The role of the TpsB transporters in Two-Partner Secretion systems of *Neisseria meningitidis*

Sadeeq ur Rahman

2014
The research described in this thesis was conducted at the Department of Molecular Microbiology, VU University, and financially supported by Higher Education Comission (HEC) of Pakistan and VU University, Amsterdam, The Netherlands.

Cover:
Type V pastel - oil chalks on paper 28/42 cm - by Ovidiu Pop (ovidius.art@gmail.com)

Printed by: Off Page
Copyright © 2014 Rahman S.u., Amsterdam. All rights reserved. No part of this thesis may be reproduced or transmitted in any form, by any means, electronic or mechanical, without the prior written permission of the author, or where appropriate, of the publisher of the articles.

Publication of this thesis was partly financed by Pfizer.
The role of the TpsB transporters in Two-Partner Secretion systems of *Neisseria meningitidis*

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. F.A. van der Duyn Schouten,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de Faculteit der Aard- en Levenswetenschappen
op dinsdag 24 juni 2014 om 13.45 uur
in de aula van de universiteit,
De Boelelaan 1105

door

Sadeeq ur Rahman

egenomen te Khall, District Dir, Pakistan
promoter: prof. dr. W. Bitter

copromotoren: dr. J.P. van Ulsen
dr. J. Luirink
Table of Contents

Preface 6

Chapter 1  Type V secretion: from biogenesis to biotechnology 14
Biochim Biophys Acta. 2013 Nov 22. pii: S0167-4889(13)00397-2

Chapter 2  System specificity of the TpsB transporters of coexpressed Two-Partner Secretion systems of Neisseria meningitidis 58

Chapter 3  The Two Partner Secretion transporter TpsB2 of Neisseria meningitidis secretes a non-cognate full-length TpsA1 but not to full functionality article in preparation 80

Chapter 4  The Polypeptide Transport-associated (POTRA) Domains of TpsB Transporters Determine the System Specificity of Two-Partner Secretion Systems 104
J Biol Chem. 2014. submitted

Chapter 5  Role of conserved N terminal motifs of TpsB transporters in Two-Partner Secretion systems 124

Chapter 6  General Discussion 144

Appendices  Samenvatting/ Dutch summary 156
Summary in Urdu 159
Summary in Pashto 161
Acknowledgments 162
Curriculum vitae 164
Publications 165
The role of the TpsB transporters of Two-partner secretion systems of *Neisseria meningitidis*

1. Meningococcal infections

*Neisseria meningitidis* (meningococcus) is a relatively common Gram-negative bacterial species that inhabits the mucosal membrane of the nose and throat. Up to 5-10% of a population can be asymptomatic carriers of this bacterium, which usually causes no harm. However, occasionally infection with *N. meningitidis* can lead to meningitis, which is an infection of the meninges, a thin layer of membranes around brain and spinal cord, or to meningococcemia, a life-threatening sepsis [1]. In rare cases, meningococcal infection can also lead to pneumonia and localized infections. As such, *N. meningitidis* is a major cause of morbidity and mortality during childhood in industrialized countries and has been causing large outbreaks and epidemics in Africa and Asia. *N. meningitidis* spreads through person-to-person contact, via infected exhaled droplets and respiratory secretions such as saliva. The incidence of meningococcal infection is higher in infancy, adolescents and adults older than 65 years [2, 3]. Risk factors include long-term close contact with an infected patient or direct contact with the respiratory secretion of an infected individual, smoking and mucosal lesions with a concomitant respiratory infection. Other high-risk conditions are complement deficiencies, community outbreaks, functional or anatomic a-splenia, and traveling to high-risk areas where meningococcal infections are prevalent.

*N. meningitidis* strains can be classified into 13 serogroups based upon their polysaccharide capsule. Of these thirteen, only five are associated with disease: serogroups A, B, C, Y and W135. Serogroup B strains cause about two-thirds of the disease cases in children younger than 6 years of age [4] and is the most important cause of endemic meningitis in industrialized countries, accounting for 30-40% of the cases in North America and for up to 80% in some European countries. The burden of annual invasive meningococcal disease associated with clinical manifestations such as meningitis and/or sepsis has been estimated to be at least 1.2 million with 135,000 deaths worldwide [5].

The onset of meningococcal meningitis disease symptoms is sudden and death can follow within hours. Diagnosis is usually based on clinical symptoms, followed by a lumbar puncture to obtain cerebrospinal fluid. Bacteria can be observed directly by microscopic analysis (Gram-negative stained diplococci) and their presence can be confirmed by culturing, agglutination or polymerase chain reaction (PCR). These last methods are also used to detect meningococcal bloodstream infections. Treatment usually involves the use of cephalosporin-like antibiotics, but even then severe side effects of the disease cannot be excluded. Therefore, vaccination appears to be the best strategy to prevent disease. Currently, conjugated polysaccharide vaccines against (combinations of) serogroups are available for serogroups A, C, Y, and W-135. However, for serogroup B a polysaccharide vaccine is not preferred, mainly because of the homology of its polysaccharide to structures in human neurologic tissue, which increases the risk of low or auto-immune responses. Therefore, research has focused on other antigens suitable to include in a vaccine against serogroup B disease isolates. Several sub-capsular but surface exposed proteins have been identified that seem to
have promising characteristics, including factor H binding protein, Neisserial adhesin A, and Neisseria-heparin binding antigen [6]. Notably, these antigens are conserved in meningococcal serogroups, suggesting the potential to protect against both serogroup B and additional serogroups. Main challenge in developing a new meningococcal serogroup B vaccine is how to cope with strain variability.

2. The cell-envelope and surface of *N. meningitidis*

The *Neisseriaceae* include two strictly human pathogens; *Neisseria meningitidis* (meningococcus) and *Neisseria gonorrhoeae* (gonococcus), while most other members of the family are considered commensals. Most of the studies in this thesis were performed using *N. meningitidis* serogroup B strain H44/76 (GenBank accession no. AEQZ00000000) [7], which was initially recovered from a patient in Norway in 1976 [8]. This strain is closely related to the serogroup B strain MC58 (GenBank access- no. AE002098) that was among the first bacterial strains whose genome was sequenced [9]. *N. meningitidis* is a Gram-negative diplococcus with a cell envelope consisting of two membranes: an outer membrane (OM) and an inner membrane (IM) separated by the periplasm, which contains the peptidoglycan layer. This cell envelope is covered by the polysaccharide capsule, which protects the bacteria against the innate immune system in the blood stream and is, as such, a major virulence determinant. The IM of the cell envelope is a symmetrical phospholipid bilayer, while the OM is an a-symmetric bilayer constituted of phospholipids in the inner leaflet and lipopolysaccharide (LPS) in the outer leaflet. Furthermore, integral IM proteins usually span the membrane by means of hydrophobic α-helical segments, whereas integral OM proteins usually con-
translocon. The TpsB then inserts in the OM and initiates the translocation of its specific TpsA partner across the OM. The processed TpsA protein contains a TPS domain at the N terminus that binds to the N terminal poly-peptide-transport-associated (POTRA) domains of the TpsB transporter to mediate secretion.

The genomes of *N. meningitidis* analyzed to date carry up to three TPS systems, indicated as systems 1-3 [11, 12]. The distribution of the three systems differs between individual isolates. System 1 is ubiquitous among *N. meningitidis* strains. In contrast, systems 2 and 3 are more frequently found in isolates from disease-causing invasive clonal complexes [12]. Functional studies have, thus far, mainly focused on system-1 TpsAs. Surprisingly, these proteins have been described to play a role in highly diverse processes, including adhesion to, invasion of and survival in cultured human cell lines [16, 17], biofilm formation [18] and bactericidal toxin activity towards other *N. meningitidis* isolates [19]. For systems 2 and 3 no functions have been described, but the proteins appear to be expressed during infection, since sera of patients recovering from the disease contain antibodies recognizing TpsAs [12].

**Scope of the thesis**

Secretion of TpsAs by their cognate TpsBs has been described to be a specific process [20]. The presence of multiple TPS systems in one strain of *N. meningitidis* [12], of which one is without a cognate TpsB, prompted the question whether and how the TpsAs target their cognate TpsBs. In the work described in this thesis we investigated how the selection of cargo by TpsB works and which parts of the protein are involved in selection and subsequent secretion of TpsA.

In **Chapter 1**, we provide a review of the current knowledge on Type V secretion, focusing on the classical monomeric autotransporters and TPS system subclasses. We provide an overview of their biogenesis and functions and provide current models for the mechanism of secretion across the cell envelope. Furthermore, we highlight biotechnical applications of autotransporters.

In **Chapter 2**, we investigated selectivity of different neisserial TPS systems. Generally, Gram-negative bacteria encode *tpsA* and *tpsB* ORFs together in an operon [ ]. However, *N. meningitidis* strains encode up to three TPS systems, of which system 1 and system 2 include a TpsB and two TpsAs (encoded by *tpsB1, tpsA1a* and *tpsA1b* and *tpsB2, tpsA2a* and *tpsA2b*, respectively), while system 3 comprises only a *tpsA3* and no dedicated *tpsB* [12]. We show that TpsB2 is less selective and can secrete all neisserial TPS domains tested, including those from *N. lactamica*. On the other hand, TpsB1 is rather specific for its cognate TPS1 domain. Thus the singular TpsA3 without a dedicated TpsB [11] is more likely secreted via TpsB2.

The findings described in Chapter 2 laid the foundation for **Chapter 3**. The specificity we had observed for the neisserial TpsBs was based on the recognition and secretion of the ~30-kDa TPS domains instead of the full length TpsA, which are considerably larger (up to > 200 kDa). In **Chapter 3**, we investigated whether TpsB2 would allow secretion of full-length non-cognate TpsA1s. We show that TpsB2 is indeed able to secrete full-length TpsA1s. Surprisingly, heterologously secreted TpsA1 is not functional.

Outer membrane-embedded TpsB proteins contains two moieties, a periplasmic protrusion, including two POTRA domains, and a C-terminal outer membrane-embedded 16-stranded β-barrel. **Chapter 4**, describes the role of the POTRA domains in the selection of TPS domains for secretion and how this influences the system-specificity...
of TpsB1 and TpsB2 (which showed a reduced specificity). Our results show that the POTRA domains are crucial for the selectivity of the secretion process.

In Chapter 5 we characterize the role of other conserved features of TpsB transporters, in particular the N-terminal conserved α-helical domain, the linker region that connects this helix to the first POTRA domain and the conserved pair of cysteines located within POTRA1. We show that the cysteines and the linker region are involved in the secretion process and all domains have a role in TpsB biogenesis. Finally, in Chapter 6 the data presented in this thesis are summarized and discussed.

Neisseria meningitidis. Cellular Microbiology 10, 2461-2482.


Preface
Type V secretion: from biogenesis to biotechnology

Peter van Ulsen¹, Sadeeq ur Rahman¹, Wouter S. P. Jong¹,², Maria H. Daleke-Schermerhorn¹,², Joen Luijrink¹,²

¹Section of Molecular Microbiology, Department of Molecular Cell Biology, VU University, 1081 HV Amsterdam, The Netherlands.
²Abera Bioscience AB, SE-111 45, Stockholm, Sweden

Biochim Biophys Acta. 2013 Nov 22. pii: S0167-4889(13)00397-2
Type V secretion: from biogenesis to biotechnology

The two membranes of Gram-negative bacteria contain protein machines that have a general function in their assembly. To interact with the extra-cellular milieu, Gram-negatives target proteins to their cell surface and beyond. Many specialized secretion systems have evolved with dedicated translocation machines that either span the entire cell envelope or localize to the outer membrane. The latter act in concert with inner-membrane transport systems (i.e. Sec or Tat). Secretion via the Type V secretion system follows a two-step mechanism that appears relatively simple. Proteins secreted via this pathway are important for the Gram-negative life-style, either as virulence factors for pathogens or by contributing to the survival of non-invasive environmental species. Furthermore, this system appears well suited for the secretion of biotechnologically relevant proteins. In this review we focus on the biogenesis and application of two Type V subtypes, the autotransporters and two-partner secretion (TPS) systems. For translocation across the outer membrane the autotransporters require the assistance of the Bam complex that also plays a generic role in the assembly of outer membrane proteins. The TPS systems do use a dedicated translocator, but this protein shows resemblance to BamA, the major component of the Bam complex. Interestingly, both the mechanistic and more applied studies on these systems have provided a better understanding of the secretion mechanism and the biogenesis of outer membrane proteins.

1. Introduction

The secretion systems in Gram-negative bacteria that are classified as Type V comprise until now the subclasses Types Va-e [1,2]. These five subclasses share structural features, in that they include for transport a β-barrel protein or domain that is embedded in the outer membrane. Moreover, they all depend on the Sec complex for translocation across the inner membrane, whereas the Bam complex in the outer membrane contributes to the translocation of the secreted protein to the cell surface. The Sec complex plays a generic role in the transport of soluble proteins to the periplasmic space and the insertion of integral inner membrane proteins into the membrane. The Bam complex facilitates the folding of outer membrane proteins into a β-barrel conformation and their insertion into the outer membrane. Of the Type V subclasses, Type Va, the classical monomeric autotransporters, and Type Vb, the two-partner secretion (TPS) systems, have been studied in greatest depth and will be the focus of this review. The other subclasses are Type Vc comprising the trimeric autotransporters [3], Type Vd comprising the patatin-like autotransporters with a distinct C-terminal transport domain that resembles the translocation unit of the TPS system [4] and Type Ve, which comprises the intimin/invasin family of proteins that resemble classical autotransporters, but with their domains in reversed order [5,6]. Autotransporters are found in all Gram-negative bacterial genera, but not in all species of which genome sequences are available [7]. They are multi-domain proteins (Fig. 1A; [8,9]) that include a signal peptide at the N terminus for targeting to the Sec machinery to mediate inner-membrane translocation. During translocation, the signal peptide is cleaved off, the matured protein is released into the periplasm and the C-terminal β-domain inserts into the outer membrane. During or after its
insertion the β-domain facilitates outer membrane translocation of the passenger domain which in the precursor protein is located between the signal peptide and the β-domain. For this reason the β-domain is also called translocator domain. Passenger translocation proceeds from C- to N-terminal direction [10] in a hairpin conformation through the translocation channel and both the insertion of the β-domain and the translocation of the passenger require the active involvement of the Bam complex. A similar involvement of the Bam complex has also been described for the trimeric autotransporters (type Vc) [11] and type Ve systems [5,12]. At the cell surface, most autotransporter passengers are proteolytically cleaved and then either remain attached to the cell surface via non-covalent interactions, or are released into the extracellular milieu. The passenger domains vary in sequence and length and carry functional subdomains that are invariably involved in interaction with the environment. Some autotransporters are post-translationally modified; e.g. the AIDA-1 adhesin of Escherichia coli is glycosylated by a dedicated glycosyltransferase that is active in the cytoplasm [13]. Another example is the NaIP protease of Neisseria meningitidis which is lipid-modified during its transfer across the cell envelope [14].

The mechanism of translocation across both membranes and the involvement of the Sec and Bam complexes will be discussed in this chapter. A more detailed understanding of the molecular details of this process is required to improve the performance of autotransporters as carriers for secretion or surface display of recombinant proteins [15,16]. Current roadblocks for these applications will be discussed.

Unlike autotransporters, TPS systems consist of two proteins: a secreted protein generically named TpsA and an outer-membrane inserted transport protein, TpsB (Fig. 1B; [17,18]). The TpsA and TpsB proteins both include an N-terminal signal peptide for Sec-mediated transport across the inner membrane. Upon arrival in the periplasm, TpsB inserts into the outer membrane as a 16-stranded β-barrel with a large periplasmic domain that includes two POTRA motifs (for polypeptide transport associated domains). The TpsB proteins show homology to the BamA protein, the major component of the Bam complex [19]. The TpsA protein, after cleavage of the signal peptide, carries at its N terminus a conserved domain called the TPS domain that targets TpsB in the outer membrane. Upon recognition, secretion of TpsA across the outer membrane by the TpsB protein is initiated. At the cell surface, the TpsA protein may be released or remain attached to the cell surface via non-covalent interactions. Similar to the autotransporters, the secreted
TpsA proteins vary in function and sequence. Furthermore, post-translational modifications of TpsAs by glycosylation, as shown for the High molecular weight (HMW) adhesin of Haemophilus influenzae [20], may add to their functionality or prevent degradation in a hostile environment.

In this chapter we first describe the functions of different autotransporter and TPS systems. We then discuss the available crystal structures and the mechanism of translocation across the cell envelope. We finish by discussing the use of autotransporters for biotechnological applications and how recent mechanistic insights in autotransporter secretion could be used to optimize their use.

2. Functions of autotransporter passengers and TpsA proteins of TPS systems

2.1 Autotransporters

Autotransporters play important roles in the virulence and survival of both pathogenic and environmental Gram-negative bacteria. The passenger domains are highly diverse and carry the specific autotransporter functions which may be enzymatic, proteolytic, (cyto)toxic or adhesive, and contribute to colonization, immune evasion and biofilm formation (Table 1). The best characterized autotransporters are the secreted serine protease autotransporters of Enterobacteriaceae (SPATEs). However, it should be noted that this versatile protein family includes a much larger repertoire of functions than those described here [7,21].

2.1.1 SPATEs and SPATE-like proteases

The SPATEs are present in pathogenic E. coli strains and other pathogenic enterobacterial relatives [21,22]. The SPATE passengers all carry a trypsin-like serine protease subdomain at their N terminus, characterized by a [GDSGS] motif (catalytic serine residue in bold), an unusually long signal peptide (see below), a highly conserved β-domain (60-99% identity at the amino acid level), and a characteristic asparagine-asparagine motif that separates the passenger domain from the β-domain [23,24]. After translocation across the cell envelope all SPATE passengers are cleaved and released into the extracellular milieu in a manner that is not dependent on the serine protease activity but is the result of an autocatalytic cleavage event inside the β-barrel [25,26,27]. Despite significant sequence similarities, the SPATEs display distinct substrate specificities [22,23,24,28]. Based on phylogenetic relationships and functional similarity, SPATEs have been divided in two groups [22]. Members of the first group, including Pet, EspC, EspP, Sat and SigA, show cytopathic effects on host epithelial cells through internalization and cleavage of intracellular host proteins [24]. For example, Pet and EspC target the actin-binding protein α-fodrin (spectrin), which affects the cytoskeleton [29,30]. In contrast, members of the second group cleave primarily extracellular proteins [24]. Examples include Pic, which is thought to promote colonization of intestinal and extra-intestinal strains by cleavage of mucin [31], and Hbp, which binds and cleaves hemoglobin [32]. The heme-binding properties of Hbp could supply bacteria with a source of iron during infection. A vital role for Hbp during infection was supported by the observation that production of Hbp promotes the growth of E. coli and B. fragilis under iron-limiting conditions, as well as the formation of abscesses in a mouse model [33]. Interestingly, in a recent report both Pic and Tsh (with only one residue being different from Hbp) were shown to cleave glycoproteins located on the surface of host immune cells, which might trigger immune evasion [28]. Unfortunately, due to the lack of suitable animal models, it is in most cases not known if the proteolytic activity of SPATEs in vitro is relevant for their function in vivo in (entero)bacterial...
Type V secretion systems

The majority of SPATES are trypsin- or chymotrypsin-like serine proteases [23]. SPATE-like proteases are also found outside the Enterobacteriaceae, and include the IgA1 proteases (IgAP) of Neisseria and Haemophilus, and Hap of non-typeable H. influenzae [34,35,36]. Unlike the SPATEs, IgAP and Hap carry an N-terminal serine protease domain that is involved in autoproteolytic cleavage [8,37]. The secreted IgAP passenger domain is further processed into an N-terminal mature domain that includes the serine protease activity, a small γ-peptide and a C-terminal α-peptide[8]. The latter subdomain remains attached to the cell surface where it contributes to biofilm pathogenesis.

Fig. 1. Schematic representation of the domain organization of autotransporters and the TpsA and TpsB proteins of TPS systems. (A) Autotransporters show a general tripartite domain organization with a signal peptide (green), a passenger (yellow-orange) and a β-domain (red). (B) Domain organization of the canonical FHA TPS system, with the secreted FhaB (TpsA; yellow-orange), which is processed into the adhesin FHA after translocation to the cell surface. Its TPS domain involved in recognition of FhaC is indicated in pale orange. FhaC (TpsB; red) functions as transporter of FhaB in the outer membrane, with the periplasmic POTRA domains indicated in pale red. Both proteins carry a signal peptide (green). Indicated above the proteins are the names of subdomains (in VacA, IgA protease and FhaB), indicated below the proteins are specific features that are discussed in the review (functional subdomains in yellow). The open arrowheads indicate the signal peptidase cleavage sites; the closed arrowheads indicate sites where proteolytic cleavages occur after translocation to the cell surface.
formation [38]. The mature protease domain cleaves secretory IgA1 [34] and the lysosomal glycoprotein LAMP-1 [39]. It also has immunostimulatory properties unrelated to the serine protease activity and may therefore promote bacterial survival during infection [40,41]. This suggestion was supported by the observation that N. meningitidis strains isolated from infected patients show a higher expression of the iga gene than those isolated from healthy carriers [42]. However, in infection studies in human volunteers an IgA protease deficient mutant of N. gonorrhoeae was as infective as the wild-type strain [43].

Hap of H. influenzae exists in two forms that appear to have different functions: a processed form that is released into the extracellular milieu and a non-cleaved and surface-exposed form that mediates bacterial aggregation and adhesion to host cells [44,45]. Interestingly, the serine protease activity of Hap is inhibited by secretory leukocyte protease inhibitor present in the mucosal secretions of human lungs. This inhibition leads to an accumulation of surface-exposed Hap and increased bacterial aggregation and adherence. It thus appears as if Hap enables H. influenza to take advantage of a host system that is meant to protect the human upper respiratory tract [44]. On the other hand, lactoferrin, present in human milk and a known antibacterial agent, inactivates Hap by cleaving the passenger at arginine rich sequences [46,47]. Apparently, the interaction with host factors is intricate and dependent on the tissue encountered.

2.1.2 Non-SPATE subtilisin-type serine proteases

A subset of autotransporters carries a subtilisin-like serine protease subdomain at the N terminus of the passenger. Bioinformatic analysis of genome sequences indicated that this group of autotransporters is quite large and diverse, but only few examples have been characterised functionally [7]. The subtilisin-type autotransporters include SspH1 and h2 of Serratia marcescens [48], SphB1 of Bordetella pertussis [49], AasP of Actinobacillus pleuropneumoniae [50] and NaIP of N. meningitidis [14]. Subtilisins often function in protein maturation and activation by proteolytic cleavage of the substrate. For example, NaIP cleaves autotransporters [14,51] and other cell surface exposed proteins of N. meningitidis, most notably heparin binding protein A [52] and lactoferrin binding protein B [53] and this results in their release from the cell surface. In this way, NaIP may regulate the presence of immunogenic proteins at the cell surface. Expression of the naIP gene is subject to a random genetic switch, known as phase-variation. Hence, in N. meningitidis the repertoire of cell surface-exposed proteins may vary during colonization of the host. Similarly, SphB1 has been shown to cleave filamentous haemagglutinin (FHA) of B. pertussis after it has reached the cell surface [49,54]. This maturation step is required for full functionality of FHA during infection of mice by B. pertussis [55].

Both NaIP and SphB1 contain a lipoprotein motif in their signal peptides (L-Al/S-G/A-C) [14,54]. Lipoproteins are post-translationally modified by the coupling of acyl chains to the N-terminal cysteine residue that remains as the first residue of the mature protein after cleavage of the signal peptide [56]. For NaIP this lipid modification has been shown for the surface-associated form [14]. However, the secreted passenger of NaIP that was released into the culture supernatant lacked the modification due to proteolytic cleavage of an N-terminal peptide from the passenger [14,54]. Nevertheless, the transient lipid modification appeared important for functioning of NaIP and SphB1. Substitution of the lipoprotein signal peptide for a heterologous non-
lipoprotein signal peptide did not affect secretion but did compromise functioning, since non-modified NalP failed to cleave its targets at the cell surface and in presence of non-lipidated SphB1 FHA maturation was absent [57]. Furthermore, processing and release of non-modified versions of NalP at the cell surface proceeded more rapidly [57], suggesting that the transient connection to the cell surface via the lipid moiety is important to bind substrates at the cell surface.

2.1.3 Self-associating autotransporter (SAAT) adhesins
The surface-exposed autotransporter AIDA-I is a multifunctional protein that promotes auto-aggregation by self-association [58], biofilm formation [59], and adherence of diffusely adherent enteropathogenic E. coli strains to a broad range of host cell types [60,61]. Similar functions have been described for Ag43 [62,63,64] and TibA, which can also mediate invasin [65,66]. These three E. coli proteins are grouped in the subfamily of self-associating autotransporters (SAAT) [60]. All three SAATs are glycosylated, in the case of AIDA-I and TibA by glycosyltransferases that are encoded in operons with the respective autotransporter genes [13,64,67]. Bioinformatic analysis showed that the glycosyl transferases are widespread among bacterial species and located near putative SAAT ORFs [68]. Furthermore, they appear to recognize a structural motif. Glycosylation appears to increase protein stability and is required for adhesion, but is dispensable for auto-aggregation and biofilm formation. This can be explained by observations that different phenotypes are linked to distinct regions of the individual SAATs [59,64,67,69,70,71].

The functional properties of SAAT proteins suggest that they play important roles in E. coli pathogenesis. For instance, SAAT-mediated aggregation might protect the bacteria from host defense mechanisms, such as phagocytosis and complement attack [59]. Interestingly, auto-aggregation of SAATs is most efficient at a low pH [59,65,72], which is probably relevant for bacteria that have to pass the acidic environment of the stomach on their route to the intestine [60]. Furthermore, auto-aggregation of AIDA-I is sensitive to sodium deoxycholate, a common bile salt, suggesting that it is modulated in response to environmental queues [58]. Other surface-structures, such as fimbriae and polysaccharide capsules, might mask the binding activity of shorter autotransporters, implying that a coordinated expression is required for the SAAT autotransporters to function efficiently [59,60]. Bioinformatic analysis of genome sequences identified a large group of yet uncharacterized autotransporters that show sequence similarity to AIDA-I [7].

2.1.4 Autotransporters with GDSL esterase/lipase passenger domains
The passenger domain of Pseudomonas aeruginosa EstA adopts a highly α-helical, globular fold, which is strikingly different from the right-handed β-helical stem that is the typical core structure of autotransporters [73](see section 3.1.1). This unusual AT encodes an esterase of the GDSL family of lipolytic enzymes, which remains covalently attached to the β-domain after translocation and is exposed on the P. aeruginosa surface [74]. EstA shows sequence similarity to a small group of autotransporters [7] that hydrolyze a variety of substrates in vitro [74,75,76,77,78,79] and are collectively known as the GDSL autotransporters [80]. Although the true substrates and physiological roles for the GDSL autotransporters have not been fully elucidated, their potential ability to hydrolyze lipids on the bacterial surface and surrounding environment suggests that they play important roles in vivo
For instance, *P. aeruginosa* EstA has been implicated in production of the biosurfactant rhamnolipid, motility and biofilm formation [81]. Other studies indicate that GDSL autotransporters supply the bacterial cell with essential nutrients [77,82] and building blocks for signaling molecules [77] or contribute to virulence by degrading (phosphor)lipids in the host cell membrane [76,83].

### 2.1.5 Examples of other autotransporters

A bioinformatic analysis of genome sequences for autotransporter-encoding genes identified several distinct groups, likely reflecting their functional diversity [7] (see Table 1 for examples). Three of these groups are typified by autotransporters that have been the subject of both functional and mechanistic studies; i.e., BrkA of *B. pertussis*, VacA of Helicobacter pylori and IcsA/VirG of Shigella flexneri.

---

![Figure 2: Crystal structures of autotransporters](https://example.com/fig2.png)

**Fig. 2. Crystal structures of autotransporters.** Cartoon representations are shown of (from top left to right bottom): the passenger of SPATE Hbp of *E. coli* (PDB code 1WXR) [142], the passenger P55 domain of toxin VacA of *H. pylori* (2QV3) [146], the passenger of the adhesin pertactin of *B. pertussis* (1DAB) [144], the autochaperone subdomain of the passenger of IcsA of *S. flexneri* (3ML3) [147], the full-length and unprocessed EstA of *P. aeruginosa* (3KVN) [73], the β-domain of NalP of *N. meningitidis* (1UYN) [151], the cleaved and pre-cleaved β-domain of the SPATE EspP of *E. coli* (2QOM; 3SLO) [26; 163]. All proteins are oriented similarly and we have indicated the N and C terminus in representative examples. For comparison we have added cartoons of the 12-stranded β-barrels of the Type Vc trimeric autotransporter Hia of *H. influenza* (2GR7) [158] and the N-terminal outer membrane domain of the Type Ve intimin of *E. coli* (4E1S) [157]. Autotransporter passengers contain a β-helical stem that adopts a triangular conformation, as illustrated by the bottom up view of the pertactin passenger. The passengers of the SPATES are proteolytically released from the β-domain by an intra-barrel cleavage [25; 26; 163]. The two structures for the β-domain of EspP represent cleaved (left) and pre-cleaved β-domain (right). The latter derives from a β-domain in which the cleavage site was mutated [163]. The top views show that the β-barrel dimensions remain similar and that the cleaved β-domain is stabilized by a loop that folds over the β-barrel and contacts the wall at the other side (arrowhead).
The BrkA autotransporter is involved in serum resistance and thus facilitates the survival of *B. pertussis* in its host [84]. The Vacuole-inducing cytotoxin for eukaryotic cells [85], although other functions have been suggested (Table 1). Its passenger is proteolytically processed in two separate proteins that both contribute to the cytotoxic activity [86](Fig. 1A) and form large functional oligomeric clusters [87]. Similarly, the passenger of the PmpD autotransporter of Chlamydia trachomatis is cleaved into multiple secreted proteins that form oligomeric complexes[88,89]. The IcsA/VirG autotransporter is involved in actin-based motility, which allows Shigella to move in the cytosol of epithelial cells [90,91]. Interestingly, this autotransporter is secreted near the old pole of the Shigella cell and this positioning is regulated intracellularly by a process that involves the cell division protein FtsQ [92,93]. Analysis of various (heterologous) autotransporters in *E. coli* also indicated that they initially emerge at the cell pole, prior to diffusion across the cell surface [94]. This localized appearance depended on the LPS produced by the *E. coli* strain and was much weaker when the O-antigen was absent. The phenomenon also appeared restricted to rod-shaped bacteria, since the neisserial autotransporter NalP appeared at the pole when produced in *E. coli*, whereas in *N. meningitidis* no specific site for surface exposure was observed [94].

### 2.2 TPS systems

Similar to the autotransporters, TPS systems are very diverse and widespread. Hundreds of genes encoding TpsAs and TpsBs have been found in genome sequencing projects in both pathogenic and environmental bacterial species[18]. TPS systems can be identified by searching for ORFs encoding TpsB-like proteins, as well as searching for ORFs that encode proteins with a TPS domain [17,95,96]. Full-length TpsAs vary in length from ~700 to over 5000 amino acid residues with a ~300-residue TPS domain in their N-terminal region as a general hallmark [18]. The few TPS systems that have been studied for function contribute to the virulence of pathogenic bacteria or facilitate bacterial adaptation to the environment (Table 2).

Although very often the *tpsB* and *tpsA* genes are organized in operons, this is not always the case [18,96,97]. For example, *N. meningitidis* strains encode up to three TPS systems [96]. Two of the three neisserial systems consist of two *tpsA* ORFs, of which only one is in an apparent operon with a *tpsB*, but both *tpsAs* are expressed and their products are secreted. Furthermore, a third system consists of a singular *tpsA*, without dedicated *tpsB*. This *tpsA3* appears to be expressed during infection and can be secreted via one of the TpsBs of the other systems [96,98]. TPS-containing operons may also encode additional proteins that modify the TpsA. For example, the adhesin HMW of *H. influenzae* is glycosylated in the cytoplasm by a glycosyltransferase encoded by *hmwC* which is part of the *hmw* operon [20].

TpsA proteins have been described to act as cytotoxins, adhesins, proteases and heme-binding proteins and as such have roles in the colonization and invasion of host tissues, dissemination from invaded tissues and biofilm formation (Table 2). Additionally, in recent years it became clear that many bacterial species apply a TPS system to inhibit growth of related bacterial species that compete for the same niche. We discuss here in detail the TPS systems that function as adhesins or as toxins. However other functions are conceivable in view of the high number of TpsA proteins found in genome analyses [18]. Well documented is the heme-binding activity of the HxuA of *H. influenzae* [99,100]. Secreted HxuA interacts with heme-hemopexin complexes to release
the heme so that it can be taken up by the pathogen.

2.2.1 TpsA adhesins
The *H. influenzae* adhesin HMW and the *B. pertussis* adhesin filamentous haemagglutinin (FHA) are the best studied secreted TPS systems [18,101]. The HMW adhesin is encoded in the *hmwA-C* operon of which two copies, encoding HMW1 and HMW2, respectively, can be found in *H. influenzae* strains [102]. The systems are widespread among strains isolated from human respiratory tract infections [103,104]. The HMW proteins are synthesized as pre-pro-protein HMWA. In the cytoplasm, HMWC adds mono- and di-hexoses to asparagine residues within the TpsA protein [20,105]. After transport to the periplasm and removal of the signal peptide, the remaining pro-protein is secreted via the HMWB1 transporter [106]. Then the N-terminal ~400 residues of the pro-protein are removed, including the TPS domain, to yield the functional HMW adhesin. Mutagenesis and labeling with polyethylene glycol maleimide revealed that HMW is anchored to the cell surface through a non-covalent interaction of its C terminus with HMWB [107]. Binding requires the C-terminal 20 amino acids of the protein and a disulphide bond between two conserved cysteine residues in this region.

FHA is found in the related species *B. pertussis* and *Bordetella bronchiseptica* and binds to carbohydrates, heparan sulfates and integrins exposed on ciliated epithelial cells, macrophages and the extracellular matrix in the upper respiratory tract [108,109,110,111]. The binding by FHA, in combination with its tendency to aggregate promotes formation of microcolonies and biofilms on these tissues [112]. Furthermore, FHA also modulates the immune response of the host by suppressing IL-17-mediated inflammation to evade innate immunity [113,114].

Like HMW, FHA is synthesized as a pre-pro-protein called FhaB [115]. Translocation of FhaB across the outer membrane is mediated by its TpsB FhaC [116]. At the cell surface, a ~1200 residue long C-terminal fragment is proteolytically removed from FhaB to generate the active FHA adhesin [49,117,118,119]. This cleavage involves the autotransporter SphB1, and possibly other unidentified proteases, yielding a slightly longer FHA. The resulting protein remains tethered to the bacterial cell surface, via non-covalent interactions with its N terminus, although a fraction is released into the extracellular milieu [117,118]. The SphB1-mediated cleavage appears essential for *B. pertussis* to cause infection in a mouse model [55].

2.2.2 TpsA toxins
The homologous group of cytolysins that includes ShlA of *Serratia marcescens* [120], HpmA of *Proteus mirabilis* [121,122] and EthA of *Edwardsiella tarda* [123,124] function as toxins that form pores in membranes of target cells. This activity has been studied in most detail for ShlA, which targets erythrocytes and epithelial cells [125,126]. ShlA is transported across the outer membrane by its TspB ShlB and during this step it is converted into an active toxin [127]. The actual pore-forming activity resides in the C-terminal part of the protein and requires the co-factor phosphatidylethanolamine [127,128]. However, the protein is only active when a conformational change occurs in the TPS domain of ShlA during the transport by ShlB [129,130,131]. The separate TPS domain is also secreted by ShlB and then shows the same conformational change. In this activated form it is even able to convert inactive full-length ShlA variants isolated from the periplasm into the active hemolysin in trans. The crystal structure of the TPS domain of the hemolysin HmpA of *P. mirabilis* showed that the β-strands in
Type V secretion systems

the structure (see section 3.2) were pairing with those in neighboring molecules, which could be instrumental in this conversion [132]. During secretion of ShIA, the conformational change is mediated by the periplasmic POTRA domains of ShhB, since peptide-insertions in this region resulted in the secretion of inactive ShIA variants [131,133].

TPS systems that confer contact-dependent growth inhibition (CDI) are toxin systems that target other bacteria [134]. They have been discovered in E. coli EC93 isolated from rats. Cells of this strain were able to kill E. coli K12 strains but only when the bacteria were in direct contact with each other, since E. coli K12 cells that expose pili were protected. The CDI phenotype was conferred by an operon encoding a TPS system consisting of the CdiA toxin, the CdiB transporter and the CdiI immunity protein [134,135,136,137]. The latter protects the producing cell against its cognate CdiA. Bioinformatic analysis showed that CDI systems are widespread in Gram-negative bacteria and very diverse [138,139]. The CdiA proteins studied thus far show nuclease activity that resides in the C terminus of CdiA. However, the sequence diversity of that region implies that CdiAs may also have other toxic activities. The CdiI proteins are equally diverse as the CdiA and protection is limited to the cognate CdiA.

CdiA is secreted with its C terminus extending from the cell surface. The receptor on the target cell is the BamA protein [140]. After binding of BamA, the C-terminal toxin domain is proteolytically cleaved from the N-terminal part by an unknown protease and transported into the periplasm of the target cell. Subsequently it enters the cytoplasm via the AcrB protein, which is part of a multidrug efflux pump [137,140]. CdiA targets only cells of the same or related species, due to the BamA polymorphisms that exist [141].

3. Structural features of autotransporter and TPS components

Crystal structures are available for the passenger and β-domains of various autotransporters, while for the TPS systems one structure of a TpsB transporter and several structures of TPS domains of TpsAs have been solved. The data illustrate convincingly that the two secretion systems at least involve related structural elements. The structures of the β-domain and the TpsB do not provide direct clues
on the mechanism of translocation of their cargo’s across the outer membrane, but they do indicate that there are limitations to the size of those cargo’s.

3.1 Structures of autotransporter domains

3.1.1 Structures of passenger domains

The crystal structures that have been reported for the β- and passenger domains of autotransporters include one complete autotransporter, combining both passenger and β-domain (i.e. EstA of *P. aeruginosa* [73]) and two SPATEs, Hbp and EspP, for which two separate structures of the passenger and β-domain are available [26,27,142,143]. The first passenger structure solved was pertactin of *B. pertussis* [144]. It revealed a characteristic β-helical stalk or stem (Fig. 2). The structures of the passengers solved since then, Hbp [142], EspP [143], IgA protease and Hap of *H. influenza* [45,145], VacA of *H. pylori* [146] and of the C-terminal region of the passenger of IcsA of *S. flexneri* (also called autochaperone domain, see below) [147] all show a β-helical stem structure, but also contain additional subdomains appended to that stalk. The β-helical conformation is thought to be of importance in the secretion process (see below) but also provides stability and protease resistance and contains binding sites to facilitate aggregation and receptor binding [58,60,144]. Remarkably, larger domains that protrude from the stem are positioned at the N terminus of the passenger which may be a requirement for efficient transport across the outer membrane (see below). The SPATEs EspP and Hbp and the related proteins Hap and IgA protease include a serine protease subdomain at their N terminus with a trypsin-like fold.

Sequence-based structure predictions indicated that most autotransporter passengers share this β-helical core structure [148] and for some time it was thought that this fold is a general feature of autotransporter passengers and perhaps even a requirement for secretion [1]. In contrast however, the EstA passenger was shown to consist of α-helices [73]. The recently elucidated Sca2 passenger structure of Rickettsia [149] also lacks a clear β-helical core although it must be noted that the C-terminal region contains sequences that resemble the so-called autochaperone domain that has been implicated in the initiation of secretion ([150] see section 6.1.2).

