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

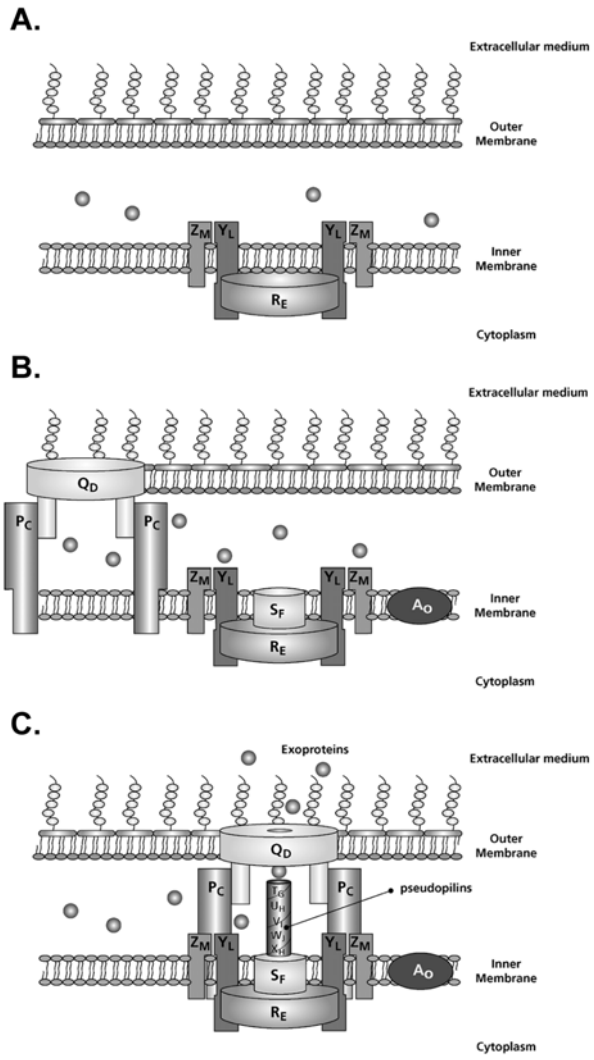

FIG. 1. Model for the assembly of the *P. aeruginosa* T2SS. (A) The formation of the stable inner membrane complex, likely, starts with the joining of XcpY with XcpZ and XcpR. (B) Subsequently, the XcpRYZ subcomplex engages in an interaction with XcpS, forming an XcpRSYZ complex. The secretin is transported 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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