Assembly of the *Pseudomonas aeruginosa* type II secretion system

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Assemblage van het *Pseudomonas aeruginosa* type II secretie systeem

(met een samenvatting in het Nederlands)

Proefschrift

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Jorik Gerardus Arts
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Promotor: Prof. Dr. J.P.M. Tommassen
(verbonden aan de Faculteit Bètawetenschappen der Universiteit Utrecht)

Co-promotor: Dr. M.C. Koster
(verbonden aan de Faculteit Bètawetenschappen der Universiteit Utrecht)

The studies described in this thesis were performed at the Department of Microbiology, Institute of Biomembranes, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands.

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voor ons pap

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Chapter 1

General introduction

Pseudomonas aeruginosa is a rod-shaped, polarly flagellated Gram-negative bacterium with filamentous pili on its cell surface. It contains a large genome of over six million base pairs with more than 9% of the assigned open reading frames encoding known or putative transcriptional regulators and two-component systems (110). This latter characteristic is thought to allow the bacterium to adapt to a wide variety of environmental conditions. Indeed, *P. aeruginosa* grows in soil, marshes, and coastal marine habitats, as well as on plant and animal tissues and is the most common pathogen responsible for acute respiratory infections in immuno-compromised patients and for chronic respiratory infections in patients suffering from cystic fibrosis (20). Besides the high incidence and the severity of infections, increased resistance of *P. aeruginosa* to conventional antibiotics forms a problem (86). Important virulence factors of this bacterium are its ability to adhere to host cells, to form biofilms, and to secrete a variety of proteins that play a role in pathogenesis, including elastase, alkaline protease, and exotoxin A (59). For proteins to be secreted, they have to travel from the cytoplasm, where they are synthesized, over the cell envelope to the exterior. Gram-negative bacteria have evolved several mechanisms to transport proteins across the entire cell envelope. In the next sections, the cell envelope of Gram-negative bacteria, translocation of proteins across the inner membrane, transport of proteins to the outer membrane, and the various secretion pathways known to date are described. Since the work described in this thesis was performed to study the type II secretion machinery of *P. aeruginosa*, this secretion pathway will be explained in more detail.

THE CELL ENVELOPE OF GRAM-NEGATIVE BACTERIA

The cell envelope of Gram-negative bacteria is composed of an inner and an outer membrane, which are separated by the peptidoglycan-containing periplasm (Fig. 1). The inner (or cytoplasmic) membrane, which encompasses the cytoplasm, is a phospholipid bilayer. It is impermeable for hydrophilic compounds and also protons are unable to migrate freely over this membrane. Inner membrane proteins may associate peripherally by means of electrostatic and/or hydrophobic interactions or be present as integral membrane proteins that span the phospholipid bilayer via hydrophobic α-helical segments. The outer membrane faces the periplasm on one side and the extracellular medium on the other. This membrane is an

asymmetrical bilayer with phospholipids on the periplasmic side and lipopolysaccharides (LPS) on the outer leaflet making it relatively resistant to detergents. The outer membrane is semi-permeable due to the presence of proteinaceous channels formed by proteins called porins, which allow for passive diffusion of small hydrophilic molecules into the cell. Integral outer membrane proteins (OMPs) are characterized by the presence of amphipathic anti-parallel β-strands that form barrel-like structures with a hydrophobic surface facing the lipids. In between the two membranes is the hydrophilic periplasmic compartment. The periplasm harbours many chaperones, degradative enzymes, and proteins involved in nutrient acquisition. It also contains a layer of peptidoglycan that shapes the cell and is important for cell rigidity. Peptidoglycan consists of linear polymeric sugar chains that are covalently linked via short oligopeptides. Proteins that are involved in the synthesis of peptidoglycan can be found in the periplasm as well.

PROTEIN TRANSLOCATION ACROSS THE INNER MEMBRANE

Most proteins that are localized to the cell envelope use the membrane-embedded Sec (sec retion) machinery for insertion into or translocation across the cytoplasmic membrane (32). The central part of the Sec apparatus is formed by a heterotrimeric complex, SecYEG (SecY, SecE and SecG), and the peripheral membrane protein SecA, but other integral membrane proteins (SecDFYajC) have been shown to be involved in Sec-mediated protein translocation as well (32). Substrates of the Sec translocon are synthesized with an N-terminal signal sequence that targets them to the translocation machinery. This may occur via a cytosolic chaperone, SecB, or via the signal-recognition particle (SRP) (78).

SecB is involved in posttranslational targeting; it interacts with the mature portion of presecretory proteins and, at least in some cases, prevents their folding. The SecB-preprotein complex subsequently docks to a membrane-bound SecA molecule, which results in the release of SecB, and SecA drives translocation of the preprotein into the SecYEG translocon in an ATP-dependent fashion (74). After passage of the preprotein over the inner membrane, the signal sequence is cleaved off by a leader peptidase, usually LepB, at the periplasmic side of the inner membrane (28). The cleavage site for LepB is characterized by the presence of two alanines at

the –3 and –1 positions, separated by any residue (AxA) (124). Particularly at the –3 position, also other small residues are allowed.

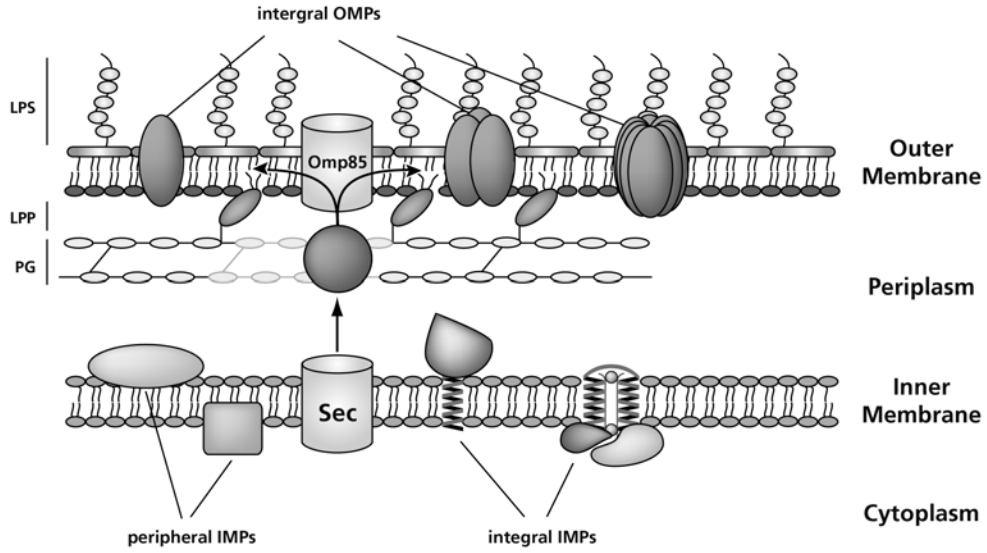

FIG. 1. Schematic representation of the cell envelope of Gram-negative bacteria. The inner and the outer membrane as well as the periplasm are indicated. The inner membrane and the inner leaflet of the outer membrane are composed of phospholipids, whereas the outer leaflet of the outer membrane is composed of lipopolysaccharide (LPS). Integral and peripherally attached inner membrane proteins (IMPs) are depicted and a model for the biogenesis of integral outer membrane proteins (OMPs) via Sec and Omp85 is shown. The peptidoglycan layer (PG) located in the periplasm is covalently attached to the outer membrane via lipoproteins (LPP). For more details, see text.

The SRP is a ribonucleoprotein complex formed by the protein P48 and a 4.5S RNA (32). SRP binds to hydrophobic sequences in ribosome-bound nascent polypeptide chains and initiates membrane targeting. The ribosome-nascent chain complex binds to the inner membrane protein FtsY, is targeted to the Sec translocon and the polypeptide is co-translationally translocated.

Typically, inner membrane proteins depend on a functional SRP pathway, whereas periplasmic proteins and OMPs predominantly use the SecB route (30). Although it is obvious that the signal sequence forms the

discriminating factor for SecB or SRP dependency, it is not entirely clear in what respect these signals differ. Classical signal sequences are characterized by a positive charge in the N-terminal domain, which is followed by a stretch of 10-15 hydrophobic residues and a more polar C terminus containing the leader peptidase cleavage site (125). In general, SRP signals are more hydrophobic (15, 67), but hydrophobicity does not seem to be the only decisive factor, since secondary structure plays a role as well (2).

Associated with the Sec complex is the integral membrane protein YidC (105). Although its precise function is unclear, YidC seems to assist Sec-dependent insertion of membrane proteins and lipoprotein translocation (39, 80). Moreover, YidC mediates Sec-independent insertion of M13 procoat and Pf3 coat *in vivo* (21, 102) and probably fulfills a similar function for the insertion of subcomponents of the major energy-transducing membrane complexes cytochrome *o* oxidase and F_1F_0 ATP synthase (121). Even though the large majority of cell envelope proteins is translocated over the cytoplasmic membrane via the Sec system, a subset of proteins uses an alternative pathway formed by the TAT (twin-arginine translocation) system. Originally, the TAT system was identified in the thylakoid membranes of chloroplasts, but homologues were subsequently characterized in bacteria and in archaea as well (12, 103, 106). Whereas the Sec translocon uses the energy of ATP hydrolysis as well as the proton-motive force to transport proteins in an unfolded conformation, the TAT system is capable of ATP-independent transport of fully folded and, often, co-factor bound substrates (77). Translocation via the TAT system is a proton-motive force-dependent process. Three genes that encode essential components of the TAT translocon, *tatA*, *tatB* and *tatC*, were identified in *Escherichia coli* (77). Typical TAT signal sequences share similarity with Sec signal sequences, but they contain a characteristic twin-arginine motif (explaining the name TAT) in the charged N-terminal region, their hydrophobic core is longer (up to 23 amino acid residues), but less hydrophobic, and the C-region frequently contains a basic residue that is proposed to act as a “Sec-avoidance” motif (11, 109).

PROTEIN TRANSPORT TO THE OUTER MEMBRANE

The outer membrane contains integral OMPs and proteins that are anchored to the membrane only via an N-terminal lipid moiety; these latter

proteins are referred to as lipoproteins. Lipoproteins can attach to both the inner and the outer membrane via an N-terminal N-acyl-diacyl-glycerylcysteine. The residues directly C-terminal to the lipidated cysteine in the mature protein determine the localization; lipoproteins that lack an inner membrane-retention signal (usually an aspartate at position +2, but the actual signal is less simplistic) are transported to the outer membrane via the Lol pathway (118). Whereas the lipoprotein-transport process to the outer membrane is largely established, the targeting and insertion of OMPs are not well understood. It has been reported that some OMPs fold and insert spontaneously into small vesicles *in vitro* (61). Nonetheless, the considerably faster kinetics of these events *in vivo* and the exclusive insertion into the outer membrane and not into the inner membrane indicate that OMP insertion is assisted *in vivo*. Voulhoux and co-workers (127) identified a key component of the OMP insertion machinery in *Neisseria meningitidis*. This component, Omp85, was found to be highly conserved in Gram-negative bacteria, and homologues were also identified in eukaryotic organelles of endosymbiotic origin, i.e. in mitochondria and chloroplasts, where they play a role in β-barrel protein insertion into the outer membrane as well (41, 90, 119). Bacterial Omp85 resides in a complex with several lipoproteins (101, 131), but the specific role of the individual components of this complex remains to be established.

PROTEIN SECRETION PATHWAYS

Gram-negative bacteria are capable of secreting a wide variety of proteins with functions ranging from nutrient acquisition to virulence. Several mechanisms have evolved to facilitate transport of proteins across the cell envelope to the cell surface (see Fig. 2). The secretion pathways known to date are described below.

Type I secretion: the ABC transporter-dependent pathway. The type I secretion pathway typically depends on an ABC (for ATP-binding cassette) transporter and is independent of the Sec translocon (52). Although secretion systems of this type are composed of only three components, they are capable of transporting polypeptides of up to 800 kDa across the cell envelope (50). The type I translocator contains two inner membrane components, the ABC-transporter and a membrane-fusion protein (MFP), and an outer membrane factor (OMF). The MFP is anchored in the cytoplasmic membrane and largely spans the periplasm. Together with the

OMF it is proposed to provide a trans-envelope channel. The ABC-transporter on its turn might supply energy to the secretion process by hydrolyzing ATP. The prototype type I secretion system is the haemolysin system in *E. coli*, which is schematically depicted in Fig. 2. The secretion signal of type I substrates is positioned at the C terminus and it is not removed during export. These secretion signals, however, are poorly conserved. Type I secretion substrates are rapidly transported, probably in an unfolded form, and no periplasmic intermediates have been described (63).

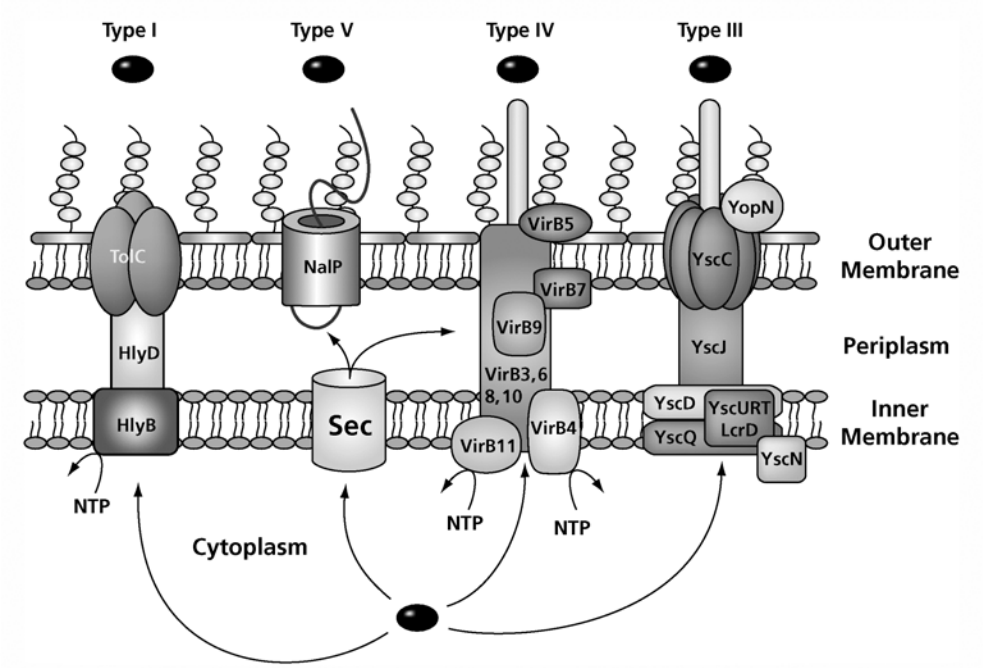

FIG. 2. Schematic representation of the different secretion pathways in Gram-negative bacteria. Sec-dependency or direct targeting of substrates to the pathways is indicated by arrows. The involvement of ATP hydrolysis to energize the systems is indicated by NTP. Type I secretion is exemplified by the haemolysin (Hly) system of *E. coli*, type III secretion by the Ysc system of *Yersinia enterocolitica*, type IV secretion by the Vir system of *Agrobacterium tumefaciens*, and type V by NalP of *N. meningitidis*. Type II secretion is depicted in Fig. 3. The recently identified type VI secretion has been omitted as well, since very little information is yet available. For more details, see text (Fig. adopted from 33).

Type II secretion. Transport of exoproteins via the type II secretion system (T2SS) occurs in a two-step procedure, in which the substrates first travel to the periplasm via the Sec or TAT machinery and, in a second step, across the outer membrane to the cell surface or the extracellular medium (48, 126). T2SSs each encompass 12-16 different components (56). Apart from the signal sequence required for transport via Sec or TAT, no linear secretion signal could be recognized in the broad range of substrates of T2SSs and it is thought that the signal required for transport across the outer membrane resides in the three-dimensional structure of the exoproteins (37). Consistently, substrates of type II secretion machineries are translocated across the outer membrane in a folded conformation (13, 46, 51, 93). Several components of the T2SS share homology with constituents of the type IV piliation machinery, of competence systems in Gram-positive bacteria, and of flagella systems in archaea (82, 91). The T2SS of *P. aeruginosa* will be described in more detail below.

Type III secretion. The complex type III secretion apparatus assembles into a syringe-like complex, which traverses both membranes (55). This needle complex is used by the bacterium to contact a host cell and to form a direct channel between the bacterial and host cell's cytoplasms, thereby allowing direct injection of type III secretion substrates into the target cell. Although the number of components that are required for this type of secretion varies among organisms, ten conserved components can be identified that probably form the minimal machinery (92). The apparatus is composed of cytoplasmic, transmembrane and extracellular segments that are morphologically well conserved. The first two segments form the basal body of the machinery, which supplies energy and controls export (55). The extracellular segment of the type III secretion apparatus is formed by the needle-like structure that protrudes from the cell surface. A schematic representation of the type III secretion system of *Y. enterocolitica* is depicted in Fig. 2. The substrates secreted via the type III pathway are divergent, but are often intimately linked with bacterial virulence. The nature of the secretion signal for type III substrates is largely unknown; it has been suggested to be contained in the N-terminal 10-15 amino acid residues (71) or in the 5' end of the mRNA (3). Components that make up the cytoplasmic and the inner membrane segments of the type III secretion apparatus share homology with components that function in the flagellum system (92).

Type IV secretion. Type IV secretion systems collectively constitute a large family of export machineries that can transport DNA, DNA-protein complex or proteins over the cell envelope (23). Some systems deliver their substrates into the extracellular environment, whereas other systems introduce their substrates directly into eukaryotic target cells. The route of protein transport across the cell envelope appears to be different among type IV secretion systems as well; the *Agrobacterium tumefaciens* and the *Helicobacter pylori* systems transport substrates in one step over the entire envelope, whereas the *Bordetella pertussis* type IV system transports pertussis toxin only across the outer membrane. In the latter case, pertussis toxin is first translocated over the inner membrane via the Sec translocon in a separate step (24). The complex multi-component type IV secretion machineries are often composed of a conjugal pilus required for establishing contact with recipient cells and of a mating channel through which DNA and/or effector proteins are exported (22). A schematic representation of the type IV secretion system of *A. tumefaciens* is depicted in Fig. 2. Type IV secretion signals are generally positioned at the C termini of the substrates (79, 122). Yet, little sequence conservation could be identified in these C termini.

Type V secretion. The type V secretion pathway is apparently the simplest route for outer membrane passage. Actually, type V secretion comprises two different types of secretion systems, the autotransporters and two-partner secretion, which will be described separately below. Both systems are two-step processes comprising a periplasmic intermediate. Many Gram-negative bacteria make use of these systems to expose a variety of often very large enzymes and adhesins.

Autotransporters. Autotransporters are composed of three domains: an N-terminal signal sequence, a passenger domain and a C-terminal β-barrel domain (49). The N-terminal signal sequence targets the protein to the Sec system for inner membrane translocation and the C-terminal β-barrel domain is involved in the outer membrane passage of the passenger domain. Whether the passenger domain is translocated across the outer membrane by passing through the central pore formed by the C-terminal barrel is still under debate (87). An alternative possibility is that transport takes place through a channel formed by the Omp85 protein, on which autotransporter secretion is dependent (127). A schematic representation of type V secretion of NalP of *N. meningitidis* is depicted in Fig. 2. After secretion, the

passenger domain may remain attached to the cell surface, or it may be released into the extracellular medium by proteolytic cleavage (116).

Two-partner secretion. Also two-partner secretion systems are simple and dedicated to the secretion of large polypeptides, which play a role in virulence (54). However, two-partner secretion involves two proteins: the secreted exoprotein (generically termed TpsA) and the outer membrane transporter (generically termed TpsB). Both these proteins are synthesized with an N-terminal signal sequence that targets them to the Sec system for translocation across the inner membrane. The mature TpsA protein contains a 110-residue conserved domain at the N terminus, which is essential for secretion to the cell surface (54). Export of TpsA across the outer membrane requires a specific interaction with its cognate transporter, TpsB. The TpsB transporters belong to the Omp85 family of proteins (128) and are large, pore-forming transporters that are predicted to form β-barrels (43, 115). After secretion, TpsA proteins may remain non-covalently associated with the cell surface, or they may be released into the extracellular medium (54).

Type VI secretion. Type VI secretion was recently described by Pukatzki *et al.* (95). They identified a novel secretion system in *Vibrio cholerae* encoded by *vas* genes (for virulence associated secretion). Homologues of these *vas* genes were found to be prevalent in a large number of Gram-negative pathogens, and, recently, the system was shown to be functional in *P. aeruginosa* (76). However, whether the products of the genes in the other cases constitute functional secretion systems remains to be determined. The exact composition of the type VI secretion system is unknown, but since a large cluster of genes is conserved, it probably consists of multiple components, likely functioning together as a molecular syringe (96). Also the nature of the secretion signal is unknown. Substrates of type VI secretion are produced without a classical signal sequence required for transport via Sec or TAT, suggesting that secretion is a one-step process without a periplasmic intermediate. Two genes in the *vas* cluster show a high degree of similarity to genes that function in type IV secretion (95).

THE TYPE II SECRETION SYSTEM OF *P. AERUGINOSA*

Except for the type IV machinery, *P. aeruginosa* has all the secretion mechanisms described above (73, 76). Most extensively used is the type II

mechanism. Elastase, exotoxin A, staphylolytic protease, chitin-binding protein D, lipases, alkaline phosphatase and low-molecular weight alkaline phosphatase are, amongst others, secreted via this pathway (38). Remarkably, *P. aeruginosa* contains two secretion machineries of the type II kind, termed Xcp (extracellular protein deficient) (130) and Hxc (homologue of Xcp) (6). Both systems are assembled from 11 core constituents (XcpP-Z and HxcP-Z) and one shared component (XcpA/PilD). The Hxc system is only produced under phosphate limitation and mediates the secretion of at least one low-molecular-weight alkaline phosphatase. The Xcp system is responsible for the secretion of the majority of the exoproteins. Disruption of any of the twelve *xcp* genes leads to the accumulation of these proteins in the periplasm (44, 130). Two divergently oriented promoters control expression of the two operons in the *xcp* gene cluster; one controls *xcpPQ* and the other *xcpR-Z*. The *xcpA* gene is not located in the *xcp* locus, but elsewhere on the chromosome. Both promoters are regulated by quorum sensing and expression starts at the late-log (*xcpR-Z*) and early stationary (*xcpPQ*) growth fases (19). The fourth letter in the designations of the homologous components of the T2SSs of different bacteria is usually identical, whereas the first three letters are different. Thus, for example PulE of the pullulanase secretion system of *Klebsiella oxytoca* is the homologue of the ExeE component of the *Erwinia* T2SS. Sometimes, for the first three letters, the generic designation Gsp (for general secretion pathway) is used. For historical reasons, however, the fourth letter of the designations of the *Pseudomonas* Xcp components deviates from that of other type II components; for example, XcpR is homologous to PulE or in the generic designations, GspE. Table 1 shows the comparison of the Xcp and the Gsp nomenclature. To facilitate comparison, the fourth letter of the Gsp nomenclature will be indicated in this chapter in subscript. For example, XcpR will be indicated as $XcpR_E$. Moreover, GspE (XcpR) will be used, when referring to XcpR homologues or to the family of XcpR proteins in general.

TABLE 1. Components of the *P. aeruginosa* T2SS and the Pil homologues

	T2SS		Molecular weight (kDa)	Pil
	Xcp	Gsp		
Major prepilin	XcpT	GspG	15	PilA
Minor prepilins	XcpU	GspH	19	PilE*
	XcpV	GspI	14	FimT*
	XcpW	GspJ	27	FimU*
	XcpX	GspK	37	PilV*
	-	-	-	PilW*
	-	-	-	PilX*
Prepilin peptidase/ N-methylase	XcpA	GspO	32	PilD
ATPase	XcpR	GspE	55	PilB
	-	-	-	PilT
	-	-	-	PilU
Secretin	XcpQ	GspD	70	PilQ
Transmembrane protein	XcpS	GspF	44	PilC
Pilotin	-	GspS	-	PilP
Others	XcpP	GspC	27	-
	XcpY	GspL	41	-
	XcpZ	GspM	19	-
	-	GspN	-	-
	-	GspA	-	-
	-	GspB	-	-
	-	-	-	PilN
	-	-	-	PilO
	-	-	-	PilY1
	-	-	-	PilZ

* minor prepilins of the Pil system are placed in random order and do not designate similarity to particular minor prepilins of the T2SS

Secretin and pilotin. $XcpQ_D$ is the only component of the Xcp system that localizes to the outer membrane (Fig. 3) (8, 16), and probably functions as the channel through which Xcp substrates are exported out of the cell (8, 81). $XcpQ_D$ belongs to a large family of homologous proteins called secretins, which function in type II and type III secretion, type IV piliation, and filamentous phage assembly (7, 40). Secretins form stable oligomers (8, 58, 64). They contain a highly conserved C-terminal region,

which is localized in the outer membrane and involved in oligomerization (16, 81). The N-terminal domain, which extends into the periplasm, is conserved only within subclasses of secretins with the same function. Multimeric $XcpQ_D$ complexes of *P. aeruginosa* were shown to have a donut-shaped structure with a central cavity of approximately 95 Å in diameter, sufficiently large to allow for the passage of folded substrates (8). Secretins are devoid of the C-terminal consensus motif that is typically found in integral OMPs (114). However, since oligomerization and outer membrane insertion of the secretin PilQ of *N. meningitis* were found to be dependent on the Omp85 protein (127), it seems that these proteins, at least to some extent, use a similar assembly pathway as other OMPs. The tendency of secretins to form large complexes severely challenges their periplasmic transport. Indeed, chaperones have been described that are essential for outer membrane localization of secretins and for their protection against proteolytic degradation. The chaperone function is typically performed by a small outer membrane-localized lipoprotein (GspS), which, in most cases, interacts with a domain at the extreme C terminus of its cognate secretin (45, 47, 107). However, GspD (XcpQ) of *Aeromonas hydrophila*, requires the inner membrane GspAB complex for assembly and outer membrane localization (4). So, multiple ways appear to exist for secretin assembly, which results in variable requirements for other proteins besides the GspC-M (XcpR-Z) core complex. No homologues of *gspAB* and *gspS* have been identified in the *P. aeruginosa* genome.

Pseudopilins and the pre-pilin peptidase. $XcpT_G$, U_H, V_I, W_J, and X_K all display N-terminal sequence similarity to the structural component of the type IV pilus, the pilin PilA, and are therefore termed pseudopilins. However, the C-terminal domains of the pseudopilins are, at least at the amino acid sequence level, rather different from pilin. Notably, the cysteines frequently present at the extreme C terminus of pilin subunits are absent in pseudopilins. The N termini of the precursors of type IVa pilins and pseudopilins contain a short positively charged leader peptide of six to eight amino acid residues followed by a hydrophobic stretch of approximately 20 amino acid residues (Fig. 4) (112).

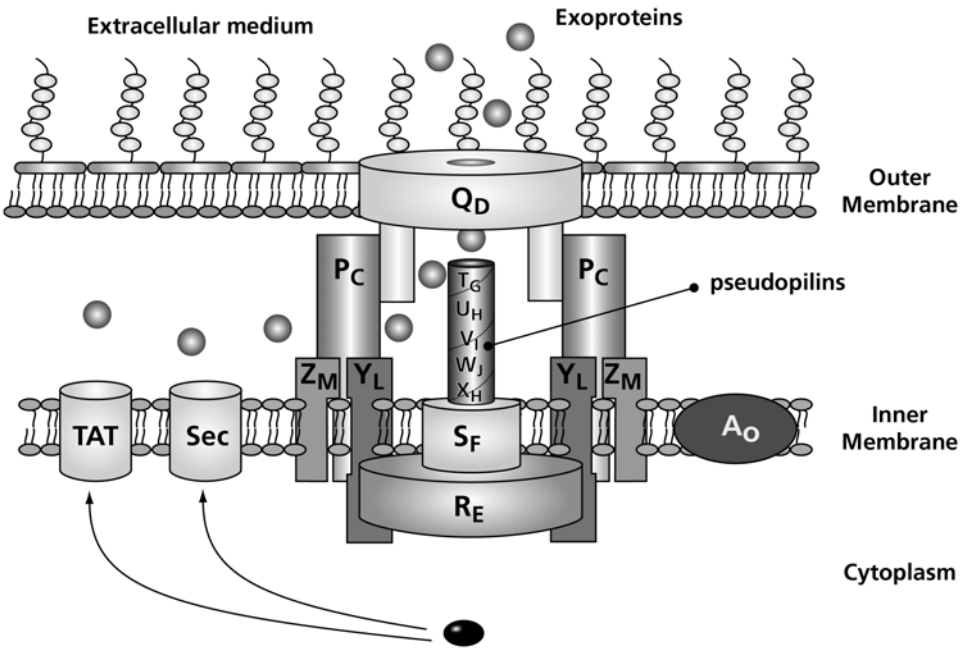

FIG. 3. Model for the Xcp secretion apparatus of *P. aeruginosa*. The Xcp-dependent exoproteins are initially exported across the IM via the Sec or Tat machinery. The folded exoproteins, shown as grey circles, are recognized by the Xcp machinery and transported across the OM via the secretin, $XcpQ_D$. $XcpZ_M$ and $XcpY_L$ mutually stabilize each other and form a stable complex. $XcpY_L$ anchors the $XcpR_E$ traffic ATPase to the inner face of the cytoplasmic membrane. $XcpS_F$ interacts with the $XcpR_EY_LZ_M$ complex. The $XcpP_C$ component interacts with the periplasmic N-terminal domain of $XcpQ_D$ and with the $XcpY_LZ_M$ complex. The pseudopilus is arbitrarily represented as a cylinder. Preceding assembly into a pseudopilus, the XcpT-X pseudopilins are processed by the prepilin peptidase, $XcpA_O$. For more details, see text (Fig. adopted from 37).

Type IVb prepilins contain longer leader peptides, 15 to 30 residues in length, have a larger average size (~190 amino acid residues as compared to ~150 residues for type IVa pilins), and lack the conserved phenylalanine at the +1 position (see below) (25). PilA of *P. aeruginosa* belongs to the family of type IVa pilins, whereas pili from *V. cholerae*, and the enteropathogenic and enterotoxic *E. coli* belong to type IVb pilins (25). The leader peptide of type IVa prepilins and pseudopilins is removed during inner membrane translocation by the dedicated prepilin peptidase $XcpA_O$ (84, 85), which is an inner membrane protein with multiple transmembrane segments. Subsequent to cleavage of the prepilins at the cytoplasmic side of

the inner membrane, this bilobed aspartate protease also catalyses the methylation of the new N-terminal amino acid residue (66, 113). In *P. aeruginosa*, the prepilin peptidase functions both in type II secretion and in type IVa piliation (84); hence, its double name XcpA/PilD. The prepilin peptidase cleavage site is characterized by a glycine residue at the –1 position, which is essential for processing (111). The glycine is frequently followed by a phenylalanine at the +1 position. However, this residue does not seem to be strictly required for functionality. Finally, a conserved glutamate residue occupies the +5 position within the hydrophobic stretch (Fig. 4). This residue has been proposed to play a central role in the helical assembly of subunits into a pilus or pilus-like structure (89). Since fractionation studies show that pseudopilins localize to the cell envelope (83, 94), the hydrophobic segment may serve as a transmembrane segment before assembly of the subunits into a pilus-like structure. From expression studies, it became apparent that the relative amounts of XcpT, XcpU, XcpV, and XcpW pseudopilins are approximately 16:1:1:4 (83), and therefore $XcpT_G$ is referred to as the major pseudopilin. Notably, also the type IV piliation system encompasses minor pilin subunits (Table 1). Inner membrane translocation of pseudopilins has been proposed to rely on the Sec translocase (69) or on a dedicated pathway formed by accessory Gsp proteins (37, 82).

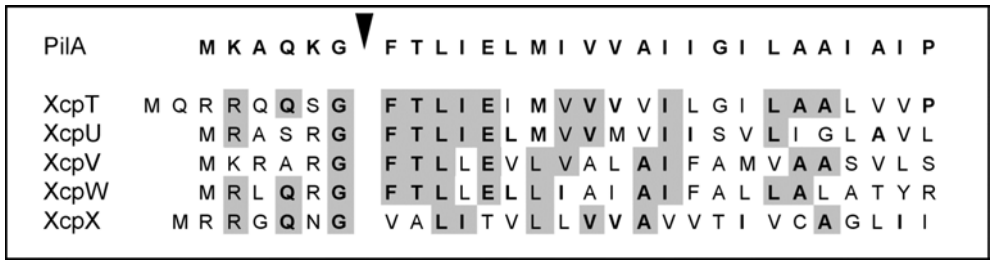

FIG. 4. Sequence alignment of the N-terminal domains of the PilA prepilin subunit and the precursors of pseudopilins of the Xcp secretory pathway from *P. aeruginosa*. Conserved residues within the pseudopilins are shaded and residues that are identical to those in the PilA sequence are in bold. The arrow shows the position of the prepilin peptidase cleavage site.

