivatives that encoded C-terminally His-tagged versions of either CupB2 or CupB4 (see Figure 3.6). The plasmids were introduced in \textit{E. coli} SHuffle cells, which constitutively express the periplasmic disulfide isomerase DsbC. It usually refolds mis-oxidized proteins into their native form (Bessette \textit{et al.}, 1999; Levy \textit{et al.}, 2001), but can also function as a chaperone and may help protein folding (Chen, 1999; Goemans \textit{et al.}, 2013).
CupB1 has two cysteine residues, not predicted to form disulfide bonds by the DISULFIND server (http://disulfind.dsi.unifi.it/), whereas CupB6 has six cysteine residues and is predicted to form three disulfide bonds (Ceroni et al., 2006).

To induce cupB gene expression in the E. coli cells, IPTG was added and bacteria were grown for 16 hours at 18°C. The bacteria were harvested and lysed by sonication. Soluble proteins were separated from cell debris by centrifugation. The His-tagged chaperones were subsequently purified using nickel affinity chromatography. Eluted fractions as well as flowthrough, soluble and insoluble, and induced and uninduced samples were analysed by SDS-PAGE and Western Blot using Coomassie-staining or CupB1 and CupB6 antibodies, respectively. If a chaperone and a pilin or adhesin interacted, the subunit would elute together with the His-tagged chaperone.

As Figure 3.7A shows, CupB1 is produced upon addition of IPTG and aggregates in the absence of a chaperone. Moreover, a degradation product below 20 kDa, the expected size for CupB1, is also clearly detectable. CupB1 was not pulled-down by the CupB4-His chaperone, but mostly accumulated in the insoluble fractions (see Figure 3.7B). In contrast, a significant amount of soluble CupB1 was pulled-down by the CupB2-His chaperone, even if it is still mainly found in the insoluble fraction (see Figure 3.7C, lanes 8 and 9).

Similar to the CupB1 antibody, the CupB6 antibody detected an unspecific band at around
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Figure 3.7: Identification of the CupB2 and CupB4 interaction partner by pull-down assays. pET28b-cupB1, pET28b-cupB1-RBS-cupB4, pET28b-cupB1-RBS-cupB2, pET28b-cupB6, pET28b-cupB6-RBS-cupB4 or pET28b-cupB6-RBS-cupB2 were transformed into E. coli strain SHuffle. Bacteria were grown to an OD\textsubscript{600} of 0.6 (sample UI) and then expression of the recombinant proteins was induced with IPTG for 16 h at 18°C (sample I). The bacteria were harvested by centrifugation and cells lysed by sonication. Soluble proteins (sample Sol) and cell debris (sample In) were separated by centrifugation. His-tagged proteins (CupB2-His and CupB4-His) were purified by nickel affinity chromatography. The experiment was carried out by Eleni Manoli, Imperial College London. The fractions, including flowthrough and elution, were analysed by SDS-PAGE and Western Blot using antibodies against CupB1, CupB6 and His-tag. UI = uninduced, I = induced, In = insoluble, Sol = soluble, FT = flowthrough, numbers 7–12 = purified fractions, small arrow = CupB1, big arrow head = CupB6. M is the marker and protein sizes are indicated in kDa. The experiments were done once. (A) Untagged CupB1. (B) His-tagged CupB4 and untagged CupB1. (C) His-tagged CupB2 and untagged CupB1. (D) Untagged CupB6. (E) His-tagged CupB4 and untagged CupB6. (F) His-tagged CupB2 and untagged CupB6.

40 kDa in the induced, uninduced, soluble, insoluble, and flowthrough fractions (compare Figure 3.7A and D). The CupB6 expression was strongly induced upon IPTG addition, as a big band around 37 kDa was detected in the induced fraction. When expressed in the absence of a chaperone, CupB6 accumulates in the insoluble fraction and only the unspecific band is present in the soluble fraction (see Figure 3.7D). Although CupB6 mostly aggregates when expressed with either chaperone (see Figure 3.7E and F), some soluble CupB6 was pulled-down by the CupB4-His chaperone (see Figure 3.7E). Notably, the unspecific band is also enriched in the elution fraction. To confirm that the band is unspecific and not CupB6-related, mass spectrometry could be carried out.
Moreover, the CupB4-His chaperone was not detected by Western Blot using the His-tag antibody, whereas CupB2-His was readily detected, making the data inconclusive. However, it appears that for the untagged CupB6, expressed in the absence of a chaperone, no traces of the adhesin were recovered in the eluted fractions (see Figure 3.7D). It suggests that, although CupB4-His is not detectable, it might be produced in small amounts, which then interact with CupB6. Overall, CupB1 must be exclusively associated with CupB2, whereas CupB6 interacts with the CupB4 chaperone.

**Bioinformatic analysis**

The P1-P4 residues on CupB2s and CupB4s G1 strand would be expected to be variable, if they indeed display a substrate specificity for either CupB1 or CupB6. The location and composition of the G1 strand in CupB2 has been identified by Cai et al. (2011), who solved CupB2s crystal structure.

Figure 3.8: Alignment of mature CupB2 and CupB4. CupB2s β-strands (purple) and α-helices (yellow) are shown as identified by Cai et al. (2011). CupB4s β-strands (green) and α-helices (yellow) have been predicted by the Quick2 Server (http://toolkit.tuebingen.mpg.de/quick2) following the PSIPRED method by Jones (1999). The P1 to P4 residues located on the G1 strand are indicated and hydrophobic residues are shown in pink.

CupB2s G1 strand is 14 residues long and displays three alternating hydrophobic residues termed
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P1 to P3: Phe (P1), Phe (P2) and Leu (P3) (see Figure 3.8). The P4 residue is a polar Asn. As described in Section 1.10, these residues are inserted into the subunits hydrophobic groove during donor strand complementation. The G1 strand of CupB4 has been predicted to be located at the same position as the one from CupB2. Notably, CupB2s and CupB4s G1 strand share 50% sequence identity. CupB4 has two alternating hydrophobic residues, Val (P2) and Leu (P3), and two polar residues, Asn (P4) and Tyr (P1) in the G1 strand. The difference in amino acid composition of the G1 strand of CupB2 and CupB4, especially in the P1 and P2 residues, might indicate that CupB2 and CupB4 recognise and interact with different subunits.

3.1.3 CupB3-independent secretion of CupB5

A study by Ruer et al. (2008) reported that the cupB3 gene is wrongly annotated on the Pseudomonas website. The annotated cupB3 start codon is a GTG, which has indeed a weak translational start (Kolaskar value: 15) and the gene product has been predicted to lack a signal peptide. Ruer et al. (2008) proposed that the actual cupB3 start codon is an ATG located 246 bp upstream of the GTG (see Figure 3.9A). The Kolaskar value for the ATG start is 29, which is a stronger translational start (Kolaskar and Reddy, 1985). The signal peptide for the longer CupB3 is 13 amino acids long (see Figure 3.9C).

Due to the new gene start, CupB3 has an N-terminal extension of approximately 80 residues, not present in CupC3 and CupA3—two other P. aeruginosa ushers. Ruer et al. (2008) reported that the N-terminal extension is a POTRA-like domain because it contains the first of two conserved glycines usually found in POTRA domains (see Figure 3.9B). However, the predicted secondary structure of the CupB3 N terminus was slightly different from those of the classical POTRA domains: $\beta$-$\alpha$-$\alpha$-$\alpha$-$\beta$ instead of $\beta$-$\alpha$-$\alpha$-$\beta$-$\beta$.

Ruer et al. (2008) proposed that the CupB3 P-usher (POTRA-like containing usher) is required for the secretion of the TpsA-like protein CupB5, having observed the loss of CupB5 secretion in a cupB3 mutant. As described in Section 3.1.1, I attempted to reproduce the data of Ruer et al. (2008) by shearing the appendages of bacteria grown on M63 agar plates (see Figure 3.3). However, in our laboratory the deletion of the cupB3 usher did not affect CupB5 secretion. This result contradicts the previous observation by Ruer et al. (2008). The reasons are unclear,
Figure 3.9: Characterisation of CupB3s DNA and protein sequence. (A) Start of cupB3 DNA sequence. The GTG start codon annotated on the *Pseudomonas* website is shown in green and the ATG start codon proposed by Ruer et al. (2008) in pink. The intergenic resion upstream of the ATG start codon is shown in grey and small letters. (B) CupB3 protein sequence. Shown are the first 101 amino acids (up to the start of the NTD) of CupB3 including the signal peptide (orange, amino acids 1-16) and the POTRA-like domain (amino acids 17-99) as proposed by Ruer et al. (2008). The secondary structure has been predicted by the PSIPRED server, E corresponds to β-strands and H to α-helices. M (pink) is the protein start suggested by Ruer et al. (2008) and V (green) annotated protein start on the *Pseudomonas* website. G (bold) is the conserved Glycine. (C) SignalP 3.0 prediction results for the CupB3 P-usher. Plot showing the C-score (raw cleavage site score), S-score (signal peptide score) and Y-score (combined cleavage site score) for the first 70 amino acids of the CupB3 P-usher. The C- and Y-scores are expected to be high at the position immediately after the cleavage site. The Y-score combines the C-score peaks and the slope of the S-score, thus predicting a accurate cleavage site.

apart from the experiments done in different laboratories and, thus, possibly in slightly different conditions. As Figure 3.3A shows, an additional problem of this method was a significant lysis and leakage, which makes conclusions unreliable. Thus, the static culture conditions (as described above) were used to probe the extracellular release of CupB5 in all cupB mutants. Except for the *cupB5* mutant, all mutants and the parental strain released CupB5 on the cell
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surface (see Figure 3.4A). It indicates that CupB5 does not require any of the CU components for secretion, thus confirming the discrepancy with the data of Ruer et al. (2008). Moreover, CupB5 was found most exclusively in the sheared fractions, while only little amounts were detectable in the cell fractions of the cupB1 and cupB3 mutants.

Strikingly, under conditions where only cupB gene expression was induced (by the pBAD promoter), CupB5 was not translocated to the cell surface by the parental strain and, thus, by none of the cupB mutants (see Figure 3.4B). This observation leads to the suggestion that, under the conditions used, CupB5 secretion involves a different (or additional) transporter, not encoded in the cupB gene cluster, and possibly induced in a RocS1-dependent manner. Chapter 4 discusses the corresponding experiments.

3.1.4 Detection of extracellular CupB proteins

Whereas the CupC1 fimbriae were readily visualised using immuno-EM (electron microscopy) and anti-CupC1 antibodies (Ruer et al., 2007), little is known about the structure and appearance of the CupB pili on the cell surface. In particular, their size and numbers are unknown. Ruer et al. (2008) proposed that the CupB1 fimbriae are responsible for CupB5 located away from the cell surface, but no immuno-EM could identify the fimbrial structures.

In an attempt to visualise the CupB pili by transmission electron microscopy for this study, PAO1ΔΔ/pRocS1 cells were grown under static conditions in M63 medium supplemented with IPTG (as described for the shearing assay) and fixed on a nickel grid. Additionally, the bacteria were immuno-gold labeled using the CupB1 antibody. Although pili-like structures were observed on the cell surface, the CupB1 antibody cross-reacted with the grid surface (data not shown), and it was unclear if the structures were CupB or CupC pili.

To monitor CupB1 pili on the cell surface and avoid any fractionation procedure, the whole-cell enzyme-linked immunosorbent assay (ELISA) was attempted. The advantage of the whole-cell ELISA is rapid simultaneous processing of several replicates and gentler (compared to the shearing assay) sample preparation, which could avoid cell lysis and periplasmic leakage. The cells were grown statically in liquid M63 medium under the same conditions used for the shearing assay. To detect the amount of CupB1 pili, MaxiSorp 96-well plates were coated with bac-
teria, blocked, and incubated with various dilutions of anti-CupB1 and alkaline phosphatase-conjugated goat anti-rabbit antibody. The results were inconclusive, and the CupB1 antibody bound to the bacteria irrespectively of the presence of CupB1 pili (see Figure 3.10A and C).

Figure 3.10: Optimisation of whole-cell ELISAs. Bacteria were either grown statically in liquid M63 medium (PAO1ΔΔ), or shaking in TSB medium (PAK and *E. coli* DH5α). MaxiSorp 96-well plates were coated with bacteria corresponding to OD$_{600}$ = 0.5 (A,B,C) or OD$_{600}$ = 0.25 (D). For the ELISA, either CupB1 or PAK antibodies were used at a dilution ranging from $-7$ to $-3$. (A) ELISA on PAO1ΔΔ/pRocS1 and PAO1ΔΔΔ $cupB1$/pRocS1 using anti-CupB1. The CupB1 antibody cross-reacts with the parental strain and the $cupB1$ mutant, as both are recognised at a similar level. (B) ELISA on PAK and *E. coli* DH5α using anti-PAK. The PAK antibody recognises PAK at $-4$ and $-3$ dilution; nonetheless high levels of *E. coli* are still unspecifically bound. (C) ELISA on induced (+IPTG) and non-induced (-IPTG) PAO1ΔΔ/pRocS1 and PAK using anti-CupB1. CupB1 antibody binds the $cupB$-induced and $cupB$-non-induced parental strain and PAK, although the latter to a lesser extent. (D) ELISA on PAO1ΔΔ/pRocS1 and PAO1ΔΔΔ $cupB1$/pRocS1 using adsorbed anti-CupB1. The adsorbed CupB1 antibody still recognises $cupB1$-expressing and $cupB1$-non-expressing strains at a similar level. ELISAs were carried out once.

Further optimisation, including (i) adsorption of the CupB1 antibody (see Figure 3.10D), (ii) use of an antibody directed against *P. aeruginosa* strain PAK (see Figure 3.10B), (iii) addition of a PAK control (see Figure 3.10C) and (iv) use of induced and non-induced instead of the parental and the $cupB1$ mutant strains (see Figure 3.10C), led to no improvements. The CupB1 antibody was adsorbed/purified with PAO1ΔΔΔ $cupB1$/pRocS1 cell-lysate to minimise unspecific interactions, but as Figure 3.10D shows, adsorbed CupB1 antibody was still cross-reacting. To
determine the correctness of the ELISA protocol, a control experiment was done using PAK antibody (see Figure 3.10B). However, the negative control E. coli strain DH5α was also recognised (although at a lesser extent than PAK, probably because the rabbit from which the antibody was derived was in contact with E. coli). Moreover, the PAK antibody, directed against whole PAK cells, might not be specific enough. Furthermore, the CupB1 antibody cross-reacted not only with the induced and non-induced parental strains but also with PAK that does not express the cupB genes (see Figure 3.10C).

Yet, whole-cell ELISA can be useful to detect CU pili, as shown for Dr fimbriae assembled by uropathogenic E. coli (Piatek et al., 2013). Instead of using MaxiSorp plates, Piatek et al. (2013) coated polystyrene microtitre plates with type IV collagen to which E. coli adhered by its Dr fimbriae. Possibly, CupB1 pili were washed off from the MaxiSorp plates, because they did not attach as tightly to the plates as they would to their natural substrate. The whole-cell ELISA assay was not developed further.

3.1.5 CupB pili are not involved in biofilm formation

CupB and CupC pili have been suggested to work together to promote bacterial clustering and micro colony formation, allowing bacteria to irreversibly attach to the surface and form a biofilm. Ruer et al. (2007) showed that without either Cup system biofilms are not as dense as in the presence of both CupB and CupC. However, the role of CupB pili in biofilm formation has been reassessed in this study. To avoid any side phenotype, which could result in the induction of additional adhesins by RocS1, the experiments used the parental strain and cupB mutants that carry the inducible pBAD promoter upstream of cupB1 on the chromosome.

First, the parental strain PAO1ΔΔ, in which all mutants were generated, was confirmed to have, due to the lack of type IV pili and flagella, a weaker biofilm phenotype than PAO1 (see Figure 3.11A and B). Cells were cultured in M63 medium supplemented with 0.4% arabinose, as used previous for the shearing assay. Biofilm assays were done in polystyrene microtiter plates, and the biofilms were visualised and measured for up to 24 hours by crystal violet staining. The control strain PAO1 is a strong biofilm former, clearly visible after 4 hours. On the other hand, the PAO1ΔΔ strain has a much slower biofilm kinetic, clearly visible only after 8 hours.
Figure 3.11: Biofilm formation of PAO1, PAO1ΔΔ (ΔΔ) and PAO1ΔΔ::pBAD (ΔΔ::pBAD). Bacteria were grown in 24-well plates in M63 minimal medium supplemented with 0.4% arabinose under static conditions at 30°C. After 4, 6, 8, and 24 hrs incubation, biofilms were stained with crystal violet (A) and resolved in ethanol to quantified biofilm formation by measuring the OD$_{600}$ (B). The wells filled with medium served as a negative control. CV is crystal violet. Shown is the average from three biological replicates. The assay was carried out once.

However, similar results were obtained for the cupB-induced PAO1ΔΔ::pBAD strain, which showed no difference in biofilm phenotype compared to PAO1ΔΔ.

Expanding the incubation time to 24 and 48 hours, biofilm formation was assessed further by the cupB-expressing PAO1ΔΔ::pBAD strain (see Figure 3.12). The conditions were similar to those of Kulasekara et al. (2005) who saw the influence of CupB and CupC1 pili on biofilm. The biofilm formation was much stronger after 48 h than after 24 h (see Figure 3.12A) for all strains, either the parental or the cupB mutant ones. A Western blot analysis confirmed that CupB1 and CupB6 were produced (see Figure 3.12B), but overall the absence or presence of CupB1 and CupB6 did not impact the biofilm formation significantly. As observed in the shearing assays (see Figure 3.4B), CupB5 is not secreted by the pBAD variants. Arguably, the lack of biofilm formation might be related to the absence of secreted CupB5. However, PAO1ΔΔ/pRocS1 and PAO1ΔΔΔcupB5/pRocS1 have the same biofilm phenotype (data not shown), excluding a role of CupB5 in biofilm formation under the conditions tested.

In conclusion, no impact of CupB1 pili or CupB6 adhesin on biofilm formation was identified under the conditions tested. Perhaps, expression of cupC genes, and possibly other genes under the control of RocS1, is required to influence significantly the biofilm formation by P. aeruginosa PAO1 on plastic surfaces.
3.2 DISCUSSION

Adhesive fibers, also called pili or fimbriae, are often assembled on the cell surface of Gram-negative bacteria by a conserved transport machinery, the CU pathway. These pili consist of many pilin subunits and can present an adhesin at their tips, essential for the specificity of attachment to diverse surfaces and tissues. The subunit assembly is catalysed by an outer membrane usher and the subunits are stabilised and targeted to the usher by their cognate periplasmic chaperone (Busch and Waksman, 2012). The *P. aeruginosa* strain PAO1 encodes four CU pathways in its genome. Little is known about their assembly mechanism and the function of the respective pilus (see Chapter 1.11). One of the CU clusters, *cupB*, is of special interest because it encodes two chaperones, whereas the classical CU clusters encode only one. More importantly, the *cupB* cluster encodes the TpsA-like protein CupB5 (Vallet *et al.*, 2001). TpsA proteins are usually transported to the cell surface by an outer membrane protein, TpsB (Jacob-Dubuisson *et al.*, 2013), which is not found in the *cupB* gene cluster. The CupB3 usher not only assembles the CupB pilus but has also been reported to secrete CupB5 (Ruer *et al.*, 2008). It is not understood how the usher can coordinate both—assembly of the CupB1 pilus

Figure 3.12: CupB1 pili and CupB6 adhesins do not promote biofilm formation. Bacteria were grown in microtiter plates in M63 minimal medium supplemented with 0.4% arabinose under static conditions at 30°C for 24 h and 48 h. The experiment was carried out once. Strains used were PAO1ΔΔ::pBAD (ΔΔ), PAO1ΔΔΔcupB1::pBAD (ΔB1), PAO1ΔΔΔcupB2::pBAD (ΔB2), PAO1ΔΔΔcupB3::pBAD (ΔB3), PAO1ΔΔΔcupB4::pBAD (ΔB4), PAO1ΔΔΔcupB5::pBAD (ΔB5) and PAO1ΔΔΔcupB6::pBAD (ΔB6) (A) Biofilm formation. Biofilms obtained after 24 h and 48 h incubation were stained with crystal violet, resolved in ethanol and quantified by measuring the OD₆₀₀. The averages from three biological replicates are shown. (B) Western Blot analysis. Planktonic bacteria, isolated from the microtiter plate wells, were centrifuged and cell pellets resuspended in SDS-loading buffer to obtain an OD₆₀₀ of 0.1. Samples were analysed by SDS-PAGE and Western Blot using antibodies against CupB5, CupB6, CupB1 and RNA-Polymerase.
and translocation of CupB5—and the primary aim of this study was to unravel the molecular details of this process.

### 3.2.1 CupB1 pilus assembly and CupB6 translocation

To study the assembly and translocation of CupB proteins on the cell surface by shearing assays and whole cell ELISA, cupB deletion mutants were constructed using the *P. aeruginosa* strain PAO1∆∆. As cupB genes are not expressed under laboratory conditions, cupB gene expression was induced by overexpression of the plasmid-encoded sensor kinase RocS1 or by placement of the arabinose-inducible $p_{BAD}$ promoter upstream of cupB1 (see Figure 3.1A and B). Both approaches gave similar results in terms of CupB1 and CupB6 translocation analysed by shearing assays (see Figure 3.4A and B). The only exception was small traces of CupB1 found in the cupB4 and cupB6 mutants when using the arabinose-inducible approach, whereas it was not detected when using the RocS1 approach. It was probably because no antibiotics were used in the arabinose-inducible approach, resulting in less stress, better growth, and healthier bacteria.

The CupB3 usher assembles the CupB pilus

Although CupB1 was readily produced and assembled in the parental strain and the cupB5 mutant (see Figure 3.4A and B), it was not detected in the cupB3 mutant lacking the usher. Deletion of the usher-encoding gene usually causes pilins to accumulate in the cells, as has been observed for P and Type 1 pili (Klemm and Christiansen, 1990; Norgren et al., 1987). That it is also so for CU pili in *P. aeruginosa* has been observed for CupC1 pili, whose subunits accumulate in the absence of the CupC3 usher (Ruer et al., 2007). So far, a single other CU exported protein is known to be degraded in the absence of the usher: the spore coat protein (MXAN3885), secreted by the MXAN3883 usher in the Gram-negative bacterium *Myxococcus xanthus* (Leng et al., 2011). The CU cluster also encodes a periplasmic chaperone (MXAN3884) and a second spore coat protein (MXAN3882). Similar to CU pili, the spore coat has some adhesive properties, as the lack of MXAN3885 results in loosely connected spores.

CupB1 was probably not detected in the cupB3 mutant because it was degraded. Likely, in the absence of the usher and therefore in the absence of assembly, not enough chaperones are
present to complement the subunits in the periplasm. Thus misfolded CupB1 subunits might accumulate in the periplasm and induce a strong Cpx stress response. The Cpx response results in the DegP-dependent degradation of the misfolded pilins in the periplasm (DiGiuseppe and Silhavy, 2003). Additionally, CupB1s Nte peptide might induce the Cpx response, as has been shown for the Nte peptide of the fibrillum subunit PapE in *E. coli* (Lee *et al.*, 2004).

Like CupB1, CupB6 was detected in the cell and sheared fraction of the parental strain and the *cupB5* mutant (see Figure 3.4A and B). CupB6 was not translocated by the *cupB3* mutant, although it accumulated in the cell fraction. In contrast to pilin domains, adhesin domains are still able to fold in the absence of (sufficient) chaperones. This has been observed for FimH, whose adhesin and pilin domains can autonomously fold in the absence of the FimC chaperone (Vetsch *et al.*, 2002). Nonetheless, the pilin domain is unstable in the absence of a chaperone, misfolds and causes FimH to aggregate. CupB6 might aggregate through a similar mechanism and because its lacking the Nte it might not induce the Cpx response as strongly as CupB1.

**CupB2 is likely the chaperone for CupB1**

Similar to the *cupB3* mutant, none or only traces of CupB1 were detected in the cell and sheared fractions of the *cupB2* and *cupB4* mutant. It indicates that CupB1 was degraded. It is well known that pilin subunits are subject to proteolytic degradation when produced in the absence of their cognate chaperone, as observed previously for the type 1 and P pili of uropathogenic *E. coli*. The absence of FimD or PapD chaperones causes periplasmic degradation of pilin subunits, because the chaperones are essential for maintaining the pilins proper fold (Jones *et al.*, 1997; Lindberg *et al.*, 1989).

Moreover, pull-down assays showed that the CupB2 and CupB4 chaperones have some level of specificity: soluble CupB1 was purified with an His-tagged CupB2, which could not be achieved using the CupB4 chaperone (see Figure 3.7C and B). However, it can not be confidently concluded that CupB1 does not interact with CupB4, as the CupB4 chaperone was not detected in the cell extracts of CupB4 overexpressing *E. coli* strains (see Figure 3.4 and below). Interestingly, the CupB1 antibody recognised two prominent protein bands, one at 21 kDa and one at 17 kDa, and some degradation products below 17 kDa in the CupB1 overexpressing strains (see
Figure 3.7A, B and C). CupB1 has a predicted size of 20 kDa, thus the 21 kDa protein might represent full-length CupB1 and the 17 kDa protein, a degradation product. Mass spectrometry could confirm the identity of the two protein bands. The hypothesis that CupB1 and CupB4 do not interact with each other has also been suggested by Cai et al. (2011) who showed that the binding affinity of CupB4 towards the C terminus of CupB1 (CupB1\textsubscript{175189}: AGTGLSRIRYL-LAYE) is low. They also showed, by limited trypsin digest and Strep-tactin pull down assays, that the CupB2 chaperone interacts with the CupB1 pilin. The results suggest that CupB2 is the preferred chaperone for CupB1, and the absence of CupB2 results in CupB1 degradation. As in the *cupB3* mutant, CupB1 was also degraded in the *cupB4* and *cupB6* deletion mutants. Assuming that CupB4 and CupB6 are required for the activation of the usher (see below), the CupB1 pilin is not exported in these mutants. As described above, accumulating CupB1 could induce the Cpx stress response and is consequently degraded.

**CupB4 is likely the chaperone for CupB6**

The results of the shearing assay indicate a key role of CupB4 towards CupB6, as CupB6 was degraded in the *cupB4* mutant but not in the *cupB2* mutant. However, CupB6 was not detected on the cell surface of either mutant. Probably, CupB6 cannot be efficiently sheared off the surface in the absence of the CupB2 chaperone, since CupB1 pilins are not present to further expose the CupB6 adhesin on their tip. Surprisingly, CupB6 was degraded in the *cupB1* deletion mutant. This result is puzzling, as CupB6 could be expected also to be degraded in all other mutants where CupB1 has been degraded. A polar effect of the *cupB1* deletion on CupB6 production can be excluded, as *cupB5*—the one before last gene in the cluster—is still expressed.

Moreover, the results of the pull-down assay suggest an interaction between CupB6 and CupB4, as CupB6 was purified together with CupB4-His (see Figure 3.4E). Although CupB4-His could not be detected in this assay, no binding of untagged CupB6 is observed in the absence of CupB4, indicating that CupB6 was bound specifically by CupB4 and not nonspecifically by the column (see Figure 3.4D). It suggests that the His-tag was not accessible for recognition by the His-tag antibody or CupB4-His was produced in very low amounts. The translational start codon of *cupB4* is assigned as GTG on the *Pseudomonas* website (see Figure 3.6). The
Kolaskar value, calculated from 18 bp up- and downstream of the GTG, is only 21, indicating a weak translational start (Kolaskar and Reddy, 1985). A potential ATG start codon is present 39 bp upstream of the annotated GTG start, however, the Kolaskar value of this ATG is also only 20. Probably the CupB4 chaperone has a weak Shino-Dalgaro sequence, as it is not required in high levels if it only interacts with CupB6. In general, the translation efficiency of the GTG start codon is much lower than that of ATG start codons and could explain the low levels of CupB4 production (Reddy et al., 1985; Romero and García, 1991). The observed interaction between CupB6 and CupB4 is in contrast to observations of Cai et al. (2011). They excluded an interaction between CupB4 and CupB6 and speculated that CupB5 is required for this interaction, as cupB5 is encoded between cupB4 and cupB6 in the cupB gene cluster.

In conclusion, CupB4 and CupB6 may interact with each other. It follows from the pull-down experiment, but also because in the absence of CupB4 or CupB6, the CupB1 pilin is not transported to the cell surface. Adhesins, such as CupB6, are known to activate the usher for pilus assembly. Thus, deletion of the adhesin or its cognate chaperone prevents usher activation (Busch and Waksman, 2012), which was observed for the cupB6 and cupB4 deletion mutants.

The role of two chaperones

The preliminary data, as well as the bioinformatic analysis of CupB2s and CupB4s G1 strand (see Figure 3.8) suggests that each chaperone has its cognate substrate. Only 50% of residues in the G1 strand are identical between CupB2 and CupB4. Moreover, the P1 and P2 residues of CupB2 are two phenylalanins, whereas the P1 residue is a tyrosine and the P2 residue a valine in CupB4. These differences in G1 strand composition might make the donor strand complementation between cognate partners more efficient and faster than between non-cognate partners. The subunit-loaded CupB2 and CupB4 chaperones might also have a different affinity for binding to the NTD of the CupB3 usher.

For the type I it is known that the subunit ordering depends on the affinity of the chaperone:subunit complexes for the usher, as well as on the DSE rate at the usher. However, the affinity of the chaperone:subunit complexes for the usher does not correspond to the actual order of subunits in the assembled pilus (Lillington and Waksman, 2013; Nishiyama and Glockshuber,
For example, FimC:FimF has a higher affinity for the usher than FimC:FimG, although FimG is being incorporated into the pilus prior to FimF. Thus, to ensure the correct assembly the DSE rate of the complexes corresponds to the order of subunits in the pilus. Nonetheless, the wrong subunits can be incorporated accidentally. Thus the presence of two chaperones might allow a faster and more efficient way of pilus assembly by preventing the off-pathway going of the subunits.

So far it has only been shown that the chaperones of CU pathways with more than one chaperone have some level of substrate specificity. The assembly of CS5 pili, for example, involves an usher (CsfC), two chaperones (CsfB and CsfF), a major subunit (CsfA), minor pilin (CsfD), and a terminator (CsfE) (Duthy et al., 2001, 2002). The minor pilin CsfD is required for the initiation of the pilus assembly and the CsfE terminator modulates the pilus length. In contrast to the CupB system, CsfA and CsfD pilins accumulate intracellularly in the usher mutant. CsfA is degraded in the absence of the CsfB chaperone, indicating the role of CsfB in CsfA targeting. Moreover, the CsfF chaperone stabilises and targets the minor pilins, CsfD and CsfE. A similar substrate specificity has been shown for the CU chaperones required for the assembly of 987P pili: the FasC chaperone binds the FasG adhesin, and the FasB chaperone interacts with the FasA major subunit (Edwards et al., 1996). Moreover, the assembly of the E. coli common pili (ECP, also known as Mat fimbriae, Garnett et al. (2012); Pouttu et al. (2001)) also requires two chaperones. This CU pathway is encoded by the ecpRABCDE operon, where ecpA encodes the major pilin, ecpB and ecpE the chaperones, ecpC the usher, ecpD the adhesin and ecpR the response regulator (Garnett et al., 2012). It is yet unknown if the chaperones have cognate or interchangeable substrates.

Model of CupB1 pilus biogenesis

Taken together, the results suggest a model of CupB pilus assembly in P. aeruginosa (see Figure 3.13). In this model, CupB1 pilin subunits are stabilised by the CupB2 chaperone, whereas the CupB6 adhesin interacts with the CupB4 chaperone. The chaperones accelerate the folding of the subunits and target them to the CupB3 usher. Supposedly, a CupB4:CupB6 complex arrives first to activate and open the usher closed by a plug. Subsequently, CupB2:CupB1 com-
plexes arrive at the usher and are assembled into a pilus. The growing pilus pushes the putative CupB6 adhesin (sitting on the tip of the pilus) away from the cell surface.

![Model of CupB1 pilus assembly](image)

Figure 3.13: **Model of CupB1 pilus assembly.** The CupB3 usher forms the pilus assembly platform in the outer membrane (OM). CupB3 is predicted to have an N-terminal domain (N), two C-terminal domains (for simplicity, only one C-terminal domain (C) is shown), a plug domain (not shown) and a pore domain. The CupB4 chaperone targets the CupB6 adhesin to the N terminus of the usher and the usher becomes activated for assembly. The CupB4:CupB6 complex is transferred to the ushers C terminus and makes space for a newly arriving CupB2:CupB1 (chaperone:pilin) complex. CupB1 and CupB6 undergo donor strand exchange and the CupB2:CupB1 complex is transferred to the ushers C terminus. The arrival of up to 1000 CupB1 subunits at the ushers N terminus and their subsequent polymerisation results into the assembly of a CupB1 pilus, which has the CupB6 adhesin on its tip. The emerging pilus supposedly folds into a right-handed and super-helical pilus. P is periplasm and E extracellular space.

### 3.2.2 CupB3-independent secretion of CupB5

In contrast to the classical CU pathways, the cupB gene cluster encodes the orphan TpsA-like protein CupB5. However, the cluster does not encode a TpsB transporter required for the secretion of TpsA proteins. Instead Ruer et al. (2008) reported that CupB3 is an unusual usher with an N-terminal POTRA-like domain.

The results of the present study contradict and conflict with the results of Ruer et al. (2008). Western Blot analysis of sheared fractions showed that CupB5 is efficiently secreted in a cupB3 deletion mutant as well as in any other cupB mutants (see Figure 3.3 and Figure 3.4A). In
contrast, Ruer et al. (2008) reported that CupB5 secretion is not only abolished in a cupB3 deletion mutant but also in a cupB4 mutant. They speculated that the reason was the inactivity of the usher that has to be activated by a chaperone:adhesin complex (CupB4:CupB6). Here, an alternative strategy was used to specifically and exclusively induce the cupB genes upon arabinose induction. The results clearly showed that the CU pathway is required for CupB1 and CupB6 transport but is insufficient to support CupB5 secretion (see Figure 3.4B). The $p_{BAD}$ promoter induces expression of the cupB genes only, whereas the sensor kinase RocS1 (used by Ruer et al. (2008)) activates or represses expression of several targets, such as the cupC genes or the genes encoding the multidrug efflux pump MexAB-Opm, respectively (Sivaneson et al., 2011). It is thus reasonable to suggest that RocS1 induces the expression of an additional transporter required for CupB5 translocation.

Ruer et al. (2008) further reported that truncations of CupB3s POTRA-like domain abolished CupB1 translocation. Yet, CupB5 is found on the cell surface, a counterintuitive result, and it was proposed that CupB5 could be jammed in the usher and thereby prevents the transport of CupB1. It was supported by the deletion of cupB5 in this same background, which unlocked the CupB3 usher and restored the CupB1 transport. Thus Ruer et al. (2008) proposed that CupB5 enters the usher pore prior to CupB1 and is then pushed out of the cell by the growing CupB1 pilus. However, this scenario is now difficult to imagine bearing in mind the complex assembly mechanism of CU pili. Here, pilin subunits are connected via their N-terminal extension with a neighbouring subunit. The N-terminal extension is pushed into a hydrophobic groove on the surface of the neighbouring subunit, thus stabilising its fold and driving the assembly of the pilus (Sauer et al., 2004). CupB5 is not predicted to have such an N-terminal extension or hydrophobic groove and cannot, therefore, be a part of the pilus. Moreover, the usher pore might be not large enough to allow passage of CupB1 and CupB5 at the same time. The activated FimD usher, for example, forms a circular pore 32 Å in diameter, which is filled entirely by the FimH lectin (Phan et al., 2011). The structural arrangements might be similar for CupB3 and CupB6 or CupB1 and, therefore, do not favour a simultaneous translocation of CupB5.

In conclusion, in the conditions tested in our laboratory, CupB3 only assembles the CupB1 pilus and translocates CupB6 at the cell surface. Although cupB5 is encoded in the cupB gene cluster, it is not required for the proper function of the CupB machinery and does not require CupB3
for its secretion. It is not clear why the present study observed different results in respect to CupB5 secretion than the study published by (Ruer et al., 2008). The experiments were done in different laboratories and, thus, possibly in slightly different conditions, for example different media and supplements. Instead the present study indicates that CupB5 is secreted via an alternative transporter, whose expression is regulated by the sensor kinase RocS1. This transporter is a TpsB outer membrane protein, encoded elsewhere on the chromosome, as will be described in the next Chapter.
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4. CupB5 secretion via the Tps4 three-partner secretion system

4.1 Results

The previous Chapter concluded that CupB3 might not be the only transporter for CupB5, if at all. Because CupB5 is a TpsA-like protein, one of P. aeruginosa’s TpsB transporters may be involved in the secretion of CupB5. CupB5 is a TpsA-like protein because it has an N-terminal TPS motif, required for the interaction between TpsA substrate and TpsB transporter in the TPS system. An amino acid alignment of the TPS domains of CupB5, FHA of B. pertussis and HMW1 of H. influenzae shows that CupB5 has the TPS domain characteristic for HMW1 family proteins: the conserved NPNG box (see Figure 1.28). Moreover, a structure prediction of CupB5, using the I-Tasser server, shows that CupB5 probably folds in a β-helix augmented by an N-terminal globular domain (c-score -0.65, low confidence). This structure is similar to those of the HAP adhesin of H. influenzae (see Figure 4.1) and the Hbp heme binding hemoglobin protease of E. coli (PDB: 1WXR). Although both proteins, HAP and Hbp, are secreted by a classical autotransporter, the secreted translocator domain folds into a right-handed parallel β-helix as observed for TpsA proteins (compare HMW1, Figure 4.1). Additionally, HAP and Hbp have an N-terminal globular serine protease domain (Meng et al., 2011; Otto et al., 2005).

4.1.1 TpsB4 is the transporter of CupB5

To meet each other in the cell, secreted proteins and cognate transporters/secretion systems should have their production coordinated and if their expression is not constitutive, the corresponding genes have to be co-regulated. As the cupB genes are under the control of the Roc1
Figure 4.1: *Comparison of CupB5s predicted structure with the solved structures of HAP and HMW1 of *H. influenzae*. CupB5s structure was modeled on the HAP and Hbp structures using the I-Tasser server. The code given after HAP and HMW1 corresponds to their PDB code. Proteins are rainbow colored from N to C terminus.

and Roc2 two-component systems (Kulasekara et al., 2005; Sivaneson et al., 2011), a study was done of whether any of *P. aeruginosa* tpsA/tpsB operons (see Figure 1.27) are also controlled in a Roc-dependent manner. qRT-PCR was used to analyse the levels of *cupB* and *tpsA1-tpsA5* expression when the sensor kinase RocS1 was overproduced. The levels were normalised by comparison with the house keeping gene *rpoD*. As Figure 4.2A shows, the expression of *cupB1* is upregulated by 123-fold, confirming the RocS1-dependent regulation of the *cupB* operon. Strikingly, the expression of only one of the five *tpsA* genes, *tpsA4*, was increased significantly by 94-fold. TpsA4, also known as LepA, functions as an exo-protease presumably secreted into the extracellular environment by the cognate TpsB4 transporter, also known as LepB (Kida et al., 2008, 2011). Expression of neither other *tpsA* genes nor of the *cupA1* negative control was up-regulated by RocS1, suggesting that TpsB4 is a good candidate for a CupB5 transporter.

The initial recognition between TpsA and TpsB has been proposed to be mediated by electrostatic potentials occurring between surface residues of the TpsBs POTRA domain and the TpsAs TPS motif (Delattre et al., 2011). This would be followed by a more intimate interaction by β-augmentation between the β-sheets of the POTRA domain and the exposed β-strands of the TPS domain. If TpsB4 recognises CupB5 and TpsA4, both proteins should have a high sim-
4.1. RESULTS

Figure 4.2: RocS1 upregulates \textit{cupB5} and \textit{tpsA4} expression and both proteins share a high sequence homology in their TPS motifs. (A) Fold induction of \textit{cupB1}, \textit{cupA1}, \textit{tpsA1}, \textit{tpsA2}, \textit{tpsA3}, \textit{tpsA4} and \textit{tpsA5} mRNA transcription in PAO1 overexpressing RocS1, from pRocS1, obtained by qRT-PCR. The negative control, cells expressing the empty vector pMMB 67EH-GW, is unity for each tested mRNA. The fold induction was normalised to the house keeping gene \textit{rpoD} which encodes the sigma factor sigma 70. The results are an average of three biological and three technical replicates. The experiment was repeated three times. (B) Amino acid alignment of conserved residues of TpsA4 and CupB5. For CupB5 amino acids 61-108 and for TpsA4 amino acids 60-107 are shown. The labels refer to the mature protein without signal peptide. These amino acids are a part of the N-terminal TPS motif and contain the conserved NPNG box (orange) required for interaction with the outer membrane transporter TpsB4 or the secretion of the TpsA. The alignment was created by ClustalOmega.

Similarity in their primary sequence and, particularly, within the TPS domain. Figure 4.2B shows an amino acid sequence alignment between the N-terminal TPS motifs of CupB5 and TpsA4, which reveals a nearly perfect match. Except for four amino acids, the regions encompassing residues S$_{69}$-D$_{108}$ in CupB5 and residues S$_{68}$-D$_{107}$ in TpsA4 are identical, supporting the idea that TpsB4 might translocate TpsA4 and CupB5.

To confirm that TpsB4 could be involved in CupB5 secretion, the \textit{tpsB4} gene was deleted in the parental strain PAO1\Delta\Delta which was conjugated with pRocS1. Bacteria were grown in liquid static cultures and fractions were collected using the shearing assay, as described in Chapter 3. The protein samples were then analysed by SDS-PAGE and Western Blot. As Figure 4.3 shows, CupB5 is not secreted in the absence of the TpsB4 transporter. Moreover, CupB5 is degraded in the cell fractions of the \textit{tpsB4} mutant. It is not surprising, since sometimes TpsA proteins are not visible and likely degraded when they accumulate within the cells. It was, for example, observed for the HMW1/HMW1B system of \textit{H. influenzae}, where HMW1 was degraded in the
absence of the TpsB transporter, HMW1B (St Geme and Grass, 1998). The degradation of HMW1 was due to the periplasmic protease DegP. However, the CupB5 stability and secretion could be readily restored upon complementation by inserting the wild type tpsB4 gene at the genomic att side in the tpsB4 mutant (see Figure 4.3). In conclusion, TpsB4 could act as transporter for CupB5 and bring it across the outer membrane of *P. aeruginosa*.

