

# **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  $\alpha$ -helical segments. The outer membrane faces the periplasm on one side and the extracellular medium on the other. This membrane is an

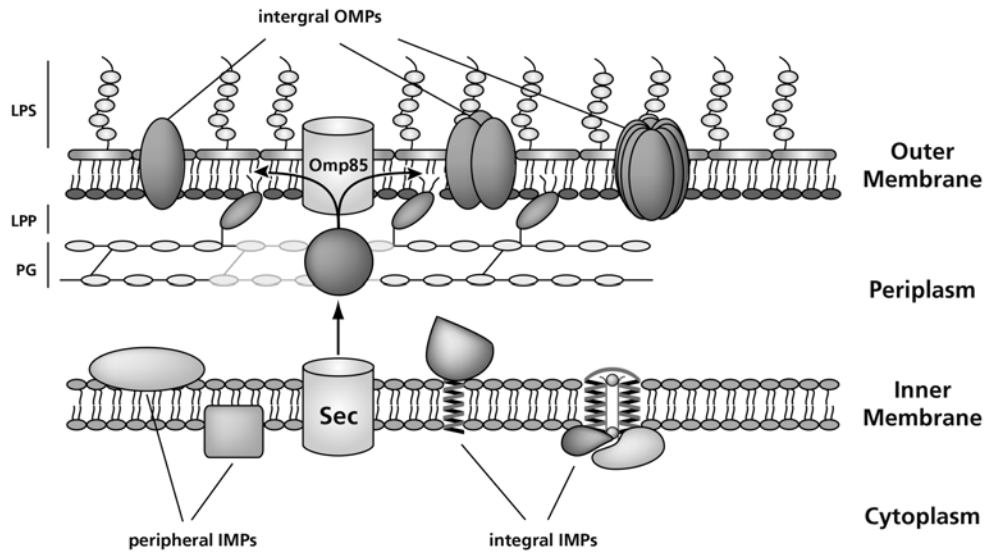
asymmetrical bilayer with phospholipids on the periplasmic side and lipopolysaccharides (LPS) on the outer leaflet making it relatively resistant to detergents. The outer membrane is semi-permeable due to the presence of proteinaceous channels formed by proteins called porins, which allow for passive diffusion of small hydrophilic molecules into the cell. Integral outer membrane proteins (OMPs) are characterized by the presence of amphipathic anti-parallel  $\beta$ -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 (secretion) 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<sub>1</sub>F<sub>0</sub> 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

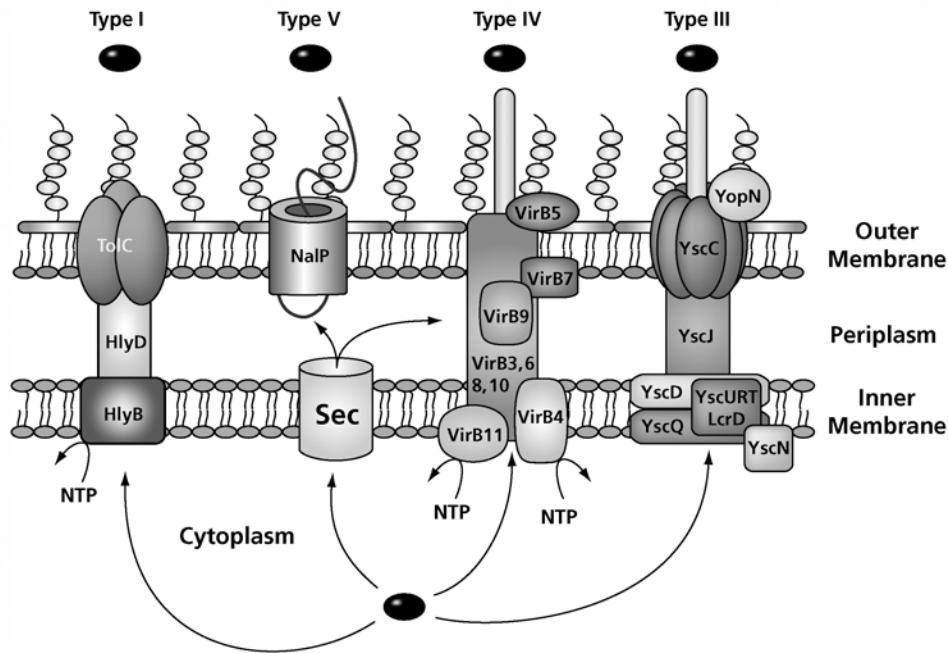
proteins are referred to as lipoproteins. Lipoproteins can attach to both the inner and the outer membrane via an N-terminal N-acyl-diacylglycerylcysteine. The residues directly C-terminal to the lipidated cysteine in the mature protein determine the localization; lipoproteins that lack an inner membrane-retention 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  $\beta$ -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 cytoplasm, 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  $\beta$ -barrel domain (49). The N-terminal signal sequence targets the protein to the Sec system for inner membrane translocation and the C-terminal  $\beta$ -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  $\beta$ -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 phases (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<sub>E</sub>. 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