The inner membrane complex. Although the T2SSs function in transport across the outer membrane, most of their constituents are localized in or associated with the inner membrane, where they supposedly form a complex (56). Several interactions between inner membrane components

have indeed been reported. $XcpY_L$ and $XcpZ_M$ are both bitopic proteins with one transmembrane segment each (10). They form a complex and mutually stabilize each other via an interaction of the periplasmic segments (75, 98, 99). $XcpY_L$ not only interacts with $XcpZ_M$, but it also docks $XcpR_E$ to the inner membrane (5). $XcpR_E$, which is devoid of apparent transmembrane segments, contains a conserved motif involved in ATP binding and hydrolysis, including a highly conserved Walker A motif, which is essential for its function (120). Therefore, this protein may supply energy to the secretion process. Proteins of the GspE (XcpR) family form part of a superfamily of ATPases, which includes a subfamily of multimeric proteins (the VirB11 subfamily) involved in, amongst others, type II and type IV secretion, and in type IV piliation (18). Recently, ATP binding by GspE (XcpR) of *Xanthomonas campestris* was shown to induce multimerization (108) and, in analogy to VirB11 of the type IV secretion system of *A. tumefaciens*, GspE (XcpR) is proposed to form hexamers (100). Modeling of the X-ray crystal structures of the cytoplasmic fragment of *V. cholerae* GspL (XcpY) together with the N-terminal fragment of GspE (XcpR) resulted in only partial filling of the groove at the binding site of GspL (XcpY) (1). This observation suggests the involvement of yet another constituent of the T2SS, which may be GspF (XcpS). GspF (XcpS) is a multispanning inner membrane protein (117) that is proposed to form multimers (27, 29, 91). With his-tagged $XcpZ_M$, Robert *et al.* (98) could co-purify $XcpR_E$, S_F, and Y_L after cross-linking, which suggests that these proteins together form an inner membrane platform (Fig. 3).

$XcpP_C$: the link between the secretin and the inner membrane complex. $XcpQ_D$ has been reported to be engaged in an interaction with $XcpP_C$ (Fig. 3) (9). $XcpP_C$ is a bitopic inner membrane protein that contains a short N-terminal cytoplasmic tail, a transmembrane segment, and a large periplasmic domain (10). Typically, GspC proteins contain a PDZ motif in their periplasmic domain (88); $XcpP_C$, however, contains a coiled-coil region instead (9). Both motifs are generally involved in protein-protein interactions, and Gérard-Vincent *et al.* (42) showed that the coiled-coil domain of $XcpP_C$ could be replaced by the PDZ motif of GspC (XcpP) of *Erwinia chrysanthemi* without loss of function. They proposed that the coiled-coil segment of $XcpP_C$ is required for the formation of homomultimers, rather than for interaction with the secretin. $XcpP_C$ together with $XcpQ_D$ might confer exoprotein recognition, since all Xcp/Gsp components of closely related bacteria could be functionally exchanged

except for $XcpP_CQ_D$ (31, 70). However, from data obtained by Bouley *et al.* (14), it appeared that exoprotein recognition differs among substrates; some exoproteins were recognized by GspC (XcpP), others by GspD (XcpQ). Apart from its interaction with $XcpQ_D$, $XcpP_C$ has been reported to interact with the inner membrane components $XcpY_L$ and $XcpZ_M$ (97). So, $XcpP_C$ could form the link between the secretin and the predicted inner membrane complex (Fig. 3).

Pseudopilus formation by the T2SS. Apart from the N-terminal sequence similarity between the type IV pilins and the pseudopilins of the T2SSs and the dual role of the prepilin peptidase in piliation and type II secretion, many components of the T2SS share homology to constituents of the type IV piliation machinery (Table 1). $XcpR_E$ is homologous to the traffic ATPase PilB, $XcpS_F$ to PilC, and also the secretins $XcpQ_D$ and PilQ display considerable sequence similarity (82, 91). Moreover, GspG (XcpT) of *K. oxytoca* was found to share structural characteristics with type IV pilins (62). Type IV pili are thin adhesive appendages on the cell surface that are synthesized by a wide variety of Gram-negative bacteria (17). They are retractile and play an important role in adherence to host cells, DNA uptake, twitching motility, and biofilm initiation and development (17). The semi-flexible rod-like type IV pili are composed of thousands of identical pilin subunits arranged in a helical manner (26). Individual pilus filaments are up to several μm long and 5 to 6 nm in diameter (26). The observation that *V. cholerae* uses its type IV piliation machinery for secretion (60) emphasizes the close relationship between the machineries involved.

Given the close homology between the Pil system and the T2SSs, it was proposed that pseudopilins would assemble into pilus-like structures, which could span the periplasm and function as a piston to push substrates out, or that could form a connection between the inner membrane platform and the secretin (38). Several groups have indeed shown that the major pseudopilin GspG (XcpT), upon overproduction, assembled into long bundled pili that protruded from the cell surface (35, 104, 123). Single filaments were up to 10 μm in length and 7 to 9 nm in diameter (35). The formation of this so-called pseudopilus was proposed to result from uncontrolled elongation of a normally shorter periplasmic structure. Upon subcellular fractionation, the GspG (XcpT) of *X. campestris* could be detected in both the membrane and the soluble fraction. In the soluble fraction, however, it was present in a large complex of approximately 400 kDa, which might reflect an intracellular pseudopilus (53). Studies by

Durand *et al.* (36) showed that only $XcpT_G$ and none of the minor pseudopilins assembled in the pseudopilus, and that the length of the pilus-like structure is controlled by the atypical pseudopilin $XcpX_K$, which lacks the conserved glutamate at the +5 position (Fig. 4). The same study also reported that XcpV is the only minor pseudopilin required for $XcpT_G$ assembly, so the roles of $XcpU_H$ and $XcpW_J$, although requisite for secretion, remain obscure. $XcpT_G$ can be cross-linked to $XcpU_H$, $XcpV_I$, $XcpW_J$, and itself (72), and, since over-expression of GspH (XcpU) and GspJ (XcpW) negatively influences pseudopilus formation in *X. campestris* (65), the minor pseudopilins may be needed for fine-tuning of $XcpT_G$ assembly. In type IV pili systems, minor pilins have been identified as well (Table 1). From results by Winther-Larsen *et al.* (129), it appears that these proteins have a function in pilus extension events, since mutants lacking single minor pilins were dramatically reduced in type IV pilation. This phenotype was overcome in the absence of the retraction ATPase PilT, and, thus, piliation does not require these proteins *per se*.

Several groups have reported the pseudopilins to participate in complexes with other Gsp proteins, positioning the pseudopilus in between the inner membrane complex and $XcpQ_D$ in the outer membrane. In *X. campestris*, GspD (XcpQ) and GspN could be co-purified with his-tagged GspG (XcpT) after cross-linking (68). Remarkably, GspD (XcpQ) also co-purified with his-tagged GspG (XcpT) upon production of these proteins in the absence of the other Gsp components. $XcpT_G$ might also take part in the inner membrane complex, since a conditional mutation in $XcpT_G$ could be suppressed by a second mutation in $XcpR_E$ (57). Yeast two-hybrid analyses with periplasmic domains of *E. chrysanthemi* Gsp proteins suggest GspJ (XcpW) to be a central component of the T2SS, since interactions with GspI (XcpV), GspJ (XcpW), and GspL (XcpY), and GspD (XcpQ) were detected (34). Whether these interactions occur simultaneously or at different stages of secretion cannot be distinguished by yeast two-hybrid studies. However, the observation that, in *X. campestris*, GspJ (XcpW) localizes to the outer membrane suggests the interactions not to occur at the same time (65).

SCOPE OF THE THESIS

Type II secretion largely contributes to the virulence of *P. aeruginosa* (59). Progressive research and structural studies have already revealed many characteristics of this type of secretion. Recent studies have

emphasized the relatedness to the type IV piliation machinery (35, 104, 123). However, the exact functioning of the type II apparatus remains enigmatic. Assembly of the machinery requires interactions between multiple components located in the cytoplasm, the inner and the outer membrane. The work described in this thesis was performed to obtain more insight in the assembly of the *P. aeruginosa* Xcp machinery. In the work described in chapter 2, we studied the inner membrane translocation route of pseudopilins. Two translocation routes have been proposed for this type of proteins: via the Sec machinery or via a dedicated translocation apparatus formed by other T2SS components. We show that the major pseudopilin, XcpT, is co-translationally transported across the inner membrane via the SRP/Sec pathway. Chapter 3 describes the construction of pilin-pseudopilin (PilA-XcpT) hybrids. The chimeras were used to study the targeting of these proteins to their cognate machineries. In support of a general translocation route for pilins and pseudopilins, we show that the hydrophobic N terminus of XcpT could be substituted by that of PilA without a loss of function. In chapter 4, our studies on the inner membrane complex constituent XcpS are described. Our results confirm that XcpS participates in the inner membrane complex. We show that interaction with XcpRY increased the stability of the XcpS protein and that this interaction requires the large cytoplasmic loop of XcpS. In chapter 5, targeting of the secretin XcpQ was studied and we describe the reconstitution of the Xcp system in the heterologous hosts *Pseudomonas putida* and *E. coli*. These studies were performed to determine whether the known Xcp components are sufficient for their assembly into a functional machine. Finally, in chapter 6, the results are summarized and discussed.

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Chapter 2

Export of the pseudopilin XcpT of the *Pseudomonas aeruginosa* type II secretion system via the SRP/Sec pathway

Jorik Arts, Ria van Boxtel, Alain Filloux, Jan Tommassen, Margot Koster

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ABSTRACT

Type IV pilins and pseudopilins are found in various prokaryotic envelope protein complexes, including type IV pili and type II secretion machineries of Gram-negative bacteria, competence systems of Gram-positive bacteria, and flagella and sugar-binding structures within the archaeal kingdom. The precursors of these proteins have highly conserved N termini, consisting of a short positively charged leader peptide, which is cleaved off by a dedicated peptidase during maturation, and a hydrophobic stretch of approximately 20 amino acid residues. Which pathway is involved in the inner membrane translocation of these proteins is unknown. We used XcpT, the major pseudopilin from the type II secretion machinery of *Pseudomonas aeruginosa*, as a model to study this process. Transport of an XcpT-PhoA hybrid was shown to occur in the absence of other Xcp components in *P. aeruginosa* and in *Escherichia coli*. Experiments with conditional *sec* mutants and reporter-protein fusions showed that this transport process involves the co-translational SRP targeting route and is dependent on a functional Sec translocon.

INTRODUCTION

The type II secretion pathway is widely used by Gram-negative bacteria to secrete proteins into the extracellular environment (18). Release of exoproteins via this pathway occurs in a two-step process, implicating a periplasmic intermediate stage in the secretion pathway. Type II secretion machineries each encompass 12-16 different components (18). Five components show sequence similarity to PilA, the structural subunit of type IV pili, and are therefore named pseudopilins. Their precursors possess a conserved N terminus, which contains a short positively charged leader peptide followed by a highly hydrophobic domain of approximately 20 amino acid residues (20, 47). The leader peptide is removed during export by a specific prepilin peptidase (4, 36). These prepilin peptidases are polytopic inner membrane proteins that cleave off the leader peptide at the cytoplasmic side of the inner membrane and catalyse the methylation of the new N-terminal amino acid residue (usually phenylalanine) of the mature protein (32, 48).

Type IV pilin-like N-terminal sequences are not only found in type IV pili and in the pseudopilin components of type II secretion systems of Gram-negative bacteria, but also in competence systems in Gram-positive bacteria, and in flagella and sugar-binding proteins of archaea (39). In all these systems, the presence of proteins with prepilin-like N termini coincides with the occurrence of accessory proteins, including a prepilin peptidase, an ATPase, and a multispanning transmembrane protein (39).

How pilins and pseudopilins are transported across the cytoplasmic membrane and recruited by their cognate machinery is unknown. Two possible pathways have been proposed: one is via the highly conserved Sec translocon, and the other is via a dedicated machinery implicating the prepilin peptidase, the ATPase and/or the multispanning transmembrane protein mentioned above. The Sec system is generally used for protein transport across the cytoplasmic membrane (13). Two targeting pathways, SRP and SecB, intersect at the Sec translocon (28). SecB interacts with the mature portion of presecretory proteins and, besides targeting of the precursor to the translocase, it prevents their folding. SRP binds co-translationally to hydrophobic sequences in its substrates. The ribosome-nascent chain complex subsequently binds FtsY and is targeted to the Sec translocon. Inner membrane proteins typically depend on a functional SRP pathway, whereas periplasmic and outer membrane proteins predominantly

use the SecB route (11). Proteins targeted to the Sec system carry signal sequences that are characterized by a positively charged N-terminal region, followed by a 10-15 residues long hydrophobic core and a more polar C terminus containing the signal-peptidase cleavage site (53). Passage of the precursor over the inner membrane is followed by cleavage of the signal sequence by the signal peptidase LepB at the periplasmic side of the inner membrane (10). The N termini of the (pseudo)pilin precursors (prepilins) share characteristics with Sec signal sequences, and previous studies have indeed shown that the N-terminal sequences of prepilins from *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae* function as export signals for alkaline phosphatase in *Escherichia coli* (14, 46). However, the N termini of the prepilins are also distinct from Sec signal sequences, since they lack the signal-peptidase cleavage site C-terminally to the hydrophobic domain and are, as mentioned above, processed by a specific prepilin peptidase N-terminally to the hydrophobic segment at the cytoplasmic side of the membrane. These differences and the fact that the pilin-like proteins are always found in concert with other proteins, has led to the proposal that pilins and pseudopilins are exported from the cytoplasm via a dedicated transport route formed by these accessory proteins (9, 18, 35).

To address the question how (pseudo)pilins are translocated across the inner membrane, we used the major pseudopilin XcpT of the *P. aeruginosa* type II secretion system as a model protein. The type II secretion machinery of this organism requires at least 12 components, XcpA and XcpP-Z (20). XcpA functions as the prepilin peptidase and is shared by the Xcp system, the type IV piliation (Pil) system, and the Hxc system, which forms a second type II secretion system dedicated to the export of low-molecular-weight alkaline phosphatases (3). We demonstrate that XcpT translocation can occur independently of the presence of other Xcp components in *P. aeruginosa* and in *E. coli* and that transport is dependent on a functional Sec apparatus. Furthermore, we show that translocation occurs co-translationally via the signal recognition particle (SRP) pathway.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains used in this study are listed in Table 1. The SRP mutant alleles *ffs69* (50) and *ffh87* (51) were introduced in MC1060 by generalized transduction with phage P1 as described (5). After the introduction of construct pJGA03, the transductants

were screened for blue coloring on LB agar plates containing X-gal. Out of six transductants, six and three were scored positive for *ffs69* and *ffh87*, respectively. *P. aeruginosa* strains and *E. coli* strains DH5α, C9, JS7131, MC1060, and the MC1060 derivatives were grown at 37°C, and other *E. coli* strains at 30°C in a modified Luria-Bertani (LB) broth (52), unless otherwise notified. For plasmid maintenance, the following antibiotics were used: for *E. coli* ampicillin 50 μg/ml; kanamycin 25 μg/ml; tetracycline 15 μg/ml; and gentamicin 15 μg/ml; for *P. aeruginosa* gentamicin 40 μg/ml.

TABLE 1. Strains used in this study

Strain	Relevant characteristics	Reference
E. coli		
DH5α	*thi-1 hsdR17 gyrA96 recA1 endA1 glnV44 relA1 phi80dlacZdelM15 phoA8* λ^-	(26)
C9	Hfr Cav, *relA1 fhuA22 pitA10 spoT1 ompF627 phoR18 creB501*	(23)
MC4100	F^-, Δ*lacU169 araD139 rpsL thi relA*	(6)
MM52	MC4100 *secAts51*	(37)
IQ86	Tn10 *thiA* Δ*lac araD rpsL rpsE relA*	(45)
IQ85	IQ86 *secYts24*	(45)
HPT244	MC4100 Δ*ara714 ffs69 zba-3054*::Tn*10*	(50)
HPT406	MC4100 Δ*ara714 pheA3141*::Tn*10*Kan *ffh87*	(51)
MC1060	Δ(*codB-lacI*)*3 galK16 galE15*(GalS) λ^- *e14*$^-$ *mcrA*$^-$ *relA1 rpsL150(strR) spoT1 mcrB1 hsdR2*	(7)
JS7131	MC1060 *attB::R6Kori* Δ*yidC* P*araBAD-yidC*	(43)
MC1060 *ffs69*	MC1060 *ffs69 zba-3054*::Tn*10*	This study
MC1060 *ffh87*	MC1060 *pheA3141*::Tn*10*Kan *ffh87*	This study
P. aeruginosa		
PAO25	PAO1 *leu arg*	(25)
PAO1ΔT	PAO1 Δ*xcpT*	(3)
DZQ40	PAO1Δ*xcpP-Z*	(2)
PAOΔ*hxc*Δ*pilAC*Δ*R*	Non polar deletion of the entire *hxc* gene cluster, of *pilA* to part of *pilC*, and of the *xcpR* gene	(16)

Plasmids and DNA manipulations. Plasmids used in this study are listed in Table 2. Recombinant DNA methods were performed essentially as described (42), using *E. coli* strain DH5α for routine cloning. Plasmids were introduced by the $CaCl_2$ procedure into *E. coli* (42) or by electroporation into *E. coli* and *P. aeruginosa* (17). PCRs were performed with the proofreading enzyme *Pwo* DNA polymerase (Roche) and PCR products

were cloned into the HincII site of vector pBC18R or into pCRII-TOPO according to manufacturer's protocol. The *lacI* gene was PCR amplified using plasmid pET16b as template and with the primers PB7 (5'-CTCCTTGCATGCACC-3') and PB8 (5'-CCCGCGCCCATGGGAAGG AGCTG-3'), thereby introducing an NcoI restriction site (underlined) downstream of the stop codon. The PCR product was cloned into pBC18R, which resulted in construct pCR-LacI and, subsequently, the SphI-NcoI fragment of pCR-LacI was introduced into the pBBR1-MCS5 vector, resulting in pYRC. The *phoA* gene without promoter and signal sequence-encoding part was PCR amplified from pPHO7 with the primers PB1 (5'-GATCCCCGGGGATCCGACTCTTATACAC-3') and PB2 (5'-CGAAAA TTCACTGTCTAGAGCGGTTTTATTTC-3'). A BamHI site (underlined) was introduced via PB1 upsteam of the fragment encoding signal-sequence-less PhoA and an XbaI site (underlined) via PB2 downstream of the stop codon. The PCR product was cloned into pBC18R, which gave pCR-PhoA. Plasmid pYRC-A was constructed by introduction of the BamHI-XbaI fragment of pCR-PhoA into BamHI-XbaI-digested pYRC. Cosmid pAX24 was used as template to amplify *xcpT* with the oligonucleotides JAXcpTfor02 (5'-CTTCCGATCCTTCGAATCAACCAACTCGTG-3') and JAXcpTrev01 (5'-GCCCGCATGTCGGATCCGTTGTCCCAGTTG-3') and the resulting product was cloned into pCRII-TOPO resulting in pCR-XcpT1-2. Underlined in JAXcpTrev01 is the BamHI site that replaced the stop codon of *xcpT* and that allowed for the construction of a translational fusion between *xcpT* and *phoA*. The PstI-BamHI fragment of pCR-XcpT1-2 was cloned into pYRC-A resulting in construct pJGA01. Plasmid pMPM-K4Ω contains an optimized Shine-Dalgarno sequence upstream of an NcoI site. To clone *xcpT* in the NcoI site, the gene was PCR amplified with primers pJAXcpTfor03 (5'-CGTGGGGTAATCCCATGGATCAGAGC CGC-3') and pJAXcpTrev01. Underlined is the NcoI site that replaces the original GTG start codon. Replacement of the start codon also led to the substitution of the 2nd residue from an asparagine to an aspartic acid in XcpT. The PCR product was cloned into pCRII-TOPO, which resulted in pCR-XcpT1-3. The NcoI-BamHI fragment of pCR-XcpT1-3 and the BamHI-XbaI fragment of pYRC-A were ligated into NcoI-XbaI-digested pMPM-K4Ω resulting in construct pJGA07. To obtain pT7-T, *xcpT* as 850-bp BssH2 fragment from pAX24 was first introduced into SmaI-digested pUC19 resulting in pUX4. Subsequently, the gene was cloned as KpnI-PstI-fragment into KpnI-PstI-digested pSPT19, which resulted in pSX4, and,

finally, the EcoRI-BamHI fragment from pSX4 was ligated into pT7-6. In construct pT7-T, *xcpT* expression is under control of the Φ10 promoter. Construct pGP1.2A is a derivative of pGP1.2 that contains *xcpA* introduced as PstI fragment from pUP2. For construct pJGA03, *lacZ* was PCR amplified from pUR292 with the primers PB3 (5'-CACAGGAAAC AGGATCCACCATGATTACGG-3'), which replaces the start codon by a BamHI site (underlined) and PB4 (5'-GGCTCGAGGTCTAGATTACC CCTGACACC-3') containing an XbaI site (underlined). The resulting product was cloned into pCRII-TOPO. The insert was subsequently excised by BamHI-XbaI digestion and introduced into BamHI-XbaI-digested pJGA01, replacing *phoA* by *lacZ*.

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristic[a]	Source or reference
pAX24	*xcpP-Z* cluster in pLAFR3	(19)
pBBR1MCS-5	Gm^r; cloning vector; P_{lac}	(30)
pBC18R	Ap^r; cloning vector	(8)
pPHO7	Ap^r; *phoA* without ss-encoding part	(24)
pET16b	Ap^r; *lacI*	Novagen
pCR-LacI	pBC18R; *lacI*	This study
pCR-PhoA	pBC18R; *phoA* without ss-encoding part	This study
pYRC	pBBR1MCS-5; *lacI*	This study
pYRC-A	pYRC; *phoA* without ss-encoding part	This study
pCRII-TOPO	Ap^r; Km^r; TOPO TA cloning vector	Invitrogen
pCR-XcpT1-2	pCRII-TOPO; *xcpT*	This study
pJGA01	pYRC; P_{lac}-*xcpT-phoA*	This study
pMPM-K4Ω	Km^r; cloning vector; P*araBAD*	(34)
pCR-XcpT1-3	pCRII-TOPO; *xcpT* (start codon replaced by NcoI site)	This study
pJGA07	pMPM-K4Ω; P_{ara}BAD-*xcpT-phoA*	This study
pUC19	Ap^r; cloning vector	(55)
pUX4	pUC19; *xcpT*	This study
pSPT19	Ap^r; cloning vector	Pharmacia
pSX4	pSPT19; *xcpT*	This study
pT7-6	Ap^r; cloning vector; T7 Φ10 promoter	(49)
pT7-T	pT7-6; PΦ10-*xcpT*	This study
pGP1.2	Km^r; T7 polymerase gene	(49)
pUP2	pUC19; *xcpA*	(12)
pGP1.2A	pGP1.2; *xcpA*	This study
pUR292	Ap^r; cloning vector; promoterless *lacZ*	(41)
pJGA03	pYRC; *xcpT-lacZ*	This study

[a]Gm, gentamicin; Ap, ampicillin; Km, kanamycin; ss, signal sequence

Enzyme assays. Secretion of elastase was analyzed qualitatively on LB plates with a top layer containing 1% elastin (Sigma). After overnight growth, the plates were screened for the presence of halos around the colonies. For alkaline phosphatase activity assays, overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.3 (*E. coli*) or 0.6 (*P. aeruginosa*) in fresh LB and grown for 2.5 (*E. coli*) or 3 h (*P. aeruginosa*). *E. coli* cultures were subsequently split into two and incubated either for another 60 min at 30°C (permissive) or at 42°C (restrictive) to induce the *sec* phenotype of IQ85. Constructs were then induced with 0.01% L-arabinose (Sigma) or 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma), respectively. After 90 min (*E. coli*) or 3 h (*P. aeruginosa*) of growth in the presence of inducer, alkaline phosphatase activity in 1 ml of cell suspension was assayed with the substrate *para*-nitrophenyl phosphate (*p*NPP) (J.T. Baker). Cells were pelleted by centifugation, resuspended in 1 ml 0.9% NaCl and permeabilized by the addition of 20 µl of chloroform and 20 µl of 0.05% sodium dodecyl sulfate (SDS). After vortexing, 420 µl of 0.5 M Tris-HCl (pH 8.0) was added and the suspension was incubated for 10 min at 30°C. The reaction was started by the addition of 50 µl of *p*NPP (30 µg/ml in 0.5 M Tris-HCl, pH 8.0). When significant yellow coloring was observed, the reaction was stopped by the addition of 500 µl 0.5 M NaOH. After centrifugation, released *p*NP was determined by measuring the OD_{420} of the supernatant. Alkaline phosphatase activity in AP units was calculated as OD_{420} x 1000 / reaction time (min) x culture volume (ml) x OD_{600}. Activity of β-galactosidase was analyzed qualitatively on LB plates containing IPTG and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Sigma) as the substrate. For quantitative β-galactosidase activity assays, overnight-grown cells were harvested from LB plates containing the appropriate antibiotic and 0.1% glucose and suspended in 0.9% NaCl. Cells were pelleted by centifugation, resuspended in 950 µl PM2 buffer (40 mM Na_2HPO_4, 26 mM KH_2PO_4, pH 7.0) and permeabelized by the addition of 50 µl chloroform. After the addition of 50 µl *o*-nitrophenyl-β-D- galacto-pyranoside (ONPG) (4 mg/ml in PM2 buffer), the hydrolysis reaction was performed at 30°C for 65 min. The reaction was stopped by the addition of 300 µl of 1 M Na_2CO_3. After centrifugation, released ONP was determined by measuring the OD_{420} of the supernatant. β-galactosidase activity was calculated as OD_{420} x 1000 / reaction time (min) x culture volume (ml) x OD_{600}. In case of strain Js7131, quantitative β-galactosidase activity assays were performed on cells grown in liquid to allow for YidC depletion. Cells

were cultured in LB broth containing 0.2% L-arabinose to an OD_{600} of 0.6. Cells were collected by centrifugation and suspended in LB containing 0.2% L-arabinose or 0.2% glucose. To induce *xcpT-lacZ* expression, 0.5 mM IPTG was added and cultures were grown for an additional 90 minutes. β-galactosidase activities were determined as described above.

SDS-PAGE and immunoblot analysis. Bacterial cells were suspended in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue 0.1 M Tris-HCl, pH 6.8). Extracellular proteins were precipitated using 5% trichloroacetic acid (TCA) and washed with acetone. The amounts of proteins loaded were equivalent to an OD_{600} of 1 of bacterial cells. Samples were heated for 10 min at 95°C and separated on SDS-PAGE gels (8% gels for XcpT-LacZ, 11% gels for XcpT-PhoA, and 14% gels for XcpT). Proteins were stained with Coomassie Brilliant Blue or transferred to nitrocellulose membranes by semidry electroblotting for immunodetection. Primary antisera used were anti-XcpT at 1:1000, and anti-PhoA and anti-LacZ at 1:10.000 dilutions. Either alkaline phosphatase- or peroxidase-conjugated goat anti-rabbit IgG antibodies (Biosource international) were used as secondary antibodies. Detection of the latter was performed with chemiluminescence (Pierce). In the case of pulse-labeled samples, polyacrylamide gels were incubated in Amplify (Amersham) after electrophoresis, vacuum dried and exposed to X-ray films at -80°C.

Pulse-labeling experiments. *E. coli* cells carrying plasmid pT7-T in combination with either pGP1.2 or pGP1.2A were grown overnight at 30°C in M9-casamino acids medium [M9 salts (42) supplemented with 1% casamino acids, 0.2% glucose, 1 mM $MgSO_4$ and 0.0001% thiamine]. Overnight cultures were diluted to an OD_{600} of 0.3 in M9 medium supplemented with all amino acids except for methionine and cysteine (all 0.12%, except aspartic acid, glutamic acid, tyrosine, 0.05% and tryptophan 0.01%) and grown for 2.5 h. Then, cells were incubated at 42°C for 60 min to induce the synthesis of T7 RNA polymerase as well as the *sec* phenotype in the case of strains MM52 and IQ85. Cells were pelleted and resuspended in 1/5 of the original volume of the growth medium. Samples were pulse-labeled for 1 min with 3 μCi Redivue L-[^{35}S]methionine (Amersham) at 42°C. Incorporation of label was stopped by placing the samples on ice and by rapidly adding one volume of a solution of 10% TCA.

Immunoprecipitation. After removal of TCA from the pulse-labeled samples by washing with acetone, the pellet was dissolved in SDS

buffer (2% SDS, 50 mM Tris-HCl [pH 8.0], 1 mM EDTA) and incubated for 10 min at 100°C. Subsequently, Triton buffer (2% Triton X-100, 50 mM Tris-HCl [pH 8.0], 0.15 M NaCl) was added and insoluble material was removed by centrifugation. Polyclonal antiserum was added and, after 3 h of incubation at room temperature, protein A CL-4B sepharose beads (Amersham) were added. After incubation for 1 h at room temperature under gentle rocking, immunocomplexes were collected by centrifugation and washed with Triton buffer. Boiling for 10 min in SDS-PAGE sample buffer eluted antigens from the sepharose beads.

Proteinase K-accessibility. Cells carrying plasmid pJGA07 were grown as described in the pulse-labeling experiments. After 1.5 h of growth, arabinose was added to a final concentration of 0.01% to induce XcpT-PhoA expression. After 1 h of induction, cells were incubated for 1 h at 42°C to induce the *sec* phenotype. Pulse-labeling was performed as described above. Directly after the pulse-labeling, an excess of non-radioactive methionine/cysteine was added, and the cells were collected by centrifugation. For spheroplasting, cells were resuspended in ice-cold buffer A (40% [wt/vol] sucrose, 1.5 mM EDTA, 33 mM Tris-HCl [pH 8.0]) and incubated with lysozyme (final concentration 5 μg/ml). After 10 min on ice, incubation was continued at 37°C for 10 min followed by the addition of 10 mM $MgCl_2$. Aliquots of the spheroplast suspension were incubated on ice for 1 h in the presence or absence of proteinase K (final concentration 50 μg/ml). Subsequently, 2 mM phenylmethylsulfonyl fluoride was added to the cell suspension and incubation was continued for 5 min on ice. Proteins were precipitated with 5% TCA and analyzed by SDS-PAGE and autoradiography.

Cell fractionation. Overnight cultures of DH5α carrying pJGA03 were diluted into fresh, pre-warmed LB medium to an OD_{600} of 0.15 and grown for 2 h at 37°C shaking at 200 rpm. Subsequently IPTG was added to a final concentration of 1 mM. After 90 min incubation, the cells were harvested and spheroplasts were prepared as described (38). After pelleting the spheroplasts by centrifugation, the supernatant was kept as periplasmic fraction. The spheroplasts were washed in 0.9% NaCl, resuspended in 2 mM EDTA, 50 mM Tris-HCl (pH 8.5) and frozen at -20°C. After thawing, they were disrupted by sonication and the membranes were collected by centrifugation for 1 h at 150.000 g at 4°C. The supernatant contained the cytoplasmic proteins.

RESULTS

Inner membrane translocation of XcpT-PhoA occurs independently of other Xcp components. To study pseudopilin transport across the inner membrane, the periplasmic reporter enzyme alkaline phosphatase (PhoA) without its signal sequence was C-terminally fused to the complete major pseudopilin XcpT of *P. aeruginosa*. Plasmid pJGA01, carrying the *xcpT-phoA* gene fusion, was introduced in wild–type PAO25 and in the *xcpT* mutant PAO1ΔT, and immunoblot analysis with an antiserum directed against PhoA confirmed production of XcpT-PhoA (Fig. 1A). Some breakdown products migrating at the same position as wild-type PhoA were detected as well. Remarkably, based on halo formation on elastin plates (Fig. 1B) and analysis of extracellular proteins (data not shown), the fusion protein appeared to restore the secretion of the Xcp substrate elastase in the *xcpT* mutant strain, indicating that the PhoA moiety did not interfere with the functionality of the protein.

PAO25 cells carrying pJGA01 displayed alkaline-phosphatase activity (Fig. 2). Since alkaline phosphatase is only active when transported out of the cytoplasm, this result shows that the XcpT-PhoA fusion protein is transported across the inner membrane. Pseudopilin transport has been proposed to occur via a dedicated transport pathway formed by the other components of the secretion machinery. To determine whether other Xcp components indeed were required for inner membrane passage of XcpT-PhoA, pJGA01 was introduced in *P. aeruginosa* strain D40ZQ lacking the *xcp* gene cluster. D40ZQ cells carrying pJGA01 still displayed alkaline phosphatase activity, although the level was somewhat lower than that found in the wild-type strain (Fig. 2). Thus, cytoplasmic membrane passage of XcpT-PhoA can occur independently of the Xcp system. Durand *et al.* (15) have reported that also the Pil system and the Hxc system can function in the assembly of the XcpT protein into a pilus-like structure. To study whether one of these two systems was responsible for the inner membrane transport of XcpT-PhoA in absence of the Xcp apparatus, pJGA01 was introduced in a strain mutated in all three pathways (PAOΔ*hxc*Δ*pilAC*Δ*R*). This strain still showed alkaline phosphatase activity, similar to that of the *xcp* deletion strain (Fig. 2). Hence, inner membrane passage of XcpT-PhoA can occur independently of the Xcp, Hxc and Pil systems.