3.1.2 Structures of autotransporter β-domains

<table>
<thead>
<tr>
<th>TpsA</th>
<th>Organism</th>
<th>Function</th>
<th>ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDI systems</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BepA</td>
<td><em>Burkholderia</em> species</td>
<td>contact dependent growth inhibition, biofilm formation</td>
<td>[135]</td>
</tr>
<tr>
<td>CdiA</td>
<td><em>Escherichia coli</em></td>
<td>contact dependent growth inhibition</td>
<td>[134]</td>
</tr>
<tr>
<td>Cytotoxins and enzymatic activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LepA</td>
<td><em>P. aeruginosa</em></td>
<td>secreted protease cleaves human protease-activated receptors</td>
<td>[287]</td>
</tr>
<tr>
<td>HpmA</td>
<td><em>Proteus mirabilis</em></td>
<td>Ca2+-dependent cytolsin</td>
<td>[121,122]</td>
</tr>
<tr>
<td>EthA</td>
<td><em>Edwardsiella tarda</em></td>
<td>Cytolsin, mediosts cellular invasion in fish</td>
<td>[123,124]</td>
</tr>
<tr>
<td>ShlA</td>
<td><em>Serratia marcescens</em></td>
<td>Cytolsin, haemolysin, pore-forming toxin</td>
<td>[120,288]</td>
</tr>
<tr>
<td>Heme-binding proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HxuA</td>
<td><em>Haemophilus influenza</em></td>
<td>binding of heme and heme-hemopexin</td>
<td>[99,100]</td>
</tr>
<tr>
<td>Adhesins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HeoA</td>
<td><em>Erwinia chrysanthemi</em></td>
<td>Adhesin to plant tissue, autoaggregation</td>
<td>[289]</td>
</tr>
<tr>
<td>HlnA (HMW)</td>
<td><em>Haemophilus influenzae</em></td>
<td>Adhesin to epithelial cells</td>
<td>[101]</td>
</tr>
<tr>
<td>EspA</td>
<td><em>Escherichia coli</em></td>
<td>Adhesin, mediating binding of flagella to host cells</td>
<td>[290]</td>
</tr>
<tr>
<td>PhaB (FHA)</td>
<td><em>Bordetella pertussis</em></td>
<td>Adhesin, binds epithelial cells, biofilm formation, immunomodulation</td>
<td>[108,112,113,291]</td>
</tr>
<tr>
<td>HpaA</td>
<td><em>Neisseria meningitidis</em></td>
<td>Adhesin, intracellular survival, biofilm formation</td>
<td>[292,293,294]</td>
</tr>
<tr>
<td>MtaA</td>
<td><em>Moraxella catarrhalis</em></td>
<td>Adhesin, binding to epithelial cells</td>
<td>[295]</td>
</tr>
</tbody>
</table>
The first published structure of a β-domain was that of NaLP of N. meningitidis [151]. This structure derived from protein that was folded in vitro and showed a twelve-stranded β-barrel with its central hydrophilic channel occupied by an α-helix (Fig 2). A β-barrel fold is the general feature of outer membrane proteins and early sequence analyses already predicted this similarity [152]. The β-domain barrels share many features with other β-barrel outer membrane proteins. They consist of amphipatic β-strands, with hydrophobic residues pointing towards the environment and hydrophilic residues lining the channel inside the barrel [153]. Other distinctive features are a girdle of aromatic residues that are juxtaposed to the hydrophilic headgroups of the membrane lipids, short periplasmic loops and turns that connect the β-strands on the periplasmic side and long and flexible loops that extend from the cell surface.

Later β-domain structures of the SPATEs EspP [26], Hbp [142] and the lipase EstA [73] were derived from domains that were purified from the outer membrane, while that of BrkA also derived from in vitro folded material [154]. All showed a very similar structure with a twelve-stranded β-barrel including an α-helix positioned in the aqueous channel. In the BrkA β-domain the α-helix was not visible perhaps due to flexibility. This basic module of the β-domains is in some cases extended by a cell surface exposed portion.
that remains attached after the proteolytic cleavage of the passenger. Examples of such extended β-domains include IgA protease of N. gonorrhoeae [155] and AIDA-I of E. coli [156].

Twelve-stranded β-barrels are also found in the trimeric autotransporters and the intimin/invasin class of proteins of the Type V secretion pathways [5,157] (Fig. 2). The crystal structure of the β-domain of the trimeric Hia autotransporter of H. influenzae showed that the three protomers of the trimer each donate 4 strands to the β barrel, snugly positioning three α-helices and their unstructured linkers in the central channel [158]. The structures of intimin and invasin [157] showed an N-terminal twelve-stranded β-barrel followed by an α-helical segment that was suggested to plug the channel of the barrel. The structures represent the intimin/invasin once the transport of the C-terminal passenger is completed. As a consequence of the order of transporter and passenger domains the intimin/invasin group of proteins are described as “reverse” autotransporters [5,6].

The β-domain structures are generally thought to reflect the end-state of the secretion process [26,151]. The β-domain of monomeric autotransporters remains inserted in the outer membrane and could have additional functions, although this has not been investigated. Interestingly, individual β-domains without a passenger are encoded in Vibrio cholerae and other species [159], but whether they are expressed and functional has not been determined yet. The inserted α-helix plugs the β-barrel and adds to its stability as suggested by molecular dynamics simulations [160] and biochemical assays [26,161,162]. In this way, the integrity and permeability of the outer membrane could be consolidated. Liposome swelling assays and black lipid conductivity measurements showed that the closed state of the barrel is fairly stable but that occasionally the pores open to a diameter of ~1.1 nm consistent with the internal barrel β-diameter deduced from crystal structures [161,162]. The channel formed by the EspP β-domain contains a very short α-helical segment of 6 residues [26] which remains upon cleavage of the passenger inside the barrel [25]. However, in that structure an external loop folds over the barrel lumen to contact the barrel wall, which adds to the stability of the barrel [26]. Deleting this loop did not affect secretion or the intra-barrel cleavage of EspP [26] and Hbp (Z. Soprova, W.Jong, J. Luirink, unpublished observation). The structures of mutants of Hbp and EspP that lack the intra-barrel cleavage site showed a second α-helical segment that is positioned in the channel (Fig. 2), while the loop that in cleaved EspP stabilizes the barrel in these mutants remains flexible and not oriented towards the barrel wall [142,163]. The non-cleaved β-domain of Hbp showed an increased pore activity when compared to the wild-type cleaved β-domain and was less stable [161], which suggests that the intra-barrel cleavage is required to obtain full stability and a closed channel.

3.2 The structures of the TPS domain and the TpsB transporter of TPS systems

The secreted proteins of the TPS systems are all very large and full-length structures are not available, yet. However, several structures of the N-terminal TPS domain have been elucidated. TPS domains are 300-350 residues long and highly conserved between TpsA proteins [17] (Fig. 1B). The structures of the TPS domains of FHA of B. pertussis [164], HMW1 of H. influenzae [165] and hemolysin A of Proteus mirabilis[132] show a rather similar β-helical structure with a short β-sheet appended to one side of the β-helix (Fig.3). In the HMW1 structure one strand of that sheet is replaced by an α-helix. The TPS domains used for crystallization derive from protein purified from the culture supernatant of
bacteria and represent the secreted form. Most likely, during translocation across the cell envelope, the TPS domains are largely unfolded [166,167]. Sequence-based structure predictions also indicated that large parts of the remaining TpsA protein also fold in a β-helical conformation similar to autotransporter passengers [148,168,169], which may be important to drive the secretion process via processive folding of the β-helix [170] (see section 8).

The single structure for a TpsB...
transporter is that of the transporter of FHA, FhaC [171] (Fig. 3). It shows a 16-stranded β-barrel with two POTRA domains at its N terminus that protrude into the periplasm. Interestingly, the structure of BamA, the major component of the Bam complex, contains five POTRA domains attached to a sixteen-stranded β-barrel as well [172]. In FhaC, an N-terminal α-helix localizes to the β-barrel channel, similar to the autotransporter β-domains. Furthermore, a large extracellular loop (Loop 6) traverses through the channel. The sequence that folds into the tip of this loop is a very conserved motif that also is present in BamA, but in BamA this tip connects to the barrel wall and does not traverse to the periplasm [172]. Substitution of the residues in this loop showed that is critical for the secretion process [171,173] and the functioning of BamA [174]. In contrast, deletion of the N-terminal α-helix of FhaC did not impair secretion of FhaB [171]. As mentioned, the complete structure of BamA has not been solved yet but the similar features of the BamA and FhaC proteins point at conserved functional properties of the two proteins [19].

4. From the ribosome to the periplasm via the Sec complex
4.1 Signal peptides
Autotransporters and TPS proteins have been shown to interact with components of the highly conserved protein conducting channel SecYEG (Sec translocon) in the inner membrane and depend on this machinery and its molecular motor protein SecA for transport from the cytosol to the periplasm [106,120,175,176,177,178,179,180]. Targeting of proteins to the Sec translocon is generally mediated by short (approximately 25 amino acids), cleavable N-terminal signal peptides classically comprising a charged N-domain, a central, hydrophobic H-domain and a C-domain containing a signal peptidase 1 (SPase1) cleavage site. The vast majority of autotransporters is equipped with a signal peptide that shows this classical architecture.

A small subset (10%) of autotransporters carry remarkably long signal peptides which often exceed 50 amino acid residues in length [7,9,181,182]. Similar long signal peptides have been identified in a number of TpsA proteins and some trimeric autotransporters [2,95,178,181,182]. These signal peptides have a dual domain organization and feature a C-terminal part that resembles a classical Sec signal peptide preceded by a conserved, approximately 25 amino acids long N-terminal extension [9,183]. Bioinformatic analysis revealed a motif with a bias for charged residues in the N-terminal part of this extension followed by a second motif containing more conserved aromatic and hydrophobic residues [181,183]. Because of its high degree of conservation and its restriction to proteins secreted via the Type V system a specific role for the extension in the biogenesis of autotransporters, TpsA proteins and trimeric autotransporters was anticipated.

4.2 Role of extended signal peptides in inner membrane targeting
Most secretory proteins reach the Sec translocon via the post-translational SecB-dependent pathway, whereas the co-translational SRP-pathway is mainly used by inner membrane proteins and secretory proteins with rather hydrophobic targeting sequences. Initially, it was speculated that the conserved signal peptide extensions may have a role in targeting pathway selection and mediate atypical targeting via the SRP pathway [9]. Indeed, the signal peptides of autotransporters Hbp and EspP, as well as TpsA protein FHA, can interact efficiently with the SRP in vitro [176,178,184]. Furthermore, Hbp was shown to require a functional SRP
targeting pathway for optimal biogenesis in vivo, although SecB could compensate for lowered cellular levels of SRP to some extent [176,185]. Optimal secretion of Hbp was observed when it was coupled to SRP-binding signal peptides, and not when coupled to classical SecB dependent signal peptides (Jong & Luirink, unpublished), suggesting that targeting via the SRP pathway is beneficial for Hbp biogenesis. The signal peptide extension of Hbp, however, appeared dispensable for the recruitment of SRP and not involved in targeting pathway selection [185], similar to the extension of the signal peptide of FHA [178]. In contrast to Hbp, a strictly post-translational mode of targeting has been claimed for IcsA, EspP, Pet and FHA [175,177,178,186]. Rather, the extension of EspP secured a post-translational targeting mode by reducing the accessibility of the signal peptide to the SRP and by modulating interactions with the Sec translocon [177]. Recent biophysical data led to a more refined scenario implying that nascent EspP polypeptide chains initially bind SRP with high affinity but are excluded from the SRP pathway at later stages of the process when arriving at the inner membrane [184].

The discrepancy between the mode of targeting of Hbp and EspP may be due to subtle structural differences in their respective signal peptides. On the other hand, the distinction between autotransporter signal peptides that promote routing via the SRP pathway and those that do not may not be very strict. Signal peptides have a variable affinity for the SRP [187], making the selection of a specific targeting pathway a delicate and balanced process. Indeed, small modifications that only slightly affected signal peptide hydrophobicity or charge, were shown to change the targeting pathway of EspP from post-translational to co-translational [177]. Also, parameters like the level of synthesis, translation rate and folding characteristics of the nascent chain may influence the preferred targeting pathway. This implies that studies relying on chimeric and truncated autotransporters, or autotransporters expressed in vitro or in a heterologous host [175,176,177,178,186,188] should be interpreted with care. For example, in contrast to chimeric proteins carrying the EspP signal peptide, targeting and secretion of wild-type EspP appeared hardly affected in a SecB-deficient background [177] suggesting a facultative use of the SRP pathway similar to Hbp.

4.2 Role of extended signal peptides beyond the inner membrane targeting step

The conserved signal peptide extension is not required for inner membrane targeting and translocation per se [9,178,186,188,189] but is clearly important for optimal biogenesis of a number of Type V system family members [180,189,190]. It was suggested that the extension functions after the initial Sec targeting step by slowing-down progression through the Sec translocon or the release of the autotransporter from the inner membrane after translocation [178,186,189]. In any case, it appeared required to prevent the non-productive folding of the SPATEs EspP [189] and Hbp (Jong & Luirink, unpublished) into a secretion-incompetent conformation in the periplasmic space. This functionality of the extension seems only relevant in the context of a full-length passenger [189,191]. In contrast to EspP and Hbp, biogenesis of the SPATE Pet appeared unaffected by the absence of the signal-peptide extension [192], but effects of such removal may depend on the experimental conditions [189]. Indeed, deleting the extension from the signal peptide of Hbp affected secretion only upon overexpression (Jong & Luirink, unpublished). Furthermore, an extended signal peptide proved important for proper
folding and translocation of the trimeric autotransporter EmaA particularly at elevated temperatures [180].

In addition to its ability to engage the SRP, the extended signal peptide of Hbp was efficiently crosslinked to the inner membrane protein insertase YidC during passage of the inner membrane in vitro. YidC is in part associated with the Sec translocon and has primarily been implicated in the biogenesis of integral inner membrane proteins and is in general targeted by the SRP pathway [193] (see chapter 8). YidC appeared critical for biogenesis of the SPATEs Hbp and EspC and its depletion resulted in the accumulation of secretion incompetent Hbp intermediates in the periplasmic space [194]. It is conceivable that YidC facilitates the secretion of autotransporters by slowing down their release from the inner membrane, but it may also have a more indirect role by recruiting or modulating the activity of other factors in the inner membrane or periplasm. It has been reported that secretion of IcsA of Shigella flexneri proceeded unaffected by YidC depletion in a heterologous E. coli background [175]. Expression of Ag43, on the other hand, was drastically affected under these conditions due to downregulation of the encoding gene flu [195]. This is consistent with the observation that Ag43 production is very sensitive to perturbations of the Sec pathway [179].

5. Periplasmic transit
5.1 Autotransporters
Upon translocation across the inner membrane via the Sec translocon, the autotransporter enters the periplasm in an unfolded conformation starting with the N terminus of the passenger domain. Transfer through the periplasm towards the outer membrane is a significant challenge because it requires the large passenger domain to be kept in an unfolded or at least soluble and translocation-competent conformation that is yet protected against degradation. At the same time, the attached β-domain needs to fold into a β-barrel structure, a process that probably starts prior to its assembly in the outer membrane [196]. Simultaneously with the folding of the β-barrel, the adjacent C-terminal region of the passenger domain must be properly positioned to allow the subsequent passenger translocation across the outer membrane [197]. Finally, any roadblocks in this process must be rapidly detected to trigger the degradation of accumulating intermediates, which might damage the integrity of the outer membrane and interfere with the regular periplasmic activities.

Several periplasmic chaperones that have been implicated in the biogenesis of outer membrane proteins, such as Skp, SurA, FkpA and DegP [198] appear also involved in autotransporter biogenesis, although to different extents. For example, secretion of IcsA is hampered in skp, surA and degP mutants [199,200]. On the other hand E. coli Hbp secretion proceeded unperturbed in skp and degP mutants, but was strongly reduced in the absence of SurA [201]. Consistently, Hbp translocation intermediates that are stuck in the outer membrane were crosslinked to SurA, and the components of the BamA and BamB components of the Bam complex [201], which corroborates with the proposed primary role for SurA in the delivery of nascent OMPs to the Bam machinery in the outer membrane [202]. Site-directed photo crosslinking revealed extensive sequential contacts of Skp (to the β-domain) and SurA (to β-domain and passenger) prior to or during the initiation of translocation of EspP across the outer membrane [203]. Interactions of EspP with Skp and SurA but also DegP were also independently reported based on yeast two hybrid and surface plasma resonance experiments [204]. Surprisingly, secretion analysis in
mutant backgrounds suggested a critical role for DegP in EspP secretion whereas surA and skp mutations only moderately affected secretion [204].

Overall, it is difficult to provide a coherent universal description of the role chaperones play in autotransporter biogenesis. Possibly, autotransporters differ in their requirement for specific chaperones due to different folding propensity and the presence or absence of specific chaperone-binding sites such as the [Aro-X-Aro] motif to bind SurA [204]. Alternatively, the different expression levels may influence the need for parallel chaperone pathways (SurA vs Skp/DegP). Finally, reduced expression of functional periplasmic chaperones is known to have strong pleiotropic effects that may complicate the interpretation of secretion defects.

The picture that emerges indicates that, like outer membrane proteins, autotransporter may recruit Skp at an early stage, perhaps even before completion of translocation across the inner membrane [205]. The jellyfish-like trimeric Skp structure [206,207] may protect the long and vulnerable AT against premature folding, inappropriate interactions and degradation. Autotransporters that are synthesized with an extended signal peptide have been shown to remain transiently tethered to the inner membrane [185,191]. SurA may play a later role and by chaperoning and targeting the β-domain to the Bam complex, but also by protecting the passenger from degradation. Dsba will catalyse the formation of disulphide bonds between closely spaced cysteines that are present in various autotransporters [208], but it does not appear to be required as a chaperone per se for cysteine lacking autotransporters; e.g. Hbp and pertactin [191,204,209]. The role of DegP remains enigmatic but it clearly plays an important role in the quality control of autotransporter biogenesis. In general, translocation-incompetent autotransporters are degraded by DegP, which relieves the cell from the toxicity that is associated with the expression of these intermediates [209].

5.2 TPS systems

TpsA proteins traverse the periplasm before interacting with the TpsB transporter. During transit, they are kept there in a (partly) unfolded and secretion competent conformation similar to the autotransporters [18,170,210]. In the absence of their cognate transporters, the pro-proteins of FHA and HMW are rapidly degraded by DegP [210,211,212]. In contrast, periplasmic full-length intermediates have been detected for ShlA [127,133], HrpA (TpsA1) of N. meningitidis [96] and OtpA of E. coli [213]. Two observations suggest that these intermediates maintain their secretion competent form. First, the secreted and activated TPS domain of ShlA could subsequently activate ShlA isolated from the periplasm into the activated full-length toxin [127,133]. Second, when full-length OtpA was expressed prior to its transporter OtpB, a significant fraction of OtpA was secreted [213].

For FHA the role of two chaperones during secretion has been addressed. Absence of DegP did severely affect the growth of B. pertussis and this growth defect was alleviated when FHA was not expressed [210,214]. DegP binds to the unfolded pro-protein of FHA and chaperones it before secretion but degrades the protein when it is not secreted. Furthermore, a denatured fragment of the FHA TPS domain binds to the peptidyl-prolyl isomerase Par27 of B. pertussis, but knocking-out the corresponding gene did not affect the secretion of FHA in vivo [215]. Involvement of other chaperones in TPS secretion remains to be shown.
6. Passage of the outer membrane
6.1 Translocation of the autotransporter passenger across the outer membrane

In the first autotransporter secretion model the passenger domain of autotransporters was proposed to translocate in a C to N direction through the β-domain in a hairpin configuration [8]. This model inspired the name ‘autotransporter’ [216] and led to the first experiments to use autotransporters for the secretion of heterologous proteins to the cell surface [217,218]. Heterologous proteins were directly fused to β-domain of AIDA-I and IgA protease with variable success. Limited folding of the passenger appeared tolerated during translocation, since some passenger domains contain one or two couples of cysteine residues that form disulfide bonded loops prior to secretion [208,209]. Furthermore, the crystal structures of passengers and β-domain posed another problem to the autotransporter model [26,142,144,151]. They revealed a diameter of ~1.1 nm for the β-domain channel, which is only large enough to fit two unfolded polypeptide chains or one α-helix and not the disulfide-bonded loops and domains present in natural and heterologous passengers that were reported to be secreted. This led to the suggestion that the active channel needs to be larger possibly involving host proteins with the Bam complex as a likely candidate [151,219]. The Bam complex is found in all Gram-negative bacteria investigated to date [220] and is involved in the assembly and membrane insertion of β-barrel outer membrane proteins [221] (reviewed in chapter 13). Therefore, it is also expected to insert the β-domain. The complex consists of the integral and essential BamA protein in complex with four conserved lipoproteins BamB-E [220]. In some bacteria the complex includes other proteins (e.g. the RmpM protein in N. meningitidis [222]). The lipoproteins are thought to be located on the periplasmic side of the membrane, but the C terminus of BamC is also detected at the cell surface [223]. The β-barrel substrates are recognized by the Bam complex through a specific C-terminal motif that includes a C-terminal aromatic residue [224,225,226]. This motif is also found at the extreme C terminus of the β-domains of autotransporters [9,152,216] and mutation of the C-terminal aromatic residue in the autotransporters Hap and Tsh interfered with secretion of the passenger, although it was not completely abolished [37,227].

6.1.1 Interactions of autotransporters with the Bam complex

The actual involvement of the Bam complex in autotransporter secretion was shown by making use of knock-out or conditional N. meningitidis and E. coli strains. Depletion of the essential BamA and BamD proteins resulted in impaired secretion of autotransporters and a failure to assemble the β-domain [201,228,229,230] while in E. coli the non-essential Bam components (BamB, C and E) appeared dispensable for secretion. Direct interaction of the autotransporters Hbp and EspP with the Bam complex was shown using crosslinking techniques [197,201,203,231,232], but only for mutant passengers that got stuck in the outer membrane or under conditions that slowed-down translocation. These experiments implied a transient proximity of the autotransporter to the BamA protein, the accessory BamB and D proteins, as well as periplasmic chaperones SurA and Skp. Site-specific photo-crosslinking using chemically modified residues indicated that crosslinking occurred from both the passenger and β-domains but to different extents. The specific Bam and chaperone proteins that were crosslinked to a stalled intermediate appeared to depend on the stage in the translocation process at which it was stalled [197,203,232] (see section 6.1.2).

The involvement of the Bam
complex prompted the question whether the Bam complex just mediates insertion of the β-domain into the outer membrane or is actively involved in secretion of the passenger domain. In an extreme version of the latter scenario the β-domain would function only as a targeting domain while the Bam complex constitutes the actual transport machine for the passenger. However, substituting the β-domain of Hbp for the twelve-stranded β-barrel of OmpLA, a Bam dependent outer membrane-based phospholipase, abolished Hbp secretion suggesting that the β-domain is needed not only for targeting to the Bam complex but...
also for the initiation of secretion [233]. Furthermore, altering the number of \(\beta\)-strands in the \(\beta\)-domain barrel negatively affected secretion even when it increased the pore diameter, suggesting that specific barrel-passenger interactions are required [233]. Mutants of the EspP \(\beta\)-domain that bound efficiently to the Bam complex but showed impaired passenger translocation supported the notion that the \(\beta\)-domain is actively involved in the translocation process [197].

In addition to the Bam complex, a second membrane protein complex appears involved in autotransporter biogenesis. The outer membrane protein TamA and inner membrane protein TamB form a trans-envelope complex that is essential for secretion of the autotransporter p1121 of *Citrobacter rodentium* and the SAAT-type autotransporters EhaA and Ag43 of *E. coli* [234]. Interestingly, the TamA protein is a member of the BamA protein family and includes a \(\beta\)-barrel and three POTRA domains [235]. The Tam complex was proposed to play a generic role in autotransporter secretion although it is unclear what this role is. However, secretion of Hbp was not unaffected in an *E. coli* tamA (ytfM) mutant [201]. Most likely, the contribution of the TamA/B complex to autotransporter secretion is restricted to a subclass of autotransporters that may not include the SPATEs.

### 6.1.2 Defined steps in translocation across the outer membrane

Mutant derivatives of the autotransporters Hbp, pertactin and EspP that are stalled in the secretion process were used to probe the sequential steps of outer membrane translocation (Fig. 4A). Introduction of pairs of cysteine residues in (cysteine free) passenger domains generated disulfide bonded loops that, when including secondary structure elements [236,237], blocked efficient secretion [10,209,219]. Stalling depended on the the periplasmic oxidireductase DsbA that is required for disulfide bond formation [209,219] and appeared reversible [209]. Using such a stalled secretion intermediate of pertactin, the C-terminal region of the passenger rather than the N terminus was detected at the cell surface [10]. This implies a vectorial C- to N-terminal mode of translocation. Furthermore, it indicates that a temporary hairpin is formed that loops through the translocation channel (Fig.4A). This is consistent with the observation that in the stalled EspP derivatives the \(\alpha\)-helix is already positioned inside a proto-form of the \(\beta\)-barrel and protected from external cleavage [196]. It is important to note that the \(\beta\)-domains of stalled secretion intermediates of Hbp and EspP are not fully folded in this proto-barrel conformation. The proto-barrels are also not fully integrated in the outer membrane and they can easily be extracted from the membrane [201,231]. Nevertheless, their passengers are already detected at the cell surface [197,201,203,209,231,232,238] indicating that passenger translocation has started at this stage. Most likely, the proto-barrels function in conjunction with the Bam complex in a flexible conformation that better fits the secretion of partly folded passengers (Fig. 4A).

The C-terminal segment of most \(\beta\)-helical autotransporters comprises a conserved subdomain termed the autochaperone [150]. Substitutions of specific residues in this domain impaired efficient secretion providing an alternative strategy to create translocation intermediates [150] [203,231,238,239,240]. In particular, mutation of the aromatic phenylalanine located at the C-terminal end of the \(\beta\)-helical segment of EspP and Hbp proved critical for secretion [231,238]. These intermediates were stalled in the outer membrane at a different, most likely earlier stage of the translocation process than the cysteine-loop mutants, as deduced from crosslinking analysis.
Site-directed photo crosslinking of the stalled EspP mutant led to a model where SurA mostly interacts with the passenger domain, whereas Skp binds the passenger and β-domain but is replaced at the β-domain by BamA, BamB and BamD once the outer membrane is engaged [203,232,238]. BamA contacts positions in both the passenger and the β-domain throughout the secretion process but at later stages these contacts seem to be less prominent than those with BamB and BamD [203].

The substitution of two conserved glycine residues in the β-domain of EspP for other residues resulted in impaired folding of the β-barrel. It also invoked slower secretion and delayed cell surface exposure of the EspP passenger [197]. Despite the folding defect these mutants engaged the Bam complex at quite similar rates as the wild-type EspP β-domain judged from photo crosslinking in combination with pulse-chase experiments.

Overall these data indicate that autotransporter secretion involves a series of coordinated steps in which the passenger domain is translocated to the cell surface and its β-domain is integrated in the outer membrane (Fig. 4A). These steps involve an intricate interplay of periplasmic chaperones and subunits of the Bam complex. The actual translocation channel through which the passenger is transported is probably formed by the β-domain but the transient interaction with the Bam complex could modulate the flexibility and size of the β-domain pore. In that case, the diameter of the translocation channel in action would be larger than the pore found in the β-domain crystal structures, either because the β-domain is not yet in its fully folded conformation, or because the channel is formed by the β-domain in conjunction with one or more components of the Bam complex. The crystal structures of BamA of N. gonorrhoeae and Haemophilus ducreyi suggest that the β-barrel of BamA can open laterally, by displacing strands 1 and 16 [172]. These strands could interact with the strands in the β-domain to increase the channel diameter. However, the lateral opening could also allow for release of the autotransporter into the outer membrane when the folding of the β-domain is completed.

What is the size of the pore during passenger translocation? A systematic analysis of the active Hbp translocation pore made use of Hbp passenger mutants with peptides of different length and structural complexity inserted in a short disulfide-bonded loop that protrudes from the main β-helical structure [236]. These experiments showed that four extended polypeptide chains, or an extended polypeptide chain and an α-helix can be accommodated in the channel, but that an β-helical hairpin is incompatible with secretion. The data indicated that the diameter of the active translocation pore is roughly between 1.7 and 2.0 nm, which is larger than found in the β-domain crystal structures. Clearly, translocation requires a more flexible, stretched or chimeric β domain as discussed above.

6.2 Translocation of TpsA proteins across the outer membrane
6.2.1 Targeting of the outer membrane-based TpsB by the TpsA
The TpsB transporter in the outer membrane is targeted by the TPS domain at the N terminus of the periplasmic TpsA protein (reviewed in [17,18]; see Fig. 4B). This TPS domain constitutes the minimal secretory unit of a TpsA as shown by deletion studies of several full-length TpsAs [106,117,126,132,164]. Initially, it was assumed that TPS domains only recognize their cognate TpsB transporter [17], since the TPS domain of FHA was not secreted by HpmB, the transporter
of hemolysin HpmA of *P. mirabilis* [211]. However, HpmB did secrete the TPS domain of the related hemolysin ShlA of *S. marcescens*. Furthermore, in *N. meningitidis* two TpsBs are co-expressed, of which one shows a strict specificity for its cognate TPS domains whereas the other is promiscuous and transports TPS domains of various neisserial TPS systems [98].

Insight in how the TPS domains interact with their TpsB transporter derives from studies on secretion of the FHA TPS domain by the FhaC transporter. The FhaC crystal structure shows two POTRA domains that are located in the periplasm. Both are essential for translocation, since deleting either of them abolishes secretion of the FHA TPS domain [171]. The TPS domains are thought to interact with the POTRA domains in an unfolded conformation (Fig. 4B). Pull-down experiments and overlay blots indicated direct binding of denatured TPS domains of FHA and HMW to their TpsBs [166,241]. Surface plasmon resonance measurements using separate POTRA domains of FhaC purified from the periplasm of recombinant *E. coli*, showed that denatured TPS domains bind with micromolar affinity and fast association and dissociation rates suggesting transient interactions [167]. Alternatively, the FhaC β-barrel, which was not included in this experimental setup, could contribute to the stability of the interactions.

Single residue substitutions in the FHA TPS domain that affect the binding to FhaC mapped to multiple regions of the TPS domain [166]. Substitutions in the POTRA domains of FhaC showed that mutations that perturb a hydrophobic groove that extends over both POTRA domains resulted in secretion defects. This groove is at the interface of a β-strand and an α-helix of each POTRA [167,171].

### 6.2.2 Transport of the TpsA across the outer membrane

The sixteen-stranded β-barrel formed by the C-terminal part of TpsB most likely forms the translocation channel through which TpsA is transported. In the crystal structure of FhaC the channel is almost completely blocked by an α-helix and the inward folded external loop 6 (Fig. 3), but TpsB proteins show pore-forming properties in both liposome swelling assays and black lipid conductivity assays [242,243,244,245]. Hence, the crystal structure presumably represents an inactive state of the protein. FhaC purified from outer membranes and reconstituted in proteoliposomes could transport the FHA TPS *in vitro* and this process required the presence of loop 6 [173,246]. Apparently, translocation does not only require an open pore but also active involvement of loop 6. The interaction of the TpsB with its substrate the TPS domain might also trigger translocation. The TpsB homologue BamA showed pore activity in the absence of a substrate but this activity increased when a peptide corresponding to the last β-strand of the β-barrel protein PhoE was added [225]. This suggests that the interaction with a substrate induces a conformational change to open the channel. Similarly, the binding of the TPS domain to the POTRA domains of the TpsB might trigger opening of the translocation pore to allow the passage of TpsA.

Transport of the TpsA appears to be a vectorial process, but there is some debate whether it occurs from N to C terminus with the TPS domain out first or the other way around [18,119,170] (Fig. 4B). The latter orientation requires a prolonged interaction of the TPS domain with the POTRA domains. Intriguingly, FHA and CdiA are anchored to the outer membrane via their N-terminal domains [117,247] whereas HMW is anchored via its C terminus and this anchor appears located in the periplasm [107]. This suggests that both directions could be followed. Recently, cysteine labeling of
FhaB, the pro-protein of FHA, showed that both the TPS domain and the C-terminal domain (called pro-domain) remain intracellular during secretion of FhaB [118,119]. Nevertheless, the pro-domain keeps FhaB in a conformation that can be proteolytically processed once secretion is completed to yield active FHA and a degraded pro-domain. This configuration of the FHA protein favors the formation of a hairpin during secretion. Provided that this configuration is a general feature, it suggests that the final orientation of the TpsA is decided by which of the two TpsA domains is released first from its TpsB binding site (Fig. 4B).

7. Release of the passengers of autotransporters after secretion

After completion of the secretion process, the autotransporter passenger domain extends from the cell surface and the β-domain is fully inserted into the outer membrane. For the EstA-type of autotransporters this structure is the end point of the secretion route, because these passengers remain covalently linked to their β domains at the cell surface [73,74]. All other monomeric autotransporters studied to date are proteolytically cleaved, disrupting the covalent linkage to the cell surface despite the fact that many of these are adhesins. For example, the SAAT AIDA-I of E. coli and pertactin of B. pertussis remain associated with the outer membrane via non-covalent interactions which can be released by mild heat or treatment with detergents [144,248].

Several different cleavage mechanisms have been described (Fig. 5). The SPATES of Enterobacteriaceae are invariably released from the cell surface via an autocatalytic cleavage mechanism that involves their β-domain [25,26,142]. A conserved motif in the linker segment that separates the β-barrel from the C-terminal end of the passenger β-helix harbors a cleavage site between two adjacent asparagine residues (Fig. 5A) [25]. The catalytic residues are also conserved and are located on the β-barrel wall pointing into the barrel channel [25,26]. Intramolecular cleavage within the β-domain is not restricted to the SPATES. The pertactin passenger is also released via an autocatalytic cleavage site within its β-domain [25] while AIDA-I is cleaved by catalytic residues that are located outside the β-barrel, but in the cell surface-exposed part of the β-domain [249].

The passengers from non-SPATE serine protease autotransporters are released from their β-domains through the proteolytic activity of the serine protease subdomain in their passengers (Fig. 5B). Cleavage occurs between adjacent molecules on the cell surface as shown for the Hap autotransporter of H. influenzae that cleaves in the unstructured linker connecting the β-helical part of the passenger with the α-helical segment of the β-domain [44,250]. As a consequence of the intermolecular cleavage mechanism, uncleaved Hap autotransporter is present at the cell surface at low expression levels, whereas the proteolytic release of the passengers into the extracellular milieu increases upon higher expression levels and higher surface density of the Hap protein. The NalP autotransporter of N. meningitidis processes both itself and other neisserial autotransporters i.e. App, IgA protease and AusI (Fig. 5C) [14,51]. NalP-mediated processing competes with autocatalytic processing and occurs between adjacent molecules. However, the cleavage by NalP results in the release of passengers that contain an extra subdomain. For example, the IgA protease passenger is extended with the α-peptide subdomain when released by NalP, whereas the cleavage by IgA protease itself results in secretion of a passenger without that α-peptide.

Passengers may also be released from their β-domains by other proteases (Fig. 5D). IcsA emerges from the outer
membrane at the pole and this position is needed to give direction to the IcsA-based motility of the cells. The IcsA passenger is released from the cell surface through cleavage by the dedicated outer membrane protease SopA to ensure that IcsA passenger molecules are only present at the poles and do not diffuse over the surface [251]. Finally, expression of heterologous autotransporters in *E. coli* sometimes results in the release of the passenger after cleavage by the outer membrane protease OmpT, as observed for the *S. marcescens* autotransporter Ssp-h1 [252].

### 8. Passenger folding and secretion

Translocation across the inner membrane via the Sec machinery is energized by ATP hydrolysis by the molecular motor SecA (see chapter 5) whereas secretion through the Tat pathway is energized by the proton-motive force that is maintained across that membrane [253]. Passage of the outer membrane cannot be driven directly by either of the two energy sources since ATP is not present in the periplasm, nor is a proton concentration gradient maintained across the outer membrane. So, how is translocation of autotransporter passenger domains across the outer membrane energized? The stacked β-helical core structure present in most autotransporters inspired a translocation model in which the sequential winding of β-strands of the β-helix at the cell surface drives translocation [10,151,197]. This process could start from the initial hairpin that is formed in the β-domain when it engages the outer membrane. *In vitro* folding experiments using the pertactin and Pet passenger domains showed that the C-terminal part of the passenger is more stable than the N-terminal part [254,255,256] and it is this part that emerges first from the cell surface during translocation of the outer membrane [10].

<table>
<thead>
<tr>
<th>Autotransporter</th>
<th>Heterologous protein</th>
<th>Function</th>
<th>Expression host</th>
<th>Display/secretion</th>
<th>Application</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA protease</td>
<td>β-subunit of cholera toxin (CTB)</td>
<td>Host receptor binding</td>
<td><em>Escherichia coli</em>, <em>Salmonella Typhimurium</em></td>
<td>Display and secretion</td>
<td>Translocation studies</td>
<td>[218]</td>
</tr>
<tr>
<td>AIDA-I</td>
<td>β-subunit of cholera toxin (CTB)</td>
<td>Host receptor binding</td>
<td><em>Escherichia coli</em></td>
<td>Display</td>
<td>Translocation studies</td>
<td>[276]</td>
</tr>
<tr>
<td>IgA protease</td>
<td>Single chain Fv fragments (scFv)</td>
<td>Virus-neutralizing immunoglobulin (Ig) domains</td>
<td><em>Escherichia coli</em></td>
<td>Display</td>
<td>Viral intervention</td>
<td>[296,297]</td>
</tr>
<tr>
<td>AIDA-I</td>
<td>Adrenoxin (Adx)</td>
<td>Steroid biosynthesis</td>
<td><em>Escherichia coli</em></td>
<td>Display</td>
<td>Whole-cell biocatalysis</td>
<td>[298,299]</td>
</tr>
<tr>
<td>AIDA-I</td>
<td>P15 peptide of C-reactive protein</td>
<td>Screening cathepsin G inhibitors</td>
<td><em>Escherichia coli</em></td>
<td>Display</td>
<td>Library screening</td>
<td>[300]</td>
</tr>
<tr>
<td>IgA protease</td>
<td>Metallothionein</td>
<td>Heavy metal adsorption</td>
<td><em>Cupriavidus metallidurans</em></td>
<td>Display</td>
<td>Bioremediation</td>
<td>[301,302]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fusions to (part of the) autotransporter passenger domains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autotransporter</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>IcsA</td>
</tr>
<tr>
<td>ExtA</td>
</tr>
</tbody>
</table>
Initial biochemical experiments that targeted the C terminus already indicated that this domain is important for the passenger to attain its final stable conformation and hence it was called the autochaperone (sub)domain [150]. Interestingly, mutants with a disabled or deleted autochaperone subdomain could be restored to folded passengers by supplying the domain in trans [150,239] suggesting some kind of template-induced folding mechanism. Deliberately perturbing the stability of the autochaperone domain by mutagenesis interfered with passenger secretion [231,238,256]. In contrast, mutations that decreased the stability of N-terminal part of the passenger domain improved secretion [256]. Apparently, a certain level of stability of the C-terminal end of the passenger once it appears at the cell surface is important for further continuation of secretion. It suggests the sequences within the autochaperone are part of an initial hairpin located in the β-barrel channel. Furthermore, a strong folding propensity in the extracellular loop of this hairpin would be a good starting point for assembly of the passenger.