Figure 4.3: CupB5 is not secreted in the absence of TpsB4. Immunoblot analysis of cell and sheared fractions obtained from PAO1ΔΔ (ΔΔ), PAO1ΔΔΔ∗cupB5 (Δ∗cupB5), PAO1ΔΔΔ∗tpsB4 (Δ∗tpsB4) and complemented PAO1ΔΔΔ∗tpsB4 (Δ∗tpsB4//miniCTX-tpsB4) using antibodies derived against CupB5, RNA-Polymerase and DsbA. Strains, all conjugated with the RocS1 expressing vector pMMBrocS1, were grown under static conditions for 24 h at 30°C in the presence of IPTG.

Probing CupB5/TpsA4 interaction with TpsB4 or CupB3 by bacterial two-hybrid assays

To further support the data showing the TpsB4-dependent secretion, and possibly CupB3-independent secretion, of CupB5, a study was done of whether CupB5 could physically interact with either one of these proteins. A commonly used approach is the bacterial two-hybrid assay (Karimova *et al.*, 1998). In this assay, the proteins of interest are fused to the two complementary fragments, T18 and T25, of the catalytic domain of the adenylate cyclase of *B. pertussis* (see Figure 4.4A-C). The adenylate cyclase is not catalytically active if both fragments are physically separated. However, if the proteins interact, the catalytic domains are brought in contact, thus reconstituting an active enzyme, which produces cAMP. cAMP binds the catabolite activator protein, in turn activating the transcription of reporter genes such as the β-galactosidase gene lacZ or the maltose operon. Utilisation of maltose, for example, can be visualised on McConkey agar plates supplemented with 1% maltose.
The cupB5, cupB3, tpsA4 and tpsB4 genes were cloned without their N-terminal signal peptide to prevent export of the gene products into the periplasm (see Figure 4.4D). TpsB4-P1 (G42–G121) encompassed POTRA-1 domain. CupB5-L (L1–Y121) and TpsA4-L (L1–Y118) both contained their N-terminal TPS-motif, and TpsA4-L was used as a positive control for the TpsA4/TpsB4 interaction. As for the cupB3 constructs, one encoded the POTRA-like domain (CupB3-P, G1–V84) and the other encoded both the N terminus and POTRA-like domain (CupB3-PN, G1–A229). All constructs were N-terminally fused to the T18 and T25 domains and plasmids were transformed into E. coli. Cells were spotted on McConkey agar plates supplemented with 1% maltose, incubated for up to 5 days and the development of red color, indicating a pH change due to the utilisation of maltose, was monitored. As Figure 4.4E shows, the positive control (3zip + 3zip), encoding the leucine zipper derived from a yeast transcriptional activator (Karimova et al., 1998), is intense red, which indicates a strong interaction, whereas the clones carrying the empty vector (EV) had no color, confirming that the assay was functional (see Figure 4.4E, first row). However, no interaction was observed in any of the combinations used (see Figure 4.4E, second row). To conclude that CupB5-L does not interact with TpsB4-P1, CupB3-PN or CupB3-P is, however, premature since no interaction was seen between TpsB4-P1 and the cognate passenger TpsA4-L (which served as a positive control).

Because recent findings (see below) have shown that TpsB4 has two POTRA domains, the interaction between with the sole POTRA-1 domain and TpsA4s TPS motif could be too weak to be detected by bacterial two-hybrid assays. Indeed, kinetic analysis carried out by Dr J. Garnett (Imperial College London) showed that the equilibrium constant for the interaction between TpsB4-P1 and TpsA479–95 is $K_d = 1.49 \text{ mM} \pm 0.18 \text{ mM}$, indicating a weak interaction. Thus, the second POTRA-2 domain might be required for a stronger interaction. Another possibility is that CupB5-L and TpsA4-L are quickly folded in the cytoplasm of E. coli, thus preventing a stable and functional interaction with TpsB4. Indeed, only the unfolded TPS motif is recognised by the POTRA domains of the TpsB transporter (Hodak et al., 2006). In conclusion, the bacterial-two hybrid assay gave inconclusive data showing that CupB5 does not interact with either TpsB4 or CupB3.
Figure 4.4: Bacterial two-hybrid assays were inconclusive. (A-C) Schematic of the bacterial two-hybrid (BTH) assay. The T18 and T25 fragments of the catalytic domain of *B. pertussis* adenylate cyclase are active when close to each other and produce cAMP (A). If both fragments are physically separated, they are inactive, and no cAMP is produced (B). The proteins of interest (CupB5 and TpsB4) are fused to the C terminus of the T18 and T25 fragments and only when both proteins interact, the T18 and T25 fragments reconstitute to an active adenylate cyclase and produce cAMP (C). (D) Comparison of full-length TpsB4, CupB3, CupB5 and TpsA4 proteins with the constructs used in the BTH assay. Constructs were N-terminally fused to the T18 and T25 fragments of the adenylate cyclase. The numbers are shown according to the mature proteins. (E) BTH assay on McConkey agar plates. *E. coli* DHM1, which are adenylate cyclase deficient, were simultaneously transformed with the indicated constructs: left fused to T25, right fused to T18 (T25+T18). Overnight cultures were spotted on McConkey agar plates, supplemented with 1% maltose and IPTG, and incubated at 30°C for 48 h and at room temperature for further 48 h. The first row, except for the positive control 3zip+3zip, shows the negative controls. The second row shows the proteins of interest. The experiment was carried out twice.
Setting up the TpsB4/CupB5 secretion system in *E. coli*

TpsB transporters can secrete TpsA proteins in an heterologous situation, such as when produced in *E. coli*. It has been shown, for example, with the *B. pertussis* FHA/FhaC pair (Delattre *et al.*, 2011), the *H. influenzae* HMW1/HMW1B pair (St Geme and Grass, 1998), and with the enterotoxigenic *E. coli* EtpA/EtpB pair (Fleckenstein and Roy, 2009). Delattre *et al.* (2011) co-produced a truncated FHA mutant, Fha44, with different FhaC variants in *E. coli* and observed relative secretion of Fha44. Fleckenstein and Roy (2009) could even purify the secreted full-length EtpA from the supernatant of *E. coli*. EtpA is an adhesin, required for the adherence to intestinal epithelial cells and the colonisation of the intestine in mice (Fleckenstein *et al.*, 2006; Roy *et al.*, 2008). It is encoded in the *etpBAC* operon. EtpB is the transporter, whereas EtpC is the glycosyltransferase that glycosylates EtpA. Fleckenstein and Roy (2009) engineered an arabinose-inducible plasmid encoding the *etpBAC* operon. All three genes could be expressed in the laboratory *E. coli* strain Top10 and successful secretion of EtpA was observed.

Thus, I studied whether TpsB4 can also secrete CupB5 when it is produced simultaneously with TpsB4 in *E. coli*. Full-length *cupB5* and *tpsB4* were cloned in tandem into the expression vector pASK-IBA3c. The *cupB5* gene was cloned upstream of *tpsB4*, and both genes were separated by a sequence corresponding to an appropriate ribosome binding site. The *tpsB4* was engineered to encode a C-terminally tagged version of the protein carrying a small Strep-tag (1 kDa) (see Figure 4.5). Expression of both proteins was induced with anhydrotetracycline. As Figure 4.6A shows, both CupB5 and TpsB4-Strep were produced upon induction, although the levels of CupB5 were much higher. CupB5 was also significantly degraded, as observed by Western Blot (Figure 4.6A, bottom), probably because of the massive overproduction.

To be functional, TpsB4-Strep has to be inserted into the outer membrane (OM). Therefore, a fractionation assay was done to study the sub-cellular localisation of the protein (see Figure 4.6B). TpsB4-Strep was expressed upon induction and upon differential solubilisation of the membrane fractions, could be seen in the OM, although a significant amount was still co-fractionating with the inner membrane (IM). A large amount of TpsB4-Strep could be associated with the IM, because overexpressed TpsB4-Strep might still be exported into the periplasm by the Sec-YEG translocon. Alternatively, it could be a limitation of the fractionation pro-
Figure 4.5: **Schematic of TpsB4-Strep.** Amino acid sequence of full-length TpsB4 with a C-terminal Strep-tag. The codon optimised TpsB4 (TpsB4 opt) is connected to the Strep-tag via a short linker. The terminal phenylalanine is highlighted with a green star.

procedure. The cytoplasmic RNA-polymerase was also found partly associated with the inner membrane. It might indicate that the IM fraction still contained soluble protein. However, no RNA-polymerase was found associated with the OM thus suggesting that the amount of TpsB4 found in this fraction showed correct targeting of the protein. The full-length CupB5 and its degradation products were detected both in the IM and OM fractions. Usually, TpsA proteins progress unfolded through the periplasm and are folded only at the cell surface during secretion (Hodak et al., 2006; Jacob-Dubuisson et al., 2013). During overproduction, more CupB5 might be present than active TpsB4 transporters. Thus, CupB5 prevails in the periplasm and might start to misfold and aggregate. Misfolded CupB5 seems to be proteolytically degraded, likely by periplasmic proteases, such as DegP (Baneyx and Mujacic, 2004).

A separate secretion assay monitored the presence of CupB5 in the extracellular milieu. CupB5 was expressed in tandem with TpsB4 or, as a control, alone from pASK-IBA3c-CupB5 (see Figure 4.6C). A small amount of CupB5 was released into the supernatant, but even in the absence of the TpsB4 transporter. Moreover, the presence of high levels of RNA-polymerase in the supernatant suggests that CupB5 is released unspecifically upon partial cellular lysis and not in a TpsB4-dependent manner.

A possible reason why CupB5 was not secreted by TpsB4 in this heterologous context is that TpsB4-Strep was not inserted properly into the OM. OMP have at their C terminus a signature
motif consisting of mainly hydrophobic residues (HR): C term- Phe- X2-Tyr/HR-X4-HR-X6- HR-X8-HR (Robert et al., 2006). This motif is recognised by the Bam complex that inserts OMP into the OM (see Chapter 1.6.1). TpsB4 also has this signature motif with a C-terminal phenylalanine. The addition of the Strep-tag, although only consisting of the eight amino acids Trp-Ser-His-Pro-Gln-Phe-Glu-Lys, could have disturbed the recognition, and thus prevented correct TpsB4 assembly by the Bam complex. The deletion or substitution of the Phe residue can decrease dramatically the efficiency of membrane insertion, as has been observed for the OM protein PhoE in the *E. coli* strain K-12 (de Cock et al., 1997; Struyve et al., 1991). Delattre et al. (2011) and Fleckenstein and Roy (2009) did not engineer a tagged FhaC or EtpB, probably allowing the proper insertion of the transporter in the OM. Thus, future experiments should aim to create an untagged TpsB4 and to produce antibodies against TpsB4, which will allow to observe its localisation.

The genetic organisation of *cupB5* and *tpsB4* might also be important. On the *P. aeruginosa* genome, *tpsB4* is the first gene in the *tpsB4*/tpsA4* operon and *cupB5* is the next-to-last gene in the *cupB* operon. However, on the pASK-IBA3c-CupB5-RBS-TpsB4 vector, *cupB5* is transcribed first. Expression of, first, *tpsB4* and, then, *cupB5* might ensure that some of the TpsB4 transporters are assembled in the OM before CupB5 is produced. So, CupB5 can be readily recognised by the transporter, and aggregation, misfolding, and degradation could be restricted.

### 4.1.2 Solving the structure of TpsB4s POTRA-1 domain

To date the only available 3D structure for a TpsB transporter is that of FhaC of *B. pertussis*. The structure shows a 16-stranded β-barrel, which has two POTRA domains, POTRA-1 and POTRA-2, at its N terminus and is closed by an α-helical plug (see Figure 1.23A) (Clantin et al., 2007). In collaboration with Prof. Steve Mathews and Dr. James Garnett (Imperial College London), the structure of the TpsB4 POTRA domains was solved based on experimental and modeling data (Garnett et al., submitted). The structure of TpsB4 was modeled by using the I-Tasser server (Roy et al., 2010): TpsB4 has a high structural homology with FhaC and is predicted to have two N-terminal POTRA domains and a C-terminal 16-stranded β-barrel (see Figure 4.7A). The barrel is closed by an α-helical plug (in the absence of the substrate) and the
Figure 4.6: TpsB4 does not secrete CupB5 in E. coli strain DH5a omnimax. TpsB4-Strep (62 kDa) and CupB5 (100 kDa) were expressed from pASK-IBA3c-CupB5-RBS-TpsB4. Bacteria were grown shaking in TSB at 37°C, induced with 200 µg/L (A,B) or 100/200 µg/L (C) anhydrotetracycline [aTc] and further grown for 5 h at 37°C (A,C) or for 16 h at 18°C (B). Fractions were analysed by Western Blot using CupB5, Strep-tag, and RNA polymerase antibodies. Star = unspecific band. (A) CupB5 and TpsB4-Strep are expressed in E. coli. Pre- and postinduction cell fractions were harvested by centrifugation and analysed by Coomassie-staining of SDS-PAGE gels (top) and Western Blot (bottom). M = marker, EV = empty vector control. (B) TpsB4-Strep is localised in the inner membrane (IM) and outer membrane (OM) of DH5a omnimax. IM and OM samples were obtained by fractionation. Pre = preinduction, post = postinduction, SN = supernatant, sol = soluble fraction. (C) CupB5 is released into the supernatant (SN) through cellular lysis. Cell and supernatant fractions were isolated via a secretion assay. Untagged CupB5 was expressed from pASK-IBA3c-CupB5. Samples shown for [aTc] = 0 were taken from cells expressing TpsB4-Strep and CupB5.

Based on the I-Tasser prediction, Dr J. Garnett engineered several His-tagged TpsB4 variants: TpsB4-NT (amino acids (aa) 1-194) encompasses the N terminus; TpsB4-P12 (aa 42-194) both POTRA domains; TpsB4-α1P1 (aa 1-121) the plug, linker and POTRA-1; TpsB4-P1 (aa 42-121) POTRA-1 and TpsB4-P2 (aa 122-194) POTRA-2 (Garnett et al., submitted). However, NMR spectroscopy showed that only TpsB4-α1P1 and TpsB4-P1 were properly folded. Both
Figure 4.7: **Model of full length TpsB4 and structure of POTRA-1 domain.** Raw data by Dr J. Garnett, figures by D. Muhl. (A) Composite of the TpsB4 model generated by the I-Tasser server (Roy et al., 2010) and the solved structure of POTRA-1. TpsB4 has a high tertiary homology with *B. pertussis* FhaC transporter. TpsB4 is predicted to form a 16-stranded \(\beta\)-barrel pore, closed by an \(\alpha\) helical plug. The periplasmic N terminus of TpsB4 has two POTRA domains. POTRA-1 is connected to the plug by a linker and POTRA-2 is connected to the barrel. (B) Comparison of the NMR solution structure of the TpsB4 POTRA-1 from *P. aeruginosa* with the POTRA-1 of *B. pertussis* FhaC (PDB: 2QDZ) and POTRA-4 of *E. coli* BamA (PDB: 3OG5). TpsB4s POTRA-1 encompasses residues G42–G121 and has the typical POTRA domain structure \(\beta\)-\(\alpha\)-\(\alpha\)-\(\beta\)-\(\beta\), also observed in POTRA-4 of BamA. The POTRA-1 of TpsB4 has two additional antiparallel \(\beta\)-sheets, \(\beta\)1 and \(\beta\)3, usually not observed in POTRA domains. FhaC also does not have the typical POTRA fold, as it is missing the second \(\alpha\) helix.

TpsB4 variants showed a well-defined POTRA-1 structure. The only exception was two flexible regions: a loop between strands \(\beta\)4-\(\beta\)5 and a N-terminal region prior to the \(\beta\)1-strand (see Figure 4.7B, left). POTRA-1 displays the usual core \(\beta\)-\(\alpha\)-\(\alpha\)-\(\beta\)-\(\beta\) structural motif of POTRA domains. Moreover, POTRA-1 has an anti-parallel \(\beta\)1-\(\beta\)3-sheet at the base of its N terminus. This sheet is usually not found in POTRA domains (compare FhaC and BamA POTRAs in...
In the absence of a substrate, the TpsB transporter is closed by a plug. For FhaC, it is closed by the H1 helix and L6 loop, and the H1 helix is connected to POTRA-1 via a linker (see Figure 1.23A). For TpsB4, NMR experiments indicated that the α3-helix and the β4-β5 loop of POTRA-1 might interact with the N-terminal linker, which would be displaced during substrate recognition.

**TpsB4 POTRA domains recognise two adjacent TPS motifs**

The recognition between TpsB and TpsA in the two-partner secretion system involves the POTRA domains of the TpsB transporter and the N-terminal TPS motif of the TpsA substrate (Clantin *et al.*, 2004; Schönherr *et al.*, 1993). As both TpsA4 and CupB5 are considered to be a TpsB4 substrate, they have an almost identical TPS domain, shown by amino acid alignments (see Figure 4.2B). Thus, we investigated next if the TPS domain of either protein directly interacts with TpsB4. To identify the amino acids involved in an interaction with TpsB4, Dr J. Garnett engineered a TpsA4 peptide library by trypsin digest of a TpsA4₁–242 protein. The library was separated by gel filtration chromatography, and the peptides were used for NMR spectroscopy. Although generated from TpsA4, the engineered peptides can represent both CupB5 and TpsA4, because of the high homology between their TPS domains. Analysis of the interacting peptides by MALDI-MS and further NMR experiments using synthesised peptides narrowed down the TPS binding interface to residues TpsA4₇₉–₉₅. Figure 4.8A shows the chemical shift perturbations observed by NMR when TpsB4-P1 was incubated in the absence and in the presence of TpsA₄⁷₉–₉₅. Moreover, a CupB5 peptide (residues 80-96) was also shown to interact with POTRA-1 (see Figure 4.8B). Figures 4.8C and D show examples of chemical shift perturbations for TpsB4 residues L₇₆, Q₉₃ and Q₁₁₀ and their localisation in POTRA-1, respectively. Q₉₃ is located at the C terminus of α3-helix, Q₁₁₀ in the β₄-β₅ loop and L₇₆ at the C terminus of the β₃ strand. The chemical shift perturbations indicate that POTRA-1 and TpsA4/CupB5 TPS domain (residues 79-95/80-96) interact directly with each other (Garnett *et al.*, submitted).

TpsB4 has a second POTRA domain, POTRA-2. This domain might also be important for the
Figure 4.8: NMR analysis of TpsB4 POTRA-1 domain and TpsA4/CupB5 interactions. Figures A–C were obtained by Dr J. Garnett. (A) TpsA4$^{79-95}$ interacts with TpsB4-P1. Comparison of $^1$H-$^15$N HSQC spectra of $^15$N-labeled TpsB4-P1 incubated with either 0 (black) or with 20 (red) molar equivalents of TpsA4$^{79-95}$. (B) CupB5$^{80-96}$ interacts with TpsB4-P1. Comparison of $^1$H-$^15$N NMR spectra for $^15$N-labeled TpsB4-P1 incubated with either 0 (black) or 2 (purple) molar equivalents of CupB5$^{80-96}$ peptide. (C) Examples of chemical shift perturbations of TpsB4 residues Q$^{93}$, L$^{76}$ and Q$^{110}$. The shifts for CupB5 and TpsA4 are overlayed. TpsA4 shifts are shown in red and CupB5 shifts in pink. (D) Composite TpsA4$^{79-95}$ and CupB5$^{80-96}$ interactions mapped onto POTRA-1 of TpsB4 using PyMol.

secretion of TpsA4/CupB5, as has been shown for the POTRA-2 of FhaC from B. pertussis (Delattre et al., 2011). Thus, a second TPS-POTRA-2 binding interface was speculated to be located C-terminal of residue 95 in TpsA4 (or 96 in CupB5). This idea was based on the observation that TpsA4 S$^{68} - D^{107}$ residues are almost identical with CupB5 S$^{69} - D^{108}$ residues (see Figure 4.2B). This possibility could not be assessed using NMR, because the second POTRA domain either alone in TpsB4-P2 or together with POTRA-1 in TpsB4-P12 was misfolded. A genetic approach was used instead introducing point mutations in the TPS motif of CupB5 and
in the POTRA domains of TpsB4. These mutations are expected to prevent the recognition between TpsB4 and CupB5 and thus abolish CupB5 secretion. The point mutations were generated in CupB5 and not TpsA4, because antibodies against CupB5 were already available. I introduced seven single point mutations in CupB5 TPS motif: S\textsubscript{69}A, N\textsubscript{85}I, N\textsubscript{87}D, F\textsubscript{91}A, R\textsubscript{93}E, Q\textsubscript{96}E and N\textsubscript{98}D (see Figure 4.9A). Basically, surface-exposed polar or positively charged amino acids were substituted with negatively charged ones to prevent the binding of the TPS motif into the binding groove on the surface of TpsB4s POTRA domains. Whereas, residues 85 to 98 are expected to bind POTRA-1 and POTRA-2 (see Figure 4.8B), residue S\textsubscript{69} is not part of the expected TPS-POTRA binding interface and should not prevent the recognition between CupB5 and TpsB4. More specifically, N\textsubscript{85}I and N\textsubscript{87}D are part of the conserved NPNG motif in CupB5 and N\textsubscript{98}D is expected to bind POTRA-2. Substitution of these polar residues with a hydrophobic isoleucine and negatively charged aspartic acid should prevent the interaction with TpsB4s POTRA domains, as observed for the homologue residues in FHA of \textit{B. pertussis} (Hodak \textit{et al.}, 2006). The aromatic phenylalanine was substituted with a smaller alanine. This might interrupt the interaction of the hydroxyl group with residues in the POTRA-1 domain. The positive charged R\textsubscript{93} and the polar Q\textsubscript{96} were both mutated to a negatively charged glutamic acid and thus expected to disturb the interaction of the TPS motif with POTRA-1 (R\textsubscript{93}) and POTRA-2 (Q\textsubscript{96}). The negatively charged aspartic acid in N\textsubscript{98}D could abolish the binding of CupB5s TPS motif towards POTRA2, if the amine group of asparagine mediates the interaction with POTRA-1.

These mutations were introduced into the plasmid-encoded \textit{cupB5}, pBBR1-Mcs4-\textit{cupB5}, and the plasmid was conjugated into the \textit{cupB5} deletion mutant, PAO1\textDelta\textDelta\textit{cupB5}/pRocS1. CupB5 secretion was monitored by the occurrence of the protein in the appendage preparation, which was subsequently analysed by Western Blot. Substitution of S\textsubscript{69} to alanine did not affect CupB5 secretion, as CupB5\textsubscript{S\textsubscript{69}A} was still detected on the cell surface (see Figure 4.9B and C). The levels of CupB5\textsubscript{S\textsubscript{69}A} vary in the two different blots, probably because of the number of samples used. In Figure 4.9C only five samples were processed simultaneously, whereas in Figure 4.9B ten samples were prepared at the same time. The use of less samples allows for quicker processing and prevents the degradation of protein. Contrary to the expectations, all of the CupB5 variants (except for N\textsubscript{85}I) were detected in the sheared fractions (see Figure 4.9B). Residue N\textsubscript{85} is close
Figure 4.9: Effect of single point mutations on CupB5 secretion. (A) Location of the single point mutations in CupB5’s TPS domain. (Top) Shown is an excerpt of CupB5’s TPS domain (amino acids 61-108 of mature CupB5). The conserved NPNG is colored orange and amino acids targeted for mutagenesis are shown in bold blue and labeled with their residue number. (Bottom) POTRA-1 (yellow) and POTRA-2 (light purple) interact with CupB5’s TPS motif (pink and green). Amino acids targeted for mutagenesis are shown as blue sticks and labeled. Residue G42 is part of TpsB4 POTRA-1. (B and C) Immunoblot analysis of cell and sheared fractions obtained from PAO1ΔΔ (ΔΔ) and PAO1ΔΔΔcupB5 (ΔcupB5) using antibodies derived against CupB5, RNA polymerase. PAO1ΔΔΔcupB5 was conjugated with pBBR1-MCS5-cupB5 variants. Strains, all conjugated with pRocS1, were grown under static conditions for 24 h at 30°C in the presence of IPTG. The experiments were carried out twice.

The proline allows the TPS motif to bend around POTRA-1. In wild type CupB5, the amine group of asparagine might stabilise the bending of the TPS motif by interacting with residue G42 in POTRA-1. Consequently, a substitution of N85 to isoleucine could disturb this interaction.
interaction and thus the TPS motif is not stably anchored to POTRA-1.

Mutations Q\textsubscript{96}E and N\textsubscript{98}D do not affect the secretion of CupB5 (see Figure 4.9B), because the TPS motif might still be anchored to the groove in POTRA-2 via the neighboring residues V\textsubscript{97} and V\textsubscript{99} (see Figure 4.9A). Similar, mutation of N\textsubscript{87} to aspartic acid might not effect CupB5s translocation because the TPS motif still interacts with POTRA-1 via N\textsubscript{85} and P\textsubscript{86}. Residue R\textsubscript{93} does not seem to bind directly into the groove as it points away from POTRA-1. Lastly, the substitution of F\textsubscript{91} to alanine might not be drastic enough to effect CupB5 secretion, as both residues are hydrophobic amino acids. Additionally, single point mutation might not as strongly affect the interaction between POTRA and TPS as double point mutations would do.

Consequently, double point mutations were introduced into the TPS motif of CupB5. The residues N\textsubscript{85} and P\textsubscript{86}, in the conserved NPNG motif, were substituted to alanine (see Figure 4.10A, center). These residues are expected to be recognised by POTRA-1, as indicated by the NMR chemical shift perturbations (see Figure 4.8B). Also, V\textsubscript{97}W and V\textsubscript{99}W substitutions should prevent an interaction between POTRA-2 and TPS motif (Figure 4.10A, right). Replacement of the aliphatic valine with aromatic and bulky tryptophans might prevent access of the TPS motif into the POTRA-2 groove. Indeed, introduction of the double point mutations V\textsubscript{97}W/V\textsubscript{99}W or N\textsubscript{85}A/P\textsubscript{86}A into CupB5 prevented its extracellular release. In contrast, the wild type CupB5 and the S\textsubscript{69}A variant are detectable in the sheared fractions. Interestingly, the CupB5 variants, which are not secreted, were also not detected in the cell fractions, again indicating that CupB5 is systematically degraded when its secretion is impaired. Similar observations have been made for HMW1 (St Geme and Grass, 1998).

If the key residues of the TPS domain of CupB5 involved in TpsB4 recognition can be identified, the reciprocal experiments should be possible: key POTRA residues were mutated and, as a consequence, CupB5 secretion should be abrogated. Thus, double point mutations were introduced into the POTRA domains of TpsB4, expected to interrupt the interaction with the TPS motif. The H\textsubscript{82}W and P\textsubscript{105}A mutation is located in POTRA-1, just at the edge of the TpsB4/CupB5 interface (see Figure 4.10C, center). For POTRA-2, mutations were introduced into the α4-helix and the β7-strand, because these regions are required for the secretion of FHA by FhaC in *B. pertussis* (Delattre *et al.*, 2011). Mutations G\textsubscript{154}W and E\textsubscript{158}W are situated
in the α4-helix, and the mutation of glycine and glutamic acids to bulky tryptophans should block the access of the TPS motif within this groove (see Figure 4.10C, right). The residues K173 and T175, found in the β7-strand of POTRA-2, were substituted for alanine and proline,
respectively, to prevent β-sheet augmentation within POTRA-2 (see Figure 4.10C, right). For a negative control, we also mutated D61 (POTRA-1) and D149 (POTRA-2) to alanine. These residues are opposite of the TPS-POTRA interface and should not interfere with their interaction. These double point mutations were introduced into a plasmid-encoded tpsB4 gene, and the mutated gene was re-cloned into the integration-proficient vector miniCTX-1. The plasmid integrates at the att site on the chromosome. The new TpsB4 variants can thus be tested for functionality in complementation experiments, upon introduction of the miniCTX1-tpsB4 variants into the PAO1ΔΔΔtpsB4 mutant. cupB gene expression was induced by overexpressing RocS1 in PAO1ΔΔΔtpsB4::tpsB4/pRocS1. CupB5 secretion was then monitored using standard shearing assays, as described before, and analysed by SDS-PAGE and Western Blot (see Figure 4.10C) (Garnett et al., submitted).

The function of the TpsB4 variants G154W/E158W and K173A/T175P was highly affected, as CupB5 was not secreted, confirming the importance of POTRA-2 in CupB5 secretion. In contrast, the POTRA-1 mutant (H82W/P105A) still secreted various amounts of CupB5. As Figure 4.10C (center) shows, the POTRA-1 residues H82W and P105A are in contact with P86 and N85 of TPS motif of CupB5. Because of P86, a part of the conserved NPNG box, CupB5 bends around POTRA-1. When mutating H82W and P105A in POTRA-1, CupB5 can still bend around POTRA-1, and it might explain why it is still secreted. To confirm the importance of POTRA-1 in TPS domain recognition, additional point mutations should be introduced. For example, residues A106 and Q110 could be substituted with aspartate and alanine, respectively. Both residues showed strong chemical shift perturbations when titrated with TpsA4 or CupB5 (see Figure 4.8C and D). A106D is located in the β4-β5 loop and addition of a negatively charged aspartate might disrupt the binding between POTRA-1 and TPS domain. Q110A, also in the β4-β5 loop, might disturb the binding as well, by removing the polar side chain of glutamine.

In conclusion, a model is proposed where CupB5 and TpsA4 have two TPS motifs: TPS1 and TPS2 (see Figure 4.11A). TPS1 contains the conserved NPNG box and is recognised by POTRA-1, whereas TPS2 contains the conserved asparagine N98 in CupB5 (N97 in TpsA4), recognised by POTRA-2 (see Figure 4.11B and C).
4.2 Discussion

Chapter 3.1.3 showed that CupB5 does not require the CupB3 usher to reach the cell surface (under the conditions tested). This Chapter showed that, instead, (i) it can be translocated across the outer membrane by the TpsB4 transporter and (ii) its TPS motif is recognised by TpsB4.

4.2.1 Tps4 is a three-partner secretion system

TpsB4 belongs to one of six Tps systems, encoded in P. aeruginosa genome, and the tpsB4 gene is organised in an operon together with the gene encoding its cognate cargo, tpsA4. Kida et al. (2008) showed that TpsA4 is secreted by clinical P. aeruginosa strains, but not by PAO1. Here, qRT-PCR showed that in PAO1 the tpsB4/A4 operon is under control of the Roc1 two-component system (see Figure 4.2A). As the expression of the cupB operon is also under control of Roc1, CupB5 and TpsB4 are co-expressed, and therefore the relationship between CupB5

Figure 4.11: TPS1 and TPS2 of TpsA4/CupB5 interact with POTRA-1 and POTRA-2 of TpsB4. (A) Tps1 (pink) and Tps2 (green) motifs of TpsA4 and CupB5. The conserved NPNG and N residues are shown in bold. The numbers represent the amino acid of the mature proteins. (B and C) Modeled interaction between TpsB442−194 and TpsA479−110. As in A, the Tps1 motif is shown in pink and the Tps2 motif in green. POTRA-1 is shown in yellow and POTRA-2 in light purple. (B) shows both POTRA domains as a cartoon and the TPS motifs as ball and sticks. (C) shows the same views as B, but POTRAs are shown in surface representation. Raw data was obtained by Dr J. Garnett.
and TpsB4 is likely to be physiologically relevant. Although not shown directly using bacterial cell cultures, the NMR spectroscopy data showed that TpsB4 interacts directly with TpsA4 and CupB5. Thus, the Tps4 system should be reconsidered as being a three-partner secretion system, assuming that TpsB4 translocates both CupB5 and TpsA4.

*P. aeruginosa* Tps4 is not the only known three partner secretion system, as other bacteria can also encode one TpsB transporter and several TpsA proteins. For example, *B. bronchiseptica* encodes the two TpsA proteins FhaB and FhaS, both secreted by FhaC (Julio and Cotter, 2005). FhaB and FhaS are 95% identical in the area that makes up 80% of the proteins. Despite the high amino acid similarity, FhaS and FhaB have different functions, as FhaS is not required for the attachment to epithelial cells *in vitro*. The bacterium *N. meningitides* has three TPS systems scattered across its genome but nonetheless co-expressed. The Tps1 and Tps2 systems each encode for one TpsB transporter and two TpsA proteins (see Chapter 1.8.4 and Figure 1.26). TpsB1 releases only the TpsAs from its own system, TpsB2 however can secrete all four TpsAs (ur Rahman and van Ulsen, 2013; van Ulsen *et al.*, 2008). Thus, the distant localisation of the *cupB5* gene from the *tpsB4/A4* operon is not unusual.

There are also examples of Tps systems encoded within CU pathway gene clusters, for example for *P. fluorescens* and *B. pertussis*. In *B. pertussis*, fhaB (*tpsA*) and fhaC (*tpsB*) are separated by the fimbrial cluster fimA-D (Spears *et al.*, 2003). In a closer relationship, *P. fluorescens* encodes a *cupB*-like cluster (PFL_1462-PFL_1469) within which a TpsB (PFL_1466) transporter and a TpsA (PFL_1467) protein are encoded in tandem (Nuccio and Bäumler, 2007; Ruer *et al.*, 2008). Ruer *et al.* (2008) even showed that PFL_1466 can complement a *cupB3* mutant and thus secretes *P. aeruginosa* CupB5 in an heterologous context. This observation suggested that during evolution *P. aeruginosa* lost an alternative CupB5 transporter encoded among the *cupB* genes. Although these data disagree with our observation that CupB5-secretion is CupB3-independent, they suggest that CupB5 can be transported and recognised by a classical TpsB. It will then fit better with our hypothesis that the original *tpsB* gene from the *cupB* cluster could have been lost, because another highly similar transporter, TpsB4, is a suitable alternative to secrete CupB5, and thus could be used for both CupB5 and TpsA4 secretion. From the evolution point of view, the results of Ruer *et al.* (2008) suggest that the CupB3 usher begun to implement a way to translocate CupB5 by displaying a POTRA-like domain at its N terminus. However, two
CupB5 transporters might not have been advantageous. Interestingly, *Burkholderia ambifaria* also encodes a *cupB* gene cluster (BamMC406_1532 - BamMC406_1538), with the same genetic organisation as the *P. aeruginosa* one. However, the CupB3 homologue, BamMC406_1534, does not seem to have the POTRA-like domain at the N terminus (see Chapter 5 and Figure 5.4). In conclusion, under the conditions tested, CupB5 is secreted by the TpsB4 transporter and not by the CupB3 P-usher, despite *cupB5*’s genetic localisation in the *cupB* operon.

### 4.2.2 TpsB4 POTRA\(s\) have unique functions

The NMR structure of POTRA-1 of TpsB4 revealed that POTRA-1 has the usual \(\beta\)-\(\alpha\)-\(\alpha\)-\(\beta\)-\(\beta\) core structure, observed in FhaC of *B. pertussis*. However, a new feature is the two-stranded \(\beta\)-\(\beta\) sheet located at its N terminus (see Figure 4.7). Only a single other TpsB transporter, Omp85 of Cyanobacteria, has been shown to have a similar \(\beta\)-sheet pair. Omp85 has three POTRA domains and POTRA-1 is capped by the \(\beta\)A-\(\beta\)B sheet pair (Koenig *et al.*, 2010). Moreover, NMR spectroscopy and point mutagenesis showed that CupB5/TpsA4 have two adjacent TPS-motifs, TPS1 and TPS2, which interact with the two POTRA domains of TpsB4, POTRA-1 and POTRA-2, respectively (see Figure 4.11). In POTRA-2 residues G\(_{154}\), E\(_{158}\) (both \(\alpha\)-helix), K\(_{173}\) and T\(_{175}\) (both \(\beta\)-strand) form a groove where TPS2 can bind. The mutation of these residues prevented the access of the TPS2 motif to the groove and \(\beta\)-augmentation within POTRA-2, resulting in the loss of CupB5 secretion by TpsB4. Moreover, mutation of residues V\(_{97}\) and V\(_{99}\) in CupsB5 TPS2 motif, which binds within the groove, also blocked the extracellular release of CupB5. TPS1 binds within the \(\beta\)-\(\beta\) loop and N-terminal to the \(\beta\)1 strand of POTRA-1 and bends, due to a conserved proline in the NPNG box, around POTRA-1. Substitution of N\(_{85}\) and P\(_{86}\) with alanine could indeed abolish CupB5 secretion. However, both mutants CupB5\(_{V_{150}W,V_{152}W}\) and CupB5\(_{N_{138}A,P_{139}A}\) were also not detected in the cell fractions. This was either due to degradation of CupB5 in the absence of secretion or because the introduced point mutations rendered CupB5 unstable. To exclude stability or folding problems, the mutants should be expressed in *E. coli*, purified and their folding checked by NMR.
4.2.3 Model of CupB5 and TpsA4 secretion by TpsB4

Collectively our data suggest the following model of CupB5 and TpsA4 secretion by TpsB4 (see Figure 4.12). Note that our model disregards any role of CupB3 in CupB5 secretion as proposed by Ruer et al. (2008). CupB5, TpsA4 and TpsB4 are secreted across the inner membrane by the SecYEG translocon. TpsB4 forms a β-barrel pore in the OM. In a resting state, the pore lumen is blocked by an α1-helix. Data obtained by Guérin et al. (2014) suggests that the α1-helix periodically moves into the periplasm thereby unblocking the pore. Importantly, the α1-helix is connected to POTRA-1 via a linker. Our data and data obtained by Guérin et al. (2014) suggests that the linker interacts with the POTRA domains, making them unaccessible for an interaction with the TpsA. The movement of the α1-helix into the periplasm might also shift the linker away from the POTRA domains. The POTRA domains would then be accessible for an interaction with the TpsA.

As the TPS1 motif reaches the periplasm first, it might be key for the initial recognition with the TpsB4 transporter. Also POTRA-1 might be important for the substrate selectivity. The affinity between transporter and substrate is further increased by an interaction between POTRA-2 and TPS2. This interaction adds another level of specificity, making sure the correct cargo is recognised and secreted by the TpsB4 transporter. It has been suggested that the POTRAs recognise the cargo’s TPS domain by β-augmentation (Kim et al., 2007; Koenig et al., 2010). Whereas no evidence of it was obtained for POTRA-1, our data suggest that POTRA-2 could interact by β-augmentation with TPS2 (Garnett et al., submitted).

According to the hairpin model (Mazar and Cotter, 2006), the region C-terminal to the TPS motif will form a hairpin in the pore lumen of TpsB4 and secretion is driven by the release of free energy during the β-helical folding of TpsA4/CupB5 on the cell surface. Folding also prevents the back sliding of the secreted protein into the cell. TpsA proteins stay anchored to the cell surface or are released into the extracellular space, as observed for HMW1 of *H. influenzae* and FHA of *B. pertussis*, respectively. HMW1 is kept anchored to its HMW1B transporter by its C terminus that blocks the HMW1B pore for another translocation (Buscher et al., 2006). In contrast, FHA stays initially connected to its FhaC transporter, but can then be released by an unknown mechanism (Noël et al., 2012). For the Tps4 three-partner secretion system, TpsA4
Figure 4.12: **Model of CupB5 and TpsA4 secretion by TpsB4.** TpsB4 forms a $\beta$-barrel in the OM. Its pore lumen is closed by a helical plug (dark blue) that is connected to POTRA-1 via linker (blue line). CupB5/TpsA4 are secreted across the inner membrane (IM) via the SecYEG translocon. Upon reaching the periplasm (PP) the N-terminal TPS motifs interact with TpsB4s POTRA domains. The initial recognition is carried out between POTRA-1 and TPS1 (pink) and further specificity is added by an interaction between POTRA-2 and TPS2 (green). The interaction between POTRA and TPS motifs also activates TpsB4 for translocation resulting in the shift of the plug into the periplasm (PP, 1). The region downstream of the TPS2 motif forms a hairpin in TpsB4s pore lumen and starts to fold at the cell surface into a $\beta$-helical fold (2). Folding of the emerging protein drives its secretion across the outer membrane (OM, 3). CupB5/TpsA4 might stay anchored to the cell surface or are released upon translocation.

and CupB5 might also be released from the cell surface, enabling TpsB4 to secrete more than one protein. According to Ruer *et al.* (2008), CupB5 could then localise with the CupB pilus.

It is unknown whether a hierarchy between CupB5 and TpsA4 exists that dictates which of these proteins is secreted first. It probably depends on the affinity of their respective TPS domains for the POTRA domains of TpsB4. But as their TPS domains are highly homologue, CupB5 and TpsAs affinities for TpsB4 might be similar. Determination of the affinity by NMR spectroscopy or surface plasmon resonance using CupB5s and TpsA4s TPS1 domain and TpsB4s POTRA-1 domain might answer this question. Chapter 7.1 discusses future experiments on the role of TpsA4 and CupB5.
5. Structural insights on the CupB components

5.1 Results

Although CU pathways are well studied in UPEC, little structural data is available about the components of the Cup pathways in *P. aeruginosa*. So far the only structure solved is that of the CupB2 chaperone (Cai et al., 2011). CupB2 has the typical boomerang-like fold of CU chaperones made of two Ig-like domains (see Chapter 1.10.1, Figure 1.30B). Like the PapD and FimC chaperones in UPEC, CupB2 can be classified as a FGS chaperone that has a short loop between its F1 and G1 β-strands (Hung et al., 1996; Zav’yalov et al., 1995). To improve the knowledge about the CupB pili assembly mechanism, we attempted, in collaboration with Prof G. Waksman (Birkbeck College, London) and Prof S. Matthews (Imperial College London), to solve the structures of the CupB1 pilin, CupB6 adhesin and CupB3 usher.

5.1.1 Outer membrane usher CupB3

So far, the only full-length usher structure solved is the FimD usher that assembles type 1 pili in UPEC (Geibel et al., 2013; Phan et al., 2011). The crystal structure showed FimD interacting with the FimC:FimH (chaperone:adhesin) complex and gave insight into the molecular mechanism by which FimD assembles type I pili. We aimed at solving the structure of the CupB3 usher and first conducted a bioinformatic analysis, using the Pfam (Punta et al., 2012) and I-Tasser servers (Roy et al., 2010; Zhang, 2008), to obtain preliminary information about CupB3.
Figure 5.1: Structure prediction of *P. aeruginosa* CupB3 usher. (A) The domain structure of CupB3 has been predicted by the Pfam server (http://pfam.sanger.ac.uk) and the position of the signal peptide by the SignalP server (http://www.cbs.dtu.dk/services/SignalP/). The numbers represent the amino acids. (B,C) CupB3 structure was modeled by the I-Tasser server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/), based on the FimC usher structure (PDB: 3RFZ). The protein domains are colored as in (A) and do not display the signal peptide. The plug domain is shown in limegreen. Shown are the two models with the highest confidence-scores: Model 1 (B) = -1.41 and model 2 (C) = -1.52.