XcpT-PhoA activity in *E. coli* depends on a functional Sec system. Given the similarity between the N termini of prepilins and Sec

signal sequences, the most likely inner membrane translocation pathway for XcpT-PhoA, since it is not transported via accessory Xcp components, would be via the Sec machinery. To investigate this possibility, XcpT-PhoA was produced in the heterologous host *E. coli*, of which several well-characterized *sec* mutant strains are available. Construct pJGA07, which contains *xcpT-phoA* under control of P*araBAD*, was introduced in the temperature-sensitive *secY* mutant IQ85 and its parental strain IQ86. Expression of the XcpT-PhoA protein was verified by immunoblot analysis with an antiserum against PhoA (results not shown) and inner membrane transport of the fusion protein was studied by measuring alkaline phosphatase activity. At the permissive temperature of 30°C, IQ85 produced similar alkaline phosphatase activity as did the parental strain (Fig. 3). However, at the restrictive temperature of 42°C, PhoA activity was drastically lower in the *secY* mutant as compared with the parental strain. This result shows that XcpT-PhoA is transported over the inner membrane of *E. coli* in a SecY-dependent manner.

Processing and inner membrane transport of XcpT requires a functional Sec system. To study the inner membrane translocation of native XcpT in *E. coli*, pulse-labeling experiments were performed. In these experiments, processing of XcpT by the prepilin peptidase XcpA was used as an indicator of inner membrane transport. Processing of XcpT results in the removal of eight residues from the N terminus and can be visualized by a small mobility shift on acrylamide gels. To allow for simultaneous induction of the *sec* phenotype and XcpT expression, the *xcpT* gene was introduced in pT7-6 under the control of the T7 promoter. The resulting construct, pT7-T, was combined either with helper plasmid pGP1.2, which contains the gene for T7 RNA polymerase under the control of the λ P_L promoter and the *cI857* gene encoding a temperature-sensitive λ repressor, or with pGP1.2A, which additionally contains *xcpA*. These constructs were introduced into the temperature-sensitive *secA* and *secY* mutant strains MM52 and IQ85, respectively, and their cognate parental strains. To induce the synthesis of T7 RNA polymerase and the Sec phenotype, cells were shifted to 42ºC for 1 h before pulse-labeling was performed. In these experiments, processed XcpT was only detected upon co-expression of XcpA (data not shown). In the XcpA-expressing parental strains MC4100 and IQ86, processing of the pseudopilin XcpT was nearly complete after 1 min pulse-labeling (Fig. 4). However, maturation of XcpT was clearly

reduced in the *secA* and *secY* mutants, which shows that processing requires a functional Sec translocase.

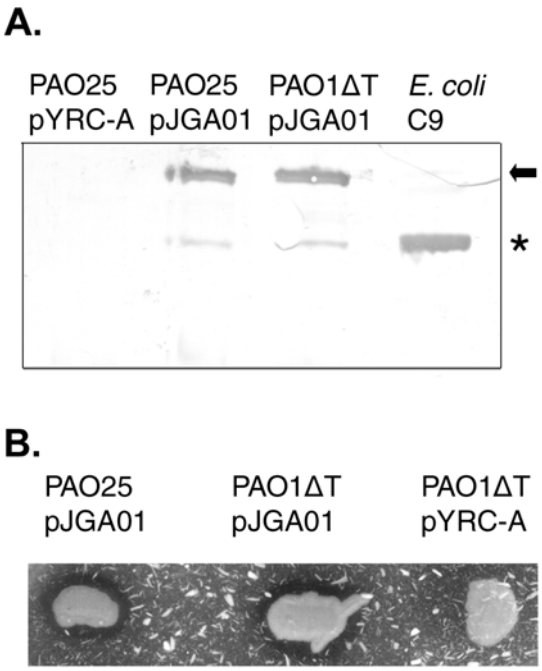

FIG. 1. Production and functionality of XcpT-PhoA fusion protein. (A) Immunoblot analysis of whole cell extracts of *P. aeruginosa* strain PAO25 and an *xcpT* mutant PAO1ΔT both expressing XcpT-PhoA from pJGA01, or containing the fusion vector pYRC-A. Immunodetection was carried out with PhoA-specific antibodies. The position of XcpT-PhoA is indicated by an arrow. Total cell lysate of *E. coli* strain C9 constitutively producing PhoA (indicated by an asterisk) was included for reference. (B) PAO25 and PAO1ΔT expressing XcpT-PhoA from pJGA01 or containing the vector pYRC-A were grown on LB agar containing 1% elastin. Secretion of elastase is visualized by clearance of elastin around the colonies.

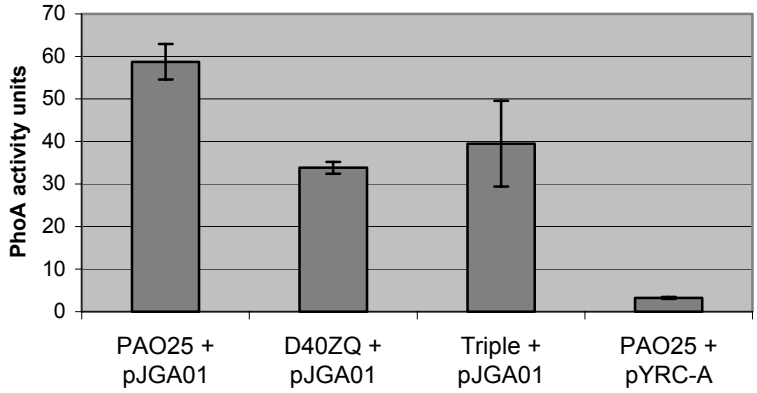

FIG. 2. Enzyme activity of XcpT-PhoA in *P. aeruginosa*. Alkaline phosphatase activities were measured in *P. aeruginosa* strains expressing XcpT-PhoA from pJGA01, or containing the vector pYRC-A. The strains used were the wild-type strain PAO25, strain D40ZQ, which lacks the *xcp* cluster, and strain PAOΔ*hxc*Δ*pilAC*Δ*R* (triple), which lacks *hxcP-Z, pilA-C*, and *xcpR*. Bars represent the averages of three independent assays and standard deviations are indicated.

The results above suggest that the Sec translocase is required for transport of XcpT and that transport is a prerequisite for processing to occur. Lack of transport in the *sec* mutants was further evaluated in proteinase K-accessibility experiments on spheroplasted cells. Since XcpT is intrinsically very resistant to the protease (results not shown), the XcpT-PhoA fusion protein was employed in these experiments. When the fusion protein was expressed from pJGA07 in the parental strains MC4100 and IQ86, it was almost completely degraded and thus accessible for proteinase K after spheroplasting of the cells (Fig. 5A). In these experiments, proteinase K treatment did not yield the stable PhoA moiety, likely because kinetics were not fast enough to allow PhoA to obtain its mature conformation. When produced under the restrictive conditions, the XcpT-PhoA protein was completely protected from the protease in spheroplasts of strain IQ85, showing that the protein remains at the cytoplasmic side of the membrane in the absence of a functional Sec system. In these cells, also the precursor of a well-known Sec substrate, OmpA, was found to accumulate in a form inaccessible to proteinase K after spheroplasting (Fig. 5B). In spheroplasts of MM52 cells grown at the restrictive temperature, XcpT-PhoA was also protected against the proteinase K treatment, although not completely (Fig.

5A). In these cells, also some mature OmpA was detected, indicating that the SecA phenotype was not complete. As expected, the proteinase K treatment resulted in the degradation of the mature form only of OmpA (Fig. 5B).

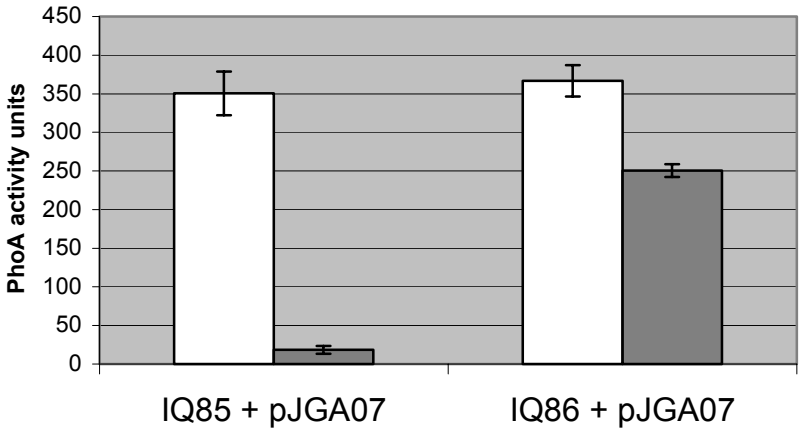

FIG. 3. Enzyme activity of XcpT-PhoA is dependent on SecY in *E. coli*. Alkaline phosphatase activities were measured in the temperature-sensitive *secY* mutant strain IQ85 and its parent IQ86, both expressing XcpT-PhoA from pJGA07. Activities were measured in cultures incubated for 2.5 h at 30°C (white bars) or at 42°C (gray bars). Bars represent the averages of three independent assays and standard deviations are indicated.

Co-translational transport of XcpT-LacZ. Transport of XcpT across the inner membrane is largely affected in the temperature-sensitive *secY* mutant and, albeit to a lesser extent, also in the *secA* mutant. The cytoplasmic protein β-galactosidase (LacZ) can be used as a reporter to distinguish between the SRP and the SecB pathways (5), which both intersect at the Sec translocon. Cells expressing LacZ fused to a SecB substrate display a Lac^+ phenotype because of the cytoplasmic accumulation of β-galactosidase. They are also sensitive to induction of the expression of the fusion protein because of jamming of the Sec translocon due to rapid folding of the LacZ moiety of the fusion protein. In contrast, when LacZ is fused to an SRP substrate, cells are Lac^- because β-galactosidase is exported. Since export is co-translational, the enzyme cannot fold in the cytoplasm and no jamming of the translocon occurs. To some extent, the cells can still be sensitive to induction of the expression of the fusion

protein, because of the formation of toxic aggregates of LacZ in the periplasm (5).

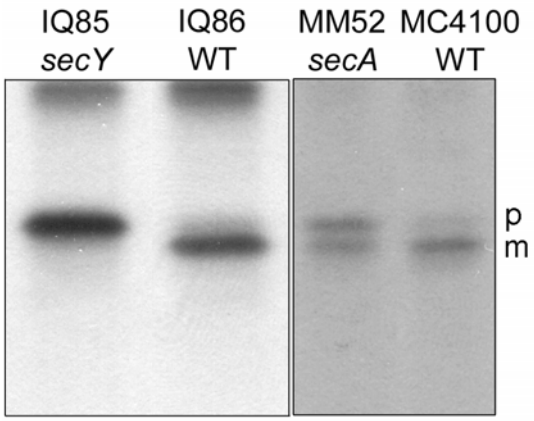

FIG. 4. Inhibitory effect of *sec* mutations on XcpT processing. XcpT was expressed from plasmid pT7-T in the *E. coli* strains MC4100 and IQ86 and their isogenic temperature-sensitive *secA* and *secY* mutants MM52 and IQ85, respectively, both co-expressing XcpA from pGP1.2A. After pulse-labeling at 42°C and immunoprecipitation with anti-XcpT, samples were analyzed by SDS-PAGE, followed by autoradiography. The location on the autoradiogram of mature XcpT and its precursor are indicated by m and p, respectively.

To make use of this system, plasmid pJGA03 was constructed, encoding an XcpT-LacZ fusion protein. Production of this chimaeric protein from pJGA03 in DH5α resulted in a Lac^- phenotype as indicated by the white colony phenotype on plates containing the β-galactosidase substrate X-gal (Fig. 6A), even after two days of incubation. Immunoblot analysis with antisera against LacZ and XcpT revealed that the fusion protein was produced (Fig. 6B), although the majority was detectable as a smaller degradation product only recognized by the LacZ antibodies. The degradation product was not always observed (see Fig. 7B) and probably reflects degradation during sample preparation. However, the appearance of the degradation product allowed us to determine the localization of XcpT-LacZ. Cell fractionation showed that the fusion protein was associated with the membranes (Fig. 6B), whereas the degradation product corresponding to the LacZ part was found mostly in the periplasmic fraction, showing that the fusion protein is transported over the inner membrane. DH5α cells carrying pJGA03 were not sensitive to induction of the expression of the chimeric

gene with IPTG. The efficient export of the XcpT-LacZ fusion protein across the inner membrane without jamming the Sec pathway is consistent with co-translational targeting via the SRP pathway.

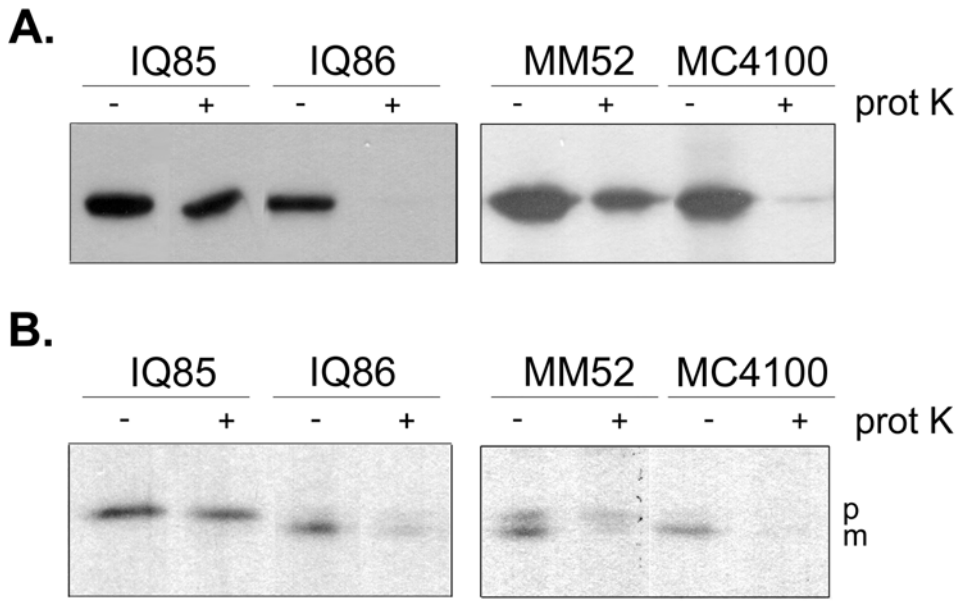

FIG. 5. Proteinase K accessibility of XcpT-PhoA and OmpA in spheroplasts. (A) Cultures of temperature-sensitive *secA* and *secY* mutants MM52 and IQ85, respectively, and their parental strains MC4100 and IQ86 were induced for XcpT-PhoA expression from pJGA07 and incubated at the restrictive temperature. Cells were subsequently pulse-labeled with [^{35}S]methionine, converted to spheroplasts and, where indicated, treated with proteinase K (prot K). After immunoprecipitation with anti-PhoA serum, samples were analyzed by SDS-PAGE, followed by autoradiography. Because of the large size of XcpT-PhoA (~65 kDa), removal of the short XcpT leader peptide during processing is not visible as a mobility shift. (B) The same samples were also used for immunoprecipitation with anti-OmpA serum. Samples were analyzed by SDS-PAGE and autoradiography. The location on the autoradiogram of mature OmpA and its precursor are indicated by m and p, respectively.

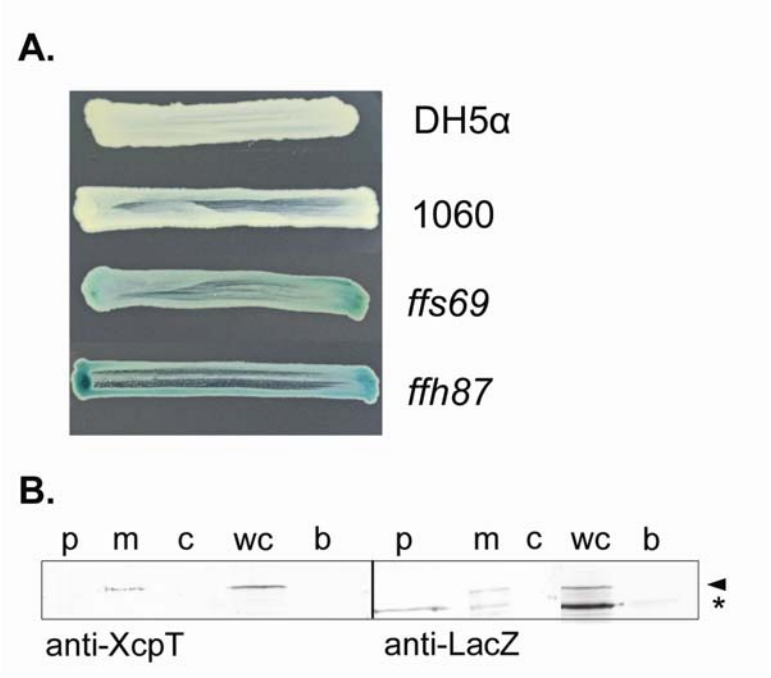

FIG. 6. XcpT-LacZ production and localization in *E. coli*. (A) *E. coli* strains DH5α, MC1060 and the MC1060 derivatives with the alleles *ffs69* and *ffh87*, respectively, producing XcpT-LacZ from pJGA03 were grown overnight on LB plates with X-gal. White colonies correspond to a Lac$^-$ phenotype; blue colonies to a Lac$^+$ phenotype. (B) DH5α cells carrying pJGA03 were induced for the expression of XcpT-LacZ, and whole cell extracts (wc) or cell fractions were analyzed by SDS-PAGE and Western blotting. Immunodetection was performed with antibodies against LacZ and XcpT. The positions of XcpT-LacZ and of LacZ are indicated with an arrowhead and an asterisk, respectively. p, periplasmic fraction; m, membrane fraction; c, cytoplasmic fraction; wc, whole cell extract; b, whole cell extract of plasmid-less DH5α cells; the weak band detected by the anti-LacZ serum in this extract likely corresponds to the inactive LacZdelM15 protein encoded from the chromosome (31).

To further substantiate this conclusion, pJGA03 was introduced into MC1060 and its derivatives containing the SRP mutant alleles *ffs69* (50) and *ffh87* (51), which confer mild defects on SRP-dependent secretion. The strains with the SRP mutant alleles formed light blue colonies on X-gal-containing plates, while the parental strain remained white (Fig. 6A), showing that the SRP mutations interfere with efficient translocation of XcpT-LacZ to the periplasm. This effect was quantified by measuring β-

galactosidase activity. As shown in Fig. 7A, the strains with the mutant alleles displayed a five-fold increase in β-galactosidase activity compared to the wild-type strain, whereas the production of XcpT-LacZ was the same in all the strains (Fig. 7B). These results underscore the SRP dependency of XcpT targeting to the Sec translocon. To test the effect of YidC on translocation of XcpT-LacZ, pJGA03 was introduced into strain Js7131, which carries a ParaBAD-yidC operon. No effects on β-galactosidase activities were measured upon removal of arabinose, which results in YidC depletion (43) (data not shown).

A.

Strain	Miller units	Normalized[a]
MC1060	0,42 (0,03)	100
MC1060 *ffs69*	1,92 (0,03)	457
MC1060 *ffh87*	2,01 (0,06)	479

B.

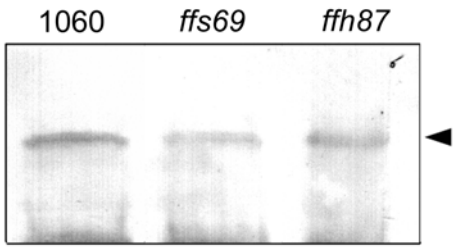

FIG. 7. XcpT-LacZ production in the presence of mutant SRP alleles. (A) β-galactosidase activity (in Miller units) of cells grown on LB plates. The activity values represent the mean of four independent experiments, with standard deviation indicated in brackets. [a]Values of β-galactosidase activity normalized against the wild-type strain in percentage. (B) Immunoblot analysis of whole cell lysates of *E. coli* strains MC1060 and and the MC1060 derivatives with the alleles *ffs69* and *ffh87*, respectively, producing XcpT-LacZ from pJGA03. Immunodetection was carried out with LacZ-specific antibodies. The position of XcpT-LacZ on the immunoblot is indicated by an arrowhead.

DISCUSSION

Sequences resembling the N terminus of prepilins are frequently detected in proteins from Gram-negative and -positive bacteria, as well as from archaea (39). Although these sequences mediate transport across the inner membrane, the pathway involved in this process was largely unknown. To study this transport process, alkaline phosphatase was fused to XcpT, the major pseudopilin of the type II secretion machinery of *P. aeruginosa,* and alkaline phosphatase activity was used to monitor transport. Export of XcpT-PhoA did occur in a *P. aeruginosa* strain lacking functional Xcp, Hxc, and Pil systems as well as in *E. coli*. These findings argue against the hypothesis that accessory Xcp components or their homologues form a dedicated inner membrane transport system for the pseudopilins. This idea was based on the observation that proteins with prepilin-like N termini are always found in concert with an ATPase (XcpR in *P. aeruginosa*), an integral inner membrane protein (XcpS), and a prepilin peptidase. In support of this hypothesis, Chung and Dubnau (9) reported that the prepilin peptidase ComC of the *Bacillus subtilis* competence system is required for translocation of the pilin-like protein ComGC. Moreover, Kagami *et al.* (27) showed that a conditional mutation in XcpT could be suppressed by a secondary mutation in the cytoplasmic ATPase XcpR, indicating that these proteins interact during assembly and/or functioning of the machinery.

However, our conclusion that the pseudopilin XcpT is not transported via accessory Xcp proteins is consistent with the observation that a mutant of the archaeon *Sulfolobus solfataricus*, in which the genes encoding the accessory ATPase (homologue of XcpR) and the integral inner membrane component (homologue of XcpS) are deleted, is not affected in its ability to insert sugar-binding proteins with prepilin-like N termini into the membrane (B. Zolghadr, personal communication). Moreover, although pilins and pseudopilins are unable to functionally replace each other, it is possible to exchange their leader peptides and hydrophobic domains without loss of function (29). Even in *P. aeruginosa*, where the Pil and Xcp systems function side by side, the N termini of PilA and XcpT could be exchanged without affecting function (our unpublished results). Thus, apparently, the N termini do not contain the information for targeting of the (pseudo)pilins to the cognate machinery, which is in agreement with export of these proteins via a general route. An explanation for the afore mentioned results described by Kagami *et al. (27)* and Chung and Dubnau (9) might be offered by the

possibility that the accessory proteins, although not required for the transport of the pseudopilins across the inner membrane, are required for their assembly into a pilus-like structure (16, 44). In the case of ComGC, translocation was assayed in NaOH-solubility studies and it is conceivable that this method distinguishes ComGC assembled in a pilus-like structure from membrane-embedded ComGC rather than showing translocation.

Since the N termini of (pseudo)pilins to a certain degree resemble Sec signal sequences, we reasoned that inner membrane translocation of XcpT would occur via the Sec machinery. The involvement of the Sec translocon was studied in the heterologous host *E. coli*. In a temperature-sensitive *secY* mutant, translocation of XcpT-PhoA was blocked at the restrictive temperature, indicating that indeed the Sec pathway is involved. The requirement for SecA and SecY in XcpT transport could also be demonstrated in pulse-labeling experiments. Both XcpT processing and protease accessibility were affected in the *secA* and *secY* mutants at the restrictive temperature. Maturation of XcpT in these experiments was strictly dependent on the presence of the *P. aeruginosa* prepilin peptidase XcpA. Nonetheless, immunoblot analysis with anti-XcpT serum of extracts of overnight grown *E. coli* cells expressing XcpT showed partial processing (unpublished observation). Apparently, previously described *E. coli* prepilin peptidase homologues (22, 54) are functional, but their ability to process XcpT was not sufficiently efficient to be detected in the short pulse-labeling experiments. These experiments showed that processing of the pseudopilin occurs after transport, despite the fact that the prepilin peptidase has its active centre at the cytoplasmic side of the inner membrane (32). Apparently, membrane insertion of XcpT is required to expose its processing site accurately to the XcpA peptidase.

In order to distinguish between targeting via the SecB or the SRP route, XcpT was fused to the reporter LacZ, which was previously shown to be a useful reporter for this purpose (5). This fusion protein was efficiently transported to the periplasm without jamming the Sec translocon, consistent with co-translational transport via the SRP pathway. Moreover, efficient inner membrane translocation of the XcpT-LacZ fusion was dependent on fully functional Ffh and Ffs, but not on YidC. Taken together, our results demonstrate that XcpT is targeted to the Sec system via the SRP route, which is common for inner membrane proteins. The precise requirements for SRP signals are still not completely clear, but hydrophobicity and secondary structure have been shown to be important determinants (1, 5,

33). Indeed, the N termini of prepilins are highly hydrophobic and contain an extended N-terminal α-helix. Since pseudopilins have a high tendency to associate (40), co-translational translocation may be important to circumvent premature interactions.

This report shows for the first time the Sec and SRP dependency of export of a pseudopilin. Given the strong conservation of their N-terminal sequences, it is likely that also other pilins and pseudopilins use the SRP/Sec pathway. Indeed, similar conclusions were reached by Francetic *et al*. in the accompanying paper on the export of the pseudopilin PulG of the type II secretion system of *Klebsiella oxytoca* (21). We propose that, after translocation, the (pseudo)pilins laterally leave the Sec translocon, after which they become recruited by their cognate machinery. The polytopic inner membrane component will subsequently act as a platform for the assembly of the subunits into a pilus (-like) structure, using the energy provided by the ATPase.

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Chapter 3

Targeting of *Pseudomonas aeruginosa* pilin-pseudopilin chimeras to the type II secretion machinery

Jorik Arts, Gaby Smits, Jan Tommassen, Margot Koster

ABSTRACT

Several proteins involved in type II secretion share homology with components of the type IV piliation apparatus. Among these are proteins that are termed pilins in the Pil system and pseudopilins in case of the type II secretion machinery, both categories being synthesized as precursors with a characteristic short leader peptide. Despite their similarity, pilins and pseudopilins cannot functionally replace each other. Thus, targeting of these subunits to their cognate assembly machinery needs to be efficient, especially when the two pathways co-exist in the same cell, as is the case in the opportunistic pathogen *Pseudomonas aeruginosa*. To assess which part of the pseudopilin XcpT contains such targeting information, we constructed gene fusions in which parts of *xcpT* were substituted by the corresponding part of *pilA*, which encodes the pilus subunit. We found that the leader peptide and the N-terminal 17 amino acid residues of mature XcpT could be functionally replaced by the corresponding part of PilA, which shows that this domain of the protein does not contain information for targeting to the Xcp system. Disruption of the processing site in XcpT or in the functional hybrids resulted in proteins with a strong dominant-negative effect in the wild-type strain on type II secretion, but not on type IV piliation. Production of a similar processing-defective PilA variant inhibited type IV piliation, showing that specific blocking of these systems can be accomplished.

INTRODUCTION

Gram-negative bacteria use several different pathways for the export of proteins into their extracellular environment. One of these pathways involves the type II secretion system (T2SS) (9). Assembly of the T2SS requires 12-16 different components, generically referred to as Gsp proteins, or, in the case of the main T2SS of *Pseudomonas aeruginosa*, as Xcp proteins. Many of these proteins share sequence similarity with constituents of the type IV piliation system (24). Type IV pili are filamentous appendages with various functions, including adherence to host cells, twitching motility, DNA uptake, and biofilm formation (4). Both type II secretion and type IV piliation require (i) a secretin, probably forming a channel in the outer membrane (3, 21), (ii) a nucleotide-binding protein of the traffic ATPase family, (iii) a multi-spanning integral inner membrane component, (iv) a group of proteins that are termed pilins in case of the Pil system or pseudopilins in case of the T2SS and that are synthesized as precursors with a short positively charged leader peptide, and (v) a dedicated prepilin peptidase that is required for the maturation of pilins and pseudopilins by cleaving off the leader peptide and methylating the new N-terminal residue (31). The prepilin peptidase processing site is characterized by a glycine residue at the –1 position, which is essential for cleavage (29). The processing site is followed by a hydrophobic stretch of approximately 20 amino acid residues, which is considered to be of importance for transport across the inner membrane (1, 12, 30) and for subunit interactions within the pilus (6). The hydrophobic region frequently starts with a phenylalanine at the +1 position, although this residue is not strictly required for proper biogenesis or functionality (29).

The sequence similarity between proteins involved in type IV piliation and type II secretion led to the idea that pseudopilins might assemble into a pilus-like structure (11), which indeed could be shown upon over-expression of the entire system, or of only the major pseudopilin GspG (XcpT) (7, 27, 33). The generation of these long pseudopili was considered to result from unrestricted elongation of a structure that normally would only span the periplasm.

Sequence similarity between pilins and pseudopilins is mainly found in their N termini. However, progressive insights in the three dimensional structures of type IV pilins from several organisms and of the pseudopilin PulG from *Klebsiella oxytoca* revealed that also their overall structures are

substantially conserved (5, 17, 18, 23). Functional conservation is reflected in the interchangeability of type IV pilins between heterologous Pil systems (14, 26, 34). Pseudopilin assembly via the T2SS seems to be rather promiscuous as well, since GspG (XcpT) homologues can be readily exchanged between T2SSs without loss of function (7, 33). Moreover, assembly of pseudopili by the Pil system has been demonstrated (7). However, assembly of pilin subunits into a pilus via the T2SS seems more problematic and has only be shown for *Escherichia coli* K-12 type IV pilin PpdD, which was assembled via the T2SS of *K. oxytoca* (18).

In *P. aeruginosa*, the T2SS and the type IV piliation system are produced simultaneously in the cell. Durand *et al.* (7) have shown that in the absence of the T2SS, the major pseudopilin XcpT can be assembled into a pilus-like structure by the Pil system. However, the pilin subunit cannot functionally replace XcpT and *vice versa*, indicating that under normal conditions targeting of the subunits to the cognate machinery should be efficient.

In this study, hybrid proteins between XcpT and the structural component of the type IV pili, PilA, were constructed to study the targeting of XcpT to the Xcp machinery. Further insight into the targeting process was obtained by blocking the Xcp machinery with processing–defective variants of these chimeras.

MATERIALS AND METHODS

Strains and growth conditions. *E. coli* strain DH5α (16) was used for routine cloning. *P. aeruginosa* strains PAO25 (15), and PAO1ΔT (2) are *leu arg* and *xcpT* mutant derivatives, respectively, of strain PAO1. Strain PAK-NP (34) is a mutant derivative of strain PAK (35), carrying a tetracycline-resistance cassette in the *pilA* gene. Strains were grown at 37°C in a modified Luria-Bertani (LB) broth (32). For selection or plasmid maintenance, antibiotics were added in the following concentrations: for *E. coli* ampicillin 50 μg/ml, and gentamicin 10 μg/ml; for *P. aeruginosa* gentamicin 40 μg/ml, tetracycline 40 μg/ml, and carbenicillin (300 μg/ml). For induction of genes from the *lac* or the *tac* promoter, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a concentration of 1 mM.

TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristic*	Source or reference
pCRII-TOPO	Ap^r; Km^r; TOPO TA cloning vector	Invitrogen
pCRII-PilA	pCRII-TOPO; *pilA*	This study
pCRII-XcpT	pCRII-TOPO; *xcpT*	This study
pUC19	Ap^r; cloning vector	(37)
pUC-PilA	pUC19; *pilA*	This study
pUC- $XcpT_{lpA\#2}$	pUC19; encoding hybrid $XcpT_{lpA}$ with disrupted processing site	This study
pUC- $XcpT_{17A}$	pUC19; $xcpT_{17A}$	This study
pUC- $XcpT_{65A}$	pUC19; $xcpT_{65A}$	This study
pYRC	pBBR1-MCS5; *lacI*	Chapter 2
pYRC- $XcpT_{65A}$	pYRC; $xcpT_{65A}$	This study
pYRC- $XcpT_{lpA}$	pYRC; $xcpT_{lpA}$	This study
pYRC- $XcpT_{17A}$	pYRC; $xcpT_{17A}$	This study
pYRC- $XcpT_{65A\#2}$	pYRC; encoding hybrid $XcpT_{65A}$ with disrupted processing site	This study
pYRC- $XcpT_{lpA\#2}$	pYRC; encoding hybrid $XcpT_{lpA}$ with disrupted processing site	This study
pYRC- $XcpT_{17A\#2}$	pYRC; encoding hybrid $XcpT_{17A}$ with disrupted processing site	This study
pYRC-$PilA_{\#2}$	pYRC; encoding PilA with disrupted processing site	This study
pYRC-PilA	pYRC; *pilA*	This study
pMMB67HE	Ap^r; cloning vector	(13)
pMMB-$PilA_{\#2}$	pMMB67HE; encoding PilA with disrupted processing site	This study
pMMB-$XcpT_{lpA\#2}$	pMMB67HE; encoding hybrid $XcpT_{lpA}$ with disrupted processing site	This study
pAX24	XcpP-Z cluster in pLAFR3	(10)

*Gm, gentamicin; Ap, ampicillin; Km, kanamycin. The proteins encoded by the *pilA-xcpT* hybrids are schematically depicted in Fig. 1.