Of note, not all autotransporter passengers are β-helical as discussed above. Furthermore, direct fusion of non β-helical heterologous proteins to the β-domain is often compatible with secretion, albeit in most cases at reduced efficiency. Possibly, other elements with folding propensity are able to start the translocation process at the cell surface. On the other hand, a strong folding propensity in the periplasm may lead to premature folding obstructing outer membrane translocation.

9. Implications of the autotransporter secretion mechanism for biotechnological applications

9.1 The use of autotransporters for biotechnological applications

Recombinant proteins for industrial purposes are most commonly produced intracellularly in bacteria, often using E. coli as host. However, secretion into the extracellular milieu or expression on the bacterial cell surface offers significant advantages, including easier purification and beneficial effects on protein folding, activity and stability [15,257,258]. Of the type V classes the monomeric autotransporters have attracted most attention as carriers to transport heterologous proteins to the cell surface or beyond [15,259,260]. We are not aware of any use of the Type Vb-d for biotechnological applications, but the intimins (type Ve) have been used for such purposes [261,262,263,264]. Similar to autotransporters, secretion of bulky passengers by intimins appears limited [263]. Furthermore, a recent comparison of recombinant proteins fused N-terminally to an autotransporter or C-terminally to an intimin led to comparable levels of surface exposure [264]. Nevertheless, the monomeric autotransporters are by far the most applied to produce secreted or cell surface exposed recombinant proteins.

Two main fusion strategies have been applied to insert the sequences of heterologous targets in autotransporters (reviewed in [260]). In most cases, heterologous proteins were directly fused to the β-domain of the autotransporter used (Table 3). Alternatively, heterologous proteins were either fused to the complete passenger or placed within the passenger at the position of one of the subdomains or loops that protrude from the β-helical stem. Both strategies have limitations in the structural complexity of the proteins that can be included and the secretion yields that can be achieved.

Autotransporter-mediated secretion or surface display (also referred to as autodisplay) has been used in a variety of biotechnological applications including live vaccine development, whole-cell biocatalysis, biosorbent and biosensor development, epitope mapping, and protein library screening.
A variety of fusion partners have been explored, ranging from small peptides to relatively large complete proteins [15,259,260]. It is important to realize that autotransporter mediated secretion comes with certain limitations with respect to size, complexity and folding propensity of the cargo molecules. Encouragingly, recent mechanistic insight in periplasmic transit and OM translocation as well as novel structural data, provide clues how to optimize the application of the type V secretion pathway.

9.2 Signal peptides and the use of autotransporters
Despite the clear role that signal peptides have at several stages of autotransporter biogenesis, the extent to which they influence the secretion of recombinant proteins via the autotransporter pathway is unclear. Only a few of the recombinant chimeric autotransporters studied to date were expressed from constructs that included their cognate autotransporter signal peptide. In most cases, the signal peptide of the heterologous fusion partner or a completely unrelated and often heterologous Sec signal peptide was used, such as the CtxB or PelB signal peptide, respectively [15,259,260]. Extended signal peptides may only be beneficial when fused in the context of the cognate full-length AT passenger [189,191]. Consistent with this supposition, replacement of the native extended signal peptide of Ag43 by regular Sec signal peptides sustained secretion of a heterologous protein that was fused to the Ag43 β-domain [269]. On the other hand, the presence of a conserved autotransporter signal peptide extension may directly influence the conformation of fused heterologous proteins in the E. coli periplasm [181] and thus affect their secretion via the autotransporter pathway. In general, it appears advisable to include the native extended signal peptide in chimeric constructs [267].

9.3 Influence of folded domains on the translocation of heterologous proteins fused to autotransporters
One of the major unresolved issues in the use of autotransporters as carriers for the transport of heterologous proteins concerns their conformation in the periplasm and during translocation across the outer membrane. While in some cases successful display of structurally complex heterologous proteins such as single-chain antibodies has been reported [219,268,269,270,271] (Table 3), similar constructs failed to be secreted in other studies [209,218,272,273,274]. This discrepancy might be explained by the use of different expression hosts, autotransporters, heterologous fusion partners, promoters and signal peptides. These studies frequently lack quantitative data which makes it difficult to compare the different reports. Furthermore, expression of chimeric constructs is often toxic for host cells and requires a rigorous assessment of specific secretion versus leakage from lysed cells.

Similar to the natural passengers, secretion of recombinant proteins may fail when a rigidly folded structure is included in the passenger. As a general rule, small proteins are the preferred fusion partners and widely spaced cysteines and folding prone domains should be avoided. Alternatively, secretion of cysteine containing fusion partners can be accomplished by growth in the presence of a reducing agent or by introducing the constructs in strains that lack the major oxidoreductase DsbA [209,218,268,272,275]. In addition, co-expression of periplasmic chaperones might help to maintain the translocation competent state of fusion constructs during periplasmic transit [259,265].

9.4 Selecting an autotransporter secretion
A variety of autotransporters and experimental conditions have been described in the literature and as well as a vast array of techniques to assess cell surface exposure or secretion [15,259,260]. This complicates the choice for the “optimal” autotransporter as carrier for display or secretion and the optimal fusion strategy. For example, autotransporter display was pioneered by the use of Neisseria IgA protease in E. coli [218]. However, it soon became clear that the use of an endogenous E. coli autotransporter such as AIDA-I, was superior to heterologous expression [276]. Possibly, a cognate Bam complex is required for the efficient biogenesis of an autotransporter biogenesis and including recombinant proteins in a heterologous autotransporter may further reduce efficient BamA-dependent secretion. Species-specific variations to the C-terminal motif recognized by Bam exist [225,226] and may be important to consider. For instance, in Pseudomonas stutzeri the EstA autotransporter and some other outer membrane proteins have a leucine as C-terminal residue and substituting it for the canonical phenylalanine decreased the stability of its β-barrel [273].

Thus far, most recombinant proteins have been fused directly upstream of the C-terminal β-domain, which in AIDA-I and IgA protease constructs includes the α-helical segment and cell surface exposed regions, and sometimes a small part of the passenger domain [15,259,260]. In a comparative study, a range of heterologous proteins were fused to either the complete EstA autotransporter of P. stutzeri or its β-domain [273], of which only one (β-lactamase) was secreted in significant amounts. In this system, it appeared to make no difference whether the recombinant protein was fused to the β-domain or to the passenger. By contrast, fusion of β-lactamase to the N terminus of full-length IcsA resulted in superior display compared to fusion to the IcsA β-domain [277].

A slightly different strategy has been applied to introduce recombinant proteins in the passenger of the SPATEs Hbp and Pet [259,267,268]. In the case of Hbp, the crystal structure allowed the identification of subdomains and loops that protrude from the β-helical stem structure [142]. Replacement of these side-domains by the Mycobacterium tuberculosis antigen ESAT-6 was shown to improve stability and yield compared to direct fusion of ESAT-6 to the C-terminal domain of Hbp [267]. These studies demonstrate that autotransporter display and secretion can benefit from structure-based platform design and minimal perturbation of the passenger structure.

In conclusion, although autotransporters are already successfully used for display and secretion in many biotechnical applications, recent advances in our understanding of the secretion mechanism and its limitations will certainly contribute to further improvement of this promising system.

Acknowledgements:
S.U.R. gratefully acknowledges funding by the Higher Education Commission of Pakistan. J.L. and W.S.P.J. were supported by a grant from the Dutch Technology Foundation STW. In addition, M.D. was supported by the European Commission FP7 ADITEC program (HEALTH-F4-2011-280873).

Competing interests
J.L., W.S.P.J. and M.D. are (in part) employed by Abera Bioscience AB. Stockholm, Sweden.


[48] Y. Ohnishi, T. Beppu, and S. Horinouchi, Two genes encoding serine protease homologues in Serratia marcescens and characterization of


Type V secretion systems

800.


Type V secretion systems


Type V secretion systems


System-specificity of the TpsB transporters of co-expressed Two-Partner Secretion systems of *Neisseria meningitidis*

Sadeeq ur Rahman, Peter van Ulsen

Department of Molecular Microbiology, Institute of Molecular Cell Biology, VU University, de Boelelaan 1085, 1081 HV Amsterdam, the Netherlands

System-specificity of the TpsB transporters of co-expressed Two-Partner Secretion systems of *Neisseria meningitidis*

The two-partner secretion (TPS) systems of Gram-negative bacteria consist of a large secreted exoprotein (TpsA) and a transporter protein (TpsB) located in the outer membrane. The TpsA targets the TpsB for transport across the membrane via its ~30-kDa TPS domain located at its N terminus and this domain is also the minimal secretory unit. *Neisseria meningitidis* genomes encode up to five TpsAs and two TpsBs. Sequence alignments of TPS domains suggested that these are organized into three systems, while there are two TpsBs, which raised questions on their system-specificity. We show here that the TpsB2 transporter of *N. meningitidis* is able to secrete all types of TPS domains encoded in *N. meningitidis* and related species *Neisseria lactamica*, but not domains of *Haemophilus influenzae* and *Pseudomonas aeruginosa*. By contrast, the TpsB1 transporter seemed specific for its cognate *N. meningitidis* system and did not secrete TPS domains of other meningococcal systems. However, TpsB1 did secrete the TPS2b domain of *N. lactamica*, which is related to the meningococcal TPS2 domains. Apparently, the secretion depends on specific sequences within the TPS domain, rather than the overall TPS domain structure.

Introduction

The two-partner secretion (TPS) pathway is a widespread protein secretion route in Gram-negative bacteria, which consists of a large and secreted exoprotein (TpsA) of typically more than 100 kDa and a ~60 kDa transporter protein (TpsB) (17). Both proteins are synthesized with an N-terminal signal sequence for transport across the inner membrane via the Sec machinery. After transport to the periplasm, the TpsB transporter integrates in the outer membrane to function in the secretion of TpsA. Targeting of this transporter by the TpsA exoprotein is mediated by a conserved targeting domain, called TPS domain, which is located downstream of the signal peptide at the N terminus of the mature TpsA (16, 17). TpsA and TpsB molecules are generally encoded in a single operon. The secreted TpsA proteins often function as virulence factors by acting as adhesins or toxins to host cells or other bacteria (15, 16). *Neisseria meningitidis* (meningococcus) is a Gram-negative diplococcus that survives in the human body by colonizing the nasopharynx. Infection can lead to meningitis and sepsis (38). *N. meningitidis* genomes encode up to three distinct TPS systems (40). Of these, system 1 is ubiquitous, whereas systems 2 and 3 were significantly more prevalent among isolates of hyper invasive clonal complexes than those of poorly invasive clonal complexes. Several functions have been attributed to the system-1 TpsAs of *N. meningitidis*. TpsA1 has been shown to promote adherence to cultured epithelial cell lines derived from the human upper respiratory tract (31). Other *in vitro* experiments have suggested that TpsA1 contributes to intracellular survival and escape from cultured epithelial cells (35). Recently, based upon sequence comparisons it was proposed that TPS system 1 of *N. meningitidis* may act as a contact-dependent bacterial killing system (27), but this has not been confirmed experimentally. The latter class of TpsAs bind to the surface of a cell of a related bacterial species, which is subsequently killed when they do not express an immunity protein. Specific functions have
Two-Partner Secretion systems specificity in Neisseria meningitidis

strains carry two copies of a genetic island encoding TPS system 1, probably as a result of a duplication event. Both copies contain ORFs encoding TpsBs and TpsAs, but one of the islands encodes a truncated TpsB lacking a signal peptide/sequence, which, when expressed, cannot reach the outer membrane. Nevertheless, expression data indicated that both TpsA1 proteins are expressed and secreted (40). The system-2 ORFs are located on two chromosomal islands and both are linked to one of the two system-1 islands. One island contains a putative operon that includes \( tpsA_{2a} \) and \( tpsB_2 \) (NMB1762), while the other contains a singular \( tpsA_{2b} \). The third system only contains a \( tpsA \) and lacks a \( tpsB \) and is located on a separate genetic island. Expression data \textit{in vitro} is lacking, but antibodies recognizing the TPS domain of the system-3 TpsA have been detected in serum of a patient recovering from meningococcal disease, which indicates expression during infection (40).

The transport of the secreted TpsA by the TpsB transporter requires the interaction of the TPS domain with the TpsB, and, in fact an isolated TPS domain is the minimal secreted unit of the canonical TpsA filamentous haemagglutinin (FhaB) of \textit{Bordetella pertussis} (28). Recognition of the FhaB TPS domain is very specific and replacing the cognate TpsB FhaC by a TpsB of another TPS system resulted in a blocked secretion (19). Multiple TPS systems co-exist in several bacterial species, \textit{e.g.} \textit{Haemophilus influenzae} (39), \textit{Pseudomonas aeruginosa} (5), \textit{Neisseria lactamica} and \textit{N. meningitidis} (35). Our previous results indicated that in \textit{N. meningitidis} three TPS systems are co-expressed (40). Furthermore, the third neisserial TPS system appears to lack a cognate TpsB, since it has never been identified in analyses of available neisserial genomes (40) and unpublished results). Of note, several singular \( tpsA \) genes without a dedicated \( tpsB \) gene nearby have been found in other bacterial

---

Fig 1 (A) Chromosomal organization of the TPS systems in \textit{N. meningitidis} H44/76, as identified in the genome sequence (6, 26). The proteins encoded by the ORFs are indicated below. The red colours designate system 1, the blue colours system 2 and the green colours system 3. The dark line indicates the part of the \( tpsA \) genes used for the truncated TpsA ORFs, which comprise the signal peptide and the TPS domain. The white arrows in between the ORFs encoding TpsA2a and TpsB2 are ORFs not encoding TPS proteins. The ORFs are believed to be part of an operon (40). (B) Graphical representation of the various meningococcal expression constructs used for this study. On the left the constructs containing a truncated TpsA are given, on the right the corresponding constructs of a truncated TpsA ORF in combination with a TpsB ORF. Colours designate the different systems, as in panel (A).

not been attributed to systems 2 and 3.

The \textit{N. meningitidis} strains that encode all three TPS systems show a similar genetic organization of the TPS ORFs, as exemplified by a comparison of the sequenced genomes of \textit{N. meningitidis} MC58 and H44/76 (6, 26, 36, 41). The
species, including \textit{N. lactamica} and \textit{P. aeruginosa} (5, 40). Therefore, the question arises how and if TPS domains are selected by their cognate TpsBs. We therefore, investigated the transport of neisserial TPS domains by the available TpsBs, as well as their possible redundancies. We conclude that the TpsB transporter of system 1 was rather selective in its recognition of meningococcal TPS domains. In contrast, the TpsB transporter of system 2 (TpsB2) appears less specific in target selection than TpsB1, since it transported TPS domains of \textit{N. meningitidis} and \textit{N. lactamica}, although not of other species.

\textbf{Materials and methods}

\textbf{Bacterial strains and growth conditions.} The \textit{N. lactamica} strain 26793, \textit{N. meningitidis} strains H44/76, its unencapsulated derivative HB-1 and the knock-out derivatives of HB-1, HB-1 \textit{tpsB1::kan}, HB-1 \textit{tpsB2::kan} (40) and HB-1 \textit{tpsB1::kan tpsB2::gen} were grown on GC agar (Oxoid) supplemented with Vitox (Oxoid) at 37°C, 5% carbon dioxide. Liquid cultures of \textit{N. meningitidis} strains were grown at 37°C in tryptic soy broth (Gibco-BRL). Gene disruptions were selected on GC/Vitox plates supplemented with 100 mg/ml kanamycin and/or 10 mg/ml gentamycin. Chloramphenicol was added to a final concentration of 8 mg/ml for plasmid selection. \textit{H. influenza} strain A950006 was grown on brain heart infusion (Oxoid) supplemented with 10 mg/ml Haemin and 10 mg/ml NAD. The \textit{Escherichia coli} strains Top10F' (Invitrogen), DH5α, and MC1061 \textit{degP::S210A} (34) were grown on Luria Bertani broth (LB) or agar plates, supplemented with 100 µg/ml ampicillin or 30 mg/ml chloramphenicol for plasmid maintenance and with 0.5% glucose for full repression of the \textit{lac} operator, when appropriate. \textit{P aeruginosa} PA01 was grown on LB agar plates at 37°C.

\textbf{Cloning of the TPS constructs.} Truncated \textit{tpsA} ORFs and combinations of these with \textit{tpsB} ORFs (Table 1) were cloned into the pEN vector, which is a neisserial expression vector (42). The DNA fragments were obtained by PCR using chromosomal DNA obtained from lysed bacterial cells as template, the Phusion DNA polymerase (Finnzymes) according to the recommendations of the manufacturer and the primer combinations that are listed in Table S1 in the supplementary material. To prepare the chromosomal DNA, colonies were scraped from freshly grown plates, resuspended in sterile de-ionized water to an optical density at 600 nm (OD\textsubscript{600}) of ~2.0 and boiled for 5 min. The lysate was subjected to full-speed centrifugation in a microtube centrifuge for 5 min and 5 ml of the supernatant was added to the PCR mixture. The resulting amplicons were cloned into the pGEM-T cloning vector (Promega) and confirmed by sequencing (Macrogen). The ORFs encoding truncated \textit{tpsA}s were first subcloned into the pET11a plasmid using the BamHI and NdeI restriction sites in that plasmid and included in the primer sequences. The ORF was then digested out of the resulting plasmid using the restriction enzymes NdeI and AatII and ligated into the pEN plasmid vector cut with the same enzymes, yielding the neisserial expression vectors that encoded the truncated TpsA ORFs. Restriction enzymes used for this study were all purchased from New England Biolabs. To combine a truncated \textit{tpsA} with a \textit{tpsB}, the ORF was obtained by PCR, cloned into pGEM-T and sequenced. The \textit{tpsB2} gene was then digested from the pGEM-T plasmid using the BamHI and EcoRI restriction sites that were included in the primers and cloned into the target pET11a-truncated \textit{tpsA} plasmid of choice that was cut with the same enzymes. This procedure places the \textit{tpsB2} ORF downstream of the truncated \textit{tpsA} in that vector. Because the \textit{tpsB1} ORF contained internal EcoRI and NdeI sites, we used a different strategy to obtain combinations...
of this gene and truncated \( \text{tpsA} \). First, we introduced by PCR a silent mutation removing the internal NdeI site in \( \text{tpsB}1 \) and cloned it into the pGEM-T vector, which was then used as a template for further cloning. The mutated \( \text{tpsB}1 \) ORF was excised from pGEMT using BamHI/AflIII and EcoRI/AflIII and these two fragments were ligated into the target pET11a-truncated \( \text{tpsA}1a \) plasmid digested with BamHI and EcoRI, resulting in a full-length \( \text{tpsB}1 \) downstream of the truncated \( \text{tpsA} \). Subsequently, the \( \text{lac} \) promoter region from the pEN vector was cloned upstream of the combined combined ORFs, replacing an NcoI-ApaI fragment from the pET11a derivative by that of plasmid pEN100 plasmid (encoding the truncated \( \text{tpsA}1 \) ORF; Table 1). The complete construct was then transferred into the pEN vector using ApaI-AatII, yielding the plasmid listed in Table 1.

**Construction of the HB-1 \( \text{tpsB}1::\text{kan} \rightleftharpoons \text{tpsB}2::\text{gen} \) double knock-out strain.** To construct an HB-1-derivative that lacked the encoded \( \text{tpsB} \) genes, we performed gene disruption of the \( \text{tpsB}2 \) gene in the already available HB-1 \( \text{tpsB}1::\text{kan} \) strain. From plasmid pKO-TpsB2::kan (40), which contains a kanamycin resistance cassette in between fragments that are located up- and downstream of the \( \text{tpsB}2 \) gene, we excised the \( \text{kan} \) cassette using BamHI and replaced it for a cassette encoding gentamycin resistance from pBSL141 (1). The construct selected for gene replacement contained the \( \text{gen} \) gene in the same orientation as \( \text{tpsB}2 \) in the chromosome. The \( N. \ meningitidis \) HB-1 \( \text{tpsB}1::\text{kan} \) was transformed by introducing the \( \text{tpsB}2::\text{gen} \) construct. The \( \text{tpsB}2 \) was subsequently disrupted by homologous recombination, which was verified by PCR using primers annealing up and downstream of the target gene and immuno-blots analysis using specific antibodies against TpsB2.

**SDS-PAGE and Western blotting.** All procedures were carried out as described earlier (40). Briefly, \( N. \ meningitidis \) HB-1 cultures were grown for 6 h to an \( \text{OD}_{660} \) of ~3.0-4.0, in the presence of 0.1-1 mM isopropyl-beta-d-thiogalactopyranoside (IPTG) where indicated. Cells were harvested by centrifugation (4,500xg, 5 min) and the pellet was resuspended in phosphate-buffered saline pH 7.4 (PBS) to a final \( \text{OD}_{660} \) of 10. Culture supernatants were centrifuged (16,000xg, 5 min) to remove residual cells and proteins were precipitated from the supernatants with 5% trichloroacetic acid and dissolved in a volume of PBS corresponding to a cell density of \( \text{OD}_{660} \) of 100 (10' concentrated compared to cells). In some experiments the supernatants were subjected to 200,000xg in a benchtop ultracentrifuge (Beckman and Coulter). TCA was added to the supernatant of this step and further treated as above, whereas the high-speed pellet was dissolved in an equal volume of PBS. Cultures of \( E. \ coli \) strains containing pEN plasmids were grown in LB broth to an \( \text{OD}_{660} \) of ~0.6. IPTG was added to a final concentration of 1 mM and incubation was resumed for another 2 h. Samples were collected as described above.

Protein samples were separated on 10% or 4-15% SDS-PAGE gels (BioRad) and stained with Coomassie brilliant blue G250, or proteins were blotted onto nitrocellulose for Western-blot analyses. Blots were pre-incubated in blocking buffer (PBS with 0.5% nonfat dried milk (Protifar; Nutricia, The Netherlands) and 0.1% (vol/vol) Tween 20 (Merck) for at least 4 h. Sera were diluted 1:5,000 (anti-TPS1 and anti-TPS2a) or 1:10,000 (anti-TpsB1 and anti-TpsB2), 1:10,000 (anti-RmpM monoclonal) or 1:2000 (anti-His; Biosource International) in blocking buffer and incubated for 1 h. After that, blots were washed three times with blocking buffer and incubated for 1 h with the secondary antibody diluted in blocking buffer. The secondary antisera
were goat anti-rabbit immunoglobulin G serum conjugated to horseradish peroxidase (Biosource International), which was diluted 1:10,000, or anti-mouse immunoglobulin G (Biosource International), which was diluted 1:2,000. The binding of antibodies to the blots was visualized using Lumilight Plus (Roche). The relative molecular weight of proteins was deduced from the positions of the bands of pre-stained Precision Plus Protein Standard (BioRad) included in each SDS-PAGE gel.

**In silico sequence analyses and structural modelling.** Pairwise sequence alignments of TPS domains were performed using amino acid sequences of 283-305 residues (Table S2). The length was based upon alignments of the known neisserial tpsA genes to the crystal structure of the FhaB TPS domain (9). The encoded protein sequences of the analysed N. meningitidis tpsA genes were first analysed for the presence and cleavage site of the signal peptide using SignalP V4.0 (25) and the ~300-residue stretch downstream of the signal peptide was used for the analyses. The sequences of the N. lactamica TPS domains were derived from the sequences of the cloned PCR amplicons. The sequences were compared using the BL2Seq server at http://blast.ncbi.nlm.nih.gov/ using standard parameters. Structure-informed multiple-sequence alignments were performed using the Expresso program at http://tcoffee.crg.cat/apps/tcoffee/index.html (22) and the same sequences used for the pairwise sequence alignments, including the sequences of the three solved TPS domain structures. Models for the structure of the neisserial TPS domains were obtained by submitting the sequences to the Phyre website at http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index (23). Additional models were obtained from the i-Tasser website at http://zhanglab.ccb.med.umich.edu/I-TASSER/ (24), but these yielded comparable outcomes. To avoid biases derived from different modeling strategies, we compared only the Phyre-derived models. The models were tested for quality by comparing them to the known structure of the FHA TPS domain using the TM-align program at http://zhanglab.ccb.med.umich.edu/TM-align/ (25) (see Table S3 in the supplemental material).

Nucleotide sequence accession numbers. GenBank accession numbers for the cloned regions of N. lactamica 26793 are KC182755 (TPS-NL2a) and KC182756 (TPS-NL4). ((46); Table S3).

<table>
<thead>
<tr>
<th>Construct</th>
<th>Plasmid name</th>
<th>TPS ORFs</th>
<th>Relevant characteristics</th>
<th>source of TPS sequences</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPS1</td>
<td>pEN1000</td>
<td>tpsA1a-tr</td>
<td>expression construct</td>
<td>N. meningitidis H44/76</td>
<td>this study</td>
</tr>
<tr>
<td>TPS1+TpsB1</td>
<td>pEN1030</td>
<td>tpsA1a-tr + tpsB1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPS1+TpsB2</td>
<td>pEN1050</td>
<td>tpsA1a-tr + tpsB2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPS2a</td>
<td>pEN1120</td>
<td>tpsA2a-tr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPS1+TpsB2</td>
<td>pEN11220</td>
<td>tpsA2a-tr + tpsB2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPS2b</td>
<td>pEN1130</td>
<td>tpsA2b-tr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPS2b+TpsB2</td>
<td>pEN11320</td>
<td>tpsA2b-tr + tpsB1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPS1</td>
<td>pEN1140</td>
<td>tpsA3-tr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPS1+TpsB2</td>
<td>pEN11420</td>
<td>tpsA3-tr + tpsB1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPS-NS2a</td>
<td>pEN-TPS-NS2a</td>
<td>tpsA2a-tr</td>
<td></td>
<td>N. lactamica 26793</td>
<td></td>
</tr>
<tr>
<td>TPS-NL4</td>
<td>pEN-TPS-NL4</td>
<td>tpsA4-tr</td>
<td></td>
<td>P. aeruginosa PAO1</td>
<td></td>
</tr>
<tr>
<td>TPS-Pa</td>
<td>pEN-TPS-Pa690</td>
<td>PA0690-tr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPS-Pa+TpsB2</td>
<td>pEN-PA0690-B2</td>
<td>PA0690-tr + tpsB2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPS-HMW1</td>
<td>pEN-TPS-HMW1</td>
<td>kmw1-tr</td>
<td>cloning vehicle</td>
<td>N. meningitidis H44/76</td>
<td>(35)</td>
</tr>
<tr>
<td>TPS-HMW1+TpsB2</td>
<td>pEN-TPS-HMW1-B2</td>
<td>kmw1-tr</td>
<td>cloning vehicle for gene disruption</td>
<td>this study</td>
<td>Novagen</td>
</tr>
<tr>
<td>pEN</td>
<td>pEN</td>
<td></td>
<td>expression vector, cam, Plac</td>
<td>source gen-cassette</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Neisserial expression vectors and other plasmids used in this study
Results

Construction of a minimal TPS expression system in *N. meningitidis*. It had been shown for several TPS systems that a truncated TpsA protein consisting of the signal peptide and the TPS domain is efficiently secreted into the culture supernatant (8, 13, 28, 32, 44). Previously, we had assigned TPS domains within the neisserial TpsAs based upon sequence homologies (40). Here, we constructed a set of neisserial expression plasmids encoding C-terminally truncated TpsAs of around ~400 amino acid residues encompassing the signal peptide and the predicted TPS domain (Fig. 1). The ORFs were cloned from *N. meningitidis* H44/76 by PCR and placed under the control of an IPTG-inducible promoter (Table 1). The expression vectors included truncated *tpsA1a* (TPS1), *tpsA2a* (TPS2a), *tpsA2b* (TPS2b) and *tpsA3* (TPS3) ORFs. Truncated *tpsA1b* was not included, because the encoded signal peptide and TPS domain are identical to those of TpsA1a, except for three residues at the C-terminus that are absent in TpsA1b (40). The TPS3 domain was extended with a C-terminal His tag to enable detection by anti-His antibodies. Sequence analysis of the cloned ORFs and the H44/76 TPS loci in the two available genome sequences (6, 26) confirmed that H44/76 encodes three TPS systems that have a similar genetic organization as found in MC58.

Secretion of the meningococcal TPS domains. We transformed *N. meningitidis* HB-1, an un-ecapsulated derivative of H44/76 (42) and its knock-out derivatives HB-1 *tpsB1::kan* and *tpsB2::kan* (40) with the constructed plasmids to test the expression and secretion of the TPS domains. Cultures were grown in presence or absence of 1 mM IPTG and cells and culture supernatants were collected. Neither the presence of the plasmids, nor the induction of gene expression by IPTG influenced the growth curves of the strains. Protein samples were run on SDS-PAGE and blotted for immuno-detection. Blots incubated with antisera raised against the TpsB1 and TpsB2 proteins confirmed the absence of the transporters in the respective knock-out strains (Fig. 2 and results not shown). To analyze the secretion of the respective TPS domains, blots were incubated with antisera against TPS1, TPS2 and the His-tag that was attached to TPS3. Bands of 32-40 kDa were detected in HB-1 culture supernatants (Fig. 2). The sizes of the TPS1 and TPS2b domains detected corresponded to the calculated sizes for the constructs after cleavage of the predicted signal peptides (40); see Table 2). The TPS3 domain showed the expected size of 39 kDa, but also a 37-kDa band that perhaps is a degradation product. When we expressed the TPS2a construct, we observed a band of ~39 kDa in the supernatant, which was larger than the expected size of ~34 kDa (Table 2). This might indicate modification of the protein during biogenesis, as has been shown for TPS systems in other bacterial species (10, 12, 23) and has also been suggested for neisserial TPS systems (40). Of note, the *tpsA2a* operon includes an ORF encoding a putative glycosyl transferase.

Expression of the TPS1 construct resulted in detection of proteins in the cell fractions of HB-1 and its knock-out derivatives (Fig. 2), which in size corresponded to unprocessed (calculated to be ~41 kDa) and processed versions of the TPS1 domain. A similar accumulation had previously been observed for endogenous full-length TpsA1 in the cell fraction of a HB-1 *tpsB1::kan* strain in which its secretion is blocked (40). Cellular intermediates were not detected for the other constructs suggesting that these were either efficiently secreted, or that non-secreted TPS domains were subjected to intracellular degradation. The recombinant TPS domains were detectable in culture supernatants on Coomassie-stained SDS-PAGE gels (results not
shown) with the exception of TPS3 suggesting a lower level of expression or secretion of this domain. The intracellular accumulation and secretion of the TPS1 domain appeared IPTG dose-dependent (Fig. 2B). Furthermore, the induction of the TPS1 construct resulted in a competition with secretion of the full-length TpsA1, resulting in the accumulation of a ~240-kDa band in the cell fraction and decrease of detection of the secreted ~240- and ~200-kDa bands in the culture supernatant, similar to the accumulated TpsA1 in the HB-1 tpsB1::kan strain (Fig. 2C). At high levels of IPTG (1mM) the amount of secreted TPS1 in the culture supernatant seemed to decrease (Fig. 2B), likely also as a result of the competition for TpsB. In contrast, expression of the TPS2a, TPS2b and TPS3 constructs did not result in a block of the secretion of the full-length TpsA2a and Tpsa2b proteins (results not shown). Overall, our results indicated that truncated TpsA constructs are expressed and secreted into the neisserial culture medium, similar to what was observed...
Fig 3 Complementation of TPS constructs. Immunoblots of cell lysates (C) and concentrated culture supernatants (S) of HB-1 tpsB1::kan/tpsB2::gen containing TPS and TPS + TpsB constructs that were induced for expression by IPTG (+) or not (−), as indicated on the top. The blots were incubated with antisera indicated on the right. (A) Immunoblot containing samples of cells expressing the TPS2 and TPS2+TpsB2 constructs incubated with antisera against the TpsB2 and TPS2 domain as indicated on the right. To analyze the leakage of cellular content to the supernatant we ultra-centrifuged the supernatant and included samples of the high-speed supernatant (hsS) and high-speed pellet (hsP) on the blot. The black arrowheads indicate the detected TPS2b domain and TpsB2. The “M” indicates a marker lane included in the blot. (B) Immunoblots containing samples of cells expressing the TPS1, TPS1+TpsB1 and TPS1+TpsB2 constructs. Of the supernatant of the TPS1+TpsB1 construct less was loaded on the gel to prevent overexposure. (C) Immunoblots of HB-1 tpsB1::kan tpsB2::gen expressing the TPS3 and TPS3+TpsB2 constructs incubated with antisera against the TpsB2 and the His tag. Indicated on the right are the positions of the Mw markers.

for other TPS systems. Furthermore, our results suggested that the secretion of a TpsA3 may not require the expression of a dedicated tpsB.

Specificity for TpsB1 and TpsB2 of meningococcal TPS domains

It has been suggested that recognition of a TPS domain by its TpsB transporter is system specific (17, 19), but this was only tested for a limited number of combinations of TPS domain and TpsB transporters. In fact, the observed secretion of the TPS3 domain suggested a reduced system-specificity of one or both of the N. meningitidis TpsBs. We therefore investigated secretion of the TPS domain in strains that lack either TpsB1, TpsB2 or both by transforming HB-1 tpsB1::kan, HB-1 tpsB2::kan (40) and HB-1 tpsB1::kan/tpsB2::gen (this study) with the TPS expression vectors and tested the transformants for secretion of the TPS
domains.

When the TPS constructs were expressed in HB-1 tpsB1::kan tpsB2::gen double knockout strain none of the TPS domains was detected in the culture supernatant (results not shown, see also Fig. 3) indicating that a TpsB protein is required for secretion. The TPS2a and TPS2b were clearly detected in the culture supernatant of HB-1 tpsB1::kan but not in that of HB-1 tpsB2::kan (Fig. 2) suggesting that the domains are specifically recognized by the TpsB2 transporter and not by TpsB1. Furthermore, TPS2a and TPS2b domains that were not secreted in the tpsB2::kan mutant appeared to be degraded, presumably in the periplasm, since no accumulation of the TPS domains was detected in the cell fractions. Of note, endogenous full-length TpsA2a and TpsA2b had also not been detected in a HB-1 tpsB2::kan background(40). The requirement of the TpsB2 for secretion was confirmed when we transformed the HB-1 tpsB1::kan tpsB2::gen strains with a neisserial expression vector that contained both TPS2b (of TpsA2b; Fig. 1) and tpsB2 under control of the lac promoter (construct TPS2b+TpsB2; Table 1). The TpsB2 was detected in cell fractions of cells harbouring this construct, confirming expression (Fig. 3A). We detected TPS2b in the concentrated supernatant fractions of cells that produced the TpsB2 from the TPS2b+TpsB2 construct and not in that of HB-1 tpsB1::kan tpsB2::gen cells containing the TPS2b construct. Similar results were obtained with a TPS2a+TpsB2 construct (results not shown). We detected trace amounts of the overproduced TpsB2 protein in the supernatant, suggesting that a low-level of leakage of cellular content had occurred. Such leakage may derive from the formation of outer membrane vesicles or blebs, for which N. meningitidis is well-known. To test this supposition and to exclude that the TPS2 proteins detected in the supernatant did result from mere leakage, we subjected the culture supernatant to ultracentrifugation at 200,000×g to pellet blebs and cellular debris (Fig. 3A). The results clearly indicated that the TpsB2 protein detected in the culture supernatant was included in the high-speed pellet fraction and absent from the high-speed supernatant fraction. Importantly the TPS2b protein remained almost totally in the high-speed supernatant, indicating secretion rather than leakage. In support, we tested the distribution of the periplasmic protein RmpM over these fractions (Fig. S1). RmpM associates with outer membrane

<table>
<thead>
<tr>
<th>Construct</th>
<th>Source</th>
<th>NCBJ Protein acc. no.</th>
<th>length TPSa</th>
<th>positionb</th>
<th>Mw (kDa)</th>
<th>observed Mwb</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPS1</td>
<td>N. meningitidis H44/76</td>
<td>EFV63348</td>
<td>307</td>
<td>73-379</td>
<td>31,3</td>
<td>32</td>
</tr>
<tr>
<td>TPS2a</td>
<td></td>
<td>EFV64269</td>
<td>325</td>
<td>81-405</td>
<td>33,8</td>
<td>39</td>
</tr>
<tr>
<td>TPS2b</td>
<td></td>
<td>EFV63446</td>
<td>375</td>
<td>78-452</td>
<td>38,9</td>
<td>39</td>
</tr>
<tr>
<td>TPS3</td>
<td></td>
<td>EFV64019</td>
<td>379</td>
<td>78-456</td>
<td>38,9</td>
<td>39/37</td>
</tr>
<tr>
<td>TPS-NL2a</td>
<td>N. lactamica 26793</td>
<td>KC182755</td>
<td>391</td>
<td>76-467</td>
<td>40,7</td>
<td>41</td>
</tr>
<tr>
<td>TPS-NL4</td>
<td></td>
<td>KC182756</td>
<td>311</td>
<td>78-389</td>
<td>31,1</td>
<td>32</td>
</tr>
<tr>
<td>TPS-HMW1</td>
<td>H. influenzae A950006</td>
<td>AAD56660</td>
<td>298</td>
<td>69-365</td>
<td>32,0d</td>
<td>33</td>
</tr>
<tr>
<td>TPS-PA</td>
<td>P. aeruginosa PAO1</td>
<td>NP_249381</td>
<td>323</td>
<td>39-361</td>
<td>34,24</td>
<td>35</td>
</tr>
</tbody>
</table>

a Expected length of the sequence that folds into the TPS domain after passage of the IM
b Position of the TPS sequence in the full-length TpsA

<table>
<thead>
<tr>
<th>Source</th>
<th>NCBJ Protein acc. no.</th>
<th>length TPSa</th>
<th>positionb</th>
<th>Mw (kDa)</th>
<th>observed Mwb</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPS1</td>
<td>N. meningitidis H44/76</td>
<td>EFV63348</td>
<td>307</td>
<td>73-379</td>
<td>31,3</td>
</tr>
<tr>
<td>TPS2a</td>
<td>EFV64269</td>
<td>325</td>
<td>81-405</td>
<td>33,8</td>
<td>39</td>
</tr>
<tr>
<td>TPS2b</td>
<td>EFV63446</td>
<td>375</td>
<td>78-452</td>
<td>38,9</td>
<td>39</td>
</tr>
<tr>
<td>TPS3</td>
<td>EFV64019</td>
<td>379</td>
<td>78-456</td>
<td>38,9</td>
<td>39/37</td>
</tr>
<tr>
<td>TPS-NL2a</td>
<td>N. lactamica 26793</td>
<td>KC182755</td>
<td>391</td>
<td>76-467</td>
<td>40,7</td>
</tr>
<tr>
<td>TPS-NL4</td>
<td>KC182756</td>
<td>311</td>
<td>78-389</td>
<td>31,1</td>
<td>32</td>
</tr>
<tr>
<td>TPS-HMW1</td>
<td>H. influenzae A950006</td>
<td>AAD56660</td>
<td>298</td>
<td>69-365</td>
<td>32,0d</td>
</tr>
<tr>
<td>TPS-PA</td>
<td>NP_249381</td>
<td>323</td>
<td>39-361</td>
<td>34,24</td>
<td>35</td>
</tr>
</tbody>
</table>
Two-Partner Secretion systems specificity in Neisseria meningitidis

in combination with either \textit{tpsB1} or \textit{tpsB2} (TPS1+TpsB1 or TPS1+TpsB2, respectively; Fig. 1 and Table 1). HB-1 \textit{tpsB1::kan/tpsB2::gen} cells expressing the TPS1+TpsB1 construct efficiently secreted TPS1 into the culture supernatant (Fig. 3B). However, cells that express the TPS1+TpsB2 construct also secreted TPS1 into the culture supernatant. Again, this secretion appeared less efficient since TPS1 protein accumulated in the cell fractions. The secretion of the TPS1 domain by TpsB2 led us to re-assess the secretion of full-length TpsA1 in HB-1, HB-1 \textit{tpsB1::kan}, HB-1 \textit{tpsB2::kan}, and HB-1 \textit{tpsB1::kan/tpsB2::gen}. Previously, we had published that in a HB-1 \textit{tpsB1::kan} knock-out strain full-length TpsA1 accumulated in the cell fraction, while no secreted protein was detected in the culture supernatant. When we re-analysed secretion of the endogenous full-length TpsA1 and included the analysis of a high-speed supernatant, we observed a similar pattern as previously published (40). Absence of TpsB1 in both the \textit{tpsB1::kan} and \textit{tpsB1::kan/tpsB2::gen} strains resulted in accumulation of a cellular ~240-kDa form of full-length proteins and is thought to connect the outer membrane to the peptidoglycan layer (14). Low amounts of RmpM were detected in the supernatant. Unlike the TPS2b domain, this RmpM protein was found to split between the high-speed pellet and the high-speed supernatant fractions. Overall, the results supported our conclusion that the TPS2b domain is secreted by the TpsB2 protein.