The Pfam server predicted that CupB3 has the expected periplasmic N-terminal and C-terminal domains, as well as the outer membrane usher pore (see Figure 5.1A). Additionally, CupB3 has the POTRA-like domain, as identified by Ruer *et al.* (2008). Further analysis by SignalP (Petersen *et al.*, 2011) predicted the signal peptide, required for the export of CupB3 across the inner membrane via the SecYEG machinery, to be located in the first 17 amino acids. The I-Tasser server modelled the CupB3 structure based on the FimC usher (PDB: 3RFZ).
5.1. RESULTS

Figures 5.1B and C show the two models with the highest confidence score. Although the structures of the N-terminal and C-terminal domain, the plug and the usher pore are similar among the two models, the predicted structure of the POTRA-like domain varies. The POTRA domain is not predicted as a part of the standard usher structure; model 1 (see Figure 5.1B) shows it extended from the usher, whereas model 2 (see Figure 5.1C) shows it to form a helical plug at the bottom of the β-barrel of the usher. Interestingly, the POTRA is not predicted to form a β-α-α-α-β fold as suggested by Ruer et al. (2008), but rather folds into four α-helices.

A comparison of the predicted CupB3 structure with the predicted domain and 3D structures of CupA3, CupC3 and CupE5 shows that these P. aeruginosa ushers also contain the typical usher components: an N-terminal domain, a plug, a pore, and two C-terminal domains (see Figure 5.2A and B). Interestingly, the CupB3 usher has been predicted to have only one C-terminal domain, whereas CupA3, CupC3 and CupE5 are predicted to have two C-terminal domains, typical for...
ushers. Thus, the POTRA-like domain of CupB3 could be significant for CupB pilus assembly in the absence of a second C-terminal domain. Attempts have been made to solve the structure of the POTRA-like domain by NMR in collaboration with Prof S. Matthews. However, the POTRA was unstable in isolation and did not fold properly (personal communication).

We then attempted to solve the full-length 3D structure of the CupB3 usher in collaboration with Prof G. Waksman. The full-length cupB3 gene, including the signal peptide DNA sequence, was cloned into the overexpression vector pASK-IBA3c using the BsaI restriction enzyme and transformed into E. coli strain BL21Star. Protein expression was induced with anhydrotetracycline at 16°C overnight. The C-terminally Strep-tagged CupB3 was purified from cell lysates by Strep-tag affinity chromatography, followed by size exclusion chromatography on a Superdex 200 10/300 GL column (see Figure 5.3). As the inset of Figure 5.3 shows, a small band corresponding to CupB3-Strep (100 kDa size) was detected by Coomassie-staining of a SDS-PAGE gel. However, protein yields of the purified CupB3-Strep were low (3.4 mg/ml) and the usher was not stable, as degradation products were detectable by SDS-PAGE (results obtained by Dr A. Busch). As we could not obtain the P. aeruginosa CupB3 structure, we investigated whether the structure of a homologue could be obtained. We first chose the CupB3 usher homologue BamMC1534, called here BamMC1534, from B. ambifaria MC40-6 (www.burkholderia.com), since the genetic organisation of the cupB cluster in this organism is strictly similar to the cupB cluster in P. aeruginosa.

5.1.2 B. ambifaria CupB3 homologue BamMC1534

The B. ambifaria cupB gene cluster (bamMC406_1532–bamMC406_1537) encodes one pilin subunit - the first gene in the operon - followed by genes encoding a chaperone, usher, a second chaperone, a TpsA-like protein (CupB5-like) and an adhesin (see Figure 5.4A). An amino acid alignment of CupB3 and BamMC1534 by Clustal Omega showed that both ushers have an identity of 47% (data not shown). BamMC1534 has an amino acid length of 880 residues; whereas CupB3 is 926 residues long (see Figure 5.4B). It is because the P. aeruginosa CupB3 has the N-terminal POTRA-like domain (aa 17–99), absent in the B. ambifaria usher. A sequence search by the Pfam server (Punta et al., 2012) predicts that BamMC1534 has the N-terminal and
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Figure 5.3: Purification of CupB3-Strep. Elution profile of CupB3-Strep on a Superdex 200 10/300 GL column. The blue line represents the absorption at 280 nm and C1, C3, C7 and C13 are the fractions that were used for SDS-PAGE. The inset shows the Coomassie-stained SDS gel. E is the input (Elution from the Strep-tag affinity chromatography) and M the BenchMark protein ladder. Protein sizes are given in kDa. Results were obtained by Dr A. Busch.

C-terminal domain structure from the PapC usher, as well as the outer membrane β-barrel of usher proteins. It was confirmed by a structure prediction using the Phyre2 server (Kelley and Sternberg, 2009) that models BamMc_1534 as a typical usher (see Figure 5.4C).

![Figure 5.3: Purification of CupB3-Strep](image)

Figure 5.4: Bioinformatic analysis of the cupB-like gene cluster of B. ambifaria and the BamMC_1434 usher. (A) Comparison of the organisation of the P. aeruginosa cupB operon and the homologue B. ambifaria cupB operon (bamMC1532–1538). (B) Comparison of full-length CupB3 with BamMc_1534 and BamMc_1534-opt-Strep. Shown are the preproteins with their N-terminal signal peptides. The numbers represent amino acids. Domains were predicted by the Pfam server (http://pfam.sanger.ac.uk) and signal peptides by SignalP (http://www.cbs.dtu.dk/services/SignalP/). (C) Structure prediction of BamMC_1534 by Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2). The domains are coloured as in B, and the plug domain is shown in limegreen.

![Figure 5.4: Bioinformatic analysis](image)
To maximise the expression of BamMC\textsubscript{1534} in \textit{E. coli}, a codon optimised \textit{bamMC\textsubscript{1534}} gene was constructed (GeneArt gene synthesis, Life technologies) and cloned into the overexpression vector pASK-IBA3c, yielding pASK-IBA3c-BamMC\textsubscript{1534-opt-Strep}. The plasmid was transformed into \textit{E. coli} DH5\textalpha omnimax and expression induced with anhydrotetracycline. Cell extracts were analysed by SDS-PAGE and Coomassie staining (see Figure 5.5A). A band of 95 kDa, the expected size of BamMC\textsubscript{1534-opt-Strep}, was detected in the cell extracts of the cells induced for expression. In addition, a protein of 20 kDa was detected, probably a degradation product.

Further Dr A. Busch analysed soluble and insoluble fractions of BamMC\textsubscript{1534-opt-Strep} expressing \textit{E. coli} DH5\textalpha omnimax and Top10 cells. BamMC\textsubscript{1534-opt-Strep} was mostly present in the insoluble fractions (see Figure 5.5B). Insoluble fractions contain aggregated proteins and inclusion bodies, but may also include proteins from the inner and outer membranes, such as the outer membrane usher. However, treatments of the membranes with the detergent Lauryl-\beta-D-maltoside did not solubilise the usher as it was still detected in the solubilised membrane pellet. It indicates that the usher is insoluble when overexpressed and maybe aggregates in inclusion bodies. This study was abandoned and alternative usher proteins were searched for crystallisation.

### 5.1.3 \textit{P. aeruginosa} ushers CupA3, CupC3 and CupE5

Three more ushers have been identified in \textit{P. aeruginosa} PAO1: CupA3, CupC3 and CupE5. As described above (see Figure 5.2A and B), all three ushers are predicted to have the classical usher domain structure: the periplasmic N- and C-terminal domains, the plug, and the outer membrane usher pore. To express these proteins in \textit{E. coli}, the genes were codon-optimised (GeneArt gene synthesis, life technologies) and cloned into the overexpression vector pASK-IBA3c, yielding C-terminally Strep-tagged recombinant proteins. As the Coomassie-stained SDS gel in Figure 5.6A shows, the proteins were expressed at various levels in \textit{E. coli} DH5\textalpha omnimax: CupA3 (96 kDa) and CupC3 (92 kDa) express at similar high levels, whereas CupE5 (87 kDa) is not detectable by Coomassie staining.

Dr A. Busch expressed the recombinant ushers in \textit{E. coli} DH5\textalpha omnimax and Top10 to identify the best expression strain and then purified the ushers via Strep-tag affinity chromatography.
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Figure 5.5: Purification of *B. ambifarias* BamMC\_1534 usher from *E. coli*. (A) Coomassie stained SDS gel of *E. coli* DH5α cells expressing BamMC\_1534-opt-Strep. Bacteria were transformed with pASK-IBA3c-BamMC\_1534-opt-Strep. Protein expression was induced with anhydrotetracycline at 37°C for 5 h, shaking. Uninduced (pre) and induced (post) cell samples were harvested by centrifugation and proteins separated by SDS-PAGE and visualised by Coomassie-staining. Two clones (#1,#2) were tested. The arrow indicates the band corresponding to BamMC\_1534-opt-Strep, and the asterisk indicates degradation products. M = marker. The experiment was carried out once. (B) Coomassie-stained SDS-PAGE gel of soluble (Sol), insoluble (In), and solubilised membrane pellet (SM) fraction of *E. coli* DH5α and Top10 cells. The arrow shows the band corresponding to BamMC\_1534-opt-Strep. M = marker. Figure B was obtained by Dr A. Busch.

CupC3 appeared to be stable, as no degradation products were detectable (see Figure 5.6B). In contrast, CupE5 was degraded to a slightly smaller product, whereas CupA3 could not be purified at all. The expression levels of the recombinant ushers are similar in the two different *E. coli* strains. As CupC3 appeared to be the most stable usher, expression was up-scaled and CupC3 was purified via Strep-tag affinity chromatography, followed by size exclusion chromatography using a Superose-6 10/300 GL column (see Figure 5.6C). The eluted fractions, E8-F14, contained pure CupC3. However, the yield was low: 2.8 mg/ml obtained from DH5α and 2 mg/ml obtained from Top10. In conclusion, although CupC3 purifies as a stable and soluble protein, crystallisation trials have not been carried out due to a low yield.

5.1.4 CupB1 and CupB6 pilin subunits

The pili assembled by the CU pathway consist of thousands of pilin subunits with usually one tip adhesin. The adhesin recognises certain substrates and promotes the attachment of bacteria to a biotic or abiotic surface. The 3D structures available, such as for FimH and PapG of type I and P pili (Dodson *et al.*, 2001; Wellens *et al.*, 2008), shed light on the molecular binding
mechanism between the substrate and adhesin. For example, P pili bind to Galα-1,4-Galβ moieties of glycolipids, present in epithelial kidney cells (Kuehn et al., 1992; Roberts et al., 1994), whereas type I pili interact with D-mannosylated receptors on the surface of epithelial bladder cells (Korea et al., 2011). Moreover, the structure of the FimA pilin (PDB: 2M5G) has been solved and enhanced the understanding of the assembly of CU pili. By solving the structure of CupB1 and CupB6 in collaboration with Prof S. Matthews, we aimed at confirming that CupB1 is likely to assemble pili and, more importantly, to obtain insights in the CupB6 adhesin domain and its possible involvement in promoting host recognition by *P. aeruginosa*.

The pilin subunits of CU pathways have an incomplete Ig-like fold due to the absence of a β-strand. It creates a hydrophobic groove, which renders the subunit unstable if not stabilised by a CU chaperone or a neighbouring subunit (see Chapter 1.10.1). However, the pilin subunits also have an N-terminal extension (Nte) peptide, inserted into the hydrophobic groove of the neighbouring subunit, a process called donor strand exchange. Insertion of the Nte stabilises
the receiving subunit and subsequently leads to the assembly of a pilus at the usher. For this reason, pilin and adhesin subunits are not stable when expressed independently and have to be expressed together with a cognate chaperone. An elegant alternative is to move the Nte peptide of a given pilin subunit from the N to the C terminus in such a way that it will fold into the hydrophobic groove of the recombinant protein and will thus prevent its improper folding (Anderson et al., 2004; Garnett et al., 2012).

Figure 5.7: Expression of the recombinant CupB1 and CupB6. (A) Comparison of *P. aeruginosa* CupB1 and CupB6 with CupB1-opt-dscB1 and CupB6-dscB1. The chimeric *cupB1-opt-dscB1* gene has been codon optimised for expression in *E. coli*, and was cloned without its signal and Nte peptide. CupB6-dscB1 was cloned without its signal peptide. Both proteins are C-terminally fused to a SNDK linker, CupB1s codon-optimised Nte and a 6 x His-tag. Amino acids are labeled according to the preprotein. (B, C) Expression of CupB1-opt-dscB1 (18.5 kDa) or CupB6-dscB1 (40 kDa) from pET28b in *E. coli* SHuffle cells. Protein expression was induced with 0.4 mM IPTG at 30°C for 4 hours. Uninduced (PI) and induced (post) cell samples were harvested by centrifugation and proteins separated by SDS-PAGE. M = marker. (B) Coomassie-stained SDS-PAGE gel. Insoluble (In) and soluble (sol) fractions were prepared by sonication of cell samples and subsequent centrifugation. Arrows indicate the overexpressed protein. (C) Western Blot using anti-His-tag antibody. Star = unspecific bands.

In this study, the Nte of CupB1 (amino acids 33-41, including the P2 (Val), P3 (Phe), P4 (Gly) and P5 (Ile) residues, see Figure 1.32) was fused to the C terminus of both CupB1 and CupB6 (see Figure 5.7A), yielding CupB1-dscB1 and CupB6-dscB1. To give the Nte space for structural reorganisation, the C terminus of the subunits and the Nte were separated by a SDNK linker.
For purification purposes, both recombinant proteins were tagged with a C-terminal His-tag. Moreover, to improve expression of CupB1 in *E. coli*, the *cupB1-dscB1* chimeric gene has been codon-optimised, yielding *cupB1-opt-dscB1*.

The chimeric genes were cloned into the overexpression vector pET28b and transformed into the *E. coli* strain SHuffle. SHuffle cells constitutively express the periplasmic disulfide isomerase DsbC that refolds mis-oxidized proteins (Bessette *et al*., 1999; Levy *et al*., 2001) or functions as a chaperone supporting protein folding (Chen, 1999). CupB1 is not predicted to form disulfide bonds by the DISULFIND server (http://disulfind.dsi.unifi.it/); however, CupB6 has six cysteine residues, which probably form disulfide bonds. Protein expression was induced with IPTG and as can be seen in Figure 5.7B and C, CupB1-opt-dscB1 and CupB6-dscB1 were expressed upon induction and detectable by Coomassie-staining and Western Blot. Moreover, both recombinant proteins were present in soluble cell fractions.

While no attempts were made to solve the structure of the CupB1 pilin, the crystal structure of CupB6 was solved (results obtained by Dr M. Rasheed). CupB6 was purified first by affinity chromatography on a nickel column, followed by size exclusion chromatography on a Superdex 75 column and crystals obtained using a protein concentration of 17 mg/ml. The CupB6 structure was then solved by X-ray crystallography with the resolution of 3.16 Å. CupB6 has the typical two domain structure of CU adhesins, i.e. an N-terminal putative adhesin domain and a C-terminal pilin domain (see Figure 5.8A). The pilin domain consists of six anti-parallel β-strands, whose incomplete Ig-like fold is complemented by the addition of CupB1s Nte peptide. Pilin and adhesin domains are connected via a linker, which forms a left-handed helix, also known as poly-L-proline type II (PPII) helix (Adzhubei *et al*., 2013). These helices do not always contain prolines; however, in the case of CupB6, the PPII contains a poly-proline motif, consisting of six proline residues (PPPPPIP). Next to the linker is another left-handed PPII helix, containing only one proline residue (see Figure 5.8B).

A comparison of CupB6s structure with the structures available from the protein data bank via the Dali Server (Holm and Rosenström, 2010), revealed that the CupB6 pilin domain has structural similarities with the FimA pilin domain (PDB: 2JTY, Z-score 14.7), whereas the CupB6 putative adhesin domain is similar to GafD (PDB: 1OIO, Z-score 8.7) and F17-G adhesin
Figure 5.8: Crystal structure of CupB6-dscB1 solved by X-ray crystallography. (A) Cartoon presentation of the structure of full-length CupB6-dscB1. CupB6-dscB1 crystals were obtained using a protein concentration of 17 mg/ml in 20 mM Tris-HCl pH 8 and 200 mM NaCl. Crystals were grown by hanging drop vapor diffusion in a 1:1 ratio against reservoir solution containing 1.6 M NaH$_2$PO$_4$ / 400 mM K$_2$HPO$_4$, 100 mM Na$_2$HPO$_4$ / citric acid pH 3.4 and triglycine. Crystals were obtained at 20°C after a fortnight and the structure solved with the resolution of 3.3 Å. CupB6-dscB1 has two domains, the N-terminal adhesin and C-terminal pilin domain, separated by a poly-proline linker. The groove in the pilin domain is complemented by the Nte peptide of CupB1 (dscB1, including the P2 (Val), P3 (Phe), P4 (Gly) and P5 (Ile) residues). CupB6-dscB1 is rainbow colored from N to C terminus. (B) The poly-proline linker forms a left-handed helix (yellow/red), next to a second left-handed helix (blue). The CupB6-dscB1 structure was obtained by Dr M. Rasheed, and figures were made by D. Muhl.

domain (PDB: 4K0O, Z-score 8.6). FimA is the main pilin subunit of the type I pilus in UPEC and its comparison with CupB6 pilin domain shows that the overall structure, such as orientation of β-sheets, α-helices and loops, is highly similar except for two additional β-strands in CupB6, located towards the end of the pilin domain (see Figure 5.9, green arrows).

The F17-G adhesin is found at the tip of F17 fimbriae assembled by enterotoxigenic E. coli and the GafD adhesin on G fimbriae produced by UPEC (Buts et al., 2003; Saarela et al., 1995). Both fimbriae belong to the γ-clade of CU fimbriae and are thus predicted to be assembled by the classical CU pathway (Nuccio and Bäumler, 2007; Wurpel et al., 2013). Although the overall
structural similarity between CupB6s, GafDs and F17Gs adhesin domain is obvious, various differences such as the orientation and number of $\beta$-sheets are also apparent (see Figure 5.10A). GafD and F17-G have, for example, three additional $\beta$-sheets near the C terminus. Moreover, all three adhesin domains have a single $\alpha$-helix, located at opposite positions when comparing GafD/F17-G and CupB6. Both, GafD and F17-G are known to bind N-acetylglucosamine at a lateral binding side (see Figure 5.10A). As Figure 5.10B shows, the binding side resembles a pocket formed by the surrounding $\beta$-sheets and the $\alpha$-helix. In contrast, CupB6 seems not to have a similar binding pocket, probably because the $\alpha$-helix is not close to the same $\beta$-sheets as in GafD and F17-G.

In conclusion, CupB6 is a typical CU adhesin with a N-terminal putative adhesin and a C-terminal pilin domain. Remarkably, the CupB6 adhesin domain displays a unique fold not observed in other CU adhesins, which involves two left-handed poly-proline helices.
Figure 5.10: Comparison of CupB6s, GafDs and F17-Gs adhesin domain structures. (A) Cartoon representation of the crystal structures of GafD (PDB: 1OIO), F17-G (PDB: 4K00) and CupB6 adhesin domains. Structures are rainbow colored from N to C terminus. The N-acetylglucosamine binding site for GafD (Merkel et al., 2003) and F17-G (Buts et al., 2003) are indicated by arrows. (B) Composite of the surface and cartoon representations of the crystal structures of GafD, F17-G and CupB6 adhesin domains. The N-acetylglucosamine binding pocket for GafD (Merkel et al., 2003) and F17-G (Buts et al., 2003) are indicated by circles.
5.2 Discussion

Although the CU pathway in *E. coli* is well studied, data about the *P. aeruginosa* Cup proteins structure and functions are scarce. To understand the molecular assembly mechanism of the CupB pilus biogenesis, we attempted to solve the structures of the CupB3 usher and the CupB6 tip adhesin.

5.2.1 Crystallisation of the ushers

The purification of a recombinant CupB3 usher was unsuccessful, and the protein could not used for crystallisation trials. The original idea was to resolve the structure of the CupB3 P-Usher to assess the structure and position of the POTRA-like domain within the usher. The predicted CupB3 structure, using I-Tasser, offers two possibilities: first, POTRA could expand from the pore and, second, it could be located at the periplasmic entrance to the pore where it forms a plug-like structure. In the former case, it could be a real POTRA as described in FhaC for example (Busch and Waksman, 2012). But in the later prediction, it could simply be an added domain involved in CupB1 pilus assembly, and it could be independent of any standard POTRA function, such as the recognition of a TpsA. These observations, thus, cannot confirm whether the P-usher POTRA-like domain is a real POTRA and whether it could be used as a motif for recognising the CupB5 protein. The data presented in Chapter 3.1.3 and Chapter 4 suggest that CupB3 is not required for CupB5 secretion, and it remains unclear which of these two possibilities is more relevant. We also attempted to obtain the structure of a CupB3 homologue in *B. ambifaria*, since the gene encoding CupB3 is located exactly in the same genetic context as the one of *P. aeruginosa*. However, the *B. ambifaria* CupB3, as well as CupB3 orthologs in, for example, *H. influenzae* F3047 and *P. fluorescens* Pf-5, have no predicted POTRA-like domain, which speaks against the idea of CupB3 carrying a functional POTRA (even though one might consider it as a part of an evolution process) and the *cupB3* gene being wrongly annotated by Ruer *et al.* (2008). Yet, no soluble proteins were obtained from *B. ambifaria* CupB3 to use for crystallisation trials. Since no *P. aeruginosa* usher structures are available, and any novel fold could increase our knowledge on CU pathways, we further attempted to purify other *P. aeruginosa* ushers, CupA3, CupC3, and CupE5. Again, due to problems with
solubility and expression, no protein for crystallisation trials was produced. Nonetheless, the CupC3 usher appears as a stable and soluble protein, although expressed at low levels. Up-scaling the protein production might finally result in obtaining a *P. aeruginosa* usher structure. This work is currently being pursued in the laboratory of Prof. G. Waksman.

**5.2.2 Crystallisation of CupB6**

In contrast, purification of CupB6 resulted in high yields of homogeneous protein and allowed us to solve the CupB6 structure by X-ray crystallography (see Figure 5.8). CupB6 has a two-domain structure with the pilin domain connected to the adhesin domain via a linker. The pilin domain has an incomplete Ig-like fold, with a hydrophobic groove at the surface. In an assembled CupB pilus, consisting of one CupB6 tip adhesin and many CupB1 pilin subunits, this groove would be filled by the Nte peptide of the neighbouring CupB1 pilin in a process called donor-strand exchange. The CupB6 linker region contains one of two PPII helices, with the second PPII helix located next to the linker.

**Function of the PPII helices**

Interestingly, PPII helices have been reported to be involved in signaling, cell motility, immune response and, most importantly, host-pathogen recognition (Adzhubei *et al.*, 2013). For example, the intracellular pathogen *Listeria monocytogenes* displays a PPII-containing protein, ActA, on its surface. ActA interacts with the mammalian Enabled (Mena) protein, which displays an N-terminal and proline motif-binding EVH1 domain on its surface (Niebuhr *et al.*, 1997). These proline motifs are usually found in mammalian proteins that participate in the transfer of external signals to the actin skeleton. Upon binding of such proteins, Mena enhances the polymerisation of actin filaments and promotes the rearrangement of the cytoskeleton (Beresio and Vitagliano, 2012). *L. monocytogenes* uses this mechanism by localising Mena proteins close to its cell surface with the help of ActA. The Mena proteins induce a rapid actin polymerisation, resulting in the formation of so-called actin tails. These tails allow *L. monocytogenes* to travel with the cytoskeleton and to colonise adjacent cells. The structure of the EVH1 domain in complex with a PPII motif (FPPPP) has been solved and showed that the PPII helix neatly fits into a
concave groove that is formed by an aromatic triad on the surface of the EHV1 domain (see Figure 5.11A) (Prehoda et al., 1999).

Figure 5.11: **Role of PPII helices.** Proteins are shown as cartoons and proline residues as red sticks. (A) EHV1 domain of Mena (green, PDB: 1QC6) in complex with a poly-proline motif (red). The aromatic triad, which forms a groove into which the poly-proline motif binds, is shown as blue sticks. (B) Crystal structure of the A3VP1 domains of AgI/II antigen encompassing the PPII helix (P1), an $\alpha$-helix (A3) and a globular $\beta$-sheet head (V, PDB: 3IPK). The PPII and $\alpha$-helix are intertwined and form a stalk-like structure. (C) Crystal structure of FctB (PDB: 3KLQ) displaying the C-terminal PPII helix and the N-terminal Ig-like fold.

However, the interaction between the PPII-containing protein and the receptor does not have to be direct. Instead, the PPII can contribute to the three-dimensional structure of the protein and indirectly favor the interaction with the host. It has been well studied in *Streptococcus mutans,* whose surface-located adhesin antigen AgI/II has a PPII helix (Larson et al., 2010). The PPII helix is intertwined with an $\alpha$-helix, creating a stem with the adhesin on top (see Figure 5.11B). Thus, the adhesin can interact with the host surface receptor salivary agglutinin.

Another example is the FctB basal pilin of *Streptococcus pyogenes,* which anchors the major pilus shaft in the cell wall (Linke et al., 2010a,b). Thereby, FctB is cleaved by a sortase (transpeptidase), which transfers and covalently links FctB to the peptidoglycan (Kline et al., 2010). PPII acts as a spacer, making sure that the sortase can access the cleavage site (Berisio and Vitagliano,
5.2. DISCUSSION

An interesting parallel to the CU pilins is that FctB folds into an Ig-like structure at the N terminus, whereas its C terminus is made from a PPII helix tail (see Figure 5.11C).

These examples show that the PPII helices of CupB6 could be involved in the recognition of the host either by binding directly to a host receptor or by positioning CupB6’s adhesin domain for receptor binding (see Figure 5.12).

Figure 5.12: Model of CupB6 function in *P. aeruginosa* pathogenesis. CupB6 is found at the tip of the CupB1-containing pilus, where it might mediate binding to abiotic or biotic surfaces, for example host cells, via its PPII helices or adhesin domain. The CupB1 pilus is shown in pink, CupB6 in blue and the PPII helix linker in red (the second PPII helix is not shown for simplicity). (A) The PPII helix linker interacts directly with the host cell envelope. (B) The PPII helix promotes a certain 3D structure of CupB6 and allows its adhesin domain to bind to the host cell surface.

**Function of the putative adhesin domain**

CupB6 putative adhesin domain has a significant structural similarity with the lectin domains of the F17-G and GafD adhesins. F17-G and GafD are the tip adhesins of F17 and G fimbriae in *E. coli*, respectively, and are predicted to be assembled by the classical CU pathway (Nuccio and Bäumler, 2007; Wurpel et al., 2013). Both adhesins recognise N-acetylglucosamine, and GafD has also been shown to bind to the glycoprotein laminin (Saarela et al., 1995, 1996; Tanskanen et al., 2001). The interaction with N-acetylglucosamine relies on a lateral binding site present in the adhesin domains of F17-G and GafD (see Figure 5.10). N-acetylglucosamine is, for example, found on surface receptors of microvilli of the intestinal epithelium. Buts et al. (2003) speculated
that the F17 fimbriae can reach between the microvilli, and the F17-G lectin can then bind to
the N-acetylglucosamine-containing receptors via its lateral binding pocket.

A comparison of the surface representation of GafDs, F17-Gs and CupB6s adhesin structures
did not indicate that CupB6 has a similar binding pocket. CupB6 also does not seem to have
an affinity for carbohydrates. Neoglycolipid-based microarrays (Marchant et al., 2012) were
used to probe the binding affinity of CupB6 towards glycolipids and neoglycolipids, such as
N-acetylglucosamine. However, no interaction was observed (Prof S. Matthews, personal com-
munication). That the CupB6 substrate choice is different from that of GafD and F17-G might
be because of different binding pocket specificities, for example, a smaller substrate. The speci-
ficity of CupB6 is likely the reason why no difference was observed in biofilm formation of
*P. aeruginosa* cupB mutants on abiotic surfaces (see Chapter 3.1.5).

In conclusion, CupB6 has the typical two-domain structure of CU adhesins: the N-terminal
adhesin domain and the C-terminal pilin domain. Both domains are connected via a linker,
which for CupB6 contains a proline-rich motif and folds into a PPII helix. The helix might have
a role in adhesin by either binding directly to the host or by promoting the adhesin domains
interaction with a receptor (see Figure 5.12). Further experiments that probe the interaction
between host cells and CupB pili are discussed in Chapter 7.1.
6. Conclusions

One of the aims of this study was to understand the molecular mechanism of CupB pilus biogenesis by the CupB CU pathway in *P. aeruginosa*. The results of Chapter 3 suggested that the CupB chaperones, CupB2 and CupB4, have their cognate pilin subunits: CupB4 targets the CupB6 adhesin to the usher, whereas CupB2 targets the major pilin subunit, CupB1. The first complex to arrive at the CupB3 usher is CupB4:CupB6, activating the pore for CupB1 pilus assembly. The CupB6 putative adhesin is probably the first subunit that arrives at the usher and thus might be present at the tip of the pilus.

Chapter 5 presented the structure of the CupB6 adhesin and showed that it has the typical CU adhesin structure with a C-terminal pilin domain and a putative N-terminal adhesin domain. The pilin domain is lacking an Nte peptide, which indicates that CupB6 is the first subunit that enters the usher and subsequently constitutes the tip of the CupB1 pilus. Whereas CupB6s pilin domain is essential for its incorporation into the pilus, the adhesin domain is usually believed to have adhesive properties. Both domains are connected via a linker, which consists of a PPII helix. Although adhesin domains and PPII helices are essential for attachment to biotic or abiotic surfaces (Berisio and Vitagliano, 2012; Buts *et al.*, 2003; Sauer *et al.*, 2000), the function of both in CupB6 is yet unknown. However, since the CupB-dependent biofilm assay on abiotic surfaces was inconclusive, the CupB fimbriae might have a role in specific interactions with eukaryotic cells, and the adhesin domain of CupB6 might be involved.

The further investigation of how CupB5 is secreted to the cell surface challenged the previous data showing it mostly depends on the CupB3 P-usher. Indeed, CupB5 is a TpsA-like protein and these proteins are usually translocated by their cognate TpsB transporters. Although the *cupB* gene cluster does not encode such a transporter, a previous study by Ruer *et al.* (2008) reported that the CupB3 P-usher is required for CupB5 secretion. We could not confirm this, but
showed in Chapter 3 that the TpsA-like protein, CupB5, is secreted by the TpsB4 transporter. Remarkably, these genetic data are supported by structural and biochemical data confirming by NMR that both proteins interact with each other (Chapter 4). The gene encoding TpsB4 is located next to the \textit{tpsA4} gene, which encodes a large exoprotease modulating the human host response by protease-activated receptors (PARs). These receptors are found on the surface of various human cell types, and their cleavage activates the NF-\(\kappa\)B pathway (Kida \textit{et al.}, 2008). Furthermore, TpsA4 degrades haemoglobin to release peptides and iron, which \textit{P. aeruginosa} uses as a nutrient source. TpsA4 has also been shown to act together with the hemolytic phospholipase C (PlcH), allowing the bacterium to grow on erythrocytes (Kida \textit{et al.}, 2011).

The role of CupB5 and whether it acts synergetically with TpsA4 or is associated with the CupB pili is unknown.

We also studied the regulation of the \textit{cupB} and \textit{tpsB4/A4} gene clusters by showing that they are both under the control of the Roc1 two-component system (Chapter 3). Moreover, Roc1 upregulates the expression of the \textit{cupC} operon, encoding the CupC1 pilus in \textit{P. aeruginosa} (Kulasekara \textit{et al.}, 2005). CupC1 pili are involved in biofilm formation, where they support microcolony formation and cell clustering. CupB1 and CupC1 pili have been proposed to cooperate in biofilm formation by promoting cell-cell interactions (Kulasekara \textit{et al.}, 2005; Ruer \textit{et al.}, 2007). However, the specific role of the CupB1 pilus and its CupB6 tip adhesin is yet unknown, and this study could not confirm their role in biofilm formation. As indicated above, the absence of adherence to abiotic surfaces might be due to CupB6 specificity to bind a host cell receptor. As Chapter 7 discusses, this hypothesis could be studied by cell attachment assays.

**Hypothetical model for a multifactorial attachment process.**

Hypothetically, CupC1 and CupB1 pili could help \textit{P. aeruginosa} to reach a surface, which could be abiotic (e.g. catheters, tubing) or biotic (host cells in the lung of CF patients, see Figure 6.1). Depending on the length of the pili (which is yet unknown), CupC1 pili might be involved in the initial and aspecific interaction. The \textit{cupC} cluster does not encode an adhesin gene, supporting CupC1s role in aspecific binding. Upon the initial attachment of the bacterium via CupC1, the CupB pilus might promote a specific and irreversible binding on a biotic surface.
Figure 6.1: Model of the role of CupB, CupC and Tps4 in *P. aeruginosa* pathogenesis. The hypothetical model shows the attachment of a flagellated *P. aeruginosa* bacterium (pink) to an eukaryotic host cell (yellow). *P. aeruginosa* assembles CupB1 and CupC1 pili and the CupB1 pili display the CupB6 tip adhesin. At the same time, TpsA4 and CupB5 are secreted by the TpsB4 transporter. Top: *P. aeruginosa* initially attaches via its CupC1 pili to the host cell. It allows the bacterium to get closer to the host cell, where the CupB6 adhesin attaches specifically to host cell receptors. Magnification: CupB5 interacts with the CupB pilus tip and is so brought close to the host cell surface, where it carries out its unknown function. TpsA4 has proteolytic activity and degrades protease-activated receptors (purple circles). Degradation of these receptors activates the NF-κB pathway.

Then, CupB6 might bind a yet unknown receptor on the surface of an eukaryotic cell. At the same time, the TpsA proteins CupB5 and TpsA4 might be transported at the cell surface. TpsA4 has a RGD cell attachment motif, which might target it to specific cells to carry out its proteolytic function. Whereas in our conditions the CupB5 transport seems to be synergic with TpsA4, for the study of Ruer *et al.* (2008) it seemed to be synergic with CupB fimbriae assembly. Indeed, they observed that CupB5 localises at the CupB pilus tip. The CupB pilus could bring CupB5 close to a substrate, avoiding its diffusion into the extracellular space. It may even happen if CupB5 is secreted by TpsB4: the CupB pili and the CupB5 protein would come into contact on the outer membranes extracellular side. Overall, one may speculate that the CupB1 and CupC1 pili together with the Tps4 system are important for *P. aeruginosa* colonisation and might enhance the bacterium’s ability to survive in and to defeat the human host.
7. Future work

Although this study aimed at unraveling further the molecular mechanism of CupB pilus assembly and CupB5 secretion in *P. aeruginosa* our knowledge of the function of CupB proteins is far from complete. This Chapter discusses several experimental approaches that might provide more insight into the assembly of CupB pili, their regulation and role in *P. aeruginosa* pathogenesis.

7.1 Molecular assembly mechanism of the CupB pilus

**Role of the chaperones.** The pull-down assays performed in this study suggest that CupB2 and CupB4 have both their cognate substrate: CupB2 targets CupB1 and CupB4 interacts with CupB6. Yet, the results are not entirely conclusive and could be refined.

- The CupB4 chaperone, for example, was neither detectable on Coomassie-stained SDS-PAGE gels nor by Western Blot, suggesting it might not be expressed. As discussed in Chapter 3, future experiments could first aim at improving CupB4 expression by engineering a *cupB4* gene with an ATG start codon.

- To increase the yields of chaperone and pilin subunits, the chaperone:pilin complexes could be purified from the periplasm by osmotic shock (Zav’yalov *et al.*, 1995). Briefly, bacteria are resuspended in 20 % (w/v) sucrose, 20 mM Tris-HCl (pH 8.0) and 5 mM EDTA at 20°C. Bacteria are then removed by centrifugation, resuspended in cold 10 mM MgCl₂ and incubated on ice. Periplasmic proteins are released in the supernatant and can be isolated by centrifugation, followed by fraction on a MonoQ column. Ammonium sulphate is then used to precipitate the proteins in the unbound fractions. This approach has been successfully used by Zav’yalov *et al.* (1995) and MacIntyre *et al.* (2001) to purify Caf1M and Caf1M:Caf1 complexes from the periplasm of *E. coli* cells.
• The stability of chaperone:subunit complexes in *E. coli* can be increased by deleting the Nte peptide of the pilin subunit. Zavialov *et al.* (2003a) replaced 12 amino acids at the N terminus of the Caf1 pilin with a six-histidine-tag and co-expression of the modified Caf1 with the Caf1M chaperone in *E. coli* led to the accumulation of highly stable complexes in the periplasm. A similar approach could be used to purify and crystallise, for example, CupB1:CupB2 and CupB6:CupB4 complexes.

• Another possibility to investigate the chaperone/pilin subunit interaction is the bacterial two-hybrid assay. It was used unsuccessfully to probe the interaction between CupB5/TpsA4 and TpsB4, but the reason might have been an unwanted folding of the TPS domain.

**Role of the CupB6 adhesin in usher activation.** Studies of the assembly of the P and type 1 pili in UPEC have shown that the adhesin is required for the activation of the usher (Waksman and Hultgren, 2009). We showed that CupB6 has the typical CU adhesin structure constituting a putative N-terminal adhesin domain and the C-terminal pilin domain. It suggests that CupB6 is required for activating the CupB3 usher in *P. aeruginosa*.

• To show that CupB6 indeed activates the usher, surface plasmon resonance spectroscopy could be carried out. Using this method, Saulino *et al.* (1998) showed that a complex of FimC:FimH (chaperone:adhesin) bound with high affinity to the usher. For this experiment, CupB3 and a CupB4:CupB6 complex could be produced in *E. coli* and then purified. CupB3 would be attached to a biosensor chip, and complexes of CupB4:CupB6 would be added. The association and dissociation constants of the CupB4:CupB6 complex can be compared with those of a CupB2:CupB1 complex. The affinity of the adhesin should be higher. The limitation of this approach will be the CupB3 purification since, as described previously, the yield was too low to carry out crystallisation.

• Dodson *et al.* (1993) used an ELISA to investigate the binding affinities of chaperone:subunit complexes for the PapC usher. They coated microtiter plate wells with the PapC usher and incubated them with PapD in complex with PapG, PapA or PapK. The wells were then incubated with PapD antibody to detect binding of the complexes towards
PapC. Incubation of the wells with alkaline phosphatase-linked anti-rabbit IgG and addition of \( \rho \)-nitrophenol phosphate gave a measure of adsorption. Similar experiments with CupB3 and the CupB2:CupB6 or CupB4:CupB6 complexes could be considered.

**Structure of a *P. aeruginosa* usher.** Work is still in progress on solving the structure of *P. aeruginosa* ushers. The yield could be improved. Apart from codon optimisation, we are also considering producing the proteins in *P. aeruginosa*, instead of *E. coli*, using a strain with an engineered T7 RNA polymerase gene on the chromosome. Production in the native host may help prevent inappropriate folding and aggregation.

**Complementation studies.** As Chapter 5 describes, *B. ambifaria* encodes a *cupB* gene cluster homologue (BamMC\_1532 - BamMC\_1537). Analysing whether the different CupB components can function in a heterologous context may provide information about the evolution of the CupB system. It can also help challenge the concept of transport of CupB5 by CupB3/TpsB4 further.

- Both ushers, CupB3 and BamMC\_1534, have an identity of 47%. It has been predicted that BamMC\_1534 is smaller than CupB3, because it lacks the POTRA-like domain at its N terminus. It would be important to assess whether BamMC\_1534 could complement a *P. aeruginosa cupB3* deletion mutant despite the lack of this domain.

- *B. ambifaria* also encodes a TpsB4 homologue: BamMC\_3283 which has a 50% sequence identity with *P. aeruginosa* TpsB4. BamMC\_3283 is encoded in an operon together with BamMC\_3284. A SMART search predicts that BamMC\_3284 has a haemagglutination activity domain (Letunic *et al.*, 2012; Schultz *et al.*, 1998). Moreover, BamMC\_3284 has a 45% identity with CupB5. The high similarity between transporters and their cognate substrates thus suggests that BamMC\_3283 can secrete CupB5. One could assess whether a *P. aeruginosa tpsB4* deletion mutant could be complemented with BamMC\_3283.

**Visualisation of CupB pili.** So far the CupC1 pili are the only *P. aeruginosa* Cup pili visualised by immuno electron microscopy (Ruer *et al.*, 2007).
• We also attempted to visualise CupB pili by electron microscopy and immuno electron microscopy using the protocol by Ruer et al. (2007). However, the results were inconclusive as only a few pili were observed and the CupB1 antibody bound unspecifically to the grid. Because CupB pili are fragile, they might have easily been sheared off during sample preparation. Thus, future work could aim at optimising the protocol for electron microscopy. One possibility would be to grow the bacteria directly on the electron microscopy grid. This method has been successfully used by Brown et al. (2001) and Weber et al. (2005) who visualised Hrp pili on the cell surface of Xanthomonas campestris and Pseudomonas syringae. Additionally, the CupB1 antibody could be purified by adsorption to prevent unspecific interactions with the grid.

7.2 Role of the CupB pilus in abiotic and biotic attachment

This study could not confirm the earlier suggestion that the CupB pilus plays a role during early stages of biofilm formation (Ruer et al., 2007). In future experiments, one may try to optimise the biofilm assays on biotic and abiotic surfaces.

• Korea et al. (2010) showed that Cup pili in UPEC attach with various affinities to different abiotic surfaces. Type 1 pili, for example, formed more biofilms on PVC than on polystyrene, depending on the temperature. The present study investigated biofilm formation only on polystyrene microtiter plates at 30°C (see Chapter 3). Different abiotic surfaces, different temperatures and incubation time should be used in future to investigate the role of CupB pili in biofilm formation on abiotic surfaces.

• P. aeruginosa might be forming biofilms in the lung of CF patients (Bjarnsholt et al., 2009). If CupB pili indeed promote biofilm formation, they might be expressed by clinical strains isolated from the lung of CF patients. To test this hypothesis, cupB gene expression in clinical strains could be verified by qRT-PCR or by Western Blot analysis using whole cell and sheared fractions.
- One could also investigate the role of CupB pili in the attachment to pulmonary cells. The attachment of PAO1ΔΔ and PAO1ΔΔΔcupB6 to pulmonary +A549 cells is currently investigated by Dr M. Guenot (Imperial College London). The attachment is expected to be specific due to the CupB6 adhesin, and thus the cupB6 mutant was first considered. However, in subsequent experiments, cupB1 and cupB5 mutants could also be tested. Dr M. Guenot used the standard strain (which does not express cupB genes under laboratory conditions) for first experiments. It was expected that the cupB gene expression might be induced upon host cell contact (see Figure 7.1). Preliminary results show that a PAO1ΔΔΔcupB6 mutant adheres less than the parental strain does. Subsequent experiments, will also use the strains where the pBAD promoter has been inserted upstream of cupB gene cluster can be used. cupB gene expression would then be induced upon arabinose addition.