Plasmids and DNA manipulations. Plasmids used in this study are listed in Table 1. Recombinant DNA methods were performed essentially as described (25). Plasmids were introduced by the $CaCl_2$ procedure into *E. coli* (25) or by electroporation into *P. aeruginosa* (8). PCRs were performed with the proofreading enzyme *Pwo* DNA polymerase (Roche) and PCR products were cloned into pCRII-TOPO according to manufacturer's protocol. Oligonucleotides used in this study are listed in Table 2. Chromosomal DNA from strain PAO1 was used as the template to amplify *pilA* with the oligonucleotides JAPilAfor and JAPilArev. The resulting product was cloned into pCRII-TOPO resulting in pCRII-PilA.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5'→ 3')	Restriction site
JAPilAfor	AAGCTTAGTTTCCTTGATCGTGGCG	HindIII
JAPilArev	GAGCTCTACCGACTGAGCTAATCCG	SacI
pUC19for	TCAGTGAGCGAGGAAGCGGAAGA	
pilA_rev09#2	CGATCAAGTCTAGACCTTTTTGAGC	XbaI
pilA_rev10#2	GAATGGCATCTAGAGCCAGGATAC	XbaI
JAXcpTshrev01	CGAGCTCTACGCTGATGATGACCATCACC	SacI
JAXcpTfor02	CTTCCGATCCTTCGAATCAACCAACTCGT G	
JAXcpT_for11	TCGCCAACATCTAGACTTCACCCTGATCG AA	XbaI
JAXcpT_for12	ATCCTCGGCATTCTAGACGCCCTGGTGGT	XbaI
SDM_A9T11#3for	GAAAGCTCAAAAAGGCTTCACCCTGATCG	
SDM_A9T11#3rev	CGATCAGGGTGAAGCCTTTTTGAGCTTTC	
SDM_A10T12#3for	GGCACCACCAGGGCGGCCAGGATACCGA TG	
SDM_A10T12#3rev	CATCGGTATCCTGGCCGCCCTGGTGGTGC C	
SDM_A9T11for	GAAAGCTCAAAAAGGCTCTAGATTTACCT TGATCGAAC	
SDM_A9T11rev	GTTCGATCAAGGTAAATCTAGAGCCTTTT TGAGCTTTC	
JAPilAfor01	GATATTAAGCTTGGTAAGTGCTTGTTGAG G	HindIII
JAPilArev02	TTCGGTCGCTCTAGAAGCAGTAGTACC	XbaI
JAXcpT_for4	CATGTACTCTAGAGACAACTTCGCCTATC CG	XbaI
JAPilAfor23	CATGAAAGCTCATCTAGACTTTACC	XbaI

The HindIII-SacI fragment of pCRII-PilA was introduced into HindIII-SacI-digested pUC19 as well as in similarly digested pYRC, resulting in pUC-PilA and pYRC-PilA, respectively. The pUC-PilA construct was used as template to amplify *pilA* fragments with pUC19for as the forward primer and either pilA_rev09#2 or pilA_rev10#2 as the reverse primer to generate the *pilA* parts of the *pilA-xcpT* hybrid genes $xcpT_{lpA}$, and of $xcpT_{17A}$, respectively (Fig. 1). For the construction of $xcpT_{65A}$, a fragment of the *pilA* gene was amplified with the oligonucleotides JAPilAfor01 and JAPilArev02. The resulting products were cloned into pCRII-TOPO generating pCRII-A9, pCRII-A10#3, and pCRII-A2. Cosmid pAX24 was used as the template to amplify *xcpT* with the oligonucleotides JAXcpTshrev01 and JAXcpTfor02 and the resulting product was cloned

into pCRII-TOPO, resulting in pCRII-XcpT. This construct was used as a template to amplify *xcpT* fragments with JAXcpTshrev01 as the reverse primer and JAXcpT_for11, JAXcpT_for12, or JAXcpT_for4 as the forward primer to generate the *xcpT* parts of the hybrid genes $xcpT_{lpA}$, of $xcpT_{17A}$, or of $xcpT_{65A}$, respectively. The resulting PCR fragments were introduced into pCRII-TOPO, resulting in pCRII-T11, pCRII-T12, or pCRII-T4, respectively. The HindIII-XbaI fragment of pCRII-A9 and the XbaI-SacI fragment of pCRII-T11 were ligated into HindIII-SacI-digested pUC19 resulting in pUC-$XcpT_{lpA\#2}$. The HindIII-XbaI fragment of pCRII-A10#3 and the XbaI-SacI fragment of pCRII-T12 were ligated into HindIII-SacI-digested pUC19 resulting in pUC-A10#3. The HindIII-XbaI fragment of pCRII-A2 and the XbaI-SacI fragment of pCRII-T4 were ligated into HindIII-SacI-digested pYRC yielding pYRC-$XcpT_{65A}$ and in HindIII-SacI-digested pUC19 yielding in pUC-$XcpT_{65A}$. The HindIII-SacI fragment of pUC-$XcpT_{lpA\#2}$ was cloned into HindIII-SacI-digested pYRC, resulting in pYRC-$XcpT_{lpA\#2}$ and into HindIII-SacI-digested pMMB67HE, resulting in pMMB-$XcpT_{lpA\#2}$. To remove the XbaI site from the insert of pUC-$XcpT_{lpA\#2}$, the QuikChange site-directed mutagenesis kit (Stratagene) was used with the complementary oligonucleotides SDM_A9T11#3for and SDM_A9T11#3rev and pUC-$XcpT_{lpA\#2}$ as template DNA. After nucleotide sequencing to verify removal of the XbaI site, the HindIII-SacI insert was ligated into HindIII-SacI-digested pYRC, resulting in pYRC-$XcpT_{lpA}$. Similarly, the XbaI site was removed from the insert of pUC-A10#3 by site-directed mutagenesis using the complementary oligonucleotides SDM_A10T12#3for and SDM_A10T12#3rev and pUC-A10#3 as template DNA. The HindIII-SacI fragment of the resulting construct, pUC-$XcpT_{17A}$, was ligated into HindIII-SacI-digested pYCR, which resulted in pYRC-$XcpT_{17A}$. An XbaI site was introduced between the nucleotides that encode the processing site of $XcpT_{17A}$ and $XcpT_{65A}$ by site-directed mutagenesis with the complementary oligonucleotides SDM_A9T11for and SDM_A9T11rev, and pUC- $XcpT_{17A}$ and pUC-$XcpT_{65A}$, respectively, as template DNA. After nucleotide sequencing to verify the introduction of the XbaI site, the HindIII-SacI fragments were ligated into HindIII-SacI-digested pYCR, yielding pYRC-$XcpT_{17A\#2}$ and pYRC-$XcpT_{65A\#2}$, respectively. With the primers JAPilAfor23 and JAPilArev and chromosomal DNA from strain PAO1 as the template, part of the *pilA* gene was amplified. After cloning of this PCR fragment into pCRII-TOPO, the XbaI-SacI fragment was cloned together with the HindIII-XbaI fragment of

pCRII-A9 into HindIII-SacI-digested pYRC resulting in pYRC-PilA$_{\#2}$. The *pilA*$_{\#2}$ gene was subsequently inserted into HindIII-SacI-digested pMMB67HE resulting in pMMB-PilA$_{\#2}$.

Enzyme assays. Secretion of elastase was analyzed qualitatively on plates with a top layer containing 1% elastin (Sigma). For quantitative analysis, the colorimetric elastin-Congo red assay (20) was used. Briefly, 250 µl of culture supernatant of cells grown overnight in the presence of IPTG were incubated for 2 h at 37°C with 500 µl of 10 mg/ml elastin-Congo red (Sigma) dissolved in assay buffer (45 mM Tris-HCl, 1.5 mM $CaCl_2$ pH 7.2). The reaction was stopped by the addition of 500 µl of 0.7 M NaH_2PO_4 pH 6.0. After removal of elastin-Congo red by centrifugation, absorbance was measured at 495 nm.

SDS-PAGE and immunodetection. Bacterial cells were suspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue, 0.1 M Tris-HCl pH 6.8). Extracellular proteins were precipitated from culture supernatants by the addition of trichloroacetic acid to a final concentration of 5% (wt/vol) and, after washing in acetone, proteins were dissolved in SDS-PAGE sample buffer, boiled and analyzed by SDS-PAGE. Whole cell lysates were heated for 10 min at 95°C and proteins were separated on gels containing 14% acrylamide. The proteins were transferred onto nitrocellulose membranes by semidry electroblotting. Immunoblots were incubated with polyclonal antisera against XcpT (1:1000) (7), or against PilA (1:10.000) (28). Alkaline phosphatase-conjugated goat anti-rabbit IgG antiserum (Biosource international) was used as secondary antiserum, unless stated otherwise. Detection was performed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

Phage sensitivity. To test the sensitivity of bacterial strains to bacteriophage PO4, a high-titer suspension of the phage was streaked on LB agar plates and, subsequently, bacteria were cross-streaked through the phage suspension. After incubation at 37°C, clear lysis zones indicated sensitivity.

RESULTS

Complementation of the secretion defect in an *xcpT* mutant by PilA-XcpT fusions. To obtain insight in the targeting of the pseudopilin

XcpT to the Xcp machinery, hybrids of *pilA* and *xcpT* were constructed and introduced into the broad-host-range vector pYRC. The leader peptide alone, the leader peptide including the hydrophobic domain, or the first 65 amino acid residues of XcpT were replaced by the corresponding segments of PilA. These chimeras are referred to as $XcpT_{lpA}$, $XcpT_{17A}$, and $XcpT_{65A}$, respectively. A schematic representation of the PilA-XcpT hybrid proteins encoded by the gene fusions is depicted in Fig. 1.

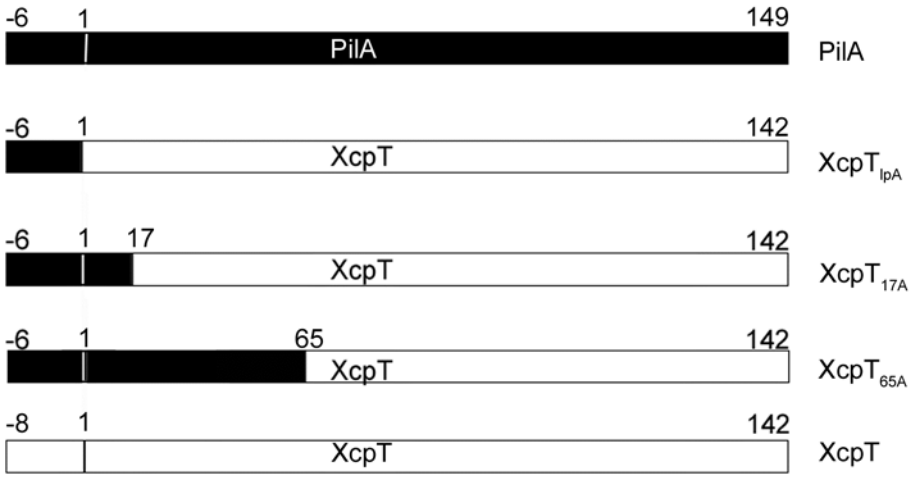

FIG.1. Schematic representation of PilA-XcpT hybrids. PilA sequences are depicted in black and XcpT sequences in white. Numbers indicate the amino acid residues relative to the prepilin peptidase cleavage site. The nomenclature of the hybrids is shown at the right.

The *pilA-xcpT* constructs were introduced into the non-polar *xcpT* mutant PAO1ΔT. Production of the three hybrid proteins was confirmed by immunoblot analysis with antiserum raised against XcpT (Fig. 2A). However, detection of fusion $XcpT_{65A}$ required considerably longer exposure times than that of hybrids $XcpT_{lpA}$ and $XcpT_{17A}$. Whether this fusion was more prone to proteolytic degradation or whether it was recognized less well by the XcpT-specific antiserum due to the loss of epitopes is not known. Functionality of the hybrid proteins was examined on elastin-containing plates where clear halos are visible around colonies of secretion-proficient strains. Like the production wild-type XcpT (results not

shown), the production of the fusions $XcpT_{lpA}$ and $XcpT_{17A}$ restored secretion in PAO1ΔT (Fig. 2B). In contrast, $XcpT_{65A}$ was not functional (Fig. 2B). These results show that the leader peptide and the N-terminal hydrophobic segment of 17 amino acid residues of mature XcpT can be substituted by those of PilA without loss of functionality. However, replacing the first 65 amino acid residues resulted in a non-functional protein.

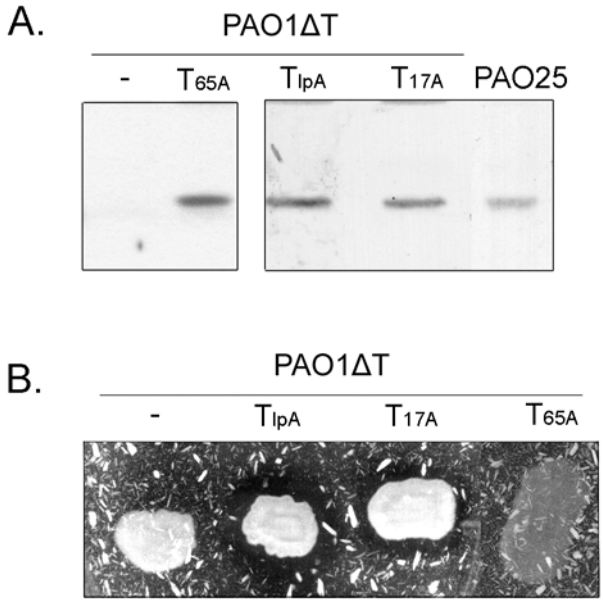

FIG. 2. Production and functionality of PilA-XcpT hybrids. (A) Immunoblot analysis of whole cell lysates of the *P. aeruginosa xcpT* mutant PAO1ΔT containing the empty vector (-), or constructs pYRC-$XcpT_{65A}$ (T_{65A}), pYRC-$XcpT_{lpA}$ (T_{lpA}), or pYRC-$XcpT_{17A}$ (T_{17A}) grown overnight in the presence of IPTG. A whole cell lysate of PAO25 was included as a reference. Immunodetection was performed with XcpT-specific antiserum, peroxidase-conjugated goat anti-rabbit IgG antiserum and chemiluminescence (Pierce). Exposure time for the left panel was 30 min and for the right panel 4 min. (B) The same strains were grown on LB agar containing 1% elastin. Secretion of elastase is visualized by clearance of elastin from the plate around the colonies.

Production of processing-defective variants of the chimeras interferes with secretion. In *P. aeruginosa*, PilA and the pseudopilins are both processed by the same dedicated prepilin peptidase XcpA/PilD (22). We hypothesized that subunits with a defect in processing may specifically

block their cognate pathway, and that blocking of either T2SS-mediated secretion or piliation could be used as an indicator for efficient targeting to either one of the corresponding machineries. Therefore, genes were constructed encoding processing-defective PilA-XcpT chimeras. The variant proteins contain two amino acid residues (leucine and aspartic acid) inserted in between the -1 glycine and the +1 phenylalanine and are indicated in the nomenclature by the addition of #2. Maturation of the wild-type PilA or the original PilA-XcpT hybrids results in the removal of six amino acid residues from the N terminus, which can be visualized as a small mobility shift on acrylamide gels. Expression of the altered proteins in a *P. aeruginosa xcpT* mutant PAO1ΔT resulted in the production of forms with a slightly lower electrophoretic mobility (shown for hybrid $XcpT_{lpA}$ in Fig. 3A), which appeared similar in both wild-type and an *xcpA* mutant strain (data not shown), consistent with a processing defect. As expected, production of the processing-defective hybrids $XcpT_{lpA\#2}$ or $XcpT_{17A\#2}$ did not complement the secretion defect of the *xcpT* mutant (results not shown). Interestingly, upon production of these proteins in wild-type *P. aeruginosa* PAO25 strong interference with secretion was observed (Fig. 3B). This dominant-negative phenotype was even observed when the expression of the hybrid genes was repressed in the absence of IPTG and by the addition of glucose to the growth medium, showing that even small amounts of unprocessed subunit precursors obstructed the secretion process. Production of mutant fusion $XcpT_{65A\#2}$ did not have such a dominant-negative effect on elastase secretion. However, production of this protein could not be detected (results not shown).

To determine whether specific targeting to the Xcp machinery was required to block secretion, a defective processing site was also introduced into the PilA protein. Production of this protein was confirmed in a *pilA* mutant by immunoblotting (results not shown). Based on halo formation on elastin-containing plates, production of $PilA_{\#2}$ somewhat reduced elastase secretion in the wild-type strain PAO25 (results not shown), but not to the same extent as did the $XcpT_{lpA\#2}$ and $XcpT_{17A\#2}$ proteins. Quantification of the elastase activity in culture supernatants with an elastin-Congo red assay showed that production of $PilA_{\#2}$ in strain PAO25 reduced elastase secretion by 40%, while $XcpT_{lpA\#2}$ or $XcpT_{17A\#2}$ reduced elastase secretion by 80-90% (Fig. 4). These results are consistent with the notion that $XcpT_{lpA\#2}$ and $XcpT_{17A\#2}$ are more efficiently targeted to the Xcp secretion machinery than $PilA_{\#2}$.

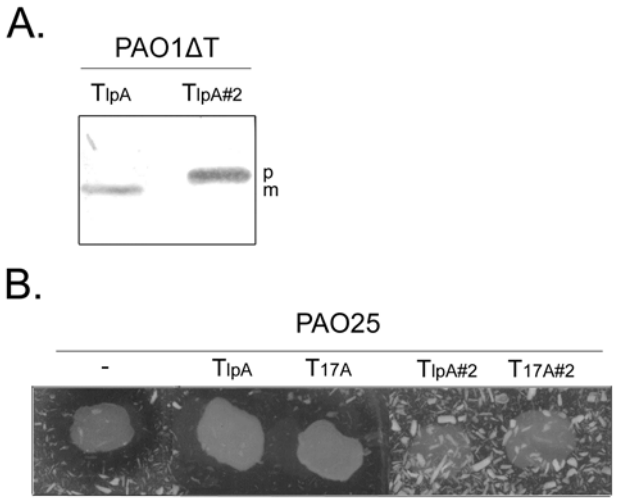

FIG. 3. Negative dominance of processing-defective fusion proteins. (A) Whole cell lysates of the *xcpT* mutant PAO1ΔT producing PilA-XcpT hybrid from pYRC-XcpT$_{lpA}$ (T$_{lpA}$), or its variant with the disrupted processing site from pYRC-XcpT$_{lpA\#2}$ (T$_{lpA\#2}$) were analyzed by SDS-PAGE, followed by Western blotting. Immunodetection was carried out with XcpT-specific antiserum. The positions of mature XcpT and its precursor on the immunoblot are indicated with m and p, respectively. (B) Wild-type *P. aeruginosa* strain PAO25 containing the empty vector (-), or producing PilA-XcpT hybrids from pYRC-XcpT$_{lpA}$ (T$_{lpA}$) and pYRC-XcpT$_{17A}$ (T$_{17A}$), or the variants disrupted for processing from pYRC-XcpT$_{lpA\#2}$ (T$_{lpA\#2}$) and pYRC-XcpT$_{17A\#2}$ (T$_{17A\#2}$) were grown on LB agar containing 1% elastin. Secretion of elastase is visualized by clearance of elastin from the plate around the colonies.

Defective processing of pilin subunits affects piliation. To test whether production of the chimeric proteins and the processing-defective variants, PilA$_{\#2}$, XcpT$_{lpA\#2}$, XcpT$_{17A\#2}$ and XcpT$_{65A\#2}$ affected type IV piliation, *P. aeruginosa* strain PAK producing the different proteins from plasmids was tested for sensitivity to phage PO4. PO4 is a PAK-specific phage, which infects the cells by interacting with the type IV pili and loss of piliation results in phage resistance (36). Production of the PAO1 pilin subunit PilA in PAK does not interfere with PO4 sensitivity (34). Phage-sensitivity was assayed by streaking the phage on a LB agar plate and cross streaking of the bacterial strains. When no IPTG was added to the growth medium, neither the original, nor their processing-defective variants affected phage sensitivity (results not shown). In the presence of IPTG, however,

production of $PilA_{\#2}$ resulted in phage resistance, indicating that the processing-defective pilin interferes with type IV piliation (Fig. 5A).

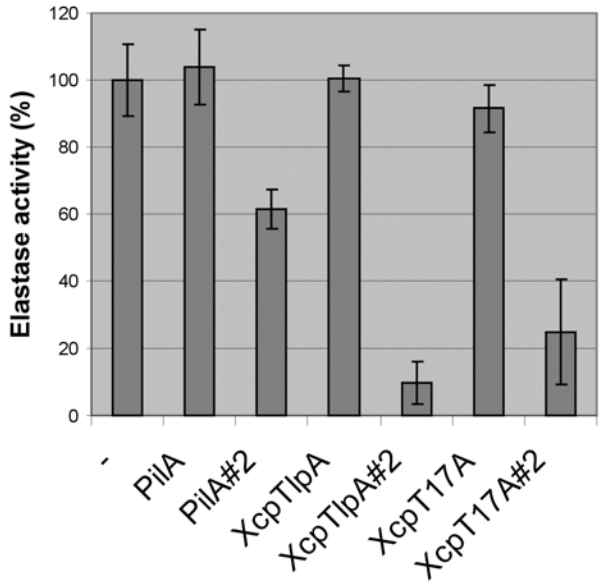

FIG. 4. Quantitative analysis of the extracellular elastase activity. Wild-type *P. aeruginosa* PAO25 producing from the pYRC derivatives PilA, hybrid $XcpT_{lpA}$, hybrid $XcpT_{17A}$, or their non-processed variants ($PilA_{\#2}$, $T_{lpA\#2}$, and $T_{17A\#2}$) from the pYRC-based plasmids were grown overnight in the presence of IPTG. Extracellular elastase activity was determined with the elastin-Congo red assay. The activity of the wild-type strain PAO25 with the empty vector (-) was set at 100%. Bars represent the averages of three independent experiments and standard deviations are indicated.

Immunoblotting showed substantial accumulation of the unprocessed pilin subunit within the cells (Fig. 5B). Phage sensitivity was not affected when processing-defective hybrid proteins were produced (shown for $XcpT_{lpA\#2}$ in Fig. 5A).

DISCUSSION

Since type IV piliation and the T2SS co-exist in *P. aeruginosa*, efficient targeting of the constituents to their cognate apparatus is required. However, from previous studies it is clear that the two systems can interfere when out of balance. For example, when overproduced in the absence of the T2SS, XcpT can be assembled into a pilus-like structure by the Pil system

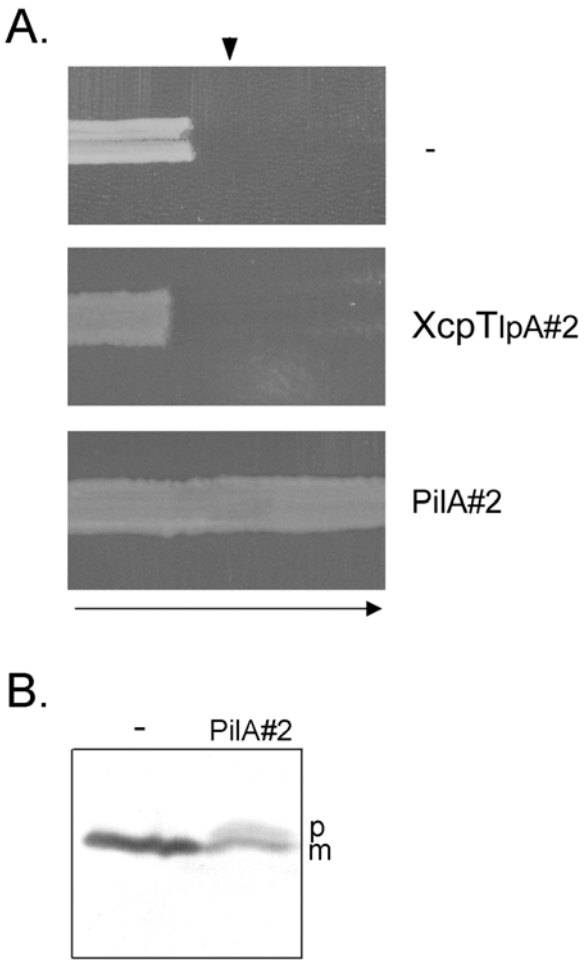

FIG. 5. Phage PO4 sensitivity and production of processing-defective PilA. (A) Wild-type *P. aeruginosa* strain PAK containing the empty vector (-), or producing hybrid $XcpT_{lpA\#2}$ or $PilA_{\#2}$ from the pMMB67HE-derived plasmids were cross-streaked against phage PO4 on LB agar plates containing 1 mM IPTG and grown overnight. The arrowhead indicates the position of the phage suspension. The arrow shows the direction of inoculation of the bacterial strains. Phage sensitivity is visualized by the absence of bacterial growth after contact with the phage suspension. (B) Whole cell lysates of wild-type *P. aeruginosa* containing the empty vector (-) or producing processing-defective PilA from pMMB-$PilA_{\#2}$ (PilA#2) were analyzed by SDS-PAGE, followed by Western blotting. Immunodetection was carried out with PilA-specific antiserum. The positions of mature PilA and its precursor on the immunoblot are indicated with m and p, respectively.

(7). Moreover, Lu *et al.* (19) reported that disruption of the *pilA* gene resulted in a significant delay in protein secretion in *P. aeruginosa*, which may be explained by targeting of pseudopilins to the Pil system when the natural substrate, PilA, is not available. Since cross talk between the two systems does occur, it is conceivable that correct targeting of pilins and pseudopilins to their cognate machineries relies on subtle differences in affinity and on an accurate stoichiometry. The presence of both systems makes *P. aeruginosa* a good model organism to identify regions within (pseudo)pilins that determine recognition by their cognate assembly machinery.

By using chimeric PilA-XcpT proteins, we found that the leader peptide and the N-terminal 17 residues of the mature pseudopilin XcpT could functionally be replaced by the corresponding part of pilin PilA. This result is in agreement with findings of Köhler *et al.* (18) who showed that the leader peptide and the N-terminal 17 amino acid residues of the pseudopilin PulG can be substituted by the corresponding segments of the pilins PpdD or PilE without loss of function. In addition, we observed that production of low amounts of processing-defective variants of the two functional chimeras completely blocked the secretion of elastase by the wild-type *P. aeruginosa* strain. Apparently, these fusions are still efficiently recognized by the Xcp system, indicating that the information needed for targeting of the (pseudo)pilins to the appropriate machinery is contained in another part of the protein. The third chimeric protein, in which the N-terminal 65 amino acid residues of mature XcpT were replaced by those of PilA, was non-functional and its processing-defective variant did not interfere with elastase secretion. Possibly, this protein is not targeted to the Xcp machinery, which would indicate that the segment C-terminally to the hydrophobic domain is important for recruitment by the Xcp machinery. Together with the hydrophobic domain, this segment forms an extended α-helix in PilA, and these long helices of the subunits form the central core of the assembled pilus structure (6). Thus, efficient packing of subunits is dependent on this region, and may be required for targeting as well. Unfortunately, $XcpT_{65A}$ was considerably less well detected than the functional hybrids, which makes it difficult to draw conclusions from the experiments with this chimeric protein.

In wild-type *P. aeruginosa*, low amounts of $XcpT_{lpA\#2}$ and $XcpT_{17A\#2}$ were sufficient to obstruct secretion of elastase. The production of these proteins did not interfere with the processing of PilA and, *vice*

versa, accumulation of unprocessed PilA did not interfere with the processing of XcpT (results not shown). Hence, this phenotype cannot be explained by titration of prepilin peptidase. More likely, incorporation of processing-defective subunits blocks elongation of the pseudopilus. Interestingly, also production of processing-defective PilA in the wild-type strain PAO25 reduced elastase secretion to some extent, suggesting a low affinity of this protein for the Xcp machinery.

Consistent with the negative dominance of processing-defective XcpT variants, production of $PilA_{\#2}$ interfered with the type IV piliation. Such a phenotype was not observed when any of the processing-defective PilA-XcpT hybrids was produced. IPTG induction was necessary to obstruct the Pil system, whereas the T2SS could be blocked even in the absence of IPTG and the presence of glucose. This difference may relate to a higher prevalence of PilA in the cell. An alternative explanation is provided by the fact that the type IV piliation apparatus contains not only an assembly ATPase, PilB, but also two retraction ATPases, PilU and PilT (35, 36). One of these proteins may remove aberrant subunits from the Pil system.

This study shows that the leader peptide and the hydrophobic region of XcpT are not required for the targeting of this pseudopilin to the Xcp system and, thus, that the targeting information is contained within another part of the protein. In addition, production of processing-defective variants of functional PilA-XcpT chimeras specifically obstructed the Xcp secretion system, whereas processing-defective PilA specifically blocked the Pil system. The latter findings can be used as valuable tools for the further identification of the targeting domains in the pseudopilins and pilins.

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Chapter 4

Interaction domains in the *Pseudomonas aeruginosa* type II secretory apparatus component XcpS

Jorik Arts‡, Arjan de Groot‡, Geneviève Ball, Eric Durand, Mohamed El Khattabi, Alain Filloux, Jan Tommassen and Margot Koster

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‡These authors contributed equally to this work.

ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen, which secretes a wide variety of enzymes and toxins into the extracellular medium. Most exoproteins are exported by the type II secretion machinery, the Xcp system, which encompasses 12 different proteins. One of the core components of the Xcp system is the inner membrane protein XcpS, homologues of which can be identified in type II secretion machineries as well as in type IV piliation systems. In this study, XcpS was shown to be stabilized by co-expression of the XcpR and XcpY components of the machinery, demonstrating an interaction between these three proteins. By replacing segments of *P. aeruginosa* XcpS with the corresponding parts of its *Pseudomonas putida* counterpart, XcpS domains were identified that are important for species-specific functioning and thus represent putative interaction domains. The cytoplasmic loop of XcpS was found to be involved in the stabilization by XcpR and XcpY.

INTRODUCTION

The ability to secrete proteins into the extracellular medium is important for the virulence of many plant, animal or human pathogens. In recent years, it has become clear that Gram-negative bacteria use a limited number of secretion mechanisms to secrete a large variety of extracellular proteins (45). One of these mechanisms is the type II secretion system (T2SS), which is widely distributed among Gram-negative bacteria, including pathogens such as *Vibrio cholerae*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Xanthomonas campestris*, *Erwinia chrysanthemi* and *Klebsiella oxytoca* (10). This system allows for the secretion of a range of degradative enzymes, including cellulases, pectinases, proteases, lipases, and toxins, such as aerolysin and cholera toxin.

Type II secretion is a two-step process. First, signal sequence-bearing exoproteins are translocated across the cytoplasmic membrane via the Sec or the Tat machinery (28, 48). After release in the periplasm, unfolded exoproteins adopt their tertiary conformation. Transport of the folded proteins across the outer membrane is the second step and takes place via the so-called secreton. The secreton is assembled from 12-16 different components, which are generically referred to as Gsp (general secretory pathway) proteins. In *P. aeruginosa*, type II secretion requires the products of 12 xcp genes, *xcpA* and *xcpP-Z* (22).

Homologues of several Xcp components are not only present in T2SSs, but also in type IV pilus biogenesis systems (29), in competence systems of Gram-positive bacteria (9), and in flagella and sugar-binding systems of various archaea (4, 36), suggesting a common evolutionary origin of these systems. The XcpTUVWX proteins show N-terminal sequence similarity to the type IV pilus subunit PilA and, therefore, they are designated pseudopilins (7, 44). Consistently, they have been demonstrated to be processed by the dedicated prepilin peptidase PilD/XcpA, which also processes the PilA precursor (35), and XcpT has been shown to assemble into a pilus-like structure upon over-production (16). The ATPase XcpR and the multispanning inner membrane component XcpS show considerable sequence similarity to PilB and PilC, respectively, which are both required for the formation of type IV pili (36). This similarity suggests that XcpR and XcpS may play key roles in the assembly of the pilus-like structure formed by the pseudopilins.

The cytoplasmic protein XcpR was shown to associate to the inner membrane via the N-terminal domain of the bitopic inner membrane component XcpY (2). XcpR contains a conserved Walker A-box motif that was shown to be indispensable for its function (47). Binding of ATP was recently shown to trigger oligomerization of the *X. campestris* XcpR homologue XpsE (43) probably into hexamers (11, 42). Knowledge of the role of XcpS in the secreton and its interactions with other Xcp components is rather limited. Recently, the components XcpRSY were shown to co-purify with his-tagged XcpZ after cross-linking (40), and yeast two-hybrid studies with *E. chrysanthemi* T2SS components revealed interactions of the N terminus of OutF, the XcpS homologue, with OutE, the XcpR homologue, and with the cytoplasmic segment of OutL, the XcpY homologue (39).