Fig 4 Expression and secretion of \textit{N. lactamica} TPS constructs in \textit{N. meningitidis} HB-1, HB-1 \textit{tpsB1::kan} \textit{tpsB2::gen}, HB-1 \textit{tpsB1::kan} and HB-1 \textit{tpsB2::kan}. Immunoblots of cell lysates (C) and culture supernatants (S) of cells grown in the presence or absence of IPTG to induce expression of the TPS construct. The bacterial strains are indicated on the top, while the TPS constructs with which the strains were transformed are indicated on the left. The blots were incubated with antisera against the His-tag included in the TPS domains, as indicated on the right. Indicated on the right are also the positions of the Mw markers. The “*” indicates the TPS-NL2b domain secreted by the meningococcal TpsB1 protein.
TpsA1, despite of the presence of TpsB2 in the former strain (Fig. S3A). The high-speed culture supernatant of these two knock-out strains lacked the secreted ~240-, ~200- and ~75-kDa forms of TpsA1. Apparently, the levels of endogenous TpsA1 were too low to be secreted via endogenous TpsB2, or secretion of full-length TpsA1 by TpsB2 is hampered because additional sequences within TpsA1 contribute to the secretion process. Finally, we tested the secretion of the TPS3 domain in the tpsB1::kan and tpsB2::kan and tpsB1::kan tpsB2::gen knock-out strains. The TPS3 domain was detected in the culture supernatant of the tpsB1::kan strain, but not in that of the tpsB2::kan (Fig. 2) or tpsB1::kan tpsB2::gen strains (Fig. 3C), suggesting efficient secretion by the TpsB2 transporter. We confirmed this observation by transforming HB-1 tpsB1::kan tpsB2::gen with a neisserial expression vector that co-expressed TPS3 and tpsB2 (TPS3+TpsB2; Table 1). It resulted in the presence of TpsB2 in the cell fraction and the secretion of the TPS3 in the culture supernatant of these strains (Fig. 3C and results not shown). Overall, the results indicated that the TpsB2 transporter of N. meningitidis H44/76 is able to secrete all TPS domains encoded in the meningococcal genome.

Species-specificity of the TpsB transporters
Our results indicated a relaxed specificity of the TpsB2 transporter for TPS domains, while the TpsB1 appeared to be only committed to transport TpsA1 proteins. To investigate the extent of the observed relaxed specificity of TpsB2, we assessed whether TPS domains from systems of other bacterial species could be secreted by N. meningitidis. We first cloned truncated ORFs encoding the signal peptide and the TPS domains of the TpsAs HMW1 of H. influenzae A950006 (39) and PA0690 of P. aurigonosa PAO1 (22) and included a C-terminal His tag for immunodetection (Table 1). In sequence alignments these TPS domains scored similarity scores of 23-33% when compared to the meningococcal domains (Table S2). After introduction of the constructs into N. meningitidis HB-1, the TPS domains were not detected in samples of the culture supernatants and the whole-
Two-Partner Secretion systems specificity in Neisseria meningitidis

Two-Partner Secretion systems specificity in Neisseria meningitidis

N. meningitidis (80% identity/85% similarity), while that encoded by NL-TPS4 is more distantly related to the N. meningitidis systems (62%/78% to its closest homologue TP3). The constructs were introduced into N. meningitidis HB-1, HB-1 tpsB1::kan, HB-1 tpsB2::kan and HB-1 tpsB1::kan tpsB2::gen. Immunoblot analysis using anti-His antiserum on whole cell lysates and culture supernatants of N. meningitidis cells expressing the constructs showed efficient secretion of TPS-NL2a and TPS-NL4 in HB-1 and HB-1 tpsB1::kan (Fig. 4A), indicating that the meningococcal TpsB2 is efficiently transporting these TPS domains of N. lactamica. Remarkably, we observed secretion of TPS-NL2b and not of TPS-NL4 in the culture supernatant of HB-1 tpsB2::kan, albeit with lower efficiency. Apparently, the N. lactamica TPS2 domain (44%/61% homologous to TPS1) contains sequence information that allows recognition by the TpsB1, whereas such information is absent in the N. meningitidis TPS2 domains (40%/59% and 40%/57% homologous to TPS1). As expected, no secretion was observed in the tpsB1::kan tpsB2::gen double knock-out strain. Similar to the TPS1 domain, the TPS-NL2b domain in the cell fraction appeared more stable or less prone to periplasmic degradation (Fig. 4). Therefore, the secretion via the TpsB1 could be forced by the higher numbers of TPS-NL2b available. However, when we tested whether the secretion of the TPS-NL2b domain would block the secretion of the endogenous full-length TpsA1 in HB-1, we observed that, despite the accumulation of TPS-NL2b in the cell fraction and in contrast to the observations for the TPS1 domain (Fig. 2C), the full-length TpsA1 protein was normally secreted. Accumulation of the TPS-NL2b did not result in accumulation of full-length TpsA1 in the cell fraction and normal levels of the secreted TpsA1 proteins were detected in the supernatant (Fig. S3B). Apparently, the recognition of the meningococcal TpsB2 transporter is not completely promiscuous in transporting TPS domains.

We then investigated the ability of the meningococcal TpsBs to secrete TPS domains of N. lactamica, since this is a closely related bacterial species that occupies the same niche in the human body. Like in N. meningitidis several TPS systems have been identified in N. lactamica and not all appear to have a dedicated TpsB encoded (35, 36). Furthermore, sequence comparisons of TPS domains showed that the systems in N. meningitidis and N. lactamica are related (Table S2). To test the secretion of N. lactamica TPS domains in N. meningitidis, we cloned the sequences encoding the signal peptide and TPS domains of tpsA2b (TPS-NL2b) and tpsA4 (TPS-NL4) from N. lactamica strain 26793 into the neisserial expression vector. The TPS proteins were extended by a His tag for detection. The TPS-NL2b construct encodes a TPS domain that is related to TPS2b of N. meningitidis (80% identity/85% similarity), while that encoded by NL-TPS4 is more distantly related to the N. meningitidis systems (62%/78% to its closest homologue TP3). The constructs were introduced into N. meningitidis HB-1, HB-1 tpsB1::kan, HB-1 tpsB2::kan and HB-1 tpsB1::kan tpsB2::gen. Immunoblot analysis using anti-His antiserum on whole cell lysates and culture supernatants of N. meningitidis cells expressing the constructs showed efficient secretion of TPS-NL2a and TPS-NL4 in HB-1 and HB-1 tpsB1::kan (Fig. 4A), indicating that the meningococcal TpsB2 is efficiently transporting these TPS domains of N. lactamica. Remarkably, we observed secretion of TPS-NL2b and not of TPS-NL4 in the culture supernatant of HB-1 tpsB2::kan, albeit with lower efficiency. Apparently, the N. lactamica TPS2 domain (44%/61% homologous to TPS1) contains sequence information that allows recognition by the TpsB1, whereas such information is absent in the N. meningitidis TPS2 domains (40%/59% and 40%/57% homologous to TPS1). As expected, no secretion was observed in the tpsB1::kan tpsB2::gen double knock-out strain. Similar to the TPS1 domain, the TPS-NL2b domain in the cell fraction appeared more stable or less prone to periplasmic degradation (Fig. 4). Therefore, the secretion via the TpsB1 could be forced by the higher numbers of TPS-NL2b available. However, when we tested whether the secretion of the TPS-NL2b domain would block the secretion of the endogenous full-length TpsA1 in HB-1, we observed that, despite the accumulation of TPS-NL2b in the cell fraction and in contrast to the observations for the TPS1 domain (Fig. 2C), the full-length TpsA1 protein was normally secreted. Accumulation of the TPS-NL2b did not result in accumulation of full-length TpsA1 in the cell fraction and normal levels of the secreted TpsA1 proteins were detected in the supernatant (Fig. S3B). Apparently, the recognition of the meningococcal TpsB2 transporter is not completely promiscuous in transporting TPS domains.

We then investigated the ability of the meningococcal TpsBs to secrete TPS domains of N. lactamica, since this is a closely related bacterial species that occupies the same niche in the human body. Like in N. meningitidis several TPS systems have been identified in N. lactamica and not all appear to have a dedicated TpsB encoded (35, 36). Furthermore, sequence comparisons of TPS domains showed that the systems in N. meningitidis and N. lactamica are related (Table S2). To test the secretion of N. lactamica TPS domains in N. meningitidis, we cloned the sequences encoding the signal peptide and TPS domains of tpsA2b (TPS-NL2b) and tpsA4 (TPS-NL4) from N. lactamica strain 26793 into the neisserial expression vector. The TPS proteins were extended by a His tag for detection. The TPS-NL2b construct encodes a TPS domain that is related to TPS2b of N. meningitidis (80% identity/85% similarity), while that encoded by NL-TPS4 is more distantly related to the N. meningitidis systems (62%/78% to its closest homologue TP3). The constructs were introduced into N. meningitidis HB-1, HB-1 tpsB1::kan, HB-1 tpsB2::kan and HB-1 tpsB1::kan tpsB2::gen. Immunoblot analysis using anti-His antiserum on whole cell lysates and culture supernatants of N. meningitidis cells expressing the constructs showed efficient secretion of TPS-NL2a and TPS-NL4 in HB-1 and HB-1 tpsB1::kan (Fig. 4A), indicating that the meningococcal TpsB2 is efficiently transporting these TPS domains of N. lactamica. Remarkably, we observed secretion of TPS-NL2b and not of TPS-NL4 in the culture supernatant of HB-1 tpsB2::kan, albeit with lower efficiency. Apparently, the N. lactamica TPS2 domain (44%/61% homologous to TPS1) contains sequence information that allows recognition by the TpsB1, whereas such information is absent in the N. meningitidis TPS2 domains (40%/59% and 40%/57% homologous to TPS1). As expected, no secretion was observed in the tpsB1::kan tpsB2::gen double knock-out strain. Similar to the TPS1 domain, the TPS-NL2b domain in the cell fraction appeared more stable or less prone to periplasmic degradation (Fig. 4). Therefore, the secretion via the TpsB1 could be forced by the higher numbers of TPS-NL2b available. However, when we tested whether the secretion of the TPS-NL2b domain would block the secretion of the endogenous full-length TpsA1 in HB-1, we observed that, despite the accumulation of TPS-NL2b in the cell fraction and in contrast to the observations for the TPS1 domain (Fig. 2C), the full-length TpsA1 protein was normally secreted. Accumulation of the TPS-NL2b did not result in accumulation of full-length TpsA1 in the cell fraction and normal levels of the secreted TpsA1 proteins were detected in the supernatant (Fig. S3B). Apparently, the recognition
and secretion of the TPS-NL2b domain by TpsB1 was specific and efficient enough to allow for normal TpsA1 secretion to take place. Overall the results show that the TpsB2 transporter of *N. meningitidis* showed relaxed specificity in recognition of TPS domains that is not restricted to *N. meningitidis* TPS systems. By contrast, the TpsB1 showed a more restricted specificity, although it was engaged by the non-cognate TPS-NL2b.

**Structural modelling of the neisserial TPS domains.**

Structurally, the TPS domains are very similar (9, 44, 45). The crystallized domains show a β-helical stem structure to which α-helical and or β-sheet appendages are attached (Fig. 5). A multiple sequence alignment incorporating structure-derived information suggested that two major groups of TPS domain structures exist (45), one including the TPS domains of FHA of *B. pertussis* (9) and hemolysin A of Proteus mirabilis (44), the other including the TPS domain of HMW1 of *H. influenzae* (45). Sequence gazing already indicated that the neisserial TPS domains would fall into the FHA/HlyA class of domains. This was corroborated by the results of a multiple sequence alignment using the Expresso program (37), which incorporates structural information (Fig. S5). This sequence alignment showed a high level of sequence homology, but interspersed there are regions of sequence divergence. We have used the same TPS domain sequences to build structural models using the Phyre modelling website (21). The resulting models (Fig. 5) all were largely based upon the FHA TPS domain structure, with the exception of TPS-NL2b (Table S3). To assess the quality of the obtained models, we compared them to the FHA TPS domain structure using the TM-align algorithm (46). It resulted in scores of 0.89-0.97 (Table S3), indicative of a high level of confidence. We then mapped the sequences with the highest divergence in the alignments on these models and found these clustered to a specific region that includes the last strand of the β-sheet that extends from the β-helical stem and the following loop. It suggested that this region might be involved in the interaction with the TpsB transporter.

**Discussion**

*N. meningitidis* and *N. lactamica* strains encode multiple TPS systems in a complex chromosomal organisation that includes the presence of singular tpsAs (40, 41). Here, we investigated the specificity of the meningococcal TpsB proteins for a range of TPS domains. Our results show that the TpsB1 transporter transports its cognate TPS domains and not the other meningococcal TPS domains. By contrast, the TpsB2 transporter is able to secrete all TPS domains encoded in the meningococcal genome, as well as the two *N. lactamica* TPS domains tested. Surprisingly, the TpsB1 was also able to secrete a *N. lactamica* TPS domain.

Although many TPS systems are organized in one operon (16, 17), our results clearly indicate that clustering is not necessary for TpsA secretion to occur. Interestingly, it was shown for the *E. coli* O157:H7 OtpA/OtpB TPS system that a TpsB and TpsA need not to be co-expressed for the TpsB to be functional (7). Meningococcal isolates contain up to five different tpsA genes distributed over different chromosomal loci, whereas only two tpsB genes were found. Based upon chromosomal location of tpsAs and tpsBs (Fig. 1) and the homology of TPS domains (Table S2) we proposed that these genes are part of three systems (35). The tpsB genes are both in an apparent operon with a tpsA and were designated system 1 and 2. To each of these two systems a singular tpsA was assigned that was located elsewhere on the chromosome (Fig. 1). We confirm here that these singular TpsAs can be secreted via their designated TpsBs.
third meningococcal TPS system consists of a singular *tpsA* gene encoding a protein that carries a TPS domain with a sequence distinct from the other systems (Table S2). In the five sequenced *N. meningitidis* genomes that contain a *tpsA*3 no third *tpsB* could be identified (40, 41), and results not shown). Here we show the TPS3 domain is secreted by TpsB2, which suggests that a cognate TpsB may not be required or may not exist at all. The latter notion is supported by the observation that five sequenced *N. lactamica* genomes contain an almost identical *tpsA*3 gene whereas they all lack an obvious *tpsB*3 gene (results not shown). Most likely, the system evolved without a dedicated TpsB.

The presence of multiple TPS systems and of singular *tpsA* genes has been described for other bacterial species. For example, strain O35E of *Moraxella catarrhalis* contains a TPS operon encoding the TpsA-like MhaB2 and the TpsB-like MhaC that co-localize with a gene encoding a TpsA-like protein called MhaB1 in opposite orientation (2). The perfectly conserved signal peptide and TPS domains within MhaB1 and MhaB2 suggest that they both use the MhaC protein for transport across the outer membrane. A similar organisation was found in *Haemophilus ducreyi* for the LspA1 and LspA2 TPS proteins (43). Furthermore, in *Pseudomonas* genomes multiple TPS systems have been detected including a singular *tpsA*6 (5). However, in contrast to the meningococcal singular TpsA3, the TpsA6 of *P. aeruginosa* PAO1 is secreted via a dedicated usher that also functions in the secretion of pilins and not via a TpsB (30).

The TpsB2 transporter appeared promiscuous in the recognition and secretion of different TPS domains, albeit that the efficiency appeared decreased for the distantly related TPS1 and TPS-NL4 domains. By contrast, the TpsB1 transporter showed a restricted specificity by transporting its cognate TPS1 domain and not the other meningococcal TPS domains. Surprisingly, TpsB1 also secreted the TPS-NL2b domain from *N. lactamica*, whereas on sequence and predicted structural level it is very similar to the meningococcal TPS domains that were not secreted by TpsB1 (Table S2; Fig. S4).

Apparently, the recognition of a TPS domain by TpsB transporters depends on specific motifs or residues within the TPS domains and not on the overall homology between these domains. Within the TpsB proteins the periplasmic POTRA domains (for Polypeptide transport-associated) seem important for the recognition of the TPS domains and their deletion renders the TpsB inactive (11, 18). The exact interaction interface between TPS domain and TpsB protein is unknown and, apparently, cannot be deduced from sequence alignments. Structurally, the TPS domains are very similar (9, 44, 45). The crystallized domains show a β-helical stem structure to which α-helical and or β-sheet appendages are attached (Fig. 5) and the most conserved region within the TPS domains appeared critical for the overall structure (9). Structure-informed alignments of the neisserial TPS domains indicated that the differences between them cluster to a specific region in structural models that is outside of the β-helical stem (Fig. 5). Exploiting the overall similarity of the meningococcal TPS domains, the observed specificity in TpsB selection, and the detected local differences that may confer this specificity could be instrumental in mapping the interaction interface in detail. For now, we do not know whether the observed redundancy of meningococcal TpsBs for secretion of TPS domains extends to full-length TpsAs. We did not observe secretion of full-length TpsA1 by TpsB2 when expressed at endogenous levels in HB-1. This may be because of too low expression levels, or because of additional sequences within the TpsA1 that may contribute to the secretion process, as
observed for FHA (24). Nevertheless, it has been widely accepted that TPS domains are absolutely required for TpsA secretion, due to their targeting function and the redundancy of meningococcal TpsBs for TPS domain recognition may not be trivial, because the meningococcal TPS systems are encoded on genomic islands that carry hallmarks of horizontal gene transfer. Furthermore, the presence of specific TPS systems appears to associate with invasive N. meningitidis isolates (4, 40). Our results suggest that TPS systems may be able to use a local TpsB to be secreted and active. For example, we have identified meningococcal disease isolates that lack a tpsB1, but encode a TpsA1 and a TpsB2 (40), which then would allow for its secretion. In addition, our analysis of the N. lactamica ST-640 genome encodes two identical copies of a tpsB gene in an operon with a tpsA. The ORFs were distinct from the known neisserial TPS systems and hence were classified as TPS system 4 (40). The ST-640 strain also encodes full-length TpsA2 and TpsA3 proteins, but no tpsB2. Of course, it needs to be confirmed whether the TpsB4 of ST-640 would be able to secrete TpsAs of other systems, but our findings support a model where neisserial TpsAs may use a wider range of TpsBs for secretion than just their designated TpsBs. This flexibility, combined with a propensity to be distributed via horizontal gene transfer could result in a competitive advantage over other bacterial strains and species. The meningococcal TpsA1 proteins have recently been suggested to act as toxins that kill other bacteria via contact-dependent inhibition (27), which would support such a role.

Acknowledgements: Joen Luijink is acknowledged for critically reading the manuscript. Peter van der Ley (RIVM Bilthoven, The Netherlands) is acknowledged for providing the anti-RmpM monoclonal. S. u R. gratefully acknowledges funding by the Higher Education Commission of Pakistan.

Two-Partner Secretion systems specificity in Neisseria meningitidis


SUPPLEMENTAL DATA

Fig S1 Immunoblot of samples of cultures of *N. meningitidis* HB-1 and HB-1 *tpsB1::kan/tpsB2::gen* cells containing the TPS2+TpsB2 construct and induced with IPTG (+) or not (-). The blot was incubated with a monoclonal antibody against RmpM. To analyze the leakage of cellular content to the supernatant of HB-1 *tpsB1::kan/tpsB2::gen* cultures we ultra-centrifuged the supernatant and included samples of the high-speed supernatant (hsS) and high-speed pellet (hsP) on the blot. The “M” indicates a marker lane included in the blot.

Fig S2 Immunoblot of samples of cultures of *N. meningitidis* HB-1 *tpsB1::kan* containing the TPS1 construct induced with different amounts of IPTG as indicated on top. The blot was incubated with antisera against the TPS1 domain. The TPS1 bands representing unprocessed (41 kDa) and processed (32 kDa) forms are indicated by black arrowheads.
Fig S3 Immunoblot of samples of cultures of (A) *N. meningitidis* HB-1, HB-1 *tpsB1::kan*, HB-1 *tpsB2::kan* and HB-1 *tpsB1::kan/tpsB2::gen* cells and of (B) HB-1 cells containing the TPS2, TPS3, TPS-NL4, or TPS-NL2b constructs and induced with IPTG (+) or not (-). The blots were incubated with an antiserum against TPS1.

### Table S1: Primers used in this study

<table>
<thead>
<tr>
<th>Expressed protein</th>
<th>gene</th>
<th>strain</th>
<th>Primers used</th>
</tr>
</thead>
</table>
| sp'TPS1a         | tpsA1a | *N. meningitidis* H44/76 | F: GCGATATGATCGAGAATTACACGCGAGCGAGTTCA
| sp'TPS2a         | tpsA2a | *N. meningitidis* H44/76 | R: CAGAACGAGTAACAATTGACACCGCGAGAATTACACGCGAGCGAGTTCA
| sp'TPS2b         | tpsA2b | *N. meningitidis* H44/76 | F: CCGATATCAGGATGATCGAGAATTACACGCGAGCGAGTTCA
| sp'TPS3          | tpsA3  | *N. meningitidis* H44/76 | F: GCGATATGATCGAGAATTACACGCGAGCGAGTTCA
| TpsB1            | tpsB1  | *N. meningitidis* H44/76 | F: CCGATATCAGGATGATCGAGAATTACACGCGAGCGAGTTCA
| TpsB2            | tpsB2  | *N. meningitidis* H44/76 | R: CAGAACGAGTAACAATTGACACCGCGAGCGAGTTCA
| sp'TPS-NL2a      | tpsA2b | *N. lactamica* 26793  | F: CCGATATCAGGATGATCGAGAATTACACGCGAGCGAGTTCA
| sp'TPS-NL4       | tpsA4  | *N. lactamica* 26793  | R: CAGAACGAGTAACAATTGACACCGCGAGCGAGTTCA
| sp'TPS-PA0690    | PA0690 | *P. aeruginosa* PA01 | F: GCGATATGATCGAGAATTACACGCGAGCGAGTTCA
| sp'TPS-HMW1      | hmwi  | *H. influenzae* A95006  | R: GCGATATGATCGAGAATTACACGCGAGCGAGTTCA

...
Two-Partner Secretion systems specificity in Neisseria meningitidis

Table S2: Pairwise alignments of Neisserial TPS domains

<table>
<thead>
<tr>
<th>N. meningitidis H44/76</th>
<th>TPS1a</th>
<th>TPS2a</th>
<th>TPS2b</th>
<th>TPS3</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. meningitidis H44/76</td>
<td>100 E=0.0</td>
<td>41/60/10 E=1e-60</td>
<td>40/57/10 E=9e-60</td>
<td>44/64/7 E=6e-69</td>
</tr>
<tr>
<td>N. lactamica ST-640</td>
<td>40/59/11 E=1e-59</td>
<td>100 E=0.0</td>
<td>89/92/7 E=0.0</td>
<td>57/74/6 E=5e-114</td>
</tr>
<tr>
<td>N. lactamica 26793</td>
<td>40/57/10 E=3e-59</td>
<td>88/92/3 E=0.0</td>
<td>100 E=0.0</td>
<td>58/73/4 E=4e-112</td>
</tr>
<tr>
<td>P. aeruginosa PA01</td>
<td>44/64/7 E=6e-69</td>
<td>58/75/3 E=3e-116</td>
<td>59/73/3 E=1e-114</td>
<td>100 E=0.0</td>
</tr>
<tr>
<td>B. pertussis</td>
<td>41/60/11 E=7e-61</td>
<td>92/96/0 E=0.0</td>
<td>89/93/0 E=5e-170</td>
<td>58/73/3 E=6e-115</td>
</tr>
<tr>
<td>H. influenzae N50006</td>
<td>41/61/8 E=1e-66</td>
<td>73/82/8 E=3e-153</td>
<td>80/85/5 E=2e-167</td>
<td>61/75/1 E=1e-119</td>
</tr>
<tr>
<td>TPS2a</td>
<td>42/61/8 E=1e-60</td>
<td>57/71/6 E=5e-110</td>
<td>57/70/4 E=3e-111</td>
<td>61/77/1 E=4e-122</td>
</tr>
<tr>
<td>TPS4a</td>
<td>32/47/18 E=5e-05</td>
<td>24/39/14 E=3e-04</td>
<td>25/39/16 E=7e-05</td>
<td>23/46/24 E=0.004</td>
</tr>
<tr>
<td>TPS3b</td>
<td>33/56/10 E=0.32</td>
<td>32/42/24 E=0.063</td>
<td>31/39/24 E=0.46</td>
<td>32/48/16 E=0.41</td>
</tr>
<tr>
<td>TPS3c</td>
<td>283</td>
<td>3F3Y</td>
<td>3</td>
<td>0.96228</td>
</tr>
<tr>
<td>TPS-NL2b Nlac</td>
<td>286</td>
<td>1RWR</td>
<td>5</td>
<td>0.96795</td>
</tr>
</tbody>
</table>

The length of the aligned sequences are indicated between brackets.

Values given are: %identity/%similarity/%gaps E-values for the alignment

1RWR is the sequence of the crystallized FhaB TPS domain (Clantin et al., 2004)

Table S3: Overview of the structural models of the neisserial TPS domains generated by Phyre

<table>
<thead>
<tr>
<th>TPS domain</th>
<th>length sequence</th>
<th>Phyre2/best hit</th>
<th>Phyre2/ab initio</th>
<th>TM-align score to 1RWR</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPS1 Nmen</td>
<td>302</td>
<td>1RWR</td>
<td>28</td>
<td>0.69045</td>
</tr>
<tr>
<td>TPS2a Nmen</td>
<td>301</td>
<td>1RWR</td>
<td>7</td>
<td>0.96280</td>
</tr>
<tr>
<td>TPS2b Nmen</td>
<td>301</td>
<td>1RWR</td>
<td>16</td>
<td>0.93297</td>
</tr>
<tr>
<td>TPS3 Nmen</td>
<td>301</td>
<td>1RWR</td>
<td>11</td>
<td>0.94719</td>
</tr>
<tr>
<td>TPS-NL2b Nl</td>
<td>283</td>
<td>3F3Y</td>
<td>3</td>
<td>0.96228</td>
</tr>
<tr>
<td>TPS-NL4 Nl</td>
<td>286</td>
<td>1RWR</td>
<td>5</td>
<td>0.96795</td>
</tr>
</tbody>
</table>

1 Results obtained from http://www.ebi.org.uk/phyre2/html/page.cgi?id=index
2 Number of residues for which modelling was not based upon structural homology

1RWR is the sequence of the crystallized FhaB TPS domain (Clantin et al., 2004)


The Two-Partner Secretion transporter TpsB2 of Neisseria meningitidis secretes a non-cognate full-length TpsA1 but not to full functionality

Sadeeq ur Rahman¹, Jesus Arenas², Jan Tommassen² and Peter van Ulsen¹

1 Section of Molecular Microbiology, Department of Molecular Cell Biology, VU University, de Boelelaan 1085, 1081 HV Amsterdam, The Netherlands
2 Section Molecular Microbiology, Department of Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

Manuscript in preparation
The Two-Partner Secretion transporter TpsB2 of *Neisseria meningitidis* secretes a non-cognate full-length TpsA1 but not to full functionality

The TpsB transporters of Two-partner secretion (TPS) systems of Gram-negative bacteria are responsible for the secretion of large exoproteins, generically called TpsAs, across the outer membrane. Various Gram-negative bacteria, including *Neisseria meningitidis*, encode multiple copies of TpsAs often in combination with specific TpsBs. However, the TpsB2 of *N. meningitidis* binds and secretes TPS domains of TpsAs from other neisserial TPS systems. This redundant recognition may be relevant in vivo, because *N. meningitidis* and *N. lactamica* genomes encode TpsAs that lack a cognate TpsB. Whether this redundancy also applies to full-length TpsAs has not been tested. Here, we show that TpsB2 can transport full-length and non-cognate TpsA1 proteins to the cell surface. Interestingly, we observed a considerable difference for TpsB2-dependent secretion of TpsA1s both in efficiency and in forms of protein secreted when compared to their TpsB1-mediated secretion. Furthermore, although TpsB2 allowed secretion of TpsA1, this secreted TpsA1 substrate did not show the antibacterial activity of the wild-type TpsA1 again suggesting a difference in the form that is secreted.

**Introduction**

Gram-negative bacteria have evolved a number of pathways with sophisticated machineries to secrete proteins across their cell envelope, which consists of the inner and outer membranes separated by the periplasm. The Two-Partner Secretion (TPS) systems utilize a dedicated transporter in the outer membrane, the TpsB transporter, to secrete a specific substrate, the TpsA exoprotein, across the outer membrane (1). TpsAs are typically proteins of > 100 kDa that are secreted into the extracellular milieu or to the cell surface to which they attach via non-covalent interactions. They function as bacterial adhesins, as toxins for bacterial or eukaryotic cells, or in obtaining nutrients from the environment. TpsAs contain an N-terminal region of ~250-300 residues called the TPS domain that is crucial for secretion (2). The TpsB transporters are characterized by a C-terminal 16-stranded β barrel inserted in the outer membrane and two polypeptide transport-associated (POTRA) domains in the periplasm (3,4). Multiple TPS systems co-exist in several bacterial species, including *Neisseria meningitidis*, *Haemophilus influenzae*, *Moraxella cattharalis* and *Pseudomonas aeruginosa* (5-9). TpsBs identify and select their cognate TpsA proteins for secretion by interacting with the TPS domains (10-12) and this interaction determines whether a TpsA protein is secreted across the outer membrane (12).

The Gram-negative diplococcus *N. meningitidis* (meningococcus) is a major cause of meningitis and sepsis worldwide. It is a strictly human pathogen that colonizes the nasopharynx without causing disease, but can cross the nasopharyngeal epithelium to cause sepsis and meningitis (13,14). Secreted proteins are considered crucial for pathogenesis of meningococcal invasive disease. *N. meningitidis* genomes encode up to three TPS systems and these are thought to be involved in virulence (6,15). TPS system one is ubiquitous in all *N. meningitidis* strains and promotes adherence to and intracellular survival in cultured human epithelial cell lines (16). Recently, TpsA1 proteins have been shown to act as contact-dependent-growth
inhibition (CDI) toxins that kill other neisserial isolates (17). No specific functions have been attributed to meningococcal TPS systems two and three, but they are more prevalent in hyperinvasive clonal complexes suggesting at least a role in virulence (6).

Our recent studies on the substrate specificity of the two meningococcal TpsB transporters TpsB1 and TpsB2 showed that the TpsB1 specifically recognizes the TPS domain of its cognate TpsA1 partners while TpsB2 is more promiscuous and able to secrete the TPS domains of all TpsA proteins of *N. meningitidis* and *N. lactamica*, albeit with different efficiencies (18). Such broad substrate specificity may explain the secretion of the TpsA of TPS system 3 of *N. meningitidis*, for which no TpsB has been identified (6). Recognition and secretion of TpsA appeared completely dependent on the POTRA domains, since exchange of these domains between TpsB1 and TpsB2 resulted in a switch in TpsA preference (18). Of note, many of the observations that resulted in the current model for the secretion mechanism of TpsA proteins have been obtained using truncated TpsA constructs consisting only of the signal sequence and TPS domain of a given TpsA (10,18). Our previous results with exchanged POTRA domains showed that the interaction between these domains and the TPS domain determines whether a full-length TpsA is secreted or not (18). Here, we investigated whether the relaxed specificity of the TpsB2 for the TPS domain extends to the secretion of the corresponding full-length TpsAs. Our study indicates that TpsB2 can, indeed, transport full-length TpsA1s of system 1, but the secreted TpsA1 appeared not fully functional, since it was not active as a toxin.

Materials and Methods

Bacterial strains and growth conditions. The *N. meningitidis* strains used in this study (Table 1) were grown on GC agar (Oxoid) supplemented with Vitox (Oxoid) at 37°C, 5% CO₂. Liquid cultures of *N. meningitidis* strains were grown at 37°C in tryptic soy broth (TSB) (Gibco-BRL). If necessary, chloramphenicol was added to a final concentration of 8 µg/ml for plasmid selection. *Escherichia coli* strains Top10F’ (Invitrogen) and DH5a were grown on Luria Bertani broth (LB) or agar plates, supplemented with 100 mg/ml ampicillin or 30 mg/ml chloramphenicol for plasmid maintenance and with 0.5% glucose for full repression of the lac operator, when appropriate.

Cloning and plasmid construction. The ORFs of *tpsB1* and *tpsB2* of *N. meningitidis* H44/76 were cloned into a neisserial expression vector (pEN) (19) using PCR and Phusion DNA polymerase (Finnzymes) according to the recommendations of the manufacturer. As a template chromosomal DNA was obtained from lysed bacterial cells as described earlier (19) and the primer combination used were pr_TpsB1_F(CACATATGGGATCCGCTAAAATATATTGATGCTAGAATT) and pr_TpsB1_R(GAGATCTGAATTCTAGAAACTGTAATTCAAGTTGAAAGCCGT) for *tpsB1* and pr_TpsB2_F(TCCGTGTATTGAATGCCATTGGT) and pr_TpsB2_R(GAGATCTGAATTCCGATCGTATTGGATTATCGTATCGTAACTGTAATTCAAGTTGAAAGCCGT) for *tpsB2*, respectively (12). The resulting amplicon was cloned into the pGEM-T (Promega) cloning vector and confirmed by sequencing (Macrogen). The *tpsB1* ORF was subcloned into pET11a (Invitrogen) cut with BamHI and EcoRI, using the BamH1/AgeI and AgeI/EcoR1 restriction sites in the ORF and yielding pET-TpsB1. The *tpsB2* ORF was subcloned into pET11a using BamHI and EcoRI yielding
protein was deduced from the Precision Plus Protein Standard (BioRad) included in each SDS-PAGE gel.

**Protease digestion assay.** Cultures of the *N. meningitidis* strains to be tested were grown in TSB, induced with 0.5 mM IPTG for expression of *tpsB* ORFs when appropriate and grown further for another hour. Cells were harvested by centrifugation and washed once with TSB. Cells were resuspended in TSB to a final OD$_{600}$ of 10 and kept for 5 min at 25°C to adapt. Trypsin (Promega) was added to a final concentration of 5 µg/ml of cells and further incubated for 15 min at 25°C, after which the protease activity was stopped by adding trypsin inhibitor (Sigma) and incubating on ice for 10 min. As a control served untreated cells resuspended in TSB and incubated at 25°C prior to addition of trypsin inhibitor. After the reaction was stopped, samples were analysed by SDS-PAGE as described above.

**Mass spectrometry analysis.** Analysis by MALDI-MS/MS was performed as described earlier (20). Briefly, gel pieces were excised from CBB-stained SDS-PAGE gels and destained using 100 µl of 50% acetonitrile solution containing 25 mM ammonium bicarbonate and washed in 100% acetonitrile. Then, the samples were dried and rehydrated for 45 min at 4°C in 15 µl of 20 ng/µl trypsin (sequencing grade, Promega) in 50 mM ammonium bicarbonate (pH 8). Proteins were then digested for 16 h at room temperature. Peptides were extracted by incubating the gel particles in 100 µl of acetonitrile solution containing 1% trifluoroacetic acid and concentrated using a vacuum centrifuge (Eppendorf). A concentrated sample of 1 µl was applied onto an Applied Biosystems MALDI plate. MS/MS spectra analysis was performed by using Mascot software (Matrix Science) and a database of *N. meningitidis* peptides. Parameters
used were a peptide mass tolerance of 0.15 kDa, a fragment mass tolerance of 0.1 Da, allowing a single miscleavage and allowing oxidation of methionine.

**Neisserial growth inhibition assay.** Rifampicin-resistant BB-1 and its ΔtpsB1 mutant derivative carrying either pEN-TpsB1 or pEN-TpsB2 were mixed 1:1 with the BB-1 ΔtpsA-tpsC strain carrying plasmid pFP10 with a gentamicin-resistance gene (17). Bacteria of the two strains were mixed and spotted on GC plates without antibiotics. GC plates either contained IPTG to induce tpsB expression or not. After 48 h of incubation, the number of viable bacteria on the plates was determined by plating serial dilutions on GC media containing rifampicin (50 µg/ml) or gentamicin (60 µg/ml) and counting colony-forming units. Results are expressed as the ratio of colonies of BB-1 ΔtpsA-tpsC over wild-type BB-1 colonies, or those of the BB-1 ΔtpsB mutants. Experiments were performed in triplicate.

**Sequence comparisons.** The amino-acid sequences for the TPS ORFs of H44/76 were extracted from the published genome sequences (21,22). The sequences of the *N. meningitidis* B16B6 TpsA1 (acc. no. AEC12886) and TpsB1 (acc. no. AEC12885) were extracted from the GenBank database (17). Sequences were aligned using the Clustal Omega alignment program at http://www.ebi.ac.uk/Tools/msa/clustalo/.

**Results**

**Secretion of TpsA1 by TpsB2 in *N. meningitidis* HB-1 requires its overexpression**

The TPS system 1 is ubiquitous in *N. meningitidis* strains and genomes include one or two copies of the genomic island encoding the system (6,15). In disease isolate H44/76, TPS system 1 consists of TpsB1 and two TpsAs: TpsA1a and TpsA1b, respectively (6,21). Furthermore, H44/76 TPS system 2 comprises TpsB2, TpsA2a and TpsA2b, and TPS system 3 contains TpsA3 without a dedicated TpsB. We studied the secretion of both full-length TpsA1s by TpsB1 and TpsB2 using a set of strains derived from strain HB-1, an unencapsulated derivative of disease isolate H44/76 (19). The set included knockout mutants of HB-1 in which genes tpsB1, tpsB2, or both had been replaced by antibiotic resistance markers (6,18) (Table 1). Expression experiments in single tpsA1a and tpsA1b knockout HB-1 strains had previously shown that the two TpsA1s appear similar on immunoblots (6). This is consistent with the fact that the two HB-1 TpsA1s are identical with the exception of their C-terminal ~100 amino acid residues, which differ considerably (Suppl. Fig. S1). Immunoblots probed with antiserum directed against the system 1 TPS domain (aTPS1) revealed multiple TpsA1-derived bands: at ~240 and ~200 kDa in cellular samples and at ~240, 200 and 75 kDa in concentrated samples of the culture supernatant (Fig. 1A). Of note, the ~200 kDa band in the supernatant is probably associated with spontaneously generated outer membrane vesicles known as blebs, since it is absent from the culture supernatant that has been subjected to an ultracentrifugation step (see also below) (18). Absence of the TpsB1 transporter in strains HB-1 tpsB1::kan and HB-1 tpsB1::kan tpsB2::gen resulted in accumulation of the ~240 kDa band detected in cell samples, whereas the bands in the culture supernatants disappeared (Fig. 1A) (6).

