Summary and General Discussion

The cell envelope of Gram-negative bacteria protects the bacteria from the harsh and potentially dangerous conditions encountered in the environment they live in. It exists of an inner membrane (IM), the periplasm, which contains the peptidoglycan layer that provides form and structure to a bacterial cell, and the outer membrane (OM). The latter is an asymmetric bilayer of phospholipids in the inner leaflet and lipopolysaccharides in the outer leaflet (1). On top of this cell envelope, bacterial surfaces may be shielded by a polysaccharide capsule. For *Neisseria meningitidis* the capsule is a major virulence determinant that allows for survival in the blood stream (2). Secreted virulence factors are synthesized in the cytoplasm and have to pass this formidable barrier to become active in the bacterial environment. *N. meningitidis* genomes encode several secretion systems, among which Type V Secretion Systems take a prominent place (3). The Type V secretion pathway includes the classical monomeric autotransporters (Type Va), the trimeric autotransporters (Type Vc) and the Two-Partner Secretion (TPS) systems (Type Vb). In **Chapter 1** we present a comprehensive overview of the functions, biogenesis, secretion mechanism and biotechnical applications of the Type V secretion Pathway (4). The remainder of the thesis focuses on the TPS system of *N. meningitidis*, in particular how a substrate for secretion is targeted to and recognized by its transporter, the TpsB.

The TPS systems of *N. meningitidis*

TPS systems usually consist of a secreted TpsA protein, which is generally large (> 150 kDa), and an OM-embedded TpsB transporter (5). Both TpsA and TpsB proteins are synthesized with an N-terminal signal peptide and transported across the IM via the Sec machinery. The TpsB then inserts into the OM and binds and secretes the TpsA protein across the OM to the cell surface where it may be released to the extracellular milieu or remain attached to that surface. The two proteins are often, but not always encoded in one operon and appear to interact in a specific manner. TpsA proteins have at their N-terminus a conserved domain, called the TPS domain, that targets the cognate TpsB transporter in the outer membrane.

In *N. meningitidis* the organization of the TPS systems appears less straightforward. Cluster analysis of the sequences of the TPS domains of TpsAs and the TpsBs respectively revealed that *N. meningitidis* disease isolates encode one to three TPS systems on large genomic islands (3,6). TPS system 1 is ubiquitous among *N. meningitidis* isolates, while systems 2 and 3 are found in a subset of the isolates studied. Interestingly, the latter two are found primarily in isolates that belong to hypervirulent clonal complexes (6). Furthermore, TPS systems 1 and 2 may contain multiple *tpsA*s, depending on the strain analyzed. For example, analysis of the genome sequence of the disease isolate H44/76 (7,8), which was used for many of the experiments described in this thesis, showed that TPS system 1 is encoded on two genomic islands (Fig. 1A). Of these, one island contains a *tpsB1* and *tpsA1* (designated *tpsA1a*) and the second island harbors a truncated *tpsB1* encoding a non-functional protein and another *tpsA1* (*tpsA1b*). Adjacent to these two genetic islands the system 2 ORFs are encoded (Fig. 1A). This system includes one *tpsB2* and two *tpsA2*s (designated *tpsA2a* and *tpsA2b*, respectively). The genetic island encoding system 3 is not linked and only encodes a single *tpsA3*, without a cognate *tpsB3*. Such a *tpsB3* has not been identified in any of the other *N. meningitidis* genome sequences available (6).