Here, we show that XcpS is highly unstable in the absence of other Xcp components, a characteristic that was used to establish interactions between this central component of the secreton and other Xcp proteins. In addition, hybrid proteins composed of *P. aeruginosa* XcpS and *Pseudomonas putida* XcpS were used to identify possible interaction domains.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains used in this study are listed in Table 1. *P. aeruginosa* and *Escherichia coli* strains were grown at 37°C in a modified Luria-Bertani (LB) broth (46). For plasmid maintenance, the following antibiotics were used: for *E. coli* ampicillin 50 μg/ml, kanamycin 25 μg/ml, tetracycline 15 μg/ml, and gentamicin 15 μg/ml; for *P. aeruginosa* gentamicin 40 μg/ml, carbenicillin 300 μg/ml. To induce the expression of genes cloned behind the *lac* or *tac* promoter, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM.

Plasmids and DNA manipulations. Plasmids used in this study are listed in Table 2. Recombinant DNA methods were performed essentially as described (41) using *E. coli* strain DH5α for routine cloning. Plasmids were introduced by the $CaCl_2$ procedure into *E. coli* (41) or by electroporation into *E. coli* and *P. aeruginosa* (18). PCRs were performed with the proof-reading enzyme *Pwo* DNA polymerase (Roche) and PCR products were cloned into pCRII-TOPO or pCR2.1-TOPO according to the manufacturer's

TABLE 1. Strains used in this study

Strain	Relevant characteristics	Source or reference
E. coli		
DH5α	*thi-1 hsdR17 gyrA96 recA1 endA1 glnV44 relA1 phoA8 phi80dlacZdelM15* λ^-	(27)
BL21(DE3)	F^- *ompT* $hsdS_B(r_B^-m_B^-)$ *dcm gal*	Novagen
P. aeruginosa		
PAO25	PAO1 *leu arg*	(26)
PAO1ΔP	PAO1Δ*xcpP*	(6)
PAN1	PAO25Δ*xcpQ*	(5)
PAN11	PAO25Δ*xcpR*Δ*lasB*	(8)
PAO1ΔS	PAO1Δ*xcpS*	This study
PAO1ΔT	PAO1Δ*xcpT*	(3)
PAOΔU	PAO1Δ*xcpU*	This study
PAOΔV	PAO1Δ*xcpV*	This study
PAOΔW	PAO1Δ*xcpW*	This study
PAO1ΔX	PAO1Δ*xcpX*	(7)
KS910	*xcpY51* mutant from PAO503	(49)
KS902	*xcpZ5* mutant from PAO503	(49)
DZQ40	PAO1Δ*xcpP-Z*	(2)

protocol. The oligonucleotides used are associated as an online supplement. With pAX24 as template, the oligonucleotide Osup, which primes upstream of the *xcpS* gene sequence, and either one of the oligonucleotides OscI, OspI, OscII, and OspII, which prime at different positions within the *xcpS* gene sequence, were used to PCR amplify 3'-truncated *xcpS* genes. These oligonucleotides were designed in such a way that a SmaI site was introduced at the 3' end of the PCR product. Each DNA fragment was subsequently cloned into pCR2.1-TOPO yielding the pOScI, pOSpI, pOScII and pOSpII plasmids, respectively. These plasmids were linearized by SmaI-XbaI digestion. The 2.6-kb *phoA* gene cassette obtained by SmaI-XbaI digestion from pPHO7 was cloned into the linearized plasmids yielding the pOScIPA, pOSpIPA, pOScIIPA and pOSpIIPA plasmids, which encode 'PhoA fused at positions E114, G216, K310, and V404, respectively, of the XcpS protein. The *lacI* gene was PCR amplified with plasmid pET16b as a template using the primers PB7 and PB8, thereby introducing an NcoI restriction site downstream of the stop codon. The PCR product was cloned into the HincII site of pBC18R, which resulted in construct pCR-LacI and subsequently the SphI-NcoI fragment was introduced into the pBBR1-MCS5 vector, resulting in pYRC. With the oligonucleotides JAXcpS01for and JAXcpS02rev *xcpS* was amplified from

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristic*	Source or reference
pCR2.1-TOPO	Ap^r; Km^r; TOPO TA cloning vector	Invitrogen
pPHO7	Ap^r; *phoA* without ss-encoding part	(25)
pOScIPA	pCR2.1; *xcpSE114-'phoA*	This study
pOSpIPA	pCR2.1; *xcpSG216-'phoA*	This study
pOScIIPA	pCR2.1; *xcpSK310-'phoA*	This study
pOSpIIPA	pCR2.1; *xcpSV404-'phoA*	This study
pMMB67HE, EH	Ap^r; cloning vector; P_{tac}	(23)
pMMB67HE-S	pMMB67HE; *xcpS*	This study
pCRII-TOPO	Ap^r; Km^r; TOPO TA cloning vector	Invitrogen
pCRII-S, -RS, -RZ	pCRII-TOPO; *xcpS*, *xcpRS*, *or xcpR-Z*	This study
pUC19	Ap^r; cloning vector	(50)
pUAWE6	pUC19; *xcpRSTUVW*	(12)
pAX24	*xcpP-Z* cluster in pLAFR3	(21)
pMPM-T4Ω	Tet^r; cloning vector; P_{araBAD}	(32)
pMPM-T4S_{xx}	pMPM-T4Ω; *xcpS*	This study
pMPM-K4Ω	Km^r; cloning vector; P_{araBAD}	(32)
pMPM-K4S1	pMPM-K4Ω; *xcpS*; *Nco*I site removed	This study
pBBR1-MCS5	Gm^r; cloning vector	(31)
pBC18R	Ap^r; *lacI*	Novagen
pCR-lacI	pBC18R; *lacI*	This study
pYRC	pBBR1-MCS5; *lacI*	This study
pYRC-R, -YZ, -RY, -RYZ	pYRC; *xcpR*, *xcpYZ*, *xcpRY*, or *xcpRYZ*	This study
pBluescript II SK(-)	Ap^r; cloning vector	Stratagene
pMEK45	pBluescript SK(-); truncated *xcpSI165*	This study
pET16b	Ap^r; expression vector; T7 promoter	Novagen
pMEK49	pET16b; encoding His_6-XcpSI165	This study
pEMBL18, 19	Ap^r; cloning vector	(15)
pAG102	pMMB67HE; *P. putida xcpRS*	(14)
pAG55	pEMBL19; *P. putida xcpS*	This study
pAG403	pEMBL18; *P. aeruginosa xcpS*	This study
pESH5, -6	pEMBL19; *xcpS* hybrid gene 5, or 6	This study
pESH7, -104, -106, -108, -109, -110	pEMBL18; *xcpS* hybrid gene 7 , 104, 106, 108, 109, or 110	This study
pMSP31	pMMB67EH; *P. putida xcpS*	This study
pMSA21	pMMB67HE; *P. aeruginosa xcpS*	This study

pMSH-6, -104, -106, -108, -109, -110	pMMB67EH; *xcpS* hybrid gene 6, 104, 106, 108, 109, or 110	This study
pMPM-6, -104, -106, -108, -109, -110	pMPM-K4Ω; *xcpS* hybrid gene 6, 104, 106, 108, 109, or 110	This study
pKNG101	Sm^r; *oriR6K sacBR mobRK2*	(30)
pKN-ΔU	pKNG101; *xcpT'V'*	This study
pKN-ΔV	pKNG101; *xcpU'W'*	This study
pKN-ΔW	pKNG101; *xcpV'X'*	This study
pRK2013	Km^r; *ori* ColE1, Tra^+ Mob^+	(19)

*Ap, ampicillin; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Tet, tetracycline; ss, signal sequence. The *xcpS* hybrid 5 contains the first 626 bp of *P. aeruginosa xcpS*, and the last 585 bp of *P. putida xcpS*; hybrid 7 contains the first 626 bp and the last 132 bp of *P. aeruginosa xcpS* and 485 bp of *P. putida xcpS*. The proteins encoded by the other *xcpS* hybrids are schematically depicted in Fig. 3.

pAX24 as template and cloned into pCRII-TOPO resulting in construct pCRII-S. The XbaI-EcoRI insert was subsequently ligated into XbaI-EcoRI-digested pMMB67HE resulting in pMMB67HE-S. With the oligonucleotides JAXcpR01for and JAXcpR02rev *xcpR* was amplified from pAX24 as template and cloned into pCRII-TOPO resulting in construct pCRII-R. The HindIII-XbaI, and the XbaI-EcoRI inserts from pCRII-S and pCRII-R were ligated together in HindIII-EcoRI-digested pRCII-TOPO yielding pCRII-RS. With oligonucleotides JAXcpY02for and JAXcpZ02rev a DNA fragment containing the genes *xcpYZ* was amplified from pAX24 as template and the product was cloned into pCRII-TOPO resulting in pCRII-YZ. The EcoRI-XbaI product of pCRII-YZ was ligated into EcoRI-XbaI-digested pUC19, resulting in pUC-YZ. The HindIII-EcoRI product of pCRII-RS and the EcoRI-XbaI product of pUC-YZ were ligated into HindIII-XbaI-digested pUC19, which resulted in pUC-RSYZ. The HindIII-EcoRI product of pCRII-RS was inserted in HindIII-EcoRI-digested pUC19, resulting in pUC-RS. Ligation of the 3.5-kb BamHI-EcoRI and the 2.1-kb EcoRI-NotI product of pAX24 into BamHI-NotI-digested pUC-RSYZ resulted in pUC-RZ. The HindIII-XbaI insert of pUC-RZ was introduced in HindIII-XbaI-digested pCRII-TOPO, resulting in pCRII-RZ. The *xcpS* gene was transferred as an XbaI-XhoI fragment from pCRII-S to XbaI-XhoI-digested pMPM-T4Ω, which resulted in pMPM-T4S_{xx}. To remove the additional start codon generated by the NcoI site, this construct was digested with NcoI and incubated with T4 polymerase. Removal of the NcoI-site was confirmed by restriction analysis. The BamHI-PstI fragment of this construct was introduced into BamHI-PstI-digested pMPM-K4Ω resulting in

pMPM-K4S1. Construct pYRC-R contains *xcpR* from pCRII-R inserted as a HindIII-XbaI fragment into pYRC. Construct pYRC-YZ contains *xcpYZ* from pCRII-YZ inserted as an EcoRI-XbaI fragment into pYRC. Introduction of *xcpR* as a HindIII-EcoRI fragment from pCRII-R into HindIII-EcoRI-digested pYRC-YZ resulted in pYRC-RYZ. The genes *xcpRY* were subsequently introduced into pYRC as a HindIII-PstI fragment from pYRC-RYZ, resulting in pYRC-RY. Constructs pMSA21 and pAG403 contain *P. aeruginosa xcpS* as a 2.0-kb SalI-XhoI fragment from pAX24 inserted in pMMB67HE and pEMBL18, respectively. With the oligonucleotides MK01 and MK02, the first 495 bp of *xcpS* was amplified from pAG403 and a stop codon was generated. The resulting PCR fragment was introduced into SmaI-digested pBluescript SK(-) yielding pMEK45. Construct pMEK45 was digested with BamHI and the fragment with truncated *xcpS* was introduced into BamHI-digested pET16b, resulting in pMEK49. Constructs pMSP31 and pAG55 contain *P. putida xcpS* as a 2.3-kb SphI fragment from pAG102 inserted in pMMB67EH or pEMBL19, respectively. The hybrid on pESH5 was constructed in two steps. First, a PCR fragment was obtained containing the last 585 bp of *P. putida xcpS* with the use of oligonucleotides PPCS and the pUC reverse primer with pAG55 as template, and the HincII-HindIII-digested PCR product was cloned in HincII-HindIII-digested pEMBL19. The resulting construct was linearized with HincII, and a 1.2-kb HincII fragment from pAG403, containing the first 626 bp of *P. aeruginosa xcpS* was inserted in the correct orientation. The hybrid on pESH6 was obtained by replacing the 1.1-kb AsuII fragment of pESH5 by the corresponding fragment of pAG403. Similarly, pESH7 was obtained by replacing the 1.1-kb AsuII fragment of pAG403 by the corresponding fragment of pESH5. A HindIII-BamHI-digested PCR fragment obtained with template pAG403 and oligonucleotides AGA1 and AGA3, and a BamHI-EcoRI-digested PCR fragment, obtained using oligonucleotides AGP1 and AGA2 with pESH7 as template, were cloned together in pEMBL18 resulting in pESH110. To construct pESH106, PCR was performed using oligonucleotides PPNS2 and AGP8 with pAG55 as template to amplify the first 393 bp of *P. putida xcpS*. This product was used in a second PCR containing further *P. aeruginosa xcpS* on pAG403 as a template and oligonucleotides AGA9 and PPNS2. Finally, the product of the second PCR was digested with HindIII and SphI and cloned in HindIII-SphI-digested pAG403. The hybrid genes on pESH108, pESH104, and pESH109 were constructed using a 3-step PCR

protocol as described before (24). Plasmids pAG55 and pAG403 were used as templates for *P. putida* and *P. aeruginosa* DNA, respectively. Final products were obtained using oligonucleotides AGA7 and AGA2, digested with BamHI and EcoRI and cloned into pEMBL18. Specific oligonucleotides were as follows: AGP2, AGP7, AGP2B and AGP7B for pESH108; AGP6, AGP3, AGP6B and AGP3B for pESH104; AGP4, AGP5, AGP4B and AGP5B for pESH109. All fusions were verified by nucleotide sequencing. In the pESH-series of plasmids, the *xcpS* gene fusions are cloned on pEMBL18 or -19 in the orientation opposite to the *lac* promoter. The fusions were recloned in the proper orientation behind the *tac* promoter into pMMB67EH resulting in pMSH-6, or into pMMB67HE resulting in pMSH-104, -106, -108, -109, and –110. The hybrid gene 110 was PCR amplified with the oligonucleotides JAXcpS01for and JAXcpS02rev, the genes 104, 108 and 109 with the oligonucleotides JAXcpS03for and JAXcpS02rev with the pMSH plasmids as template DNA. The resulting products were ligated into pCRII-TOPO. The inserts were subsequently recloned as HindIII-EcoRI fragments into HindIII-EcoRI-digested pMPM-K4S1. The hybrid gene 6 was cloned as a HindIII-EcoRI fragment from pMSH-6 into pUC19. Hereafter, hybrid 6 was introduced as PvuII-HindIII fragment in PvuII-HindIII-digested pMPM-K4S1. The resulting constructs were named pMPM-6, -104, -106, -108, -109, and -110.

Construction of chromosomal *P. aeruginosa xcp* mutants. The mutants PAOΔU (Δ*xcpU*), PAOΔV (Δ*xcpV*) and PAOΔW (Δ*xcpW*) were constructed using the approach previously described (16, 17). Briefly, 500-bp DNA fragments upstream and downstream the target genes were PCR amplified. The oligonucleotides were designed for amplifying fragments with overlapping 3’ and 5’ ends. For the *xcpU* deletion, the upstream fragment was obtained with the oligonucleotides XcpT'5 and XcpT'6, and the downstream region with XcpV’3 and XcpV’4. For the *xcpV* deletion, the upstream fragment was obtained with the couple XcpU’6 and XcpU’7, and the downstream region with XcpW’3 and XcpW’4. For the *xcpW* deletion, the upstream fragment was obtained with the couple XcpV’5 and XcpV’6, and the downstream region with XcpX’3 and XcpX’4. In each case, the two fragments obtained were fused by performing an overlap PCR: the two fragments were mixed, melted and annealed with the most upstream and downstream primers to perform a second PCR. The resulting PCR product was cloned into pCR2.1-TOPO. A 1.000-bp BamHI-ApaI DNA fragment was then subcloned into the suicide vector pKNG101. The resulting

constructs were transferred to *P. aeruginosa* by mobilization with pRK2013. The strains in which the chromosomal integration event occurred were selected on *Pseudomonas* isolation agar plates containing 2 mg/ml streptomycin. Excision of the plasmid, resulting in the deletion of the chromosomal target gene, was performed after selection on LB plates containing 5 % (wt/vol) sucrose. Clones that became sucrose resistant and streptomycin sensitive were confirmed to contain the gene deletion by PCR analysis. For the construction of PAO1ΔS, an internal 1.1-kb AsuII fragment in *xcpS* on plasmid pUAWE6 was deleted. The gene with the deletion was cloned into the suicide vector pKNG101. The pKNG101 derivative was introduced in PAO1, and an *xcpS* deletion mutant resulting from double crossover was obtained as previously described (14).

Enzyme assays. Alkaline phosphatase activity was assayed by growth of strains on LB agar plates containing 0.4 mg/ml 5-bromo-4-chloro-3-indolylphosphate (XP). Secretion of elastase was analyzed qualitatively on LB agar plates with a top layer containing 1% elastin (Sigma). After overnight growth, the plates were screened for the presence of haloes around the colonies. For quantitative measurements, the colorimetric elastin-Congo red assay (34) was used. Briefly, 250 µl of culture supernatant of cells grown overnight in the presence of IPTG were incubated for 2 h at 37°C with 10 mg/ml elastin-Congo red (Sigma) dissolved in assay buffer (0.045 M Tris/HCl, 1.5 mM $CaCl_2$, pH 7.2), the reaction was stopped by the addition of 500 µl of 0.7 M NaH_2PO_4, pH 6.0. After centrifugation, absorbance in the supernatant was measured at 495 nm.

SDS-PAGE and immunoblot analysis. Bacterial cells were suspended in SDS-PAGE sample buffer (2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue, 0.1 M Tris/HCl, pH 6.8). Whole cell lysates were heated for 10 min at 95°C and proteins were separated on gels containing 10 % acrylamide. Proteins were transferred to nitrocellulose membranes by semi-dry electroblotting for immunodetection. The primary antiserum directed against XcpS was used at a 1:1000 dilution. Alkaline phosphatase-conjugated goat anti-rabbit IgG antiserum (Biosource international) was used as secondary antibody, unless otherwise indicated. Alkaline phosphatase-conjugated antibodies were detected by staining with XP and nitroblue tetrazolium. When peroxidase-conjugated goat anti-rabbit IgG antiserum (Biosource international) was used, detection was carried out with chemiluminescense (Pierce). The XcpS antiserum was raised in a rabbit against His-tagged XcpS produced in BL21(DE3) from pMEK49.

Briefly, BL21(DE3) carrying pMEK49 was grown overnight, cells were harvested by centrifugation, resuspended in TEN buffer (100 mM NaCl, 1 mM EDTA, and 50 mM Tris/HCl, pH 8.0), and sonicated. The lysate was centrifuged for 30 min at 10,000 g at 4 ºC and the pelleted inclusion bodies were resuspended in 8 M urea in TEN buffer. After centrifugation for 15 min at 3,000 g, the solubilized His-tagged XcpS was purified with Ni-NTA beads (Qiagen) according to the manufacturer's protocol.

Bioinformatic predictions. For bioinformatic predictions, the TopPred program (bioweb.pasteur.fr/seqanal/interfaces/toppred.html) was used.

RESULTS

Topology of the XcpS protein. Bioinformatic predictions suggested that XcpS contains three transmembrane segments, which separate a large cytoplasmic N-terminal domain (residues 1-173), a short periplasmic loop (residues 193-220), a large cytoplasmic loop (residues 240-376), and a short periplasmic C-terminal domain (residues 396-405) (Fig. 1). We constructed *xcpS-phoA* hybrid genes to experimentally test this topology. The leaderless *phoA* gene was fused to the 3'-end of *xcpS* fragments truncated at positions corresponding to residues E114, G216, K310, and V404 in the XcpS protein, yielding the *xcpSE114-'phoA*, *xcpSG216-'phoA*, *xcpSK310-'phoA*, and *xcpSV404-'phoA* gene fusions (Fig. 1). The recombinant plasmids were introduced into *E. coli* DH5α and the strains were plated on LB agar containing XP to probe alkaline phosphatase activity. Colonies of cells carrying the plasmids encoding XcpSG216-'PhoA and XcpSV404-'PhoA were blue on these plates, whereas those of cells producing XcpSE114-'PhoA and XcpSK310-'PhoA remained white. This observation located the G216 and V404 residues on the periplasmic side of the cytoplasmic membrane, and residues E114 and K310 in the cytoplasm, in agreement with the predicted topology (Fig. 1).

XcpS is stabilized by other Xcp components. When pMMB67HE-S carrying the *xcpS* gene under control of the *tac* promoter was introduced in a *P. aeruginosa xcpS* mutant (PAO1ΔS), the secretion defect was complemented (results not shown) and production of XcpS was readily detectable by immunoblot analysis (Fig. 2A). However, when the plasmid

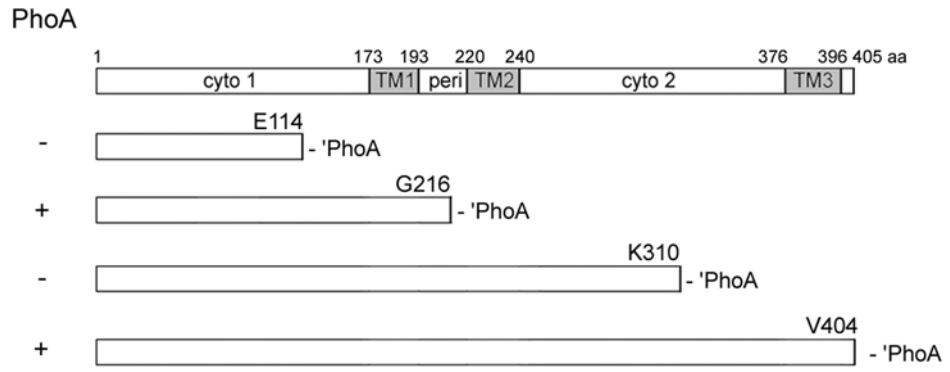

FIG. 1. Topology of the XcpS protein. A topology model is shown at the top. Transmembrane segments (TM) are indicated in grey, and the cytoplasmic (cyto) and periplasmic (peri) domains in white. To verify the topology, plasmids containing various *xcpS-phoA* fusions (fusion sites are indicated) were introduced into *E. coli* DH5α and activity of the periplasmic marker alkaline phosphatase (PhoA) was assayed by growth on LB agar containing XP. +, blue colouring of colonies; -, absence of blue colouring.

was introduced in strain DZQ40, which lacks the entire *xcp* gene cluster, XcpS was not detectable (Fig. 2A), which indicates that the protein is unstable in the absence of other Xcp components. To identify the Xcp protein(s) involved in XcpS stabilization, the levels of XcpS were determined in various non-polar *xcp* mutants. Remarkably, similar amounts of XcpS as in the wild-type strain were detected in all mutant strains, except that the *xcpQ* mutant produced reproducibly more XcpS than the other strains (Fig. 2B). The latter phenomenon was not further investigated. The observation that XcpS was not detected in the DZQ40 strain when expressed from a plasmid, but was present in all single mutants suggested that more than one component can interact with and stabilize XcpS.

XcpS is stabilized by XcpRY. XcpS production was also studied in the heterologous host *E. coli*. XcpS was detectable in cells with the *xcpS* gene on the high copy-number construct pCRII-S (Fig. 2C). However, the levels largely increased when the protein was produced from plasmid pCRII-RZ, which contains the entire *xcpR-Z* operon (Fig. 2C). Although other interpretations are possible, this result is consistent with the idea that production of other Xcp proteins can stabilize XcpS. An interaction between

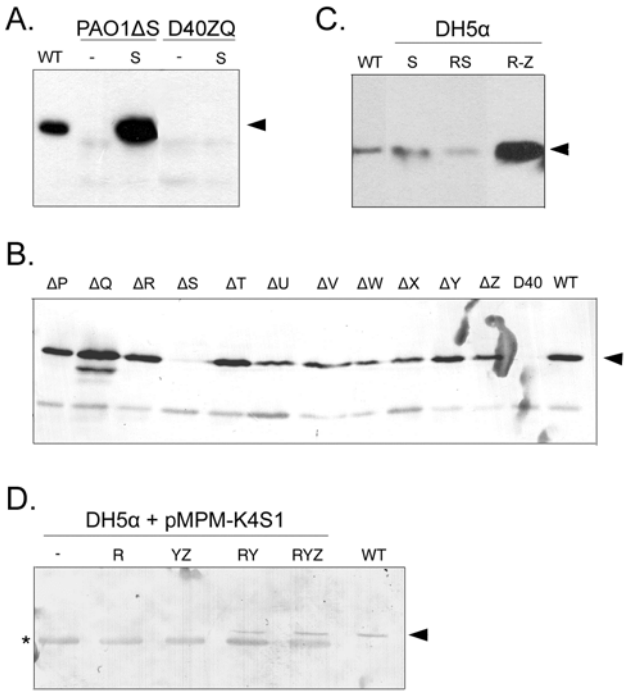

FIG. 2. XcpS production in the presence or absence of other Xcp components. Whole cell lysates were analysed by SDS-PAGE, followed by Western blotting using anti-XcpS antiserum. Immunoanalysis was performed as described in Methods (B and D) or with peroxidase-conjugated goat anti-rabbit IgG antiserum and chemiluminescense (A and C). PAO25 (WT) was included in all panels as reference and the position of XcpS is indicated with an arrow. (A) *P. aeruginosa xcpS* mutant PAO1ΔS and strain DZQ40, which lacks the entire *xcpP-Z* operon, either expressing XcpS from pMMB67HE-S (S) or containing the empty vector (-). Cells were grown overnight with IPTG to induce the *tac* promoter. (B) Chromosomal expression of XcpS in the wild-type strain PAO25 (WT), the DZQ40 (D40) mutant lacking *xcpP-Z* and different non-polar *xcp* mutants (indicated with the last letter of the gene designation) of *P. aeruginosa.* (C) *E. coli* DH5α with the constructs pCRII-S (S), pCRII-RS (RS), or pCRII-RZ (RZ). Overnight cultures were diluted to an optical density of 0.1 at 600 nm, grown for 1 h and subsequently induced for 2.5 h by the addition IPTG. (D) *E. coli* DH5α carrying plasmid pMPM-K4S1 combined with the empty vector pYRC (-), pYRC-R (R), pYRC-YZ, pYRC-RY (RY), or pYRC-RYZ (RYZ). A prominent cross-reacting protein in *E. coli* is indicated with an asterisk (*).

OutF and OutE, the *E. chrysanthemi* homologues of XcpS and XcpR, respectively, has been established by the yeast two-hybrid system (39). Production of XcpR together with XcpS from construct pCR-RS, however, did not increase XcpS levels compared to its production from pCRII-S (Fig. 2C). Other good candidates for an interaction with XcpS are the inner membrane components XcpY and XcpZ. To study if these proteins are important for XcpS stabilization, an experiment was performed in *E. coli* with *xcpS* under the control of an arabinose-inducible promoter on the high copy-number plasmid pMPM-K4S1 in combination with a second plasmid encoding XcpR, XcpYZ, XcpRY, or XcpRYZ. When cells were grown with arabinose, considerable amounts of XcpS were already detected in the strain carrying only pMPM-K4S1 (data not shown). However, at low expression levels, *i.e.* in the absence of inducer, XcpS was barely detectable in this strain (Fig. 2D). Co-production of XcpR or XcpYZ did not alter XcpS levels, but the presence of constructs pYRC-RY or pYRC-RYZ containing the *xcpRY* and *xcpRYZ* genes, respectively, considerably increased the quantity of XcpS. The construct with the *xcpRYZ* genes was slightly more effective in increasing the XcpS levels than the one carrying only the *xcpRY* genes. Production of XcpY and/or XcpZ from pYRC-RY, pYRC-YZ and pYRC-RYZ was confirmed by immunoblotting (results not shown). The amount of XcpR could not be determined because no antiserum was available. It can be concluded from these experiments that XcpR and XcpY together can, at least partially, stabilize XcpS.

Analysis of *P. aeruginosa* - *P. putida* XcpS chimeras. The XcpS proteins of *P. aeruginosa* and *P. putida* are similar in size and share 44% amino acid sequence identity. In contrast to a plasmid carrying *P.aeruginosa xcpS* (pMSA21), introduction of a construct carrying the *xcpS* gene of *P. putida* (pMSP31) into the *P. aeruginosa xcpS* mutant did not restore elastase secretion (see below), presumably because the heterologous XcpS does not properly interact with other components of the Xcp machinery. To identify putative interaction domains in XcpS, a series of chimeric genes was constructed in which various parts of *P. aeruginosa xcpS* were replaced by the corresponding parts of *P. putida xcpS*. A schematic representation of the proteins encoded by the hybrid genes is depicted in Fig. 3A. Production of all hybrid proteins, except for hybrid 106, could be confirmed by immunoblotting (Fig. 3B). The XcpS antiserum used was raised against the N-terminal cytoplasmic region of *P. aeruginosa* XcpS

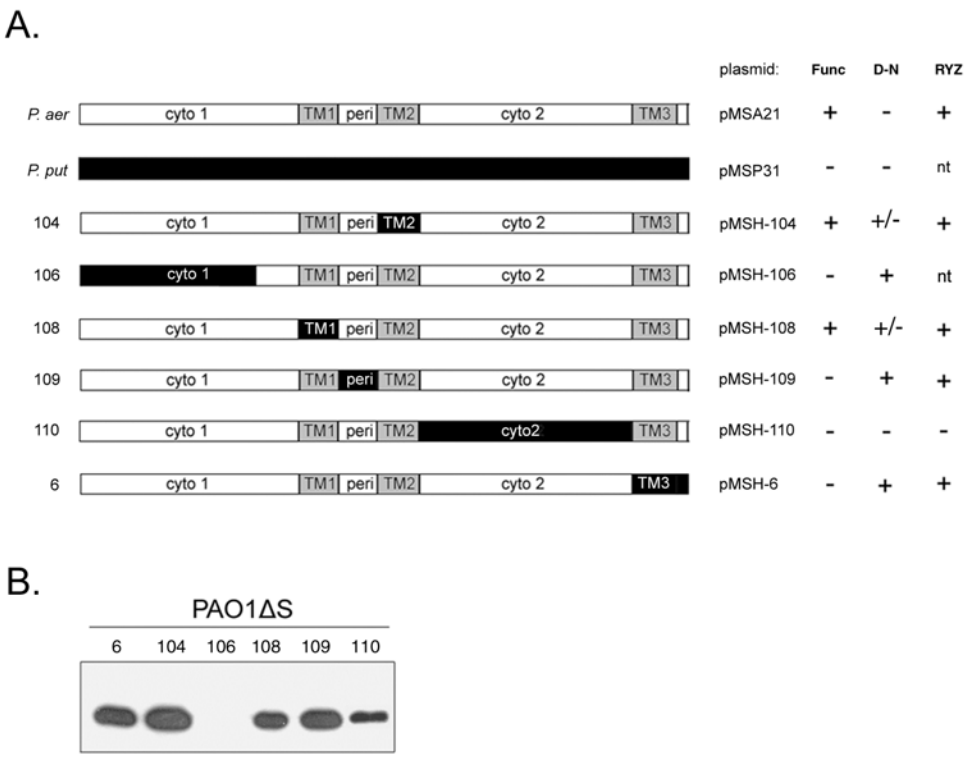

FIG. 3. *P. aeruginosa / P. putida* XcpS hybrids. (A) Schematic representation of XcpS hybrids. *P. aeruginosa* sequences are depicted in white or in gray for transmembrane segments, *P. putida* sequences in black. Transmembrane segments (TM), and the cytoplasmic (cyto) or periplasmic (peri) domains are indicated. The nomenclature of the hybrid proteins is shown on the left, and of plasmids encoding the proteins on the right. Functionality of the hybrids (func) is based on their ability to complement the defect in elastase secretion of PAO1ΔS. +, complementation; -, no complementation. The dominant-negative effect (D-N) of the hybrids is based on their effect on elastase secretion in the wild-type strain. –, no effect on secretion; +, strong inhibition of secretion; +/-, partial inhibition of secretion. The column RYZ indicates the stabilization of the hybrids by XcpRYZ in *E. coli.* +, stabilization; -, no stabilization; nt, not tested. (B) Production of XcpS hybrids in the *P. aeruginosa xcpS* mutant. Whole cell lysates of *P. aeruginosa xcpS* mutant PAO1ΔS expressing *xcpS* hybrids from pMSH-6 (6), pMSH-104 (104), pMSH-106 (106), pMSH-108 (108), pMSH-109 (109), or pMSH-110 (110) were analysed by SDS-PAGE, followed by Western blotting. Immunodetection was carried out using XcpS-specific antiserum, peroxidase-conjugated goat anti-rabbit IgG antiserum and chemiluminescense. Cells were grown overnight in the presence of IPTG.

and did not cross-react with *P. putida* XcpS. Hybrid 106 contains the N-terminal domain of *P. putida xcpS* (Fig. 3A), which explains the lack of detection of the corresponding protein on the immunoblot (Fig. 3B). However, the presence of plasmid pMSH-106 in the wild-type strain affected secretion (see below), showing that also this chimeric protein was produced.