We have shown previously that TpsB2 is able to secrete truncated TpsA1-derived constructs that consist of the TPS1 domain (TPS1), albeit with reduced efficiency (18). Here, we examined whether TpsB2 can transport full-length TpsA1s by comparing the secretion profiles of HB-1 tpsB1::kan and HB-1 tpsB1::kan tpsB2::gen. However, we did not detect a clear difference on immunoblots (Fig. 1A)
Fig. 1. Secretion of TpsA1s of N. meningitidis HB-1 by TpsB1 and TpsB2. A. Immunoblots of whole cell lysates (C) and concentrated culture supernatants (S) of HB-1, HB-1 tpsB1::kan, HB-1 tpsB2::kan and HB-1 tpsB1::kan tpsB2::gen cultures incubated with αTPS1 antiserum. In all blots, TpsA1-derived bands are indicated by closed arrowheads, whereas open arrowheads represent background proteins (6). Indicated on the left are molecular weight markers. B. Top panel: immunoblots of whole cell lysates (left) and ultracentrifuged and concentrated culture supernatant samples (right) of HB-1 tpsB1::kan tpsB2::gen carrying pEN_TpsB1, pEN_TpsB2 or no plasmid (empty) incubated with antisera αTPS1, αTpsB1, αTpsB2 and αRmpM. Induction of expression by IPTG is indicated above the lanes. Lower panels: immunoblot of whole cell lysates of HB-1 (wild type) or HB-1 tpsB1::kan tpsB2::gen carrying either pEN_TpsB1 (+ tpsB1) or pEN_TpsB2 (+ tpsB2) incubated with αTpsB1 or αTpsB2, as indicated. C. Immunoblots of whole cell lysates of HB-1 tpsB1::kan tpsB2::gen carrying pEN_TpsB1, or no plasmid, treated with trypsin. Strains used are indicated above the blots. Blots were incubated with αTPS1 and αRmpM. The ~200-kDa cell-associated band appears surface exposed, since it is readily degraded by trypsin. Degradation products are marked (*). D. Effect of IPTG concentration on secretion of TpsA1 by TpsB2 analysed by immunoblots of whole cell lysates (C) and ultracentrifuged and concentrated culture supernatant samples (S) of HB-1 tpsB1::kan tpsB2::gen carrying pEN_TpsB2. Concentrations of IPTG used are indicated above the lanes.
TpsB2 transporter secretes non cognate full length TpsA

Table 1: N. meningitidis strains and expression plasmids used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Remark</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>strains:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HB-1</td>
<td>unencapsulated H44/76</td>
<td>(19)</td>
</tr>
<tr>
<td>HB-1 tpsB1::kan</td>
<td></td>
<td>(6)</td>
</tr>
<tr>
<td>HB-1 tpsB2::kan</td>
<td></td>
<td>(6)</td>
</tr>
<tr>
<td>HB-1 tpsB1::kan tpsB2::gen</td>
<td></td>
<td>(18)</td>
</tr>
<tr>
<td>BB-1</td>
<td>unencapsulated B16B6</td>
<td>(17)</td>
</tr>
<tr>
<td>BB-1 ΔtpsA-tpsC</td>
<td>lacks tpsA1 and immunity protein</td>
<td>(17)</td>
</tr>
<tr>
<td>BB-1 ΔtpsB1::kan</td>
<td>lacks tpsB1 gene</td>
<td>(17)</td>
</tr>
<tr>
<td>plasmids:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pEN_TpsB1</td>
<td>tpsB1 under control of lac promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pEN_TpsB2</td>
<td>tpsB2 under control of lac promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pFP10-gen</td>
<td>expression vector for gentamycin selection</td>
<td>(17)</td>
</tr>
<tr>
<td>pEN</td>
<td>neisserial expression vector</td>
<td>(19)</td>
</tr>
</tbody>
</table>

indicating that either full-length TpsA1 is not secreted by TpsB2, or the amounts of secreted protein are below the detection limit. To determine whether TpsB2 can secrete TpsA1 at all, we constructed neisserial expression vectors that contained the tpsB1 or tpsB2 ORFs under the control of an inducible lac promoter (pEN_TpsB1 and pEN_TpsB2, respectively). The plasmids were introduced into HB-1 tpsB1::kan tpsB2::gen and expression of the genes was induced by the addition of IPTG.

To remove blebs, we subjected the supernatant samples to ultracentrifugation prior to TCA precipitation (18). Western blot analysis using antibodies against the outer-membrane marker protein RmpM indicated that this step removed most of the blebs and cellular contaminants from the supernatant samples (Fig. 1B). The ~200 kDa band was not detected in the culture supernatant upon ultracentrifugation, indicative of localization in blebs (18). Recently, mass spectrometric analysis of outer membrane blebs also indicated the presence of TpsA1 in these vesicles (23).

Uninduced expression of the cognate tpsB1 from pEN-TpsB1 already resulted in restoration of TpsA1 secretion, as judged by the appearance of the ~240 and 75 kDa bands in the culture supernatant samples, despite the fact that the TpsB1 was not detectable under these conditions (Fig. 1B). Upon induction, TpsB1 was detected and the level of TpsA1 increased, most notable for the ~200 kDa TpsA1 band in the cell pellet and the ~75 kDa TpsA1 band in the supernatant (Fig. 1B). To confirm that the ~200 kDa cell-associated TpsA1 band is exposed to the cell surface, we treated whole cells with externally added trypsin. HB-1 tps1::kan tpsB2::gen cells that expressed tpsB1 from plasmid were incubated with trypsin (5 µg/ml) for 10 min at room temperature, after which protease activity was blocked. Western blot analysis clearly indicated that the ~200 kDa cell-associated TpsA1 protein species was susceptible to protease activity, resulting in decrease of the ~200 kDa band and the appearance of two prominent degradation products of ~180-160 kDa (Fig. 1C), which points at cell surface exposure and thus secretion of the protein. Interestingly, the ~240 kDa TpsB1-dependent cell associated-TpsA1 band was also reduced in amount after

85
Fig. 2. Secretion and functionality of TpsA1 of N. meningitidis BB-1 in presence of either TpsB1 or TpsB2. A. Immunoblots of whole cell lysates and culture supernatants of BB-1 (wild type), BB-1 tpsA1::kan, BB-1 tpsB1::kan (empty), tpsB1::kan carrying pEN_TpsB1 (+ tpsB1), or BB-1 tpsB1::kan carrying pEN_TpsB2 (+ tpsB2). Blots were incubated with αTPS1, αTpsB1 and αTpsB2. In all blots, the TpsA1-derived bands are indicated by closed arrowheads, whereas open arrowheads represent background proteins. Induction of expression by IPTG is indicated above the lanes. Indicated on the left are molecular weight markers. B. Immunoblots of whole cell lysates and ultracentrifuged and concentrated culture supernatant samples incubated with αTPS1. Strains used are the same as in panel A and indicated above the blots. C. Immunoblots of whole cell-lysates of BB1(right) and BB-1 tpsB1::kan (left) treated with trypsin. Strains used are indicated above the blots. Blots were incubated with αTPS1 and αRmpM. The ~200-kDa cell-associated band appears surface exposed, since it is readily degraded by trypsin Degradation products are indicated (*). D. Results of a growth inhibition assay in which cultures of BB-1 (wt) or BB-1 ΔtpsB1::kan containing plasmid pEN_TpsB1 or pEN_TpsB2 were mixed with BB-1 ΔtpsA-tpsC containing plasmid pFP10-gen. Cells were incubated under non-selecting conditions, after which serial dilutions were plated onto selective plates, containing either rifampicin or gentamycin to discriminate between the two strains mixed. Ratios of log CFUs are plotted as the mean of three independent experiments, with standard deviations indicated. The graph shows that cells having TpsB1 expressed from plasmid are as active as those having endogenous TpsB1. TpsA1 proteins secreted by TpsB2, by contrast, do not result in growth inhibition of the competing strain.
protease treatment more likely indicate cell surface exposure to some extent. The periplasmically exposed outer-membrane protein RmpM was not degraded under the conditions used, indicating that the cell integrity remained intact.

We then assessed secretion of TpsA1 by TpsB2 expressed from plasmid. Induction of expression of the tpsB2 from pEN-TpsB2 resulted in overproduced TpsB2 (Fig. 1B lower panel). High levels of TpsB2 resulted in the detection of the ~75 kDa and some ~240 kDa TpsA1 bands in the culture supernatant (Fig. 1B). In contrast, the ~200 kDa outer-membrane associated band was not detected. The identity of TpsA1-attributed bands in the culture supernatants of cells expressing tpsB2 from pEN_TpsB2 was confirmed by MALDI-MS/MS analysis (Suppl. Table S1). From these results, we concluded that TpsB2 could in part complement for the absence of TpsB1 secreting not only TPS1 domains (18) but also full-length TpsA1 proteins. Secretion of the ~240 and ~75 kDa proteins was dose-dependent and increased when more IPTG was added (Fig. 1D). However, the efficiency of TpsA1 secretion by TpsB2 is clearly lower than found for TpsB1, and the secretion of the ~200 kDa cell-associated TpsA1 protein species was not observed, suggesting that TpsB1 might play a role in post-secretion modification steps (6).

The H44/76 tpsB2 complements the secretion defect of a tpsB1 mutant of strain B16B6, but does not restore the function of TpsA1 as an antibacterial toxin.

Next we investigated whether TpsB2-secreted TpsA1 at the cell surface retains its function as a CDI toxin. This activity of TpsA1 has been established using N. meningitidis strain B16B6 (17). N. meningitidis strain B16B6 contains a TPS system 1 comprising one TpsB and one TpsA, while TPS systems 2 and 3 are absent and this system kills N. meningitidis B16B6 cells that do not express the toxin and its concomitant immunity protein. To test if a TpsB2-secreted TpsA1 protein can act as a CDI toxin, we introduced the plasmid encoding the TpsB2 of N. meningitidis H44/76 into BB-1 ΔtpsB1::kan, which is an unencapsulated derivative of B16B6 in which the tpsB1 gene is replaced by a kanamycin-resistance cassette (17). As a control, we introduced the pEN_TpsB1 plasmid encoding TpsB1 of strain H44/76. Western blots of cell and concentrated culture supernatant samples showed TpsA1 protein species of ~240 and ~200 kDa, but the ~75 kDa band appeared absent (Fig. 2A). As expected, the ~240 and ~200 kDa bands were absent in samples of the BB-1 ΔtpsA1-tpsC::kan knockout strain. Absence of TpsB1 in the BB-1 ΔtpsB1::kan knock-out strain resulted in accumulation of the ~240-kDa TpsA1 species in the cell samples. Expression of the heterologous H44/76 TpsB1, which shows 98% identity to that of B16B6 on sequence level (Fig. S1), restored secretion of TpsA1 and the appearance of the ~200 and ~240 kDa proteins in the cell and supernatant samples (Fig. 2A). Based on the secretion profile of HB-1, we hypothesized that the ~200 kDa protein in the culture supernatant associated with blebs, but analysis of the supernatant after an ultracentrifuge spin suggested it is secreted (Fig. 2B). Apparently, there are differences in the TpsA1 secretion pattern between the two strains, which may reflect their sequence differences (Fig. S1).

Interestingly, in the BB-1 ΔtpsB1::kan strain expressing tpsB2, a ~200 kDa band was detected in the cell and supernatant samples (Fig. 2A), which was absent from the culture supernatant after the ultracentrifuge spin, indicative of its association with the outer membrane (Fig. 2B). In contrast, the TpsB2-secreted TpsA1 in HB-1 was secreted in soluble form and not cell-associated (see Fig. 1B). To assess whether the ~200 kDa band
detected in BB-1 is exposed at the cell surface, we performed trypsin digestion on whole cells under similar conditions as used for HB-1 cells. Western blot analysis clearly indicated that the level of ~200 kDa TpsA1 protein species decreased, while degradation products of ~70 and ~80 kDa appeared (Fig. 2C).

We then assessed whether TpsB2-secreted TpsA1 was active in a growth inhibition assay towards a susceptible *N. meningitidis* strain (17). As expected, secretion of TpsA1 by the heterologous H44/76 TpsB1 resulted in killing activity. However, the TpsA1 secreted by TpsB2 was not active in the same killing assay, suggesting that the TpsB2-secreted TpsA1 is not functioning as an antibacterial toxin (Fig. 2D). This indicates that the CDI activity of TpsA1 may require an additional activation step and not just secretion. Overall our results suggest that TpsB2 can complement the secretion function of TpsB1, but that this secretion does not lead to restoration of the CDI toxin activity.

**Discussion**

*N. meningitidis* strain H44/76 encodes two TpsBs and three TPS systems (6). We have shown previously that the TpsB2 has a relaxed specificity being able to recognize and secrete all neisserial TPS domains when truncated TPS constructs comprising only the signal sequence and the TPS domain are used (18). However, additional sequences of full-length TpsAs might influence the secretion by a non-cognate TpsB. The work presented here shows that TpsB2 is able to secrete non-cognate and full-length TpsA1s of *N. meningitidis*, although only when over-expressed from a plasmid. Expression of *tpsB2* led to secretion of TpsA1 to the cell surface and the culture medium of two *N. meningitidis* strains, H44/76 and B16B6. (6,24).

The TPS3 domain of TpsA3 proteins of *N. meningitidis* needs a non-cognate TpsB for secretion, since the *tpsA3* ORFs in various strains are invariably encoded on genetic islands that do not encode a dedicated TpsB (6,18). Therefore, we hypothesize that the relaxed specificity of TpsB2 could explain the secretion of the TpsA3. Multiple TPS systems within one strain are found in *N. meningitidis* and *N. lactamica* (6,9). Furthermore, multiple TPS systems are found in species like *Haemophilus influenzae* (5), *Pseudomonas aeruginosa* (8), and *Moraxella catarrhalis* (7). Especially in *N. meningitidis* and *N. lactamica* the presence of orphan *tpsA* genes or systems have been detected that lack a dedicated *tpsB* (6,9,17,24).

Neisserial TpsA1 ORFs show a mosaic homology and many internal repeat sequences, suggesting that they are subject of genetic recombination and exchange via horizontal gene transfer (15,17). CDI killing is mediated by the C-terminal domains of TpsA proteins (25) and this region is quite similar between the TpsA1 of B16B6 and the TpsA1b of H44/76 while other regions are quite different (Fig. S1). The sequence differences may be the reason for the differences in secretion patterns observed for TpsA1s in HB-1 and BB-1. Presence of a TpsB with a more relaxed specificity might be an advantage in picking-up orphan TpsAs of other TPS systems. This is in line with the observation that secretion of a full-length TpsA is foremost dependent on the initial interaction of a TPS domain with a TpsB, which occurs via binding of the TpsB POTRA domains (12). This might promote distribution of TPS system 3, which is only present in a subset of *N. meningitidis* isolates and in the majority of cases together with TPS system 2 (6). On the other hand, not many neisserial isolates carry a *tpsA1* ORF without a cognate *tpsB1* on the chromosome (6,17).

TpsA1 may be secreted by TpsB2, but the secreted protein is in that case not active as a CDI toxin. Secretion of TpsA1 of strain BB-1 by the heterologous H44/76
TpsB2 transporter secretes non-cognate full length TpsA

TpsB1 restored TpsA1 CDI toxin-related killing activity, while its secretion by TpsB2 did not restore killing activity (Fig. 2D). This might be due to differences in the modification or folding of the secreted protein. Meningococcal TpsA1 proteins appear to be modified during translocation and multiple protein variants exist (6), *i.e.* for HB-1 secreted protein species of ~240 kDa and 75 kDa, a cell-surface-exposed species of ~200 kDa and ~240 kDa, an internal pre-secretory species of again ~240 kDa, which appears different from the secreted ~240 kDa species (Fig. 1). Furthermore, secretion of TpsA1 by TpsB1 or TpsB2 leads to a different profile of TpsA1 fragments, while the TpsA1 killing function depends on secretion by TpsB1. TpsA proteins often undergo modifications before, during or after translocation. This may involve proteolytic cleavage, as has been shown for FHA of *B. pertussis* (26,27) and HMW1 of *H. influenza* (28), or conformation changes, as has been shown for the toxin ShlA of *S. marcescens* (29). Furthermore, TpsA proteins may be glycosylated which is possibly essential for the activity as well, as shown for HMW1 of *H. influenzae* (30). Therefore, secretion via the neisserial TpsB1 and TpsB2 may result in different modifications of the secreted cargo, influencing functionality. Based upon the differences in sizes and locations observed for TpsB1 and TpsB2-secreted TpsA1, we hypothesize that the activation involves a conformational change, as observed for ShlA, which is activated during secretion by its cognate TpsB, designated ShlB (ref). In support of this supposition, the TpsB1 and TpsB2 share with ShlB the presence of a conserved pair of cysteines in the periplasmic subdomain, which is not found in the TpsBs HmwB and FhaC.

Overall our results show that TpsB2 can secrete full-length TpsA1, albeit inefficiently. However, the protein secreted by the non-cognate transporter appears differently processed and lacks the CDI killing activity. We did not test the other functional activities attributed to the TpsA1s, *i.e.* bacterial adhesion to host cells and enhancement of the intra-cellular survival of *N. meningitidis*. These functions might be less affected by the differences in secreted TpsA1 proteins. In any case, our results clearly indicate that the TpsB2 can bind and secrete a full-length TpsA1 after binding its TPS1 domain.

**Acknowledgements:** Joen Luirink and Wilbert Bitter are acknowledged for critically reading the manuscript. S. u. R. gratefully acknowledges funding by


22. Budroni, S., Siena, E., Hotopp, J. C. D.,
roles of the N-terminal and C-terminal domains. *Molecular Microbiology* **36**, 55-67
### SUPPLEMENTAL DATA

**Fig S1:**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td></td>
</tr>
<tr>
<td>TpsA1_B1686</td>
<td>MNKGLHRIIFSDKKHSHTMVAVAETANSQGKQAGSSVSVLKTSGDLCGLKKTLLTLC 60</td>
</tr>
<tr>
<td>TpsA1B_H44/76</td>
<td>MNKGLHRIIFSDKKHSHTMVAVAETANSQGKQAGSSVSVLKTSGDLCGLKKTLLTLC 60</td>
</tr>
<tr>
<td>TpsA1A_H44/76</td>
<td>MNKGLHRIIFSDKKHSHTMVAVAETANSQGKQAGSSVSVLKTSGDLCGLKKTLLTLC 60</td>
</tr>
<tr>
<td><strong>SUPPLEMENTAL DATA</strong></td>
<td></td>
</tr>
<tr>
<td>TpsA1_B1686</td>
<td>SLVLS6MLPAH9QITDSDKFSKQNNQVVLKKTNTGAPLUNIQTNQPNGRSLNRYTFQDDV 120</td>
</tr>
<tr>
<td>TpsA1B_H44/76</td>
<td>SLVLS6MLPAH9QITDSDKFSKQNNQVVLKKTNTGAPLUNIQTNQPNGRSLNRYTFQDDV 120</td>
</tr>
<tr>
<td>TpsA1A_H44/76</td>
<td>SLVLS6MLPAH9QITDSDKFSKQNNQVVLKKTNTGAPLUNIQTNQPNGRSLNRYTFQDDV 120</td>
</tr>
<tr>
<td><strong>SUPPLEMENTAL DATA</strong></td>
<td></td>
</tr>
<tr>
<td>TpsA1_B1686</td>
<td>AKGAV1LNDRRNNPFLAKGSAQLILNEVRGAAASKLNGIVTVGQKADVIIANPNGITVNG 180</td>
</tr>
<tr>
<td>TpsA1B_H44/76</td>
<td>NIKGAV1LNDRRNNPFLVKGQAQLILNEVRGAAASKLNGIVTVGQKADVIIANPNGITVNG 180</td>
</tr>
<tr>
<td>TpsA1A_H44/76</td>
<td>NIKGAV1LNDRRNNPFLVKGQAQLILNEVRGAAASKLNGIVTVGQKADVIIANPNGITVNG 180</td>
</tr>
<tr>
<td><strong>SUPPLEMENTAL DATA</strong></td>
<td></td>
</tr>
<tr>
<td>TpsA1_B1686</td>
<td>GGFKNVGRGILITAGFQIGDKGALTQDFQRTLTVGAAGWNREDQAGTDVGVLARAVLQ 240</td>
</tr>
<tr>
<td>TpsA1B_H44/76</td>
<td>GGFKNVGRGILITAGFQIGDKGALTQDFQRTLTVGAAGWNREDQAGTDVGVLARAVLQ 240</td>
</tr>
<tr>
<td>TpsA1A_H44/76</td>
<td>GGFKNVGRGILITAGFQIGDKGALTQDFQRTLTVGAAGWNREDQAGTDVGVLARAVLQ 240</td>
</tr>
<tr>
<td><strong>SUPPLEMENTAL DATA</strong></td>
<td></td>
</tr>
<tr>
<td>TpsA1_B1686</td>
<td>GKLQCNLAVSTGQPKVQDYASEGISAGTAAGKTPIALTDLTTALGMYADSDITLAINEKGV 300</td>
</tr>
<tr>
<td>TpsA1B_H44/76</td>
<td>GKLQCNLAVSTGQPKVQDYASEGISAGTAAGKTPIALTDLTTALGMYADSDITLAINEKGV 300</td>
</tr>
<tr>
<td>TpsA1A_H44/76</td>
<td>GKLQCNLAVSTGQPKVQDYASEGISAGTAAGKTPIALTDLTTALGMYADSDITLAINEKGV 300</td>
</tr>
<tr>
<td><strong>SUPPLEMENTAL DATA</strong></td>
<td></td>
</tr>
<tr>
<td>TpsA1_B1686</td>
<td>GVKNQGTEELAQLQVLSQGRENSISRIATTDGTEASPTYSLJETTEKQGAAGTFISNG 360</td>
</tr>
<tr>
<td>TpsA1B_H44/76</td>
<td>GVKNQGTEELAQLQVLSQGRENSISRIATTDGTEASPTYSLJETTEKQGAAGTFISNG 360</td>
</tr>
<tr>
<td>TpsA1A_H44/76</td>
<td>GVKNQGTEELAQLQVLSQGRENSISRIATTDGTEASPTYSLJETTEKQGAAGTFISNG 360</td>
</tr>
<tr>
<td><strong>SUPPLEMENTAL DATA</strong></td>
<td></td>
</tr>
<tr>
<td>TpsA1_B1686</td>
<td>RIESGKGLVETGEDISLRLNGAVQVNGSREPATTNLVAHGHNLIESTKNVNNAKGSANLS 420</td>
</tr>
<tr>
<td>TpsA1B_H44/76</td>
<td>RIESGKGLVETGEDISLRLNGAVQVNGSREPATTNLVAHGHNLIESTKNVNNAKGSANLS 420</td>
</tr>
<tr>
<td>TpsA1A_H44/76</td>
<td>RIESGKGLVETGEDISLRLNGAVQVNGSREPATTNLVAHGHNLIESTKNVNNAKGSANLS 420</td>
</tr>
<tr>
<td><strong>SUPPLEMENTAL DATA</strong></td>
<td></td>
</tr>
<tr>
<td>TpsA1_B1686</td>
<td>AGGTTINDATIQGSSVSSTGKDGTLENGRIATETAEVTVLSNGSISSGSAVIEAKOTH 480</td>
</tr>
<tr>
<td>TpsA1B_H44/76</td>
<td>AGGTTINDATIQGSSVSSTGKDGTLENGRIATETAEVTVLSNGSISSGSAVIEAKOTH 480</td>
</tr>
<tr>
<td>TpsA1A_H44/76</td>
<td>AGGTTINDATIQGSSVSSTGKDGTLENGRIATETAEVTVLSNGSISSGSAVIEAKOTH 480</td>
</tr>
<tr>
<td><strong>SUPPLEMENTAL DATA</strong></td>
<td></td>
</tr>
<tr>
<td>TpsA1_B1686</td>
<td>IESGKQLSLLTESTVASNIHLWNGKIGQVLMADDIGIQXASKNGLSAGNLHNVHAK 540</td>
</tr>
<tr>
<td>TpsA1B_H44/76</td>
<td>IESGKQLSLLTESTVASNIHLWNGKIGQVLMADDIGIQXASKNGLSAGNLHNVHAK 540</td>
</tr>
<tr>
<td>TpsA1A_H44/76</td>
<td>IESGKQLSLLTESTVASNIHLWNGKIGQVLMADDIGIQXASKNGLSAGNLHNVHAK 540</td>
</tr>
<tr>
<td><strong>SUPPLEMENTAL DATA</strong></td>
<td></td>
</tr>
<tr>
<td>TpsA1_B1686</td>
<td>DLNADKDLSTQISLARADNTNSSMTLTAEKNDIQAQSLSVQPQSLQSSQSNQMS 600</td>
</tr>
<tr>
<td>TpsA1B_H44/76</td>
<td>DLNADKDLSTQISLARADNTNSSMTLTAEKNDIQAQSLSVQPQSLQSSQSNQMS 600</td>
</tr>
<tr>
<td>TpsA1A_H44/76</td>
<td>DLNADKDLSTQISLARADNTNSSMTLTAEKNDIQAQSLSVQPQSLQSSQSNQMS 600</td>
</tr>
<tr>
<td><strong>SUPPLEMENTAL DATA</strong></td>
<td></td>
</tr>
<tr>
<td>TpsA1_B1686</td>
<td>AKGKNQLRNKTLNKNAAKLSTALTQLQINVSQDSLHAJADGHSVSLNANGDFTGHTNL 660</td>
</tr>
<tr>
<td>TpsA1B_H44/76</td>
<td>AKGKNQLRNKTLNKNAAKLSTALTQLQINVSQDSLHAJADGHSVSLNANGDFTGHTNL 660</td>
</tr>
<tr>
<td>TpsA1A_H44/76</td>
<td>AKGKNQLRNKTLNKNAAKLSTALTQLQINVSQDSLHAJADGHSVSLNANGDFTGHTNL 660</td>
</tr>
<tr>
<td><strong>SUPPLEMENTAL DATA</strong></td>
<td></td>
</tr>
<tr>
<td>TpsA1_B1686</td>
<td>EGDNANSVSGKRRKMDNITDIAYAGAVDVKVAGQQLLDGPLNGT----VNVGHSHLDSKGS 716</td>
</tr>
<tr>
<td>TpsA1B_H44/76</td>
<td>EGDNANSVSGKRRKMDNITDIAYAGAVDVKVAGQQLLDGPLNGT----VNVGHSHLDSKGS 716</td>
</tr>
<tr>
<td>TpsA1A_H44/76</td>
<td>EGDNANSVSGKRRKMDNITDIAYAGAVDVKVAGQQLLDGPLNGT----VNVGHSHLDSKGS 716</td>
</tr>
<tr>
<td><strong>SUPPLEMENTAL DATA</strong></td>
<td></td>
</tr>
</tbody>
</table>

92
TpsB2 transporter secretes non cognate full length TpsA
TpsA1_B16B6  VTAAGSSTAATAA---T-VTAATTTVSTAAMQTAALALYSQAAVAINNKDVQGKALKD 1550
TpsA1b_H44/76  VAASGSSTAARGAATTTAATTTVVTTATAMQTAALALYSQAAVSIINNKDVQGKALKD 1502
TpsA1a_H44/76  VAAGSSSTAARGAATTTAATTTVVTTATAMQTAALALYSQAAVSIINNKDVQGKALKD 1502

TpsA1_B16B6  LGTSDTQVIVTSALAAGALNMQAGADIAQLNKSVEKTEFSSTGNQTIANLGGRLATLNLN 1610
TpsA1b_H44/76  LGTSDTQVIVTSALTAGALNMQAGADIAQLNKSVEKTEFSSTGNQTIANLGGRLATLNLN 1562
TpsA1a_H44/76  LGTSDTQVIVTSALTAGALNMQAGADIAQLNKSVEKTEFSSTGNQTIANLGGRLATLNLN 1562

TpsA1_B16B6  AGISAGINTAVNNGSLKDNLGNALGALVNSFQGEEAGSKIKTTFTSSDYVAKQFAHAALGC 1670
TpsA1b_H44/76  AGISAGINTAVNNGSLKDNLGNALGALVNSFQGEEAGSKIKTTFTSSDYVAKQFAHAALGC 1622
TpsA1a_H44/76  AGISAGINTAVNNGSLKDNLGNALGALVNSFQGEEAGSKIKTTFTSSDYVAKQFAHAALGC 1622

TpsA1_B16B6  VSGVQGKCKDGIAAGAVEIVASILGGGRNFATLSDAEHKVIVSKIIAGSVAALNG 1730
TpsA1b_H44/76  VSGVQGKCKDGIAAGAVEIVASILGGGRNFATLSDAEHKVIVSKIIAGSVAALNG 1682
TpsA1a_H44/76  VSGVQGKCKDGIAAGAVEIVASILGGGRNFATLSDAEHKVIVSKIIAGSVAALNG 1682

TpsA1_B16B6  DNVTAAANAAEVNVNANLFNSTTPTNAKHKQFPKDFKTALEKIIQSMPAHAAAGATMFQ 1790
TpsA1b_H44/76  DNVTAAANAAEVNVNANLFNSTTPTNAKHKQFPKDFKTALEKIIQSMPAHAAAGATMFQ 1742
TpsA1a_H44/76  DNVTAAANAAEVNVNANLFNSTTPTNAKHKQFPKDFKTALEKIIQSMPAHAAAGATMFQ 1742

TpsA1_B16B6  DKDAAIWISNIWGIGPEIVITISYGYAAAGWTPALIGTAGKAAISTCMANPSGCTVMVTQ 1850
TpsA1b_H44/76  DKDAAIWISNIWGIGPEIVITISYGYAAAGWTPALIGTAGKAAISTCMANPSGCTVMVTQ 1802
TpsA1a_H44/76  DKDAAIWISNIWGIGPEIVITISYGYAAAGWTPALIGTAGKAAISTCMANPSGCTVMVTQ 1802

TpsA1_B16B6  AAEAGAGATGAVTGVNANAWEPVAGLSKAKAATAAQAIPTQTVKDEGQLLEQSKBNIGAVNT 1910
TpsA1b_H44/76  AAEAGAGATGAVTGVNANAWEPVAGLSKAKAATAAQAIPTQTVKDEGQLLEQSKBNIGAVNT 1862
TpsA1a_H44/76  AAEAGAGATGAVTGVNANAWEPVAGLSKAKAATAAQAIPTQTVKDEGQLLEQSKBNIGAVNT 1862

TpsA1_B16B6  I--NANISTRTPRMRTQGP--VSAGFHEVLGHHFHFRRPNNR---SF--TISPNELEK 1961
TpsA1b_H44/76  I--NANISTRTPRMRTQGP--VSAGFHEVLGHHFHFRRPNNR---SF--TISPNELEK 1913
TpsA1a_H44/76  I--NANISTRTPRMRTQGP--VSAGFHEVLGHHFHFRRPNNR---SF--TISPNELEK 1913

TpsA1_B16B6  RDTQDLDAWTQFGNVRKAGGKLALDSNPIITINGKTIKPQVAISSLKGAPVYSQEGEFQ 1962

TpsA1_B16B6  VILQSNKVVSPVSYTPFDEQYRMVT--DVQKVIGTSIKEGGQPTTITIKVFDTKSGNLHIT 2020
TpsA1b_H44/76  VILQSNKVVSPVSYTPFDEQYRMVT--DVQKVIGTSIKEGGQPTTITIKVFDTKSGNLHIT 1972
TpsA1a_H44/76  VILQSNKVVSPVSYTPFDEQYRMVT--DVQKVIGTSIKEGGQPTTITIKVFDTKSGNLHIT 1972

TpsA1_B16B6  ALYRQMTGQLNFVRVFZDGLRANIQISTEGAWGFD----KIALRNFKTENSTQR 1973

TpsA1_B16B6  YPVKN-------- 2026
TpsA1b_H44/76  YPV--------KGN-------- 1978
TpsA1a_H44/76  WTLQNNPSPFKGKLELKQ -------- 1995
TpsB2 transporter secretes non cognate full length TpsA

**TpsB2**

**H44/76**

MKPLELSYPNIRLSWCCCLLAGI-ATLLASNPNAEIRMMQDDIQQRHEEQRLTQM 58

**TpsB1**

B6

---------MKFFAPCLLVILAVPILTIAADENDAEAL--------RSQMQCHIADAE 45

**H44/76**

---------MKFFAPCLLVILAVPILTIAADENDAEAL--------RSQMQCHIADAE 45

**TpsB2**

H44/76

PESDVRLQNKNTGETVNLQMDGSSQCPFCAINEVUGHEGEHARFPQFALKLARTEGTGQAG 118

**TpsB1**

B6

TDANVRFEOPL--EKKNYVLSEDTEPCFTRVNYISLDDVKSKFSLPVMLKETAFTKGT 102

**H44/76**

TDANVRFEOPL--EKKNYVLSEDTEPCFTRVNYISLDDVKSKFSLPVMLKETAFTKGT 102

**TpsB2**

H44/76

KCLHAGNINQMSLNAQNLIRGGTTTRILAAPQDLSNKGQLTFLPSYLRSIRIDRSND 178

**TpsB1**

B6

MCLGSNSNLRLQKAQILMVRGVTYLSQAIQFPQMDGLKLRKLRVSEIGDTRVEEKR 162

**H44/76**

MCLGSNSNLRLQKAQILMVRGVTYLSQAIQFPQMDGLKLRKLRVSEIGDTRVEEKR 162

**TpsB2**

H44/76

DQTAGRHIAAFQNPFFRTSNDLLNRDLQGLENKRLPTAEADLQIVPVEGEPNQSDV 238

**TpsB1**

B6

GKASAEGRSLNFKPFYMRKINLNLDEVQGENLRLSVKTQIDIQIFSEE-GKSQDLQ 221

**H44/76**

GKASAEGRSLNFKPFYMRKINLNLDEVQGENLRLSVKTQIDIQIFSEE-GKSQDLQ 221

**TpsB2**

H44/76

VQWPRLLLFYVSVGMDNSEGATKGQCNITFSADNLGLSDMRYVNVRSGITDEEE 298

**TpsB1**

B6

IKWQOM-KPIFRSUGIDAGCTTGCTGKYSTGNVALSDFNLPLGLDFLYSVYGRGLAHKDTL 280

**H44/76**

IKWQOM-KPIFRSUGIDAGCTTGCTGKYSTGNVALSDFNLPLGLDFLYSVYGRGLAHKDTL 280

**TpsB2**

H44/76

SFCHHRKEEGSNYNAYVHSAPFGWKTQWAFNHLGNHGYQAVGSL7SEYDNYKSSITDFGF 358

**TpsB1**

B6

DAFTGSETSGRSYRSYVYRSVPVLRFLSFNHGNSHRHDTAGESVNYDYNQGKQSSLA 340

**H44/76**

DAFTGSETSGRSYRSYVYRSVPVLRFLSFNHGNSHRHDTAGESVNYDYNQGKQSSLA 340

**TpsB2**

H44/76

NRLYRDARKTRYLGVKLWYRTXGQALDAELTVQQRKTAGLWLAELEHKEYIGRSTADF 418

**TpsB1**

B6

ERMLWFRNHLKHTSGVRKMLWPTQYKIDAEIIEVEQRRAGSAEAEHRMLNQMDLGK 400

**H44/76**

ERMLWFRNHLKHTSGVRKMLWPTQYKIDAEIIEVEQRRAGSAEAEHRMLNQMDLGK 400

**TpsB2**

H44/76

LKYKRTGQMODALRAPEEAF---ETSRSMKINTASADVNFQIKGLYFADTVSIHQW 475

**TpsB1**

B6

LSYKRGTRQGMRQGAPAEPEEAGDIIPLGTSMRRKITTAGDAAAPFLGKQFFFFATQAQW 460

**H44/76**

LSYKRGTRQGMRQGAPAEPEEAGDIIPLGTSMRRKITTAGDAAAPFLGKQFFFFATQAQW 460

**TpsB2**

H44/76

NKBPQDRTSDKLAIQGQHTVNGEDGMLSAEAGWYRNWLDSQFKEGQYLIALQDVHVS 535

**TpsB1**

B6

NKTPPLQVDKLSLIGSIYRTVRGGVDQGSLGKEREQFYQNLTYFENHNPQSYLFQDGYSV 520

**H44/76**

NKTPPLQVDKLSLIGSIYRTVRGGVDQGSLGKEREQFYQNLTYFENHNPQSYLFQDGYSV 520

**TpsB2**

H44/76

GQSAWLGLQGLTGVAQIGIRYQLIKLNNGHLHADDFTGRALKKPEFQPSKMAWSSGFQYFTF 595

**TpsB1**

B6

GESAQVYSGCQLVQAUGVRGFRHGKVMGFAYDFAGKLPKGKFGQFTNTVYGNLMYSF 580

**H44/76**

GESAQVYSGCQLVQAUGVRGFRHGKVMGFAYDFAGKLPKGKFGQFTNTVYGNLMYSF 580

---

Fig. S1. Sequence alignments of TpsA1 and TpsB proteins of *N. meningitidis* strains H44/76 and B16B6. Sequence alignments were obtained using the Clustal Omega webserver at [http://www.ebi.ac.uk/Tools/msa/clustalo/](http://www.ebi.ac.uk/Tools/msa/clustalo/). A. Alignment of full-length TpsA1s of B16B6 (TpsA1) and H44/76 (TpsA1a and TpsA1b). B. Alignment of the full-length TpsB1 of B16B6 and the TpsB1 and TpsB2 of H44/76. The TpsB1s of the two strains are 99% homologous with only four amino acid residues being different (highlighted in yellow).
<table>
<thead>
<tr>
<th>prot_hit_num</th>
<th>prot_acc</th>
<th>prot_desc</th>
<th>peptides found</th>
<th>prot_score</th>
<th>prot_mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>gi</td>
<td>15677274</td>
<td>iron-regulated protein FrpC /A [Neisseria meningitidis MC58]</td>
<td>NALVLSDAKDEIFHFDNGKLMTDYVEAEEKVLQGQTVALLAXSGGFYGERHLMRTDYEEA \n</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>gi</td>
<td>15676598</td>
<td>IgA-specific serine endopeptidase [Neisseria meningitidis MC58]</td>
<td>ELATRQKYNMTELSRLYNHNYENNHQDDYQDRFLRFVSGATLSVSKFLTGTNNGKFLTGT \n</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>gi</td>
<td>25467200</td>
<td>hemolysin-type calcium binding protein [Neisseria meningitidis serogroup W135]</td>
<td>RNLSDLXADWNWQGGKEVUGLETYKSQINSHTLAGQKTLLUSTAEQAGIRNQGDLFASSNG \n</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>gi</td>
<td>77358708</td>
<td>haggaullitin/hemolysin-related protein [FhaB]/[TpsA]/[Neisseria meningitidis MC58]</td>
<td>GTASKLNGIVTQGGQXVSSPSVMTPGZQYMRATTADGTEASYLSETTEKKDQGTVGA \n</td>
<td></td>
</tr>
</tbody>
</table>
**Protein identified in the ~ 75 kDa band in the culture supernatant**