Without exception, the strains that only encode TPS system 1 have one genetic island in their genome containing one *tpsA1*in an operon with *tpsB1*, whereas the presence of the system 2 ORFs is associated with the duplication of the system 1 genetic island (6,9). The system 1 genetic islands are further characterized by the presence of several *tpsA*-related ORFs, referred to as *tpsC,* interspersed with small intervening ORFs (IORFs) (9). These cassettes appear to encode alternative C-terminal ends of the TpsA1s and appear the result of recombination events. If these recombination events occur, they are rare, since the genetic islands appear rather invariable. However, they have been shown experimentally to occur and then change the TpsA1s encoded in the active operon (9). Functions that are attributed to system 1 TpsAs are adhesion (10), promoting intracellular survival (11), biofilm formation (12) and contact-dependent growth inhibition (CDI) of other meningococcal isolates (9). In this latter functionality TpsAs are able to kill other *N. meningitidis* cells and they use their variable C-terminus to do so. Selfprotection is conferred by specific immunity proteins encoded by the *i*ORFs, also encoded by the same genetic island. Therefore, a function of the large genetic islands might be the accumulation of different *i*ORFs conferring protection to different TpsAs present in the meningococcal strain population.

The presence of multiple TPS systems in one strain, sometimes without a cognate TpsB, prompted the question whether and how the TpsAs target their cognate TpsBs. The absence of a TpsB3 suggests that, when expressed, at the least this TpsA should target a non-cognate TpsB.

System specificity of the two TpsBs of *N. meningitidis*

In **Chapter 2** we investigated the specificity of TpsB1 and TpsB2. We made use of truncated TpsA proteins that contain the signal sequence and TPS domain of a TpsA. The set of TPS domains included all the TpsAs of *N. meningitidis* H44/76, those of *Neisseria lactamica* and TpsA of *Haemophilus influenzae* and PA0690 TpsA of *Pseudomonas aeruginosa*. The experiments showed that the TpsB2 has a relaxed specificity and can secrete all TPS domains of *N. meningitidis* and *N. lactamica* that we tested, albeit with different efficiencies. In contrast, TpsB1 is more specific and secretes only the TPS domain of system 1 TpsAs of *N. meningitidis* and one TPS domain of the *N. lactamica* system 2. The TPS domain of TpsA3 was efficiently recognized and secreted by the TpsB2, indicating that the full-length TpsA3 might also be secreted via this TpsB2 (13).

The TPS domain is the minimal part of the TpsA that can be secreted (14) and is thought to engage the TpsB first (5). Additional regions within the TpsA might also be involved in regulating and advancing system specific secretion that could block TpsB2-mediated secretion. In **Chapter 3** we show that TpsB2 is also able to secrete full-length TpsA1, although only when overexpressed from plasmid. No secretion of the TpsA1s of H44/76 was observed at endogenous TpsB2 levels. This result suggests that the recognition is poor, as was also apparent from the decreased efficiency of TPS1 secretion by TpsB2. On the other hand these results also suggest that once an interaction between the TpsA and the TpsB is established, the secretion process can be completed.