Figure 7.1: Cell attachment assay. Bacteria, PAO1ΔΔ and PAO1ΔΔΔcupB6, were grown overnight shaking at 37°C, diluted to OD600 = 0.01 and added to 70% confluent A549 pulmonary cells. Bacteria and pulmonary cells were incubated for 1 hour at 37°C and then washed three times with PBS to remove non adherent bacteria. A549 cells were lysed with 0.1% TX-100 in PBS to release the adherent bacteria and the number of bacteria was estimated in colony-forming units (CFU). The experiment was carried out by Dr M. Guenot.

7.3 Role of CupB5 in P. aeruginosa pathogenesis

Kida et al. (2008) showed that the cognate TpsB4 substrate, TpsA4, is expressed and secreted by clinical P. aeruginosa strains, but not by the laboratory strain PAO1. Moreover, they showed
that TpsA4 works together with the phospholipase PlcH and degrades hemoglobin and erythrocytes (Kida et al., 2011).

- This study showed that \textit{cupB5} and \textit{tpsB4/A4} expression is under the control of the Roc1 two-component system. It implies that \textit{cupB5} and \textit{tpsA4} expression are both upregulated in clinical strains. qRT-PCR could be used to confirm this assumption.

- Kida et al. (2008) also showed that a \textit{tpsA4} deletion mutant is attenuated in a \textit{P. aeruginosa} mouse model of acute systemic infection. It would be interesting to know if a \textit{cupB5} deletion mutant is also attenuated.

- CupB5 has a haemagglutination activity domain, typical for TpsA proteins. This domain might be essential for the interaction of CupB5 with the host cell. Thus, assays similar to those described above for CupB6 could be used to investigate the role of CupB5 in attachment.

- CupB5 could also interact with other bacterial appendages to mediate the attachment of the bacterium to its target cell or surface. For example, ETEC secrete a TpsA protein, the EtpA adhesin, that interacts with the tip of the bacterial flagellum (Clements et al., 2012; Fleckenstein et al., 2006). It promotes the attachment of ETEC to eukaryotic cells, even if ETEC is distal from its target. However, this interaction allows the bacterium to then move closer to the target cell and to use other colonization factors to strengthen the attachment. Meanwhile, EtpA is degraded by the EatA protease. Interestingly, Roy et al. (2009) and collaborators showed that EtpA interacts with the flagellum, since they were able to pull down EtpA with the N terminus of flagellin. As for CupB5, immunoelectron microscopy indicated that CupB5 is associated with the tip of the CupB pilus (Ruer et al., 2008). Thus future work could aim at carrying out pull-down assays with CupB5 and CupB6 or/and CupB5 and CupB1.
7.4 Regulation of \textit{cupB} gene expression

The \textit{cupB} gene expression is under control of the Roc1 and Roc2 two-component systems (Kulasekara \textit{et al.}, 2005; Sivaneson \textit{et al.}, 2011). Intriguingly, both sensor kinases, RocS1 and RocS2, act on \textit{cupB} by a yet unknown regulatory protein. To identify this protein, mariner transposon mutagenesis could be carried out. The mariner transposon, which contains a \textit{p}\textsubscript{tac} promoter, would be introduced into a \textit{P. aeruginosa} strain that carries a \textit{cupB-lacZ} transcriptional fusion at the \textit{att} site on the chromosome. The strain would also have to carry a plasmid expressing \textit{rocS1} to induce \textit{cupB} gene expression, resulting in blue colony formation on X-gal plates. Transposon insertion into a gene involved in \textit{cupB} regulation downstream of RocS1 would then result in the appearance of white colonies. Amplification of the DNA upstream and downstream of the transposon followed by sequencing would reveal the nature of the protein. Moreover, one could then test if the expression of the \textit{tpsB4/A4} operon is also under the control of the unknown regulatory protein.
8. Experimental procedures

8.1 Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Table 8.1. *Escherichia coli* strains were grown at 37°C in LB medium containing the appropriate antibiotic concentration: ampicillin (Ap) 50 µg/ml, gentamycin (Gm) 50 µg/ml, kanamycin (Km) 25 µg/ml, tetracycline (Tet) 15 µg/ml, chloramphenicol (Cam) 34 µg/ml, and streptomycin (Sm) 50 µg/ml. Transformants were selected on LB agar plates containing Ap, Km, and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) 40 µg/ml. For protein expression, *E. coli* were grown at 30°C or 37°C in terrific broth or tryptone soya broth (TSB), and upon reaching OD\textsubscript{600} = 0.6 induced with (depending on the transformed vector) either 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) or 100/200 µg/l anhydrotetracycline.

*Pseudomonas aeruginosa* strains were grown at 37°C in LB medium containing the appropriate antibiotic concentration: 80 µg/ml Gm, 300 µg/ml carbenicillin (Carb), 50 µg/ml tetracycline or 2 mg/ml Sm. Recombinant plasmids were introduced in *P. aeruginosa* by three partner conjugation using the conjugative plasmid pRK2013. Transconjugants were selected on *Pseudomonas* isolation agar (PIA) plates supplemented with 2 mg/ml Sm, 50 µg/ml Tet, 150 µg/ml Gm and/or 600 µg/ml Carb.

For appendage preparation and whole-cell ELISAs using the RocS1 system, *P. aeruginosa* strains conjugated with pMMBrocS1 (or pMMBrocS1 and pBBR1-Mcs4) were grown under static conditions at 30°C for one day in liquid M63 medium supplemented with 0.4 % L-arginine, 1 mM MgSO\textsubscript{4}, 80 µg/ml Gm (or 80 µg/ml Gm and/or 300 µg/ml Carb), and 0.1 mM IPTG. When using the pBAD system for the shearing assay or whole-cell ELISAs, *P. aeruginosa* strains were grown static at 30°C for one day in liquid M63 medium supplemented with 0.4 % L-arginine,
1 mM MgSO$_4$, 0.4% glutamate and 0.4% arabinose. Alternatively, strains conjugated with pMMBrocS1 were grown at 30°C for one to four days on solid agar M63 medium supplemented with 0.4% L-arginine, 1 mM MgSO$_4$, 80 µg/ml Gm, and 0.1 or 1 mM IPTG. For expression studies of the cupB genes via the pBAD system in P. aeruginosa, bacteria were grown overnight at 30°C on M63 agar plates supplemented with 0.4% L-arginine, 1 mM MgSO$_4$, 0.4% glutamate and 0.1 to 1% arabinose or 0.4% glutamate.

**Table 8.1: Strains and plasmids used in this study.**

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<th>Description or phenotype</th>
<th>Reference/source</th>
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### 8.1. BACTERIAL STRAINS AND GROWTH CONDITIONS

**Table 8.1**

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<th>Strain or plasmid</th>
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### Table 8.1

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<td>( \text{tpsB4} ) and its promoter cloned into pCR2.1</td>
<td>this study</td>
</tr>
</tbody>
</table>
### Table 8.1

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description or phenotype</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCR2.1-<em>araC</em>P&lt;sub&gt;BAD&lt;/sub&gt;</td>
<td><em>P&lt;sub&gt;BAD&lt;/sub&gt;</em> promoter and <em>araC</em> gene cloned into pCR2.1</td>
<td>H. Mikkelson, PhD, Imperial College</td>
</tr>
<tr>
<td>pCR2.1-<em>P&lt;sub&gt;BAD&lt;/sub&gt;</em>-cup</td>
<td><em>P&lt;sub&gt;BAD&lt;/sub&gt;</em>-<em>araC</em> fragment cloned in front of the <em>cupB1</em> gene start</td>
<td>this study</td>
</tr>
<tr>
<td>pCR2.1-*ΔcupB1-*P&lt;sub&gt;BAD&lt;/sub&gt;</td>
<td><em>cupB1</em> mutator in pCR2.1 to delete <em>cupB1</em> in PAO1ΔΔ::<em>P&lt;sub&gt;BAD&lt;/sub&gt;</em> strains</td>
<td>this study</td>
</tr>
<tr>
<td>pCR2.1-<em>cupB1</em></td>
<td><em>cupB1</em> cloned into pCR2.1, without stop codon</td>
<td>this study</td>
</tr>
<tr>
<td>pCR2.1-<em>cupB6</em></td>
<td><em>cupB6</em> cloned into pCR2.1, without stop codon</td>
<td>this study</td>
</tr>
<tr>
<td>pCR2.1-<em>cupB1</em>-no-tag</td>
<td><em>cupB1</em> cloned into pCR2.1, with stop codon</td>
<td>this study</td>
</tr>
<tr>
<td>pCR2.1-<em>cupB6</em>-no-tag</td>
<td><em>cupB6</em> cloned into pCR2.1, with stop codon</td>
<td>this study</td>
</tr>
<tr>
<td>pCR2.1-<em>cupB3</em>-P</td>
<td>encodes CupB3’s POTRA (aa: 1-84), without signal peptide</td>
<td>this study</td>
</tr>
<tr>
<td>pCR2.1-<em>cupB3</em>-NP</td>
<td>encodes CupB3’s POTRA and Nte (aa: 1-229), without signal peptide</td>
<td>this study</td>
</tr>
<tr>
<td>pCR2.1-<em>tpsB4</em>-P1</td>
<td>encodes TpsB4’s POTRA1 (aa: 42-121), without signal peptide</td>
<td>this study</td>
</tr>
<tr>
<td>pCR2.1-<em>cupB5</em>-L</td>
<td>encodes CupB5’s TPS domain (aa: 1-121), without signal peptide</td>
<td>this study</td>
</tr>
<tr>
<td>pCR2.1-<em>tpsA4</em>-L</td>
<td>encodes TpsA’s TPS domain (aa: 1-118), without signal peptide</td>
<td>this study</td>
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<tr>
<td>pCR2.1-<em>rocS1</em></td>
<td><em>rocS1</em> mutator cloned into pCR2.1</td>
<td>this study</td>
</tr>
<tr>
<td>pCR2.1-<em>P&lt;sub&gt;BAD&lt;/sub&gt;</em>-<em>rocS1</em></td>
<td><em>P&lt;sub&gt;BAD&lt;/sub&gt;</em> and <em>araC</em> inserted upstream of <em>rocS1</em> mutator</td>
<td>this study</td>
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<tr>
<td>pKNG101-<em>ΔcupB1</em></td>
<td><em>ΔcupB1</em> mutator cloned into pKNG101</td>
<td>this study</td>
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<tr>
<td>pKNG101-<em>ΔcupB2</em></td>
<td><em>ΔcupB2</em> mutator cloned into pKNG101</td>
<td>this study</td>
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<tr>
<td>pKNG101-<em>ΔcupB3</em></td>
<td><em>ΔcupB3</em> mutator cloned into pKNG101</td>
<td>this study</td>
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<tr>
<td>pKNG101-<em>ΔcupB6</em></td>
<td><em>ΔcupB6</em> mutator cloned into pKNG101</td>
<td>this study</td>
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<tr>
<td>pKNG101-<em>ΔtpsB4</em></td>
<td><em>ΔtpsB4</em> mutator cloned into pKNG101</td>
<td>this study</td>
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<tr>
<td>pKNG101-<em>P&lt;sub&gt;BAD&lt;/sub&gt;</em>-cup</td>
<td><em>P&lt;sub&gt;BAD&lt;/sub&gt;</em>-<em>cup</em> mutator cloned into pKNG101</td>
<td>this study</td>
</tr>
<tr>
<td>pKNG101-<em>ΔcupB1</em>-P&lt;sub&gt;BAD&lt;/sub</td>
<td>&gt; <em>P&lt;sub&gt;BAD&lt;/sub&gt;</em>-<em>cup</em> mutator cloned into pKNG101</td>
<td>this study</td>
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</table>
## Table 8.1

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
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<tbody>
<tr>
<td>pKNG101-(PBAD)-(rocS1)</td>
<td>(PBAD)-(rocS1) cloned into pKNG101</td>
<td>this study</td>
</tr>
<tr>
<td>pMMB67EH-GW (pEV)</td>
<td>Broad host range vector, IncQ, (ptac), (lacZ\alpha), Gm(^R)</td>
<td>lab collection</td>
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<tr>
<td>pMMB(rocS1) (pRocS1)</td>
<td>(rocS1) gene cloned in pMMB67EH-GW, Gm(^R)</td>
<td>Kulasekara et al. (2005)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>helper plasmid, Km(^R)</td>
<td>Figurski and Helinski (1979)</td>
</tr>
<tr>
<td>pCR-Blunt-(cupB5)</td>
<td>(cupB5) gene and it’s RBS cloned into pCR-Blunt</td>
<td>this study</td>
</tr>
<tr>
<td>pCR-Blunt-(cupB5)-S(69A)</td>
<td>(cupB5)-TPS-(S69A) and it’s RBS cloned into pCR-Blunt</td>
<td>this study</td>
</tr>
<tr>
<td>pCR-Blunt-(cupB5)-N(85I)</td>
<td>(cupB5)-TPS-(N85I) and it’s RBS cloned into pCR-Blunt</td>
<td>this study</td>
</tr>
<tr>
<td>pCR-Blunt-(cupB5)-N(87D)</td>
<td>(cupB5)-TPS-(N87D) and it’s RBS cloned into pCR-Blunt</td>
<td>this study</td>
</tr>
<tr>
<td>pCR-Blunt-(cupB5)-R(93E)</td>
<td>(cupB5)-TPS-(R93E) and it’s RBS cloned into pCR-Blunt</td>
<td>this study</td>
</tr>
<tr>
<td>pCR-Blunt-(cupB5)-Q(96E)</td>
<td>(cupB5)-TPS-(Q96E) and it’s RBS cloned into pCR-Blunt</td>
<td>this study</td>
</tr>
<tr>
<td>pCR-Blunt-(cupB5)-N(98D)</td>
<td>(cupB5)-TPS-(N98D) and it’s RBS cloned into pCR-Blunt</td>
<td>this study</td>
</tr>
<tr>
<td>pCR-Blunt-(cupB5)-F(91A)</td>
<td>(cupB5)-TPS-(F91A) and it’s RBS cloned into pCR-Blunt</td>
<td>this study</td>
</tr>
<tr>
<td>pCR-Blunt-(cupB5)(_V97W),(_V99W)</td>
<td>(cupB5)_(V97W),(_V99W) cloned into pCR-Blunt</td>
<td>this study</td>
</tr>
<tr>
<td>pCR-Blunt-(cupB5)(_N85A),(_P86A)</td>
<td>(cupB5)(_N85A),(_P86A) cloned into pCR-Blunt</td>
<td>this study</td>
</tr>
<tr>
<td>pCR-Blunt-(cupB1)-RBS-(cupB2)</td>
<td>(cupB1) and (cupB2), separated by the ribosome binding side (RBS)</td>
<td>this study</td>
</tr>
<tr>
<td>pCR-Blunt-(cupB1)-RBS-(cupB4)</td>
<td>(cupB1) and (cupB4), separated by the RBS</td>
<td>this study</td>
</tr>
<tr>
<td>pCR-Blunt-(cupB6)-RBS-(cupB2)</td>
<td>(cupB6) and (cupB2), separated by the RBS</td>
<td>this study</td>
</tr>
<tr>
<td>pCR-Blunt-(cupB6)-RBS-(cupB4)</td>
<td>(cupB6) and (cupB4), separated by the RBS</td>
<td>this study</td>
</tr>
<tr>
<td>pCR-Blunt-(cupB3)-full length</td>
<td>full length (cupB3) and it’s RBS cloned into pCR-Blunt</td>
<td>this study</td>
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8.1. BACTERIAL STRAINS AND GROWTH CONDITIONS

continued: Table 8.1

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description or phenotype</th>
<th>Reference/source</th>
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<tr>
<td>pCR-Blunt-CupB5-RBS-TpsB4</td>
<td>encodes CupB5 and TpsB4-Strep, separated by the RBS</td>
<td>this study</td>
</tr>
<tr>
<td>pCR-Blunt-cupB5-mini</td>
<td>encodes full-length CupB5 with a stop codon</td>
<td>this study</td>
</tr>
<tr>
<td>pBBR1-Mcs4-cupB5</td>
<td>cupB5 gene and its RBS cloned into pBBR1-Mcs4</td>
<td>this study</td>
</tr>
<tr>
<td>pBBR1-Mcs4-cupB5-N85I</td>
<td>cupB5-TPS-N85I cloned into pBBR1-Mcs4</td>
<td>this study</td>
</tr>
<tr>
<td>pBBR1-Mcs4-cupB5-S69A</td>
<td>cupB5-TPS-S69A cloned into pBBR1-Mcs4</td>
<td>this study</td>
</tr>
<tr>
<td>pBBR1-Mcs4-cupB5-v97w,v99w</td>
<td>cupB5-v97w,v99w cloned into pBBR1-Mcs4</td>
<td>this study</td>
</tr>
<tr>
<td>pBBR1-Mcs4-cupB5-N85A,P86A</td>
<td>cupB5-N85A,P86A cloned into pBBR1-Mcs4</td>
<td>this study</td>
</tr>
<tr>
<td>pBBR1-Mcs4-cupB3</td>
<td>full length cupB3 and its RBS cloned into pBBR1-Mcs4</td>
<td>this study</td>
</tr>
<tr>
<td>pBBR1-Mcs4-cupB5-N87D</td>
<td>cupB5-TPS-N87D cloned into pBBR1-Mcs4</td>
<td>this study</td>
</tr>
<tr>
<td>pBBR1-Mcs4-cupB5-r93E</td>
<td>cupB5-TPS-r93E cloned into pBBR1-Mcs4</td>
<td>this study</td>
</tr>
<tr>
<td>pBBR1-Mcs4-cupB5-Q96E</td>
<td>cupB5-TPS-Q96E cloned into pBBR1-Mcs4</td>
<td>this study</td>
</tr>
<tr>
<td>pBBR1-Mcs4-cupB5-N98D</td>
<td>cupB5-TPS-N98D cloned into pBBR1-Mcs4</td>
<td>this study</td>
</tr>
<tr>
<td>pBBR1-Mcs4-cupB5-F91A</td>
<td>cupB5-TPS-F91A cloned into pBBR1-Mcs4</td>
<td>this study</td>
</tr>
<tr>
<td>miniCTX-tpsB4</td>
<td>tpsB4 and its promoter cloned into mini-CTX1</td>
<td>this study</td>
</tr>
<tr>
<td>miniCTX-tpsB4-D61A,D149A</td>
<td>tpsB4-D61A,D149A cloned into mini-CTX1</td>
<td>this study</td>
</tr>
<tr>
<td>miniCTX-tpsB4-H82W,P105A</td>
<td>tpsB4-H82W,P105A cloned into mini-CTX1</td>
<td>this study</td>
</tr>
<tr>
<td>miniCTX-tpsB4-G154W,E158W</td>
<td>tpsB4-G154W,E158W cloned into mini-CTX1</td>
<td>this study</td>
</tr>
<tr>
<td>miniCTX-tpsB4-K173A,T175P</td>
<td>tpsB4-K173A,T175P cloned into mini-CTX1</td>
<td>this study</td>
</tr>
<tr>
<td>Strain or plasmid</td>
<td>Description or phenotype</td>
<td>Reference/source</td>
</tr>
<tr>
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<tr>
<td>pET28b-cupB1-RBS-cupB4</td>
<td><em>cupB1</em>-RBS-<em>cupB4</em> cloned into pET28b using NcoI, XhoI</td>
<td>this study</td>
</tr>
<tr>
<td>pET28b-cupB6-RBS-cupB2</td>
<td><em>cupB6</em>-RBS-<em>cupB2</em> cloned into pET28b using NcoI, XhoI</td>
<td>this study</td>
</tr>
<tr>
<td>pET28b-cupB6-RBS-cupB4</td>
<td><em>cupB6</em>-RBS-<em>cupB4</em> cloned into pET28b using NcoI, XhoI</td>
<td>this study</td>
</tr>
<tr>
<td>pET28b-cupB1</td>
<td><em>cupB1</em> cloned into pET28b using NcoI, XhoI, His-tag</td>
<td>this study</td>
</tr>
<tr>
<td>pET28b-cupB6</td>
<td><em>cupB6</em> cloned into pET28b using NcoI, XhoI, His-tag</td>
<td>this study</td>
</tr>
<tr>
<td>pET28b-cupB1-no-tag</td>
<td><em>cupB1</em> cloned into pET28b using NcoI, XhoI, no tag</td>
<td>this study</td>
</tr>
<tr>
<td>pET28b-cupB6-no-tag</td>
<td><em>cupB6</em> cloned into pET28b using NcoI, XhoI, no tag</td>
<td>this study</td>
</tr>
<tr>
<td>pET28b-cupB1-dscB1-opt</td>
<td>encodes the codon-optimised <em>cupB1</em>, with an C-terminal Nte peptide, cloned into pET28b using NcoI, XhoI</td>
<td>this study</td>
</tr>
<tr>
<td>pET28b-cupB6-dscB1</td>
<td>encodes <em>cupB6</em>, with an C-terminal codon-optimised <em>cupB1</em> Nte peptide, cloned into pET28b using NcoI, XhoI</td>
<td>this study</td>
</tr>
<tr>
<td>pUT18c-tpsB4-P1</td>
<td>TpsB4’s POTRA-1 (aa: 42-121) N-terminally fused to the T18 polypeptide</td>
<td>this study</td>
</tr>
<tr>
<td>pUT18c-tpsA4-L</td>
<td>TpsA’s TPS domain (aa: 1-118) N-terminally fused to the T18 polypeptide</td>
<td>this study</td>
</tr>
<tr>
<td>pUT18c-cupB5-L</td>
<td>CupB5’s TPS domain (aa: 1-121) N-terminally fused to the T18 polypeptide</td>
<td>this study</td>
</tr>
<tr>
<td>pUT18c-cupB3-P</td>
<td>CupB3’s POTRA (aa: 1-84) N-terminally fused to the T18 polypeptide</td>
<td>this study</td>
</tr>
<tr>
<td>pUT18c-cupB3-PN</td>
<td>CupB3’s POTRA and Nte (aa: 1-229) N-terminally fused to the T18 polypeptide</td>
<td>this study</td>
</tr>
<tr>
<td>pKT25-tpsB4-P1</td>
<td>TpsB4’s POTRA-1 (aa: 42-121) N-terminally fused to the T25 polypeptide</td>
<td>this study</td>
</tr>
<tr>
<td>pKT25-tpsA4-L</td>
<td>TpsA’s TPS domain (aa: 1-118) N-terminally fused to the T18 polypeptide</td>
<td>this study</td>
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continued: Table 8.1

<table>
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<tr>
<th>Strain or plasmid</th>
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<tbody>
<tr>
<td>pKT25-cupB5-L</td>
<td>CupB5’s TPS domain (aa: 1-121) N-terminally fused to the T18 polypeptide</td>
<td>this study</td>
</tr>
<tr>
<td>pKT25-cupB3-P</td>
<td>CupB3’s POTRA (aa: 1-84) N-terminally fused to the T25 polypeptide</td>
<td>this study</td>
</tr>
<tr>
<td>pKT25-cupB3-PN</td>
<td>CupB3’s POTRA and Nte (aa: 1-229) N-terminally fused to the T25 polypeptide</td>
<td>this study</td>
</tr>
<tr>
<td>pMK-RQ-BamMC406_{1534}opt</td>
<td>encodes the codon-optimised BamMC406_{1534} (usher) from <em>B. ambifaria</em>, Kan(^R)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pMK-RQ-PA4540_{opt}</td>
<td>encodes the codon-optimised PA4540 (TpsB4) from <em>P. aeruginosa</em>, Kan(^R)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pMA-T-cupB1_{dscB1}_{opt}</td>
<td>encodes the codon-optimised <em>cupB1</em>, with an C-terminal Nte peptide, Amp(^R)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pMK-RQ-CupA3</td>
<td>encodes the codon-optimised CupA3, Kan(^R)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pMK-RQ-CupE5-{opt}</td>
<td>encodes the codon-optimised CupE5, Kan(^R)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pMK-RQ-CupC3-{opt}</td>
<td>encodes the codon-optimised CupC3, Kan(^R)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pASK-IBA3c-CupB5-RBS-TpsB4</td>
<td>encodes CupB5 and TpsB4-Strep, separated by the RBS, restriction sides: XhoI, XbaI</td>
<td>this study</td>
</tr>
<tr>
<td>pASK-IBA3c-CupB5</td>
<td>encodes non-tagged CupB5, restriction sides: BglII and XhoI</td>
<td>this study</td>
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<td>pASK-IBA3c-CupB3</td>
<td>expresses the CupB3 usher from PAO1, restriction side: BsaI</td>
<td>Dr A. Busch, Birkbeck College, London</td>
</tr>
<tr>
<td>pASK-IBA3c-CupA3</td>
<td>expresses the CupA3 usher from PAO1, codon optimised for <em>E. coli</em>, gene synthesised, restriction side: BsaI</td>
<td>this study</td>
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<tr>
<td>pASK-IBA3c-CupC3</td>
<td>expresses the CupC3 usher from PAO1, codon optimised for <em>E. coli</em>, gene synthesised, restriction side: BsaI</td>
<td>this study</td>
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<tr>
<td>pASK-IBA3c-CupE5</td>
<td>expresses the CupE5 usher from PAO1, codon optimised for <em>E. coli</em>, gene synthesised, restriction side: BsaI</td>
<td>this study</td>
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8. EXPERIMENTAL PROCEDURES

continued: Table 8.1

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<th>Description or phenotype</th>
<th>Reference/source</th>
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<tbody>
<tr>
<td>pASK-IBA3c_BamMC406_1534_opt</td>
<td>expression of the codon optimised usher homologue from <em>B. ambifaria</em>, gene synthesised, restriction side: BsaI</td>
<td>this study</td>
</tr>
</tbody>
</table>

8.2 Molecular biology techniques

DNA purification, extraction and quantification

Genomic DNA was isolated using the PureLink Miniprep Kit (Invitrogen) and plasmid DNA was isolated using the QIAprep Spin Miniprep and Midiprep Kits (Qiagen). PCR fragments were purified with the QIAquick PCR Purification Kit (Qiagen). Digested DNA fragments were extracted from agarose gels using the QIAquick Gel Extraction Kit (Qiagen). The concentration of purified DNA was determined with the NanoDrop 1000 spectrophotometer (NanoDrop Technologies).

Polymerase chain reaction

PCR was performed using primers purchased from Eurogentec (see Table 8.2) and DNA was sequenced by GATC Biotech, Germany. For cloning purposes, DNA fragments were amplified from the genomic DNA of *P. aeruginosa* strain PAO1 or from a purified plasmid. For up to 2 kb, the Expand High Fidelity<sup>PLUS</sup> DNA polymerase (Roche), whereas for fragments above 2 kb the KOD Hot Start DNA Polymerase (Merek) was used. For site-directed mutagenesis, the Pfu Ultra High Fidelity<sup>PLUS</sup> DNA polymerase from Agilent Technologies was used as indicated in the QuickChange II Site-directed Mutagenesis Kit (Stratagene). Overall, the PCR reactions and thermal cycling conditions used were according to the manufacturer instructions. However, 1 M betaine (Sigma) was added to each PCR reaction to prevent the formation of secondary structures during the amplification of *P. aeruginosa’s* GC-rich DNA.
To screen for positive clones, colony PCR was used. Single *E. coli* or *P. aeruginosa* colonies were resuspended in 100 µl molecular biology water and lysed by one (*E. coli*) or two (*P. aeruginosa*) cycles of freezing (10 min, -80°C) and boiling (10 min, 95°C). Cell debris were removed by centrifugation (13000 × g, 5 min, RT) and 5 µl of the DNA-containing supernatant was used for each PCR reaction. DMSO was added to a final concentration of 3% to prevent the formation of secondary structures. Taq polymerase (NEB) was used for the amplification. Amplification was initiated with an initial temperature step of 5 min at 95°C and then the following steps were repeated 30 times: DNA denaturation - 95°C for 30 sec, primer annealing - 55°C for 30 sec, and DNA elongation - 72°C for 1 min/kb.

**Gene synthesis**

Some genes, as indicated in Table 8.1, were synthesised and codon-optimised for expression in *E. coli* by GeneArt Gene Synthesis (Invitrogen).

**Restriction endonuclease digestion and ligation**

DNA fragments, amplified by PCR, were ligated into the cloning vectors pCR2.1 or pCR-Blunt by using the TA, Zero Blunt or Zero Blunt Topo cloning Kits (Invitrogen). Restriction endonucleases (Roche) were used according to the manufacturers instructions. DNA fragments were ligated into a linearised vector by using the T4 DNA polymerase (Roche) or the Quick-Stick Ligase (Bioline) as instructed by the manufacturers.

**Agarose gel electrophoresis**

DNA was separated on 0.8 - 2% (w/v) agarose gels in 0.5 x TAE buffer (50 x TAE buffer: 2 M Tris, 50 mM EDTA, 57.1 ml acetic acid, pH 8.5) supplemented with SYBR Safe DNA gel stain (Invitrogen). DNA samples were mixed with 6 x DNA loading buffer (0.25% (w/v) bromphenolblue, 30% glycerol in H$_2$O) loaded on the gel and separated at 110 V for 30 min. 1 kb or 100 bp DNA ladders (NEB) were used as a reference. DNA was visualized in a blue light transilluminator (Peqlab).
Quantitative real-time PCR

Total RNA was prepared from triplicate cell samples of PAO1ΔΔ and PAO1ΔΔ conjugated with pMMBrocessS1, stored in RNealater (Ambion), using the RNeasy extraction kit (Qiagen) in combination with on column DNaseI treatment (Applied Biosystems). The DNase treatment was carried out twice to remove all traces of chromosomal DNA. mRNA concentration was measured with the NanoDrop 1000 spectrophotometer (NanoDrop Technologies) and normalised to 20 ng/µl. cDNA was synthesized from 20 ng/µl RNA template using SuperScript III RNase H-Reverse Transcriptase (Invitrogen), 10 mM dNTPs (Bioline), RNAGuard (Roche), random hexamer oligonucleotides (Amersham), 0.1 M DTT, and X first strand buffer (RT Superscript Kit). Negative controls without reverse transcriptase were run with all reactions. The resulting cDNA was 1:5 diluted and stored at -20°C. For quantitative real-time PCR, SYBR green PCR master mix (ABI), an upstream and downstream primer pair (see Table 8.2) and water were used. Samples were analysed by the StepOne Real-Time PCR System (Applied Biosystems). The relative quantity gene expression was determined by defining the expression of rpoD mRNA as 1.

Construction of deletion mutants

PCR was used to amplify 500 bp DNA fragments upstream of the cupB1, cupB2, cupB3, cupB6 and tpsB4 genes from the P. aeruginosa genome using the primer pairs 1014/1015, 424/425, 950/951, 736/849 and 976/977, respectively, whereas 500 bp downstream DNA fragments were generated by using the primer pairs 1016/1017, 426/427, 952/953, 850/739 and 978/979, respectively. The generated DNA fragments were used as a matrix in an overlapping PCR using primer pairs 1014/1017, 424/427, 950/953, 736/739 and 976/979, to obtain the cupB1, cupB2, cupB3, cupB6 and tpsB4 mutator DNA, respectively. The mutator DNA fragments were ligated into the cloning vector pCR2.1 and re-cloned into the suicide vector pKNG101 using the restriction enzymes BamHI/ApaI for cupB1, cupB2, cupB3 and cupB6. For tpsB4 the DNA fragment was obtained from pCR2.1 using BamHI/XbaI and the fragment was ligated into pKNG101 linearised with BamHI/SpeI. Plasmids were conjugated in PAO1ΔΔ by three partner conjugation using the conjugative plasmid pRK2013. As previously described (Kaniga et al., 1991), mutants
were selected on LB agar plates supplemented with 5% sucrose and appropriate antibiotics. Colony PCR using primer pairs 1018/1019, 954/955, 740/741 and 980/981 was used to check for deletion of \textit{cupB1}, \textit{cupB2}, \textit{cupB3}, \textit{cupB6} and \textit{tpsB4}, respectively. A final colony PCR confirmed the construction of PAO1\textDelta\textDelta\textDelta\textit{cupB1}, PAO1\textDelta\textDelta\textDelta\textit{cupB2}, PAO1\textDelta\textDelta\textDelta\textit{cupB3}, PAO1\textDelta\textDelta\textDelta\textit{cupB6} and PAO1\textDelta\textDelta\textDelta\textit{tpsB4}.

**Insertion of the \textit{p}_{BAD} promoter upstream of \textit{cupB1}**

PCR was used to amplify a 554 bp DNA fragment upstream of \textit{cupB1}s promoter using primer pair 638/639 and a 558 bp DNA fragment downstream of \textit{cupB1}s promoter using primers 640/641. DNA fragments were used as matrix for an overlapping PCR using external primer pair 638/641 to obtain a \textit{p}_{BAD}\text{-cupB} mutator fragment. The mutator DNA fragment was ligated into pCR2.1. The \textit{araC}_{pBAD} promoter DNA fragment was obtained by restriction digest of pCR2.1-\textit{araC}_{pBAD} and ligated into linearised pCR2.1-\textit{p}_{BAD}\text{-cup}. The \textit{p}_{BAD}\text{-cup} fragment was re-cloned into the suicide vector pKNG101 and conjugated into PAO1\textDelta, PAO1\textDelta\textDelta\textDelta\textit{cupB2}, PAO1\textDelta\textDelta\textDelta\textit{cupB3}, PAO1\textDelta\textDelta\textDelta\textit{cupB4}, PAO1\textDelta\textDelta\textDelta\textit{cupB5} and PAO1\textDelta\textDelta\textDelta\textit{cupB6}. Mutants were selected on LB plates supplemented with 5% sucrose (Kaniga \textit{et al.}, 1991). To create PAO1\textDelta\textDelta\textDelta\textit{cupB1}, the \textit{cupB1} gene was deleted in PAO1\textDelta\textDelta::\textit{p}_{BAD}. The primer pairs 730/731 and 732/733 were used to amplify a DNA fragment upstream and downstream of \textit{cupB1}, respectively. An overlapping PCR was performed with these fragments using primer pairs 730/733. The deletion followed the same steps as described above.

**Insertion of the \textit{p}_{BAD} promoter upstream of \textit{rocS1}**

A 523 bp DNA fragment was amplified upstream and a 474 bp DNA fragment downstream of \textit{rocS1} using primers 1063/1064 and 1065/1066, respectively. DNA fragments were joined in an overlapping PCR using primers 1063/1066. The DNA fragment was ligated into pCR2.1, yielding pCR2.1-\textit{rocS1}. The \textit{araC}_{pBAD} promoter DNA fragment was inserted upstream of \textit{rocS1} by restriction digest with NdeI and NheI and the resulting \textit{p}_{BAD}\text{-rocS1} fragment cloned into pKNG101 using ApaI and SpeI. As described above, pKNG101-\textit{p}_{BAD}\text{-rocS1} was conjugated into PAO1\textDelta\textDelta to place the \textit{p}_{BAD} promoter upstream of \textit{rocS1}.
Construction of complementation vectors

PCR was used to amplify the full-length \( tpsB_{4} \) gene with its promoter and RBS and the \( cupB_{5} \) gene and its RBS from the chromosome of PAO1\( \Delta \Delta \) by using primer pairs 1315/1316 and 812/813, respectively. The \( cupB_{5} \) DNA fragment was ligated into pCR-Blunt and the \( tpsB_{4} \) gene into pCR2.1. \( tpsB_{4} \) was re-cloned into mini-CTX1 using the restriction enzymes SpeI/EcoRI thereby creating miniCTX-\( tpsB_{4} \). Positive clones were identified by colony PCR using the vector primers 667 and 1315. To obtain pBBR1-Mcs4-\( cupB_{5} \), \( cupB_{5} \) was transferred into pBBR1-Mcs4 by using PstI/SpeI/SmaI, whereas SmaI was used to linearise pCR-Blunt. Positive clones were identified by colony PCR using the vector primer M13-Rv and the \( cupB_{5} \) primer 813.

Table 8.2: Oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence(5’ → 3’)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Delta cupB_{1} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1014</td>
<td>TCC ATC CGG AAT GCG AGT</td>
<td>Deletion of ( cupB_{1} ) in PAO1( \Delta \Delta ), UpFw</td>
</tr>
<tr>
<td>1015</td>
<td>TTA CTC GTA CTG TTG CAT CTG ATT TCC G</td>
<td>Deletion of ( cupB_{1} ) in PAO1( \Delta \Delta ), UpRe</td>
</tr>
<tr>
<td>1016</td>
<td>ATG AAC AAG TAC GAG TAA GCG GCG GTC</td>
<td>Deletion of ( cupB_{1} ) in PAO1( \Delta \Delta ), DwFw</td>
</tr>
<tr>
<td>1017</td>
<td>AGC CGA ATC GAC ACT CTT CA</td>
<td>Deletion of ( cupB_{1} ) in PAO1( \Delta \Delta ), DwRe</td>
</tr>
<tr>
<td>1018</td>
<td>GAG TCG AAG GCA TGT TCG AT</td>
<td>External primer to proof deletion of ( cupB_{1} ) in PAO1( \Delta \Delta ), Fw</td>
</tr>
<tr>
<td>1019</td>
<td>GGC GTC GGA TTA TTT ACC TG</td>
<td>External primer to proof deletion of ( cupB_{1} ) in PAO1( \Delta \Delta ), Re</td>
</tr>
<tr>
<td>( \Delta cupB_{2} )</td>
<td></td>
<td></td>
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<tr>
<td>424</td>
<td>GAA GTC GCG TGC GAT ATC AAC GGC CAG GCG CC</td>
<td>Deletion of ( cupB_{2} ) in PAO1( \Delta \Delta ), UpFw</td>
</tr>
<tr>
<td>425</td>
<td>TTA TTT GCC CGG CGC CAT GGC CTC ACC TCT TCG</td>
<td>Deletion of ( cupB_{2} ) in PAO1( \Delta \Delta ), UpRe</td>
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<tr>
<td>426</td>
<td>ATG GGC CCG GGC AAA TAA CCC CTC CGT TTC CCC</td>
<td>Deletion of ( cupB_{2} ) in PAO1( \Delta \Delta ), DwFw</td>
</tr>
<tr>
<td>427</td>
<td>CGG CTC TTG AGC AGC AGG TCG AGG CGA TAG CGG</td>
<td>Deletion of ( cupB_{2} ) in PAO1( \Delta \Delta ), DwRe</td>
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</table>
## 8.2. MOLECULAR BIOLOGY TECHNIQUES

**continued: Table 8.2**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence ( (5' \rightarrow 3') )</th>
<th>Purpose</th>
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<tr>
<td>428</td>
<td>TCT TGC GCC CCT CGT TGC GT</td>
<td>external primer to proof deletion of ( \Delta cupB2 ) in PAO1ΔΔ, Fw</td>
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<tr>
<td>429</td>
<td>CCA GCA GGC CCC GGT CGT AG</td>
<td>external primer to proof deletion of ( \Delta cupB2 ) in PAO1ΔΔ, Re</td>
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</table>

### \( \Delta cupB3 \)

<table>
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<th>Sequence ( (5' \rightarrow 3') )</th>
<th>Purpose</th>
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<tr>
<td>950</td>
<td>GCG TCG AAT TGA TTG TCG AT</td>
<td>deletion of ( \Delta cupB3 ) in PAO1ΔΔ, UpFw</td>
</tr>
<tr>
<td>951</td>
<td>CGT CGT CTC AAC GGC CAC CGC GGG CGC</td>
<td>deletion of ( \Delta cupB3 ) in PAO1ΔΔ, UpRe</td>
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<tr>
<td>952</td>
<td>GTG GCC GTT GAG ACG ACG CCT CCC GTC</td>
<td>deletion of ( \Delta cupB3 ) in PAO1ΔΔ, DwFw</td>
</tr>
<tr>
<td>953</td>
<td>GAC GGA ACA GGA GCT TCA TCC</td>
<td>deletion of ( \Delta cupB3 ) in PAO1ΔΔ, DwRe</td>
</tr>
<tr>
<td>954</td>
<td>AGG TTC CCT TCA GTG TCA CG</td>
<td>external primer to proof deletion of ( \Delta cupB3 ) in PAO1ΔΔ, Fw</td>
</tr>
<tr>
<td>955</td>
<td>GTG ATA GGG ACT GCG GTT GT</td>
<td>external primer to proof deletion of ( \Delta cupB3 ) in PAO1ΔΔ, Re</td>
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### \( \Delta cupB6 \)

<table>
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<th>Primer</th>
<th>Sequence ( (5' \rightarrow 3') )</th>
<th>Purpose</th>
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<tr>
<td>736</td>
<td>AAC TGG TCA ACG TGG ATG C</td>
<td>deletion of ( \Delta cupB6 ) in PAO1ΔΔ, UpFw</td>
</tr>
<tr>
<td>849</td>
<td>GGT AAA CGT CAT GCT CAT GGA ACT CTC</td>
<td>deletion of ( \Delta cupB6 ) in PAO1ΔΔ, UpRe</td>
</tr>
<tr>
<td>850</td>
<td>ATG AGC ATG ACG TTT ACC TTC CCC TGA</td>
<td>deletion of ( \Delta cupB6 ) in PAO1ΔΔ, DwFw</td>
</tr>
<tr>
<td>739</td>
<td>CCC CGG TTC AAA GTG AAC AT</td>
<td>deletion of ( \Delta cupB6 ) in PAO1ΔΔ, DwRe</td>
</tr>
<tr>
<td>740</td>
<td>CAT GCT CGG AAA CCT GAA AG</td>
<td>external primer to proof deletion of ( \Delta cupB6 ) in PAO1ΔΔ, Fw</td>
</tr>
<tr>
<td>741</td>
<td>CAT CTC CAA AGA ACC GCA CT</td>
<td>external primer to proof deletion of ( \Delta cupB6 ) in PAO1ΔΔ, Re</td>
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### \( \Delta tpsB4 \)

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<tr>
<td>976</td>
<td>CTT AAA CCG AGA ACG GCA TT</td>
<td>deletion of ( \Delta tpsB4 ) (PA4540) in PAO1ΔΔ, UpFw</td>
</tr>
<tr>
<td>977</td>
<td>TCA GAA GTA CAT TCC CAT GAG GCC CGT</td>
<td>deletion of ( \Delta tpsB4 ) (PA4540) in PAO1ΔΔ, UpRe</td>
</tr>
</tbody>
</table>
**Table 8.2**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence(5' → 3')</th>
<th>Purpose</th>
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</thead>
<tbody>
<tr>
<td>978</td>
<td>ATG GGA ATG TAC TTC TGA GCA AGC TCC</td>
<td>deletion of <em>tpsB</em>&lt;sub&gt;4&lt;/sub&gt; (PA4540) in PAO1ΔΔ, DwFw</td>
</tr>
<tr>
<td>979</td>
<td>CAG GGT GGA AGC CAC CAG</td>
<td>deletion of <em>tpsB</em>&lt;sub&gt;4&lt;/sub&gt; (PA4540) in PAO1ΔΔ, DwRe</td>
</tr>
<tr>
<td>980</td>
<td>GCC AAT CGA TCG AGA TGA AG</td>
<td>external primer to proof deletion of <em>tpsB</em>&lt;sub&gt;4&lt;/sub&gt; (PA4540) in PAO1ΔΔ, Fw</td>
</tr>
<tr>
<td>981</td>
<td>CCG TCG AAA TTG AGG TTG AT</td>
<td>external primer to proof deletion of <em>tpsB</em>&lt;sub&gt;4&lt;/sub&gt; (PA4540) in PAO1ΔΔ, Re</td>
</tr>
</tbody>
</table>