The functionality of the XcpS hybrids could not be tested in *P. putida,* since the substrates of its Xcp machinery have not been characterized (13, 14). In *P. aeruginosa*, the hybrids were tested for their ability to complement the defect of elastase secretion in an *xcpS* mutant, using elastin-Congo red as a substrate for the enzyme. Hybrids 104 and 108, in which the second and the first transmembrane segment, respectively, of XcpS is substituted, were functional in secretion, although they appeared less efficient than wild-type *P. aeruginosa* XcpS (Fig. 4A). All other hybrids were found to be non-functional. The fusions were also produced in wild-type *P. aeruginosa* PAO25 to detect any dominant-negative effect on secretion. Fusions 6, 106 and 109, in which the third transmembrane domain, the N-terminal cytoplasmic domain and the small periplasmic loop, respectively, are exchanged, all strongly inhibited secretion. Hence, these non-functional fusions were still capable of interacting with other Xcp components, although they do not assemble into a functional apparatus. The non-functional chimeric protein 110, which contains the large cytoplasmic loop of *P. putida* XcpS, did not have such a negative effect on secretion, indicating that this protein is not able to engage stably into an interaction with other Xcp components. Finally, production of the functional hybrids 104 and 108 in wild-type *P. aeruginosa* reduced secretion to some extent, suggesting that they were somewhat less functional compared with wild-type XcpS (Fig. 4B).

The cytoplasmic loop of XcpS is involved in the stabilization by XcpRY. The data presented above suggested that the cytoplasmic loop of XcpS is important for interaction with other Xcp components. To determine whether this part of the protein is required for the stabilization of XcpS by the XcpRYZ proteins, the hybrid gene 110 was cloned into pMPM-K4Ω, resulting in pMPM-110. This construct was introduced into *E. coli* and stabilization was studied by production of XcpRYZ *in trans*. The protein was detectable on immunoblots when the cells were grown with L-arabinose (data not shown). However, at low expression levels in the absence of L-

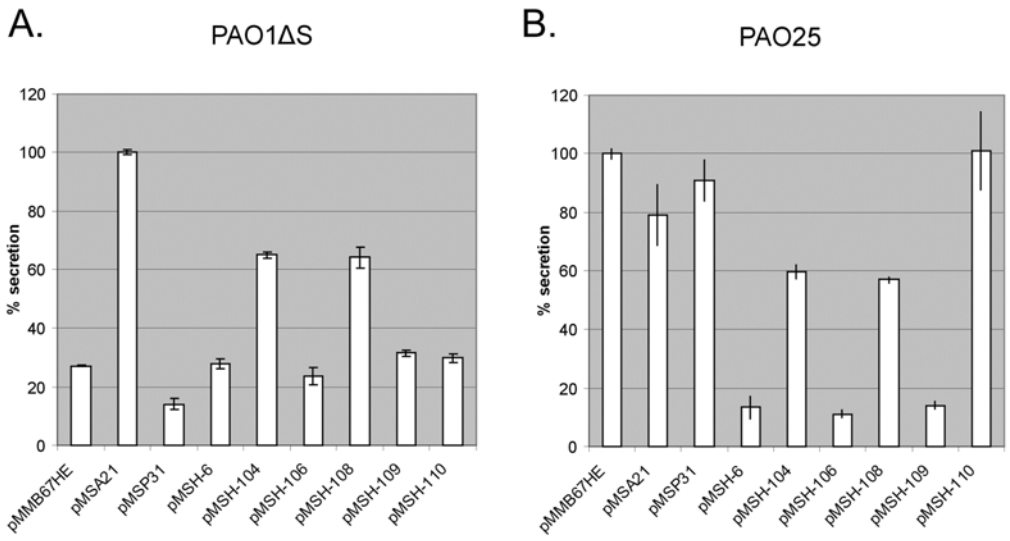

FIG. 4. Extracellular elastase activity of *P. aeruginosa* strains expressing the XcpS hybrids or containing the empty vector. Elastase activity was determined with the elastin-Congo red assay. Bars represent the average of three independent experiments and standard deviations are indicated. Expression of the hybrids was induced by the addition of IPTG. Elastase activity is presented as percentage of the activity measured in PAO1ΔS complemented with wild-type XcpS (A), or in PAO25 containing the empty vector (B).

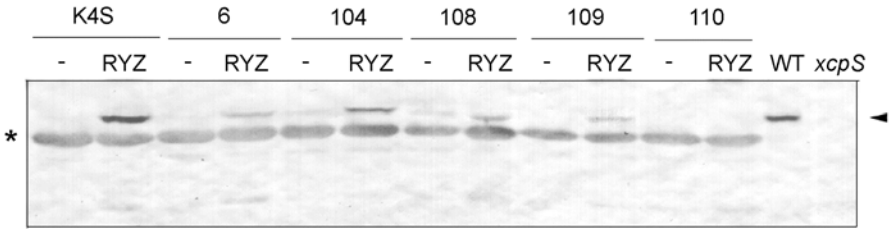

FIG. 5. Effect of production of XcpR, XcpY and XcpZ *in trans* on the stability of XcpS hybrids in *E. coli*. Whole cell lysates of *E. coli* containing plasmid pYRC-RYZ (RYZ), or the empty vector pYRC (-) combined with pMPM-K4S1 (K4S), pMPM-6 (6), pMPM-104 (104), pMPM-108 (108), pMPM-109 (109), or pMPM-110 (110) were analysed by SDS-PAGE, followed by Western blotting. Immunodetection was carried out using XcpS-specific antiserum. PAO25 (WT) and PAO1ΔS (*xcpS)* were included as references. The position of XcpS is indicated with an arrow, and a prominent cross-reacting protein with an asterisk (*).

arabinose, this chimeric XcpS was not detectable and the amounts of the protein did not increase to detectable levels upon co-production of XcpRYZ (Fig. 5). In contrast, all other chimeric proteins were stabilized by co-production of XcpRYZ (Fig. 5). Hence, the cytoplasmic domain of XcpS, between residues 240 and 376 appears to be important for interaction with XcpRYZ.

DISCUSSION

The T2SS is a complex apparatus that spans the cell envelope and is composed of up to 16 different proteins (20). The mode of action of these machines is still largely enigmatic. Insight in the interactions between the constituents of the secreton might provide important clues on the assembly of the apparatus. In this study, we used the instability of XcpS to identify interactions with other Xcp components. This approach has been successfully applied before to show association between XcpY and XcpZ (33), XcpY and XcpR (2), and XcpP and XcpQ (6).

XcpS expressed in *E. coli* was stabilized by co-expression of both XcpR and XcpY simultaneously and not when one of these proteins was co-expressed alone. These results indicate that XcpSRY form a ternary complex in which XcpS interacts directly with either one of the partner proteins or with both of them. However, since interactions between OutE and OutF, the *E. chrysanthemi* XcpR and XcpS homologues, and between OutF and OutL, the XcpS and XcpY homologues, have been reported (39), it is conceivable that both XcpR and XcpY interact directly with XcpS. In that case, the role of XcpY in the stabilization of XcpS may be dual: (i) to interact directly with XcpS and (ii) to dock XcpR to the inner membrane (2, 37, 38), thereby facilitating an interaction between XcpR and XcpS. Noteworthy in this respect is that modelling of the X-ray crystal structure of the cytoplasmic fragment of the XcpY homologue EpsL together with that of a fragment of the XcpR homologue EpsE of the *V. cholerae* T2SS resulted in only partial filling of the groove between EpsL domains II and III (1). This observation hints at a missing protein in the modelled complex, which may be the cognate XcpS homologue. The XcpR homologue OutE of the *E. chrysanthemi* system has been shown to undergo a change in conformation that requires the XcpY homologue OutL and *vice versa* (38). This conformational change may be required to enable the interaction with

the cognate XcpS homologue. We observed that production of the XcpZ protein together with XcpRY somewhat further elevated XcpS levels, which may be related to the stabilizing effect of XcpZ on XcpY (33). However, we cannot exclude a direct interaction between XcpS and XcpZ. The existence of an XcpRSYZ sub-complex is in agreement with a recent publication showing the co-purification of XcpRSY with his-tagged XcpZ after cross-linking (40). It should be stressed that XcpS production was substantially higher from a construct carrying *xcpR-Z* than upon coexpression of only *xcpRYZ*. Since this increase did not correlate with an increase in XcpY production (data not shown), other Xcp components beside XcpY and XcpR appear to play role in XcpS stabilization.

The *P. putida xcpS* gene could not complement an *xcpS* mutation in *P. aeruginosa*, probably because it fails to interact properly with other Xcp components in the heterologous host. We used this observation to identify regions in the protein that are important for the species-specific functioning and that thus likely represent interaction domains. For that purpose, a series of chimeric *xcpS* genes was constructed and the results of these studies are summarized in Fig. 3A. The first two transmembrane segments of XcpS could be replaced by those of *P. putida* XcpS without loss of function. The similarities between the amino acid sequences of the *P. aeruginosa* and *P. putida* transmembrane segments one and two are 11 and 20%, respectively. This low level of similarity and the fact that they can be functionally exchanged shows that these segments are not involved in species-specific interactions. The third transmembrane segment including the last few periplasmic residues (hybrid 6) could not be replaced, although on an elastin-containing plate this hybrid appeared still partially functional as evidenced by the formation of a small halo around the colonies (data not shown). The production level of this hybrid was similar to those of the other hybrids; therefore, augmented instability does not seem to be the reason of its non-functionality. Hence, the last membrane-spanning segment and/or the C-terminal periplasmic residues appear to be involved in the species-specific functioning of XcpS, and thus likely in the interaction with other Xcp components. Similarly, replacement of the large N-terminal cytoplasmic domain (hybrid 106) and of the short periplasmic loop (hybrid 109) resulted in loss of functionality. Production of fusions 6, 106 and 109 in the wild-type strain interfered with secretion, which shows that these proteins still have the right conformation to interact with at least one other component of the secretion machinery but interfere with the formation of a

functional complex. Consistently, these proteins were still stabilized by XcpRYZ, and the non-functionality of these hybrids must be explained by inappropriate subsequent interactions with Xcp components.

Expression of fusion 110, in which the large cytoplasmic loop is replaced, did not complement the secretion defect of the *xcpS* mutant and did not display a dominant-negative effect on secretion in the wild-type strain. Apparently, this fusion is no longer stably incorporated in the secreton. When expressed in *E. coli*, fusion 110 was found to be the only hybrid that was no longer stabilized by co-expression of XcpRYZ. This result suggests that the cytoplasmic loop of XcpS is an essential segment for interaction with these Xcp components. In contrast, the N-terminal part of the XcpS homologue OutF was found to interact with the XcpR homologue OutE of *E. chrysanthemi* in yeast two-hybrid experiments (39). Possibly, both cytoplasmic segments of XcpS participate in the interaction with XcpR, but the cytoplasmic loop between residues 239 and 379 suffices for the stabilization effect. On the other hand, these results can also reflect the dynamic nature of the secreton, in which interactions change during assembly.

In summary, multiple domains of XcpS play a role in the species-specific functioning of this protein, suggesting that XcpS interacts with several other components at both sides of the cytoplasmic membrane. Multiple interactions may also provide an explanation for our observation that XcpS is unstable when the entire *xcp* gene cluster is absent, but not when individual *xcp* genes were missing. Likely, more than one component can stabilize the XcpS protein. The sensitivity of XcpS and other Xcp proteins to proteolytic degradation may be important to ensure the correct order of interactions during assembly of the secreton. Based on the current knowledge, we propose this assembly to occur in the following steps. XcpZ recruits XcpY resulting in a more or less stable complex in the cytoplasmic membrane (33). XcpY on its turn forms a docking site for the cytoplasmic ATPase XcpR, which then associates with the inner membrane (2). Docking of XcpR to the inner membrane results in conformational changes in both XcpY and XcpR (38). The XcpRYZ subcomplex subsequently engages with XcpS, rendering the latter less prone to degradation. This results in an inner membrane complex that might act as a platform for the assembly of a pilus-like structure. Our next goal will be to identify the other interaction partner(s) of XcpS.

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Chapter 5

Species-specific outer membrane targeting of the *Pseudomonas aeruginosa* secretin XcpQ

Jorik Arts, Ria van Boxtel, Peter van Ulsen, Jan Tommassen, Margot Koster

ABSTRACT

Many Gram-negative bacteria employ a complex, envelope-spanning type II secretion system (T2SS) for exporting a broad range of proteins across the outer membrane into the extracellular medium. The T2SS of the opportunistic pathogen *Pseudomonas aeruginosa* consists of 12 different Xcp proteins, of which the oligomeric XcpQ protein is thought to form the actual transport channel in the outer membrane. In the present study, we demonstrate that the Xcp proteins assemble into a functional system when expressed in the heterologous bacterial host *Pseudomonas putida*, but not in *Escherichia coli*. In *E. coli*, XcpQ was not targeted properly to the outer membrane, since XcpQ oligomers were found associated with the inner membrane. By exchanging the C terminus of XcpQ by the corresponding segment of PulD, the secretin of the T2SS of *Klebsiella oxytoca*, and co-production of the PulD-specific pilotin PulS, outer membrane targeting could be achieved. In *P. aeruginosa*, production of the XcpQ-PulD hybrid restored secretion in an *xcpQ* mutant strain. However, the stability and functionality of this protein were strictly dependent on the presence of PulS. These results suggest that outer membrane targeting of XcpQ may require a so-far unidentified pilot protein(s), specific for *Pseudomonas* species.

INTRODUCTION

Protein secretion in Gram-negative bacteria relies on a limited number of dedicated transport machineries. Widely spread is the type II secretion pathway (13). Proteins secreted via this pathway are translocated across the cytoplasmic membrane via the Sec translocon or the TAT system (35, 55). Subsequently, the type II secretion system (T2SS) mediates the transport of the folded proteins across the outer membrane (9, 46). The opportunistic pathogen *Pseudomonas aeruginosa* secretes several enzymes and toxins via the T2SS, which is formed by the products of at least 12 *xcp* genes: *xcpA* and *xcpP-Z* (23). Disruption of any of the 12 *xcp* genes leads to the accumulation of the exoproteins in the periplasm (23, 30, 57).

Although the Xcp apparatus functions in the transport of proteins across the outer membrane, most of its constituents are found in or associated with the inner membrane. Only one component, XcpQ, localizes to the outer membrane (23). XcpQ forms oligomers, which supposedly function as the channels through which the Xcp substrates are exported out of the cell (6, 10). The inner membrane protein XcpP is thought to link XcpQ with the other components in the inner membrane (7). XcpY and XcpZ form a stable complex in the inner membrane and the cytoplasmic traffic ATPase XcpR docks to this complex by interacting with XcpY (4, 43). The polytopic integral inner membrane component XcpS also participates in this subcomplex (48). Finally, the Xcp apparatus contains several proteins with N-terminal sequence similarity to PilA, the structural component of type IV pili. These proteins are processed by the dedicated prepilin peptidase XcpA (44).

XcpQ belongs to a large family of proteins called secretins, which participate in the transport of macromolecules across the outer membrane in various systems, including type II and type III protein secretion, filamentous phage release, and type IV pilus assembly (28). Secretins form stable oligomers of 12-14 subunits (5). After their synthesis in the cytoplasm, they are targeted to the Sec machinery for translocation across the inner membrane, which is followed by removal of the N-terminal signal sequence by leader peptidase (47). Later steps in the biogenesis of these proteins are largely unknown. Secretins appear to use the same Omp85-dependent sorting pathway as other integral outer membrane proteins (56). However, secretins are devoid of the C-terminal consensus motif that is typically found in outer membrane proteins (53) and which is recognized by Omp85

(49). Furthermore, their oligomerization severely challenges their periplasmic transfer. Indeed, several secretins require specific chaperones for correct outer membrane localization and protection against proteolytic degradation. Typically, small outer membrane lipoproteins with little sequence conservation are involved in chaperoning the secretins. Such lipoproteins, collectively designated pilotins, have been identified, for example, in the T2SSs of *Klebsiella oxytoca* (PulS) and *Erwina chrysanthemi* (OutS) and they bind their cognate substrates at the extreme C terminus (15, 33, 51). On the other hand, the secretin of the T2SS of *Aeromonas hydrophila* requires the inner membrane ExeAB complex for proper localization (3). Thus, multiple mechanisms appear to exist for secretin assembly in T2SSs.

In *P. aeruginosa*, no homologues of *pulS*, *outS* or *exeAB* have been identified. Therefore, XcpQ is believed not to require a chaperone for its targeting to the outer membrane (23). However, an alternative possibility is that a putative chaperone has remained undetected so far, due to no or only limited sequence similarity to known secretin chaperones. To determine whether the twelve *xcp* genes are sufficient to assemble into a functional secretion system, we have expressed, in the present study, the *xcp* genes in the heterologous bacterial hosts *Escherichia coli* and *Pseudomonas putida* and report XcpQ assembly defects in *E. coli*.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions. *P. aeruginosa* strains PAO25 (29) and D40ZQ (4) are *leu arg* and Δ*xcpP-Z* mutant derivatives, respectively, of strain PAO1. PAN1 (6) is a mutant derivative of strain PAO25, carrying a gentamicin-resistance cassette in the *xcpQ* gene. Furthermore, *E. coli* strain DH5α (31) and *P. putida* strain WCS358 (27) were used. *P. aeruginosa* and *E. coli* strains were grown at 37°C, and *P. putida* at 30°C in a modified Luria-Bertani (LB) broth (54). For plasmid maintenance, the following antibiotics were used: for *E. coli*: ampicillin 50 μg/ml, chloramphenicol 25 μg/ml, gentamicin 15 μg/ml, and kanamycin 25 μg/ml; for *P. aeruginosa*: gentamicin 40 μg/ml, chloramphenicol 300 μg/ml, and piperacillin 20 μg/ml; for *P. putida* gentamicin 40 μg/ml, and kanamycin 40 μg/ml.

TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristic*	Source or reference
pAX24	*xcpP-Z* cluster in pLAFR3	(22)
pCRII-TOPO	Ap^r; Km^r; TOPO TA cloning vector	Invitrogen
pCRII-P	pCRII-TOPO; *xcpP*	This study
pCRII-R	pCRII-TOPO; *xcpR*	Chapter 4
pCRII-201	pCRII-TOPO; *lipA, lif*	This study
pCRII-'D65	pCRII-TOPO; *'pulD65*	This study
pCRII-'Q65	pCRII-TOPO; *'xcpQ65*	This study
pCRII-'QD65	pCRII-TOPO; *'xcpQD65*	This study
pCRII-'$Q_{\Delta C35}$	pCRII-TOPO; *'xcpQ$_{\Delta C35}$*	This study
pCRII-PulS	pCRII-TOPO; *pulS*	This study
pUC19	Ap^r; cloning vector	(58)
pUP2	PUC19; *xcpA*	(16)
pUAWE-5	PUC19; *xcpPQ*	(17)
pUC-R	PUC19; *xcpR*	This study
pUC-RZ	PUC19; *xcpR-Z*	Chapter 4
pUC-QZ	pUC-RZ; *xcpPQ* behind their own promoter	This study
pUC-QZII	pUC19; *xcpR-Z* and *xcpPQ* all in one operon	This study
pJF29	PUC19; *cbpD*	(24)
pUC-PQD_{65}	PUC19; *xcpPQD$_{65}$*	This study
pUC-$PQ_{\Delta C35}$	PUC19; *xcpPQ$_{\Delta C35}$*	This study
pUC-QD_{65}	PUC19; *xcpQD$_{65}$*	This study
pUC-$Q_{\Delta C35}$	PUC19; *xcpQ$_{\Delta C35}$*	This study
pUC-QD_{65}Z	pUC19; *xcpR-Z* and *xcpPQD$_{65}$* in one operon	This study
pUC-$Q_{\Delta C35}$Z	pUC19; *xcpR-Z* and *xcpPQ$_{\Delta C35}$* in one operon	This study
pYRC	pBBR1-mcs5; *lacI*; Gm^r; P_{lac}	Chapter 2
pYRC-QZII	pYRC; *xcpR-Z* and *xcpPQ* in one operon	This study
pMMB67HE, EH	Ap^r; cloning vector; P_{tac}	(26)
pB28	pMMB67HE; *xcpQ*	(6)
pMMB67-QD_{65}	pMMB67HE; *xcpQD$_{65}$*	This study
pMBK201	pMMB67HE; *lipA, lif*	This study
pULF201	PUR6500; *lipA, lif*	This study
pUR6500EH	pMMB67EH::Km^R, Ap^S; P_{tac}	(25)
pULB22	PUR6500; *lasB*	(8)
pMPM-K4Ω	Km^r; cloning vector; P_{araBAD}	(41)
pMPM-K4-22A	pMPM-K4; *lasB, xcpA*	This study
pMPM-K4-29A	pMPM-K4; *cbpD, xcpA*	This study
pMPM-K4-201A	pMPM-K4; *lipA, lif, xcpA*	This study
pCHAP580	pSU19; *pulS*	(15)
pBL7	pBluescript; *lipA, lif*	(20)
pCHAP710	pACYC184; all *pul* genes except *pulA* and *pulB*	(37)
pBBR1-MCS	Cam^R; cloning vector	(39)
pBBR1-PulS	pBBR1-MCS; *pulS*	This study

* Ap, ampicillin; Cam, chloramphenicol; Gm, gentamicin; Km, kanamycin

DNA manipulations. Plasmids used in this study are listed in Table 1. Recombinant DNA methods were performed essentially as described (50), using *E. coli* strain DH5α for routine cloning. Plasmids were introduced with the $CaCl_2$ procedure (50) into *E. coli* or by electroporation into *P. aeruginosa* and *P. putida* (21). PCRs were performed with the proofreading *Pwo* DNA polymerase (Roche) and PCR products were cloned into pCRII-TOPO according to the manufacturer's protocol. Oligonucleotides used in this study are listed in Table 2.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5'→ 3')*	Restriction site
JAXcpP01for	CGGCCAGTCAATCGATTTGATAGAAGTAGG	ClaI
JAXcpP02rev	CGTACGAAAAGCTTGAAGGGGCAAACAGGG	HindIII
BK101	CCGCTCGAGATGACCAGGAGAACTGCATATGA AGAA	XhoI
BK104	CATGGATCCCGGTCAGCGCTGCTCGGCCTG	BamHI
D65for	TATCTGGCCAACGGTGATCCGCGACCGC	MscI
D65rev	ACGAGCTCAGCGGCGGGGGTCATAGATTG	SacI
Q65for	CGACCGAGCAACATCCTCATCC	
Q65rev	AGGCCTCAGGAAGACCATCAGGTTG	StuI
Qtrunc_rev	AGAGCTCTCTTAGACCCGGATGTCGCTG	SacI
pulSfor	GAGCTCAGATTTTCTGATGACTACGG	SacI
pulSrev	GAGCTCCGATTGAGGAGAGTCCGCAG	SacI

* Restriction sites are underlined

The *xcpR* gene on a HindIII-XbaI fragment of pCRII-R was ligated into HindIII-XbaI-digested pUC19, which resulted in pUC-R. The SacI-XbaI fragment of pUAWE-5 with the *xcpPQ* genes was inserted into SacI-XbaI-digested pUC-RZ behind the *xcpR-Z* operon, resulting in construct pUC-QZ. With the oligonucleotides JAXcpP01for and JAXcpP02rev *xcpP* was amplified from pAX24. The product was introduced into pCRII-TOPO, resulting in pCRII-P. The XbaI-SbfI product of pCRII-P was ligated into XbaI-SbfI-digested pUC-QZ, resulting in pUC-QZII, which carries the two *xcp* operons in tandem behind the *lac* promoter. The HindIII-XbaI fragment of pUC-QZII was ligated into the broad-host-range vector pYRC digested with the same enzymes, resulting in pYRC-QZII. Plasmid pMPM-K4-29A was constructed by ligation of *cbpD* on an SphI-EcoRI fragment of pJF29 and *xcpA* on an SphI-PstI fragment from pUP2 into EcoRI-PstI-digested pMPM-K4Ω. Plasmid pMPM-K4-22A was constructed by ligation of *lasB* on an SphI-EcoRI fragment of pULB22 and *xcpA* on a SphI-PstI fragment

from pUP2 into pMPM-K4Ω. A DNA fragment containing the *lipA* and *lif* genes was PCR amplified with the primers BK101 and BK104 and pBL7 as template. The product was cloned into pCRII-TOPO resulting in pCRII-201. The *lipA* and *lif* genes were excised from this construct by XhoI-BamHI digestion and the fragment was ligated into XhoI-BamHI-digested pMMB67HE resulting in construct pMBK201. Construct pULF201 contains *lipA* and *lif* on SacI-DrdI and KpnI-DrdI fragments of pMBK201 ligated into KpnI-SacI-restricted pUR6500HE. Construct pMPM-K4-201A was obtained by the insertion of *lipA* and *lif* as an XbaI-SacI fragment from pMBK201 and *xcpA* as a SacI-HindIII fragment from pUP2 into XbaI-HindIII-digested pMPM-K4Ω. For the construction of the *xcpQD*$_{65}$ hybrid, part of *pulD*, encoding the C-terminal 65 amino acid residues of the protein, was amplified from pCHAP710 with the oligonucleotides D65for and D65rev. The PCR product was inserted into pCRII-TOPO resulting in pCRII-'D65. Part of *xcpQ*, encoding amino acid residues 319-603 of the protein, was amplified from pAX24 with primers Q65for and Q65rev. The PCR product was ligated into pCRII-TOPO resulting in construct pCRII-'Q65. The MscI-SacI fragment of pCRII-'D65 was inserted into StuI-SacI-digested pCRII-'Q65, which resulted in pCRII-'QD65. This construct was digested and *xcp'QD65* on an EcoRV-SacI fragment was inserted into EcoRV-SacI-digested pUAWE-5, which resulted in pUC-PQD$_{65}$. To place *xcpQD*$_{65}$ under control of the *lac* promoter, *xcpPQ'* on a HindIII-EcoRV fragment from pUC-PQD$_{65}$ was replaced by the HindIII-EcoRV fragment of pB28, which resulted in pUC-QD$_{65}$. The HindIII-SacI fragment of pUC-QD$_{65}$ was introduced in HindIII-SacI-digested pMMB67HE resulting in pMMB67-QD$_{65}$. Digestion of pUC-QD$_{65}$ with SbfI-SacI followed by ligation into SbfI-SacI-digested pUC-QZII resulted in pUC-QD$_{65}$Z. To construct the gene encoding XcpQ$_{\Delta C35}$, part of *xcpQ*, encoding amino acid residues 319-623 of the protein, was amplified from pAX24 with the primers Q65for and Qtrunc_rev. The PCR product was ligated into pCRII-TOPO resulting in pCRII-'Q$_{\Delta C35}$. The EcoRV-SacI fragment of pCRII-'Q$_{\Delta C35}$ was ligated into EcoRV-SacI-digested pUAWE-5 resulting in pUC-PQ$_{\Delta C35}$. To remove *xcpP* and upstream DNA, and to place *xcpQ*$_{\Delta C35}$ under control of the *lac* promoter, the HindIII-EcoRV fragment of pB28 was introduced into HindIII-EcoRV-digested pUC-PQ$_{\Delta C35}$, which resulted in pUC-Q$_{\Delta C35}$. The SbfI-SacI fragment of pUC-PQ$_{\Delta C35}$ was inserted into SbfI-SacI-digested pUC-QZII, resulting in pUC-Q$_{\Delta C35}$Z. With the primers pulSfor and pulSrev *pulS* was amplified from pCHAP710 and the product

was introduced into pCRII-TOPO, resulting in pCRII-PulS. The HindIII-XbaI fragment of pCRII-PulS was subsequently ligated into HindIII-XbaI-digested pBBR1MCS, resulting in pBBR-PulS.

Enzyme assay. Secretion of elastase was analyzed qualitatively on LB plates with a top layer containing 1% elastin (Sigma). After overnight growth, the plates were examined for the presence of clear haloes around the colonies.

SDS-PAGE and immunodetection. Bacterial cells were suspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue, 0.1 M Tris-HCl pH 6.8). Extracellular proteins were precipitated using 5% trichloracetic acid (TCA; Sigma) and washed with acetone before resuspending them in SDS-PAGE sample buffer. Samples were boiled for 10 min unless otherwise indicated and proteins were separated on acrylamide gels. For immunodetection, proteins were transferred to nitrocellulose membranes by semi-dry electroblotting. Primary antisera used were anti-XcpQ (6) at 1:3000, anti-CbpD (24) at 1:10.000, anti-LipA (40) at 1:1000, anti-PspA (36) at 1:1000, anti-XcpS (2) at 1:1000, anti-XcpT (19) at 1:1000, anti-XcpY (43) at 1:5000, and anti-XcpZ (43) at 1:500 dilutions. Alkaline phosphatase-conjugated goat anti-rabbit IgG antiserum (Biosource international) was used as secondary antibody unless stated otherwise. Detection was performed by staining with 5-bromo-4-chloro-3-indolylphosphate (Sigma) and nitroblue tetrazolium (Sigma). Alternatively, where indicated, horse radish peroxidase-conjugated goat anti-rabbit IgG antiserum (Biosource international) was used as secondary antibody. Detection was performed by chemiluminescence (Pierce).

Cell fractionation. Inner and outer membranes of *E. coli* were separated as described (11). In the case of *P. aeruginosa* and *P. putida*, the membranes were separated as described by Hancock and Nikaido (32) with three modifications: (i) Dithiothreitol was added after disruption of the cells to a final concentration of 2 mM and was present throughout the remaining part of the procedure, (ii) isolation of the total membrane fractions on a 70% sucrose cushion was omitted, and (iii) sucrose gradient centrifugation was carried out in a Beckman SW28 rotor at 25,000 rpm for 16 h. NADH-oxidase activity was determined as described (45) and the presence of the outer membrane porins in the different fractions was evaluated by SDS-PAGE. Cell envelope preparations of *E. coli* and *P. aeruginosa* were

isolated from ultrasonically disrupted cells (6, 11) and analyzed on 8% acrylamide gels.

RESULTS

The *P. aeruginosa* Xcp system is not functional in *E. coli*. The T2SSs of *K. oxytoca* and *E. chrysanthemi* have been successfully reconstituted in *E. coli* (14, 34). We wanted to determine whether the twelve *xcp* genes of the more distantly related *P. aeruginosa* could assemble into a functional system in this host as well. The Xcp system of *P. aeruginosa* is encoded by two divergently transcribed operons, *xcpR-Z* and *xcpPQ*, which are under quorum-sensing control (12). The twelfth gene, encoding the prepilin peptidase XcpA, is located elsewhere on the chromosome in a cluster of genes involved in type IV pilus biogenesis. To obtain sufficient expression in *E. coli*, the two operons were cloned in tandem resulting in construct pYRC-QZII, which contains the *xcpRSTUVWXYZPQ* genes under the control of P_{lac}. When this construct was introduced into a *P. aeruginosa* strain lacking the *xcp* gene cluster, i.e. strain D40ZQ, secretion of the Xcp-dependent substrate elastase was restored as evidenced by halo formation on elastin-containing agar plates (results not shown). Production of the Xcp proteins in *E. coli* strain DH5α from pYRC-QZII was confirmed by immunoblot analysis with the available antisera (anti-XcpQ, S, T, Y and Z). When induced by the addition of IPTG to a final concentration of 0.1 mM, the production levels of these Xcp components were similar to those in wild-type *P. aeruginosa* (results not shown). To determine whether the Xcp proteins assembled into a functional system, a second construct containing *xcpA* as well as a gene for an Xcp-dependent substrate under control of the P*araBAD* was introduced into DH5α already containing pYRC-QZII. Three different substrates were tested, i.e. chitin-binding protein D (CbpD), elastase (LasB) and lipase (LipA). The substrates accumulated inside the cells and were not detected in the extracellular medium, as shown for CbpD in Fig. 1A. By using various concentrations of IPTG and arabinose, the stoichiometry of substrate and Xcp system was varied. However, Xcp-dependent secretion of the substrates was never observed (data not shown).

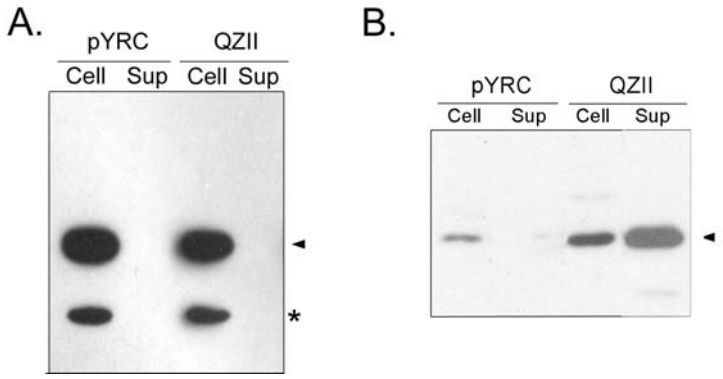

FIG. 1. Functionality of the Xcp system produced in *E. coli* DH5α and in *P. putida* WCS358. Extracellular proteins (sup) and whole cell lysates (cell) were analyzed by SDS-PAGE, followed by Western blotting using CbpD- or LipA-specific antibodies. Extracellular proteins were loaded in 5 times excess compared to cell lysates. Peroxidase-conjugated antiserum and chemiluminescence (Pierce) was used for immunodetection. (A) DH5α producing CbpD and XcpA from pMPM-K4-29A and containing either the pYRC-QZII (QZII) encoding the Xcp system or the empty vector (pYRC). Overnight grown cultures were diluted 1:10 into fresh medium and grown for 3 h. Subsequently, production of Xcp proteins was induced by the addition of 0.1 mM IPTG, and that of CbpD and XcpA by adding 1% L-arabinose. The position of CbpD is indicated with an arrowhead, and that of a degradation product with an asterisk. (B) *P. putida* WCS358 producing LipA and Lif from pULF201 and containing either the pYRC-QZII (QZII) or the empty vector (pYRC). Production of Xcp proteins and of lipase and foldase was induced by the addition of 1 mM IPTG. An arrowhead indicates the position of lipase.