The results presented in **Chapter 3** also indicated that TpsA1 protein secreted by TpsB2 is different (because ~200 kDa secreted TpsA1 specie is absent) from TpsA1 secreted by TpsB1. We therefore tested whether TpsB2-secreted TpsA1 proteins would be active as CDI toxin. Remarkably, TpsB2-secreted TpsA1 was not active as a CDI toxin, whereas the TpsB1-secreted TpsA1 protein species were. Apparently, transport to the cell surface is not sufficient for TpsA1toxin activity. Probably TpsA1 proteins are processed during
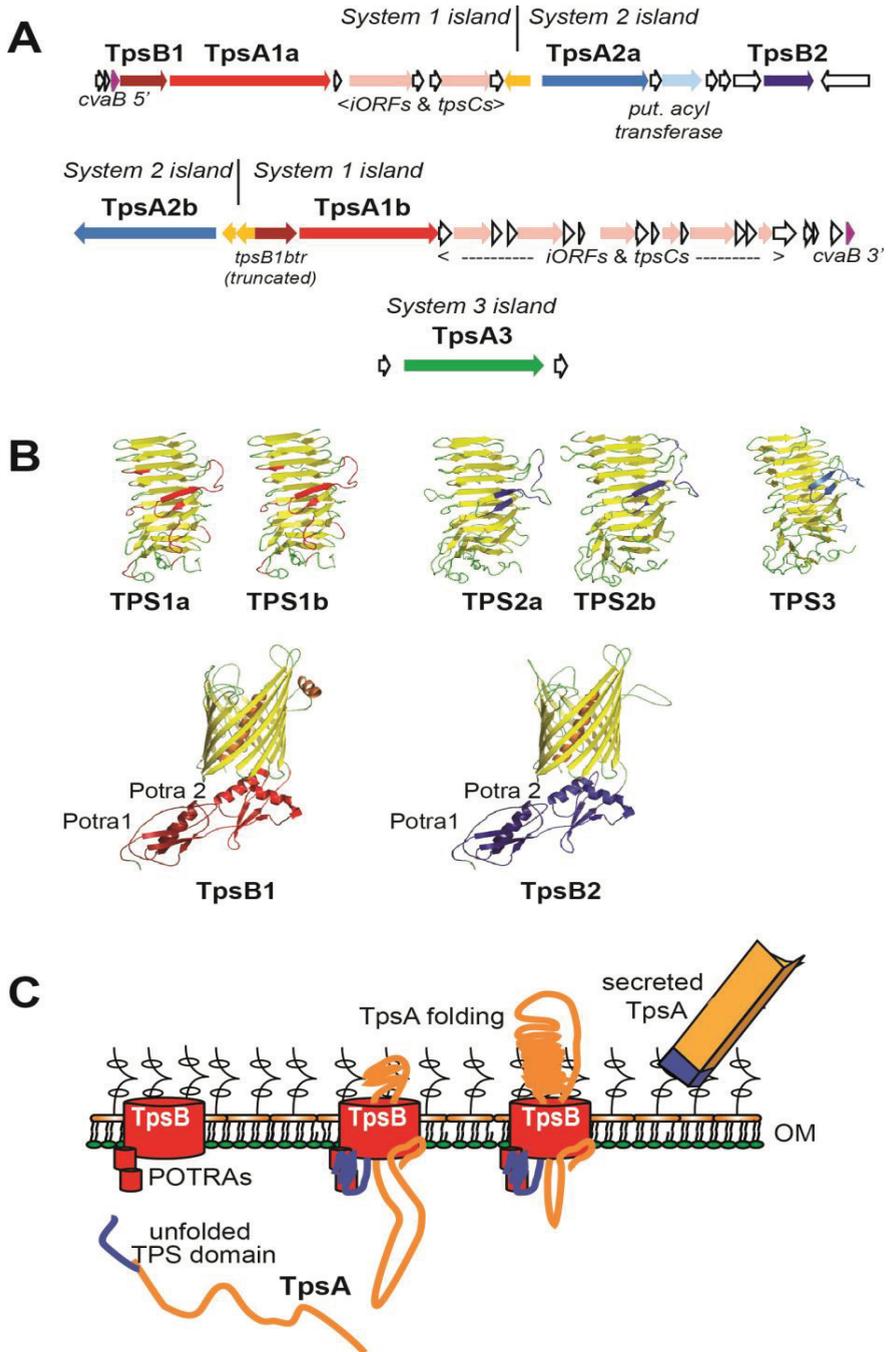
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Fig. 1 Schematic representation of the TPS secretion systems of *Neisseria meningitidis* H44/76. A. The three meningococcal TPS systems are encoded on five genetic islands, of which the two islands of

system 1 and system 2 are linked. Arrows denote the ORFs. Indicated above the genomic islands are their designation (System 1 island etc.) and the TPS system protein(s) encoded. Below the genomic islands are other interesting features; the cvaB ORF is disrupted by the insertion of genomic islands (3); the system 2 ORF encoding an acyl transferase, putatively involved in modifying TpsA2s. Colour coding: Red, system 1 ORFs; blue system 2 ORFs, green, system 3 ORFs; yellow, transposon derived sequence encoding DNA modifying enzymes (transposases etc.). B. Structural models of the TPS domains of the five TpsAs encoded in the N. meningitidis H44/76 genome (top row; (13)) and of their TpsB transporters [ur Rahman Chapter 5]. Colour-coding: red, protein sequences proposed to be involved in system-specific secretion of system 1 proteins; protein sequences proposed to be involved in the secretion of system 2 (dark) and system 3 proteins (light blue). C. Cartoon of the subsequent steps in the secretion of the TpsA (blue/yellow) across the outer membrane (adapted from (4)). The TPS domain (blue) and the POTRAS in the TpsB transporter are indicated (Red). Transport of a TpsA across the outer membrane is thought to be a folding-driven process (4). The TPS domain remains attached to the TpsB during that process (23).