<table>
<thead>
<tr>
<th>prot_hit_num</th>
<th>prot_acc</th>
<th>prot_desc</th>
<th>peptides found</th>
<th>prot_s</th>
<th>prot_mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>gi</td>
<td>15676598</td>
<td>IgA-specific serine endopeptidase [Neisseria meningitidis MC58]</td>
<td>SLITDPPKFSEFVRHAAQ5RQAOEALLRRNMTEL5R5MLNL5PYNRE1NDV 3</td>
<td>TpsB2 transporter secretes non cognate full length TpsA</td>
<td>1461</td>
</tr>
</tbody>
</table>

| gi|15677274 | iron-regulated protein FrpC [Neisseria meningitidis MC58] | GFGSGSKIVALSSDVKETLCATI5Q5D5QGHDD5NKL5MTD5VEEAK5V5Q5ET5VALLA | TpsB2 transporter secretes non cognate full length TpsA | 1056 | 193727 |

| gi|15676490 | putative iron-regulated protein FrpA [Neisseria meningitidis MC58] | GFGSGSKIVALSSDVKETLCATI5Q5D5QGHDD5NKL5MTD5VEEAK5V5Q5ET5VALLA | TpsB2 transporter secretes non cognate full length TpsA | 1003 | 141312 |

<p>| gi|15676066 | elongation factor G [Neisseria meningitidis MC58] | SFALAAKTPSYLRFEPSVEKEFSVDPKATSYMEFKAGDOIAAALGGDKOOAAALGK | TpsB2 transporter secretes non cognate full length TpsA | 991 | 119566 |</p>
<table>
<thead>
<tr>
<th>ID</th>
<th>Gene</th>
<th>Protein Name</th>
<th>Protein ID</th>
<th>E-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>gii6977941</td>
<td>NplpR (Neisseria meningitidis serogroup B)</td>
<td>NPOGHRYFGFLYFGFVNGVANDRNSQFLVSNFGCLVAVFFHESSRHMD</td>
<td>5</td>
<td>657</td>
</tr>
<tr>
<td>gii15875815</td>
<td>Isocitrate dehydrogenase</td>
<td>GVFLSMLHMVFTAFVPEFXNAELSVAQGKSAGSAVNPVLRALLAFLEQVKKNHSHM</td>
<td>6</td>
<td>641</td>
</tr>
<tr>
<td>gii15876837</td>
<td>5-Methyltetrahydropteroylglutamate--homocysteine methyltransferase</td>
<td>VLSAGVIGDRAEVPDVPQDAEIFMAKTAIEVPPQVIREDFQPAARTTFLHSGFR</td>
<td>7</td>
<td>558</td>
</tr>
</tbody>
</table>
TpsB2 transporter secretes non cognate full length TpsA
<table>
<thead>
<tr>
<th>ID</th>
<th>Name</th>
<th>Description</th>
<th>Amino Acid Sequence</th>
<th>Accession</th>
<th>Length</th>
<th>EValue</th>
</tr>
</thead>
<tbody>
<tr>
<td>gi</td>
<td>254672000 hemolysin-type calcium binding protein [Neisseria meningitidis serogroup W135]</td>
<td>AREQQRVELTAEQAKAEAAALSGLANLKEAEASLGDLANLKEAQALDLNLLHKGMLLAADLNLHSRGMGDLLLADNLSHRTATGWSADGDGLVGRFGSLFEEH TNGIRNLGNGNLTACQSGSYYTKNLGNGNTAQCGSYTKEELGCGSALDRLAYK WQDLNOQDGISOANLIRDNGNIDNGAEFGDNMTKAIGTNSMYFETLRSHK</td>
<td>55703</td>
<td>374</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gi</td>
<td>15676521 phosophoenolpyruvate synthase [Neisseria meningitidis MC68]</td>
<td>MEFINRMEFIRNRMFEFIRNRAFLNHGLSRMIFTDKAEAGKLKLDVDEVALRVP GFTAAEAYRDSGVLVESFDFERLYQARPETKVLVDEVDVAILRVPGKNAAGLMSIQLTEKNSLQGEMISQLTEKIGASYPVDVFYDKALLEDFKQDEKLSVTVNVQFEEDRINREAMEHHTVFASLYDRESEIDTEITELAHYALTIEKSNENLYVGGNYEPEHEE NPMGLFRSNEYVANLVGGNYEPEHEENPMLGFR</td>
<td>67115</td>
<td>328</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gi</td>
<td>15677326 dlpB protein [Neisseria meningitidis MC68]</td>
<td>QGDLALASKQGDLASKMLEVLHRQIDEVKAIIDLEIDASEIRVNGEVPESLYRT DRLPIEKKNPVLGEVPGVKGKLAVSDAIALLIAKISCIEINPLAKMHEYEDLEHUKAL LAGNYPASEIERRELILDEEENGLKOALAGFLOSGDHRVLVGEPSVEDITLARSGL ADPNKPYGSLFLGPTVGKALLDDQNSGAALLAHAGVNVQV JK</td>
<td>95136</td>
<td>265</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gi</td>
<td>304386642 family 65 glycosyl hydrolase [Neisseria meningitidis ATCC 13091]</td>
<td>AVEMYRNILQCRSTFVGFVRFVCLVDKENV/VIITLKYRPRDLNVRAINAINFSKV KGEP/LDQVY/GKIMEISWPTLRIEMISWPTLIFI/VQGVEDAKXAIAGVAF/TLDAH KLOESLTS/LGNYGMMDRIRPYPSALSDDPL/NQKDISS/ADVKNESNYEVENK VFITLKGEP/LDQVY/GK</td>
<td>85297</td>
<td>251</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gi</td>
<td>254670236 maltose phosphorylase [Neisseria meningitidis alpha153]</td>
<td>AVEMYRNILQCRSTFVGFVRFVCLVDKENV/VIITLKYRPRDLNVRAINAINFSKV KGEP/LDQVY/GKIMEISWPTLRIEMISWPTLIFI/VQGVEDAKXAIAGVAF/TLDAH KLOESLTS/LGNYGMMDRIRPYPSALSDDPL/NQKDISS/ADVKNESNYEVENK VFITLKGEP/LDQVY/GK</td>
<td>65347</td>
<td>240</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gi</td>
<td>15677610 hemagglutinin/hemolysin-related protein [Neisseria meningitidis MC68]</td>
<td>VGNHATDLKEXSSSDTAEKIDAPVWQGDFRRELVLGILEETYKEACVSELQLDRQSGKL NSHTLDQAGKTIUSTACQAGIYVAMAAMNAGAQYAQQTNGKNQGQLFSSGNVIA DANGRATLTTGDPCQYAGDLSFKGSQTLQSGHHJIDDITKGAQR/REAATAAEAL ANKGMETAKKRAEVIPAPAGINGGGFINASRVVQQNINSSHSSQMQNGYIEV GGRVVNQQNNSHSSQMQNGYIEVGGRGALLSNTINVSGLKNSGTAGR</td>
<td>265458</td>
<td>221</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TpsB2 transporter secretes non cognate full length TpsA

<table>
<thead>
<tr>
<th>Page</th>
<th>Line</th>
<th>Text</th>
</tr>
</thead>
<tbody>
<tr>
<td>g254804436</td>
<td>19</td>
<td>chaperone Hsp70 [Neisseria meningitidis alpha14]</td>
</tr>
<tr>
<td>g14884687</td>
<td>20</td>
<td>lactoferrin-binding protein precursor [Neisseria meningitidis]</td>
</tr>
<tr>
<td>g15676052</td>
<td>21</td>
<td>elongation factor Tu [Neisseria meningitidis MC58]</td>
</tr>
<tr>
<td>g45187</td>
<td>22</td>
<td>unnamed protein product [Neisseria meningitidis]</td>
</tr>
<tr>
<td>g15677288</td>
<td>23</td>
<td>outer membrane protein PorA [Neisseria meningitidis MC58]</td>
</tr>
<tr>
<td>g260160</td>
<td>24</td>
<td>porin (class 3 serotype 15) [Neisseria meningitidis, MC58, Peptide, 312 aa]</td>
</tr>
<tr>
<td>g121635155</td>
<td>25</td>
<td>CipB protein [Neisseria meningitidis FAM18]</td>
</tr>
<tr>
<td>g254669474</td>
<td>26</td>
<td>ATP-dependent protease ATPase subunit [Neisseria meningitidis alpha153]</td>
</tr>
<tr>
<td>g261392212</td>
<td>27</td>
<td>lactoferrin-binding protein B (LbpB) [Neisseria meningitidis 8013]</td>
</tr>
<tr>
<td>g77358708</td>
<td>28</td>
<td>hemagglutinin/hemolysin-related protein [Neisseria meningitidis MC58]</td>
</tr>
</tbody>
</table>
The Polypeptide Transport-associated (POTRA) Domains of TpsB Transporters Determine the System Specificity of Two-Partner Secretion Systems

Sadeeq ur Rahman\textsuperscript{1}, Hülya Öztürk\textsuperscript{1}, Nicole Dekker\textsuperscript{1}, Peter van Ulsen\textsuperscript{1}

\textsuperscript{1}Section of Molecular Microbiology, Department of Molecular Cell Biology, VU University, 1081 HV Amsterdam, The Netherlands;

submitted to JBC
The Polypeptide Transport-associated (POTRA) Domains of TpsB Transporters Determine the System Specificity of Two-Partner Secretion Systems

**Background:** TpsB transporters secrete large exoproteins across the outer membrane of Gram-negative bacteria.

**Results:** Exchanging the polypeptide transport-associated (POTRA) domains between TpsBs switches their substrate specificity.

**Conclusion:** POTRA domains select and bind TpsB substrates to initiate secretion.

**Significance:** These findings increase our understanding of the working mechanism of members of the Omp85 protein family, which is conserved between pro- and eukaryotes.

The Two-partner secretion (TPS) systems of Gram-negative bacteria secrete large TpsA exoproteins by a dedicated TpsB transporter in the outer membrane. TpsBs contain an N-terminal module located in the periplasm that includes two polypeptide transport-associated (POTRA) domains. These are thought to initiate secretion of a TpsA by binding its N-terminal secretion signal, called the TPS domain. Neisseria meningitidis encodes up to five TpsA proteins that are secreted via only two TpsB transporters: TpsB1 and TpsB2. Of these two, the TpsB2 recognizes the TPS domains of all TpsAs, despite their sequence diversity. By contrast, the TpsB1 shows a limited recognition of a TPS domain that is shared by two TpsAs. The difference in substrate specificity of the TpsBs enabled us to investigate the role of the POTRA domains in the selection of TPS domains. We tested secretion of TPS domains or full-length TpsAs by TpsB mutants with deleted, duplicated and exchanged POTRA domains. Exchanging the two POTRAs of a TpsB resulted in a switch in specificity. Furthermore, exchanging a single POTRA domain showed that each of the two domains contributed to the cargo selection. Remarkably, the order of POTRAs could be reversed without affecting substrate selection, but this aberrant order did result in an alternatively processed secretion product. Our results suggest that secretion of a TpsA is initiated by engaging both POTRA domains of a TpsB transporter and that these select the cognate TpsAs for secretion.

Proteins of the type V secretion pathway of Gram-negative bacteria cross the cell envelope consisting of the inner membrane, the peptidoglycan containing periplasmic space and the outer membrane in a series of consecutive steps (1). Within the type V secretion pathway, the Two-Partner Secretion (TPS) systems form a distinct subclass (1,2). TPS systems comprise a secreted TpsA protein and an outer membrane-embedded TpsB transporter. TpsAs are large exoproteins of more than 100 kDa and function as bacterial adhesins, as toxins for bacterial or eukaryotic targets and in obtaining nutrients from the environment. Both TpsA and TpsB proteins are synthesized with an N-terminal signal peptide and are transported across the inner membrane via the Sec complex. The TpsB then inserts into the outer membrane and binds and secretes the TpsA across this membrane. TpsAs target their TpsB translocator via a TPS domain located at the N terminus of the processed TpsA.

The TpsB proteins belong to the Omp-85 family of proteins that also includes the BamA protein involved in the biogenesis of outer membrane-based
Polypeptide transport associated domains determine TPS system specificity

β-barrel proteins and its eukaryotic homologs (3,4). The family is characterized by a C-terminal 16-stranded β-barrel and a soluble module of one to five polypeptide transport-associated (POTRA) domains. POTRA domains are also found outside the Omp85 family, for example in the cell division protein FtsQ (5-7). TpsB proteins contain two POTRAs, as shown by the crystal structure of FhaC, the TpsB of filamentous haemagglutinin of *B. pertussis* (FHA) (8). POTRA domains adopt a conserved βααββ configuration that folds into a three-stranded β-sheet overlaid with two anti-parallel α-helices, although the α2 is missing in the POTRA1 of FhaC (4,8-11) (Fig. 1). Several lines of evidence indicate that POTRA domains interact directly with the TpsB substrates. Two POTRA domains of BamA of *E. coli* were shown by NMR to change conformation when incubated with peptides derived from a β-barrel OMP (12). Surface plasmon resonance measurements, as well as pull-down and overlay experiments indicated binding of TPS domains to isolated POTRAs of their cognate TpsBs (8,13-15) or full-length TpsB in the case of FhaC (14,15), while deletion of POTRA domains abolished the secretion of truncated FHA constructs (8). By contrast, deletion of POTRAs of BamA proteins of *E. coli* and *N. meningitidis* rendered the proteins partially functional (9,16). In *N. meningitidis* it was possible to delete four of the five POTRA domains of BamA without affecting the viability, although it affected the efficiency of β-barrel protein assembly progressively (16). In *E. coli*, single POTRA domains 1 and 2 could be deleted from the array of five, but not 3-5 (9). A particular feature of FhaC and other TpsBs that is not shared with other members of the Omp85 family is that the channel inside the β-barrel domain is occupied by both an α-helix that precedes the two POTRA domains in the sequence and an extended external loop (loop 6) that folds inward. In the recent crystal structures of BamA (17), the β-barrel channel is not blocked but closed off by a dome formed by the extracellular loops.

The Gram-negative diplococcus *Neisseria meningitidis* (meningococcus) is a major cause of meningitis and sepsis world-wide (18). *N. meningitidis* genomes encode up to three TPS systems that have been implicated in pathogenesis (19,20). Of these three, TPS system 1 is ubiquitous whereas systems 2 and 3 are more prevalent among hyperinvasive clonal complexes. Furthermore, antibodies binding to the TPS domains of the latter two systems have been detected in sera of patients recovering from meningococcal disease. Functions have only been attributed to system 1. TpsA1 (HrpA) promotes adherence to and the intracellular survival and escape from cultured human epithelial cell lines (21,22), is involved in biofilm formation (23) and acts as contact-dependent toxin involved in bacterial fratricide against other meningococcal strains (24).

Remarkably, the genomes encode only two *tpsB* genes, *i.e.* *tpsB1* and *tpsB2*, which are located in an operon with either a system 1 or 2 *tpsA*, respectively (19,25). The system 3 TpsA is encoded on a genetic island that lacks a gene encoding a dedicated TpsB. Our previous study showed that the TPS domain of TpsA3 is efficiently secreted by TpsB2 (26). In fact, the TpsB2 showed a relaxed system specificity and was able to recognize and secrete TPS domains of *N. meningitidis* and *N. lactamica*. In contrast, the TpsB1 of system 1 transported only cognate TPS domains, with the exception of one TPS domain of *N. lactamica*. Here, we have used this difference in substrate specificity between TpsB2 and TpsB1 to investigate the function of the POTRA domains in the recognition and selection of TPS domains of secreted TpsA proteins. We found that both POTRA domains are crucial for and define this specificity. Interestingly, the order of the POTRA domains seems
irrelevant for secretion.

**Experimental procedures**

**Bacterial strains and growth conditions.** The *N. meningitidis* strain HB-1 *tpsB1::kan/*tpsB2::gen (26) was grown on GC agar (Oxoid) supplemented with Vitox (Oxoid) at 37°C, 5% CO₂, supplemented with 8 µg/ml chloramphenicol for plasmid maintenance when needed. Liquid cultures of *N. meningitidis* strains were grown at 37°C in tryptic soy broth (Gibco-BRL). *Escherichia coli* strains Top10F' (Invitrogen) and DH5a were grown on lysogeny broth (LB) or LB agar plates supplemented with 100 µg/ml ampicillin or 30 µg/ml chloramphenicol for plasmid maintenance and with 0.5% glucose for full repression of the lac operator when appropriate.

**Plasmid construction.** The wild-type *tpsB* ORFs analysed here were obtained by PCR using chromosomal DNA obtained from lysed *N. meningitidis* HB-1 cells as template and Phusion DNA polymerase (Finnzymes) as the synthesizing enzyme according to the recommendations of the manufacturer. The ORFs of mutant *tpsBs* were obtained by either using chromosomal DNA or pGEM-T plasmids (Promega) containing *tpsB* ORFs as the template. The primers used are listed in Table 1, the constructed ORFs are depicted in Fig. 1C. PCR amplicons of full-length ORFs were cloned into the pGEM-T cloning vector and confirmed by sequencing (Macrogen).

The ORFs encoding TpsB2 lacking POTRA1 or POTRA2 were cloned in two PCR steps using chromosomal DNA as template. First, the 3'end of the ORFs were obtained by either using chromosomal DNA or pGEM-T plasmids (Promega) containing *tpsB* ORFs as the template. The primers used are listed in Table 1, the constructed ORFs are depicted in Fig. 1C. PCR amplicons of full-length ORFs were cloned into the pGEM-T cloning vector and confirmed by sequencing. The ORFs encoding TpsB2 with duplicated POTRAs (TpsB2_P1P1 and TpsB2_P2P2) were constructed by combining three PCRs. For TpsB2_P1P1, primer pairs pr_TpsB2_P1P1_1 and pr_tpsB2_F, and pr_TpsB2_P1P1_2 and pr_tpsB2_R were used with the pGEM-T containing *tpsB* ΔP2 as template to obtain the 5' and 3' ends of the ORFs, respectively. For TpsB2_P2P2, primer pairs pr_TpsB2_P2P2_1 and pr_tpsB2_F, and pr_TpsB2_P2P2_2 and pr_tpsB2_R were used with the pGEM-T containing *tpsB* ΔP1 as template to obtain the 5' and 3' ends of the ORFs, respectively. The amplicons were combined in a PCR with the primers pr_tpsB2_F and pr_tpsB2_R to yield the full-length ORFs.

The ORFs encoding TpsB1 with the POTRAs of TpsB2 (TpsB1_ExP1P2B2) was cloned in three PCR steps. First, the *tpsB1* up to the POTRA1 location was amplified using pr_tpsB1_F and pr_P1P2B2_1 and chromosomal DNA as template. The latter primer included an overlap with POTRA1-encoding sequence of *tpsB2*. The resulting fragment was combined with pr_P1P2B2_2 and pGEM-T containing wild type *tpsB2* as a template to extend the fragment with the sequence encoding the POTRAs of *tpsB2*. The pr_P1P2B2 also included sequences overlapping with the region directly upstream of those encoding POTRA2 in *tpsB2* and the final step involved combining the amplicon with primer pr_tpsB1_R and pGEM-T containing wild type *tpsB1* as template to obtain the full-length ORF.

The ORFs encoding the TpsB2_ExpP2B1, TpsB2_ExpP1B1, TpsB2_ExpP2B1 and TpsB2_RevP2P1 (Fig. 1C) were cloned using a similar approach. For TpsB2_ExpP1B2, primers pr_tpsB2_F and
Polypeptide transport associated domains determine TPS system specificity

pr_P1P2B1_1 and chromosomal DNA as template yielded the first PCR amplicon. That was combined with pr_P1P2B1_2 and pGEM-T containing wild-type tpsB1 to yield an extended fragment, which was then combined with primer pr_tpsB2_R and pGEM-T containing wild-type tpsB2 to yield the full-length ORF. For the TpsB2_ExP1B1, the primers pr_tpsB2_F and pr_P1B1_1 were combined with pGEM-T containing tpsB2-ExP1P2B1 as template to yield an amplicon that extended up to the region encoding the POTRA1 of TpsB1. In a second PCR, primers pr_P1B1_2 and pr_tpsB2_Rev and chromosomal DNA yielded the 3’ end of the ORF. The amplicons of the two PCRs were linked in a PCR that included primers pr_tpsB2_F and pr_P2B1_1. For TpsB2_RevP2P1, primers pr_tpsB2_F and pr_RevP2P1B2_1 were incubated with pGEM-T containing tpsB2_P2P2 as template to yield a 5’ fragment of the ORF that included the POTRA2 sequence. Primers pr_tpsB2_R and pr_RevP2P1B2_2 and the pGEM-T containing tpsB2_P1P1 as the template yield the 3’ fragment of the ORF including the POTRA1 sequence. The two amplicons were combined with primers pr_tpsB2_F and pr_tpsB2_R to yield a full-length ORF.

The tpsB s ORFs were cloned as single gene or in combination with truncated tpsA ORFs into the pEN vector, which is a neisserial expression vector (27) (Table 2). The constructs of wild-type tpsB2 and tpsB1 ORFs in combination with truncated tpsA1 or tpsA2 ORFs have been described earlier (26). The mutated ORFs were first subcloned downstream of the truncated tpsA1 ORF in pPU100, the truncated tpsA2a ORF in pPU1200, or the truncated tpsA2b ORF in pPU1300 (19) using the EcoRI and BamHI restriction sites inserted in the primers. The combination of the mutant tpsB ORFs and the truncated tpsA were then transferred to the pEN vector using NdeI and AatII restriction enzymes. To include the tpsB2_ExprP1P2B1 and tpsB1_ExprP1P2B2 ORFs in the pEN vector as a single gene they were directly cloned from the pGEM-T vector using NdeI and AatII.

**SDS-PAGE and Western blotting.** All procedures were carried out as described earlier (19,26). Briefly, *N. meningitidis* HB-1 cultures were grown for 4-6 h to an optical density at 600 nm (OD$_{600}$) of ~2.5-3.5 in the presence or absence of 0.1 or 0.25 mM IPTG. Cells were harvested by centrifugation (4,500 × g, 5 min) and the pellet was resuspended in PBS pH 7.4 to a final OD$_{600}$ of 10. The whole cell lysates were obtained by adding an equal volume of 2’ sample buffer (125 mM M Tris–HCl pH 6.8, 20% (v/v) glycerol, 0.02% (w/v) bromophenol blue, 40 mM DTT and 4% (w/v) SDS and boiling the samples for 10 min. Culture supernatants were centrifuged (16,000 × g, 10 min) to remove residual cells and the culture supernatant was then subjected to ultracentrifugation (200,000 × g, 1 h) in a bench top ultracentrifuge (Beckman and Coulter). Proteins were precipitated from the supernatants with 5% TCA and dissolved in a volume of PBS corresponding to a cell density of OD$_{600}$ 100 (10’ concentrated compared to cell samples), further diluted in 2’ sample buffer and boiled for 10 min. Protein samples were separated on 7.5-10- or 12-% SDS-PAGE gels and stained with Coomassie brilliant blue G250, or proteins were blotted onto nitrocellulose for Western-blot analyses. Blots were pre-
incubated in blocking buffer (PBS with 0.5% skim milk powder (Fluka) and 0.1% (vol/vol) Tween 20 (Merck)) for at least 4 h. Sera were diluted 1:5,000 (anti-TPS1 and anti-TPS2) or 1:10,000 (anti-TpsB1 and anti-TpsB2) in blocking buffer and incubated for 1-2 h. Blots were washed and incubated for 1 h with goat anti-rabbit immunoglobulin G serum conjugated to horseradish peroxidase (Biosource International) diluted 1:10,000 in blocking buffer. The binding of antibodies to the blots was visualized using Lumilight-normal or Plus (Roche). The indicated relative molecular weight of the protein was deduced from the Precision Plus Protein Standard (BioRad) including in each SDS-PAGE gel.

**Outer membrane isolation and heat modifiability.** Outer membrane fractions were isolated according to (28). Cells expressing the tpsB gene to be analysed were harvested by centrifugation (4,500 g, 5 min). The pellet was stored in the freezer (>18 h) to kill the bacteria and washed. Cells were resuspended in a 50 mM Tris-HCl pH 8.0 and 2 mM EDTA buffer and were passed 2 times through a One Shot Cell disrupter (Constant Systems Ltd) at 30,000 psi. Unbroken cells were pelleted by centrifugating the lysate (4,500 g, 5 min). The supernatant was then subjected to ultracentrifugation (200,000×g, 30 min). The resulting pellet containing a crude outer membrane fraction was resuspended in PBS to represent a culture of OD600 ~20. The denatured samples that were further diluted in 2’ sample buffer and boiled for 10 min. The native samples were diluted in 2’ semi-native sample buffer (i.e. 2’ sample buffer with 0.4% SDS and lacking DTT) and kept at room temperature for 10 min. Samples were loaded on semi-native SDS-PAGE gels prepared without SDS in the gel and run at 12 mA for at least 3 h while cooled in ice. Blotting was performed as described above.

**In silico sequence analyses.** Sequence analyses were performed with amino acid sequences of mature TpsBs, which lack the sequence of the signal peptide. The signal peptides were identified by analyzing the full length sequence with SignalP 4.0 (29). Initial pairwise sequence alignments of TpsB proteins were performed using the BL2Seq server at http://blast.ncbi.nlm.nih.gov/ using standard parameters. Additional multiple sequence alignments were performed with the mature TpsB sequence and the sequences encompassing the POTRA domains by using the M-Coffee programme at http://tcoffee.crg.cat/apps/tcoffee/do:mcoffee (30). The encoded protein sequences Models of the mature TpsBs of *N. meningitidis* and their POTRA domains were generated by the Phyre2 website at http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index (31). The quality of the obtained model was assessed by comparing them to the crystallized FhaC the TM-align programme at http://zhanglab.ccmb.med.umich.edu/TM-align/ (32).

**Results**

**POTRA domain assignment in TpsB1 and TpsB2**

To construct various POTRA mutants of TpsB1 and TpsB2 derived from *N. meningitidis* strain H44/76 we used the crystal structure of FhaC (8) to build structural models. TpsB1 and TpsB2 show a reasonable similarity to FhaC with an identity/similarity of 26%/42% for TpsB1 and 27%/44% for TpsB2, respectively. Sequence alignments and structural modeling identified amino acid residues 69-148 and 149-226 of TpsB1 and 85-164 and 165-244 of TpsB2 as POTRA1 and POTRA2, respectively (Fig. 1A and 1B). Differences between the POTRAs cluster in two specific regions of the POTRA domains and include α-helix 3 of the structural elements, which is not part of the
Polypeptide transport associated domains determine TPS system specificity

When we mention POTRA1 or 2 in the remainder of the study, we refer to these assigned sequences (Fig. 1C, Table 2). We focused primarily on the TpsB2 protein as this protein showed a relaxed specificity in the secretion of minimal TPS constructs that consist of the signal peptide and TPS domain of one of the TpsAs of the neisserial TPS systems (26).

Deletion of POTRA domains from TpsB2 prevents secretion

To investigate the role of the POTRA domains in secretion, we first deleted POTRA1, POTRA2, or both POTRAs of TpsB2 (Fig. 1C). The ORFs encoding these mutated TpsB proteins were cloned in a neisserial expression vector downstream of a truncated tpsA2a gene under the control of a lac promoter (26). This truncated tpsA2a includes the TPS domain and represents the minimal TpsA protein that is still secreted. Plasmids carrying the wild-type tpsB2 or the truncated derivatives were used.

---

Fig 1 (A) Alignment of the TpsB1 and TpsB2 of N. meningitidis H44/76 with FhaC of Bordetella pertussis and ShlB of Serretia marcescens using M-Coffee (30). The colour coding represents the quality of the alignment from bad (blue/green) to good (red) and the % scores are given. (B) cartoon models of the crystallized POTRA domains of FhaC and the modeled POTRA domains of TpsB1 and TpsB2 obtained using the Phyre2 program (31). The areas that showed a lower score in the alignment are colored blue in the models. (C) Graphical representation of the various TpsB2 and TpsB1 constructs used in the article. For expression they are combined with either TPS1 or TPS2 domains, as indicated in the text.
to transform H44/76 derivative *tpsB1*kan *tpsB2::gen* (19,26). In these strains, the *tpsB2* and/or *tpsB1* genes are replaced by kanamycin and gentamycin resistance cassettes, respectively. Cultures were grown in the presence or absence of 0.1 mM IPTG M (Fig. 2). Samples of whole-cell lysates and concentrated culture supernatants were analysed by Western blotting using anti-TpsB2 and TPS2a antisera. As expected, the combination of the TPS2a construct with the wild type *tpsB2* resulted in an efficient secretion of TPS2a (26), which is detected in the culture supernatant (Fig. 2A). Truncated TpsB2, however, was not able to secrete the TPS2a construct as there was no TPS2a detected in the concentrated culture supernatant. The non-secreted TPS2a did not accumulate inside the cells but appeared to be degraded, similar to what we observed previously (26). The growth curves of the cultures did not change upon the induction of gene expression with IPTG (Results not shown).

The secretion defect was not the result of absence or improper localization of the TpsB2 mutants, since these mutants were detected in whole cell lysates at comparable levels (Fig. 2A). Furthermore, wild-type and truncated TpsB2 variants showed a similar running behavior on semi-native PAGE gels when heated (10 min at 100 °C) and non-heated (10 min at ambient temperature) outer membrane preparations were compared (Fig. 3). The non-heated samples showed increased mobility of the TpsB protein on gel, at a position of ~47 kDa for wild-type TpsB2, which shifted towards the position corresponding to the expected molecular weight of ~64 kDa for wild-type TpsB2 when samples were heated. This heat-modifiability is considered to be indicative of folding of a β-barrel protein (33-35). Both the wild type TpsB and the truncated variants lacking one POTRA domain showed this behavior. The samples incubated at room temperature already contained some protein running at the position of denatured protein in the gel, likely indicating some instability of the protein under these mild conditions. All TpsB2 variants migrated as a folded protein, although the relative amounts of denatured and folded material suggested that truncated TpsBs were a bit less stable. In particular, the mutant that lacks two POTRA domains showed a decreased amount of the faster running band, when compared to wild-type TpsB2. Overall, the results indicated that deleting a POTRA domain resulted in a decreased amount of the faster running band, when compared to wild-type TpsB2. Overall, the results indicated that deleting a POTRA domain resulted in an inactive TpsB2 transporter, suggesting that two POTRA domains are needed for secretion.

**TpsB2 with a duplicated POTRA2 domain are not functional**

We then tested whether secretion required the presence of a specific set of POTRA domains, or two random POTRA domains. To test this, we constructed *tpsB2* mutants that encoded TpsB2 with either a duplicated POTRA1 (TpsB2-P1P1) or a duplicated POTRA2 (TpsB2-P2P2) and cloned them downstream of the TPS2a construct in the neisserial expression vector (Fig. 2B). Both mutants were expressed (Fig. 2B) and appeared properly localized judged from the heat-modifiability of the proteins (Fig. 3). However, the denatured sample of the TpsB2-P1P1 variant ran at a position in the gel of ~52 kDa, which is much lower than the calculated ~63kDa. Apparently, a periplasmic part of the TpsB was cleaved off, resulting in an inactive protein (Fig. 2B). Indeed we could not detect TPS2a in the culture supernatants of strains carrying TpsB2-P1P1 (Fig 2B). The TpsB2_P2P2 variant, despite having similar expression and folding characteristics as wild type, was also inactive. The results led us to conclude that a tandem of POTRA domains is not sufficient for secretion, but that secretion requires a specific couple of POTRA domains.
Swapping POTRA domains between TpsB2 and TpsB1 changes substrate specificity

We previously observed that the meningococcal TpsB1 operates system specific, i.e. only on cognate TPS domains. By contrast, TpsB2 is able to secrete TPS domains of all neisserial TpsAs tested (26). Isolated POTRA domains have been shown to bind TPS domains in vitro, but with a rather low affinity and this has only been tested for cognate TpsBs (14,15). We, therefore, investigated the role of the POTRA domains in substrate selection by exchanging sequence encoding the POTRA domains between the two meningococcal tpsBs, yielding hybrids TpsB2_Exp1P2B1 and TpsB1_Exp1P2B2, respectively (Fig. 1C). The two hybrids were cloned downstream of the TPS2a or the TPS1 construct, to be able to analyse the effect on secretion of system-1 and system-2 derived proteins, and introduced into N. meningitidis HB-1 tpsB1::kan tpsB2::gen. The hybrid proteins were difficult to detect when the corresponding sera were applied (Fig. 4). Apparently, the POTRA domains are immunodominant regions in the proteins, because the sera did clearly detect the hybrids carrying the POTRAs of the protein it was raised against. Overall levels of hybrid and wild type TpsBs appeared comparable and the hybrid TpsBs showed the characteristic heat-modifiability on semi-native gels indicative of proper localization and folding (see Fig. 3 for TpsB2_Exp1P2B1).

As shown previously, co-expression of TPS2a and wild-type TpsB2 resulted in secretion of TPS2a, while TPS2a secretion was absent in the presence of TpsB1 (Fig. 4A). Simultaneous production of TPS2a and the TpsB2-Exp1P2B1 hybrid did not result in secreted TPS2a in the medium. Interestingly, co-expression of the reciprocal TpsB1-Exp1P2B2 hybrid with TPS2a resulted in secretion of significant amounts of TPS2a. We concluded that the recognition of the TPS2a construct by the TpsB2 POTRA domains is sufficient for secretion, while the lack of recognition

Fig. 2 The influence of deletion or duplication of the POTRA domains of TpsB2 on TPS2a secretion. (A) Immunoblots of whole cell lysates (C) and culture supernatants (S) of N. meningitidis tpsB1::kan/tpsB2::gen cells carrying plasmids encoding TpsB2 or its mutant derivatives with either one or two POTRA domains deleted as indicated above the lanes in combination with the TPS2a construct. (B) Immunoblots of whole cell lysates (C) and culture supernatants (S) of N. meningitidis tpsB1::kan/tpsB2::gen cells carrying plasmids encoding TpsB2 or its mutant derivatives with either POTRA1 or POTRA2 duplicated as indicated above the lanes in combination with the TPS2a construct. The cells were grown in the presence (+) or absence (-) of 0.01 mM IPTG for expression. The blots were incubated with antisera against the TpsB2 and TPS2 domains as indicated on the right. Indicated on the left are the Mw markers.
by the TpsB1 POTRA domains prevents secretion.

We then analysed the secretion of TPS1 (Fig. 4B). As expected, co-expression of TPS1 together with TpsB2 resulted in secretion of TPS1, albeit with a reduced efficiency (26). In line with the results obtained for TPS2, the TpsB1_ExP1P2B2 hybrid secreted only very limited amounts of TPS1, again suggesting a major role for the POTRA domains in substrate selection and the initiation of secretion. The expression of TPS1 with TpsB2-ExP1P2B1 resulted in secretion of TPS1 to a level comparable to secretion of TPS1 by wild-type TpsB2, but lower than that of wild-type TpsB1. This could be caused by the absence of other TpsB1 domains that may contribute to TPS1 secretion or suboptimal functioning of the chimeric protein.

The decisive role of the POTRA domains in the secretion of truncated TPS constructs prompted us to investigate whether the hybrids could select and support secretion of full-length TpsAs. We cloned the ORFs encoding TpsB1_ExP1P2B2 and TpsB2_ExP1P2B1 into the neisserial expression vector as singular genes and introduced them in HB-1 tpsB1::kan tpsB2::gen. This double knockout strain mutant lacks the neisserial TpsBs and as a result the full-length TpsAs remain intracellular, where they accumulate as a ~240 kDa band (TpsA1) or are degraded (TpsA2) (26). Upon induction with IPTG, the complemented strains express the hybrid tpsBs in comparable amounts (Fig. 4B). Presence of the hybrid TpsB2_ExP1P2B1 results in the secretion of TpsA1, as judged from the appearance of bands of ~240 kDa, ~200 kDa and ~75 kDa on blots that were also found in wild type HB-1, albeit in different relative amounts (19). The presence of these proteins in the supernatants were not the result of leakage, as judged from the absence of the marker protein RmpM in the concentrated culture supernatants (Fig. 4B). Furthermore, in the whole cell lysates a cell-associated band at ~200 kDa is detected that was also observed in wild-type cells (19). Similar to what we observed for the truncated TPS2a construct, the full-length TpsA2a and Tps2b proteins do not appear to be secreted by the TpsB2_ExP1P2B1 hybrid. Note that the antiserum detects both full-length system-2 TpsAs (19). Analogous to what we observed for the truncated TPS constructs, the expression of the TpsB1_ExP1P2B2 hybrid resulted in the detection of full-length TpsA2a or TpsA2b-derived bands in the whole cell lysates and the culture supernatant samples, but the cell-associated or secreted TpsA1 bands that result from an active TpsB1 were not detected. Overall, the results clearly indicate that the POTRA domains have an important and decisive role in substrate recognition, but they also indicate that other domains within the complete TpsB are needed for efficient completion of the subsequent steps in secretion.

Single POTRA domains contribute to substrate selection

Next, we investigated whether a single POTRA domain within the POTRA pairs was decisive in target selection. For that we constructed two hybrid TpsB2s in which a single POTRA domain was swapped for the corresponding POTRA of TpsB1, yielding TpsB2_ExP1B1 and TpsB2_ExP2B1, respectively (Fig. 1C). Subsequently, these hybrids were expressed in combination with either the TPS1 or the TPS2a constructs. The hybrid TpsB2 proteins were expressed and showed heat-modifiable running behavior on semi-native PAGE (results not shown). In contrast to TpsB2_ExP1P2B1 hybrid that was unable to secrete the TPS2a construct, the single-POTRA exchange hybrids TpsB2_ExP1B1 and TpsB2_ExP2B1 were able to secrete TPS2a, although the levels were lower than with wild-type TpsB2. The two single-POTRA hybrids were
Polypeptide transport associated domains determine TPS system specificity

**Fig. 3** Heat modifiability of TpsB2 and mutant derivatives of TpsB2. Immunoblot of outer membrane preparations of of *N. meningitidis* tpsB1::kan/tpsB2::gen cells expressing the TpsB variants indicated above the lanes. Samples were either heated for 10 min at 100 °C (D) or kept at room temperature (N). The blot was incubated with anti-TpsB2. The positions of the bands representing folded TpsBs are indicated on the left side, the Mw markers on the right. The panels were taken from the same blot. The TpsB2-ExP1P2B1 was co-expressed with either the TPS2a or the TPS1 construct, as indicated.

**Fig. 4** The influence of swapping the POTRA domains of TpsB1 and TpsB2 on the secretion of TPS2a and TPS1 constructs and full-length TpsA1 and TpsA2 proteins. (A) Immunoblots of whole cell lysates (C) and culture supernatants (S) of *N. meningitidis* tpsB1::kan/tpsB2::gen cells carrying plasmids encoding a wild-type or mutated TpsB as indicated above the lanes in combination with the TPS1 or TPS2a construct. (B) Immunoblots of whole cell lysates and culture supernatants of *N. meningitidis* tpsB1::kan/tpsB2::gen cells carrying plasmids encoding a wild-type or mutated TpsB without TPS construct to analyze secretion of full-length TpsAs. The cells were grown in the presence (+) or absence (-) of 0.01 mM IPTG for expression of the TpsB. The blots were incubated with antisera against a TpsB or a TPS domain as indicated on the right. On the left, the Mw markers are indicated. The full-length TpsA-derived bands in (B) are indicated by closed arrowheads (~240, 200 and 75 kDa for TpsA1 and ~250 and 260 kDa for TpsA2, respectively). A distinct background band detected by the TPS1 antiserum is indicated by an open arrowhead (19).
also able to secrete TPS1 to levels that were higher than that obtained by the double-POTRA hybrid TpsB2_ExP1P2B1, or wild-type TpsB2 (Fig. 5A). Overall, these results indicate that both POTRA domains contribute to the binding site of TPS domains and that there is some flexibility in binding for secretion to occur. Nevertheless, the differences in efficiency also suggest that the binding is influenced by the context of the POTRA domains and the interaction between these two domains.