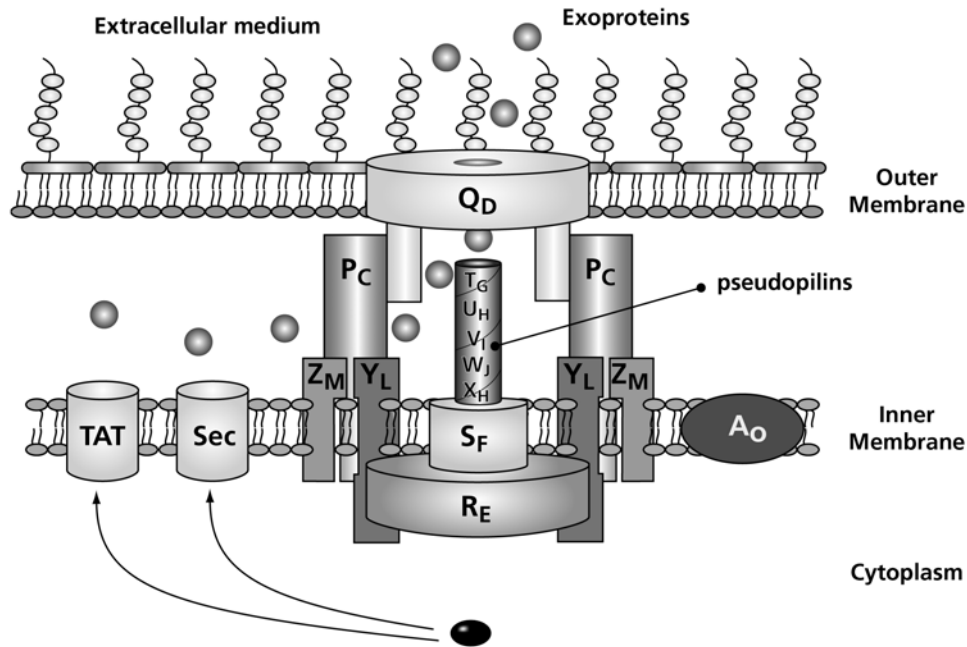
	T2SS		Molecular weight (kDa)	Pil
	Xcp	Gsp		
Major prepilin	XcpT	GspG	15	PilA
Minor prepilins	XcpU	GspH	19	PilE*
	XcpV	GspI	14	FimT*
	XcpW	GspJ	27	FimU*
	XcpX	GspK	37	PilV*
	-	-	-	PilW*
	-	-	-	PilX*
Prepilin peptidase/ N-methylase ATPase	XcpA	GspO	32	PilD
	XcpR	GspE	55	PilB
	-	-	-	PilT
	-	-	-	PilU
Secretin	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<sub>D</sub> 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<sub>D</sub> 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<sub>D</sub> 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.