**Δ*cupB*<sub>1</sub>::*p*<sub>BAD</sub>**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence(5' → 3')</th>
<th>Purpose</th>
</tr>
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<tbody>
<tr>
<td>730</td>
<td>GTC GGC AAA CAA ATT CTC GT</td>
<td>Insertion of <em>p</em>&lt;sub&gt;BAD&lt;/sub&gt; in Δ<em>cupB</em>&lt;sub&gt;1&lt;/sub&gt;, UpFw</td>
</tr>
<tr>
<td>731</td>
<td>TTA CTC GTA CTT GTT CAT CTG ATT TCC</td>
<td>Insertion of <em>p</em>&lt;sub&gt;BAD&lt;/sub&gt; in Δ<em>cupB</em>&lt;sub&gt;1&lt;/sub&gt;, UpRe</td>
</tr>
<tr>
<td>732</td>
<td>ATG AAC AAG TAC GAG TAA GCG GCG GTC</td>
<td>Insertion of <em>p</em>&lt;sub&gt;BAD&lt;/sub&gt; in Δ<em>cupB</em>&lt;sub&gt;1&lt;/sub&gt;, DwFw</td>
</tr>
<tr>
<td>733</td>
<td>CCC CCT GAC TCC AGA AAC TT</td>
<td>Insertion of <em>p</em>&lt;sub&gt;BAD&lt;/sub&gt; in Δ<em>cupB</em>&lt;sub&gt;1&lt;/sub&gt;, DwRe</td>
</tr>
<tr>
<td>734</td>
<td>ATG ATT TGC CCA AAC AGG TC</td>
<td>External primer to proof insertion of Δ<em>cupB</em>&lt;sub&gt;1&lt;/sub&gt;, Fw</td>
</tr>
<tr>
<td>735</td>
<td>TAT TTG CCG AGT GCC CTA TC</td>
<td>External primer to proof insertion of Δ<em>cupB</em>&lt;sub&gt;1&lt;/sub&gt;, Re</td>
</tr>
</tbody>
</table>

***cupB*::*p*<sub>BAD</sub>**

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<tr>
<th>Primer</th>
<th>Sequence(5' → 3')</th>
<th>Purpose</th>
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<tr>
<td>638</td>
<td>AAA GGA TCC CGG GAA GGA CGT GGT TGT C</td>
<td>Insertion of <em>p</em>&lt;sub&gt;BAD&lt;/sub&gt; upstream of <em>cupB</em>&lt;sub&gt;1&lt;/sub&gt;, UpFw</td>
</tr>
<tr>
<td>639</td>
<td>ACA GCT AGC TTT CAT ATG TCC TGG CTG CTC CCT GGA G</td>
<td>Insertion of <em>p</em>&lt;sub&gt;BAD&lt;/sub&gt; upstream of <em>cupB</em>&lt;sub&gt;1&lt;/sub&gt;, UpRe</td>
</tr>
<tr>
<td>640</td>
<td>GGA CAT ATG AAA GCT AGC TGT TCC ACA ACT CCA AAG G</td>
<td>Insertion of <em>p</em>&lt;sub&gt;BAD&lt;/sub&gt; upstream of <em>cupB</em>&lt;sub&gt;1&lt;/sub&gt;, DwFw</td>
</tr>
<tr>
<td>641</td>
<td>AAA GGG CCC TGG ACA CAT AGG AGG CCT TG</td>
<td>Insertion of <em>p</em>&lt;sub&gt;BAD&lt;/sub&gt; upstream of <em>cupB</em>&lt;sub&gt;1&lt;/sub&gt;, DwRe</td>
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<tr>
<td>642</td>
<td>GAG TCG AAG GCA TGT TCG AT</td>
<td>External primer to proof insertion of <em>p</em>&lt;sub&gt;BAD&lt;/sub&gt; upstream of <em>cupB</em>&lt;sub&gt;1&lt;/sub&gt;, Fw</td>
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<tr>
<td>643</td>
<td>GAG GTA GCG AAT ACG GGA CA</td>
<td>External primer to proof insertion of <em>p</em>&lt;sub&gt;BAD&lt;/sub&gt; upstream of <em>cupB</em>&lt;sub&gt;1&lt;/sub&gt;, Re</td>
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8.2. MOLECULAR BIOLOGY TECHNIQUES

continued: Table 8.2

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence(5′ → 3′)</th>
<th>Purpose</th>
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<tr>
<td>PAO1ΔΔ::pBAD-rocS1</td>
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</tr>
<tr>
<td>1063</td>
<td>GTT GCT GAC CGA GTT CCA C</td>
<td>Insertion of $p_{BAD}$ upstream of rocS1, UpFw(1)</td>
</tr>
<tr>
<td>1064</td>
<td>GCA GCT AGC TTT CAT ATG GTT GTA TGG AAA GGA AAC G</td>
<td>Insertion of $p_{BAD}$ upstream of rocS1, UpRe(2)</td>
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<tr>
<td>1065</td>
<td>AAC CAT ATG AAA GCT AGC TGC CGG CCC TGC CGC ACA G</td>
<td>Insertion of $p_{BAD}$ upstream of rocS1, DwFw(3)</td>
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<tr>
<td>1066</td>
<td>GAG ATC CTC CGG CGA GTT</td>
<td>Insertion of $p_{BAD}$ upstream of rocS1, DwRe(4)</td>
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<tr>
<td>1067</td>
<td>TGT TCT GCC AAC TGT TCA GC</td>
<td>External primer to proof insertion of $p_{BAD}$ upstream of rocS1, Fw(5)</td>
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<tr>
<td>1068</td>
<td>ACA GGT CGG TAT GTC CGA AA</td>
<td>External primer to proof insertion of $p_{BAD}$ upstream of cupB1, Re(6)</td>
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<tr>
<td>qPCR cupB1up</td>
<td>GCG CGG ACC CAT TTC AC</td>
<td>qPCR</td>
</tr>
<tr>
<td>qPCR cupB1down</td>
<td>GCG ATG GAA ACC TTC TTG TCA</td>
<td>qPCR</td>
</tr>
<tr>
<td>qPCR cupA1 F</td>
<td>GCG GCA AAC ACT ATC ACA TTC A</td>
<td>qPCR</td>
</tr>
<tr>
<td>qPCR cupA1 R</td>
<td>CCG GTA CGC TGT CGA GGA T</td>
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</tr>
<tr>
<td>qPCR tpsA4fw</td>
<td>GGC GCC ACG GTC GTT</td>
<td>PA4541</td>
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<tr>
<td>qPCR tpsA4rev</td>
<td>TGA ACT CGT TCC AAT TGG TGA T</td>
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<tr>
<td>qPCR tpsB4fw</td>
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<td>ATC GCG AAG TCG CTT GTT TT</td>
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<td>ACC ATT TCC GCG ACT ACA AC</td>
<td>PA2462</td>
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<td>qTps1_Rv</td>
<td>ACC TGG TTG AGG ATC ACC TG</td>
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<tr>
<td>qTps2_Fw</td>
<td>GGA AGG TGC TGA AAG ACA GC</td>
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### Table 8.2

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<th>Primer</th>
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<tbody>
<tr>
<td>qTPS2_RV</td>
<td>GGT CTA CCG TGA CCT TTC CA</td>
<td>PA0041</td>
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<tr>
<td>qTPS3_Fw</td>
<td>GAC GCC TAC GTC AAC AGT CA</td>
<td>PA4625 ($cdrA$)</td>
</tr>
<tr>
<td>qTPS3_Rv</td>
<td>GTT ACC GGT GAT CGA GTA CT</td>
<td>PA4625 ($cdrA$)</td>
</tr>
<tr>
<td>qTPS5_Fw2</td>
<td>CCG TCA GGT CAT CCT GTC TT</td>
<td>PA0690</td>
</tr>
<tr>
<td>qTPS5_Rv2</td>
<td>TAG AGG GAT CTG GCA TCC AC</td>
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<tr>
<td>rpoD Fwd</td>
<td>AGG CCG TGA GCA GGG ATA C</td>
<td>house keeping gene</td>
</tr>
<tr>
<td>rpoD Rev</td>
<td>TCC CCA TGT CGT TGA TCA TG</td>
<td>house keeping gene</td>
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### Complementation

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<tr>
<td>1315</td>
<td>AAA GAA TTC CGG AAT CGC GCT CAG CCG C</td>
<td>cloning of $tpsB4$ and its promoter into mini-CTX1</td>
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<tr>
<td>1316</td>
<td>TTT ACT AGT CGG GAG CTT GCT CAG AAG TA</td>
<td>cloning of $tpsB4$ and its promoter into mini-CTX1</td>
</tr>
<tr>
<td>812</td>
<td>GAA TAG GTC GCG GGG TTT</td>
<td>cloning of $cupB5$ and its RBS into pBBR1-Mcs4</td>
</tr>
<tr>
<td>813</td>
<td>CCA CAC TCC AGG CGA GAG</td>
<td>cloning of $cupB5$ and its RBS into pBBR1-Mcs4</td>
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<tr>
<td>1140</td>
<td>GCC GGT ACC CGC GCC ATT CTC GAC</td>
<td>cloning of $cupB3$ and its RBS into pBBR1-Mcs4, KpnI</td>
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<td>1094</td>
<td>GCT CTA GAC GGT CAC AGC GTG CG</td>
<td>cloning of $cupB3$ and its RBS into pBBR1-Mcs4, XbaI</td>
</tr>
</tbody>
</table>

### Point mutations

<table>
<thead>
<tr>
<th>Line</th>
<th>Sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1819</td>
<td>TTT CCT GTG CCG CGA GGA AGG CGC G</td>
<td>Introduction of a SPM in TpsB4 P105A ($c352g_{antisense}$)</td>
</tr>
<tr>
<td>1820</td>
<td>CTC AAT CTG AGC CAA TTG TGG GCC GCC GCC GCC</td>
<td>Introduction of a SPM in TpsB4 H82W ($c283t_{284g_{285g}}$)</td>
</tr>
<tr>
<td>1821</td>
<td>CGC GCC TTC CTC GCG GCA CAG GAA A</td>
<td>Introduction of an double point mutation in TpsB4 P105A ($c352g$)</td>
</tr>
</tbody>
</table>
### Table 8.2

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence ((5' \rightarrow 3'))</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1822</td>
<td>GGC GGC GGC GGC CCA CAA TTG GCT CAG ATT GAG</td>
<td>Introduction of a double point mutation in TpsB4 H82W ((c283t, a284g, c285g, \text{antisense}))</td>
</tr>
<tr>
<td>1823</td>
<td>CCG CGG TCC AGT GGT CGG AGC TGT GGC GTG CGC TTC</td>
<td>Introduction of a double point mutation in TpsB4 G154W E158W ((g499t, c501g, g511t, a512g))</td>
</tr>
<tr>
<td>1824</td>
<td>GAA GCG CAC GCC ACA GCT CCG ACC ACT GGA CCG CGG</td>
<td>Introduction of a double point mutation in TpsB4 G154W E158W ((g499t, c501g, g511t, a512g, \text{antisense}))</td>
</tr>
<tr>
<td>1825</td>
<td>GGC CTC CAG GCC GCA GCC CCC CTG CTG CCG G</td>
<td>Introduction of a double point mutation in TpsB4 K173A T175P ((a556g, a557c, a562c))</td>
</tr>
<tr>
<td>1826</td>
<td>CCG GCA GCA GGG GGC CTG CGG CCT GGA GGC C</td>
<td>Introduction of a double point mutation in TpsB4 K173A T175P ((a556g, a557c, a562c, \text{antisense}))</td>
</tr>
<tr>
<td>1827</td>
<td>CAG CAG GCG TCG AGC GTC GAA CTG CCT</td>
<td>Introduction of a SPM in TpsB4 D61A ((a221c, \text{antisense}))</td>
</tr>
<tr>
<td>1828</td>
<td>CTG GAC CGG GGT AGC TCG TTC GAG TGC</td>
<td>Introduction of a SPM in TpsB4 D149A ((a485c, \text{antisense}))</td>
</tr>
<tr>
<td>1829</td>
<td>AGG CAG TTC GAC GCT CGA CGC CTG CTG</td>
<td>Introduction of a SPM in TpsB4 D61A ((a221c))</td>
</tr>
<tr>
<td>1830</td>
<td>GCA CTC GAA CGA GCT ACC GCG GTC CAG</td>
<td>Introduction of a SPM in TpsB4 D149A ((a485c))</td>
</tr>
<tr>
<td>1802</td>
<td>GAA GCC ACC AGG CCG CCC CAA TTC CAC TGG GCG CCG CGA CCG A</td>
<td>Introduction of a double point mutation in CupB5 V97W V99W ((g481t, t449g, a450g, g454t, c455g, c456g, \text{antisense}))</td>
</tr>
<tr>
<td>1803</td>
<td>TCG GTC GCG GGC CCC AGT GGA ATT GGG GCG GCC TGG TGG CTT C</td>
<td>Introduction of a double point mutation in CupB5 V97W V99W ((g481t, t449g, c450g, g454t, c455g, c456g))</td>
</tr>
<tr>
<td>1804</td>
<td>CAG GTC TTC CTG GTC GCC GCC AAC GGC GTG CTC T</td>
<td>Introduction of a double point mutation in CupB5 N85A P86A ((a412g, a413c, c415g))</td>
</tr>
<tr>
<td>1805</td>
<td>AGA GCA CGC CGT TGG CGG CGA CCA GGA AGA CCT G</td>
<td>Introduction of a double point mutation in CupB5 N85A P86A ((a412g, a413c, c415g, \text{antisense}))</td>
</tr>
<tr>
<td>814</td>
<td>CGG CAC CAA GGC CGC CGA TAT CCA GGG C</td>
<td>SPM in the TPS motive of CupB5 S69A ((a364g, g365c))</td>
</tr>
</tbody>
</table>
### Table 8.2

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence($5' \rightarrow 3'$)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>815</td>
<td>GCC CTG GAT ATC GGC GGC CTT GGT GCC G</td>
<td>SPM in the TPS motive of CupB5 S69A (a364g_g365c_antisense)</td>
</tr>
<tr>
<td>816</td>
<td>CCA GGT CTT CCT GGT CAT CCC CAA CGG</td>
<td>SPM in the TPS motive of CupB5 N85I (a413t)</td>
</tr>
<tr>
<td>817</td>
<td>CCG TTG GGG ATG ACC AGG AAG ACC TGG</td>
<td>SPM in the TPS motive of CupB5 N85I (a413t_antisense)</td>
</tr>
<tr>
<td>1235</td>
<td>CCT GGT CAA CCC CGA CGG CGT GCT CTT</td>
<td>SPM in the TPS motive of CupB5 N87D (a418g)</td>
</tr>
<tr>
<td>1236</td>
<td>AAG AGC ACG TCG GGG TTG ACC AGG</td>
<td>SPM in the TPS motive of CupB5 N87D (a418g_antisense)</td>
</tr>
<tr>
<td>1237</td>
<td>GCG CCC AGG TCG ATG TCG GCG GC</td>
<td>SPM in the TPS motive of CupB5 N98D (a451g)</td>
</tr>
<tr>
<td>1238</td>
<td>GCC GCC GAC ATC GAC CTG GGC GC</td>
<td>SPM in the TPS motive of CupB5 N98D (a451g_antisense)</td>
</tr>
<tr>
<td>1241</td>
<td>CAA CGG CGT GCT CTT CGG TGA CGG CGC CCA GG</td>
<td>SPM in the TPS motive of CupB5 R93E (c436g_g437a_g438g)</td>
</tr>
<tr>
<td>1242</td>
<td>CCT GGG CGC CCT CAC CGA AGA GCA CGC CGT TG</td>
<td>SPM in the TPS motive of CupB5 R93E (c436g_g437a_g438g_antisense)</td>
</tr>
<tr>
<td>1243</td>
<td>GGT CGC GGC GCC GAG GTC AAT GTC G</td>
<td>SPM in the TPS motive of CupB5 Q96E (c445g)</td>
</tr>
<tr>
<td>1244</td>
<td>CGA CAT TGA CCT CGG CGC CGC GAC C</td>
<td>SPM in the TPS motive of CupB5 Q96E (c445g_antisense)</td>
</tr>
<tr>
<td>1245</td>
<td>CAA CGG CGT GCT CGC CGG TCG CGG CGC C</td>
<td>SPM in the TPS motive of CupB5 F91A (c530g_c531c)</td>
</tr>
<tr>
<td>1246</td>
<td>GCC GCC GCG ACC GCC GAG CAC GCC GTT G</td>
<td>SPM in the TPS motive of CupB5 F91A (c530g_c531c_antisense)</td>
</tr>
</tbody>
</table>

**Expression**

| 1522   | CCA TGG CAC TGA ACG AGG TCG CCC TGA ACT GCT CG | cloning of cupB6_\_dsc1 optimised |
| 1523   | CTC GAG TTC GGT AAT ATT GCC GCT AAA ATT CAC GAT GCC ATC CAC TTT ATT ATC GCT GGG GAA GGT AAA CGT GAA CTC GGC GGC GCC GTC | cloning of cupB6_\_dsc1 optimised |
| 1831   | CTC GAG GGG GAA GGT AAA CGT G | amplification of cupB6 to clone into pET28b, Re |
**8.2. MOLECULAR BIOLOGY TECHNIQUES**

continued: Table 8.2

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence ($5' \rightarrow 3'$)</th>
<th>Purpose</th>
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</thead>
<tbody>
<tr>
<td>1832</td>
<td>CTC GAG CTC GTA CGC CAG GAG</td>
<td>amplification of <em>cupB1</em> to clone into pET28b, Re</td>
</tr>
<tr>
<td>1908</td>
<td>CTC GAG TCA CTC GTA CGC CAG GAG</td>
<td>amplification of <em>cupB1</em> with stop to clone into pET28b</td>
</tr>
<tr>
<td>1909</td>
<td>CTC GAG TCA GGG GAA GGT AAA CGT G</td>
<td>amplification of <em>cupB6</em> with stop to clone into pET28b</td>
</tr>
<tr>
<td>1694</td>
<td>AAC CAT GGC AAT GAA CAA GTT CAA ACG</td>
<td>cloning of CupB1 and CupB2 in tandem into pET28b, tanB1-B2-up-Fw</td>
</tr>
<tr>
<td>1695</td>
<td>GCG CCA TTG GTA TAT CTC CTT CTT ACT CGT ACG CCA GGA GGT AG</td>
<td>cloning of CupB1 and CupB2 in tandem into pET28b, tanB1-B2-up-Rv</td>
</tr>
<tr>
<td>1696</td>
<td>CGA GTA AGA AGG AGA TAT ACC AAT GGC GCC GCT AAT GCA TCG</td>
<td>cloning of CupB1 and CupB2 in tandem into pET28b, tanB1-B2-dw-Fw</td>
</tr>
<tr>
<td>1697</td>
<td>TTC TCG AGT TTG CCG AGT GCC CTA TCG</td>
<td>cloning of CupB1 and CupB2 in tandem into pET28b, tanB1-B2-dw-Rv</td>
</tr>
<tr>
<td>1698</td>
<td>AAC CAT GGC AAT GAG CAT GAG GTT ATT TC</td>
<td>cloning of CupB6 and CupB4 in tandem into pET28b, tanB6-B4-up-Fw</td>
</tr>
<tr>
<td>1699</td>
<td>GGT CAC TGG TAT ATC TCC TTC TCA GGG GAA GGT AAA CGT GAA CT</td>
<td>cloning of CupB6 and CupB4 in tandem into pET28b, tanB6-B4-up-Rv</td>
</tr>
<tr>
<td>1700</td>
<td>CCC TGA GAA GGA GAT ATA CCA GTG ACC GCC GTC GCC CTG GGC CG</td>
<td>cloning of CupB6 and CupB4 in tandem into pET28b, tanB6-B4-dw-Fw</td>
</tr>
<tr>
<td>1701</td>
<td>TTC TCG AGC GGG TAT CTC TCT GCC GCC GC</td>
<td>cloning of CupB6 and CupB4 in tandem into pET28b, tanB6-B4-dw-Rv</td>
</tr>
<tr>
<td>1708</td>
<td>CGG TCA CGG TAT ATC TCC TTC TTA CTC GTA CGC CAG GAG GTA G</td>
<td>cloning of CupB1 and CupB4 in tandem into pET28b, tanB1-B4-up-Rv</td>
</tr>
<tr>
<td>1709</td>
<td>CGA GTA AGA AGG AGA TAT ACC GTG ACC GCC GTC GCC CTG GGC C</td>
<td>cloning of CupB1 and CupB4 in tandem into pET28b, tanB1-B4-dw-Fw</td>
</tr>
<tr>
<td>1710</td>
<td>GCG CCA TTG GTA TAT CTC CTT CTC AGG GGA AGG TAA ACG TGA AC</td>
<td>cloning of CupB6 and CupB2 in tandem into pET28b, tanB6-B2-up-Rv</td>
</tr>
<tr>
<td>1711</td>
<td>CCC CTG AGA AGG AGA TAT ACC AAT GGC GCC GCT AAT GCA TCG</td>
<td>cloning of CupB6 and CupB2 in tandem into pET28b, tanB6-B2-dw-Fw</td>
</tr>
</tbody>
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## Table 8.2

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′ → 3′)</th>
<th>Purpose</th>
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</thead>
<tbody>
<tr>
<td>1712</td>
<td>AAT CTA GAG AAG GAG ATA TAC CAT GAA CAA ATG CTA TGC ACT GG</td>
<td>cloning of CupB5 and TpsB4 in tandem into pASK-IBA3c, CupB5-TpsB4-up-Fw</td>
</tr>
<tr>
<td>1713</td>
<td>CAT ACC CAT GGT ATA TCT CCT TCT TAC CAG ATG TTG CCG TAG TCG</td>
<td>cloning of CupB5 and TpsB4 in tandem into pASK-IBA3c, CupB5-TpsB4-up-Rv</td>
</tr>
<tr>
<td>1714</td>
<td>CTG GTA AGA AGG AGA TAT ACC ATG GGT ATG GCA ATG CTG AGC AG</td>
<td>cloning of CupB5 and TpsB4 in tandem into pASK-IBA3c, CupB5-TpsB4-dw-Fw</td>
</tr>
<tr>
<td>1715</td>
<td>TTC TCG AGA AAA TAA CGG GTG GCC TGC AGC CAC</td>
<td>cloning of CupB5 and TpsB4 in tandem into pASK-IBA3c, CupB5-TpsB4-dw-Rv</td>
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### Screening

<table>
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<tr>
<th>RpKN</th>
<th>CAT ATC ACA ACG TGC GTG GA</th>
<th>pKNG101-specific primer</th>
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<tbody>
<tr>
<td>UpKN</td>
<td>CCC TGG ATT TCA CTG ATG AG</td>
<td>pKNG101-specific primer</td>
</tr>
<tr>
<td>M13-Rev</td>
<td>CAG GAA ACA GCT ATG ACC</td>
<td>pCR2.1/ pBBR1-Mcs4-specific primer</td>
</tr>
<tr>
<td>M13-Fw</td>
<td>TGT AAA ACG ACG GCC AGT</td>
<td>pCR2.1/ pBBR1-Mcs4-specific primer</td>
</tr>
<tr>
<td>1514</td>
<td>GAG TTA TTT TAC CAC TCC CT</td>
<td>pASK-IBA3c_FW</td>
</tr>
<tr>
<td>1515</td>
<td>CGC AGT AGC GGT AAA CG</td>
<td>pASK-IBA3c_Rv</td>
</tr>
</tbody>
</table>

### BTH

| 1203   | AAA TCT AGA GGG CGG CCC GAC GGT GCT G | cloning of TpsB4’s POTRA1 (aa: 42-121) |
| 1204   | TTT GGT ACC TTA TCC CTC GAG CAC TCC GAT | cloning of TpsB4’s POTRA1 (aa: 42-121) |
| 1207   | AAA TCT AGA GCT GCC AAG CGG GGG AAC G | cloning of CupB5’s TPS domain (aa: 1-121) |
| 1208   | TTT GGT ACC TTA GTA TCT GGA GGA GTT GCC | cloning of CupB5’s TPS domain (aa: 1-121) |
| 1209   | AAA TCT AGA GCT GCC CAG CGG CGC CAC G | cloning of TpsA’s TPS domain (aa: 1-118) |
| 1210   | TTT GGT ACC TTA GTA GTT GCC GGC GAG GAA | cloning of TpsA’s TPS domain (aa: 1-118) |
| 1224   | AAA TCT AGA GGG GCA GGT CGC CCT GTT G | cloning of CupB3’s POTRA (aa: 1-84) |
Introduction of point mutations in the plasmid-encoded *cupB5* and *tpsB4*

To perform the amino acid substitutions in *tpsB4* and *cupB5*, point mutations were introduced into pCR2.1-*tpsB4*mi and pCR-Blunt-*cupB5* by using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene). To create *tpsB4*<sub>G154W,E158W</sub> and *tpsB4*<sub>K173A,T175P</sub> the primer pairs 1823/1824 and 1825/1826 were used (see Table 8.2). To clone *tpsB4*<sub>D61A,D149A</sub> and *tpsB4*<sub>H82W,P105A</sub> two rounds of amplification were carried out. Use of the primer pairs 1819/1821 and 1827/1830 introduced the first point mutation H59W and D47A into *tpsB4*, respectively. A second PCR using the primers 1820/1822 and 1828/1830 introduced the second mutation into *tpsB4*<sub>H82W,P105A</sub> and *tpsB4*<sub>D61A,D149A</sub>. Amplified plasmids were transformed into *E. coli* strain XL1-Blue and successful mutation was confirmed by sequencing. The mutated *tpsB4* genes were cloned into mini-CTX1 using the restriction enzymes SpeI and EcoRI. The miniCTX-*tpsB4* variants were then conjugated into PAO<sub>1ΔΔΔtpsB4*/pMMBrocS1</sub>.

To engineer *cupB5*<sub>N85A,P86A</sub> and *cupB5*<sub>V97W,V99W</sub> primer pairs 1804/1805 and 1802/1803 were used, respectively. For cloning *cupB5*<sub>S69A</sub> primers 814/815, for *cupB5*<sub>N85I</sub> primers 816/817, for *cupB5*<sub>N87D</sub> primers 1235/1236, for *cupB5*<sub>N98D</sub> primers 1237/1238, for *cupB5*<sub>R93E</sub> primers 1241/1242, for *cupB5*<sub>Q96E</sub> primers 1243/1244 and for *cupB5*<sub>F91A</sub> primers 1245/1246 were used. Amplified pCR-Blunt-*cupB5* variants were transformed into *E. coli* strain XL1-Blue and successful mutation was confirmed by sequencing. A 600 bp *cupB5* DNA fragment containing the point mutations was transferred into pBBR1-MCS4-*cupB5* by using the restriction enzymes SfaAI and SgrDI. Positive clones were identified by sequencing. pBBR1-MCS4-*cupB5*<sub>N85A,P96A</sub>, pBBR1-MCS4-*cupB5*<sub>V107W,V109W</sub>, pBBR1-MCS4-*cupB5*<sub>S69A</sub>, pBBR1-MCS4-*cupB5*<sub>N85I</sub>, pBBR1-MCS4-*cupB5*<sub>N87D</sub>, pBBR1-MCS4-*cupB5*<sub>R93D</sub>, pBBR1-MCS4-*cupB5*<sub>Q96E</sub>,...
pBBR1-MCS4-cupB5\textsubscript{N98D} and pBBR1-MCS4-cupB5\textsubscript{F91A} were conjugated into PAO1\Delta\Delta\Delta cupB5/pMMBrocS1.

Construction of pET28b variants

PCR was used to amplify an up DNA fragment, encoding $cupB1$ or $cupB6$, using primer pairs 1694/1695 ($cupB1$), 1694/1708 ($cupB1$), 1698/1699 ($cupB6$) and 1698/1710 ($cupB6$). A down DNA fragment, encoding $cupB2$ or $cupB4$, was amplified with primers 1696/1697 ($cupB2$), 1711/1697 ($cupB2$), 1700/1701 ($cupB4$) and 1709/1701 ($cupB4$). The up and down fragments were spliced together by an overlapping PCR using primers 1694/1697 ($cupB1$-$cupB2$), 1698/1701 ($cupB6$-$cupB4$), 1694/1701 ($cupB1$-$cupB4$) and 1698/1697 ($cupB6$-$cupB2$). DNA fragments were ligated into pCR-Blunt and recloned into pET28b using NcoI and XhoI restriction enzymes. To clone untagged $cupB1$ and $cupB6$, both genes were cloned, with their respective stop codons, into pET28b. To amplify $cupB1$ primer pairs 1908/1694 and for $cupB6$ pairs 1909/1698 were used. DNA fragments were ligated into pCR2.1 and recloned into pET28b using NcoI and XhoI. To clone pET28b-cupB1\textsubscript{\textasciitilde}dsc1\textsubscript{\textasciitilde}opt the $cupB1$\textsubscript{\textasciitilde}dsc1\textsubscript{\textasciitilde}opt DNA fragment was recloned from pMA-T into pET28b using NcoI and XhoI. To obtain pET28b-cupB6\textsubscript{\textasciitilde}dsc1\textsubscript{\textasciitilde}opt $cupB6$ was amplified from the chromosome of PAO1 using the primers 1522/1523. Primer 1523 contains the codon-optimised Nte of $cupB1$. The DNA fragment was ligated into pCR-Blunt and transferred into pET28b using NcoI and XhoI.

Engineering of pASK-IBA3c-CupB5 and pASK-IBA3c-CupB5-RBS-TpsB4

To clone pASK-IBA3c-CupB5-RBS-TpsB4, $cupB5$ was amplified from the genome of \textit{P. aeruginosa} PAO1 using primers 1712/1713 and $tpsB4$ from pMK-RQ-PA4540\textsubscript{\textasciitilde}opt (encodes a codon-optimised $tpsB4$) using primers 1714/1715. Both DNA fragments were joined by an overlapping PCR using primers 1712/1715. The resulting DNA fragment was gel purified and ligated into pCR-Blunt, yielding pCR-Blunt-CupB5-RBS-TpsB4. The CupB5-RBS-TpsB4 DNA fragment was recloned into pASK-IBA3c using the restriction enzymes XhoI and XbaI. To clone pASK-IBA3c-CupB5, pCR-Blunt-cupB5\_mini and pASK-IBA3c-CupB5-RBS-TpsB4 were digested with the enzymes BglII and XhoI. The $cupB5$ DNA fragment was ligated into the linearised pASK-IBA3c-CupB5-
RBS-TpsB4 vector, resulting in pASK-IBA3c-CupB5.

8.3 Preparation of competent cells and transformation

Plasmids were transformed into chemically competent *E. coli* cells (see Table 8.1). Except for *E. coli* Top10, which were bought from Invitrogen, all other competent cells were home-made. To make competent cells, bacteria were grown in 50 ml LB medium until an OD$_{600}$ of 0.4 - 0.6. Cells were harvested by centrifugation (10 min, 4°C, 4000 x g) and resuspended in 25 ml (1/2 of the culture volume) ice-cold washing buffer (50 mM CaCl$_2$, 15% glycerol). The bacterial resuspension was chilled on ice for 30 min and cells were harvested by another centrifugation (10 min, 4°C, 4000 x g). The cell pellet was resuspended in 2.5 ml (1/20 of the culture volume) ice-cold washing buffer and bacteria were aliquoted and stored at -80°C. To transform home-made competent cells, bacteria were thawed on ice and 100 µl were added to the ligation mix or plasmid. Bacteria were chilled on ice for 20 min, then heat-shocked at 42°C for 1–2 min and chilled on ice for 5 min. 500 µl SOC (Super Optimal broth with Catabolite repression) medium was added to 100 µl bacterial culture, bacteria were incubated shaking at 37°C for one hour and then plated on LB agar plates supplemented with appropriate antibiotics.

8.4 Biofilm assays in microtiter plates

Quantification of biofilm formation was performed in 24-well or 96-well polystyrene microtiter plates. M63 medium (900 µl/well) with 0.4% arabinose was inoculated to a final OD$_{600}$ of 0.2. The plates were incubated for different time periods at 30°C under static conditions. Biofilms were stained with 100 µl of concentrated crystal violet (CV, Gram Crystal Violet, BD, 212525) that was added to the 900 µl culture. Biofilms were stained for 10 min under agitation. The CV was removed and biofilms washed twice with 1.8 ml water before being solubilized in 1 ml 96% ethanol. CV staining was measured by reading the optical density at 600 nm. To measure the
planktonic growth, the OD$_{600}$ of 900 µl of the planktonic cultures was read. For Western Blot analysis, the content of one well was resuspended and 900 µl resuspended culture centrifuged for 5 min, 4°C, 13000×g. The cell pellet was resuspended in 1×SDS-loading buffer to obtain an OD$_{600}$ of 1.

8.5 Biochemical techniques

Shearing experiments

Bacterial cultures, obtained from the liquid static culture, were diluted to an equivalent of 10 U of OD$_{600}$ and appendages were sheared off by gentle agitation for 3 h at 4°C. Alternatively, bacteria were scraped from M63 solid agar plates and resuspended in LB broth containing 10 mM MgCl$_2$ and shearing was performed overnight by gentle agitation at 4°C. Subsequently, the cell density was measured and adjusted to OD$_{600}$ of 5 with LB broth. Bacteria and sheared fraction were separated by centrifugation: first, 4000 × g for 15 min at 4°C, second, 4000 × g for 20 min at 4°C and third, 13000 × g for 15 min at 4°C. Cell pellets, obtained after the first centrifugation step, were washed in 10 mM Tris, pH 8, sedimented by centrifugation (5 min, 13000 × g, 4°C) and resuspended in 1 x SDS-loading buffer. The sheared fraction was then subjected to an ammonium sulphate precipitation at a concentration of 50% for 5 min at room temperature and sheared fraction pellets were harvested by centrifugation (75 min, 13000×g, 4°C). The pellet was resuspended in 10 mM Tris, pH 8 and 5 x SDS-loading buffer was added. Appendage and cell pellets, resuspended in SDS loading buffer, were boiled for 10 min at 95°C. When carrying out the shearing assay after Ruer et al. (2008), the first slow centrifugation step (to separate cell and sheared fractions) was followed by ultracentrifugation (17600 × g, 15 min, 4°C). A second ultracentrifugation (70400 × g, 1h, 4°C) was carried out after the ammonium sulphate precipitation of the sheared fractions. Otherwise, all steps followed the above protocol.

Fractionation assay

Bacteria (20 OD units) were harvested by centrifugation (4000 × g, 20 min, 4°C). The su-
permatant was kept to check for secreted proteins (supernatant sample). The cell pellet was resuspended in 1 ml 50 mM Tris pH 8, supplemented with complete protease inhibitor cocktail (Roche) and 1 mM EDTA. Cells were lysed by sonication (3 x 30 sec, amplitude 35%). Intact cells were removed by three cycles of centrifugation (4000 × g, 5 min, 4°C). The clear supernatant was subjected to ultracentrifugation (100000 × g, 60 min, 4°C) to separate soluble and insoluble fractions. The resulting supernatant corresponds to the soluble fraction (soluble sample) and the remaining pellet to the membrane fraction. The membrane pellet was resuspended in 500 µl 15 mM Tris (HCl) pH 7.4, 2% (w/v) sodium N-lauroylsarcosinate to extract inner membrane proteins. Samples were incubated for 1 h at 4°C with gentle shaking. Soluble and insoluble membrane proteins were separated by ultracentrifugation (100000 × g, 60 min, 4°C). The supernatant (inner membrane sample) and the soluble sample were mixed with 50 µl 5×SDS-loading buffer and the pellet (outer membrane sample) resuspended in 500 µl 1×SDS-loading buffer. Samples were boiled at 95°C for 10 min before SDS-PAGE.

**Secretion assay**

Cells and culture supernatants were separated by centrifugation of bacterial cultures at 4000 × g for 15 min at 4°C. Cells were directly resuspended in 1×SDS loading buffer. The supernatant was subjected to a second centrifugation at 4000 × g for 20 min at 4°C to remove residual cells. Proteins contained in the culture supernatant were precipitated using 6 M trichloroacetic acid at a final concentration of 10%. Protein pellets were washed in 90% acetone, dried, and resuspended in 1×SDS loading buffer for analysis by SDS-PAGE. Samples were boiled at 95°C for 10 min before SDS-PAGE.

**Whole-cell ELISA**

Bacteria were diluted to an OD₆₀₀ = 0.25 or 0.5 in phosphate buffered saline (PBS), pelleted by centrifugation (15 min, 4°C, 4000 × g) and resuspended in PBS to an OD₆₀₀ = 0.25 or 0.5. Nunc-MaxiSorp 96-well plates (Thermo Fisher Scientific) were coated with 100 µl cell suspension/well and plates were incubated overnight at either 37°C or 4°C. Wells were washed three times with 150 µl washing buffer (PBS, 0.05% Tween 20). Then 100 µl primary anti-
body (see Table 8.3), diluted in dilution buffer (PBS, 1% Tween20), was added per well and plates were incubated at room temperature for 2 h. Primary antibody dilutions ranged from $10^{-3}$ to $10^{-7}$. Wells were washed three times with washing buffer and then 100 µl of alkaline phosphatase-conjugated goat-anti-rabbit antibody, diluted in dilution buffer, was added. Plates were incubated for 2 h at room temperature in the dark. Excess antibody was washed off three times with washing buffer. Next, 100 µl 1-Step PNPP ($\rho$-nitrophenyl phosphate disodium salt, Thermo Fisher Scientific) was added to the wells and left for 15–30 min in the dark. To stop the reaction, 50 µl stop solution (1 N NaOH) was gently mixed into the wells. Finally the absorbance of each well was measured at 405 nm with FLOUstar Omega plate reader (BMG labtech). Absorbances shown are an average of duplicates.

Immuno-electron microscopy

Bacteria were scraped from from M63 agar plates, resuspended in 10 mM PBS and diluted to an OD$_{600} = 1$. 20 µl of the bacterial suspension were spotted on Formvar- and carboncoated nickel grids and incubated for 5 min at room temperature. Excess PBS was removed with filter paper and bacteria fixed on the grid with 20 µl 1 % paraformaldehyde for 5 min. Grids were washed four times for 1 min with 10 mM PBS. The grid was blocked at room temperature with 5 % BSA in PBS for 10 min and then incubated with the CupB1 antibody at a dilution of 1:100 in 0.5 % BSA and 10 mM PBS for one hour. Next the grid was washed three times for three minutes each with 0.5 % BSA/ 10 mM PBS. The grid was incubated with the 5 or 10 nm gold-coupled secondary antibodies, GAR5 or GAR10, (Tebu) for 30 min. Samples were washed three times for 1 min first with PBS and then with water. Finally grids were incubated for 1 min in 1 % uranyl acetate and examined in a Philips CM200 (FEG) transmission electron microscope.

Nickel affinity chromatography

_E. coli_ were grown at 30°C in 500 ml terrific broth, and upon reaching OD$_{600} = 0.6$ induced with 0.4 mM IPTG and further grown at 18°C for 16 h. Bacteria were harvested by centrifugation (20 min, 4°C, 4000 × g) and the cell pellet resuspended in 10 ml of 50 mM Tris, 500 mM NaCl and 20 mM imidazole, pH 8. Bacteria were lysed by sonication (8 cycles, 30% amplitude, 30 sec).
Cell debris (insoluble fraction) were removed by centrifugation (60 min, 4°C, 13000 × g) and the supernatant (soluble fraction) filtered with a 0.45 mm syringe filter. The supernatant was loaded on a 1 ml nickel column that was equilibrated with binding buffer (50 mM Tris, 500 mM NaCl, 20 mM Imidazole, pH 8). The column was washed with binding buffer and His-tagged proteins bound to the nickel column were eluted with elution buffer (50 mM Tris, 500 mM NaCl, 500 mM imidazole, pH 8) in a 20 ml volume. Flowthrough and elution fractions were analysed by SDS-PAGE and Western Blot.

**Preparation of soluble and insoluble fractions**

Cells and supernatant were separated by centrifugation (5 min, 4°C, 13000 × g) and the cell pellet resuspended in 1×SDS-loading buffer. 20 µl were kept for the post-induction sample. The residual suspension was sonicated (5 times, 10 sec, amplitude 35%) to lyse cells. Insoluble and soluble fractions were separated by centrifugation (10 min, 4°C, 13000 × g). The pellet, containing the insoluble fraction was resuspended in 20 µl 1×SDS-loading buffer, whereas the soluble fraction was mixed with 5× SDS loading buffer. Samples were boiled for 2 min prior SDS-PAGE.

**Arabinose-induced expression of the cupB gene cluster**

*P. aeruginosa* mutants were scrapped from M63 solid agar plates and resuspended in LB broth. The cell density was adjusted to OD_{600} of 5 and cells were harvested by centrifugation (15 min, 4000 × g, 4°C). Cell pellets were washed in 10 mM Tris, pH 8, sedimented by centrifugation (5 min, 13000 × g, 4°C) and resuspended in SDS-loading buffer. Cell samples corresponding to the equivalent of 0.1 U of OD_{600} were loaded on a 12% SDS gel.

**SDS-PAGE and Western blot analysis**

Proteins were separated by electrophoresis on a 10 to 12% polyacrylamide gel at 160 V. The proteins were either stained with Coomassie blue or transferred to a nitrocellulose membrane (Whatman) using a semi-dry transfer system (Biorad). The membrane was blocked by incubation overnight at 4°C in 5% (w/v) milk containing 0.01% (v/v) Tween in PBS (PBST). The membrane was incubated for two hours at room temperature with the primary antibody, see
Table 8.3, diluted in 5% (w/v) milk in PBST. Membranes were washed three times for 5 min in PBST and then incubated for 45 min at room temperature in horseradish peroxidase (HRP)-conjugated goat-anti-rabbit/rabbit-anti-mouse secondary antibody at a dilution of 1:5000 in 5% (w/v) milk in PBST. Membranes were again washed three times for 5 min in PBST. Proteins were detected using a chemiluminescence revelation kit (Thermo Fisher Scientific). Western Blot analysis using the StrepMAB-Classic Hrp-conjugated antibody varied slightly: the membrane was blocked in PBS-blocking buffer (PBS, 3% BSA, 0.5% v/v Tween 20) and washed with PBS-Tween (PBS, 0.1% v/v Tween 20). The StrepMAB-Classic Hrp-conjugated antibody was first 1:100 diluted in dilution buffer (PBS, 0.2% BSA, 0.1% Tween 20) and then 10 µl of the 1:100 dilution were added to 10 ml of PBS-Tween. Membranes were incubated for one hour at room temperature, washed twice with PBS-Tween and twice with PBS buffer prior to protein detection with the chemiluminescence revelation kit.