TpsB2 is functional when the order of POTRA domains is switched

Because our results indicated that the combined POTRAs determine substrate specificity of the TpsB transporter and contribute to the binding site for the TPS domain, we next wondered if the order of the two POTRAs is important for initiation of secretion. To test this, we cloned a tpsB2 variant that encodes a TpsB2 with its POTRA domains in reversed order (TpsB2_RevP2P1; Fig. 1C) and placed it downstream of the TPS2a and TPS1 constructs. In contrast to wild-type TpsB2, this TpsB2_RevP2P1 mutant was not able to secrete TPS1 (Fig. 5B). Co-expression of the TPS2a or TPS2b construct with TpsB2_RevP2P1 resulted in efficient secretion of TPS2a and TPS2b to levels comparable to wild type, clearly indicating that the TpsB2 mutant is functional. Remarkably, however, the secreted TPS2a and TPS2b proteins run at a lower position in the gel when secreted by the TpsB2_RevP2P1. Our previous work already suggested that the TPS2a and TPS2b constructs undergo a processing step during translocation (26). Apparently, the interaction of the system-2 TPS domains with the POTRAs in reversed order corrupts the concomitant processing step. This could suggest a different alignment of the TPS domain in the TpsB2 protein during secretion.

Discussion

A distinctive feature of the TPS systems of N. meningitidis and N. lactamica is that their TPS systems include multiple tpsA copies that appear secreted by a single TpsB. Furthermore, both species encode a homologous TPS system that lacks a specific tpsB (19,20). Recently, we have reported on the redundancy of the meningococcal TpsB transporters and showed that the TpsB1 is more specific in binding system-specific substrates while the TpsB2 shows a more promiscuous binding of TPS domains (26). Here we have used this difference in secretion specificity to investigate the role of the periplasmic POTRA domains of the TpsB transporters in the recognition and secretion of TpsAs. By exchanging POTRA domains between the neisserial TpsB1 and TpsB2, we could swap the specificity of the respective transporters for their substrates. Our results show clearly that the POTRA domains of TpsB transporters not only contribute to binding of the substrates, but that they select the substrates that are secreted by the TpsB. These results were reciprocal between TpsB1 and TpsB2 and, therefore, appear to be a general feature of the POTRA domains. The relaxed specificity of the TpsB2 transporter seems also determined by its POTRA domains, since the TpsB2_ExP1P2B1 mutant carrying the POTRA domains of B1 did not secrete the TPS2 construct, whereas the TpsB1_ExP1P2B2 mutant carrying those of TpsB2 still secreted some TPS1 (Fig. 4). Importantly, the secretion of full-length TpsAs strictly depended on the selection of its TPS domain by the POTRA domains of the TpsB, since secretion of full-length TpsAs in presence of the hybrid TpsB switched to the mutant carrying the corresponding POTRA domains. The sequences of the TpsAs of N. meningitidis differ considerably (19) and, apparently, the TPS domains fully determine the specificity for the TpsB transporter they use for secretion.

All members of the Omp85 protein
family carry one or more POTRA domains (3,7) and our results add to the notion that POTRA domains are the initial binding sites of the substrates of these transporters (4,12,15). Deletion of either POTRA domain of the meningococcal TpsB2 abolishes secretion of the truncated TPS2a construct (Fig. 2), similar to what was found for FhaC of B. pertussis (8). Furthermore, the results with the TpsB mutants that carried duplicated and swapped POTRA domains show that efficient secretion requires the presence of two POTRA domains. Each POTRA appears to have a specific role in the selection process, since a mere duplication of the POTRA2 domain of TpsB2 did not result in a functional protein. However, their order seemed less relevant since placing them in reversed order did not affect secretion, although it did change the concomitant modification of the TPS domain. Furthermore, both POTRA domains appeared to contribute to system specificity, since exchanging only one POTRA domain between TpsB2 and TpsB1 resulted in intermediate substrate specificities (Fig. 5). Overall, these results indicate that the binding site for TPS domains stretches over the two POTRA domains.

The FhaC transporter of FHA is the most studied TpsB and its crystal structure has been solved (8). Mutational studies of FhaC targeted a continuous hydrophobic groove that extends over the two POTRA domains and involves α-helix 1 and β-strand 2 for POTRA1 and α-helix 3 and β-strand 5 for POTRA2 (see Fig. 1). The results indicated that this region is involved in substrate binding and...
secretion (8,15). However, the mutated residues are in the conserved regions of the POTRA domains suggesting that they have a general role in TPS domain binding (Fig. 1A). Based upon our results with the hybrid TpsB mutants, we hypothesize that the specificity of the binding is determined by the non-conserved regions of the domains; e.g. the region comprising β-strand 2 and α-helix 2 (Fig. 1). This surface is close to the hydrophobic groove in the POTRA domains, but may constitute a second binding site. Of note, TPS domains interact with TpsBs in an unfolded fashion (14,15), making a stretched interface likely. Ongoing work in our laboratory focuses on the role of the non-conserved regions in system-specific secretion of TpsAs.

Surprisingly, when we reversed the order of the POTRA domains we observed fairly normal secretion of TPS2 domains. An explanation could be that the two POTRA domains have a symmetric role in the binding of the TPS domain, but this seems less likely since duplicating POTRA2 did not result in a functional TpsB2. Alternatively, the POTRAs may bind the TPS domains in two steps, first at a non-specific general level and then second in a more specific and intricate way. Such a bimodal interaction complies with the observation that the exchange of a single POTRA domain also resulted in a switch in binding specificity.

Reversing the order of the POTRA domains changed the modification of the secreted TPS domains. Previously, we had observed that the position on blot of the secreted TPS2 constructs was lower than what was expected from sequence, already suggesting a modification event (26). The results presented here clearly indicate that, indeed, a modification step occurs, which depends on how the TPS domain interacts with the POTRA domains. Two totally different types of modifications of TPS domains have been described. Firstly, proteolytic cleavage of the N-terminal TPS domain of HMW1A of H. influenzae results in the release of the N-terminal end of the protein from the cell surface (36). Secondly, the TPS domain of ShlA of Serratia marcescens undergoes a conformational switch when secreted by ShlB (37). This switch is needed to activate the hemolysin activity of ShlA and for the TPS domain results in an altered mobility on gels and blots. However, the different sizes we observe point to a proteolytic cleavage event and seem incompatible with a conformational change. This cleavage must occur during secretion, while the protein interacts with the TpsB and the switch of the POTRA domains, apparently, changes the position of the TPS domain in the TpsB so that a new site is cleaved.

Additional binding of TpsAs to other regions of the TpsBs, for example the β-barrel, or the preceding linker region, could contribute to the formation of the secreting complex. Mutations in the FhaC POTRAs that abolish binding in vitro still supported secretion of the FhaB TPS domain in vivo (15). Furthermore, full-length TpsA2 in our experiments appeared protected from degradation by binding to the hybrid TpsB2_ExP1P2B1. In Pseudomonas aeruginosa a hybrid secretion system exists in which a TpsA is secreted by an usher-like OMP that carries POTRA domains (38). This P-usher can also transport and assemble pilin subunits into a pilus. Deletion of the P-usher POTRA domains blocked TpsA secretion, but not its binding to the usher. As a result the pilus assembly was blocked, whereas pili normally assembled when TpsA was absent.

In conclusion, our results clearly indicate that interaction between the POTRA domains and the TPS domain is the decisive step for secretion to occur. The POTRA domains of a TpsB thereby act as specificity filter and their interaction with the TPS domain initiates the secretion process.
Acknowledgements: Joen Luirink and Wilbert Bitter are acknowledged for critically reading the manuscript. S. u. R. gratefully acknowledges funding by the Higher Education Commission of Pakistan.

is essential for intracellular survival of Neisseria meningitidis. Cell. Microbiol. 10, 2461-2482
Table 1: Primers used in this study

<table>
<thead>
<tr>
<th>TpsB mutant</th>
<th>primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TpsB1 (wt)</td>
<td>pr_tpsB1_F</td>
<td>CACATATGGGATCCCTGAGCTAAAATTATTATTTATGTGATGCTAGAAATC</td>
</tr>
<tr>
<td></td>
<td>pr_tpsB1_R</td>
<td>GAGATCTTGAATTTCTGAAAACCTGATAATTCAAGATTGGAAGGCGT</td>
</tr>
<tr>
<td>TpsB2 (wt)</td>
<td>pr_tpsB2_F</td>
<td>CACATATGGGATCCCTGAGCTAAAATTATTATTTATGTGATGCTAGAAATC</td>
</tr>
<tr>
<td></td>
<td>pr_tpsB2_R</td>
<td>GAGATCTTGAATTTCTGAAAACCTGATAATTCAAGATTGGAAGGCGT</td>
</tr>
<tr>
<td>TpsB2_ΔP1</td>
<td>pr_tpsB2_ΔP1</td>
<td>AGCGGAGAATCTGGTTGCGCTGTTGGCA</td>
</tr>
<tr>
<td>TpsB2_ΔP2</td>
<td>pr_tpsB2_ΔP2</td>
<td>ACCTTGGATATGCATCGGTATCAGGGTTAATGGAAGCT</td>
</tr>
<tr>
<td>TpsB2_ΔP1P2</td>
<td>pr_tpsB2_ΔP1P2</td>
<td>ACCTTGGATATGCATCGGTATCAGGGTTAATGGAAGCT</td>
</tr>
<tr>
<td>TpsB2_P1P1</td>
<td>pr_tpsB2_P1P1_1</td>
<td>ACGGCAATAGCTCGGAGGTCCTGCCCATGACACCAGCA</td>
</tr>
<tr>
<td></td>
<td>pr_tpsB2_P1P1_2</td>
<td>TGGCGCGAACGTCAGCTAGCTCGGCTCCATTGGAAGC</td>
</tr>
<tr>
<td>TpsB2_P2P2</td>
<td>pr_tpsB2_P2P2_1</td>
<td>ACGGCAATAGCTCGGAGGTCCTGCCCATGACACCAGCA</td>
</tr>
<tr>
<td></td>
<td>pr_tpsB2_P2P2_2</td>
<td>TGGCGCGAACGTCAGCTAGCTCGGCTCCATTGGAAGC</td>
</tr>
<tr>
<td>TpsB2_ExtP1P2B1</td>
<td>pr_P1P2B1_1</td>
<td>TTTACCGGAACCGTGCTGCTGCTGCTATGACCCAGCA</td>
</tr>
<tr>
<td></td>
<td>pr_P1P2B1_2</td>
<td>ACGGCAATAGCTCGGAGGTCCTGCCCATGACACCAGCA</td>
</tr>
<tr>
<td>TpsB1_ExtP1P2B2</td>
<td>pr_P1P2B2_1</td>
<td>TGGCGCGAACGTCAGCTAGCTCGGCTCCATTGGAAGC</td>
</tr>
<tr>
<td></td>
<td>pr_P1P2B2_2</td>
<td>TGGCGCGAACGTCAGCTAGCTCGGCTCCATTGGAAGC</td>
</tr>
<tr>
<td>TpsB2_ExtP1B1</td>
<td>pr_P1B1_1</td>
<td>GAGGCAATAGCTCGGAGGTCCTGCCCATGACACCAGCA</td>
</tr>
<tr>
<td></td>
<td>pr_P1B1_2</td>
<td>TACCGGATATGCATCGGTATCAGGGTTAATGGAAGCT</td>
</tr>
<tr>
<td>TpsB2_ExtP2B1</td>
<td>pr_P2B1_1</td>
<td>TGGCGCGAACGTCAGCTAGCTCGGCTCCATTGGAAGC</td>
</tr>
<tr>
<td></td>
<td>pr_P2B1_2</td>
<td>TACCGGATATGCATCGGTATCAGGGTTAATGGAAGCT</td>
</tr>
<tr>
<td>TpsB2_RevP2P1</td>
<td>pr_RevP2P1B2_1</td>
<td>TGGCGCGAACGTCAGCTAGCTCGGCTCCATTGGAAGC</td>
</tr>
<tr>
<td></td>
<td>pr_RevP2P1B2_2</td>
<td>TGGCGCGAACGTCAGCTAGCTCGGCTCCATTGGAAGC</td>
</tr>
</tbody>
</table>

*) Restriction sites used for cloning are underlined.
## Table 2: Expression and cloning vectors used in this study

<table>
<thead>
<tr>
<th>Construct</th>
<th>Plasmid name</th>
<th>TPS ORF</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TPS2a with TpsB2 and its derivatives</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPS2a + TpsB2</td>
<td>pEN1220</td>
<td>tpsA2a-tr</td>
<td>(26)</td>
</tr>
<tr>
<td>TPS2a + TpsB2_ΔP1</td>
<td>pEN1222</td>
<td>tpsA2a-tr</td>
<td>this study</td>
</tr>
<tr>
<td>TPS2a + TpsB2_ΔP2</td>
<td>pEN1223</td>
<td>tpsA2a-tr</td>
<td></td>
</tr>
<tr>
<td>TPS2a + TpsB2_ΔP1P2</td>
<td>pEN1244</td>
<td>tpsA2a-tr</td>
<td></td>
</tr>
<tr>
<td>TPS2a + TpsB2_P1P1</td>
<td>pEN1226</td>
<td>tpsA2a-tr</td>
<td></td>
</tr>
<tr>
<td>TPS2a + TpsB2_P2P2</td>
<td>pEN1227</td>
<td>tpsA2a-tr</td>
<td></td>
</tr>
<tr>
<td>TPS2a + TpsB2_ExP1P2B1</td>
<td>pEN1225</td>
<td>tpsA2a-tr</td>
<td></td>
</tr>
<tr>
<td>TPS2a + TpsB2_ExP1B1</td>
<td>pEN1232</td>
<td>tpsA2a-tr</td>
<td></td>
</tr>
<tr>
<td>TPS2a + TpsB2_ExP2B1</td>
<td>pEN1233</td>
<td>tpsA2a-tr</td>
<td></td>
</tr>
<tr>
<td>TPS2a + TpsB2_RevP2P1</td>
<td>pEN1234</td>
<td>tpsA2a-tr</td>
<td></td>
</tr>
<tr>
<td><strong>TPS2a with TpsB1 and its derivatives</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPS2a + TpsB1</td>
<td>pEN1250</td>
<td>tpsA2a-tr</td>
<td>(26)</td>
</tr>
<tr>
<td>TPS2a + TpsB1_ExP1P2B2</td>
<td>pEN1229</td>
<td>tpsA2a-tr</td>
<td>this study</td>
</tr>
<tr>
<td><strong>TPS2b with TpsB2 and its derivatives</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPS2b + TpsB2</td>
<td>pEN1320</td>
<td>tpsA2b-tr</td>
<td>(26)</td>
</tr>
<tr>
<td>TPS2b + TpsB2P2P1</td>
<td>pEN1334</td>
<td>tpsA2b-tr</td>
<td>this study</td>
</tr>
<tr>
<td><strong>TPS1 with TpsB1, TpsB2 and their derivatives</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPS1 + TpsB1</td>
<td>pEN1030</td>
<td>tpsA1a-tr</td>
<td>(26)</td>
</tr>
<tr>
<td>TPS1 + TpsB2</td>
<td>pEN1050</td>
<td>tpsA1a-tr</td>
<td>(26)</td>
</tr>
<tr>
<td>TPS1 + TpsB1_ExP1P2B2</td>
<td>pEN1029</td>
<td>tpsA1a-tr</td>
<td>this study</td>
</tr>
<tr>
<td>TPS1 + TpsB2_ExP1P2B1</td>
<td>pEN1028</td>
<td>tpsA1a-tr</td>
<td></td>
</tr>
<tr>
<td>TPS1 + TpsB2_ExP1B1</td>
<td>pEN1032</td>
<td>tpsA1a-tr</td>
<td></td>
</tr>
<tr>
<td>TPS1 + TpsB2_ExP2B1</td>
<td>pEN1033</td>
<td>tpsA1a-tr</td>
<td></td>
</tr>
<tr>
<td>TPS1 + TpsB2_RevP2P1</td>
<td>pEN1034</td>
<td>tpsA1b-tr</td>
<td></td>
</tr>
<tr>
<td><strong>not combined with TPS construct</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TpsB2_ExP1P2B1</td>
<td>pEN_tpsB2_ExtP1P2B1</td>
<td></td>
<td>this study</td>
</tr>
<tr>
<td><strong>cloning vectors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPS1</td>
<td>pPU1000</td>
<td>tpsA1a-tr</td>
<td>(19)</td>
</tr>
<tr>
<td>TPS2a</td>
<td>pPU1200</td>
<td>tpsA2a-tr</td>
<td>(19)</td>
</tr>
<tr>
<td>TPS2b</td>
<td>pPU1300</td>
<td>tpsA2b-tr</td>
<td>(19)</td>
</tr>
<tr>
<td>pGEM-T</td>
<td>Finnzymes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pEN300</td>
<td>(27)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* The suffix “–tr” indicates that the construct comprises a truncated *tpsA* ORF that encodes the signal peptide and the TPS domain (26).
Polypeptide transport associated domains determine TPS system specificity
Role of the conserved N-terminal motifs of TpsB transporters on secretion by Two-partner Secretion systems of *Neisseria meningitidis*

Sadeeq ur Rahman, Peter van Ulsen

Section of Molecular Microbiology, Department of Molecular Cell Biology, VU University, de Boelelaan 1085, 1081 HV Amsterdam, The Netherlands

*Manuscript in preparation*
Role of conserved N terminal motifs of TpsB transporters on secretion by Two-partner Secretion systems of Neisseria meningitidis

In Two-Partner Secretion (TPS) systems of Gram-negative bacteria, the outer membrane-embedded TpsB transporter is dedicated to the transport of a large exoprotein, generically called TpsA. TpsBs consist of a C-terminal 16-stranded β barrel that is filled by an N-terminal α-helix. An unstructured 30-residue linker region connects the C terminus of α-helix with two periplasmic polypeptide transport-associated (POTRA) domains. The first POTRA domain often contains a conserved pair of cysteines. The distance between the two cysteine residues and the periplasmic location of the POTRA domains of TpsBs suggest that these cysteines may form a disulfide bond. Secretion of TpsA is initiated when the POTRA domains interact with the N-terminal TPS domain of a TpsA. Here, we investigated the influence of the N-terminal α-helix, the linker region and the cysteines on the functioning of TpsB1 and TpsB2 of Neisseria meningitidis. Our data indicate that deletion of the helix domain and the linker region resulted in instability of the TpsB transporters. Despite this increased instability, deletion of the helix domain did not affect TpsA secretion. In contrast, deleting the linker region or mutating the cysteine pair in POTRA1 did affect secretion efficiency.

Introduction

The Two-Partner Secretion (TPS) systems of Gram-negative bacteria form a distinct subclass of the Type V secretion pathway, which also includes the classical and trimeric autotransporters (1,2). TPS systems consist of minimally two proteins; a secreted TpsA protein and a dedicated TpsB transporter that is embedded in the outer membrane. TpsA proteins are typically over 100 kDa and have a predominantly β-helical structure (3,4). They have been reported to function as adhesins, toxins with bacterial or eukaryotic targets or receptors/scavengers of nutrients from the environment (2). The TpsB transporter secretes the TpsA protein across the outer membrane in a process that is not fully understood. Both TpsA and TpsB are produced with an N-terminal signal peptide and are transported across the inner membrane to the periplasmic space via the Sec translocon. TpsB subsequently inserts in the outer membrane to facilitate the secretion of the TpsA. The TpsA N-terminal domain of ~250-300 residues, called the TPS domain, is crucial for this interaction (5).

TpsB is a member of the Omp85 family of proteins, which also includes BamA of Gram-negative bacteria and its eukaryotic homologs (6-10). The Omp85 family consists of a C-terminal 16-stranded β-barrel domain, which is embedded in the membrane, and an N-terminal periplasmic domain that includes one to five polypeptide transport-associated (POTRA) domains. The first POTRA domain often contains a conserved pair of cysteines. The distance between the two cysteine residues and the periplasmic location of the POTRA domains of TpsBs suggest that these cysteines may form a disulfide bond. Secretion of TpsA is initiated when the POTRA domains interact with the N-terminal TPS domain of a TpsA. Here, we investigated the influence of the N-terminal α-helix, the linker region and the cysteines on the functioning of TpsB1 and TpsB2 of Neisseria meningitidis. Our data indicate that deletion of the helix domain and the linker region resulted in instability of the TpsB transporters. Despite this increased instability, deletion of the helix domain did not affect TpsA secretion. In contrast, deleting the linker region or mutating the cysteine pair in POTRA1 did affect secretion efficiency.
autotransporters results in instability of the β-barrel (13,14).

Sequence alignments of TpsB proteins showed that these proteins can be clustered in two major groups that are discriminated by the presence or absence of two cysteine residues (5) that are conserved in position and in distance. When modeled onto the FhaC structure, they map to the POTRA1 domain. Cysteines are able to form disulfide bonds, which in Gram-negative bacteria is usually a periplasmic event mediated by the disulphide bond (Dsb) system (15). Interestingly, these cysteines are conserved among the transporters that secrete TpsA with toxin activities. For example, the contact dependent growth inhibition (CDI) toxins of *Escherichia coli* and *Neisseria meningitidis* (16,17) and cytolsin ShlA of *Serratia marcescens* are secreted by TspBs that have the conserved cysteines (18). Secreted cytolsins are activated upon secretion across the outer membrane and this activation involves their cognate TpsBs (18-20).

The Gram-negative diplococcus *Neisseria meningitidis* (meningococcus) is a major cause of meningitis and sepsis and the neisserial TPS systems are considered to be contributing to its virulence (17,21-24). The genetic organization of neisserial TPS systems appears complex and genomes may encode 1-3 TPS systems that are co-expressed (22,25). *N. meningitidis* genomes encode TpsB1 and/or TpsB2. Of these, TpsB1 showed substrate specificity, whereas TpsB2 was shown to secrete TpsAs of other TPS systems as well (25,26). We have shown previously that system specificity is conferred by the periplasmic POTRA domains (27). Interestingly, our hybrid constructs indicated that other TpsB domains contribute to the secretion process. We therefore investigated the influence of deleting the N-terminal H1 and linker region, as well as mutating the conserved cysteines on TpsA secretion. Our results indicate that conserved N-terminal motifs contribute to the stability and function of TpsBs.

**Materials and methods**

**Bacterial strains and growth conditions.** The *N. meningitidis* and *E. coli* strains used for this study are listed in Table 1. *N. meningitidis* strains were grown on GC agar (Oxoid) supplemented with Vitox (Oxoid) at 37°C, 5% CO₂. Liquid cultures of *N. meningitidis* strains were grown at 37°C in tryptic soy broth (Gibco-BRL). Chloramphenicol was added to a final concentration of 8 μg/ml for plasmid selection. *E. coli* strains were grown on lysogeny broth (LB) or grown on LB agar plates supplemented with 100 μg/ml ampicillin or 30 μg/ml chloramphenicol for plasmid maintenance and with 0.5% glucose for full repression of the lac operator, when appropriate.

**Construction of HB-1 degQ::kan tpsB2::gen**

HB-1 _degQ::kan_ (28) was transformed with pKOtpsB2::gen (25) to obtain HB-1 _degQ::kan tpsB2::gen_. Transformants resistant to gentamycin were screened by PCR to confirm the replacement of _tpsB2 ORF_ by the gentamycin cassette.

**Cloning and construction.** The various TpsB mutants that were analyzed (Table 1) were obtained by PCR using either chromosomal DNA or pGEM-TpsB1 or pGEM-TpsB2 as template in combination with Phusion DNA polymerase (Finnzymes) and primers (Table 2) (27). In general, we apply an overlapping-PCR strategy, by first amplifying the 5’ and 3’fragments of the ORFs by separate PCRs. For the 5’fragment the _pr_tpsB1_F or _pr_tpsB2_F_ (forward primer for _tpsB1_ or _tpsB2_) was combined with the first primer listed for the construct to be made (Table
2), whereas for the 3’ fragment the pr_tpsB1_R or pr_tpsB2_R (reverse primer) with the second primer listed (Table 2). The construct-specific primers contain overlapping sequences and in the third PCR the 5’ and 3’ fragments are combined and the intact gene is amplified by adding the forward and reversed primers. The resulting amplicons were cloned into the pGEM-T cloning vector (Promega) and confirmed by sequencing (Macrogen). The ORFs were subcloned into pET11a (Invitrogen) using the BamHI/EcoRI restriction sites and, subsequently, into the neisserial pEN expression vector using the same enzymes (29). The resulting pEN vectors contain the ORFs as single genes under control of the IPTG-inducible lac promoter. Where the tpsB1 mutant was combined with a truncated tpsA1a ORF (25), the ORF was first cloned into pPU1000 using the BamHI/EcoRI restriction sites. The combined ORFs were then cloned from the resulting plasmid into the pEN expression vector using NdeI/AatII. In case that the tpsB2 mutant was combined
Role of N terminal conserved motifs of TpsB transporters

Table 2: Primers used in this study

<table>
<thead>
<tr>
<th>TpsB mutant</th>
<th>primer name</th>
<th>Primer sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type TpsB1</td>
<td>pr_tpsB1_F</td>
<td>CACATATGGGATCCGCTAAATATATGTATGCTAAATGAAATT</td>
</tr>
<tr>
<td></td>
<td>pr_tpsB1_End</td>
<td>GAGATCTGAAATTTGTAAAGCTGTAATTGCATAGTTAGAGCCGTT</td>
</tr>
<tr>
<td>wild type TpsB2</td>
<td>pr_tpsB2_F</td>
<td>CACATATGGGATCCGCTAAATATATGTATGCTAAATGAAATT</td>
</tr>
<tr>
<td></td>
<td>pr_tpsB2_End</td>
<td>GAGATCTGAAATTTGTAAAGCTGTAATTGCATAGTTAGAGCCGTT</td>
</tr>
<tr>
<td>TpsB1_ΔH1L</td>
<td>pr_TpsB1_ΔHL_1</td>
<td>ACACGGTGTCTTCATGGGCACTAAGGTATTTTAAGGGG</td>
</tr>
<tr>
<td></td>
<td>pr_TpsB1_ΔHL_2</td>
<td>CTTAGCTGCGATGAAACACCTGTAATGCGGTTAATT</td>
</tr>
<tr>
<td>TpsB1_ΔH1</td>
<td>pr_TpsB1_ΔH1L_1</td>
<td>TGCTAGTTAAGGCACTAAGGTATTTTAAGGGG</td>
</tr>
<tr>
<td></td>
<td>pr_TpsB1_ΔH1L_2</td>
<td>TTAGCTGCGCTTAACGTGAAACACCTGGTTAATT</td>
</tr>
<tr>
<td>TpsB1_ΔL</td>
<td>pr_TpsB1_ΔL_1</td>
<td>CGGTACACGGGCATTTGCAGATCTATTGCGTCT</td>
</tr>
<tr>
<td></td>
<td>pr_TpsB1_ΔL_2</td>
<td>AATTGTACCGGTGACTGCGGTTAAATTCA</td>
</tr>
<tr>
<td>TpsB2_ΔH1L</td>
<td>pr_tpsB2_ΔL_1</td>
<td>AACCAGTTGGGCTGGCGAGGGTTGGGGG</td>
</tr>
<tr>
<td></td>
<td>pr_tpsB2_ΔL_2</td>
<td>CCCGCAACGCGCAACGTTGGTTGGCA</td>
</tr>
<tr>
<td>TpsB2_ΔH1</td>
<td>pr_tpsB2_ΔH_1</td>
<td>ATCCGCTTCAAGGGCGAGGGTTGGGGAGCCCAAAC</td>
</tr>
<tr>
<td></td>
<td>pr_tpsB2_ΔH_2</td>
<td>TCCCCAAACCGCTGCCCTGAAACCGATGCGGTTGCAAT</td>
</tr>
<tr>
<td>TpsB2_ΔL</td>
<td>pr_TpsB2_ΔL_1</td>
<td>AACCACGGCTGACATGCTTTTGGCGCAGCTG</td>
</tr>
<tr>
<td></td>
<td>pr_TpsB2_ΔL_2</td>
<td>AACCATGCGCCGTGGTTGGCATTACGAAGTG</td>
</tr>
<tr>
<td>TpsB1_His</td>
<td>pr_tpsB1_His_1</td>
<td>GAGGCCGTGGGTGGGTTGGGCTGCGCCAGCTAAGGTATTTTAAGGGG</td>
</tr>
</tbody>
</table>

with a truncated tpsA2a ORF (25), the ORF was first cloned into pPU1200 using the BamH1/EcoRI restriction sites and then into the pEN expression vector using NdeI/AatII. Of note, His tags (6×His) were inserted at the N terminus of tpsB1-ΔH1 and tpsB2Δ_H1 ORFs immediately after the predicted signal peptides, using the three-PCR strategy.

**SDS-PAGE and Western blotting.** All procedures were carried out as described earlier [22, 25]. Briefly, *N. meningitidis* HB-1 cultures were grown for 4-5h to an OD₆₀₀ of ~2.5-0.3, in the presence of 1- to 0.1mM of isopropyl-beta-d-thiogalactopyranoside (IPTG) where indicated. Cells were harvested by centrifugation (4,500× g, 5 min) and the pellet was resuspended in phosphate-buffered saline pH 7.4 (PBS) to a final OD₆₀₀ of 10. Culture supernatants were centrifuged (16,000×g, 10 min) to remove residual cells. Occasionally the culture supernatant was further subjected to ultracentrifuge (200,000×g) in a bench top ultracentrifuge (Beckman and Coulter). Proteins were precipitated from the supernatant samples using 5% trichloroacetic acid (TCA) and dissolved in a volume of PBS corresponding to a cell density of OD₆₀₀ 100 (10× concentrated compared to cells). Samples were then
mixed 1:1 with 2x sample buffer (mix). In specific cases DTT was omitted to keep disulfide bonds intact. Cultures of E. coli strains containing pEN plasmids were grown in LB broth to an OD$_{600}$ of ~0.4-0.6. IPTG was added to a final concentration of 0.1- to 1- mM and incubation was resumed for another 1-2 hrs. Samples were collected as described above. Protein samples were separated on 7.5-10- or 12-% SDS-PAGE gels (BioRad) and proteins were blotted onto nitrocellulose for Western-blot analyses. Blots were treated as described earlier (25) and incubated with sera diluted 1:5,000 (anti-TPS1 and anti-TPS2a) or 1:10,000 (anti-TpsB1 and anti-TpsB2), 1:10,000 (anti-DegP). The secondary antibody used was goat anti-rabbit immunoglobulin G serum conjugated to horseradish peroxidase (Biosource International), diluted 1:10,000. The binding of antibodies to proteins on the blots was visualized using Lumilight-normal or Plus (Roche). The indicated relative molecular weight of the protein was deduced from the Precision Plus Protein Standard (BioRad) including in each SDS-PAGE gel.

**Outer membrane isolation and heat modifiability.** Outer membrane fractions were isolated according to (30). Cells were harvested by centrifugation (4,500× g, 5 min). The pellet was resuspended in a 50 mMTris-HCl pH 8.0 and 2 mM EDTA buffer and were passed 2 times through a One Shot Cell disrupter (Constant Systems Ltd) at 30,000 psi. Unbroken cells were pelleted by centrifugating the lysate (4,500× g, 5 min). The supernatant was then subjected to ultracentrifugation (200,000×g, 30 min). The resulting pellet containing a crude outer membrane fraction was resuspended...
Fig. 2: Effect of deletion of H1 and linker region of TpsB1 and TpsB2 on expression of TpsBs and secretion of full-length TpsAs in *N. meningitidis*. A. Immunoblots incubated with anti-TPS1 (top panel), which recognizes TpsA1, anti-TpsB1 (middle panel) and anti-RmpM (bottom panel) of whole cell lysates (C) and ultracentrifuged and concentrated culture supernatants (S) of HB-1 tpsB1::kan tpsB2::gen cells carrying no plasmid (empty) or plasmids expressing wild type TpsB1, TpsB1-∆H1, TpsB1-∆L or TpsB1-∆H1L. In all blots, induction of expression by adding IPTG, or not, is indicated above the lanes. The (*) indicates the cell-associated TpsA1 bands secreted by TpsB1-∆L. The TpsA1-derived bands are indicated by closed arrowheads (~240 and ~200 kDa in the cell, and ~240 and ~75 kDa in the supernatant), whereas the open arrowhead represents a background protein (22). The periplasmic RmpM protein is only detected in the cell fractions, indicating the absence of leakage of cellular material to the culture supernatant. B. Immunoblots incubated with anti-TPS2 and anti TpsB2 serum of whole cell lysates (C) and ultracentrifuged and concentrated culture supernatants (S) of HB-1 tpsB1::kan tpsB2::gen cells carrying no plasmid (empty) or plasmids expressing wild type TpsB2, TpsB2-∆H1, TpsB2-∆L or TpsB2-∆H1L. The TpsA2-derived bands are indicated by closed arrowheads (~260 and ~250 kDa in the cell, and ~260, ~250 and ~90 kDa in the supernatant). C. Heat modifiability of TpsB2 and mutant derivatives of TpsB2. Immunoblot of outer membrane preparations of of *N. meningitidis* tpsB1::kan/tpsB2::gen cells expressing the TpsB variants indicated above the lanes. Samples were either heated for 10 min at 100 ºC (D) or kept at room temperature (N) (22).
in PBS to represent a culture of OD\textsubscript{600} \sim 20. The denatured samples were further diluted in 2\times sample buffer and boiled for 10 min. The native samples were diluted in 2\times semi-native sample buffer (i.e. 2\times sample buffer with 0.4\% SDS and lacking DTT) and kept at room temperature for 10 min. Samples were loaded on semi-native SDS-PAGE gels, that are prepared without SDS in the gel and run at 12 mA for at least 3 h while cooled in ice. Blotting was performed as described above.

**Antibiotics sensitivity assay.** All procedures were performed as mentioned (13) with slight modifications. Briefly, a lawn of bacteria was inoculated on IPTG-containing GC agar or LB-agar plates by pouring 5 ml of soft agar containing 200 \mu l of 1 OD\textsubscript{600} of bacteria grown overnight on plates. Standard antibiotic discs (Rosco Diagnostica) were placed on the top-layer-agar and the plates were incubated at 37 °C for 18 h. Sensitivity to the antibiotics were assessed by measuring the ring of growth inhibition around the disc. Experiments were performed in triplicate.

**In silico sequence analyses.** Sequence analyses were performed with amino acid sequences of mature TpsBs. These signal peptides were identified by analyzing the full length sequence with SignalP 4.0 (31) and confirmed with PrediSci(32). Initial pairwise sequence alignments of TpsB proteins were performed using the Bl2Seq server at http://blast.ncbi.nlm.nih.gov/ using standard parameters. Secondary structure predictions were performed with PsiPred at http://bioinf.cs.ucl.ac.uk/psipred/ (32). Additional multiple sequence alignments were performed using the assigned \alpha-helix and linker region within the mature TpsB sequences by using the M-Coffee programme at http://tcoffee.crg.cat/apps/tcoffee/do:mcoffee (33). Models of the mature TpsBs of *N. meningitidis* were generated by the Phyre2 website at http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index (34). The quality of the obtained model was assessed by comparing them to the crystallized FhaC the TM-align programme at http://zhanglab.ccmb.med.umich.edu/TM-align/ (35).

**Results**

**H1 and linker regions of meningococcal TpsB transporters**

We have used the primary sequence of the TpsB of *N. meningitidis* strain H44/76 to produce structural models of the TpsB1 and TpsB2 transporters, which are based upon the FhaC structure (12) (Fig. 1A). Based upon these models and sequence alignments we assigned H1 to residues 24-44 of TpsB1 and 35-57 of TpsB2 and the linker region to 45-68 of TpsB1 and 58-82 of TpsB2, respectively. When we mention H1 or linker region in the remainder of this study, we refer to these regions. A multiple sequence alignment indicated a low sequence similarity between the H1 of TpsBs when compared to that of the POTRA domains (27) and the linker region (Fig. 1B). However, secondary structure predictions and the structural modeling predicted the \alpha-helical structure of H1.

**Deletion of the linker region inactivates TpsB1**

To investigate the function of H1 and the linker region in the functioning of the TpsB1 transporter, we constructed deletion mutants that lacked the H1 \alpha-helix (TpsB1-\DeltaH1), the linker region (TpsB1-\DeltaL) or both (TpsB1-\DeltaH1L) (Table 1). Neisserial expression vectors carrying wild-type *tpsB1* or the deletion mutant derivatives were introduced in the double knock-out *N. meningitidis* strain HB-1 *tpsB1::kan tpsB2::gen* (25) to analyze their effect on production of the TpsBs and secretion of the TpsAs of TPS system 1. HB-1 is an unencapsulated derivative of disease isolate H44/76. Its genome contains ORFs of two *tspA1* variants (*tspA1a* and *tspA1b*)
Role of N terminal conserved motifs of TpsB transporters

and also two tspA2 variants (tspA2a and tspA2b) (22). Because of the tpsB1::kan knock-out, full-length TpsA1 proteins accumulate in the whole cell lysates, as indicated by the accumulation of a ~240 kDa band in immunoblots (Fig. 2A). Complementing the strain with wild type tpsB1 from pEN_TpsB1 restored secretion of full length TpsA1s, as indicated by the appearance, apart from the ~240 kDa band, of a processed ~200 kDa cell-associated band in whole cell lysates and ~240 and ~75 kD bands in the culture supernatants (Fig 2A)(26).

We tested whether the TpsB1-ΔH1L, TpsB1ΔH1 and TpsB1-ΔL mutants are able to restore secretion of full-length TpsA1s in HB-1 tpsB1::kan tpsB2::gen when expressed from plasmid. The TpsB proteins were detected in the cell samples of the double knock-out strain, but the amounts produced seemed lower than those of wild-type TpsB1 expressed from plasmid (Fig. 2A). Furthermore, the deletions of H1

Fig. 3: Effects of deletion of the H1 and linker region of TpsB1 and TpsB2 on expression of TpsBs and secretion of truncated TpsAs in N. meningitidis and E. coli. A. Immunoblots of whole cell lysates (C) and culture supernatants (S) of N. meningitidis tpsB1::kan/tpsB2::gen cells carrying plasmids encoding TpsB1, TpsB-ΔH1, or TpsB1-ΔH1L as indicated above the lanes in combination with the TPS1a construct. B. Immunoblots of whole cell lysates (C) and culture supernatants (S) of N. meningitidis tpsB1::kan/tpsB2::gen cells carrying plasmids encoding TpsB2, TpsB2-ΔH1, TpsB2-ΔL, or TpsB2-ΔH1L as indicated above the lanes in combination with the TPS2a construct. C. Immunoblots of samples of whole cell lysates and concentrated culture supernatants of E. coli MC1061 cells and its degP mutant derivative, In all blots cells were grown in the presence (+) or absence (-) of 0.01 mM IPTG for expression. The blots were incubated with antisera indicated on the right.
alone or the linker region alone apparently rendered the TpsB1 more prone to degradation, since the proteins ran at a lower position in the gel than was expected from calculated molecular weights (Fig 2A). The sensitivity of TpsB1 to degradation is further underlined by the degradation products of ~52-55 kDa appearing in the blots. Nevertheless, the proteins appeared properly localized, since they were present in isolated membrane fractions (results not shown). Our functional results for TpsB1-ΔH1 corroborated this notion (see below).