secretion (6,13). We, therefore, hypothesize that the TpsB1 mediates a processing event that activates TpsA1. Such a function has been described for ShlB, the TpsB that mediates secretion of ShlA of *Serratia marcescens* (15). ShlA is active as a hemolysin. The secreted protein in its active form results from a processing step that alters the ShlA conformation. This conformational change seems to be induced by the ShlB, since mutants of ShlB have been described that can still secrete the ShlA, but only in an inactive conformation. A similar processing event may require the interaction of the neisserial TpsA1 with a TpsB1 to activate the toxin activity, while the TpsB2 is not able to perform that step, despite its ability to transport the TpsA1 across the OM to the cell surface. However, the TpsA1 secreted by TpsB2, although not active as CDI toxin, might be able to perform the other functions attributed to TpsA1, such as adhesion [10] intracellular survival [11] and biofilm formation [12]. The repeated sequences within the TPS-containing genetic islands indicate at possible genetic recombination events and horizontal gene transfer. If that occurs, the presence of a TpsB transporter with broader range of substrates might be beneficial, as observed with the secretion of TpsA3.

The POTRA domains and linker function in binding of and specificity for TpsAs
TpsB proteins belong to the Omp85 protein family, which also includes BamA proteins. BamA is part of the protein complex that inserts β-barrel proteins into the outer membrane (16). This family of proteins is characterized by a C-terminal 16-stranded membrane-embedded β-barrel domain preceded by one to five so-called polypeptide transport-associated domains (POTRA) that protrude into the periplasm. There are crystal structures available for four Omp85 family members: *i.e.* FhaC of *Bordetella pertussis* (17), BamA proteins of *Hameophilus ducreyi* and *Neisseria gonorrhoeae* (18) and TamA of *E. coli* (19). TpsBs contain an array of two POTRA domains, an α helical domain (H1) located within the barrel and an unstructured linker region that connects H1 to the first POTRA domain. The linker region and H1 domain are not shared with other members of the Omp85 family, but can be found in the majority of TpsBs.

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There are no crystal structures for the TpsBs of *N. meningitidis*, but in **Chapter 4** we have used molecular models to assign these POTRA domains to TpsB1 and TpsB2 sequences (Fig. 1B). POTRA domains are thought to bind the TPS domains of TpsAs, since the POTRA domains of HMWB and FhaC have been shown to interact with their cognate TPS domains in *in vitro* experiments (20-22). Furthermore, for FhaC and FhaB

this interaction is prolonged during the complete secretion process (23). To establish the functions of the two POTRA domains of the neisserial TpsB1 and TpsB2 in the secretion of their cognate TpsAs, we have made use of the difference in substrate specificity. We constructed TpsB mutants with deleted, duplicated and exchanged POTRA domains and tested whether these were able to secrete either the TPS domains present in the truncated TpsA constructs or full-length TpsAs. Similar to what was observed for FhaC of *B. pertussis* (17), deletion of one or two POTRAs, or duplicating either POTRA1 or POTRA2 of a neisserial TpsB resulted in absence of secretion. However, exchanging the two POTRAs of a TpsB for those of the other TpsB resulted in a switch in specificity for TpsA substrates. Furthermore, exchanging a single POTRA domain showed that each of the two domains contributed to the selection of a TPS domain (Chapter 4). Previous studies on TPS systems showed that FhaC of *B. pertussis* only recognizes its cognate TpsA partner FhaB and not the hemolysin HmpA of *Proteus mirabilis*. However, the very closely related TpsB ShlB of *Serretia marcescens* was able to secrete and activate HpmA (24), suggesting a relaxed specificity within similar systems. Remarkably, the order of POTRAs in TpsB2 could be reversed without affecting substrate selection, but this change did result in alternative processing of the secretion product. This flexibility of POTRA domains is not a general feature in the Omp85 protein family, since the POTRA domains of the BamAs of *E. coli* and *N. meningitidis* could not be swapped, suggesting that additional interactions are needed when these proteins function to insert OMPs in the OM of the respective organisms (25).