Table 8.3: Antibodies used in this study

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<tr>
<td>CupB1</td>
<td>rabbit 93</td>
<td>1:5000</td>
<td>Eurogentec</td>
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<tr>
<td>CupB6</td>
<td>rabbit 88</td>
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<td>Eurogentec</td>
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<tr>
<td>CupB5</td>
<td>rabbit 26</td>
<td>1:500</td>
<td>Eurogentec</td>
</tr>
<tr>
<td>RNA-Polymerase</td>
<td>mouse</td>
<td>1:5000</td>
<td>Neoclonne W0023, MAb to β subunit of E. coli RNA-polymerase</td>
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<tr>
<td>PolyHistidine</td>
<td>mouse</td>
<td>1:5000</td>
<td>SIGMA</td>
</tr>
<tr>
<td>StrepMAB-Classic Hrp-conjugated</td>
<td>1:1000</td>
<td>IBA</td>
<td></td>
</tr>
<tr>
<td>DsbA</td>
<td>rabbit</td>
<td>1:10000</td>
<td>provided by Dr. Karl E. Jaeger (IMET, Juelich, Germany)</td>
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<tr>
<td>PAK (Serotype 06) -  #MF83-1</td>
<td>rabbit</td>
<td>1:3000 - 1:7000</td>
<td>(ELISA) provided by Peter C. Y. Lau, PhD (University of Toronto, Canada)</td>
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<tr>
<td>Rabbit-anti-mouse HRP-conjugated</td>
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<td>SIGMA A9044</td>
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<tr>
<td>Goat-anti-rabbit HRP-conjugated</td>
<td>goat</td>
<td>1:5000</td>
<td>SIGMA A6154</td>
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8.5. BIOCHEMICAL TECHNIQUES

continued: Table 8.3

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<th>Antibody</th>
<th>Derived from</th>
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<th>Manufacturer</th>
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</thead>
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<tr>
<td>Anti-rabbit alkaline phosphatase-conjugated goat</td>
<td>1:20000, 1:1000 (ELISA)</td>
<td>SIGMA A3687</td>
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<tr>
<td>Goat-anti-rabbit GAR5, 5 nm gold particle size goat</td>
<td>1:50</td>
<td>Tebu</td>
<td></td>
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<tr>
<td>Goat-anti-rabbit GAR10, 10 nm gold particle size goat</td>
<td>1:50</td>
<td>Tebu</td>
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</table>

Production of antibodies directed against CupB1, CupB5 and CupB6

Two peptides present in the amino acid sequence of CupB1, CupB5 and CupB6 were synthesized by Eurogentec. For CupB1 peptides CTNDKKVSIAFDQVTN (amino acids 90-105) and TEVACDINGQAPGAGN (40-55), for CupB5 peptides AEFNGNSSRYFTGPS (165-181) and AEATGKVSGGSNSRVG (822-838) and for CupB6 peptides KNFPRVNDTSTERSFD (263-278) and IPVDNQPHALDKRVT (135-149) were chosen (see Figure 8.1 for binding sites on folded proteins). The polyclonal peptide antibodies were produced by Eurogentec with the Speedy 28-days program. Briefly, two rabbits were injected with the two corresponding peptides, followed by three boosters after 7, 10 and 18 days. The rabbits were bled after 28 days, sera collected and purified against the peptides. The specificity of the antibodies was tested by Western Blotting.

Bacterial two-hybrid assays (BTH)

DNA fragments, encoding truncations of the protein of interest, were cloned into the BTH vectors pKT25 and pUT18C (Karimova et al., 1998). This creates proteins that have the T25 or T18 fragments of the CyaA protein of B. pertussis fused to their N terminus. One pKT25 and one pUT18C construct were simultaneously transformed into the competent E. coli strain DHM1 that does not express an adenylate cyclase. As a positive control, bacteria were also transformed with the pKT25-zip and pUT18C-zip plasmids, which express the leucine zipper domains from the yeast transcriptional activator. Cotransformants were selected on LB agar plates supple-
Figure 8.1: **Binding sites of CupB antibodies.** The structures of full-length CupB1 and CupB5 have been predicted by the Phyre or I-Tasser server, respectively. The full-length CupB6 structure has been identified in this study. N and C termini are indicated. The peptides that are recognised by the polyclonal antibodies are shown in red.

mented with Ap and Km. Single colonies were used to inoculate 5 ml of an overnight culture and 10 µl of the inoculum were spotted on McConkey (Difco) agar plates supplemented with 1 mM IPTG, 1% maltose, Ap100 and Km50. Plates were first incubated at 30°C for 2 days and then at room temperature for further 2 days. An interaction between the proteins of interest resulted in the development of red colonies (as early as after one day of incubation) due to the acidification of the medium through the cAMP-dependent degradation of maltose.

### 8.6 Bioinformatic analysis

PAO1 and PA14 genome sequences were obtained from the *Pseudomonas* website (www.pseudomonas.com) (Winsor *et al.*, 2011). Primers were designed by using Primer3 (Untergasser *et al.*, 2012) and the QuikChange Primer Design Program (Agilent Technologies). DNA sequence alignments were performed using Geneious (Biomatters Limited). Sequencing results were analysed using Chromas Lite (Technelysium Pty Ltd) and Geneious.
8.6. BIOINFORMATIC ANALYSIS

Clustal Omega (Sievers et al., 2011) and Geneious were used to align protein sequences, Phyre2 (Kelley and Sternberg, 2009) and I-TASSER (Roy et al., 2010; Zhang, 2008) to predict protein 3D structures, the Dali server (Holm and Rosenström, 2010) for protein structure comparison, SignalP (Petersen et al., 2011) to predict signal peptides and the Pfam server (Punta et al., 2012) to predict protein domain structures. PyMOL (Schrödinger, LLC) was used to visualize three dimensional structures and the DISULFIND server to predict disulfid bonds (Ceroni et al., 2006).
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BIBLIOGRAPHY


# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ADPRT</td>
<td>adenosine diphosphate ribosyltransferase</td>
</tr>
<tr>
<td>AHL</td>
<td>acyl-homoserine lactone</td>
</tr>
<tr>
<td>Big</td>
<td>bacterial immunoglobulin-like</td>
</tr>
<tr>
<td>BTH</td>
<td>bacterial-two hybrid</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>c-di-GMP</td>
<td>cyclic di-guanosine monophosphate</td>
</tr>
<tr>
<td>CF</td>
<td>cystic fibrosis</td>
</tr>
<tr>
<td>CDI</td>
<td>contact-dependent growth inhibition</td>
</tr>
<tr>
<td>CP</td>
<td>cytoplasm</td>
</tr>
<tr>
<td>CTD</td>
<td>C-terminal domain</td>
</tr>
<tr>
<td>CU</td>
<td>chaperone-usher</td>
</tr>
<tr>
<td>Cup</td>
<td>chaperone-usher pathway</td>
</tr>
<tr>
<td>DGC</td>
<td>diguanylate cyclases</td>
</tr>
<tr>
<td>DSC</td>
<td>donor strand complementation</td>
</tr>
<tr>
<td>DSE</td>
<td>donor strand exchange</td>
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<tr>
<td>eDNA</td>
<td>extracellular DNA</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>ETEC</td>
<td>enterotoxig <em>E. coli</em></td>
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<tr>
<td>FGL</td>
<td>long F1 and G1 loop</td>
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<tr>
<td>FGS</td>
<td>short F1 and G1 loop</td>
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Abbreviations

FuBa functional bacterial amyloids
FUP fimbrial usher protein
GAP GTPase activating
GMP guanosine monophosphate
GTP guanosine-5’-triphosphate
H-NS histone-like nucleoid structuring protein
Hpt histidine phophotransfer
HRP horseradish peroxidase
Ig immunoglobulin
IHF integration host factor
IM inner membrane
IPTG isopropyl β-D-thiogalactopyranoside
Lrp leucine-responsive regulatory protein
MFP membrane fusion protein
NTD N-terminal domain
Nte N-terminal extension
OM outer membrane
OMP outer membrane protein
PCR polymerase chain reaction
PD passenger domain
PDB protein data bank
PDE phosphodiesterase
pGpG 5’- phosphoguanylyl-(3’→5’)-guanosine
PIA Pseudomonas isolation agar
PLP patatin-like protein
POTRA polypeptide transport-associated
P or PP periplasm
PPII poly-L-proline type II
qRT-PCR quantitative real-time PCR
RBS ribosome binding side
RT room temperature
Sbp3 solute binding proteins
SCV small colony variants
SOC super optimal broth with catabolite repression
SRP signal recognition particle
T1SS type I secretion system
T2SS type II secretion system
T3SS type III secretion system
T4SS type IV secretion system
T5SS type V secretion system
T6SS type VI secretion system
T7SS type VII secretion system
T4a pili type IVa pili
T4b pili type IVb pili
TAT twin-arginine transporter
TCS two-component system
TD translocator domain
TEM transmission electron microscopy
TPS two-partner secretion
TSB tryptone soja broth
UPEC uropathogenic *E. coli*
X-gal 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
Appendices

Research Publications


Book Chapters


Permissions
Structure-function analysis reveals a dual role of the *Pseudomonas aeruginosa* Tps4 two-partner secretion system

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† contributed equally
* corresponding authors

ABSTRACT

*Pseudomonas aeruginosa* is a Gram-negative opportunistic bacterium, synonymous with cystic fibrosis patients, which can cause chronic infection of the lungs. This pathogen is a model organism to study biofilms: bacterial accretions that provide protection from environmental pressures and lead to persistence. A number of chaperone-usher pathways (CUPs), namely CupA-CupE, play a key role in these processes by assembling adhesive pili on the bacterial surface. One of these, encoded by the *cupB* operon, is unique in that it contains a non-chaperone-usher gene product, CupB5. Two-partner secretion (TPS) systems are comprised of a C-terminal integral membrane β-barrel pore with tandem N-terminal POTRA (polypeptide transport associated) domains located in the periplasm (TpsB) and a cognate secreted substrate (TpsA). Using cellular and solution NMR studies we show that TpsB4 secretes both CupB5 and its usual cargo TpsA4, an extracellular protease. This is the first documentation of a TPS system that can transport non-cognate cargo. We also report that the *tps4* and *cupB* operons are induced by overexpression of RocS1, as is *cupC*, which suggests that *P. aeruginosa* has developed synergy between these systems to exploit a community based lifestyle under specific conditions. Furthermore, we have calculated the solution structure of the TpsB4 POTRA1 domain and together with restraints from NMR paramagnetic relaxation enhancement experiments, chemical shift mapping and *in vivo* mutational analysis we have calculated a structural
ensemble for the entire TpsB4 periplasmic region in complex with the TpsA4 secretion motif. The data highlight the specific residues for TpsA4/CupB5 recognition by TpsB4 in the periplasm and suggest distinct roles for each POTRA domain.

AUTHOR SUMMARY

*Pseudomonas aeruginosa* is a bacterium that usually lives in the soil and stagnant water, although it is also opportunistic and can infect individuals with impaired immune systems. During infection it forms structures called biofilms, which are cellular accretions encased in a self-produced matrix that protects the bacteria from dehydration, antibacterial compounds and many other environmental pressures. Here we unravel the translocation mechanism used by a two-partner secretion (TPS) system of *P. aeruginosa*, that helps it thrive within biofilms under specific conditions. We show that it is unique in that it transports two proteins (TpsA4 and CupB5), rather than the usual single cargo into the extracellular space. Furthermore, many other bacterial pathogens use TPS systems to cause disease and this work highlights a general secretion mechanism that could be exploited in the fight against emerging bacterial resistance to antibiotics.

INTRODUCTION

*Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen of animals, including humans, which can infect the eyes, lungs, kidneys and urinary tract and lead to fatal consequences. *P. aeruginosa* is considered to be a model organism for studying bacterial biofilms [1, 2] and uses a large number of extracellular appendages and adhesins. A number of chaperone-usher (CU) pathways (CUPs) [3-6] have been identified in *P. aeruginosa* (CupA to CupE) [7-12] that sustain this extracellular lifestyle, although for many of them their specific targets still remain unknown. However, it has been shown that CupA pili promote adherence to solid surfaces [9], CupB/CupC pili act in synergy to support microcolony formation [12], CupD pili function to increase biofilm formation and decrease motility [11, 13] and CupE pili aid in colony formation during early biofilms whilst also shaping the 3-dimensional architecture of maturing biofilms [10]. Furthermore, these structures are differentially regulated [14], which supports the notion that they play distinct roles during biofilm formation and/or allow the colonization of specific environmental niches [7, 8, 10-
The CupC pili form hair-like structures on the bacterial surface [15] and their production is tightly controlled by the Roc systems [16], specifically the RocS1 sensor [17]. CupB pili are also controlled by the Roc system but are assembled by an atypical CU-pathway. This system contains two chaperones (CupB2 and CupB4) [18] and a ~95 kDa gene product, CupB5, that is not found in other CU pathways [19]. In *P. aeruginosa* there are also five known type Vb secretion (commonly referred to as two-partner secretion; TPS) systems: Tps1-Tps5 [20], which secrete specific high molecular weight substrates that usually play an important role in virulence [21]. The Tps3 system transports a large adhesive structure, CdrA, onto the surface of *P. aeruginosa*, which promotes inter-bacterial aggregation through interactions with Psl polysaccharides [22]. CupB5 has been shown to possess sequence similarity with the N-terminal ‘secretion domain’ of TpsA4, a protease also known as LepA and the substrate for the Tps4 system [23, 24].

Historically, TPS systems have been thought to be composed of just two components: a dedicated outer membrane transporter belonging to the Omp85 superfamily (TpsB) [25] and a passenger exoprotein (TpsA) [26, 27]. The translocation unit is comprised of a membrane-embedded, β-barrel pore and two N-terminal periplasmic POTRA (polypeptide transport associated) domains [28]. At the extreme N-terminus is an α-helix that is located with a loop within the barrel, which function together to gate the pore [29]. TpsB POTRA domains recognize extended conformers of TpsA passengers within their secretion domain [30, 31]. This induces channel opening, threading of the polypeptide through the pore and folding at the cell surface [28, 29]. It is believed that POTRA domains, formed of a three-stranded β-sheet packed against two α-helices, function through augmentation of this sheet with its cargo, although there is no direct structural evidence for this to date [32-35]. Whilst the atomic details of these events still remain elusive, the formation of β-helical architectures outside of the cell is thought to generate the force for translocation [28].

Using solution state NMR and genetic manipulation of the *cupB* and *tps4* operons we show that TpsB4, the translocation apparatus of TpsA4, also secretes CupB5. Our data reveal a new variation of the TPS system in that TpsB4 is able to secrete more than one dedicated cargo. Furthermore, this is the first molecular characterization of
interactions with POTRA domains and indicates that each domain plays a unique role in substrate recognition and coordinating translocation.

RESULTS

CupB3-independent secretion of the TpsA-like protein CupB5. Secretion of the TpsA-like protein CupB5 was previously thought to be dependent on a P-usher [19], CupB3, which was an original observation, suggesting a subtle mix and match between CU pathways and TPS systems. In an attempt to refine this mechanism, we used alternative conditions, in which the strains are grown in static culture instead of on agar plates. A full set of cupB mutants were engineered (PAO1ΔΔΔcupB1-B6) and secretion of CupB5 was assessed in these various strains overexpressing the RocS1 sensor-encoding gene [17]. Interestingly, using western blot and CupB5-specific antibodies, it was found that CupB5 is systematically released in the sheared fraction, including in the PAO1ΔΔΔcupB3 mutant, which lacks the P-usher (Fig. 1A,B) and suggests that under these conditions alternative transporters are involved in CupB5 secretion. The phenotype of the cupB mutants was confirmed using CupB1 (major pilin subunit) and CupB6 (tip adhesin) specific antibodies. Western blot analysis showed that cell surface release of these two pilins is indeed blocked in the PAO1ΔΔΔcupB3 mutant and can only occur in the PAO1ΔΔΔcupB5 mutant, which is in agreement with the notion that CupB5 does not contribute to the CU pathway (Fig. 1B).

In strains that overexpress RocS1, it is expected not only that the cupB genes are induced, but that the change in expression of other genes also contribute to the observed phenotypes. We used an alternative strategy aiming at overexpressing solely the cupB genes. We introduced an arabinose inducible promoter (pBAD), upstream of the first gene in the cluster, cupB1, and this was engineered in the wild-type strain and each of the individual cupB mutants following procedures previously described [36]. Secretion of CupB5 was assessed as described above upon addition of arabinose. In this new genetic context, CupB5 is not transported to the cell surface of the parental strain, while CupB1 and CupB6 are (Fig. 1C). Furthermore, CupB1 and CupB6 are not surface exposed in any of the cupB mutants, except for the cupB5 mutant. These observations confirmed that in these growth conditions CupB3 is not sufficient for
CupB5 transport and another transporter is likely involved.

**TpsB4 is the transporter of CupB5.** TpsA proteins are usually transported by cognate TpsB transporters, and it is plausible that CupB5 could use either one of the five TpsBs that have been identified in *P. aeruginosa* [37]. qRT-PCR analysis was performed to investigate whether any of the five *tpsa/tpsB* gene couples could be up-regulated upon overexpression of *rocS1* and thus be co-regulated with the *cupB5* gene. Strikingly, whereas *cupB1* gene expression was readily induced upon introduction of the *rocS1* gene, the *tpsA4* gene also displayed an over 80-fold increase in expression (Fig. 2A). TpsA4 has been recently shown to be part of the LepA/LepB TPS system, in which TpsA4 is a protease (LepA) secreted by the cognate transporter TpsB4 (LepB) [23, 24]. None of the other *tps* genes, or the *cupA1* gene, responded to the RocS1 control, suggesting that TpsB4 is the best candidate for CupB5 transport.

TPS-dependent transport requires the presence of a secretion or TPS motif within the secretion domain at the N-terminus of TpsA proteins [30, 31]. We hypothesized that TpsB4 is the likely transporter for CupB5 and would therefore have a very similar TPS pattern to TpsA4. Amino acid sequence alignment reveals that the N-terminal region of CupB5 shares significant homology within the secretion domain of TpsA4, with residues S68-D107 (TpsA4) and S69-D108 (CupB5) being identical or conserved (Fig. 2B). Overall, our data led us to re-evaluate previous observations that the CupB3 usher is the sole transporter of CupB5 [19] and test whether TpsB4, the transporter for TpsA4, plays a role in this process.

The *tpsB4* gene was deleted from the PAO1ΔΔ chromosome and, upon overexpression of RocS1, secretion of CupB5 was assessed as described above. Our data showed that lack of *tpsB4* readily abrogates CupB5 secretion, which could then be recovered by reintroducing the *tpsB4* gene at the att site on the chromosome of the PAO1ΔΔΔtpsB4 strain (PAO1ΔΔΔtpsB4::tpsB4) (Fig. 2C). We thus conclude that in static growth condition, CupB5 secretion is dependent on the TpsB4 transporter.

**Solution structure of the TpsB4 POTRA1 domain.** To address the molecular mechanism by which TpsB4 recognizes both TpsA4 and CupB5, we used the multiple-threading alignment combined with fragment assembly simulation protocol of I-Tasser to model TpsB4 and facilitate the design of constructs for interactional
Two adjacent periplasmic POTRA domains with overall tertiary homology with the *Bordetella pertussis* TpsB protein, FhaC [28], were predicted in TpsB4 (C-score 0.58). Based on these predictions we produced recombinant TpsB4 His<sub>6</sub> tagged proteins containing the entire periplasmic region (TpsB4-NT; residues 1-194), POTRA1-2 domains (TpsB4-P12; residues 42-194), the N-terminal plug helix, linker and POTRA1 domain (TpsB4-a1P1; residues 1-121), POTRA1 domain (TpsB4-P1; residues 42-121) and POTRA2 domain (TpsB4-P2; residues 122-194) (Fig. 3A). These were expressed in *Escherichia coli* K12 strain and purified by nickel affinity and gel filtration chromatography. 1D <sup>1</sup>H NMR spectra were recorded on purified proteins and showed that only TpsB4-α1P1 and TpsB4-P1 to be correctly folded under our conditions. The mis-folding of recombinant material for the second POTRA domain is likely due to lack of interactions with the periplasmic face of the outer membrane β-barrel that would contribute to its stability.

We next used heteronuclear NMR methods to elucidate the structure of the POTRA1 domain in solution. Using a combination of manual and ARIA (Ambiguous Restraints for Iterative Assignment) NMR assignment methods [39], a total of 1818 nuclear Overhauser effects (NOEs) were assigned in TpsB4-P1 <sup>15</sup>N/<sup>13</sup>C-edited NOESY spectra at pH 7.0. The structure determination was also supplemented with φ/ψ dihedral angles and hydrogen bond restraints. The average pair-wise root-mean squared deviation (RMSD) for the water-refined final structures is 0.17 ± 0.04 Å for the backbone atoms and 0.49 ± 0.06 Å for the heavy atoms of residues within secondary structure. Structural statistics are shown in Table S1.

All areas of secondary structure are well defined, however, there is less precision in the β4-β5 loop and at the N-terminus preceding the β1-strand (Fig. 3B,C). The enhanced flexibility of these regions is supported by measurement of <sup>1</sup>H-<sup>15</sup>N heteronuclear NOEs, which report on ns-ps timescale motions (Fig. S2). TpsB4-P1 exhibits the usual β-a-α-β POTRA-domain topology (β2-α2-α3-β4-β5) [28, 32] but also forms an antiparallel β1-β3 sheet pairing at the N-terminal base that is not observed in other TPS POTRA domain structures (Fig. 3C). Another interesting feature is that we observe NOE correlations between the N-terminal residues of the POTRA domain and the C-terminal pole of the α3-helix. This results in this region
(directly adjacent to the N-terminal plug helix linker in the intact TpsB4 structure) creating a pseudo-continuous helix, mediated by electrostatic interactions from the α3-helix dipole, the α3-helix residue S79 and the carbonyl groups of G42 and P44.

In the crystal structure of FhaC [28], electron density for the linker that connects the N-terminal plug helix and POTRA1 was not observed suggesting that it is somewhat flexible. We therefore used NMR to compare $^1$H-$^15$N HSQC spectra of $^15$N-labelled TpsB4-α1P1 and TpsB4-P1 are compared substantial chemical shift perturbations (CSPs) are observed (Fig. S3). The most significant of these are mapped to the N-terminus, α3-helix and β4-β5-loop, indicating that the linker to the plug helix folds back interacts transiently within this region (Fig. 3D).

**TpsB4-P1 recognizes a conserved sequence in TpsA4/CupB5.** We next examined how the TpsB4 POTRA1 recognizes its cargo. It has been shown in other systems that these events can only be established when the secretion domain is unfolded [31]. To identify which TpsA4 residues interact, a His$_6$ tagged secretion domain (TpsA4$^{1-242}$; residues 1-242) was purified under denaturing conditions and diluted into non-denaturing buffer in the presence of TpsB4-P1, before treatment with the protease trypsin. The TpsA4$^{1-242}$ peptide library was separated by gel filtration and used in NMR titration experiments by monitoring peak perturbations in $^1$H-$^15$N HSQC spectra of $^15$N-labelled TpsB4-P1 (data not shown). Samples that showed positive interactions were sent for MALDI-MS analysis. Whilst a number of TpsA4 sequences were detected in all samples, repeat titrations with synthetic peptides showed that only V$_{80}$FLVNPNGVVF GSAQVNVGGGLVASTLDLAD$_{111}$ (TpsA4$^{80-111}$) bound $^15$N-labelled TpsB4-P1 (data not shown). Furthermore, when this was truncated at the C-terminus, the sequence K$_{79}$VFLVNPNGVVF GSAQ$_{95}$ (TpsA4$^{79-95}$) produced identical CSPs in $^1$H-$^15$N HSQC peak positions as observed for TpsA4$^{80-111}$ (Fig. 4A).

The sequence of TpsA4$^{79-95}$ is almost identical in CupB5 and the corresponding peptide (CupB5$^{80-96}$; Q$_{80}$VFLVNPNGVLFGRGAQ$_{96}$) also interacts with TpsB4-P1 (Fig. 4B and Fig. S4), suggesting that these regions share a similar function. However, due to the poor solubility of CupB5$^{80-96}$ in aqueous buffer it was not possible to derive a dissociation constant for this peptide with TpsB4-P1, whilst for TpsA4$^{79-95}$ we could calculated an average $K_d = 1.49$ mM ($\pm 0.18$ mM) (Fig. 4C). The affected residues in POTRA1 are localized to a distinct, contiguous region (Fig. 4D), the β4-β5-loop, the
adjoining β5-strand and the C-terminal region of the adjacent α3-helix and N-terminus/β1-strand. Interestingly, some of the largest CSPs are located to the extreme N-terminus of the POTRA domain (G42-T45) and into the neighbouring N-terminal His₆-tag. Furthermore, some of these CSPs coincide with those observed between TpsB4-α1P1 and TpsB4-P1, implying that interactions between TpsA4 or CupB5 and the POTRA domains of TpsB4 would displace its N-terminal linker connected to the plug helix, thereby initiating opening of the pore.

**TpsB4 POTRA domains recognize tandem TpsA4/CupB5 TPS motifs.** Interactions between TpsB4 POTRA1 and TpsA4⁷⁹-⁹⁵ or CupB5⁸⁰-⁹⁶ are in fast exchange on the NMR timescale, precluding the elucidation of any high-resolution structures for these complexes using NOE restraints alone. We therefore adopted a site-directed spin labelling approach [40], in which paramagnetic centres were introduced into TpsA4⁷⁹-⁹⁵ peptides and the induced relaxation enhancements in amide resonances of TpsB4-P1 were used to derive intermolecular distances to the spin labelled position.

Residues in TpsA4⁷⁹-⁹⁵ were chosen for mutation to cysteine (V80C, V88C) and subsequently coupled to the paramagnetic spin label MTSL. ¹H-¹⁵N HSQC peak intensities were recorded for each mutant separately and then again after reducing MTSL to its diamagnetic state with ascorbic acid. NMR paramagnetic relaxation enhancement (PRE) data were derived from ratios of the peak intensities for the two states. Whilst CSPs matched unlabelled TpsA4⁷⁹-⁹⁵ peptide, indicating that the spin label does not interfere with binding, it was not possible to fully reduce MTSL at peptide concentration greater than 2-fold molar excess (20% in bound state). Therefore only the peaks that were broadened beyond detection in the presence of paramagnetic MTSL were used in the docking calculation, with intermolecular distances between the paramagnetic centre and the POTRA1 backbone amide set as 8 Å ± 4 Å [40].

As residues S68-D107 in TpsA4 are nearly identical in CupB5 (Fig. 2B), we postulated that the sequence C-terminal to TpsA4⁷⁹-⁹⁵ would represent a binding motif for the second TpsB4 POTRA domain. Mutational data from *Bordetella pertussis* FhaC has also highlighted a region within the second POTRA domain, between the β7-strand and α4-helix in TpsB4, which plays a role in the secretion of its FHA cargo.
We therefore created a number of mutants in TpsB4 POTRA2 and CupB5 and monitored the secretion of CupB5.

Firstly, we generated mutations in the conserved TPS motif of CupB5. We introduced the double point mutation N85A/P86A, part of the conserved NPNG box (Fig. 2B), and V97W/V99W, designed to disrupt interactions between the CupB5 TPS motif and TpsB4 POTRA2 groove, and thus disrupt secretion. In addition we created an S69A mutant outside of the expected TPS motif as a negative control. The point mutations were generated in the CupB5-encoding plasmid, pBBR-MCS4-cupB5. The plasmids were introduced in the cupB5 mutant strain overexpressing RocS1, PAO1△△△cupB5/pMMBrocS1, and secretion of CupB5 or mutant derivatives monitored. Western blot analysis using anti-CupB5 antibodies clearly show that the N85A/P86A and V97W/V99W substitutions abrogate secretion, whilst S69A can still be recovered from the sheared fraction, thus supporting the role of the CupB5 TPS motif in the interaction with the TpsB4 POTRA domains (Fig. 5A).

Secondly, we engineered mutations in both TpsB4 POTRA domains at positions that are likely to interact with the TPS motif of CupB5, with additional mutations predicted not to affect binding. A double point mutation H82W/P105A was successfully created in POTRA1, localized to the periphery of the TpsB4/CupB5 interface based on NMR CSPs and designed to test the importance of these residues at this interface. We also introduced double point mutations G154W/E158W in the α4-helix and K173A/T175P in the β7-strand of the POTRA2. Substitutions of G154 and E158 were expected to block CupB5 binding within this groove, whilst mutation of K173 and T175 would prevent β-sheet augmentation within POTRA2. A negative control, residues D61 (POTRA1) and D149 (POTRA2), located on opposite side of the CupB5/TpsB4 interaction surface, were each substituted by alanine. The point mutations were generated in the plasmid-encoded tpsB4 gene, and the chimeric genes were recloned into the integration-proficient vector mini-CTX1. For complementation, the mini-CTX1-tpsB4 or its derivatives were conjugated into the tpsB4 mutant overexpressing rocS1, PAO1△△△tpsB4/pMMBrocS1, thus placing the tpsB4 allele at the att site on the chromosome. Western blot analysis to monitor CupB5 secretion showed that both the G154W/E158W and K173A/T175P double substitution severely impaired CupB5 transport to the cell surface (Fig. 5B) further supporting the
interaction model between CupB5 and TpsB4. At the same time, and as hypothesized, the control had no influence on CupB5 secretion (Fig. 5B), although this was also apparent for the H82W/P105A double mutant.

We next refined our structural model of TpsB by incorporating our experimental NMR structure for TpsB4-P1 and docking of the whole TPS motif (TpsA479-100) using a HADDOCK approach [42, 43] into the periplasmic region containing the two POTRA domains (residues 42-194). Restraints were incorporated from CSPs, PREs and mutational experiments and the 200 final water-refined structures for the TpsB442-194/TpsA479-100 complex were clustered according to a pair-wise RMSD cut-off of 3.0 Å, producing a single cluster of 22 structures. The average intermolecular interaction energy for this cluster was \(-261\pm51\) kcal mol\(^{-1}\). Figure 5C shows an overlay of the ten water-refined structures with the lowest interaction energies from the top cluster. Structural statistics are shown in Table S2.

**Experimental derived model of the TpsB442-194/TpsA479-100 complex.** The TpsB442-194/TpsA479-100 complex can be considered as an interaction between adjoining POTRA domains (POTRA1 and POTRA2) and two adjacent TPS motifs (TPS1 and TPS2) (Fig. 5C,D). Most strikingly TPS1 contains the conserved NPNG box whilst TPS2 contains the completely conserved N97 residue (N98: CupB5). POTRA1 is recognized by TPS1, which kinks under the N-terminal base of this POTRA domain at position P85 (P86: CupB5). Here, the first substantial interaction is observed between TPS1 F81 (F82: CupB5) and POTRA1 in a putative pocket created by the \(\beta_4-\beta_5\)-loop and \(\beta_1\)-strand (Fig. 5D). In the unbound POTRA domain structure, this loop is ‘pinned’ back with the side chains of Q110 in the loop and R115 in the \(\beta_5\)-strand (Fig. S5). Our model for the complex suggests that when the peptide binds, TPS1 F81 is accommodated by a rearrangement of this loop region.

TPS1 P85 (P86: CupB5) binds between the C-terminal pole of the \(\alpha_3\)-helix and the extreme N-terminus of POTRA1 (Fig. 5D), disrupting intra-POTRA1 interactions between G42-P44 and the \(\alpha_3\)-helix, whilst TPS1 N84 (N85: CupB5) is stabilized by the dipole of the POTRA1 \(\alpha_3\)-helix. In this model, TPS1 binds around the POTRA1 residue H82, with its side chain pointing away from the peptide, and P105 has minimal hydrophobic interactions with TPS1 (Fig. 5D). These observations are
consistent with all models within this cluster and together suggest an explanation as to why the double mutant H82W/P105A did not abrogate secretion of CupB5 (Fig. 5B).

The TPS2 motif (K92:G100 in TpsA4; R93:G101 in CupB5) binds between the β7-strand and α4-helix of POTRA2 (Fig. 5D). Although a fewer number of restraints are present, β-sheet augmentation of the region between the β7-strand and TPS2 is observed in the lowest energy structures of this cluster.

**DISCUSSION**

*Tps4 is a ‘three-partner’ secretion system.* In this study, we show that under static growth conditions the TpsB4 transporter and not the P-usher CupB3 translocates CupB5 across the *P. aeruginosa* outer membrane. Although this is in contrast with previous observations, evidence for CupB3-independent transport of CupB5 was suggested in this work as a deletion of the CupB3 N-terminus, the POTRA domain, did not prevent CupB5 from reaching the surface [44]. We have revealed here that the TPS motif in CupB5 is highly similar to the TpsA4 secretion signal and we confirm using biochemical and genetic approaches that both motifs can be recognized by the POTRA domain of the TpsB4 transporter, one of five TpsBs identified in *P. aeruginosa* [20, 37]. The existence of such a three-partner secretion system reflects a co-evolution between chaperone usher pathways and two-partner secretion systems, which can be genetically linked. Usually, when a *tpsA* gene is clustered with CU genes, it is also found with a cognate *tpsB* gene. Both genes can be separated by other genes in the CU cluster as in *B. pertussis*, or organized in tandem and embedded in the CU cluster as in *Pseudomonas fluorescens* [45]. Our observation that the product of the orphan *P. aeruginosa tpsA* gene, *cupB5*, can be transported by a distally encoded TpsB, reflects that an anciently-linked *tpsB* gene may have been lost because of the presence of a highly similar TpsB, which provided an alternative. Although the modification reported in the P-usher CupB3 could also account for an alternative evolution, we have now collected clear evidence that the interaction between CupB5 and TpsB4 is effective and functional, involving key residues in the TPS of CupB5 and the POTRA domain of TpsB4 as described here.

*TpsB4 POTRA domains have distinct functions.* The solution structure of TpsB4-P1 contains the usual core β-α-α-β-β structural motif, but also possesses an additional
β1-β3 sheet pairing at the N-terminal base of the domain. This was not observed in FhaC [28, 46], the only other TpsB structure, although it is present in one other POTRA domain containing structure: POTRA1 from a Cyanobacterial Omp85 [47] (pdb: 3mc8). Interestingly, significant conformational dynamics exist within the β4-β5 loop and at the N-terminus preceding the β1-strand (Fig. 3B,C), which is where the TpsA4 TPS1 motif binds and are likely important for selection of the cargo. A conserved proline in the NPNG box of TPS1 allows TpsA to be kinked around POTRA1, which would set the initial register of the TPS motif on the POTRA domains. The main interactions between TpsA4 and POTRA1 are localized to this N-terminal pole of TpsB4 and do not extend substantially along the face of the domain (Fig. 3D). In fact, in our structural ensemble of the TpsB4<sup>42-104</sup>/TpsA4<sup>79-100</sup> complex, TPS1 residues K79-V88, interact and follow a similar trajectory through this region, whilst V89-G91, located towards the C-terminal pole of the domain are less well converged (Fig. 5D). We also show that the linker adjoining the TpsB4 N-terminal α1 helix (involved in gating the β-barrel pore) specifically interacts with POTRA1 and would be displaced during recognition of TpsA4 TPS1. These observations indicate that recognition of TPS motifs by POTRA1 may coordinate pore opening through signalling release of the N-terminal plug helix.

Using the entire periplasmic region of FhaC and an extended 30 kDa FHA TpsA-secretion domain, low micromolar dissociation constants have been reported [41]. In TpsB transporters the interaction with single POTRA domains and corresponding TPS regions are significantly weaker. The presence of two POTRA domains increases the affinity between TpsB transporters and their cargo substantially, whilst enabling greater specificity. It has been suggested that POTRA domains recognize their substrates through β-sheet augmentation [32-35], although there is no direct structural evidence for this. Our NMR structure of POTRA1 clearly shows that β-sheet augmentation would not be possible, as chemical shift data show that the TPS1 motif primarily interacts with the β4-β5 loop of the domain. However, in our model of the whole periplasmic region, POTRA2 the β7-strand is exposed and forms the edge of a groove with the α4-helix and we do observe β-sheet augmentation in a number of low energy structures. When the TpsA4<sup>79-100</sup> conformation is transposed onto the POTRA domains from the FhaC crystal structure [28] (pdb: 2qdz), the FHA TPS motif follows a similar trajectory through grooves with complementary electrostatic
surface potential (Fig. S6) and it is likely that our observations with TpsA4/B4 can be generalized for other TPS systems.

The TPS motif from TpsA4 used in our modelling is almost identical to that for CupB5. Differences occur between at positions K79, K92, S93 in TpsA4, which are replaced by Q80, R93 and G94 in CupB5. In our model of the TpsB4/tpsA4 complex the side chains of the CupB5 residues are either exposed to solvent or can be readily accommodated in our model. It is also worth noting that G100 in TpsA4 is positioned at the C-terminal pole of POTRA2, which in the context of intact TpsB4 would sit at the opening of the barrel. The ten residues that follow are either identical or highly conserved between TpsA4 and CupB5 (Fig. 2B). This corresponds to approximately 35 Å of extended polypeptide, which is sufficient to cross the outer membrane and interact within the barrel of TpsB4 to facilitate transport. These observations suggest that once the TPS motif of the cargo binds the POTRA domains (TpsA N-terminus facing into the periplasm) a hairpin could form within the TpsB4 pore and polymerization at the tip of this hairpin result in translocation of the remaining C-terminus across the outer membrane. In addition to our work here, others have shown that interactions between POTRA domains and TPS motifs are quite dynamic, which will be essential for substrate release upon folding [41]. Whilst the specific cargo release mechanism is unknown, it is interesting to note that extracellular TpsA4 is processed at it N-terminus and begins at His22, just prior to the TPS1 motif. Therefore, proteolysis of TpsA4, albeit slightly upstream of the TPS1 motif, may destabilize POTRA-TPS interactions and contribute to its release.

**Tps4, CupB and CupC systems act in synergy to promote *P. aeruginosa* virulence.** TpsB4 (LepB) has recently been reported to transport a cognate cargo molecule TpsA4 (LepA), which functions as an extracellular protease [23, 24, 48]. It has been shown to activate the NF-κB pathway through cleavage of protease-activated receptors (PARs), allowing the modulation of host responses against bacterial infection [24]. Furthermore, in cooperation with a *P. aeruginosa* haemolysin (PlcH), TpsA4 can degrade haemoglobin to liberate peptide fragments and iron as a source of nutrients for bacterial growth. In this study and in previous investigations [12] we have shown that tps4, cupB and cupC operons are induced by overexpression of RocS1. Whilst CupC fimbriae promote the development of microcolonies during
early biofilm formation, the specific role for the CupB pilus and CupB5 have yet to be determined. However, CupB pili display the usual tip adhesin (CupB6) and it is plausible that this play a role in initiating biofilms on biotic surfaces. Analysis of CupB5 using the I-Tasser server (Fig. S7) [38] identifies a number of β-helical structures with excellent alignment scores, including the E. coli Hbp protease (sequence coverage: 0.9, normalized Z-score: 4.0) [49] and the Haemophilus influenzae Hap adhesion (sequence coverage: 0.9, normalized Z-score: 2.0) [50]. It has previously been shown by transmission electron microscopy and immunogold labelling that CupB5 is localised on the surface of P. aeruginosa alongside CupB pili. In enterotoxogenic Escherichia coli, the TpsA-like molecule EtpA is secreted into the extracellular space where it associates with the flagella and mediates adhesion to host cells [51]. It is tempting to speculate that CupB5 plays a similar role here by bridging or modulating adherence between the CupB pilus and specific host receptors.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains and growth conditions.** The strains and plasmids used in this study are listed in Table S3. P. aeruginosa strains were grown at 37°C in LB medium containing the appropriate antibiotic concentration: 80 μg/ml gentamicin (Gm), 300 μg/ml carbenicillin (Carb), 50 μg/ml tetracycline (Tet) or 2 mg/ml streptomycin (Sm). Recombinant plasmids were introduced in P. aeruginosa by three-partner conjugation using the conjugative plasmid pRK2013. Transconjugants were selected on Pseudomonas isolation agar (PIA) plates supplemented with 2 mg/ml Sm, 150 μg/ml Gm, 100 μg/ml Tet or 600 μg/ml Carb. For appendage preparation using the RocS1 system, P. aeruginosa strains conjugated with pMMBrocS1 were grown under static conditions at 30°C for 24 hours in liquid M63 medium supplemented with 0.4 % L-arginine, 1 mM MgSO₄, 80 μg/ml Gm and 0.1 mM IPTG. For appendage preparation using the pBAD system, P. aeruginosa strains were grown static at 30°C for 24 hours in liquid M63 medium supplemented with 0.4 % L-arginine, 1 mM MgSO₄, 0.4 % glutamate and 0.4 % arabinose.

**Construction of PAO1ΔΔΔcupB1, PAO1ΔΔΔcupB2, PAO1ΔΔΔcupB3, PAO1ΔΔΔcupB6, and PAO1ΔΔΔtpsB4.** The oligonucleotides used in this study are listed in Table S4. Polymerase chain reaction (PCR) was used to amplify 500-bp
DNA fragments upstream of the \textit{cupB1}, \textit{cupB2}, \textit{cupB3}, \textit{cupB6} and \textit{tpsB4} genes from the \textit{P. aeruginosa} genome using the primer pairs 1014/1015, 424/425, 950/951, 736/849 and 976/977, respectively, whereas 500-bp downstream DNA fragments were generated by using the primer pairs 1016/1017, 426/427, 952/953, 850/739 and 978/979, respectively. The generated DNA fragments were used as a matrix in an overlapping PCR using primer pairs 1014/1017, 424/427, 950/953, 736/739 and 976/979, to obtain the \textit{cupB1}, \textit{cupB2}, \textit{cupB3}, \textit{cupB6} and \textit{tpsB4} mutator DNA, respectively. The mutator DNA fragments were ligated into the cloning vector pCR2.1 and re-cloned into the suicide vector pKNG101 by restriction digest. Plasmids were conjugated in PAO1\Delta\Delta by three-partner conjugation. As previously described \cite{52}, mutants were selected on LB plates supplemented with 5\% sucrose and appropriate antibiotics.