We also tested the possibility to reconstitute the *P. aeruginosa* Xcp system in *P. putida*, which is more closely related to *P. aeruginosa* than is *E. coli*. Therefore, pYRC-QZII and a second plasmid, pULF201, encoding *P. aeruginosa* lipase (LipA) and its cognate foldase (Lif) were introduced into *P. putida* strain WCS358. It was not considered necessary to introduce the *P. aeruginosa xcpA* gene, since *P. putida* has been shown to produce a functional prepilin peptidase that is able to process *P. aeruginosa* XcpT (18). Immunoblot analysis showed that considerable amounts of lipase were secreted into the extracellular medium, whereas in the absence of the *P. aeruginosa* Xcp system, lipase was detected only inside the cells (Fig. 1B). The total amount of lipase was considerably lower in the strain lacking the *P. aeruginosa* Xcp system, indicating that intracellular lipase is prone to proteolytic degradation. These results show that the *P. aeruginosa* Xcp system can be functionally expressed in *P. putida*.

XcpQ oligomers localize in the inner membrane in *E. coli*. To identify the reason for the non-functionality of the Xcp system in *E. coli*, we first studied oligomerization and localization of the secretin XcpQ. XcpQ oligomers are stable in SDS-PAGE sample buffer and only dissociate upon boiling (6). Non-boiled cell lysates of *E. coli* producing XcpQ from pYRC-QZII were compared with those of wild-type *P. aeruginosa* overproducing the Xcp system from pAX24. As shown in Fig. 2A, XcpQ oligomers were detected in both samples. Thus, oligomerization of XcpQ appears unaffected in *E. coli*. Subsequently, the localization of the XcpQ oligomers produced from pYRC-QZII in *P. aeruginosa* D40ZQ *, P. putida* WCS358, and *E. coli* DH5α, was determined by separating inner and outer membranes with sucrose gradient centrifugation. Whereas in *P. aeruginosa* and *P. putida*, the XcpQ oligomers were mainly found in the fractions that contained markers for the outer membrane, the majority of the XcpQ oligomers co-localized with the inner membrane marker NADH-oxidase in *E. coli* (Fig. 2B). Hence, XcpQ is not properly targeted to the outer membrane in *E. coli*.

Mislocalization of secretins often results in the induction of the synthesis of phage shock response protein A (PspA) (1, 15, 33, 38). However, PspA production was found to be below detection levels in DH5α cells induced for the expression of *xcpQ* from pYRC-QZII by the presence of 0.1 mM IPTG (data not shown).

PulS-dependent outer membrane localization of an XcpQ-PulD hybrid in *E. coli*. Studies by Shevchik *et al.* (52) and by Burghout *et al.* (11) showed that C-terminally truncated derivatives of the secretins OutD and YscC were still functional. In contrast to the intact proteins, these truncates were targeted to the outer membrane in the absence of their dedicated pilotins. To investigate whether the removal of the C terminus of XcpQ may facilitate its outer membrane targeting in *E. coli*, a truncated XcpQ lacking the C-terminal 35 amino acid residues was produced in *E. coli* from pUC-$Q_{\Delta C35}Z$ (Fig. 3A). The mutant protein formed oligomers, which, however, fractionated with markers for the inner membrane in sucrose gradients (results not shown).

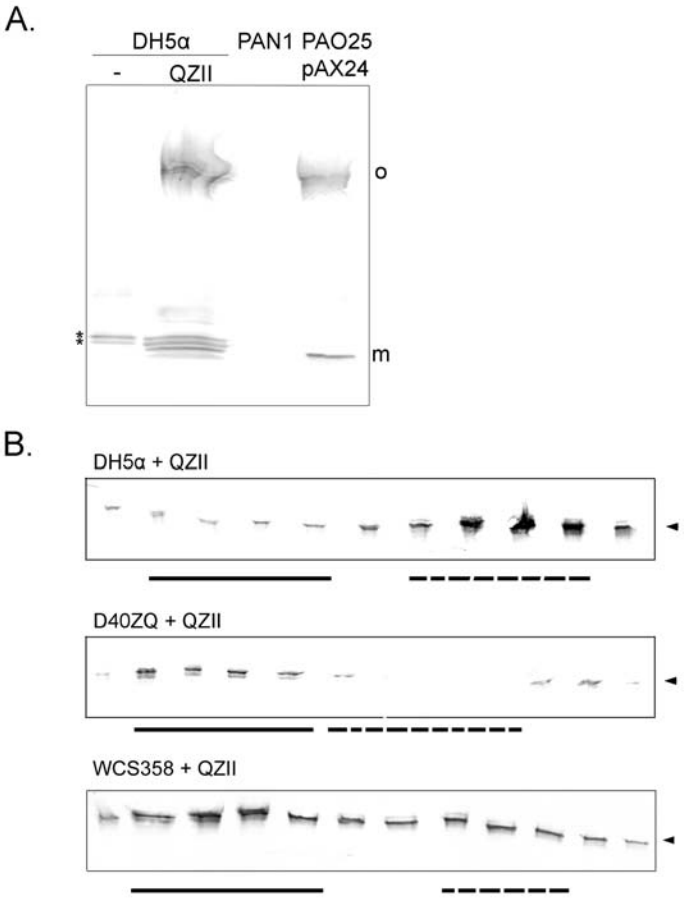

FIG. 2. Oligomerization and localization of XcpQ. (A) Non-boiled whole cell lysates of DH5α containing the empty vector pYRC (-), or producing XcpQ from pYRC-QZII (QZII), the *P. aeruginosa xcpQ* mutant (PAN1), and the wild-type *P. aeruginosa* strain PAO25 overproducing the Xcp system from pAX24 (pAX24) were separated by SDS-PAGE on a 3-9% acrylamide gradient gel and analyzed by Western blotting using XcpQ-specific antiserum. The position of oligomeric XcpQ is indicated with 'o', of the monomeric form with 'm', and of cross-reacting bands with asterisks. (B) Cell envelope fractions of French press lysates of DH5α, *P. aeruginosa* lacking the *xcpP-Z* gene cluster (D40ZQ), and *P. putida* WCS358, all producing XcpQ from pYRC-QZII were applied onto 30-55% sucrose gradients, which were centrifuged and fractionated as described in experimental procedures. Samples from all fractions were analyzed on 3-9% acrylamide gradient gels and Western blotting with anti-XcpQ antiserum. The samples representing the inner (dashed lines) and outer membranes (solid lines) were identified based on NADH-oxidase activity and the presence of porins, respectively. Arrowheads indicate the position of oligomeric XcpQ.

Subsequently, another strategy was employed to target XcpQ to the *E. coli* outer membrane, employing a well-known pilotin. PulD is the XcpQ homologue of the T2SS of *K. oxytoca* and PulS is the cognate pilotin, which stabilizes PulD and promotes its outer membrane localization (33). Daefler *et al.* (15) showed that fusion of the C-terminal 65 amino acid residues of PulD to the secretin pIV of the filamentous phage f1 rendered the fusion protein $pIVD_{65}$ dependent on PulS for stability and for proper localization. Analogously, a gene fusion was constructed encoding a chimeric protein in which the C-terminal 55 amino acid residues of XcpQ are replaced by the corresponding C-terminal 65 amino acid residues of PulD. Upon production of the hybrid protein $XcpQD_{65}$ from pUC-QD_{65}Z in *E. coli*, a product was detected by immunoblotting that migrated faster in the gel than wild-type XcpQ (Fig. 3A), suggesting that the C-terminal PulD domain was proteolytically removed. Co-production of PulS from pCHAP580 resulted in the detection of $XcpQD_{65}$ migrating at a position similar to that of wild-type XcpQ (Fig. 3A). Thus, PulS appears to protect the C-terminal PulD domain of $XcpQD_{65}$ against proteolytic degradation. Interestingly, co-production of $XcpQD_{65}$ and PulS also resulted in pronouncedly increased levels of monomeric $XcpQD_{65}$ in non-boiled samples, without concomitant increase in the amount of oligomers (Fig. 3B). Localization studies showed that the $XcpQD_{65}$ oligomers were predominantly present in the inner membrane fractions in the absence of PulS (Fig. 3C). When $XcpQD_{65}$ and PulS were produced simultaneously, the $XcpQD_{65}$ oligomers (Fig. 3C) and PulS (results not shown) co-localized predominantly with the outer membrane markers. Thus, $XcpQD_{65}$ can be targeted to the outer membrane of *E. coli* in a PulS-dependent manner.

Next, we tested whether the Xcp system produced from pYRC-QD_{65}Z was functional when PulS was co-produced. Therefore, pMPM-K4-201A, which encodes LipA, Lif, and XcpA, was introduced as a third construct into DH5α already containing pYRC-QD_{65}Z and pCHAP580, and extracellular lipase activity was determined under several induction conditions. However, no lipase activity was detected in the culture supernatants (data not shown). Apparently, mistargeting of XcpQ is not the only obstacle in the functioning of the Xcp apparatus in *E. coli*.

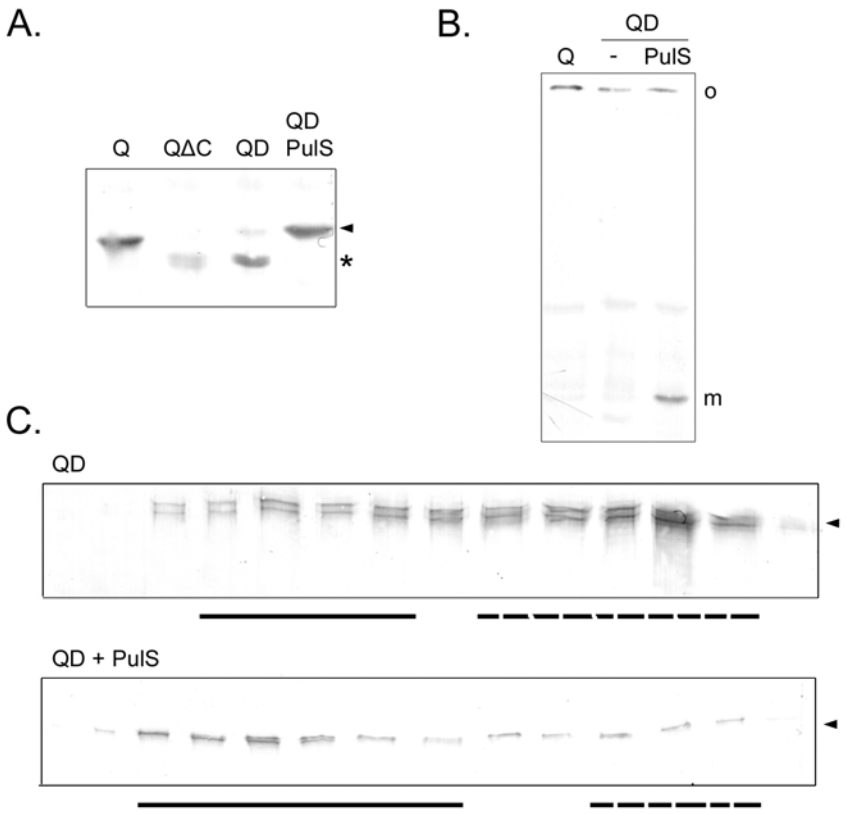

FIG. 3. Stability and localization of different XcpQ variants in *E. coli*. (A) Cell envelopes of DH5α containing pUC-QZII carrying wild-type *xcpQ* (Q), pUC-$Q_{\Delta C35}Z$ carrying *$xcpQ_{\Delta C35}$* (QΔC), pUC-$QD_{65}Z$ carrying *$xcpQD_{65}$* (QD), or pUC-$QD_{65}Z$ together with pCHAP580 carrying *pulS* (QD PulS) were boiled for 10 min in sample buffer and analyzed on an 8% acrylamide gel, followed by Western blotting and immunodetection with XcpQ-specific antiserum. The position of monomeric XcpQ is indicated with an arrowhead, and of a degradation product with an asterisk. (B) Non-boiled cell envelopes of DH5α containing pUC-QZII (Q), pUC-$QD_{65}Z$ (QD) with or without pCHAP580 (PulS) were separated on an 8% acrylamide gel and analyzed by Western blotting and immunodetection with XcpQ-specific antiserum The position of oligomeric XcpQ is indicated with 'o', and of the monomeric form with 'm'. (C) Cell envelope fractions of French press lysates of DH5α containing pUC-$QD_{65}Z$ (QD) with or without pCHAP580 (PulS) were applied onto 30-55% sucrose gradients, which were centrifuged and fractionated. Samples from all fractions were analyzed on 3-9% acrylamide gradient gels, followed by Western blotting using XcpQ-specific antibodies. The samples representing the inner (dashed lines) and outer membranes (solid lines) were identified based on NADH-oxidase activity and the presence of porins, respectively. An arrowhead indicates the position of oligomeric XcpQ.

Functionality of $XcpQD_{65}$ in *P. aeruginosa*. The functionality and PulS dependency of $XcpQD_{65}$ were also analysed in the *P. aeruginosa xcpQ* mutant strain PAN1, into which pMMB67-QD_{65} was introduced for this purpose. The C terminus of PulD appeared to render the fusion protein prone to degradation. In contrast to *E. coli*, where the XcpQ moiety of the fusion protein was protected (Fig. 3A), the complete protein appeared to be degraded in *P. aeruginosa*, since no degradation products were detected (Fig. 4A). When PulS was co-produced from pBBR1-pulS, the amount of $XcpQD_{65}$ detected was similar to wild-type XcpQ levels (Fig. 4A). To study functionality, PAN1 producing $XcpQD_{65}$ in the presence or absence of PulS was grown on LB agar plates containing elastin and halo formation was evaluated (Fig. 4B). $XcpQD_{65}$ complemented the secretion defect of the *xcpQ* mutant when PulS was co-produced, but was not functional in the absence of PulS. In conclusion, replacement of the C-terminal part of XcpQ by that of PulD resulted in a protein that requires PulS for stability and functionality. Whether the chimeric secretin also depended on PulS for outer membrane targeting in *P. aeruginosa* could not be determined, because of the instability of $XcpQD_{65}$ in the absence of PulS.

DISCUSSION

By placing the *P. aeruginosa xcp* genes under control of the *lac* promoter, the components of the Xcp system could be produced in *E. coli*. However, the proteins did not assemble into a functional secretion system. In contrast, the *P. aeruginosa* Xcp system could be reconstituted in *P. putida*. Thus, the assembly of the Xcp secreton from pYRC-QZII appeared host-specific. The two heterologous hosts differed in their ability to target the secretin XcpQ to the outer membrane. While XcpQ was correctly localized in the outer membrane in *P. putida*, it was associated with the inner membrane in *E. coli*. In *E. coli*, the XcpQ oligomers appeared to be present in the membranes and not in aggregates, since they remained associated with the inner membrane fractions in flotation experiments and were not solubilized by 8 M urea (data not shown). Remarkable in this respect is the observation that, in our hands, incorrectly localized XcpQ oligomers did not induce PspA production.

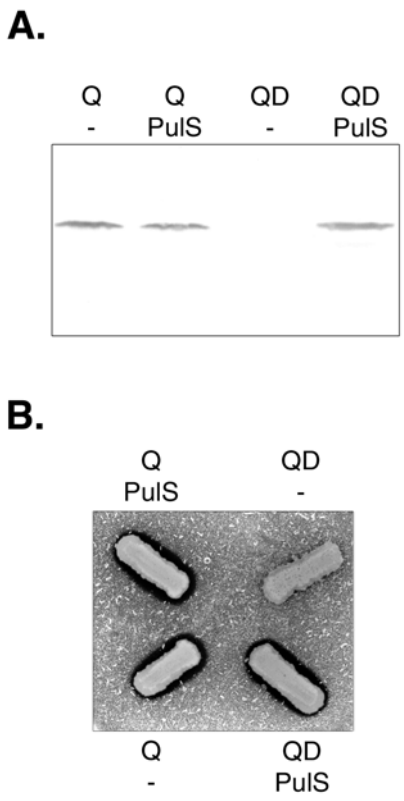

FIG. 4. Production and functionality of XcpQD$_{65}$ in *P. aeruginosa.* (A) Cell envelopes of the *P. aeruginosa xcpQ* mutant PAN1 producing wild-type XcpQ from pB28 (Q) or XcpQD$_{65}$ from pMMB67-QD$_{65}$ (QD), and co-producing PulS from pBBR1-pulS (PulS) or containing the empty vector pBBR1-MCS (-) were analyzed by SDS-PAGE, followed by Western blotting using XcpQ-specific antibodies. (B) The same strains were grown on LB agar containing 1% elastin. Secretion of elastase is visualized by clearance of elastin from the plate around the colonies.

Induction of PspA synthesis is a common response to conditions that affect the integrity of the inner membrane and has been reported to occur in response to mistargeting of various secretins (1, 15, 33, 38). Akrim *et al.* (1) have shown that production of *P. aeruginosa* XcpPQ from the T7 promoter did induce PspA synthesis. Whether this discrepancy relates to differences in the production levels or to the co-production of the other Xcp components is currently under investigation.

In an attempt to achieve proper targeting of XcpQ in *E. coli*, we deleted the C-terminal 35 amino acid residues of XcpQ, or, in another approach, we replaced a C-terminal segment by the corresponding part of the secretin PulD. $XcpQ_{\Delta C35}$ still accumulated in the inner membrane, but $XcpQD_{65}$ was targeted to the outer membrane in a PulS-dependent manner. Remarkably, co-production of PulS with $XcpQD_{65}$ also resulted in a significantly increased monomer:oligomer ratio compared to that found for wild-type XcpQ. This result indicates that either PulS or the C-terminal segment of PulD functions by delaying oligomerization, thereby possibly facilitating the transport of $XcpQD_{65}$ in a monomeric state across the peptidoglycan layer.

Although $XcpQD_{65}$ localized to the outer membrane when PulS was co-produced, the Xcp proteins still failed to assemble into a functional T2SS in *E. coli*. The non-functionality might result from abnormal stoichiometry of the components. Normally, *xcpR-Z* and *xcpPQ* are expressed from different promoters (12), which regulate both onset and production levels. However, the Xcp system produced from pYRC-QZII was functional in *P. aeruginosa* and in *P. putida*. Possibly, the problem relates to the production level of the prepilin peptidase XcpA relative to the other components. Although the vast majority of XcpT was found in the mature form in overnight grown *E. coli* cells (data not shown), we cannot exclude the possibility that processing was not sufficiently efficient to generate a functional Xcp system. Of note, we observed that small amounts of processing-defective pseudopilin subunits can strongly interfere with secretion in an otherwise wild-type *P. aeruginosa* strain (Chapter 3). Alternatively, a host-specific factor may be required for proper functioning of the machinery. For example, the structure of the LPS in the outer membrane has been shown to influence the functionality of the Xcp machinery (42).

The *xcpQD*$_{65}$ hybrid gene was able to complement an *xcpQ* mutation in *P. aeruginosa*, showing that the chimeric protein is functional. The functionality and stability of this protein were, however, strictly dependent on PulS. In the absence of the pilotin, the $XcpQD_{65}$ secretin was completely degraded. In stark contrast, the major portion of the protein was stable in *E. coli*, where only the PulD moiety appeared to be removed. Similarly, the C-terminally truncated XcpQ was detectable in *E. coli* but not in *Pseudomonas*. Possibly, oligomerization occurs fast in *E. coli*, thereby protecting XcpQ against proteolytic degradation and, at the same time,

resulting in aberrant targeting of the XcpQ oligomers, e.g. by preventing passage of the peptidoglycan layer. In *P. aeruginosa*, oligomerization may be delayed until after the passage of the peptidoglycan, thereby rendering the $XcpQD_{65}$ protein more susceptible to degradation. In agreement with this assumption substantial amounts of XcpQ are present in the monomeric form in *Pseudomonas* (6, 10). Maintaining the monomeric state may be accomplished by the activity of a chaperone, which, in that case, would still bind to the mutant XcpQ lacking the C-terminal segment, as has also been reported for the binding of pilotin YscW to secretin YscC (11).

Does the secretin XcpQ require a so-far unidentified pilotin for targeting to the outer membrane? The fact that the protein is not correctly localized to the outer membrane in *E. coli* points in that direction, especially since targeting could be achieved artificially by co-expression of PulS with the $XcpQD_{65}$ hybrid. Interestingly, deletion of the C terminus renders XcpQ highly unstable in *Pseudomonas*, but not in *E. coli*. The latter finding may indicate that XcpQ contains multiple interaction sites, e.g. an interaction site to maintain a monomeric state, which does not require the C terminus, and a C-terminal interaction site that stabilizes the protein in *Pseudomonas*. Interestingly, Hamood *et al.* (30) reported the isolation of a *P. aeruginosa* TT2S-defective mutant, which could neither be complemented by *xcpP-Z* nor by *xcpA*, hinting at the existence of additional gene(s) required for secretion. The putative pilotin should also be present in *P. putida*, since XcpQ was localized properly in this host. *P. putida* does possess a T2SS, but this system is not very related to that of *P. aeruginosa*. The homology between *P. putida* XcpQ and *P. aeruginosa* XcpQ is not high (37% identity) and their C termini are very different (18). Thus, in contrast to the known pilotins, *Pseudomonas* may use a more general system for targeting secretins to the outer membrane.

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Chapter 6

General and summarizing discussion

The type II secretion pathway is widely spread among Gram-negative bacteria (13), where it is used to secrete a range of different substrates across the outer membrane (22). Type II secretion systems (T2SSs) consist of 12-16 different proteins (22), many of which interact and multimerize (5, 25, 43, 44, 47). Therefore, these complex systems appear to be extremely large structures in the bacterial cell envelope. T2SSs have been identified in several pathogenic bacteria, including *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Legionella pneumophila*, and *Yersinia enterocolitica*, and are important determinants of bacterial virulence (13, 22). Therefore, they are attractive targets for the development of novel antimicrobial therapies (30). The T2SSs may also be of interest for the production of biocatalysts, for which folding is a bottleneck. The exoproteins pass through the periplasm, which contains many folding catalysts, and are secreted in a folded conformation, which is a unique feature of this secretion pathway. More insight into the functioning and assembly of these secretion systems will facilitate the development of pharmaceutical and biotechnical applications for T2SSs. The work described in this thesis was performed to study the assembly of the main T2SS of *P. aeruginosa*, designated Xcp.

INNER MEMBRANE TRANSLOCATION OF THE MAJOR PSEUDOPILIN XcpT

Transport of exoproteins via the T2SS occurs in a two-step procedure, in which the substrates are first translocated to the periplasm via the Sec or TAT machinery and, in a second step, across the outer membrane to the cell surface or the extracellular environment (28, 56). In *P. aeruginosa*, the Xcp system is responsible for the secretion of the majority of the exoproteins. This system is assembled from 12 constituents, XcpA (also designated PilD) and XcpP-Z, of which XcpQ is thought to form the actual channel in the outer membrane through which the exoproteins are exported (5, 7).

Many Xcp proteins share sequence similarity with constituents of the type IV piliation system, designated the Pil system in *P. aeruginosa* (40). Type IV pili are filamentous appendages with various functions, including adherence to host cells, twitching motility, DNA uptake, and biofilm formation (10). The Xcp system contains five proteins, XcpTUVWX, with sequence similarity to the structural component of the type IV pilus, PilA. Therefore, these components are referred to as pseudopilins (23). Moreover,

XcpR is homologous to the traffic ATPase PilB, XcpS to PilC, and also the outer membrane proteins XcpQ and PilQ display considerable sequence similarity (38, 40). One component, the prepilin peptidase XcpA/PilD, which processes the precursors of (pseudo)pilins, is even shared by the Xcp and Pil systems in *P. aeruginosa* (39). Homologues of the pseudopilins, the prepilin peptidase, XcpS and XcpR are also found in type IV piliation and competence systems in Gram-positive bacteria, and in the flagellum and sugar-binding systems in archaea (40, 45), suggesting that these proteins form an ancient apparatus involved in the assembly of pilus(like) structures.

The peculiar N-terminal sequences of prepilins can mediate transport across the inner membrane and act as transmembrane segments (54). However, the pathway involved in translocation was unknown and a matter of debate. Two possible pathways have been proposed; i.e. via the highly conserved Sec translocon or via a dedicated system most likely involving the traffic ATPase XcpR and the multispanning transmembrane protein XcpS. The Sec system is generally used for protein transport across the cytoplasmic membrane (18). The N termini of prepilins share the characteristics of Sec signal sequences, and previous studies have shown that the N-terminal sequences of prepilins from *P. aeruginosa* and *Neisseria gonorrhoeae* function as export signals when fused to alkaline phosphatase in *Escherichia coli* (19, 54). However, the N termini of the prepilins are also distinct from Sec signal sequences, for example by the presence of a conserved glutamate within the hydrophobic domain and they lack a characteristic signal-peptidase cleavage site C-terminally to the hydrophobic domain. Instead, they are processed N-terminally to the hydrophobic segment at the cytoplasmic side of the membrane by the specific prepilin peptidase, which is an integral inner membrane protein. These differences and the fact that the pilin-like proteins are always found in concert with members of the XcpR/PilB, XcpS/PilC and XcpA/PilD families, led to the proposal that pilins and pseudopilins are exported from the cytoplasm via a dedicated transport route formed by these accessory proteins (12, 22, 38). In support of this hypothesis, Chung and Dubnau (12) reported that the prepilin peptidase ComC of the *Bacillus subtilis* competence system is required for translocation of the pilin-like protein ComGC. Moreover, Kagami *et al.* (29) showed that a conditional mutation in XcpT could be suppressed by a secondary mutation in the cytoplasmic ATPase XcpR, indicating that these proteins interact during assembly and/or functioning of the machinery.

Our data (chapter 2) show that inner membrane translocation of XcpT coupled to alkaline phosphatase (XcpT-PhoA) was independent of functional type IV piliation and T2SSs in *P. aeruginosa*, which indicated transport via a general export route. Experiments performed with *E. coli* revealed that translocation of XcpT and XcpT-PhoA was dependent on the Sec translocon and that XcpT-LacZ was co-translationally targeted to the Sec apparatus via the signal-recognition particle (SRP). Pseudopilins have a strong tendency to associate with each other, even when their amino acid sequence in their C-terminal moiety is changed and the proteins are no longer functional (42). Therefore, co-translational transport may be needed to circumvent premature association of subunits within the cytoplasm.

Considering the strong conservation of their N-terminal sequences, it is likely that also other pilins and pseudopilins use the SRP/Sec pathway. Indeed, similar conclusions were reached simultaneously by Francetic *et al*. in their study on the export of the pseudopilin PulG of the T2SS of *Klebsiella oxytoca* (24).

The SRP pathway is mainly used for the insertion of inner membrane proteins (17). Hence, transport of XcpT is likely followed by the lateral insertion of the pseudopilin from the Sec translocon into the inner membrane, after which processing by the prepilin peptidase can take place. Although processing occurs at the cytoplasmic side of the membrane, our experiments showed that transport is a prerequisite for this event to occur. Subsequently, XcpT presumably enters the Xcp machinery laterally to assemble into a pilus-like structure. Interestingly, recycling of Cy3-labeled pilin subunits has been shown in a time-lapse experiment (53), indicating that the bacterium makes use of a pool of subunits in the membrane. Recycling of subunits is difficult to imagine if the pilins would enter the machinery directly from the cytoplasm, and therefore argues against the model of transport and assembly via the same machinery, involving XcpR and XcpS.

Also our finding that the N-terminal sequences of the XcpT and PilA precursors could be exchanged without affecting the functionality of XcpT (chapter 3) supports the notion that XcpT is transported across the inner membrane independent of other components of the Xcp machinery. If the machinery was involved in transport across the inner membrane, one would expect the information to be present in the part of the protein important for inner membrane transport.

TARGETING OF XcpT TO THE TYPE II SECRETION MACHINERY

After transport across the inner membrane, the XcpT protein has to be targeted to the Xcp machinery. In *P. aeruginosa*, T2SS and type IV piliation machineries co-exist in the cell and the structural component of type IV pili and the major pseudopilin of T2SSs share extensive structural similarity (20, 31, 49, 55). Nonetheless, pilins and pseudopilins cannot substitute for each other and it appears that the secretion and the piliation systems efficiently identify their cognate subunits. However, several studies have shown that the two systems can interfere when out of balance and that correct targeting likely depends on subtle differences in affinity and accurate stoichiometry (20, 34). In chapter 3, a hybrid protein approach was used to obtain more insight into the importance of the N-terminal segments of XcpT and PilA in targeting. Our data show that the leader peptide and the N-terminal 17 amino acid residues of mature XcpT could be replaced by the corresponding residues of PilA without affecting functionality. These data are consistent with the findings by Köhler *et al.* (31), who studied, in the absence of a type IV piliation system, whether the *K. oxytoca* T2SS could assemble pilin-pseudopilin hybrids into a pseudopilus. It appeared that the *K. oxytoca* XcpT homologue PulG was assembled into such structure also when its leader peptide and its N-terminal 17 amino acid residues were replaced by those of pilin PpdD. Taken together, targeting information is not contained in the N-terminal segment, but within another part of the protein.

In chapter 3, we also showed that disruption of the processing site in XcpT and in PilA resulted in the specific obstruction of the Xcp and Pil system, respectively. Production of processing-defective XcpT variants dramatically affected secretion, while production of PilA with a disrupted processing site interfered with type IV piliation. Obstruction did not occur at the level of the prepilin peptidase, since accumulation of processing-defective PilA did not result in the accumulation of XcpT precursor. Thus, blocking appeared to be caused by the incorporation of unprocessed subunits in the systems. The production of processing-defective PilA slightly reduced T2SS-mediated secretion, which may reflect occasional aberrant targeting of PilA to the Xcp system. The finding that specific interference with secretion and piliation can be accomplished by disruption of the processing site offers a convenient tool to further investigate where the targeting information, which discriminates pilins from pseudopilins, is located.

ASSEMBLY OF XcpS INTO THE INNER MEMBRANE PLATFORM

Since pilins and pseudopilins are always found in concert with homologues of the XcpR and XcpS proteins, these proteins likely fulfil an essential role in the assembly of the pilus-like structures. As mentioned before, Kagami *et al.* (29) showed that a conditional mutation in XcpT could be suppressed by a secondary mutation in the cytoplasmic ATPase XcpR, suggesting that these two proteins interact. However, based on our results and those of others, T2SS proteins are not involved in the transport of the pseudopili (2, 24), and XcpR likely acts at a later stage, during the elongation of the pseudopilus. One could speculate that XcpR plays a role in the assembly of membrane-anchored pseudopilin subunits into the periplasmic pseudopilus. The conditional mutant described by Kagami *et al.* (29) produces XcpT with a substitution of the serine at position 121 with a leucine. Given the topology of XcpT, with the C terminus in the periplasm, their finding suggests that XcpR somehow penetrates through the cytoplasmic membrane, which might occur via a structure formed by the other Xcp proteins.

The multispanning membrane component XcpS would be the most likely candidate for such a structure, since it is present in all systems mentioned above. XcpS is predicted to contain three transmembrane segments, which separate a large N-terminal cytoplasmic domain, a short periplasmic loop, a large cytoplasmic loop, and, finally, a short periplasmic C terminus. This topology was supported by the analysis of fusions of XcpS with the reporter alkaline phosphatase (chapter 4). We observed that XcpS was largely instable in the absence of other Xcp proteins. This instability was used to identify interacting Xcp components. In addition, chimeras of *P. aeruginosa/ Pseudomonas putida* XcpS were constructed to identify domains in the protein that are important for the species-specific functioning and that, thus, likely represent interaction domains. We showed that XcpS was, at least partially, stabilized by the simultaneous co-production of the components XcpR and XcpY and that this stabilization was dependent on the presence of the large cytoplasmic loop of *P. aeruginosa* XcpS (chapter 4). XcpY is an inner membrane protein with one transmembrane segment that docks the cytoplasmic ATPase XcpR to the membrane via its cytoplasmic domain (4). Whether both XcpY and XcpR directly interact with XcpS, or that XcpY is merely involved in the association of XcpR with

the membrane, after which an interaction between XcpR and XcpS can be established, is not known. However, yeast two-hybrid studies showed an interaction between the XcpS homologue OutF of *Erwinia chrysanthemi* and OutE (XcpR) as well as OutL (XcpY) (44). Moreover, three-dimensional modeling of the crystal structures of the *V. cholerae* homologues of XcpR and XcpY demonstrated only partial filling of the binding groove, allowing an additional interaction (1). Thus, most likely, both XcpR and XcpY interact with XcpS. Surprisingly, the two-hybrid studies showed these interactions with the N-terminal cytoplasmic domain of the XcpS homologue. Although stabilization by XcpRY only required the cytoplasmic loop of *P. aeruginosa* XcpS, other parts of XcpS, including the N terminal domain, may interact as well. Production of the XcpZ protein together with XcpRY somewhat further elevated XcpS levels, which may be related to the stabilizing effect of XcpZ on XcpY (37). Nonetheless, we cannot exclude an additional direct interaction between XcpS and XcpZ.