Overall, our results underscore the pivotal role of the TpsB POTRA domains in specific interaction with the TPS domain of the substrates. Furthermore, this interaction initiates secretion and, as was suggested by the results of **Chapter 3** as well, once secretion of a TpsA has started, it is completed. Apparently, both the β-barrel domain of TpsB, and the TpsA sequences downstream of the TPS domain are not required for a specific interaction. Of note, the β-barrel domain of TpsB includes the highly conserved loop 6, which folds inwards into the β-barrel channel (17). This loop contains highly conserved motifs that are important for the secretion process, since their mutation abolishes secretion of truncated TpsA constructs (17,26). Our results suggest that this loop may not provide cargo-specific binding pocket, since hybrid TpsB1 with POTRA domains of TpsB2 secretes TPS2a domain, which is not secreted by wild type TpsB1 (**Chapter 2**)

In **Chapter 5** we extended our analysis of the role of conserved structures of the neisserial TpsBs in the secretion process to the H1 domain, the linker region and the pair of cysteines that are in the POTRA1 domain. The H1 α-helix in the FhaC crystal structure is inserted in the β-barrel channel axis and partially blocks it (17). Deleting the H1 region of FhaC did not affect secretion and we show here that also deleting H1 from TpsB1 or TpsB2 did not affect the secretion of neisserial TpsAs. However, deletion of H1 did affect the biogenesis of the TpsBs, since the amount of TpsBs detected was reduced. Nevertheless, the TpsB2-ΔH1 mutant appeared well-folded in the OM, since the mutant showed a heat-modifiability similar to the wild-type protein. This could indicate that the α-helical domain has a role in targeting of the TpsB to the OM. On the other hand also the stability of the protein in the OM might have been affected. For autotransporters, which also have an α-helical segment inserted in the channel of their C-terminal β-barrel domain, deletion of this helix affects the stability of the domain (27,28). Furthermore, protease-protection assays suggested that the α-helix is inside the barrel channel early in the biogenesis, prior to full insertion of the β-barrel in the OM (29).

Deleting the linker region that connects H1 to POTRA1 did affect the secretion of TpsA1s by TpsB1, since only a reduced amount of cell-associated TpsA1 was detected and

no TpsA1 in the culture supernatant. In contrast, the analogous deletion in TpsB2 did not affect the secretion TpsA2s. Apparently, the linker region contributes to the interaction interface with the TPS domains of TpsA1s, for example by having additional contacts with the TPS domain, or by aligning the POTRA domains in such a way that they promote full secretion. The antibiotic sensitivity assay indicated that the pore of TpsB1 is still partly closed upon deletion of only the linker region in the TpsB1-ΔL, when compared to TpsB1-ΔH1L, suggesting that the linker region still interacts with the β-barrel domain, which could facilitate the positioning of POTRA domains. Such a facilitating activity is clearly not needed for secretion of TpsA2s by TpsB2 and it could be one of the reasons that TpsB1 is more specific than the TpsB2. However, it is not a decisive feature in the secretion process, since in the TpsB mutants with exchanged POTRAs (30) the linker region remained unchanged.

We have shown in **Chapter 5** that mutating the conserved disulfide bond present in TpsB proteins affected TpsB expression, but did not abolish secretion. The expression level of the TpsB2-C86A mutant was reduced, indicating a problem in biogenesis, or an increased degradation due to a reduced stability of the protein in absence of the disulfide bond.