**Construction of PAO1\Delta\Delta::pBAD, PAO1\Delta\Delta\Delta\textit{cupB1}::pBAD, PAO1\Delta\Delta\Delta\textit{cupB2}::pBAD, PAO1\Delta\Delta\Delta\textit{cupB3}::pBAD, PAO1\Delta\Delta\Delta\textit{cupB4}::pBAD, PAO1\Delta\Delta\Delta\textit{cupB5}::pBAD and PAO1\Delta\Delta\Delta\textit{cupB6}::pBAD.** The oligonucleotides used in this study are listed in Table S4. PCR was used to amplify a 554-bp DNA fragment upstream of \textit{cupB1} using primer pair 638/639 and a 558-bp DNA fragment downstream of \textit{cupB1} using primers 640/641. DNA fragments were used as matrix for an overlapping PCR using external primer pair 638/641 to obtain a \textit{cupB1}-prom mutagenic fragment. The mutagenic DNA fragment was ligated into pCR2.1. The \textit{araCpBAD} promoter DNA fragment was obtained by restriction digest of pCR2.1-\textit{araCpBAD} and ligated into linearised pCR2.1\textit{cupB1–prom}. The \textit{pBAD-cupB1-prom} fragment was re-cloned into the suicide vector pKNG101 and conjugated into PAO1\Delta\Delta, PAO1\Delta\Delta\Delta\textit{cupB2}, PAO1\Delta\Delta\Delta\textit{cupB3}, PAO1\Delta\Delta\Delta\textit{cupB4}, PAO1\Delta\Delta\Delta\textit{cupB5} and PAO1\Delta\Delta\Delta\textit{cupB6}. Mutants were selected on LB plates supplemented with 5\% sucrose \cite{52}. The same strategy was used to insert the \textit{pBAD} promoter and the \textit{araC} gene into PAO1\Delta\Delta\Delta\textit{cupB1}. Briefly, differences were that the primer pairs 730/731 and 732/733 were used to amplify a DNA fragment upstream and downstream of \textit{cupB2}, respectively. An overlapping PCR was performed with these fragments using primer pairs 730/733.
Construction of complementation vectors. PCR was used to amplify the tpsB4 gene with its promoter and ribosome binding site (RBS) and the cupB5 gene and its RBS from the chromosome of PAO1ΔΔ by using primer pairs 1315/1316 and 812/813, respectively (Table S4). The cupB5 DNA fragment was ligated into pCR-Blunt and the tpsB4 gene into pCR2.1. tpsB4 was re-cloned into mini-CTX1 using the restriction enzymes SpeI/EcoRI thereby creating miniCTX-tpsB4. To obtain pBBR-MCS4-cupB5, cupB5 was transferred into pBBR1MCS-4 by using PstI/SpeI/SmaI, whereas SmaI was used to cut pCR-Blunt.

Introduction of point mutations in the plasmid-encoded cupB5 and tpsB4. To perform the amino acid substitutions in tpsB4 and cupB5, point mutations were introduced into pCR2.1-tpsB4 and pCR-Blunt-cupB5 by using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene). To create tpsB4_{G154W,E158W} and tpsB4_{K173A,T175P} the primer pairs 1823/1824 and 1825/1826 were used (Table S4). To clone tpsB4_{D61A,D149A} and tpsB4_{H82W,P105A} two rounds of amplification were carried out. Use of the primer pairs 1819/1821 and 1827/1830 introduced the first point mutation H59W and D47A into tpsB4, respectively. A second PCR using the primers 1820/1822 and 1828/1830 introduced the second mutation into tpsB4_{H82W} and tpsB4_{D61A}, resulting in tpsB4_{H82W,P105A} and tpsB4_{D61A,D149A}. Amplified plasmids were transformed into E. coli XL1-Blue and successful mutation was confirmed by sequencing. The mutated tpsB4 genes were cloned into mini-CTX1 using the restriction enzymes SpeI and EcoRI. miniCTX-tpsB4_{xxx} was then conjugated into PAO1ΔΔΔtpsB4/pMMBrocS1.

To engineer cupB5_{S69A}, cupB5_{N85A,P86A} and cupB5_{V97W,V99W} primer pairs 2001/2002, 1804/1805 and 1802/1803 were used, respectively. Amplified plasmids, pCR-Blunt-cupB5_{S69A}, pCR-Blunt-cupB5_{N85A,P86A} and pCR-Blunt-cupB5_{V97W,V99W}, were transformed into E. coli XL1-Blue and successful mutation was confirmed by sequencing. A 600 bp cupB5 DNA fragment containing the point mutations was transferred into pBBR1-MCS4-cupB5 by using the restriction enzymes SfaAI and SgrDI. Positive clones were identified by sequencing. pBBR1-MCS4-cupB5_{S69A}, pBBR1-MCS4-cupB5_{N85A,P86A} and pBBR1-MCS4-cupB5_{V97W,V99W} were conjugated into PAO1ΔΔΔcupB5/pMMBrocS1.
**Quantitative real-time PCR.** Total RNA was prepared from triplicate cell samples of PAO1ΔΔ and PAO1ΔΔ conjugated with pMMBrocS1, stored in RNAlater, using the Total RNA Isolation kit (Promega) in combination with on column DNaseI treatment (Promega). The DNase treatment was carried out twice to remove all traces of chromosomal DNA. mRNA concentration was measured with the NanoDrop 1000 spectrophotometer (NanoDrop Technologies) and normalised to 20 ng/µl. Reverse transcription and qPCR was carried out as previously described. Briefly, cDNA was synthesized using SuperScript III Rnase H-Reverse Transcriptase (Invitrogen), 10 mM dNTPs, RNAguard (Roche), random hexamer oligonucleotides (Amersham), 0.1 M DTT, and X first strand buffer (RT Superscript Kit). Negative controls without reverse transcriptase were run with all reactions. The resulting cDNA was 1:5 diluted and stored at −20° C. For quantitative real-time PCR, SYBR green PCR master mix (ABI), an upstream and downstream primer pair (Table S4) and water were used. The relative quantity gene expression was determined by defining the expression of rpoD mRNA as 1.

**Shearing experiments.** Bacterial cultures were diluted to an equivalent of 10 U of OD600 and appendages were sheared off by gentle agitation for 3 h at 4° C. Bacteria and sheared fraction were separated by centrifugation: first, 4000 × g for 15 min at 4°C, second, 4000 × g for 20 min at 4° C and third, 13000 × g for 15 min at 4°C. Cell pellets, obtained after the first centrifugation step, were washed in 10 mM Tris, pH 8, sedimented by centrifugation (5 min, 13000 × g, 4°C) and resuspended in 1 x SDS-loading buffer. The sheared fraction was then subjected to an ammonium sulfate precipitation at a concentration of 50% for 5 min at room temperature and sheared fraction pellets were harvested by centrifugation (75 min, 13000 × g, 4°C). The pellet was resuspended in 10 mM Tris, pH 8 and 5 x SDS-loading buffer was added. Appendage and cell pellets, resuspended in SDS loading buffer, were boiled for 10 min at 95° C.

**SDS-PAGE and Western blot analysis.** Proteins were separated by electrophoresis on a 12 % polyacrylamide gel at 160 V. The proteins were either stained with Coomassie blue or transferred to a nitrocellulose membrane (Whatman) using a semidry transfer system (Biorad). The membrane was blocked by incubation
overnight at 4 °C in 5 % (w/v) milk containing 0.01 % (v/v) Tween in PBS (PBST). The membrane was incubated for two hours at RT with the primary antibody, see Table S5, diluted in 5 % (w/v) milk in PBST. Membranes were washed three times for 5 min in PBST and then incubated for 45 min at RT in horseradish peroxidase (HRP)-conjugated goat-anti-rabbit/rabbit-anti-mouse secondary antibody at a dilution of 1:5000 in 5 % (w/v) milk in PBST. Membranes were again washed three times for 5 min in PBST. Proteins were detected using a chemiluminescence revelation kit (Thermo Fisher Scientific).

**Cloning, expression and purification.** The mature N-terminal periplasmic region (TpsB4-NT; residues 1-194), POTRA domains 1-2 (TpsB4-P12; residues 42-194), N-terminal helix, linker and POTRA domain 1 (TpsB4-α1P1; residues 1-121) PORTRA domain 1 (TpsB4-P1; residues 42-121) and PORTRA domain 2 (TpsB4-P2; residues 122-194) of TpsB4, and TpsA4 TPS domain (TpsA41-242; residues 1-242) were amplified by PCR using *P. aeruginosa* PAO1 gDNA as template with primers B4-1/B4-2, B4-3/B4-2, B1-5/B4-4, B4-3/B4-4, B4-2/B4-5 and A4-1/A4-2, respectively (Table S4). These were subsequently cloned into Ek/LIC pET46 vector (Novagen) (Table S3) and expressed in *E. coli* strain BL21 (DE3) in either LB, minimal media containing 0.07% 15NH₄Cl or minimal media containing both 0.07% 15NH₄Cl and 0.2% 13C-glucose. TpsB4 samples were purified using nickel affinity chromatography followed by gel filtration with a Superdex-75 column. TpsA41-242 was purified in the presence of 8 M urea using first nickel affinity chromatography followed by gel filtration with a Superdex-75 column.

**NMR solution structure determination.** NMR measurements were performed at 310 K on a 15N13C-labelled TpsB4-P1 sample in 50 mM NaPO₄ pH 7.0, 150 mM NaCl, 10% D₂O. NMR experiments for backbone and side-chain assignment were performed on three different Bruker spectrometers, a DRX500, DRX600 and DRX800, equipped with TCI and TXI cryoprobes. Assignments were completed using standard triple-resonance assignment methodology [53] and data were analysed using in-house algorithms with the program NMRView [54, 55]. Heteronuclear NOE cross-relaxation rates on a 15N-labelled TpsB4-P1 sample were recorded at 600 MHz. NOE data for structure calculations of TpsB4-P1 were obtained from an 800-MHz edited 1H-15N/13C nuclear Overhauser effect spectroscopy (NOESY)-heteronuclear
single/multiple quantum coherence (HSQC/HMQC) experiments. The ARIA protocol for automated NOESY assignment interfaced with the CNS program was used for structure calculation [39]. Secondary structure in the TpsB4-P1 POTRA domain was first identified using the chemical shift-based dihedral angle prediction software TALOS [56]. For residues located in secondary structure, experimentally derived hydrogen bonds and φ/ψ backbone dihedral angles from TALOS, were introduced as restraints in the ARIA structure calculation. The frequency window tolerances for assigning NOEs were ±0.03 ppm for direct proton dimensions, ±0.06 ppm for indirect proton dimensions, and ±0.65 ppm for nitrogen and carbon dimensions. One hundred structures were calculated in the final iteration and these had no NOE violations >0.3 Å and no dihedral angle violations >5°. The ten lowest energy structures have been deposited in the Protein Data Bank with PDB ID code 2mhj and a summary of NMR-derived restraints and statistics is reported in Table S1.

**Production and analysis of TpsA4 TPS domain fragments.** TpsA4<sup>1-242</sup> (0.5 mM) in 20 mM Tris-HCl pH 8, 8 M urea was diluted 100-fold in 20 mM Tris-HCl pH 8, 150 mM NaCl, 2.5 mM CaCl<sub>2</sub> and incubated with 1 µM TpsB4-P1 for 30 min at 22°C, followed by the addition of 0.5 nM trypsin for a further 30 min. This was followed by addition of 1 mM PMSF and incubation for 20 min at 70°C. This was loaded onto a Superdex-30 gel filtration column equilibrated in 25% (v/v) trifluoroacetic acid and eluted peaks were pooled and samples sent for commercial analysis by MALDI-MS (University of Leeds, UK). The remaining samples were freeze dried, washed in H<sub>2</sub>O, freeze dried again and then resuspended in 20 mM NaPO<sub>4</sub> pH 7.0, 150 mM NaCl, 10% DMSO, 10% D<sub>2</sub>O. These were mixed with <sup>15</sup>N-labelled TpsB4-P1 (200 µM) in the same buffer and <sup>1</sup>H–<sup>15</sup>N HSQC NMR spectra measured.

**Peptide synthesis.** To increase solubility all peptides were produced with free N- and C-termini. Initial peptides used in this study (including TpsA4<sup>80-111</sup>) were synthesized by ThermoScientific to >95% purity, whilst all other peptides (TpsA4<sup>79-95</sup>, TpsA4<sup>79-95</sup> Cys mutants and CupB5<sup>80-96</sup>) were synthesized using standard Fmoc solid phase methods, using an automated ResPepSL synthesiser (Intavis) running Multipep software, adapted from [57].
To spin label peptides, lyophilized, purified peptide containing a single Cys residue was dissolved in a 50:50 (v/v) mix of 20 mM MOPS (pH 7.0) and acetonitrile to a concentration of 10 mg/ml and chilled to 4 °C in the dark. 2.5 molar equivalents of MTSL was added from a 100 mM stock in acetonitrile, and the reaction incubated at 4°C with vigorous shaking for 18 hours. The spin-labelled peptide was then separated from the unlabelled starting material and free MTSL by semi-preparative LC-MS. Analyses of peptide purity is shown in Table S6.

**NMR titrations.** $^{15}$N-labelled TpsB4-P1 (200 µM) in 50 mM NaPO$_4$ pH 7.0, 150 mM NaCl, 10% DMSO, 10% D$_2$O with the addition of 0, 0.5, 2, 3, 5, 7.5, 10, 15, 20, 25 or 50 molar equivalents of TpsA4$^{79-95}$ or 0, 0.5, 2 molar equivalents of CupB5$^{80-96}$ peptide, were used to measure $^1$H-$^{15}$N HSQC NMR spectra. Experiments were performed at 310 K on a Bruker DRX600. Kinetic analysis was performed in NMRView [55].

**NMR PRE experiments.** $^{15}$N-labelled TpsB4-P1 (200 µM) in 50 mM NaPO$_4$ pH 7.0, 150 mM NaCl, 10% DMSO, 10% D$_2$O with the addition of 2 molar equivalents of MTSL labelled TpsA4$^{79-95}$ peptide, were used to measure NMR $^1$H-$^{15}$N HSQC NMR spectra. These samples were then incubated overnight with 10 mM ascorbic acid and the $^1$H-$^{15}$N HSQC spectra recorded again. PRE distances were deduced using an established method based on the modified Solomon–Bloembergen equation for transverse relaxation [40, 58] and the distances were corrected for the fraction of bound peptide at a 1:1 molar ratio.

**Experimental driven docking.** Nine unambiguous, intermolecular distance restraints calculated from PREs, supplemented with ambiguous distance restraints from chemical shift perturbations were used to drive a structural calculation of a TpsB4$^{42-194}$/TpsA$^{79-100}$ complex using the ‘conformational selection and induced fit protein-peptide HADDOCK docking’ approach [59]. For the calculation, a composite NMR/I-Tasser [38] model of TpsB4$^{42-194}$ and an extended conformation of a 22 residue TpsA$^{79-100}$ peptide were used with a 1:1 stoichiometry. Superposing our NMR structure of TpsB4$^{42-121}$ onto residues 42-121 from the I-Tasser [38] model of TpsB4 and then combining this with residues 122-194 produced the model of TpsB4$^{32-194}$. PyMOL [60] was used, inputting $-139^\circ$ for phi and $-135^\circ$ for psi, to generate the
extended 22 residue TpsA\textsuperscript{79-100} peptide conformation. Based on chemical shift perturbation, filtered for a relative solvent accessibility greater than 50\%, 17 residues in TpsB4-P1 (42, 43, 44, 45, 46, 78, 79, 82, 83, 86, 104, 105, 106, 107, 109, 110, 111) were identified as active residues. In this study we have shown that mutants in CupB5 (equivalent positions 84, 85, 96, 98 in TpsA4) and positions 154, 158, 173, 175 in TpsB4 POTRA2 abrogate CupB5 secretion and these were therefore assigned as active residues. Residues juxtaposed to these in the monomer structures that have a relative solvent accessibility greater than 50\% were termed passive residues. TpsA\textsuperscript{79-100} was allowed to be completely flexible and the interfacial residues in TpsB4\textsubscript{42-194} were allowed to move during the simulated annealing and water refinement. 3000 initial complex structures were generated by rigid body energy minimization and the best 1000 by total energy were selected for refinement and analysis with 2500 followed by 5000 molecular dynamic cycles. The best 200 by total energy were selected for torsion angle dynamics and subsequent Cartesian dynamics in an explicit water solvent. Default scaling for energy terms was used as described previously [42]. Details of the structural statistic are shown in Table S2.

**ACKNOWLEDGEMENTS**

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Figure Captions

Fig. 1: CupB1 fimbriae assembly and translocation of CupB6 adhesins and CupB5 TpsA-like proteins. (A) Schematic representation of the cupB and tps4 operons. (B,C) Immunoblot analysis of cell and sheared fraction (SF) obtained from PAO1ΔΔ (ΔΔ), PAO1ΔΔΔcupB1 (ΔB1), PAO1ΔΔΔcupB2 (ΔB2), PAO1ΔΔΔcupB3 (ΔB3), PAO1ΔΔΔcupB4 (ΔB4), PAO1ΔΔΔcupB5 (ΔB5) and PAO1ΔΔΔcupB6 (ΔB6) using antibodies derived against CupB5, CupB6, CupB1, RNA-Polymerase and DsbA. (B) CupB5 secretion is CupB3-independent. cupB gene induction was induced via the sensor kinase RocS1. CupB5 is released by the cupB3 mutant, whereas CupB1 and CupB6 aren’t. (C) CupB5 secretion requires a transporter not encoded by the cupB operon. All strains have a chromosomal insertion of the pBAD promoter upstream of cupB1. Under these conditions, CupB5 is not released by the parental strain or any cupB variant. CupB1 pilus assembly and CupB6 translocation are as observed for RocS1-induction.

Fig 2: TpsB4 transports CupB5. (A) Fold induction of cupB1, cupA1, tpsA1, tpsA2, tpsA3, tpsA4 and tpsA5 mRNA transcription in PAO1ΔΔ in the absence (pEV) or presence (procS1) of RocS1. The fold induction was normalised to the house keeping gene rpoD, which encodes the sigma factor sigma 70. (B) Primary sequence alignment between the N-terminus of mature P. aeruginosa TpsA4 and CupB5 using ClustalW2 [61]. Identical residues are red (*), highly similar residues are blue (:), weakly similar residues are green (.) and none homologous residues are orange. The conserved NPNG box and Asn residue required for interaction with the outer membrane transporter TpsB4 or secretion of TpsA4 are boxed. (C) Immunoblot using the anti-CupB5 antibodies. CupB5 is released in the sheared fraction (SF) of the parental PAO1ΔΔ (ΔΔ) strain, but not in the isogenic tpsB4 mutant (ΔtpsB4). CupB5 secretion is restored in the complemented strain PAO1ΔΔΔtpsB4::tpsB4. Each strain was conjugated with pMMBrocS1 to induce cupB and tpsB4/A4 gene expression.

Fig 3: Solution NMR structure of the TpsB4 POTRA1 domain from P. aeruginosa. (A) Schematic representation of the constructs used in this study. (B) Backbone superimposition of the ten best NMR structures of TpsB4-P1 shown in two orientations. (C) Secondary structure of one of the final TpsB4-P1 structures shown as
cartoon. (D) Chemical shift mapping on TpsB4-P1 of peak perturbation between $^1$H-$^{15}$N HSQC NMR spectra of $^{15}$N-labelled TpsB4-P1 and TpsB4-$\alpha$1P1. Deeper red areas indicate residues in closer vicinity to the TpsB4-$\alpha$1P1 N-terminal plug helix linker.

**Fig 4: NMR analysis of TpsB4 POTRA1 domain and TpsA4/CupB5 interactions.** (A) $^1$H-$^{15}$N HSQC spectra of $^{15}$N-labelled TpsB4-P1 (black) with 20 molar equivalents of TpsA4$^{79-95}$ (red). (B) Expanded $^1$H-$^{15}$N HSQC spectra for $^{15}$N-labelled TpsB4-P1 with two molar equivalents of TpsA4$^{79-95}$ (red) or CupB5$^{80-96}$ (purple). (C) Representative regions of $^1$H-$^{15}$N HSQC spectra for $^{15}$N-labelled TpsB4-P1 titrated against TpsA4$^{79-95}$. Examples of binding curves are displayed below. (D) Composite TpsA4$^{79-95}$ and CupB5$^{80-96}$ interactions mapped onto the surface of TpsB4-P1. Red areas indicate residues in close vicinity to TpsA4$^{79-95}$ and CupB5$^{80-96}$.

**Fig 5: Structural modelling of a TpsB4/TPS motif complex.** (A) Immunoblot analysis of cell and sheared fractions obtained from PAO1ΔΔ (ΔΔ), PAO1ΔΔΔcupB5 (ΔcupB5) using antibodies derived against CupB5, RNA-Polymerase and DsbA. PAO1ΔΔΔcupB5 was complemented with wild type cupB5 and the cupB5 variants cupB5$^{S69A}$, cupB5$^{N85A/P86A}$, and cupB5$^{V97W/V99W}$. Strains were conjugated with pMMBrocS1. (B) Determination of the secretion efficiency of the TpsB4 variants. Immunoblot analysis of cell and sheared fractions obtained from PAO1ΔΔ (ΔΔ), PAO1ΔΔΔcupB5 (ΔcupB5) and PAO1ΔΔΔtpsB4 (ΔtpsB4) using antibodies derived against CupB5, RNA-Polymerase and DsbA. PAO1ΔΔΔtpsB4 was complemented with wild type tpsB4 and the tpsB4 variants tpsB4$^{H82W/P105A}$, tpsB4$^{G154W/E158W}$, tpsB4$^{K173A/T175P}$ and tpsB4$^{D61A/D149A}$. Strains were conjugated with pMMBrocS1. (C) Overlay of the 10 water-refined structures with the lowest interaction energies from the top cluster of docking between TpsB4$^{42-194}$ and TpsA4$^{79-100}$. TpsB4 POTRA1 is green, TpsB4 POTRA2 is teal, TpsA4 TPS1 is orange and TpsA4 TPS2 is magenta. (D) Details of TpsB4$^{42-194}$/TpsA4$^{79-100}$ interactions for the structure with the lowest interaction energy. Equivalent residues in CupB5 are labelled in parenthesis and annotations are coloured based on (C).
References


FIGURES

Figure 1

A. 

**cupB operon**

- **chaperone**
  - B1
  - B2
- **chaperone**
  - B3
  - B4
- **putative tip adhesin**
  - B5
  - B6

- **major pilin**
- **P-usher**
- **TpsA-like**

**tps4 operon**

- **transporter** (LepB)
- **TpsA-like** (LepA)

B.

- **cells**
  - ΔA
  - ΔB1
  - ΔB2
  - ΔB3
  - ΔB4
  - ΔB5
  - ΔB6

- **SF**
  - ΔA
  - ΔB1
  - ΔB2
  - ΔB3
  - ΔB4
  - ΔB5
  - ΔB6

C.

- **cells**
  - ΔA
  - ΔB1
  - ΔB2
  - ΔB3
  - ΔB4
  - ΔB5

- **SF**
  - ΔA
  - ΔB1
  - ΔB2
  - ΔB3
  - ΔB4
  - ΔB5
  - ΔB6
Figure 2

A.

B.

C.

Figure 3

A.

B.
Figure 4
D.
Figure 5

A.

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| 100 kDa | α-CupB5 |
| 150 kDa | α-RNA-P |
| 25 kDa  | α-DsbA  |

B.

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| miniCTX6x84 | 
| 100 kDa |
| 150 kDa |
| 25 kDa  |

C.

[Diagram showing POTRA2 and TPS2 structures]
D.
Supporting Information

Structure-function analysis reveals a dual role of the *Pseudomonas aeruginosa* Tps4 two-partner secretion system

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† contributed equally

* corresponding authors

Table S1: Summary of NMR structural restraints and statistics

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<td>Intra-residue</td>
<td>480</td>
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<tr>
<td>Sequential (</td>
<td>i-j</td>
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<tr>
<td>Short-range (</td>
<td>i-j</td>
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<td>ψ</td>
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<td>Hydrogen bonds restraints</td>
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**Structural statistics**

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<td>Distance restraints (Å)</td>
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<tr>
<td>Dihedral angle restraints (°)</td>
<td>0.25 ± 0.016</td>
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</table>

Deviation from idealized geometry

| Bond length (Å) | 0.0014 ± 0.000064 |
| Bond angle (°)  | 0.313 ± 0.0034 |
| Improper (°)    | 0.173 ± 0.0040 |

Average pairwise rmsd(Å)

| Heavy atoms     | 0.17 ± 0.04 |
| Backbone atoms  | 0.49 ± 0.06 |

**Structural quality**

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<td>Favoured regions (%)</td>
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<td>Allowed regions (%)</td>
<td>99.8</td>
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<tr>
<td>Disallowed regions</td>
<td>4 residues</td>
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Average values and standard deviations over the 10 lowest-energy conformers with respect to the average structure.

† Percentage of residues in the Ramachandran plot regions determined by MOLPROBITY [1] using an average structure from the 10 lowest-energy conformers.

* All outlier are situated in highly dynamic regions.
**Table S2: Structural statistics for the TpsB4<sub>42-19</sub>/TpsA4<sub>79-100</sub> complex**

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<td>Total ambiguous CSP-derived</td>
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<td>Total ambiguous mutation-derived</td>
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<th>RMSD from experimental restraints and idealized covalent geometry</th>
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<td>Bonds (Å)</td>
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<td>Angles (°)</td>
<td>0.523 ± 0.0167</td>
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<td>9.7 ± 5.4</td>
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<tr>
<td>Ebond</td>
<td>22.7 ± 0.9</td>
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<tr>
<td>Eangle</td>
<td>101.3 ± 6.5</td>
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<td>Evdw†</td>
<td>-647 ± 20.6</td>
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<th>Coordinate RMSD (Å)</th>
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<td>All backbone atoms within the interface</td>
<td>1.6 ± 0.9</td>
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<tr>
<td>All backbone atoms</td>
<td>1.7 ± 0.98</td>
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*Evdw is the Lennard–Jones van der Waals energy and is not included in the target function for simulated annealing.*

**Table S3: Strains and plasmids used in this study**

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<td>Lysogen of λ DE3 carrying a chromosomal copy of the T7 RNA polymerase gene under control of the lacUV5 promoter</td>
<td>Novagen</td>
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<tr>
<td>CC118(λpir)</td>
<td>Host strain for pKNG101 replication Δ(ara-leu ) araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE(Am) recA1 Rf&lt;sup&gt;1&lt;/sup&gt;(λpir) F-mrcA Δ(mrr-hsdRMS-mcrBC) Φ80 lacZΔ15ΔlacX74 nupG recA1 araD139</td>
<td>Lab collection</td>
</tr>
<tr>
<td>TOP10</td>
<td>Δ(ara-leu)7697 galE15 galK16 rpsL(Str&lt;sup&gt;R&lt;/sup&gt;) endA1 λ-recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>[F’ proAB lac&lt;sup&gt;IQ&lt;/sup&gt; ZΔM15 Tn10 (Tet&lt;sup&gt;R&lt;/sup&gt;)] F’ proAB lac&lt;sup&gt;IQ&lt;/sup&gt; ZΔM15 Tn10 (Tet&lt;sup&gt;R&lt;/sup&gt;) Δ(ccdAB) mrcA Δ(mrr-hsdRMS-mcrBC) Φ80 lacZΔ15 Δ(lacZYA-argF) U169 endA1 recA1 supE44 thi-1 gyrA96 relA1 tonA panD</td>
<td>Stratagene</td>
</tr>
<tr>
<td>DH5α omnimax</td>
<td></td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

**P. aeruginosa**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description or phenotype</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1ΔpilADΔfliC</td>
<td>PAO1 deletion mutant for the pilA and fliC genes (PAO1ΔΔ)</td>
<td>[2]</td>
</tr>
<tr>
<td>PAO1ΔΔΔcupB1</td>
<td>PAO1ΔΔ deletion mutant for the cupB1 gene</td>
<td>this study</td>
</tr>
<tr>
<td>PAO1ΔΔcupB2</td>
<td>PAO1ΔΔ deletion mutant for the cupB2 gene</td>
<td>this study</td>
</tr>
<tr>
<td>PAO1ΔΔcupB3</td>
<td>PAO1ΔΔ deletion mutant for the cupB3 gene</td>
<td>this study</td>
</tr>
<tr>
<td>PAO1ΔΔΔcupB4</td>
<td>PAO1ΔΔ deletion mutant for the cupB4 gene</td>
<td>[2]</td>
</tr>
</tbody>
</table>
PAO1ΔΔcupB5  PAO1ΔΔ deletion mutant for the cupB5 gene [2]
PAO1ΔΔcupB6  PAO1ΔΔ deletion mutant for the cupB6 gene this study
PAO1ΔΔtpsB4  PAO1ΔΔ deletion mutant for the tpsB4 gene (PA4540) this study
PAO1ΔΔ::pBAD PAO1ΔΔ deletion mutant with an arabinose-inducible promoter upstream of the cupB1 gene this study
PAO1ΔΔcupB1::pBAD PAO1ΔΔcupB1 deletion mutant with an arabinose-inducible promoter upstream of the cupB1 gene this study
PAO1ΔΔcupB2::pBAD PAO1ΔΔcupB2 deletion mutant with an arabinose-inducible promoter upstream of the cupB1 gene this study
PAO1ΔΔcupB3::pBAD PAO1ΔΔcupB3 deletion mutant with an arabinose-inducible promoter upstream of the cupB1 gene this study
PAO1ΔΔcupB4::pBAD PAO1ΔΔcupB4 deletion mutant with an arabinose-inducible promoter upstream of the cupB1 gene this study
PAO1ΔΔcupB5::pBAD PAO1ΔΔcupB5 deletion mutant with an arabinose-inducible promoter upstream of the cupB1 gene this study
PAO1ΔΔcupB6::pBAD PAO1ΔΔcupB6 deletion mutant with an arabinose-inducible promoter upstream of the cupB1 gene this study

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description or phenotype</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCR2.1</td>
<td>TA cloning vector for PCR products, lacZα</td>
<td>Invitrogen</td>
</tr>
<tr>
<td></td>
<td>ColE1 f1 ori, ApR , KmR</td>
<td></td>
</tr>
<tr>
<td>pCR-Blunt</td>
<td>Cloning vector for blunt-end DNA fragments,</td>
<td>Invitrogen</td>
</tr>
<tr>
<td></td>
<td>lacZα-cedB pUC ori, ZeocinR , KmR</td>
<td></td>
</tr>
<tr>
<td>pKNG101</td>
<td>Suicide vector in P. aeruginosa, sacB, SmR</td>
<td>lab collection</td>
</tr>
<tr>
<td>mini-CTX1</td>
<td>TcR, self-proficient integration vector with tet, Ω-FRT-attP-MCS, ori, int, and oriT</td>
<td>[3]</td>
</tr>
<tr>
<td>pBBR1MCS-4</td>
<td>AmpR, broad-host-range cloning vector</td>
<td>[4]</td>
</tr>
<tr>
<td>pET46 Ek/LIC</td>
<td>Expression vector</td>
<td>Novagen</td>
</tr>
<tr>
<td>pCR2.1-ΔcupB1</td>
<td>ΔcupB1 mutator cloned into pCR2.1</td>
<td>this study</td>
</tr>
<tr>
<td>pCR2.1-ΔcupB2</td>
<td>ΔcupB2 mutator cloned into pCR2.1</td>
<td>this study</td>
</tr>
<tr>
<td>pCR2.1-ΔcupB3</td>
<td>ΔcupB3 mutator cloned into pCR2.1</td>
<td>this study</td>
</tr>
<tr>
<td>pCR2.1-ΔcupB6</td>
<td>ΔcupB6 mutator cloned into pCR2.1</td>
<td>this study</td>
</tr>
<tr>
<td>pCR2.1-ΔtpsB4</td>
<td>ΔtpsB4 mutator cloned into pCR2.1</td>
<td>this study</td>
</tr>
<tr>
<td>pKNG101-ΔcupB1</td>
<td>ΔcupB1 mutator cloned into pKNG101</td>
<td>this study</td>
</tr>
<tr>
<td>pKNG101-ΔcupB2</td>
<td>ΔcupB2 mutator cloned into pCR2.1</td>
<td>this study</td>
</tr>
<tr>
<td>pKNG101-ΔcupB3</td>
<td>ΔcupB3 mutator cloned into pKNG101</td>
<td>this study</td>
</tr>
<tr>
<td>pKNG101-ΔcupB6</td>
<td>ΔcupB6 mutator cloned into pKNG101</td>
<td>this study</td>
</tr>
<tr>
<td>pKNG101-ΔtpsB4</td>
<td>ΔtpsB4 mutator cloned into pKNG101</td>
<td>this study</td>
</tr>
<tr>
<td>pCR2.1-tpsB4mi</td>
<td>tpsB4 and its promoter cloned into pCR2.1</td>
<td>this study</td>
</tr>
<tr>
<td>miniCTX-tpsB4</td>
<td>tpsB4 and its promoter cloned into mini-CTX1</td>
<td>this study</td>
</tr>
<tr>
<td>pMMB67EH-GW</td>
<td>Broad host range vector, IncQ, ptac, lacZα</td>
<td>lab collection</td>
</tr>
</tbody>
</table>
pMMB
rocS1
rocS1 gene cloned in pMMB67EH-GW [5]

pCR-Blunt-cupB5

cupB5 gene and its ribosome binding site cloned into pCRblunt

pCR-Blunt-cupB5

cupB5<sub>S69A</sub> cloned into pCRblunt

pCR-Blunt-cupB5

cupB5<sub>N85A,P96A</sub> cloned into pCRblunt

pCR-Blunt-cupB5<sub>V97W/V99W</sub>
cupB5 gene and its ribosome binding site cloned into pCRblunt

pBBR1-Mcs4-cupB5

cupB5<sub>S69A</sub> cloned into pBBR1-Mcs4

pBBR1-Mcs4-cupB5<sub>N85A,P86A</sub>
cupB5<sub>N85A,P86A</sub> cloned into pBBR1-Mcs4

pBBR1-Mcs4-cupB5<sub>V97W,V99W</sub>
cupB5<sub>V97W,V99W</sub> cloned into pBBR1-Mcs4

miniCTX-tpsB4<sub>D61A,D149</sub>
tpsB4<sub>D61A,D149</sub> cloned into mini-CTX1

miniCTX-tpsB4<sub>H82W,P105</sub>
tpsB4<sub>H82W,P105</sub> cloned into mini-CTX1

miniCTX-tpsB4<sub>G154W,E158</sub>
tpsB4<sub>G154W,E158</sub> cloned into mini-CTX1

miniCTX-tpsB4<sub>K173A,T175</sub>
tpsB4<sub>K173A,T175</sub> cloned into mini-CTX1

pB4-NT

tpsB4<sub>1-194</sub> cloned into pET46 Ek/LIC

pB4-P12

tpsB4<sub>42-194</sub> cloned into pET46 Ek/LIC

pB4-a1P1

tpsB4<sub>1-12</sub> cloned into pET46 Ek/LIC

pB4-P1

tpsB4<sub>42-122</sub> cloned into pET46 Ek/LIC

pB4-P2

tpsB4<sub>122-194</sub> cloned into pET46 Ek/LIC

pA4-TPS

tpsA<sub>4</sub> cloned into pET46 Ek/LIC

---

**Table S4: Primers used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ -&gt; 3’)</th>
<th>Purpose</th>
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</thead>
<tbody>
<tr>
<td>ΔcupB1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1014</td>
<td>TCCATCCGGAATGCGGAT</td>
<td>Deletion of cupB1 in PAO1ΔΔ, UpFw</td>
</tr>
<tr>
<td>1015</td>
<td>TTAATCAGTTTTTATCTCATTTCCG</td>
<td>Deletion of cupB1 in PAO1ΔΔ, UpFw</td>
</tr>
<tr>
<td>1016</td>
<td>ATGAAACAGTACGAGTAAGCGCGGCGGCTC</td>
<td>Deletion of cupB1 in PAO1ΔΔ, DwFw</td>
</tr>
<tr>
<td>1017</td>
<td>AGCCGAATCGACACTCTTCA</td>
<td>Deletion of cupB1 in PAO1ΔΔ, DwRe</td>
</tr>
<tr>
<td>1018</td>
<td>GAGTCGAAGGCAATCAGTGTGGAT</td>
<td>Deletion of cupB1 in PAO1ΔΔ, Fw</td>
</tr>
<tr>
<td>1019</td>
<td>GCGGTCGGATATTATTTACCTG</td>
<td>Deletion of cupB1 in PAO1ΔΔ, Re</td>
</tr>
<tr>
<td>ΔcupB2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>424</td>
<td>GAAGTCGCGTGCGATATCAACGGCAGGCGC</td>
<td>Deletion of cupB2 in PAO1ΔΔ, UpFw</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CGC
Deletion of cupB2 in PAO1∆, UpRe

Deletion of cupB2 in PAO1∆, DwFw

Deletion of cupB2 in PAO1∆, DwRe

External primer to proof deletion of cupB2 in PAO1∆, Fw

External primer to proof deletion of cupB2 in PAO1∆, Re

Deletion of cupB3 in PAO1∆, UpFw

Deletion of cupB3 in PAO1∆, UpRe

Deletion of cupB3 in PAO1∆, DwFw

Deletion of cupB3 in PAO1∆, DwRe

External primer to proof deletion of cupB3 in PAO1∆, Fw

External primer to proof deletion of cupB3 in PAO1∆, Re

Deletion of tpsB4 in PAO1∆, UpFw

Deletion of tpsB4 in PAO1∆, UpRe

Deletion of tpsB4 in PAO1∆, DwFw

Deletion of tpsB4 in PAO1∆, DwRe

External primer to proof deletion of tpsB4 in PAO1∆, Fw

External primer to proof deletion of tpsB4 in PAO1∆, Re

Deletion of cupB6 in PAO1∆, UpFw

Deletion of cupB6 in PAO1∆, UpRe

Deletion of cupB6 in PAO1∆, DwFw

Deletion of cupB6 in PAO1∆, DwRe

External primer to proof deletion of cupB6 in
### ΔcupB1::pBAD

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>730</td>
<td>GTCGGCAAAACAAATTCCTCGT</td>
<td>Insertion of pBAD in ΔcupB1, UpFw</td>
</tr>
<tr>
<td>731</td>
<td>TTACTCGTACTTTGTCATCTGAATTC</td>
<td>Insertion of pBAD in ΔcupB1, UpRe</td>
</tr>
<tr>
<td>732</td>
<td>ATGAACAAAGTACGTAAGCGGCGGTC</td>
<td>Insertion of pBAD in ΔcupB1, DwFw</td>
</tr>
<tr>
<td>733</td>
<td>CCCCTGACTCCAGAAACTT</td>
<td>Insertion of pBAD in ΔcupB1, DwRe</td>
</tr>
<tr>
<td>734</td>
<td>ATGATTTCGCAAAACAGGTTC</td>
<td>External primer to proof insertion of pBAD in PAO1ΔΔΔcupB1, Fw</td>
</tr>
<tr>
<td>735</td>
<td>TATTGCGGAGGTGCCCTATC</td>
<td>External primer to proof insertion of pBAD in PAO1ΔΔΔcupB1, Re</td>
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</table>

### cupB::pBAD

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer</th>
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<tbody>
<tr>
<td>638</td>
<td>AAAGGATCCCCGGGAGACGGTGTTTGTCC</td>
<td>Insertion of pBAD upstream of cupB1, UpFw</td>
</tr>
<tr>
<td>639</td>
<td>ACA GCT AGC TTT CAT ATG TCC</td>
<td>Insertion of pBAD upstream of cupB1, UpRe</td>
</tr>
<tr>
<td>640</td>
<td>TGGCTG CTG CCT GGA G</td>
<td>Insertion of pBAD upstream of cupB1, DwFw</td>
</tr>
<tr>
<td>641</td>
<td>GGACATGATAGCTAGCTGTTCCACAAATCCAAAGG</td>
<td>Insertion of pBAD upstream of cupB1, DwRe</td>
</tr>
<tr>
<td>642</td>
<td>GAGTCGAAAGGCATTTGAT</td>
<td>External primer to proof insertion of pBAD upstream of cupB1, Fw</td>
</tr>
<tr>
<td>643</td>
<td>GAGGTCAGCAGATACGGGACA</td>
<td>External primer to proof insertion of pBAD upstream of cupB1, Re</td>
</tr>
</tbody>
</table>

### qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>cupB1up</td>
<td>GCGCCGACCACATTTCAC</td>
<td></td>
<td>qPCR</td>
</tr>
<tr>
<td>cupB1down</td>
<td>GCGATGGGAACCTTCTTGTCA</td>
<td></td>
<td>qPCR</td>
</tr>
<tr>
<td>cupA1 F</td>
<td>GCCGCAAAACATATACATTTCA</td>
<td></td>
<td>qPCR</td>
</tr>
<tr>
<td>cupA1 R</td>
<td>CGGTACGCTGTCGAGGAT</td>
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<td>qPCR</td>
</tr>
<tr>
<td>TpsA4fw</td>
<td>GGGCCCGTGCTCTTGTCC</td>
<td>PA4541</td>
<td>qPCR</td>
</tr>
<tr>
<td>TpsA4rev</td>
<td>TGAACCTGTTCCTAATTTGAT</td>
<td>PA4541</td>
<td>qPCR</td>
</tr>
<tr>
<td>qTps1_Fw</td>
<td>ACCATTTCCGCGACTACAAC</td>
<td>PA2462</td>
<td>qPCR</td>
</tr>
<tr>
<td>qTps1_Rv</td>
<td>ACCTGGTTGAGGATACCTG</td>
<td>PA2462</td>
<td>qPCR</td>
</tr>
<tr>
<td>qTps2_Fw</td>
<td>GGAAGGTGGCTGAAGACAGC</td>
<td>PA0041</td>
<td>qPCR</td>
</tr>
<tr>
<td>qTps2_Rv</td>
<td>GGTCTACCGTGACCTTCCA</td>
<td>PA0041</td>
<td>qPCR</td>
</tr>
<tr>
<td>qTps3_Fw</td>
<td>GACGCCTACGTCACAGTCA</td>
<td>PA4625 (cdrA)</td>
<td>qPCR</td>
</tr>
<tr>
<td>qTps3_Rv</td>
<td>GTCCCCGTGATCGGTACTCTG</td>
<td>PA4625 (cdrA)</td>
<td>qPCR</td>
</tr>
<tr>
<td>qTps5_Fw2</td>
<td>CCGTCAGGCTCATCCTTCTT</td>
<td>PA0690</td>
<td>qPCR</td>
</tr>
<tr>
<td>qTps5_Rv2</td>
<td>TAGGGAGGTCGTGCACCTCC</td>
<td>PA0690</td>
<td>qPCR</td>
</tr>
<tr>
<td>rpoD Fwd</td>
<td>AGGCCGTTGAGCAGGATAC</td>
<td>qPCR, housekeeping gene</td>
<td></td>
</tr>
<tr>
<td>rpoD Rev</td>
<td>TCC CCA TGT CGT TGA TCA TG</td>
<td>qPCR, housekeeping gene</td>
<td></td>
</tr>
</tbody>
</table>
Complementation

1315  AAA GAA TTC CGG AAT CGC GCT CAG CCG C  cloning of tpsB4 and its promoter into miniCTX
1316  TTT ACT AGT CGG GAG CTT GCT CAG AAG TA  cloning of tpsB4 and its promoter into miniCTX
812   GAA TAG GTC GCG GGG TTT  cloning of cupB5 and its RBS into pBBR-MCS4
813   CCA CAC TCC AGG CGA GAG  cloning of cupB5 and its RBS into pBBR-MCS4

Point mutations

1819  TTT CCT GTG CCG CGA GGA AGG CGC G  introduction of a double point mutation in TpsB4 H82W P105A (c352g_antisense)
1820  CTC AAT CTG AGC CAA TTG TGG GCC GCC GCC GCC  introduction of a double point mutation in TpsB4 H82W P105A (c283t_a284g_e285g)
1821  CGC GCC TTC TCT CGC GCA CAG GAA A  introduction of a double point mutation in TpsB4 H82W P105A (c352g)
1822  GGC GGC GGC GCC CCA CAA TTG GCT CAG ATT GAG  introduction of a double point mutation in TpsB4 H82W P105A (c283t_a284g_e285g_antisense)
1823  CCG CGG TCC AGT GTG CGG AGC TGT GGC GTG CGC TTC  introduction of a double point mutation in TpsB4 G154 E158W (g499c_e501g_g511t_a512g)
1824  GAA GCG CAC GCC ACA GCT CGG ACC ACT GGA CCG CGG  introduction of a double point mutation in TpsB4 G154 E158W (g499c_e501g_g511t_a512g_antisense)
1825  GGC CTC CAG GCC GCA GGC CCC CTG CTG CCG G  introduction of a double point mutation in TpsB4 K173A T175P (a556g_a557c_a562c)
1826  CCG GCA GCA GGG GGC CTG CCT GGA GCC G  introduction of a double point mutation in TpsB4 K173A T175P (a556g_a557c_a562c_antisense)
1827  CAG CAG GGC TCG AGC GTC GAA CTG CCT  introduction of a double point mutation in TpsB4 D61A D149A (a221c_antisense)
1828  CTG GAC CGC GGT AGC TCG TTC GAG TGC  introduction of a double point mutation in TpsB4 D61A D149A (a485c_antisense)
1829  AGG CAG TTC GAC GCT CGA CGC CTG CTG  introduction of a double point mutation in TpsB4 D61A D149A (a221c)
1830  GCA CTC GAA CGA GCT ACC CGC GTC CAG  introduction of a double point mutation in TpsB4 D61A D149A (a485c)
introduction of a double point mutation in CupB5 V97W,V99W (g448t_t449g_c450g_g454t_t455g_c456g_antisense)

introduction of a double point mutation in CupB5 V97W,V99W (g448t_t449g_c450g_g454t_t455g_c456g)

introduction of a double point mutation in CupB5 N85A, P86A (a412g_a413c_c415g)

Introduction of S69A point mutation in CupB5 (a364g_g365c)

Introduction of S69A point mutation in CupB5 (a364g_g365c_antisense)

Structural work

B4-1 GACGACGACAAGATGGCCGCCGGATGCG GGG tpxB4<sup>1-194</sup> expression

B4-2 GAGGAGAAGCCTAGCAGGCCGCTCG GAGCGGACGACGACAAGATGGCCGCCGGATGCG

B4-3 GACGACGACAAGATGGCCGCCGGATGCG

B4-4 GAGGAGAAGCCTAGCAGGCCGCTCG

B4-5 GAGGAGAAGCCTAGCAGGCCGCTCG

A4-1 GACGACGACAAGATGGCCGCCGGATGCG GCC tpxA4<sup>1-242</sup> expression

A4-2 GAGGAGAAGCCTAGCAGGCCGCTCG

ATGCGTCGT
**Table S5. Antibodies used in this study**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Manufacturer</th>
</tr>
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<tbody>
<tr>
<td>CupB1</td>
<td>1:5000</td>
<td>Eurogentec</td>
</tr>
<tr>
<td>CupB6</td>
<td>1:1000</td>
<td>Eurogentec</td>
</tr>
<tr>
<td>CupB5</td>
<td>1:500</td>
<td>Eurogentec</td>
</tr>
<tr>
<td>RNA-Polymerase</td>
<td>1:5000</td>
<td>Neoclon W0023, MAb to β subunit of <em>E. coli</em> RNA-polymerase</td>
</tr>
<tr>
<td>DsbA</td>
<td>1:10000</td>
<td>provided by Dr. Karl E. Jaeger (IMET, Juelich, Germany)</td>
</tr>
<tr>
<td>Rabbit-anti-mouse HRP-conjugated</td>
<td>1:5000</td>
<td>SIGMA A9044</td>
</tr>
<tr>
<td>Goat-anti-rabbit HRP-conjugated</td>
<td>1:5000</td>
<td>SIGMA A6154</td>
</tr>
</tbody>
</table>

**Table S6. Characterization data for purified peptides used in this study.**

All peptides bear a free N- and C-terminus and were synthesized using standard Fmoc solid-phase protocols with pre-loaded Gln-Wang resin. Analysis of the peptides was performed with a Waters LC-MS system using an isocratic gradient of 5-98 % H₂O/MeOH (0.1 % HCOOH) over 18 min. Where relevant, the position of the MTSL spin label attachment is indicated with an asterisk.