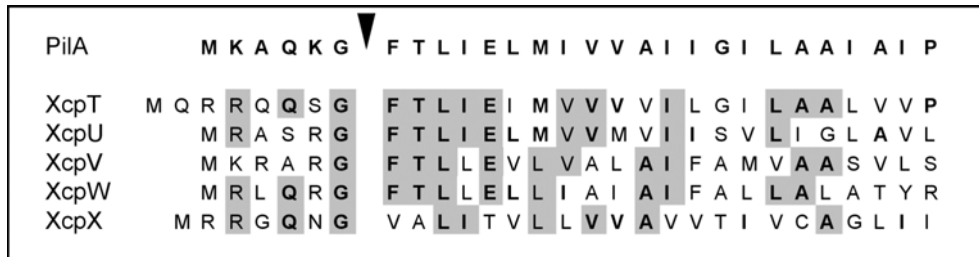
**Pseudopilins and the pre-pilin peptidase.** XcpT<sub>G</sub>, U<sub>H</sub>, V<sub>I</sub>, W<sub>J</sub>, and X<sub>K</sub> all display N-terminal sequence similarity to the structural component of the type IV pilus, the pilin PilA, and are therefore termed pseudopilins. However, the C-terminal domains 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<sub>D</sub>. XcpZ<sub>M</sub> and XcpY<sub>L</sub> mutually stabilize each other and form a stable complex. XcpY<sub>L</sub> anchors the XcpR<sub>E</sub> traffic ATPase to the inner face of the cytoplasmic membrane. XcpS<sub>F</sub> interacts with the XcpR<sub>E</sub>Y<sub>L</sub>Z<sub>M</sub> complex. The XcpP<sub>C</sub> component interacts with the periplasmic N-terminal domain of XcpQ<sub>D</sub> and with the XcpY<sub>L</sub>Z<sub>M</sub> 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<sub>O</sub>. 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 enterotoxigenic *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<sub>O</sub> (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<sub>G</sub> 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<sub>L</sub> and XcpZ<sub>M</sub> 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<sub>L</sub> not only interacts with XcpZ<sub>M</sub>, but it also docks XcpR<sub>E</sub> to the inner membrane (5). XcpR<sub>E</sub>, 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 multispansing inner membrane protein (117) that is proposed to form multimers (27, 29, 91). With his-tagged XcpZ<sub>M</sub>, Robert *et al.* (98) could co-purify XcpR<sub>E</sub>, S<sub>F</sub>, and Y<sub>L</sub> after cross-linking, which suggests that these proteins together form an inner membrane platform (Fig. 3).

**XcpP<sub>C</sub>: the link between the secretin and the inner membrane complex.** XcpQ<sub>D</sub> has been reported to be engaged in an interaction with XcpP<sub>C</sub> (Fig. 3) (9). XcpP<sub>C</sub> 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<sub>C</sub>, 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<sub>C</sub> 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<sub>C</sub> is required for the formation of homomultimers, rather than for interaction with the secretin. XcpP<sub>C</sub> together with XcpQ<sub>D</sub> might confer exoprotein recognition, since all Xcp/Gsp components of closely related bacteria could be functionally exchanged

except for XcpP<sub>C</sub>Q<sub>D</sub> (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<sub>D</sub>, XcpP<sub>C</sub> has been reported to interact with the inner membrane components XcpY<sub>L</sub> and XcpZ<sub>M</sub> (97). So, XcpP<sub>C</sub> 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<sub>E</sub> is homologous to the traffic ATPase PilB, XcpS<sub>F</sub> to PilC, and also the secretins XcpQ<sub>D</sub> 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  $\mu\text{m}$  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  $\mu\text{m}$  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<sub>G</sub> 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<sub>K</sub>, 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<sub>G</sub> assembly, so the roles of XcpU<sub>H</sub> and XcpW<sub>J</sub>, although requisite for secretion, remain obscure. XcpT<sub>G</sub> can be cross-linked to XcpU<sub>H</sub>, XcpV<sub>I</sub>, XcpW<sub>J</sub>, 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<sub>G</sub> 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 piliation. 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<sub>D</sub> 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<sub>G</sub> might also take part in the inner membrane complex, since a conditional mutation in XcpT<sub>G</sub> could be suppressed by a second mutation in XcpR<sub>E</sub> (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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