Overall, the results in **Chapter 4** and **Chapter 5** highlight the important role of the POTRA domains in recognition and binding of the TpsA and the initiation of the secretion process. Additional factors in the periplasmic domains of TpsB play an accessory role in the process, but the binding to the POTRAs appears to be the decisive step.

Outlook for the secretion process

In **Chapter 1** we have provided an overview of the current knowledge on the mechanism of secretion of TPS systems (Fig. 1C). This knowledge is largely based on the experimental work on the FhaC/FhaB system of *B. pertussis*, HmwB/HmwA system of *H. influenzae* and the ShlB/ShlA system of *Serretia marcescens* (4,31). Our work on the *N. meningitidis* TPS systems showed how in the secretion process the secreted protein is targeted to its transporter in the OM. This is an important part in the process, since multiple TPS systems can be co-expressed in a single cell. System-specific targeting is sometimes required for functioning. For example, the system 1 TpsAs in *N. meningitidis* act as CDI-toxins towards other *N. meningitidis* cells, but this activity requires secretion by the system-specific TpsB. The non-cognate TpsB2 might secrete a TpsA1, but then an essential step is missing, rendering the TpsB2-secreted TpsA1 inactive as CDI toxin.

From the work presented here (30) and by others (21,22) it is clear that the interaction between the TPS domain and the TpsB POTRA domains decide whether a TpsA is secreted or not. Similar receiver functions have also been attributed to POTRA domains of other members of the Omp85 family (32,33). Sequence analysis of the TPS domains (5) suggested a very conserved NPN/FL motif that was initially proposed to be part of the interaction interface and mutation of that motif in ShlA and FhaB, indeed, abolished secretion (5,34). However, the crystal structures of the TPS domains of FhaB, HMWA and HpmA, a hemolysin of *Proteus mirabilis*, indicated that this motif is a structural motif and mutations in this region likely affect the overall conformation (14,35,36). The solved structures indicated a highly similar β-helical fold for the TPS domains that appears to confer structural stability to the protein. This β-helical conformation has also been proposed for the remainder of the TpsA (37,38). However, on one side of the β-helical backbone the TPS domain is covered by a small extra sheet of four β-strands (or three β-strands and an α-helix in the HMWA TPS domain) (Fig. 1B). When compared, the TPS domains of the *N.*

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meningitidis TPS systems showed a fairly high degree of sequence similarity, but they are secreted via different TpsBs. When the sequence variation between the TPS domains of the different neisserial systems was mapped onto the structural models of these domains, we observed that they localize to the extra sheet and the β-strands that it is built from (Fig 1B)(13). We hypothesize that these specific differences might be indicators of the regions of the proteins that are involved in the recognition process.

Similarly, a specific variable domain can be identified in the POTRA domains of the neisserial TpsBs. TpsB POTRAs have a structural β-linker-α-β-β and β-linker-α-α-β-β configuration for POTRA1 and POTRA2, respectively (Fig. 1B), The major differences map to the linker-α-α moiety. The POTRA domains are modeled to interact with their substrates via β-augmentation; *i.e.* the aligning of β-strands from different polypeptides in one β-sheet (39). Such an arrangement was detected in the crystal structures of the BamA POTRA domains (32) and the TamA protein (19), in which dimers formed between POTRAs of adjacent polypeptides, by sliding an extra β-sheet in the three-stranded POTRA β-sheet, rendering it a 4-stranded β-sheet. We hypothesize that the interaction between the TpsB POTRAs and the TPS domains involves such β-augmentations in the individual POTRAs and thus could involve the β-sheet appendage of the TPS domain. Of note, the TPS domain is probably not fully folded during translocation, since a folded TPS domain does not interact with POTRAs *in vitro* (21). Furthermore, the differences in the POTRAs of the *N. meningitidis* TpsBs are not located in the β-sheets, but their position indicates that they could result in a different arrangement of the POTRA β-sheets, and thus have an indirect effect.

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