<table>
<thead>
<tr>
<th>Peptide name</th>
<th>Peptide sequence</th>
<th>Calculated mass (Da)</th>
<th>Rₜ (min)</th>
<th>ES+ peaks found (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TpsA4⁷⁹-⁹⁵</td>
<td>KVFLVNPNGVVFGKSAQ</td>
<td>1804.1</td>
<td>9.97</td>
<td>902.7 (m/2+1), 1804.5 (m+1)</td>
</tr>
<tr>
<td>CupB5⁸⁰-⁹⁶</td>
<td>QVFLVNPNGVLFGRAQ</td>
<td>1816.1</td>
<td>11.28</td>
<td>908.9 (m/2+1), 606.3 (m/3+1), 1817.2 (m+1)</td>
</tr>
<tr>
<td>TpsA4⁷⁹-⁹⁵</td>
<td>KC*FLVNPNGVVFGKSAQ</td>
<td>1994.4</td>
<td>10.50</td>
<td>997.3 (m/2+1), 665.3 (m/3+1)</td>
</tr>
<tr>
<td>V80C MTSL</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TpsA4⁷⁹-⁹⁵</td>
<td>KVFLVNPNGC*FGKSAQ</td>
<td>1994.4</td>
<td>10.59</td>
<td>997.1 (m/2+1), 664.8 (m/3+1)</td>
</tr>
</tbody>
</table>
Figure S1: Model of full length TpsB4. This model was generated using the I-Tasser server [6] and domains are coloured as in Fig. 3A.

Figure S2: Dynamics of the TpsB4-P1 POTRA domain. Heteronuclear NOE cross-relaxation rates are shown for residues with well-resolved peaks in $^1$H$^{15}$N-NOE NMR spectra. Secondary structure elements are displayed below.
Figure S3: The TpsB4 N-terminal helix linker interacts with POTRA1. Comparison of $^1$H-$^{15}$N HSQC NMR spectra for $^{15}$N-labelled TpsB4-P1 (red) and TpsB4α1P1 (black), with chemical shift perturbations associated with L104 and Q107 in the β4-β5 loop expanded.

Figure S4: CupB5$^{80-96}$ interacts with TpsB4-P1. Comparison of $^1$H-$^{15}$N HSQC NMR spectra for $^{15}$N-labelled TpsB4-P1 incubated with either 0 (black) or 2 (purple) molar equivalents of CupB5$^{80-96}$ peptide.
Figure S5: The TpsB4-P1 β4-β5 loop closes on TpsA4/CupB5. Structure of TpsB4-P1 is shown with residues Q110 and R115 displayed as sticks (left). Interactions within this region results in the β4-β5 loop being ‘pinned’ back in a number of the final structures within this ensemble. During titration of TpsA4\textsuperscript{79-95} peptide against \textsuperscript{15}N-labelled TpsB4-P1, residues in this region display substantial chemical shift perturbations in \textsuperscript{1}H-\textsuperscript{15}N HSQC NMR spectra (right). This suggests that the β4-β5 loop is primed for recognition of TpsA4/CupB5 through conformational selection.
Figure S6: Comparison between TpsB4^{42-194}/TpsA4^{79-100} and FhaC/FHA. POTRA domains 1-2 (residues 53-210) from the crystal structure of FhaC [7] (pdb: 2dqz) were superimposed onto TpsB4^{42-194}/TpsA4^{79-100} and residues in TpsA4^{79-100} were changed to the sequence from FHA. In this model of FhaC/FHA the FHA sequence follows a similar trajectory through a groove in FhaC POTRA1 and POTRA2. Furthermore, there is complementarity between the electrostatic surface potential of FhaC and FHA side chain functionality.
**Figure S7: Model of mature CupB5.** This model was generated using the I-Tasser server [6] with C-score 0.05 and is coloured as rainbows from N- (red) to C-terminus (blue).

**References**

Site-Directed Mutagenesis and Gene Deletion Using Reverse Genetics

Daniela Muhl and Alain Filloux

Abstract

Understanding gene function is far easier when tools are available to engineer a bacterial strain lacking a specific gene and phenotypically compare its behavior with the corresponding parental strain. Such mutants could be selected randomly, either by natural selection under particular stress conditions or by random mutagenesis using transposon delivery as described elsewhere in this book. However, with the advent of the genomic era there are now hundreds of bacterial genomes whose sequence is available, and thus, genes can be identified, chosen, and strategies designed to specifically inactivate them. This can be done by using suicide plasmids and is most convenient when the bacterium of interest is easily amenable to genetic manipulation. The method presented here will describe the use of a suicide vector, pKNG101 (Kaniga et al. Gene 109:137–141, 1991), which allows the selection of a double-recombination event. The first event results in the integration of the pKNG101 derivative carrying the “mutator” fragment onto the chromosome, and could be selected on plates containing appropriate antibiotics. The pKNG101 carries the sacB gene, which induces death when cells are grown on sucrose. Growth on sucrose plates will thus select the second homologous recombination event, which results in removing the plasmid backbone and leaving behind the mutated target gene. This method has been widely used over the last 20 years to inactivate genes in a wide range of gram-negative bacteria and in particular in Pseudomonas aeruginosa.

Key words Suicide vector, pKNG101, Sucrose sensitivity, Conjugation, Homologous recombination

1 Introduction

All bacterial geneticists have now become incredibly efficient at modifying genes of interest by exchanging a wild-type gene for a mutated allele directly on the bacterial chromosome. This has become easier with the ever-increasing publication of bacterial genome sequences, hundreds of which are readily available on the Web. Genetic manipulation has also become much easier with the advent of PCR, which allows to engineer and to clone any DNA fragment of interest by the appropriate design of primers.

The allelic exchange on the bacterial chromosome could be a preferred method when one wants to disrupt exclusively the gene of interest with a minimum impact on expression of other
genes and on the genetic environment of the target gene. Random transposon insertion [2–4], or even targeted interruption of a gene by insertion of a suicide vector carrying a small region of the gene to be disrupted may have global impact on expression of downstream genes, especially when a gene is part of a cluster and included within an operon. In this case any polar effect will prevent to associate the observed phenotypic traits with the gene of interest.

In order to perform the allelic exchange one needs to direct the new allele to the target site on the genome by homologous recombination. This will be achieved by having the new allele cloned into a suicide vector, which cannot replicate in the bacterium to be genetically modified. For example the standard pUC18 or pUC19 plasmids are ColE1 replicon and do not replicate in *P. aeruginosa* except if they are specifically modified [5]. When introducing these plasmids in *P. aeruginosa*, the only way the plasmid can be maintained, together with the resistance gene they carry, is to become stably inserted into the chromosome. If the gene of interest, or part of it, is cloned into the pUC plasmids, homologous recombination will allow this integration event to occur. However, in this case there is no possibility to impose a selection pressure for the excision of the plasmid and for leaving behind the mutated allele. This step is crucial for the allelic exchange and again several plasmids have been developed that carries a counter-selectable marker. This will allow a screen for the loss of the plasmid backbone and its excision from the chromosome via a second event of homologous recombination. Such marker, which was included in the original suicide vector pKNG101 [1, 6], is the *Bacillus subtilis sacB* gene that confers sucrose sensitivity. If this plasmid is on the chromosome and the cells spread on agar plates containing 5 % sucrose, it will impose a strong selective pressure and only those bacteria, which have expelled the plasmid backbone and the *sacB* gene will survive. This occurs via a second event of recombination, which will either reconstruct the wild-type allele or will replace it with the mutated allele as precisely described in the methods presented below. Several of these counter-selectable plasmids are now available [7]. Of note another quick method to generate mutants in *P. aeruginosa* is to make use of the lambda red recombinase system [8].

The allelic exchange will allow replacing the wild-type gene with a gene, which for example contains a nucleotide(s) change resulting in the substitution of only one single amino acid in the gene product. Furthermore the method is more generally used to delete the gene of interest in a clean and precise manner. Importantly, the deletion can be obtained without leaving behind, and in the middle of the gene, an antibiotic resistance cassette. Therefore, once the deletion has been checked by PCR, additional deletion can be introduced in this new genetic background without having the problem of choosing new and different antibiotic resistance cassette for the selection procedure. This way as many deletions as desired can be introduced in one single strain.
2 Materials

2.1 Strains and Plasmids

See Tables 1 and 2.

2.2 Amplification of the Gene of Interest and Mutator Fragment

1. Template DNA: Purified genomic *Pseudomonas aeruginosa* DNA (50–100 ng/μl).

2. Polymerase Chain Reaction (PCR) and agarose gel electrophoresis (see protocol in Chapter 35).

3. Purification of the PCR product: PCR purification kit.

4. NanoDrop to measure the concentration of the PCR product.

5. pCR2.1 TA cloning kit: pCR2.1 vector, 10× ligation buffer, T4 DNA ligase (4 U/μl), molecular biology water.

6. Transformation: Ice, competent Top10 *Escherichia coli* cells (50 μl competent cells/PCR reaction), 42 °C water bath, 500 μl SOC (Super Optimal broth with Catabolite repression) medium/ transformation, Luria–Bertani (LB) agar plates supplemented with 50 μg/ml ampicillin (Amp50), 25 μg/ml kanamycin

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### Table 1

<table>
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<th>Strain</th>
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<td><em>Escherichia coli</em>:</td>
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<tr>
<td>CC118 λpir Δ(ara-leu) araD ΔlacX74 galE galK phosphoA20 thi-1 rpsE rpoB argE(Am) recA1 Rf(λpir)</td>
<td>Host strain for pKNG101 replication</td>
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<tr>
<td>XL1-Blue recA1 endA1 gyra96 thi-1 hsdR17 supE44 relA1 lac [F proAB lacPΔM15 Tn10 (TetR)]</td>
<td>Strain used for site-directed mutagenesis</td>
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<tr>
<td>Top10 F-mrcA Δ(mrr-hsdRMS-mcrBC)Φ80 lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ-</td>
<td>Strain for pCR2.1 replication</td>
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### Table 2

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<th>Plasmid</th>
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<td>pKNG101</td>
<td>Streptomycin (Sm)</td>
<td>Suicide vector in <em>P. aeruginosa</em></td>
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<tr>
<td>pCR2.1</td>
<td>Kanamycin (Km), Ampicillin (Amp)</td>
<td>TA cloning vector for PCR products</td>
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</table>
(Kan25) and 40 μg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal40). Prepare a 40 mg/ml stock solution of X-gal in dimethyl sulfoxide (DMSO), see Note 1.

### 2.3 Site-Directed Mutagenesis

1. **Template DNA**: Purified methylated plasmid DNA that contains the gene/DNA fragment in which the mutation will be introduced (see Notes 2 and 3). This method uses plasmids that have a size up to 8 kb.

2. For the PCR reaction: Pfu Ultra High Fidelity^{PLUS} DNA polymerase (2.5 U/μl), 10× Pfu Ultra High Fidelity^{PLUS} DNA polymerase reaction buffer, 10 μM of primer 3’, 10 μM primer 5’, 20 mM dNTP mix, 5 M betaine solution, molecular biology water, 5–50 ng double stranded plasmid DNA (template). PCR machine with hot-top assembly.

3. 1 % agarose gel.

4. Digest of parental DNA: DpnI restriction enzyme (10 U/μl), 37 °C incubator.

5. Transformation: Ice, competent XL1-Blue E. coli cells (80 μl competent cells/PCR reaction, see Note 4), 42 °C water bath, 500 μl SOC medium/transformation, LB agar plates supplemented with the required antibiotic.

### 2.4 Re-cloning into pKNG101

1. LB agar plates supplemented with 50 μg/ml streptomycin (Sm50). Make a 10 ml stock solution of 50 mg/ml Sm: Weigh 0.5 g Sm in a 15 ml falcon tube. Add water to a volume of 8 ml and vortex to resuspend. Make up to 10 ml with water. Filter-sterilize (see Note 5). For 1 plate, add 20 μl of the stock solution to 20 ml hand warm LB agar and pour one LB agar plate. Dry the plate next to the Bunsen burner for 30–45 min.

2. LB agar plates supplemented with 5 % sucrose. Prepare a 50 % sucrose stock solution in a total of 50 ml. Weigh 25 g of sucrose in a 50 ml falcon tube. Top up to 50 ml with water and resuspend by vortexing (see Note 6). Top up again to 50 ml with water and filter-sterilize. To make one plate mix 2 ml of the 50 % sucrose solution with 18 ml of hand warm LB agar. Pour the plate and leave to set. Dry next to the Bunsen burner for 30–45 min.

3. Purified sucrose-sensitive pKNG101 and pCR2.1-mutator at a concentration of 200 ng/μl at least (see Notes 7 and 8).

4. Restriction enzymes and buffers to reclone the mutator fragment from pCR2.1 into pKNG101.

5. Purification of the digested vector and mutator: Two 1 % agarose gels, PCR and gel purification kit if required.


7. Transformation: Ice, competent CC118\_pir E. coli cells (100 μl per reaction), 42 °C water bath, 500 μl SOC medium/transformation, LB agar plates supplemented with Sm50.
2.5 Generation of the Mutant

1. LB agar plates supplemented with 2,000 μg/ml streptomycin (Sm2000). Make a stock solution of 100 mg/ml Sm.
2. LB agar plates supplemented with 5% sucrose. Prepare fresh on the day of use.
3. Primers for colony PCR:
   (a) Primers that anneal to pKNG101: 10 μM of primer UpKn (5′—ccctgatttctagtatgg—3′), 10 μM primer RpKn (5′—catatcacaagttgtgga—3′).
   (b) External primers that anneal next to the modified gene: 10 μM primer 3′, 10 μM primer 5′.
   (c) Protocol for colony PCR see Chapter 35.
4. PIA and LB agar plates.

3 Methods

3.1 Amplification of the Gene of Interest for Site-Directed Mutagenesis

Prior to site-directed mutagenesis, amplify a 1,000 bp DNA fragment of the gene of interest from the Pseudomonas genome using primers 1 and 2 (Fig. 1). The region of the desired mutation should be in the middle of the DNA fragment flanked by approximately 500 bp on each site.

1. Set up a PCR reaction following the protocols from Chapter 35.
2. Ligate the PCR product into the cloning vector pCR2.1. Mix on ice:
   - 2 μl of pCR2.1 vector
   - 1 μl of T4 DNA ligase
   - 1 μl of 10x ligation buffer
   - 1 μl of insert
   - 5 μl of molecular biology water to a total of 10 μl
3. Ligate overnight at 16 °C and proceed to Subheading 3.3.

3.2 Amplification of the Mutator Fragment

To engineer a clean deletion of a gene on the Pseudomonas chromosome, a fragment of approximately 500 bp on either side of the gene of interest (GOI) is amplified. The 3′ primer of the upstream fragment (primer 2), and the 5′ primer of the downstream fragment (primer 3) will contain a few bases of the gene, including the start and stop codons (see Figs. 2 and 3).

The two fragments are joined by overlapping PCR to create a mutator fragment, which is cloned into pKNG101, a Pseudomonas suicide vector. Subsequently, pKNG101 is conjugated into the Pseudomonas strain of interest. Through a series of two recombination events, the gene will be deleted.
1. Retrieve gene sequence of the GOI including roughly 800 bp upstream and downstream of GOI.

2. Design primers to amplify ~500 bp upstream and ~500 bp downstream of GOI (see Figs. 2 and 3, see Note 9). Also design

**Fig. 1** Primer design for the amplification of the gene of interest. Primer 1 and 2 (underlined) are used to amplify a 1,000-bp DNA fragment from the *Pseudomonas* chromosome. In the middle of this DNA fragment the point mutation (SPM) will be introduced. Primer 3 and primer 4 are external primers that bind approximately 200 bp upstream of primer 1 or downstream of primer 2, respectively. External primers will be used to check for the presence of the first and second crossover.

**Fig. 2** Schematic presentation of the gene of interest (GOI) and the primers used to create the mutator fragment for gene deletion. The upstream fragment is labelled up and the downstream fragment dw. ATG gene start and TAA gene stop are indicated. *Numbers in brackets* correspond to primers 1–5.
external primers that will be used to check for the deletion of the GOI on the *Pseudomonas* genome.

(a) Primer 1: Forward (Fw) primer ca. 500 bp upstream of the ATG of the GOI.

(b) Primer 2: Reverse (Rv) primer upstream fragment; consists of 9 bases + ATG + 6 bases + 9 bases tail overlapping with Primer 3 (Fig. 4).

---

**Fig. 3** Example strategy for amplification of the mutator fragment. *Underlined* is the gene of interest. ATG start codon and TAA stop codon are in *bold letters*. (1) Primer 1: Forward (Fw) primer ca. 500 bp upstream of the ATG of the gene to be deleted. (2) Primer 2: Reverse (Rv) primer upstream fragment; consists of 9 bases + ATG + 6 bases + 9 bases tail overlapping with Primer 3. (3) Primer 3: Fw primer downstream fragment; consists of 6 bases + STOP + 9 bases tail overlapping with primer 2. (4) Primer 4: Rv primer ca. 500 bp downstream of the TAA of the gene to be deleted. (5) Primer 5: Fw external primer, ca. 100 bp upstream of primer 1. (6) Primer 6: Rv external primer, ca. 100 bp downstream of primer 4. (a) Gene of interest with its upstream and downstream regions. (b) A PCR using primers 1 and 2 amplifies the upstream fragment, whereas the downstream fragment is amplified using primers 3 and 4 in a PCR. Both fragments are approximately 500 bp long and are used, together with primers 3 and 4, to create the mutator fragment by an overlapping PCR.
**b**

520 bp upstream fragment:

```
TCCATTCGGGAATGCGAGT(1)GGGCGGCACTAGGGTTCTCTCCTGCTGAGAGCCATCGTGCCT
GTCTTGTGAGTATCCCGATATCGGCACTAGGGTTCTCTCCTGCTGAGAGCGGGCGGAACATCGTGCCT
GTCTTCTGCGGCAATCGTGCCTGCTGCTGAGAGCGGGCGGAACATCGTGCCTGCTGCTGAGAGCG
```

(c) Primer 3: Fw primer downstream fragment; consists of 6 bases + STOP + 9 bases + 9 bases tail overlapping with primer 2.

(d) Primer 4: Rv primer ca. 500 bp downstream of the TAA/TGA of the GOI.

(e) Primer 5: Fw external primer, ca. 100 bp upstream of primer 1.

(f) Primer 6: Rv external primer, ca. 100 bp downstream of primer 4.

**Fig. 3 (continued)**
3. Check insert sequence (mutator fragment) for the presence/absence of restriction sites compatible with pKNG101.

4. Prepare on ice two separate PCR reactions to amplify the upstream (primer 1 and 2) and downstream (primer 3 and 4) DNA fragments following the protocol from Chapter 35.

5. After checking the size and purity of the PCR product by agarose gel-electrophoresis, purify the residual 45 μl PCR product with a PCR purification kit or by a method of your choice.

6. Measure DNA concentration with a NanoDrop and dilute the PCR product 1:20 in water and use 1.5 μl of the upstream and 1.5 μl of the downstream fragment for an overlapping PCR using primer 1 and 4. The overlapping PCR is designed to anneal the two fragments and create a mutator fragment for the gene knockout.

   Prepare on ice the following PCR mix and run a PCR following the conditions of Table 3:

   1.5 μl 1:20 diluted upstream fragment
   1.5 μl 1:20 diluted downstream fragment
   5 μl 10 μM primer 1
   5 μl 10 μM primer 4
   1 μl 20 mM dNTPs

![Fig. 4 Alignment of primers 2 and 3. Both primers contain 18 bases that overlap. This overlapping tail will be used in an overlapping PCR to unite both fragments and thereby forming the mutator fragment](image)

| Table 3 |
| Reaction conditions for the overlapping PCR |

<table>
<thead>
<tr>
<th>Step</th>
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<td>t3.4</td>
<td>Initial denaturing step</td>
<td>95 °C</td>
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<tr>
<td>t3.5</td>
<td></td>
<td><strong>Start cycle: 20 cycles</strong></td>
</tr>
<tr>
<td>t3.6</td>
<td>Denaturing</td>
<td>95 °C</td>
</tr>
<tr>
<td>t3.7</td>
<td>Annealing</td>
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<tr>
<td>t3.8</td>
<td>Extension</td>
<td>72 °C</td>
</tr>
<tr>
<td>t3.9</td>
<td><strong>End cycle</strong></td>
<td></td>
</tr>
<tr>
<td>t3.10</td>
<td>Final extension</td>
<td>72 °C</td>
</tr>
<tr>
<td>t3.11</td>
<td>Hold</td>
<td>10 °C</td>
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</table>
10 μl 5× Expand High FidelityPLUS buffer
10 μl 5 M Betaine
15.5 μl molecular biology water to a final volume of 50 μl

7. After the overlapping PCR, check PCR product on an agarose gel and ligate the PCR product into pCR2.1 by following the protocol from Subheading 3.1.

3.3 Transformation into Competent Top10 E. coli Cells

Carry out all procedures on ice unless otherwise stated! Work sterile.

1. Gently thaw your competent Top10 cells on ice and transfer 50 μl into a pre-chilled 1.5 ml eppendorf tube that contains the whole ligation mixture.

2. Incubate on ice for 30 min.

3. Heat shock the transformations for 30 s at 42 °C and immediately place the reaction on ice for 1 min.

4. Add 500 μl SOC medium to each transformation and incubate the transformations at 37 °C for 1 h shaking at 200–250 rpm.

5. Spread 100 μl of the transformation on LB agar plates supplemented with Amp50, Kan25 and X-gal40, see Notes 10 and 11.

6. Spin down the residual 400 μl for 1 min in a microcentrifuge and decant the supernatant. Resuspend the pellet in the remaining 100 μl supernatant and spread on LB agar plates supplemented with Amp50, Kan25 and X-gal40.

7. Incubate the transformation plates at 37 °C for at least 16 h.

8. Positive clones that contain the insert can be identified by blue/white screening [9]. Patch positive (white) colonies on the LB Amp50, Kan25, X-gal40 agar plates and inoculate a 5 ml culture for plasmid preparation.

9. Purify plasmids and verify insert by sequencing.

10. For gene deletion, proceed directly to Subheading 3.7.

3.4 Primer Design for Site-Directed Mutagenesis

1. Both 3’ and 5’ primer have to contain the point mutation and anneal to the same sequence of DNA.

2. The optimal length of the primers is between 25 and 45 bp and the melting temperature is about ≥78 °C.

3. The point mutation should be designed in the middle of the primer, so that 10–15 bases with the correct sequence are present on either side of the primer.

4. The GC content of the primers should not be higher than 40 % and both primers should have two or three C or G bases at their ends (see Note 12).
3.5 Mutagenesis by PCR

Carry out all procedures on ice unless otherwise stated.

1. Prepare on ice the sample reaction by mixing
   - 5 μl of 10× Pfu Ultra High Fidelity\textsuperscript{PLUS} DNA polymerase buffer
   - 1.5 μl of plasmid DNA (see Note 13)
   - 1.5 μl of 3′ primer
   - 1.5 μl of 5′ primer
   - 1 μl of dNTPs
   - 10 μl betaine
   - 28.5 μl molecular biology water to a final volume of 50 μl

2. At last add 1 μl of Pfu Ultra High Fidelity\textsuperscript{PLUS} DNA polymerase.

3. Start the PCR reaction (see Note 14). The number of cycles depends on the type of mutation that is introduced. Point mutations require only 12 cycles, codon changes 16 cycles and multiple codons deletions or insertions 18 cycles.

4. After the PCR has finished incubate the PCR reaction on ice at least for 2 min to cool down the sample.

3.6 DpnI Digestion of the Parental DNA and Transformation into XL1-Blue Competent Cells

1. To digest the non-mutated, parental DNA add 1 μl of the restriction enzyme DpnI directly into each PCR tube and mix gently by pipetting the reaction mix up and down.

2. Incubate the restriction mix for 1 h at 37 °C (see Note 15).

3. Carry out all procedures on ice! Work sterile.

4. Gently thaw your competent XL1-Blue cells on ice and aliquot 80 μl into a pre-chilled 1.5 ml eppendorf tube.

5. Add 5 μl of the DpnI-digested DNA to the competent cells and gently mix by stirring with your pipette tip.

6. Incubate the reactions on ice for 30 min.

7. Heat shock the transformations for 1:15 min at 42 °C and immediately place the reaction on ice for 2 min.

8. Add 500 μl SOC medium to each transformation and incubate the transformations at 37 °C for 1 h shaking at 200–250 rpm.

9. Spread 100 μl of the transformation on LB agar plates supplemented with the required antibiotic.

10. Spin down the residual 400 μl for 1 min in a microcentrifuge and decant the supernatant. Resuspend the pellet in the remaining 100 μl supernatant and spread on a LB agar plate supplemented with the required antibiotic (see Notes 10 and 11).

11. Incubate the transformation plates at 37 °C for at least 16 h.

12. Patch the appearing colonies on the LB agar plates with antibiotic and inoculate a 5 ml culture for plasmid preparation.

13. Verify the mutation by sequencing.
1. To reclone the mutator from pCR2.1 into pKNG101, identify restriction sites which are present on the pCR2.1-mutator and pKNG101, but not in the mutator sequence. Prepare the restriction digest in 1.5 ml eppendorf tubes. If the plasmid concentration is higher than 200 ng/μl use 10 μl vector, and if the concentration is lower than 200 ng/μl use 20 μl vector.

Restriction mix:
- 10 μl/20 μl vector
- 4 μl restriction enzyme buffer
- 1 μl enzyme A
- 1 μl enzyme B
- $X$ μl molecular biology water to a final volume of 40 μl

2. Carry out the digest at the appropriate temperature for 2–3 h (see Note 16).

3. Take 5 μl of the restriction digest and load on a 1 % agarose gel to check for restriction. Two bands should separate for pCR2.1 and the mutator fragment and one band should be visible for pKNG101.

4. Purify the digested pKNG101 in a final volume of 30 μl water with a PCR purification kit or the method of your choice.

5. Meanwhile, load the whole volume of the pCR2.1-mutator restriction digest on a 1 % agarose gel and run the gel until vector backbone and mutator are sufficiently separated. Using a scalpel cut out the mutator band from the gel and purify with a gel purification kit or the method of your choice (see Note 17).

6. Measure the concentrations of your purified vector and mutator and ligate in a 1:3 ratio by a method of your choice.

7. Transform competent CC118λpir E. coli cells with the whole ligation reaction: Pre-chill the ligation reaction on ice, then add 100 μl competent cells and incubate on ice for 30 min. Heat shock the transformation reaction for 2 min at 42 °C. Place reaction on ice immediately and chill for 1 min. Add 500 μl SOC medium and incubate at 37 °C shaking at 200–250 rpm.

8. Spread 100 μl of the transformation on LB Sm50 agar plates. Spin down the residual 400 μl for 1 min in a microcentrifuge and decant the supernatant. Resuspend the pellet in the remaining 100 μl supernatant and spread on a LB Sm50 agar plate.

9. Incubate the transformation plates at 37 °C for at least 16 h.

10. Patch single colonies on LB agar plates supplemented with Sm50 and incubate at 37 °C overnight.
11. Confirm insertion of the mutator into pKNG101 by colony PCR (see Chapter 33) and restriction digest of the purified pKNG101.

12. Conjugate pKNG101-mutator into Pseudomonas by three-partner conjugation (see Chapter 3).

Since pKNG101 derivatives cannot replicate in P. aeruginosa, a single homologous recombination event between the gene of interest on the chromosome and the mutator fragment on pKNG101 has to occur to maintain the plasmid (and corresponding antibiotic resistance), integrating the whole vector into Pseudomonas chromosome. The integration can take place via a crossover occurring in the pKNG101 mutator upstream or downstream of the gene of interest (for site-directed mutagenesis see Fig. 5a and for gene deletion see Fig. 6).

1. Patch appearing colonies as soon as possible on LB Sm2000 as PIA might cause mutation of Pseudomonas. Incubate at 37 °C overnight.

2. Confirm first event of recombination by colony PCR using external primers in combination with the plasmid primers RpKn or UpKn. Use of external primers confirms the insertion of the plasmid in the correct genomic location.

3. With a loop pick two upstream and two downstream recombinants and restreak them onto LB agar plates supplemented with Sm2000 to single colonies. Incubate at 37 °C overnight.

4. Pick a single colony of each recombinant and streak them to single colonies on LB agar plates supplemented with 5 % sucrose. Change the loop between the streaks to yield more singles colonies. Incubate the plates at room temperature for 48–72 h (see Note 18).

After 2 days on sucrose some single colonies should appear on the plates. These bacteria have ejected pKNG101 from their chromosome by a second upstream or downstream crossover event leaving behind either the mutated or the wild-type gene (see Fig. 5b for site-directed mutagenesis and Fig. 6 for gene deletion).

1. Patch each single colony onto an LB Sm2000 agar plate, a PIA agar plate and a LB agar plate, in that order, and incubate at 37 °C overnight.

2. Clones that are Sm2000 sensitive (because they have ejected pKNG101, see Notes 19 and 20), but grow on PIA and LB are checked by colony PCR using the external primers. Five μl of the PCR product are run on a 1 % agarose gel to verify purity and right size of the PCR product. For site-directed mutagenesis, residual PCR products are sequenced using primer 1 or 3 as a sequencing primer.
Fig. 5 Cloning strategy for site-directed mutagenesis of a gene of interest on the chromosome of *P. aeruginosa*. The pKNG101-mutator is introduced in *P. aeruginosa* by three-partner conjugation. Since pKNG101 derivatives cannot replicate in *Pseudomonas*, a single homologue recombination event between the functional GOI on the
3. Positive clones from the patch on the LB plate are streaked to single colonies on first LB Sm2000 and then on LB agar plates. Incubate at 37 °C overnight. Bacteria should not grow on Sm2000.

4. Single colonies are patched on LB agar plates, incubated at 37 °C overnight and checked again by colony PCR and sequencing. Glycerol stocks are prepared from positive clones.

4 Notes

1. The X-gal is light sensitive and should be kept in the dark. Wrap the tube in aluminum foil to protect the X-gal from light and store it in the freezer. The X-gal solution should be colorless; however, if it is yellow the X-gal has gone off.

2. Prepare plasmid template DNA from a dam+E. coli strain to ensure that the plasmid is methylated. Methylated template DNA and also hemi-methylated PCR products will be digested by the endonuclease DpnI which allows selection of mutagenized plasmids only.

3. Use freshly prepared plasmid DNA as a template for the PCR, as the mutagenesis of freshly prepared DNA is more efficient than the mutagenesis of defrosted DNA.

4. XL1-Blue E. coli cells are resistant to tetracycline. If your mutagenized plasmid carries the tetR resistance gene, you have to use an alternative E. coli strain that is sensitive to tetracycline. Alternatively, use a plasmid with a different resistance marker.

5. Sterile streptomycin stock solutions can be stored at −20 °C for several months or at 4 °C in the fridge for up to 1 week. Do not re-freeze defrosted solutions as repeated freezing and defrosting decreases the stability of the streptomycin.

6. Sucrose solutions have to be made fresh due to the low stability of sucrose in solution. When preparing a 50 % sucrose solution it will take 1–1.5 h before the sucrose will go into solution. Do not heat up the solution, as high temperatures decrease the

Fig. 5 (continued) chromosome and the mutator fragment on pKNG101 has to occur, integrating the whole plasmid into Pseudomonas chromosome. Primers are presented by arrows labelled with a number, e.g., Primer 1 is labelled (1). UpKn and RpKn are primers that anneal to pKNG101. (a) The integration can be caused by an upstream or downstream crossover. To check for pKNG101 integration primers 3 and UpKn or primers 4 and RpKn can be used. (b) The forced rejection of pKNG101 from the chromosome of Pseudomonas occurs via a second crossover, which can be again upstream or downstream. This second crossover generates either the wild-type gene or the mutated gene and is confirmed by colony PCR using the external primers 3 and 4. Both, wild-type and mutated gene, will yield the same size PCR product, and therefore, confirmation of successful site-directed mutagenesis is archived only by sequencing.
first crossing-over:

a. upstream

b. downstream:

second crossing-over of the:

upstream integration

downstream integration

c. deletion of GOI:

d. conversion to wild type:

**Fig. 6** Cloning strategy for gene deletion. The pKNG101-mutator is introduced in *P. aeruginosa* by three-partner conjugation and will integrate into *Pseudomonas* chromosome by homologue recombination. Primers are presented by arrows labelled with a number, e.g., Primer 5 is labelled (5). UpKn and RpKn are primers that anneal to pKNG101. GOI is the gene of interest. (a) The integration can be caused by an upstream or downstream crossover. To check for pKNG101 integration primers 5 and UpKn or primers 6 and RpKn can be used,
stability of sucrose. Also, pay attention that the LB agar has cooled down to hand temperature before adding the sucrose. You can speed down the cooling of the agar by swirling the agar bottle in a beaker with cold water.

7. Test the sucrose sensitivity of pKNG101 by streaking out a single colony of pKNG101-containing CC118λpir E. coli cells, first on LB agar supplemented with Sm50 and then using the same loop on LB agar supplemented with 5% sucrose. Incubate at room temperature for 2 days. Cells should grow on Sm50 but not sucrose verifying the sucrose sensitivity of pKNG101.

8. To yield high concentrations of pKNG101, inoculate two times 50 ml LB medium with a single sucrose sensitive colony of CC118λpir/pKNG101, taken from a LB Sm50 agar plate, and grow overnight at 37 °C shaking. Purify pKNG101 from the total 100 ml culture by the method of your choice. The obtained concentration of purified pKNG101 in water should be approximately 800 ng/μl.

9. When you design the mutator fragment, pay attention that you (a) delete in frame, (b) do not delete the Shine–Dalgarno Sequence of downstream genes, (c) do not delete START/STOP codon of surrounding genes and (d) do not delete part of another gene when genes overlap in an operon.

10. Spread the transformation reaction only on well dried agar plates to obtain single colonies. The use of wet agar plates might result in less single colonies.

11. If your plasmid contains more than one resistance marker, use only one antibiotic for selection to facilitate the growth of bacteria.

12. To simplify the design of the primers use the free online QuikChange Primer Design program Web site from Agilent technologies (https://www.genomics.agilent.com/). You have to register with Agilent for this service, but will not be charged. Paste in the DNA sequence, choose up to 7 bases that you want to mutate and you will obtain your primer sequences along with information about their length, melting temperature and energy cost of mismatches.

13. Use a range of DNA concentrations varying from 5 to 50 ng (e.g., 5, 10, and 20 ng). Do not use more than 50 ng DNA in the transformation reaction.

Fig. 6 (continued) e.g., if an upstream crossover has occurred, primers 5 and RpKn will amplify a shorter PCR product than in the case of a downstream crossover (compare length of grey arrows). (b) The forced rejection of pKNG101 from the chromosome of Pseudomonas occurs via a second crossover, which can be again upstream or downstream. This second crossover generates either the wild-type gene or gene deletion and is confirmed by colony PCR using the external primers 5 and 6. The amplified PCR product for the mutant will be smaller in size than the PCR product amplified for the wild type.
total as high DNA concentration decrease the efficiency of the mutagenesis. As a control to check for DNA amplification, also prepare a tube that contains DNA but none PfU Ultra High FidelityPLUS DNA polymerase. Check for amplification of the plasmid by agarose gel electrophoresis. Prepare a 1% agarose gel and load 5 μl of the PCR reaction on the gel. Compare the strength of the DNA band of the control reaction (without polymerase) and the sample reactions (with polymerase). A much stronger band in the sample reactions indicates successful amplification of the plasmid.

14. To improve DNA amplification, increase the annealing temperature for the primers to up to 68 °C. Higher temperatures will avoid the formation of secondary structures and also increase the binding specificity of the primers (Table 4).

15. Increase the incubation time with DpnI up to 1:30 h if you have a low mutagenesis efficiency to allow DpnI to digest more parental DNA.

16. Do not digest for longer than 3 h as the restriction enzymes may start to cut the DNA unspecific.

17. Change the TAE buffer in the gel tank prior to running your gel to avoid any cross-contamination with DNA present in the tank. Cut out the DNA band with a clean sharp scalpel. Make sure to stay as close as possible to the DNA band.

18. The temperature should not be over 22 °C, because Pseudomonas would grow too quickly and eject pKNG101 too fast giving no time for the recombination event. Also high temperatures decrease the stability of the sucrose, resulting in not enough pressure on Pseudomonas to eject pKNG101 from the chromosome. If the temperature in the summer is too high in the lab, try to find an incubator that you can set to room temperature. In winter, at lower temperatures Pseudomonas might take more time to grow but will eject pKNG101 properly.

19. Some Pseudomonas cells might grow on the LB Sm2000 agar plates. However, the growth should not be as strong as on the
LB agar plate. If that is the case, re-streak bacteria on LB and
LB Sm2000 agar plates to single colonies and incubate at
37 °C overnight. No colonies should grow on the LB Sm2000
plates. Patch colonies from the LB plate, incubate at 37 °C and
carry out colony PCR to gain samples for sequencing.

20. Strong growth on the LB Sm2000 agar plate indicates that the
selection did not work properly. Re-streak bacteria on LB 5 %
sucrose agar plates and incubate again at room temperature for
48–72 h. Proceed as described by patching single colonies on
LB Sm2000, PIA and LB agar plates.

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