Interestingly, XcpS production levels were substantially higher from a construct containing *xcpR-Z* than from a construct containing *xcpRSYZ* (data not shown). Production levels of XcpY and XcpZ from these constructs were comparable (results not shown), showing that XcpS stabilization resulted not merely from increased abundance of interaction partners, but rather from a more complete secretion system. This result suggests that other Xcp constituents also interact with XcpS. Indeed, the characterization of the XcpS chimeras demonstrated that multiple domains on both sides of the membrane are involved in species-specific functioning.

One of the interaction partners could be XcpS itself, which could be cross-linked into homo-oligomers (F. Senf, unpublished results). Other candidates are the pseudopilins. The conserved prevalence of proteins of the XcpS type together with proteins with prepilin-like sequences has led to the suggestion that XcpS plays a role in the assembly of the latter type of proteins (22). Evidence for such an interaction, however, is lacking.

BIOGENESIS OF THE SECRETIN XcpQ

Amongst others with biotechnological applications in mind, we have investigated the possibility of reconstituting the highly active T2SS of *P. aeruginosa* in a heterologous host. The Xcp systems of *Pseudomonas* species are among the T2SSs, for which no additional requirements outside of the twelve core components have been described (35). Out of the four

supplementary components that are identified in some other T2SSs, three are involved in the biogenesis of the oligomeric secretin. The GspA and GspB proteins form a complex in the inner membrane and have been shown to assist targeting of the secretin GspD in *Aeromonas hydrophila* (3). Proteins of the GspS type are small outer membrane lipoproteins required for outer membrane localization and stability of secretins (27). The component GspN, for which no role in secretin biogenesis has been described, may in fact be a XcpP-type of protein (22). To test whether production of the twelve Xcp proteins is indeed sufficient for the secretion of Xcp-dependent substrates, the *xcp* genes were cloned into a broad host-range vector in one operon under control of P_{lac}. This construct was introduced into *E. coli* and into *P. putida*. As described in chapter 5, the system was functional in *P. putida*, but not in *E. coli*. In contrast to the opportunistic pathogen *P. aeruginosa*, *P. putida* has a GRAS (generally recognized as safe) status and is, as such, an interesting organism for biotechnological application.

By studying the production and localization of the Xcp proteins in *E. coli*, we found that the secretin XcpQ, which forms stable oligomers, was not correctly targeted to the outer membrane, explaining the non-functionality of the Xcp system in *E. coli*. As described above, several members of the secretin family have been shown to require a dedicated chaperone for proper localization and for protection against proteolytic degradation (3, 9, 14, 16, 27, 32). One of them is PulD, the secretin of the T2SS of *K. oxytoca*, which requires the outer membrane lipoprotein PulS for stability and for localization to the outer membrane (27). The binding site for PulS is contained in the C-terminal amino acid residues of PulD and Daefler *et al.* (15) showed that fusion of this segment to the filamentous phage f1 secretin pIV rendered the resulting protein dependent on PulS for stability and proper localization. No such dedicated chaperone has been reported for XcpQ and, therefore, we explored two different strategies to obtain targeting of XcpQ to the outer membrane. First, we tested whether a C terminal truncation of XcpQ would facilitate outer membrane targeting, as was shown before for two other secretins, OutD and YscC, which normally require a dedicated pilotin (9, 51). C-terminally truncated derivatives of OutD and YscC were functional and correctly localized, independent of their cognate pilotin (9, 51). The truncated XcpQ formed oligomers in *E. coli*; however, these oligomers failed to localize to the outer membrane.

Next, we constructed a hybrid gene encoding XcpQ with its C-terminal 55 amino acid residues replaced by the corresponding 65 residues of PulD. This approach was successful, since the XcpQ-PulD protein was targeted to the outer membrane of *E. coli* in a PulS-dependent fashion. Remarkably, co-production of XcpQ-PulD and PulS resulted in elevated levels of the monomer of the secretin. This observation indicates that PulS interacts with and stabilizes the monomer and may act by preventing oligomerization of the secretin to allow passage through the peptidoglycan layer.

Unfortunately, the Xcp system remained non-functional in *E. coli*, despite the outer membrane localization of the XcpQ-PulD oligomers. The XcpQ-PulD hybrid was functional in *P. aeruginosa* when PulS was co-produced. Hence, the PulD segment does not interfere with the functioning of XcpQ. Consequently, the inactive state of the Xcp system in *E. coli* must have an additional cause. Possibly, processing of the pseudopilins was not efficient enough as even small amounts of unprocessed XcpT were sufficient to block the Xcp system in *P. aeruginosa* (chapter 3). Alternatively, a host-specific factor, like LPS (36), interferes with proper functioning.

As mentioned above, the XcpQ-PulD hybrid protein was functional in *P. aeruginosa* upon co-production of PulS. In the absence of PulS, the fusion protein was highly instable and could not be detected with immuno-analysis. In contrast, when produced in the absence of PulS in *E. coli*, only the PulD segment of XcpQ-PulD appeared to be degraded. Similarly, the C-terminally truncated XcpQ was detectable in *E. coli,* but not in *P. aeruginosa*. Possibly, XcpQ variants can be stabilized in two different ways: via an interaction with a chaperone-like protein or via rapid oligomerization. If true, XcpQ and XcpQ variants might rapidly oligomerize in *E. coli* resulting in stable complexes that are mistargeted, due to their inability to pass through the peptidoglycan. In *P. aeruginosa*, oligomerization may occur slower, allowing for the correct targeting, but also rendering the XcpQ variants more prone to degradation. Our data hint at the existence of a so-far unidentified chaperone or pilotin that protects the XcpQ monomer against proteolytic degradation via an interaction with the C-terminal amino acid residues and delays its oligomerization via an additional binding site elsewhere in the protein. The YscC secretin of *Y. enterocolitica* also does not require its C terminus for pilotin binding (9). The observation that the *P. aeruginosa* Xcp system is functional in *P. putida* implicates that, should

such a chaperone exist, it acts not very specifically and may be a factor more general to *Pseudomonas* species. Re-examination of the *P. aeruginosa* genome sequence failed to identify homologues of *pulS* or of other genes, which have been shown to assist T2SS secretin targeting in other organisms.

Hamood *et al.* (26) reported the isolation of a *P. aeruginosa* T2SS-defective mutant, which could not be complemented by *xcpP-Z* or by *xcpA*, hinting at the existence of additional gene(s) required for secretion. It would be interesting to determine whether this mutant is impaired in the outer membrane localization of XcpQ.

MODEL FOR THE ASSEMBLY OF THE Xcp SYSTEM

Assembly of the T2SS appears a tightly regulated process, requiring correct stoichiometry. Stability of several Xcp proteins depends on the presence of other components (2, 4, 6, 37). Moreover, overproduction of single components can result in elevated breakdown of other secreton constituents (20, 41). Sensitivity of single Xcp proteins to proteolytic degradation may be of importance to ensure the correct order of events during assembly of the T2SS and for accurate localization. The sub-cellular localization of the T2SS in *P. aeruginosa* is unknown and hard to predict, since studies on the localization of the secreton of *V. cholerae* and of *K. oxytoca* had different outcomes (8, 50). In *V. cholerae*, monitoring of green fluorescent protein (GFP) fused to the XcpZ homologue EpsM indicated that the T2SS was located at the old pole after cell division (50), whereas, in *K. oxytoca*, GFP-PulM was evenly distributed over the cell envelope, with occasional brighter foci (8). Overproduction of GFP-PulL and GFP-PulM in *E. coli* did result in polar accumulation, indicating that polar localization might be an artifact. However, since additional evidence indicates that type II secretion occurs at the cell pole of *V. cholerae* (50), it seems that the sub-cellular localization of T2SSs indeed differs among organisms.

The *xcp* genes are organized in two divergently oriented operons, and transcription from the *xcpPQ* promoter starts after initiation of *xcpR-Z* expression (11). This observation implies that the inner membrane complex is formed separately. The inner membrane complex is composed of XcpRSYZ (2, 47). Stabilization studies have shown that in the absence of XcpZ, XcpY levels are decreased, and that XcpZ cannot be detected in an *xcpY* mutant (37). XcpR levels are also diminished in an *xcpY* mutant (4). Moreover, plasmid-encoded XcpS could not be detected in a *P. aeruginosa*

strain lacking the entire *xcp* gene cluster (chapter 4). Based on these observations, we propose the following succession of events during the assembly of the inner membrane complex.

The formation of the inner membrane complex involves the interaction of XcpY and XcpZ to form a stable complex in the cytoplasmic membrane (37). In *V. cholerae*, the XcpZ protein seems to determine the polar localization (50). However, the observation that XcpZ is dispensable for pseudopilus formation (21) and that T2SSs exist in which no obvious *xcpZ* homologue is present (13), indicates that, although XcpZ is essential for Xcp functionality, it fulfils a subtle role. XcpY forms the docking-site for the cytoplasmic ATPase XcpR, which associates with the inner membrane (4). Docking of XcpR to XcpY will very likely result in conformational changes in both proteins, as has been shown for their *E. chrysanthemi* homologues (43). XcpR forms multimers upon binding of ATP, and probably assembles into a hexameric ring (52).

Overproduction of XcpY interferences with secretion (4, 37), probably because incomplete complexes are formed. This negative dominance was overcome by the co-overproduction of either XcpR (4) or XcpZ (37). Thus, association of XcpY with XcpR and with XcpZ occurs early in the assembly of the Xcp system, and an interaction with XcpR and XcpZ facilitates the further assembly of a complete system. Since XcpS is only stabilized by XcpY and XcpR together, and not by the individual proteins, the formation of the XcpRYZ subcomplex preceeds the interaction with XcpS (Fig. 1A). The resulting XcpRSYZ complex (Fig. 1B) might act as a platform for the assembly of the pseudopilus.

Pseudopilins are translocated across the inner membrane via the SRP/Sec pathway and laterally enter the XcpRSYZ system (2, 24). Subsequently, the major pseudopilin XcpT assembles into a pseudopilus (20). For the formation of this pseudopilus all minor pseudopilins, except the XcpV protein, are dispensable (21), indicating that XcpV may be important for the initiation of pilus formation. The elongation of the pilus-like structure may be halted by incorporation of the atypical pseudopilin XcpX, which lacks the conserved glutamate at the +5 position (21). The periplasm-spanning structure could function as a piston to push substrates out of the cell or play a role in the opening of the secretin (23). The function of the other minor pseudopilins is unknown, but they are indispensable for T2SS functioning. They may be needed for fine-tuning of XcpT assembly

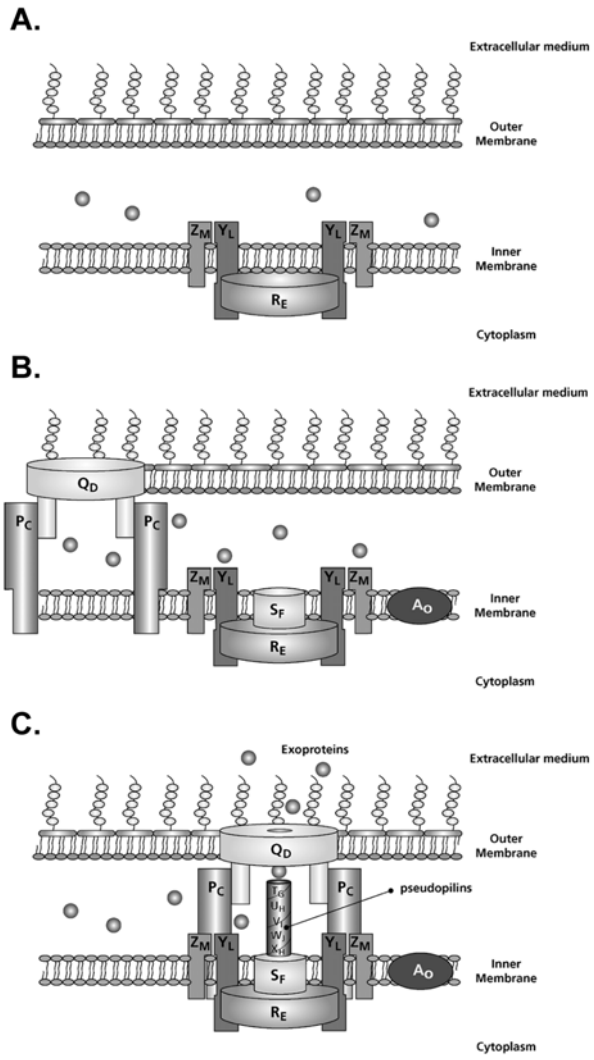

FIG. 1. Model for the assembly of the *P. aeruginosa* T2SS. (A) The formation of the stable inner membrane complex, likely, starts with the joining of XcpY with XcpZ and XcpR. (B) Subsequently, the XcpRYZ subcomplex engages in an interaction with XcpS, forming an XcpRSYZ complex. The secretin is transported to the outer membrane and interacts with XcpP. (C) Recognition of exoproteins by XcpPQ, results in conformational changes, which are signalled to the inner membrane complex via XcpP. A pseudopilus is formed, acting as a piston to push substrates out of the cell. The folded exoproteins, shown as grey circles, are transported across the OM via the secretin, XcpQ. For more details, see text.

(33) or be involved in the regulation of the pseudopilus length or strength, in analogy to the proposed role of minor type IV pilins (57).

XcpQ is exported into the periplasm via the Sec translocase. Insertion of XcpQ into the outer membrane may require the function of a pilotin (chapter 5). XcpP is protected from degradation by XcpQ (6), implying an interaction between these two components. XcpP has been reported to interact with the inner membrane component XcpZ as well (46), although it could not be co-purified with his-tagged XcpZ (47). Thus, XcpP could form a transient link between the secretin and the inner membrane complex (25, 48) (Fig. 1C). Complementation of an *xcpP* mutation by the expression of a C-terminally truncated XcpP variant has been shown to result in an increase in secretion of elastase (6). This truncated XcpP lacked the coiled-coil domain, which presumably is involved in homo-multimerization, as well as the extreme C terminus. Production of an XcpP variant with an internal deletion of the coiled-coil domain, but retaining the extreme C terminus was negative in complementation, hinting at the involvement of the latter domain in maintaining the closed conformation of XcpQ. This observation suggests that XcpP negatively controls secretion and that disengagement of XcpP and XcpQ would be required to open the channel (22). The drastic reduction of XcpP upon overproduction of XcpT might be explained by the inability of XcpP to re-associate with the secretin (20), since assembly of the artificial pseudopilus will inhibit closing of XcpQ. From results obtained by Bleves *et al.* (6), it is clear that XcpP is more prone to degradation in the absence of XcpQ. Upon prolonged disengagement of XcpP and XcpQ, increased XcpP turn-over is expected. Normally, dissociation of XcpP and XcpQ could be triggered by substrate binding.

XcpP might signal to the inner membrane components, resulting in the formation of the pseudopilus, which may energize the last step in secretion during which the exoproteins are released from the periplasm and exported out of the cell (22). After secretion, the secretin closes and elongation of the pseudopilus is stopped by the incorporation of XcpX (21).

Although progressive research has already revealed many characteristics of the T2SS, the precise composition and functioning of the apparatus remains unknown. It is noteworthy in this respect that secretion machineries, irrespective of their nature, might exist only transiently as active complexes, which would severely hamper interaction studies. For

instance, XcpP proteins have been suggested to interact with the inner membrane complex based on stabilization studies (41, 48), but direct biochemical or biophysical evidence for such interaction is, so far, lacking. Since the production of processing-defective XcpT variants in an otherwise wild-type *P. aeruginosa* strain obstructed the Xcp apparatus (chapter 3), it might be worthwhile to resolve whether in this case the machinery is trapped in an otherwise functional state. Performing cross-linking studies and co-immunoprecipitation with these cells could result in new insights.

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Samenvatting

De celenvelop van Gram-negatieve bacteriën bestaat uit een binnenmembraan, welke het cytoplasma omsluit, en een buitenmembraan, met daartussenin het periplasma. Door de aanwezigheid van porines kunnen kleine hydrofiele moleculen de buitenmembraan passeren. Translocatie over de binnenmembraan vindt echter alleen plaats via gespecialiseerde systemen. Op deze wijze wordt diffusie van componenten uit de cel voorkomen en wordt de cel beschermd tegen schadelijke stoffen buiten de cel. De celenvelop vormt echter ook een barrière voor de opname van voedingstoffen en voor het transport van celeigen moleculen, bijvoorbeeld eiwitten. Eiwit synthese vindt namelijk exclusief plaats in het cytoplasma, terwijl eiwitten niet alleen in het cytoplasma functioneren, maar ook in de binnen- en buitenmembraan, in het periplasma, of buiten de cel.

Het inserteren in-, of het passeren van de binnenmembraan is meestal afhankelijk van het Sec (voor secretion) systeem. De belangrijkste componenten van dit systeem zijn de eiwitten SecY, SecE en SecG, welke het kanaal vormen waardoor het transport van eiwitten plaatsvindt, en het perifere eiwit SecA, welke energie levert aan de machinerie. Eiwitten die gebruik maken van het Sec systeem worden gesynthetiseerd met een N-terminale signaal sequentie, waardoor ze zich onderscheiden van cytoplasmatische eiwitten. Er zijn twee routes waarop substraten naar het Sec systeem geleid kunnen worden. Eiwitten kunnen reeds tijdens de synthese een interactie aangaan met het zogenaamde SRP (voor signal recognition particle), waarna transport via de Sec machinerie plaats heeft. Daarnaast kunnen eiwitten ook na synthese herkend worden door het helpereiwit SecB, welke zorgt voor het ontvouwen houden van het substraat en voor de levering aan het Sec systeem. Binnenmembraan eiwitten en periplasmatische eiwitten bereiken na insertie of translocatie via het Sec apparaat hun uiteindelijke bestemming. Buitenmembraan eiwitten moeten daarentegen het periplasma nog passeren en in de genoemde membraan inserteren. De wijze waarop dit gebeurt, is nog grotendeels onbekend. Ten slotte kunnen eiwitten ook uit de cel getransporteerd worden. Er zijn verschillende routes bekend waarop eiwitten door Gram-negatieve bacteriën gesecreteerd kunnen worden. Eén van deze gespecialiseerde systemen is het type II secretie apparaat, welke is opgebouwd uit 12-16 verschillende componenten. Diverse van deze bouwstenen vertonen gelijkenis met eiwitten die betrokken zijn bij de vorming van zogenaamde type IV pili.

Het werk beschreven in dit proefschrift is uitgevoerd om meer inzicht te verkrijgen in de assemblage van het type II secretie apparaat van

de opportunistisch pathogene bacterie *Pseudomonas aeruginosa*. Het type II secretie systeem van deze bacterie bestaat uit 12 eiwitten, te weten XcpA en XcpP t/m Z (Hoofdstuk 1, Fig. 3). Enkele Xcp eiwitten vertonen N-terminale gelijkenis met de structurele component van de type IV pilus genaamd piline en worden daarom pseudopilines genoemd. De N termini van deze eiwitten kenmerken zich door de aanwezigheid van een 20-tal geconserveerde hydrofobe aminozuren voorafgegaan door een korte, positief geladen leader peptide, welke door het prepilin peptidase XcpA verwijderd wordt. De -1 positie ten opzichte van de XcpA knipplaats is zonder uitzondering een glycine, de +5 positie wordt in veel gevallen gevormd door een glutamaat (Hoofdstuk 1, Fig. 4). Deze prepiline sequenties worden ook aangetroffen in componenten van competentie systemen van Gram-positieve bacteriën en bij flagel systemen en suikerbindende structuren van archaea. In alle gevallen gaat de aanwezigheid van eiwitten met deze leader peptides samen met de aanwezigheid van het genoemde prepilin peptidase dat gelokaliseerd is in de binnenmembraan, een cytoplasmatisch ATPase (XcpR), en een integraal binnenmembraan eiwit (XcpS). In het verleden is gesuggereerd dat het binnenmembraan transport van pseudopilines wel eens van deze eiwitten afhankelijk zou kunnen zijn. Daarnaast is echter ook voorgesteld dat dit transport afhankelijk zou kunnen zijn van het Sec systeem.

In hoofdstuk 2 beschrijven we onze studies naar het binnenmembraan transport van de pseudopiline XcpT. Deze pseudopiline gekoppeld aan het periplasmatische marker eiwit alkalisch phophatase (XcpT-PhoA) werd ook in afwezigheid van alle andere Xcp eiwitten over de binnenmembraan getransporteerd. Experimenten met dit fusie-eiwit in een temperatuur gevoelige *Escherichia coli secY* mutant stam maakten duidelijk dat binnenmembraan transport afhankelijk is van het Sec systeem. Met pulse-label experimenten konden we dit ook laten zien voor wild-type XcpT. Bij deze experimenten laten we cellen een korte tijd radioactieve aminozuren incorporeren in nieuw gesynthetiseerde eiwitten, zodat we deze eiwitten kunnen volgen in de tijd. We tonen aan dat het verwijderen van het leader peptide door XcpA pas plaats vindt na transport over de binnenmembraan en dat dit transport afhankelijk is van zowel SecA als SecY. SRP afhankelijkheid werd bestudeerd met behulp van een XcpT-LacZ fusie. Het gen *lacZ* codeert voor β-galactosidase, een enzym dat actief is in het cytoplasma. Eerdere onderzoeken hebben laten zien dat het plaatsen van een SecB signaal sequentie voor LacZ resulteert in obstructie van het

Sec systeem doordat de vouwing van LacZ erg snel verloopt en het Sec systeem enkel ongevouwen eiwitten kan transporteren. Wanneer er een SRP signaal sequentie aan LacZ gekoppeld werd, dan werd het eiwit wel getransporteerd, omdat synthese en binnenmembraan transport gelijktijdig verlopen en LacZ geen tijd krijgt om te vouwen. In dat laatste geval was het LacZ eiwit niet actief aangezien het uit het cytoplasma getransporteerd werd. Wanneer XcpT-LacZ in wild-type *E. coli* geproduceerd werd, kon er slechts zeer geringe LacZ activiteit in de cel gemeten worden. Het fusie-eiwit werd dus uit het cytoplasma getransporteerd voordat LacZ kon vouwen, wat wijst op de betrokkenheid van SRP. Om de rol van SRP meer direct aan te tonen werd het XcpT-LacZ fusie-eiwit ook in *E. coli* stammen met een milde SRP mutatie geproduceerd en de LacZ activiteit bepaald. In deze stammen werd een vijfvoudige verhoging van de LacZ activiteit ten opzichte van de wild-type stam gemeten. Samen laten deze studies zien dat de pseudopiline XcpT via SRP/Sec in de binnenmembraan inserteert. Gezien het feit dat prepiline sequenties sterk geconserveerd zijn, gaan deze bevindingen zeer waarschijnlijk op voor alle pilines en pseudopilines.

In *P. aeruginosa* functioneren het Xcp en het type IV pilus apparaat naast elkaar in de cel. Zoals hierboven beschreven vertoont de pseudopiline XcpT N-terminale homologie met piline (PilA). Bovendien kunnen beide eiwitten tot vergelijkbare structuren assembleren en hebben de monomeren een overeenkomstige driedimensionale vorm. Toch kunnen deze eiwitten elkaar niet vervangen. Dit suggereert dat beide eiwitten efficiënt naar de juiste machinerie geleid worden. In hoofdstuk 3 laten we zien dat de informatie voor het correct sorteren zich niet in het geconserveerde N-teminale domein bevindt. Dit hebben we bepaald door fusie-genen te construeren, coderend voor piline-pseudopiline (PilA-XcpT) hybrides, waarbij de leader peptide, het hydrofobe domein, en de alpha helix-vormende residuen zijn uitgewisseld (Hoofdstuk. 3, Fig. 1). Uitwisseling van de eerste twee genoemde domeinen resulteerde in normaal functionerende eiwitten. De PilA sequenties zorgen er dus niet voor dat de hybride eiwitten in het Pil systeem terechtkomen. Het fusie-eiwit met de eerste 65 aminozuren van XcpT vervangen door de corresponderende aminozuren van PilA ($XcpT_{65A}$) was niet functioneel, maar had ook geen negatieve invloed op het Xcp of Pil systeem. Door het verstoren van de prepilin peptidase knipplaats in de hybrides was het mogelijk om de rekrutering duidelijker te volgen. Productie van een piline variant, die niet langer door XcpA geknipt werd, blokkeerde namelijk specifiek het type IV

pilus systeem, terwijl een vergelijkbare XcpT variant enkel met het type II secretie systeem interfereerde. De functionele PilA-XcpT hybrides blokkeerden het secretie apparaat en werden dus efficiënt door het Xcp systeem herkend. Productie van $XcpT_{65A}$ met een verstoorde knipplaats had geen negatieve invloed op het Xcp of het Pil systeem. Echter, productie kon niet worden gedetecteerd waardoor het onmogelijk was om hier conclusies aan te verbinden. Het belang van de alpha helix bij de juiste sortering van pilines en pseudopilines blijft daardoor onduidelijk.

Het werk beschreven in hoofdstuk 4 richtte zich op het identificeren van interacties tussen het integrale binnenmembraan eiwit XcpS en andere Xcp componenten. Eiwitten behorend tot de XcpS familie functioneren niet alleen in type II secretie systemen, maar ook in de andere systemen zoals die hierboven beschreven staan. Dit geeft aan dat eiwitten van deze familie een belangrijke rol spelen in deze uiteenlopende systemen, waarschijnlijk in de assemblage van de pilines/pseudopilines. We tonen in hoofdstuk 4 aan dat XcpS drie transmembraan domeinen bevat welke de cytoplasmatische N terminus, een korte periplasmatische loop, een grote cytoplasmatische loop en de korte periplasmatische C terminus van elkaar scheiden. XcpS was in *P. aeruginosa* sterk gevoelig voor afbraak in afwezigheid van alle overige Xcp componenten. In enkelvoudige *xcp* mutanten kon dit niet worden waargenomen. Blijkbaar zijn meerdere Xcp componenten betrokken bij de stabilisatie van XcpS. Door XcpS op een laag niveau te produceren in *E. coli* konden we aan tonen dat XcpS gestabiliseerd werd door XcpR en XcpY. XcpS is, zoals hierboven beschreven staat, een eiwit bestaande uit meerdere eiwitdomeinen. Met het gebruik van XcpS hybriden hebben we bepaald welk domein van het eiwit betrokken is bij de stabilisatie door XcpRY. Deze hybriden bestonden uit *P. aeruginosa* XcpS waarin telkens een verschillend domein vervangen was door het corresponderende gedeelte van *Pseudomonas putida* XcpS (Hoofdstuk 4, Fig. 3). *P. putida* XcpS is niet functioneel in *P. aeruginosa*, waarschijnlijk omdat interacties met de andere *P. aeruginosa* Xcp componenten verstoord zijn. Studies met deze hybriden toonden niet alleen aan welk domein betrokken is bij de XcpRY interactie, maar lieten ook zien dat XcpS waarschijnlijk met meerdere Xcp eiwitten interacties aangaat en dus een centrale component in het Xcp systeem is.

Tenslotte hebben we het gehele Xcp systeem van *P. aeruginosa* in de heterologe bacteriën *E. coli* en *P. putida* geproduceerd en de functionaliteit ervan bepaald (hoofdstuk 5). In de eerst genoemde bacterie bleek het Xcp apparaat niet te functioneren, in *P. putida* wel. De

mogelijkheid tot reconstitutie van het Xcp systeem is dus soorts afhankelijk.. Uit onze experimenten bleek in *E.coli* XcpQ, de enige buitenmembraan component van het systeem, niet juist te lokaliseren. XcpQ vormt homomultimeren en deze functioneren (zeer waarschijnlijk) als kanalen waardoor substraten de cel verlaten. Het is dus voor de functionaliteit essentieel dat dit component correct lokaliseert. Voor een aantal eiwitten van de XcpQ familie zijn gespecialiseerde helper eiwitten beschreven, die zorgen voor bescherming tegen afbraak en voor de integratie in de buiten membraan. De helper van de XcpQ homoloog van *Klebsiella oxytoca*, PulD, is PulS. Dit helpereiwit bindt aan de C terminus van PulD. Door de C terminus van XcpQ uit te wisselen voor het corresponderende deel van PulD en dit fusie-eiwit vervolgens samen met de helper PulS te produceren, kon XcpQ naar de buitenmembraan van *E. coli* gesorteerd worden. Het disfunctioneren van het Xcp apparaat was daarmee echter nog niet mee opgelost. Mogelijk was de productie van de prepilin peptidase XcpA niet hoog genoeg waardoor de pseudopilines niet efficiënt genoeg geknipt werden. Verrassend genoeg was productie van het bovengenoemde XcpQ-PulD fusie-eiwit in *P. aeruginosa* niet aan te tonen in de afwezigheid van de helper PulS. Dit in tegenstelling tot in *E. coli* waar in afwezigheid van PulS weliswaar een klein gedeelte van het fusie-eiwit werd afgesplitst, maar waar het eiwit verder net zo stabiel was als wild type XcpQ. Dit zou het gevolg kunnen zijn van verschil in protease activiteit tussen de bacterie stammen, maar het kan ook duiden op een verschil in de manier waarop de cel omgaat met XcpQ-PulD. Het lijkt erop dat XcpQ-PulD zonder PulS in *E. coli* al multimeren vormt voordat het peptidoglycaan gepasseerd is, waardoor het complex de buitenmembraan niet meer kan bereiken. Dit resulteert enerzijds in verkeerde lokalisatie, maar aan de andere kant zorgt de multimerisatie voor bescherming tegen proteases. In *P. aeruginosa* wordt XcpQ-PulD in een monomere, makkelijk te transporteren vorm gehouden, waardoor deze gevoeliger voor afbraak is. XcpQ zou in dat geval enerzijds een domein bevatten dat multimerisatie vertraagd, mogelijk door de binding van een helper eiwit, en anderzijds een C terminus bevatten die essentieel is voor de stabiliteit, wat zou duiden op een tweede bindingsplaats voor een helper. Aangezien XcpQ normaal functioneert in *P. putida*, zou(den) de nog onbekende helper(s) een algemeen *Pseudomonas* eiwit kunnen zijn. Samengevat lijkt het erop dat XcpQ net als vele andere eiwitten van de XcpQ familie een helper eiwit nodig heeft.

In hoofdstuk 6 is een model voor de assemblage van het *P. aeruginosa* type II secretie systeem te vinden. Dit model verenigt data beschreven in de literatuur met de bevindingen uit dit proefschrift.

Nawoord

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Jorik

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Steenbakkers, P.J., J. Arts, R. Dijkerman, H.R. Harangi, H.J.M. Op den Camp, C. van der Drift, and G.D. Vogels. 1999. The cellulosome-like complex of the anaerobic fungus Piromyces E2. In: Genetics, biochemistry and ecology of cellulose degradation (K. Ohmiya, K. Hayashi, K. Sakka, S. Karita and T. Kimura, eds), pp 507-510, Uni Publ. Co., Tokyo

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Curriculum vitae

De schrijver van dit proefschrift werd op 25 mei 1976 in Helmond geboren. De middelbare school werd doorlopen aan het St. Willibrord Gymnasium in Deurne en deze werd in 1994 afgesloten met het V.W.O. diploma. In september van dat jaar werd begonnen met een H.L.O. microbiologie opleiding aan de Hogeschool Eindhoven. Als onderdeel van deze opleiding werden stages doorlopen bij het laboratorium voor Pathologische Anatomie en Medische Microbiologie (PAMM) in Veldhoven en bij de vakgroep Microbiologie (Dr. H. op den Camp) aan de Radboud Universiteit in Nijmegen. Na het behalen van het diploma in juni 1998, werd gestart met een verkorte studie medische biologie aan de Vrije Universiteit in Amsterdam. Na het doorlopen van een stage bij de vakgroep moleculaire dierfysiologie (Prof. Dr. G.J.M. Martens) aan de Radboud Universiteit in Nijmegen werd in december 2000 werd het doctoraal diploma behaald. Van februari tot en met november 2001 is hij als analist werkzaam geweest bij het bedrijfslaboratorium van de Vlisco in Helmond. Vanaf december 2001 is hij werkzaam geweest als assistent-in-opleiding bij de vakgroep Moleculaire Microbiologie van de Universiteit Utrecht. Tijdens deze periode werd het in dit proefschrift beschreven onderzoek verricht onder begeleiding van Prof. Dr. J.P.M. Tommassen en Dr. M.C. Koster.