TpsB1-ΔH1L appeared unable to secrete TpsA1s, since in cellular samples a ~240 kDa band accumulated, while the cell-associated ~200 kDa and secreted ~240 and ~75 kD bands were not detected (Fig. 2A). In contrast, the TpsB1-ΔH1 mutant did show the secreted bands of TpsA1s in the cell and supernatant samples. The TpsB1-ΔL showed an intermediate phenotype, with the ~240 and ~75 kDa bands absent from the culture supernatant, but with the appearance of the ~200 kDa cell-associated band indicating that some TpsA1s may be associated with the cell surface (Chapter 3 Fig 1b), but not released. Overall, the results indicate that the absence of the H1 do not influence secretion per se, however, the absence of the linker region does affect secretion, particularly, when both H1 and linker regions are absent.

**Deletion of the H1 and linker region does not affect functioning of TpsB2**

To analyze whether the linker region of TpsB2 is of similar importance for secretion, we constructed the comparable set of mutants for TpsB2: *i.e.* TpsB2-ΔH1, TpsB2-ΔL and TpsB2-ΔH1L (Table 1). Expression vectors carrying wild type *tpsB2* or the deletion mutants were introduced in the double knock-out HB-1 *tpsB1::kan tpsB2::gen* and the effect on TpsA2 secretion was analyzed. In contrast to TpsA1, full-length TpsA2 proteins cannot be observed in the double knock-out, indicating that non-secreted protein is degraded. Complementation with wild-type *tpsB2* expressed from pEN_TpsB2 restored TpsA2 expression and secretion (Fig. 2B), which results in the presence of TpsA2 related bands of ~260 and ~250 kDa full length protein in the cells and in the detection of TpsA2 bands of ~260, ~250 and ~90 kDa bands in the supernatant. Wild-type TpsB2 was expressed efficiently, whereas again the different deletion mutants of TpsB2 seemed to be decreased compared to that of the wild type ORF. However, unlike TpsB1, the TpsB2 mutants appeared intact because the corresponding protein bands run at positions in the gel according to their calculated sizes. Even at lower expression levels, the TpsB2 deletion mutants all appeared as efficient in secreting the full-length TpsA2s as wild-type TpsB2 (Fig. 2B) and the levels of the secreted ~260, ~250 and ~90 kDa bands did not change. There were some differences in amounts detected for the cell-associated ~260 kDa band, but all showed increased presence, when compared to uninduced cells (Fig. 2B).

The TpsB2 and its deletion mutant derivatives were detected in membrane fractions and again the amounts of protein detected for the deletion mutants appeared diminished, as was also observed for the whole cell lysates (Fig. 2C). Furthermore, all TpsB2 mutants showed a heat-modifiable running behavior similar to wild type TpsB2; *i.e.* when purified outer membranes were separated on a semi-native SDS-PAGE they showed a denatured band running at ~64 kDa and native bands running at ~35 and ~33 kDa indicating that they are properly localized and folded (Fig. 2C) (27). DegQ is a homolog of the periplasmic protease DegP of *E. coli* implying that could be involved in degradation of outer membrane proteins of *N. meningitidis* (28,36). We tested whether it is responsible for the degradation of TpsB mutant derivatives, but expression
of the \textit{tpsB2-ΔH1L} ORF in a HB-1 Δ\textit{degQ} \textit{tpsB2::gen} knock-out strain was unchanged compared to the wild type HB-1 strain (Results not shown). Our results further indicate that, upon insertion, folding and stability of the TpsB2 mutants is comparable to wild-type TpsB2 (Fig. 2C). Overall, the results suggest that, unlike TpsB1-ΔH1L, the TpsB2-ΔH1L mutant is still functional suggesting linker domain is not needed for TpsB2 secretion function.

**Secretion defects of TpsB1-ΔH1L using truncated TPS1 constructs**

In previous studies, we investigated secretion of TpsA proteins by using truncated TpsA constructs comprising the signal peptide and TPS domains (25). To address whether such truncated TpsA constructs were more efficiently secreted by the different TpsB1 mutants, we combined the TpsB1 ORFs with the truncated TPS1 constructs, yielding plasmid pEN1031 and pEN1035, respectively (Table 1) and introduced these into HB-1 \textit{tpsB1::kan tpsB2::gen}. Western blot analysis showed that the mutant TpsB1s were expressed and showed bands similar to our first experiments (Fig. 3A). Like full-length TpsA1s, truncated TPS1 protein was not secreted by TpsB1-ΔH1L. We also analyzed secretion of truncated TpsA2s by TpsB2 and its deletion mutant derivatives (Table 1) and found that it was not affected by any of the deletions (Fig. 3B), similar to what we observed for full-length TpsA2s. Overall, the results corroborate a role for the linker region in secretion of TpsA1s, but not TpsA2s.

We have also tested the secretion of the truncated TPS2 construct by TpsB2-ΔH1L in \textit{E. coli} MC1061 and its \textit{degP} mutant derivative. DegP of \textit{E. coli} is a periplasmic stress factor that has been shown to degrade unfolded proteins that accumulate in the periplasm (37,38). Expression of the constructs encoding TpsB2 or TpsB2-ΔH1L in combination with truncated TPS2 resulted in the expression of TpsB2, albeit that the amounts TpsB2 mutant was again lower (Fig. 3C). The amount of TpsB2-ΔH1L did not increase when DegP was absent. Expression of the TpsBs in the \textit{degP} mutant strain resulted in a reduced growth and cellular lysis, as can be observed by the appearance of TpsB2 and TpsB2-ΔH1L in the culture supernatant. However, and in line with our previous study (25), secretion of the truncated TPS2 protein could be observed in the two \textit{E. coli} strains. Deletion of the H1 and linker region did not interfere with secretion, similar to what we found for \textit{N. meningitidis}. Absence of DegP did also not result in accumulation of intracellular TPS2, indicating that in \textit{E. coli} TPS2 secretion is relatively efficient. Overall, the results indicate that the linker region of TpsB1 is important for the secretion of TpsA1s, but that the absence of the corresponding linker region in TpsB2 influences the biogenesis of TpsB2, though not its function as transporter.

**Pore activity of TpsB1 and TpsB2 and their deletion mutants.**

The crystal structure of FhaC suggests that the conserved H1 α helix can function as a plug that closes the β-barrel (12). We therefore tested whether the deletion of the linker region and H1 would influence pore-activity of these proteins, when over expressed from plasmid in \textit{N. meningitidis} \textit{tpsB1::kan tpsB2::gen} and \textit{E. coli} Top10F. We tested antibiotic sensitivity, by placing discs with antibiotics on plates inoculated with the strain to be tested. The zone of growth inhibition around the discs was taken as a measure of pore activity (Table 3) (13). The antibiotics used were ampicillin, nalidixic acid and trimethoprim. Wild type TpsB1 and TpsB2 expressed from plasmid yielded comparable growth inhibition zones around the discs in the \textit{N. meningitidis} double knock-out strain, suggesting a similar pore size for the two
TpsBs. Expression of deletion mutants of TpsB2 resulted in a larger inhibition zone around ampicillin and nalidixic acid, indicative of larger pore sizes. These results were similar, when the TpsB2 wild type and mutant proteins were expressed in *E. coli*, indicating that the increased antibiotic sensitivity, indeed, resulted from *tpsB2* expression (Table 3). Apparently, deletion of either the linker region or the H1 or both resulted in open TpsB2 channels. For the TpsB1 deletion mutants a different picture emerged. For this protein the inhibition zones from ampicillin and nalidixic acid for strains that express TpsB1-ΔH1 and TpsB1-ΔL proteins are similar to wild type, suggesting a similar availability of the channel for diffusion of the antibiotics. The TpsB1-ΔH1L mutant showed a larger inhibition zone, but not to the extent found for TpsB2. Apparently, the channel in TpsB1 is less open which could possibly be the result of binding and obstruction by additional regions of the periplasmic domains still present in the mutants, or of other regions in the protein, e.g. the external loops. The results for trimethoprim did not fully fit with the results for the other antibiotics, since for both TpsB1 and TpsB2 the deletion of both H1 and linker region resulted in decrease of the susceptibility for the antibiotic. This could indicate that the properties of the antibiotics may influence the diffusion process, depending on the channel conformation. Overall, the results indicated that the TpsB deletion

### Table 3: Antibiotic sensitivity of *N. meningitidis* tpsB1::kan tpsB2::gen and *E. coli* Top10F expressing wild type or mutant TpsBs.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>µg in Disc</th>
<th>Mw</th>
<th>Expression host</th>
<th>TpsB derivatives</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TpsB1 wild type</td>
</tr>
<tr>
<td><em>N. meningitidis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin 33</td>
<td>349.41</td>
<td>15 ± 0.5</td>
<td>16 ± 2.3</td>
<td>15 ± 2.0</td>
</tr>
<tr>
<td>Nalidixic acid 130</td>
<td>232.23</td>
<td>16 ± 0.0</td>
<td>16 ± 0.5</td>
<td>16 ± 0.5</td>
</tr>
<tr>
<td>Trimethoprim 5.2</td>
<td>290.32</td>
<td>0 ± 0.0</td>
<td>6 ± 0.5</td>
<td>6.0 ± 0.0</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin 33</td>
<td>349.41</td>
<td>16 ± 1.2</td>
<td>21 ± 0.5</td>
<td>24 ± 0.0</td>
</tr>
<tr>
<td>Nalidixic acid 130</td>
<td>232.23</td>
<td>17 ± 0.5</td>
<td>18 ± 0.5</td>
<td>18 ± 0.5</td>
</tr>
<tr>
<td>Trimethoprim 33</td>
<td>290.32</td>
<td>0 ± 0.0</td>
<td>0 ± 0.0</td>
<td>5.0 ± 1.2</td>
</tr>
</tbody>
</table>

a) The growth inhibition zone was measured in mm from the rim of the disc until the edge of the zone of inhibition. Values represent the mean of three measurements. b) The concentration of the antibiotic as per manufacturer.
mutants, although expressed at lower levels do assemble in the outer membrane and then constitute channels for diffusion of antibiotics. These channels may be blocked by the H1 and linker region, as the structure of FhaC suggested. Their respective deletions influenced channel properties, but differently for TpsB2 and TpsB1, suggesting that additional factors may influence the availability of the pore for diffusion.

**Role of the conserved cysteine residues in meningococcal TpsB transporters**
The TpsB1 and TpsB2 proteins of *N. meningitidis* harbor a unique conserved...
pair of cysteine residues positioned within POTRA1 (Fig. 1A). POTRA1 of the neisserial TpsB was modeled to be in a β1-α1-β2-β3 configuration with a long loop connecting β1 and α1 (27). In both TpsB1 and TpsB2 the cysteines are 33 residues apart. The first cysteine residue is located just upstream of β1, at the end of the linker region, while the second cysteine residue is located in the extended loop just prior to the conserved α2 (Fig. 1A). The periplasmic location of the POTRA domains suggests that the pair could form a disulfide bond (15). Of note, N. meningitidis encodes three DsbAs, proteins that moderate disulfide bond formation, including two that are membrane-bound (39). We first assessed the presence of a disulfide bond in TpsB1 and TpsB2 expressed from plasmid in HB-1 tpsB1::kan tpsB2::gen. Whole cell lysates were prepared in sample buffer with or without DTT and analyzed on Western blot. The Western blot showed a slightly faster running form of the TpsB proteins in samples where DTT was excluded, which suggests the presence of a disulfide bond (Fig 4A).

To study the influence of the disulfide bond on the biogenesis and the function of TpsB2, we substituted the first cysteine (C86) for alanine using site-directed mutagenesis and cloned the ORF encoding TpsB2-C86A in combination with the truncated TpsA2a construct, yielding pEN1220_C86A (Table 1). We first compared the expression of the construct with that containing wild-type tpsB2 in E. coli MC1061 (Fig. 4B). The amount of the TpsB2-C86A in MC1061 suggested that the expression level of the mutant was somewhat decreased, but not as severe as for the deletion mutants of TpsB2 (compare Fig. 4B and 3C). Western blot analysis further revealed that the mutant TpsB2 was able to secrete the TPS2 construct to the culture supernatant with an efficiency that appeared similar to wild-type. Formation of disulfide bonds in the mutant and wild type TpsB2s were analysed by expression of the constructs in E. coli DHB4 and its ΔdsbA-derivative DHBA (40). The tpsB2 and tpsB2-C86A genes were expressed from plasmid in these strains and whole cell lysate samples were prepared in absence or presence of DTT. On Western blots the TpsB2-C86A mutant was detected as a band that migrated at the expected ~64 kDa position and this band did not shift when DTT was absent, indicating the absence of disulfide bonds (Fig. 4C). The wild-type TpsB2 did show a shift to a lower position when DTT was omitted, both in DHB4 and DHBA. Apparently, the cysteines were so close to each other in the protein that the disulfide bond formed spontaneously, even when DsbA was absent. Overall, the results suggest that the two cysteines in POTRA1 form a disulfide bond, but that disruption of this bond does not disrupt secretion efficiency.

**Discussion**

In recent years, it has become clear that the POTRA domains of TpsBs act as the receptors of TpsA and initiate their secretion (12,27,41,42). Our recent work showed that interaction of the POTRA domains with the TPS domain of TpsA proteins determines whether the TpsA is a suitable cargo for secretion and initiates the secretion process (27). Furthermore, the TPS domains and POTRA domains remain bound to each other during the secretion process (43). The involvement of the conserved α-helical H1 domain and linker region at the N terminus of the TpsB proteins remains less understood. Here, we studied mutant TpsB1 and TpsB2 of N. meningitidis in which these parts were deleted.

The TpsB models based upon the FhaC crystal structure (12) suggest that the H1 domain inserts in the β-barrel channel while the linker region attains no defined structure and connects the H1 to the first POTRA (Fig. 1A). Previous
research has shown that deletion of the H1 region of either FhaC (44) or ShlB did not influence secretion (45). Here we show that deleting the H1 domain from the neisserial TpsBs also did not affect the secretion of the full-length TpsAs of both neisserial system 1 and system 2. Apparently, H1 is not essential for the TpsB secretion function. The HMW1B TpsB of Haemophilus influenzae even appears to lack an N-terminal α-helix (46). In this particular case, the HMW1B channel appears to be plugged by a C-terminal loop of its substrate, the HMW1A TpsA. Although the H1 deletion did not affect TpsA secretion levels, the amount of TpsB protein was affected. Furthermore, the TpsB2 deletion mutants showed a larger pore activity in an antibiotics diffusion assay, indicating that a channel is formed in the outer membrane, but not plugged by the α-helix. These results are consistent with a plug function of H1 and a role in β-barrel assembly (10). A similar functional role has been attributed to the α-helical segment that is inserted in the 12-stranded β-barrel channel of the β-domain of autotransporters (13,14,47). In contrast to autotransporters, the absence of the α-helix in the channel of the TpsBs of N. meningitidis affects the levels of proteins in the membrane, but not the stability per se, since the similar heat-modifiability of the TpsB2 mutants and wild type suggested a similar stability. Therefore, the absence of the H1 α-helix could affect the efficiency of membrane insertion.

In contrast to H1, the linker region of a TpsBs may have a function in the secretion process, since deleting this region did affect secretion of TpsA1 proteins. The reduced secretion was observed for both the full-length and truncated TpsA1 proteins, indicating that the initial phases of the secretion process are affected. It is unclear whether the effect of the linker region deletion is direct or indirect. An indirect effect may be that the deletion results in a different TpsB conformation, for example by misplacing the POTRA domains with respect to other interacting surfaces. TpsB1 mutant lacking the linker region and H1 is clearly inactive. However, TpsB1 mutant lacking only linker region represents an intermediate functional phenotype as indicated by the appearance of cell surface exposed ~200 kDa TpsA1 specie (see also Chapter 3 Figure 1b) and absence of secreted (~240 and ~200 kDa) TpsA1 specie. Therefore, we favor a model in which the linker contributes to the interaction interface between the TPS1 domain and the TpsB1 POTRA domains by placing the POTRA domains in the right context.

Finally, we have investigated the importance of the two conserved cysteine residues in POTRA1 of TpsB2 for TpsA secretion. Our results suggest that these cysteines form a disulfide bond when present in N. meningitidis cell envelopes. Furthermore, substituting one of the two cysteines for alanine rendered the TpsB2 mutant less efficient in assembling in the outer membrane, both in E. coli and N. meningitidis. The mutation, however, did not block secretion. Apparently, the disulfide bond contributes to POTRA1 conformation and without this bond the binding affinity for TPS domains decreases. The pair of cysteines is primarily found in TpsBs that secrete TpsA with cytotoxic activity; e.g. the hemolysin ShlA from Serratia marcescens. To become active as a hemolysin, the ShlA undergoes a conformational change during or shortly the translocation across the outer membrane by the TpsB ShlB (48,49). Recent results indicated that POTRA2 of ShlB is involved in this activation (45). Overall, our results indicate that of the conserved features of TpsBs, the linker region contributes to the secretion efficiency, whereas the other features rather contribute to TpsB biogenesis.
Acknowledgements: Joen Luirink and Wilbert Bitter are acknowledged for critically reading the manuscript. S. u. R. gratefully acknowledges funding by the Higher Education Commission of Pakistan.


Summary and General Discussion

Sadeeq ur Rahman
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 *tpsAs*, 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 *tpsA2s* (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 iORFs, also encoded by the same genetic island. Therefore, a function of the large genetic islands might be the accumulation of different iORFs 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 TpsA1 toxin activity. Probably TpsA1 proteins are processed during
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 POTRA domains 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).

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.

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.
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.


**Summary and General Discussion**


Samenvatting
Summary in Urdu
Summary in Pashto
Acknowledgments
Curriculum Vitae
Publications
Nederlandse Samenvatting

Bacteriën brengen een grote verscheidenheid aan eiwitten naar hun oppervlak om hun leefomgeving te kunnen koloniseren. Deze leefomgevingen kunnen zich in het milieu bevinden, maar ook op of in het menselijk lichaam (en in feite op of in alle levende organismen). Weefsels van mensen die in contact staan met de buitenwereld worden gekoloniseerd door een veelvoud aan bacteriesoorten en hun aanwezigheid kan bijdragen aan gezondheid, maar ook ziekteverwekkend (pathogeen) zijn. Alle bacteriesoorten hechten aan menselijke weefsels en nemen voedingsstoffen op om te kunnen overleven. Maar de pathogene bacteriën produceren vaak meerdere kolonisatiefactoren tegelijk en hebben bovendien systemen die schade aan weefsels kunnen veroorzaken (door middel van toxines), of dienen om het afweersysteem van de mens te ontwijken of te ontregelen. Dit proefschrift beschrijft hoe een pathogene bacteriesoort, Neisseria meningitidis, een specifiek eiwitsysteem, het Twee-partner Secretiesysteem (afgekort tot TPS systeem), gebruikt om eiwitten naar zijn oppervlakte te brengen die bijdragen aan een infectie van mensen.

N. meningitidis is een pathogene bacterie die in de bovenste luchtwegen van mensen koloniseert zonder merkbare ziekteverschijnselen te veroorzaken. In zeldzame gevallen, meestal veroorzaakt door een verlaagde weerstand, kan deze bacteriesoort vanuit de luchtwegen doorbreken naar de bloedbaan en daar bloedvergiftiging (sepsis) veroorzaken, of de hersenvliezen bereiken en daar hersenvliesontsteking (meningitis) veroorzaken. Het TPS systeem van N. meningitidis zorgt ervoor dat de bacterie de zogenaamde TpsA eiwitten uitscheidt en aan zijn oppervlakte bindt, wat kan bijdragen aan de N. meningitidis infectie. Hoe de TpsA eiwitten bijdragen is onbekend, maar patiënten met een N. meningitidis infectie hebben soms antistoffen in hun bloed die het TpsA eiwit herkennen, wat alleen kan als dat ook daadwerkelijk bij de infectie aanwezig is. Bovendien brengen Neisseria stammen die een hoger aantal ziektegevallen veroorzaken meerdere TPS systemen naar hun oppervlakte. Daarnaast blijkt uit laboratoriumexperimenten dat het meest voorkomende TPS systeem betrokken bij het hechten aan en binnendringen van luchtwegepitheel (de buitenste cellaag van de luchtwegen). Bovendien is het TpsA eiwit een toxine dat andere N. meningitidis stammen doodt. Dit lijkt misschien onlogisch, maar kan voor een infecterende stam nuttig zijn in de competitie met concurrerende bacteriestammen om nutriënten en leefomgeving.

Na het transport door de membraan komt het TpsA eiwit in de omgeving van de bacterie terecht en oefent zijn functie uit.

Pathogene *N. meningitidis* stemmen bevatten meerdere TPS systemen (genummerd 1-3) en deze lijken tussen *N. meningitidis* stammen uitgewisseld te worden. Het onderzoek beschreven in dit proefschrift richt zich op het mechanisme waarmee het TpsB transporteiwit het TpsA eiwit in het periplasma van de bacterie herkent en zo het transportmechanisme start. Bovendien is bestudeerd wat er gebeurt wanneer er meerdere TPS systemen naast elkaar gemaakt worden: werken dan de systemen onafhankelijk van elkaar werken en hoe selecteren de TpsB transporteiwitten dan hun te transporteren TpsA eiwitten.

Hoofdstuk 1 is een overzicht van de bestaande kennis over twee veelvoorkomende en aan elkaar verwante eiwitsecretiesystemen, het TPS systeem en de autotransporters. Hoofdstukken 2-5 bevatten de beschrijving van het eigenlijke wetenschappelijke onderzoek dat aan dit proefschrift ten grondslag ligt en waarvoor we de *N. meningitidis* stam H44/76 hebben bestudeerd. Deze stam bevat drie TPS systemen en maar twee verschillende TpsB transporteiwitten, behorend bij systeem 1 en 2. In Hoofdstuk 2 en 3 laten we zien de twee TpsB transporteiwitten een verschillende specificiteit hebben. De TpsB van systeem 1 is erg specifiek en herkent en secreteert alleen de TpsA eiwitten van dit systeem aan de specifieke eiwitsequenties van hun TPS domein. De TpsB van systeem 2 werkt veel minder selectief en kan de TpsA eiwitten van zijn eigen systeem en dat van de twee andere systemen herkennen. In hoofdstuk 2 laten we zien dat het geldt voor de TPS domeinen van deze systemen, die daarvoor apart geproduceerd werden en in Hoofdstuk 3 dat ditzelfde geldt voor de volledige TpsA eiwitten. Bovendien laten we zien dat deze minder specifieke TpsB ook TPS domeinen afkomstig uit een andere bacteriesoort transporteert. Deze komen uit *Neisseria lactamica*, een aan *N. meningitidis* verwante bacteriesoort die ook voorkomt in de bovenste luchtwegen van mensen. Deze observatie suggereert dat TPS systemen tussen deze bacteriesoorten kunnen worden uitgewisseld.

In Hoofdstuk 4 laten we zien dat een onderdeel van de TpsB transporteiwitten verantwoordelijk is voor de selectie van TpsA eiwitten voor transport door de buitenste membraan. Deze selectie vindt plaats door de zogenaamde POTRA domeinen. Deze onderdelen van het TpsB transporteiwit steken in het periplasma en kunnen de TPS domeinen van het TpsA eiwit binden. Het werk in Hoofdstuk 4 laat zien dat het mogelijk is de POTRA domeinen tussen de twee TpsB transporteiwitten van *N. meningitidis* uit te wisselen. De eiwitten blijven dan functioneren als transporteiwit, maar hun specificiteit verandert. De verandering volgt de herkomst van de POTRA domeinen. Dat wil zeggen; een TpsB transporteiwit van systeem 1 met de POTRA domeinen van de TpsB van systeem 2 heeft ook de eigenschappen van het gehele TpsB2 transporteiwit en transporteert TpsA eiwitten van verschillende systemen. Een TpsB transporteiwit van systeem 2 die de POTRA domeinen het transporteiwit van systeem 1 draagt heeft de omgekeerde eigenschappen; in dit geval kan deze TpsB alleen heel goed de TpsA eiwitten van systeem 1 herkennen, terwijl de TpsA eiwitten van systeem 2 veel slechter herkend worden. Dit werk laat zien dat de POTRA domeinen niet alleen de TPS domeinen binden, maar ook selecteren en dat na selectie en interactie, het transport van de TpsA plaatsvindt. Andere delen van de TpsB en TpsA eiwitten lijken daarbij een minder belangrijke rol te spelen; wanneer er een interactie tussen POTRA en TPS domein is vindt er secretie plaats.

In het laatste hoofdstuk, Hoofdstuk 5, bestuderen we de invloed van andere onderdelen van de TpsB transporteiwitten, die ook in het periplasma van de bacterie
lokaliseren. Deze delen komen voor bij alle TpsB eiwitten die zijn beschreven in de literatuur, maar hun rol in het secretieproces was nog onduidelijk. Wij hebben deze delen uit de TpsB eiwitten verwijderd en gekeken of dit de lokalisatie in de buitenste membraan en hun activiteit als transporteiwit van TpsA eiwitten beïnvloedde. Uit de resultaten bleek dat deze domeinen bijdragen aan de stabiliteit van de eiwitten in de membraan en ook aan de efficiëntie van het secretieproces. Maar het zijn geen essentiële onderdelen in dit proces, omdat de TpsB eiwitten ook zonder deze onderdelen het TpsA eiwit nog secreteerden.

Het werk beschreven in dit proefschrift toont aan dat de TpsB transporteiwitten van *N. meningitidis* een verschillende specificiteit hebben voor de te transporteren TpsA eiwitten en dat dit reguleert welke TpsA eiwitten deze bacterie naar zijn oppervlakte secretieert. Bovendien laat het zien dat de POTRA domeinen van deze TpsB eiwitten de onderdelen van het eiwit zijn die de selectie uitvoeren. Daarmee bepalen de TpsB eiwitten het arsenal aan TpsA eiwitten dat de bacterie op zijn oppervlakte draagt tijdens een infectie.
Summary in Urdu
خلاصة
منگو کوکس ایک خاص قسم کا گرام منفی جرثومہ ہے۔ جو ناک اور گلی کی اندرونی ہوکے خلیوں میں پا رہا ہے۔ پانچ سے دس فیصد متاثر افراد میں، جرثومہ کونی علامات پینا نہیں کرتا ابتدائی طور پر، ایک جھلی میں موجود ہے۔ خصوصی کلیئے مخصوص علامات اور ریزوکٹ بنئے اسے رنج گلدی ہوئی ہے جتنا کا تجزیہ کیا گیا ہے۔ علاج کے دوران مرض کو متفرق اینڈز اور بین (شمول سیالوسپورین) دی جاتی ہو کبھی ہی کبھی ضعیف اشراق رکھتی ہے۔ اسی مرض کے سبب کا بہت سار طرفہ۔ ویکسین کی استعمالیہ ہے جس میں کے بارے میں سب سے زیادہ اہمیت کی میںیوں کے اصل ہے۔

55 برس سے زائد عمر کے لوگوں میں پانی جاتی ہے۔ منگو کوکس پس ملال بارہ لاکھ افراد کو متاثر اور 13,500 افراد کی جان لنگی ہے۔

عمرے بیماری کی علامات اجناس نور نور پر بہت اکثر علاج کے بغیر چند ہبنگت میں جان لوہا ثابت ہو سکتی۔

تشفیع کلیئے مخصوص علامات اور ریزوکٹ بنئے اسے رنج گلدی ہوئی ہے جتنا کا تجزیہ کیا گیا ہے۔ علاج کے دوران مرض کو متفرق اینڈز اور بین (شمول سیالوسپورین) دی جاتی ہو کبھی ہی کبھی ضعیف اشراق رکھتی ہے۔

اس مقامی میں بیان کر دے رہا تحقیق منگو کوکس کی اپنی بی/1 قسم پر کی گئی ہے جس کا ماحول نارود میں پہنچی بیماری کا جرثومہ ایک 1/44-1/47 بے جرثومہ کی بیماری پہلی والی ہے۔ پہلی جرثومہ نے ایک تنازع بکر بکر منفی کر دی جاتی ہے بیماری پہلی والی ہے۔ پہلی جرثومہ کو ان پر زیادہ کو اندرونی اور پہلی جرثومہ نے ایک منفی کر دی جاتی ہے۔ پہلی جرثومہ کی جرنی بس سے رطوبتی نظام پناہ سے تاہم، سے رطوبتی نظام نہ سے ممکن ہے۔ اس نظام میں کیشمالی تھا۔

9.5-15، سـ: سـ/لیکھی (نئی 5-سـ) اور بابی روٹینی نظام (نئی 5-سـ) 5-15 دوسرے سے شامل ہیں۔

ثیسا ایس نظام منگو کوکس جرثومہ کا سب سے موثر پتھر ہے جو مرض کی ابتدائی ہی پہلی جرثومہ کی جرنی یا پہلی جرثومہ کی خلیوں نے زیادہ جرثوم کی ابتدائی اور پہلی جرثوم میں مدد دیتا ہے۔ پہلی جرثوم
جهلی سے خارج کردے ایک پروٹین پر مشتمل ہے جس کو تیّ پی ایس لیٽ کي بین، اس پروٹین کی تخلیق جینز کے ایک گروہ کی مدد سے بھیتی بیا ہے۔ اس پروٹین کے "این سری" پر 200 سے 032 کیلی گیا ایک جاتہ بھیتی ہے جو برومنی خلی جھلی کے ترانسپورٹر جنہیں سے ملکر پروٹین کے خلی سے اخراج کو ممکن بناتا ہے۔ اس مقام کا باب اول ناتھ-03 رقمنی نظام کی حیاتیاتی تخلیق کو اخراج کا طریقہ کار اور اسے حیاتیاتی تکنیکی اطاق کا جائزہ لیتا ہے، ممکن کہ یہ حمص مینگوکوس کی تیّ پی ایس نظام، اس کے اخراج کا طریقہ کار اباف کے نشانیاتی اور خاصجاتی تیّ پی ایس پر ترانسپورٹر کے ذریعے اسکی پچان کی گردگھومنا ہے。

دوسرے گرام منفی ہروغمون کے برعکس مینگوکوس کا ذی این لے بہت سے تیّ پی ایس نظام (نظام ایک تین) بنا سکتا ہے جن کے بطن میں صرف دو تیّ پی ایس بی(تی پی ایس بی(0 اور تیّ پی ایس بی(01 پاہ جاتے ہیں جو اس نظام کے بروکس کی تربو ایس نظام (تی پی ایس بی(00 سے تشوکل بہتے ہیں، ان کے برعکس تیّ پی ایس نظام(3 کی بدلیات صرف ایک جین (تی پی ایس بی(3 ایس) بم یا صفحہ جاتی ہے۔ باب دوم اور سوم مین دکھائی گیا ہیں کہ تیّ پی ایس بی(2 نظام کثرت مین ہے اور ایک خطرہ منقطع، پروٹین (تی پی ایس بی(3) خارج کردتا ہے جسکی بدلیات ایچ بی(1 جر تو سے کے جین میں ملنی بین، البته تیّ پی ایس بی(1 صرف منقطعیہ تیّ پی ایس بی(3 کی خلو خلی جھلی کے اطراف منطیقہ کسکا پی، باب چھوٹے مین تجویدی ہیں۔ یہ بنیادی گاہی ہی ہوک کس طرح تیّ پی ایس بی(0 کا پہلا جز منصوب کرده پروٹین کی اخراج مین اس کردار اس کردتا ہے۔ ہیں۔ باب پہچم مین ہی گاہی کہ تیّ پی ایس ترانسپورٹر کے این سرے کے کچھ مضوام اجزا اخراج کے عمل مین اس کردار اکیا کردا۔

اس ضمن مین ایڈاف پر پروٹین کے بانی روابط اور تیّ پی ایس پر ترانسپورٹر نظم کے عمل کی طریقہ کار پر مزید خاندان کی پروٹین کے طریقہ کار کو بہت طور پر سمجھ جا تحقیق کی ضرورت ہے تاکہ اس پر ایسم/087 تیّ پی اس سمگھا ازین اس تحقیق کی مدد سے جرمنی کی سطح پر موجود انتہائی اور خارج شدہ پروٹین کے نظام کو بھی سمچھا اور مین ملدے گی تا کہ مستقبل مین مینگوکوس کے خلاف ویکسین تیار کی جا سکتا۔
تمین کړی (Gram negative) (Neisseria meningitidis) دا کوم چې د پیو زي او مری په دنیا کولی کوي. دا دغه لیومیز کې دی، چې د سب سره د بائیونریما لپاره خویښت کیږي. "پورپوز" په ځان کولو لپاره چرکاوه او دغه کې سینکریت نه کړی. دا دغه نوی په یې کېږي، چې د سینکریت نه یې کړی او دا دغه لیوه لپاره خویښت کیږي.
Acknowledgments

Higher Education Commission (HEC) of Pakistan and the visionaries who realized this mega project that provides funds to thousands of Pakistani scholars abroad deserve first place for thanking of. NUFFIC administration was quite co-operative and friendly to facilitate me in this regard, I am thankful to them. And yes, Joen Luijrink, my Co-promoter, you took care of my early days in Amsterdam: from housing to my pocket money, and for the grant that enabled me to survive the last 6 months of my PhD tenure, I highly appreciate that; and just saying thank you might not be enough. Bundle of thanks go to my promotor Wilbert Bitter, who not only did inspire and kept my momentum during PhD, but in fact materialized on-time submission of this thesis book to the examination committee and sharply fixing date for thesis defence.

Indeed, my supervisor, Peter van Ulsen effectively translated my abilities to work as an independent scientist in the future, an utmost quality of PhD product. Peter, you had a very pragmatic approach towards Science and your’s on-time suggestions made me out of the sinking boats many times. I appreciate your patience in the beginning when I started over in the lab as I was very new to all these kinds of techniques. Your input from lab to finishing up writing made me able to the last part (acknowledgments) of this book. Not only in the science, but I always enjoyed our conversation related to non-scientific life. You were quite friendly and helpful and this made me feel to enjoy my work. It was September 2008 that you received me from Schipol airport and made me a visit of the lab (although I was very tired of ~24 hr travelling). I don’t doubt that you would offer to see me off on the airport as well.

I was very lucky to have very nice colleagues in MolMic and MMI, who added to the joyful atmosphere in our green and blue lab. Greg, (bossssss ♀) you were always there when I needed to ask anything: from Dutch translation to where is the x-chemical?. You are great colleague and friend. I was lucky to have you around. I appreciate your company during lunch and all sorts of other breaks. Thank you for being my paranymph. Ana Sauri (Sauriiiiiiiiiiiiiiiiiiiiii) you were not only my colleague, but a good friend. You certainly impressed me by your excellent and valuable suggestions on improving my experiments. Beside science, we shared and discussed many other issues and I always learnt from and enjoyed your friendly discussion. Ovi, my first roommate during working time, you were great. You invited me the first time on dinner and then you dropped me home late at night. I still remember the good taste of pumpkin soup and fried fish you and Ana prepared. It was sad that you left MolMic very soon, but the good thing is we are still friends® and thanks for putting efforts in designing the cover of this book. Thank you Corinne! for everything and your all sorts of help in the lab and arranging flowers and ballons on my birthdays. I have your basket ball, so I will always remember you when I go to basket. Zhong, conversations with you was always enjoyable, though most of the time it used to be quite hard to convince you on most of the points. Zora, it was great to have you as my roommate. I still have the tastes of your cakes (and cake prepared by your Mom) and soup. I enjoyed our scientific-and non-scientific conversations. Aniek, you were working across my bench for quite a while, it was nice working with you and being your colleague. I highly appreciate your help for generously providing the template for laying out thesis book, which was indeed a great gift. Karlijn, I will not forget the number of overnight cultures you used to do and then the next morning your bench full of culture tubes®. Good luck for your project. It was indeed wonderful to talk on many issues especially on writing-up. Wout, it was great time being your colleague.
and thanks for the wonderful scientific and non-scientific discussions. Bart, I enjoyed your company in the lab and during movie nights. Maria, your bench was across mine, and I liked the calmness prevailed at your bench. Alexander was born to you during the last year of my PhD and this made you off the lab for a couple of months, but I hope to see you on my defence. Edith, discussions were always joyful with you, and combination of your smile and laugh always made it lively. Mathijs, your presence in the coffee room made our discussion quite spicy. I hope you keep it up with your new colleagues. Marjoelein (lekker), you were smiley and pleasant to talk with. Good time when we used to make jokes and thanks for being my parnamph. Trang, though I changed a couple of roommates, but since, you were the last one during PhD, a nice and polite colleague, who was curious about many things. Good luck for your project and keep in touch. Markus Peschke and Mélanie le Goff, I hope we can make it into a dinner before I leave, good luck for your projects. A big thanks goes to all my colleagues Ben, Ester, Astrid, Sandra, Louis, Maikel and Eveline at MMI. I highly appreciate our useful scientific discussions with my collaborators Jan Tommassen and Jesus at Utrecht University, Utrecht, The Netherlands. I am highly grateful to my Pakistani friends, those who are currently in The Netherlands or completed their projects successfully, for their nice company and joyful moments (the list is pretty long :)). Good luck for your theses Saif, Soaib, Qaisar, Adnan and Altaf.

While working abroad and away from family, it would have been certainly impossible to carry on the lab work without moral support and material help of my wife (Aalia Sadeeq), parents, and other family members specially my brothers, Aziz ur Rahman and Ata ur Rahman. My good friends, Mukaram Khan, Farooq Ahmed and Ali Khan were always supportive whenever I needed their help in Pakistan. Particularly, thank you Farooq, for every time receiving me at the airport and being with me until home.

Sadeeq ur Rahman
April 30th 2014
CURRICULUM VITAE

Sadeeq ur Rahman was born in Khall, District Dir, Khyber Pakhtunkhwa, Pakistan in March, 1980. After his early education from GHSS Khall, he moved to Islamia College Peshawar for secondary school in 1996. He moved to Lahore in 1999 for studying Doctor of Veterinary Medicine (D.V.M) from University of Veterinary and Animal Sciences, Lahore, Pakistan. He graduated in 2003, after which, he immediately started over M. Phil in Microbiology. After completion of course work, he worked in the cell culture lab for his thesis under supervision of Prof. Dr. Khushi Muhammad and Prof. Dr. Masood Rabbani. He optimized the baby hamster kidney-21 (BHK-21) cell line growth and maintenance for cultivation and propagation of Foot-and-Mouth disease virus. During his Master programme, he got stipend and fellowship for two years (2005-06) from Higher Education Commission (HEC) of Pakistan under support to scientific talent programme. After defending his thesis, he graduated in 2006. During and after thesis defence, he worked in a couple of private vaccine productions units and diagnostic labs in Lahore and Islamabad.

In 2007, Sadeeq was inducted as a lecturer in the department of Animal Health, University of Agriculture, and Peshawar, where he taught Microbiology for almost two years. In 2007, once again, Sadeeq won a fellowship for Master leading to PhD, from HEC of Pakistan under overseas scholarship scheme. Under the scheme, he moved to The Netherlands in 2008, and synchronized with the MSc biomedical sciences courses in September 2008 at VU University, Amsterdam, The Netherlands. After the deficiency courses for almost a year, he started over with his PhD project in the lab of dr. Joen Luirink and Prof. dr. Wilbert Bitter under the supervision of dr. Peter van Ulsen and dr. Joen Luirink. The results obtained are described in this thesis. During his PhD project, Sadeeq, presented his work in many inter-national conferences including a couple of workshops in abroad and in the Netherlands.

Sadeeq is currently enjoying hiking, fishing and relaxing. He has applied for temporary placement under the IPFP programme to HEC for a teaching post as an Assistant Professor and also looking for a PostDoc position.
Publications

