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Review

Type V secretion: From biogenesis to biotechnology ☆

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A B S T R A C T

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. This article is part of a Special Issue entitled: Protein trafficking and secretion in bacteria. Guest Editors: Anastassios Economou and Ross Dalbey.
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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. Both complexes are reviewed in detail elsewhere in this issue of BBA [Chapters 5, 6 and 13 this issue of BBA]. 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

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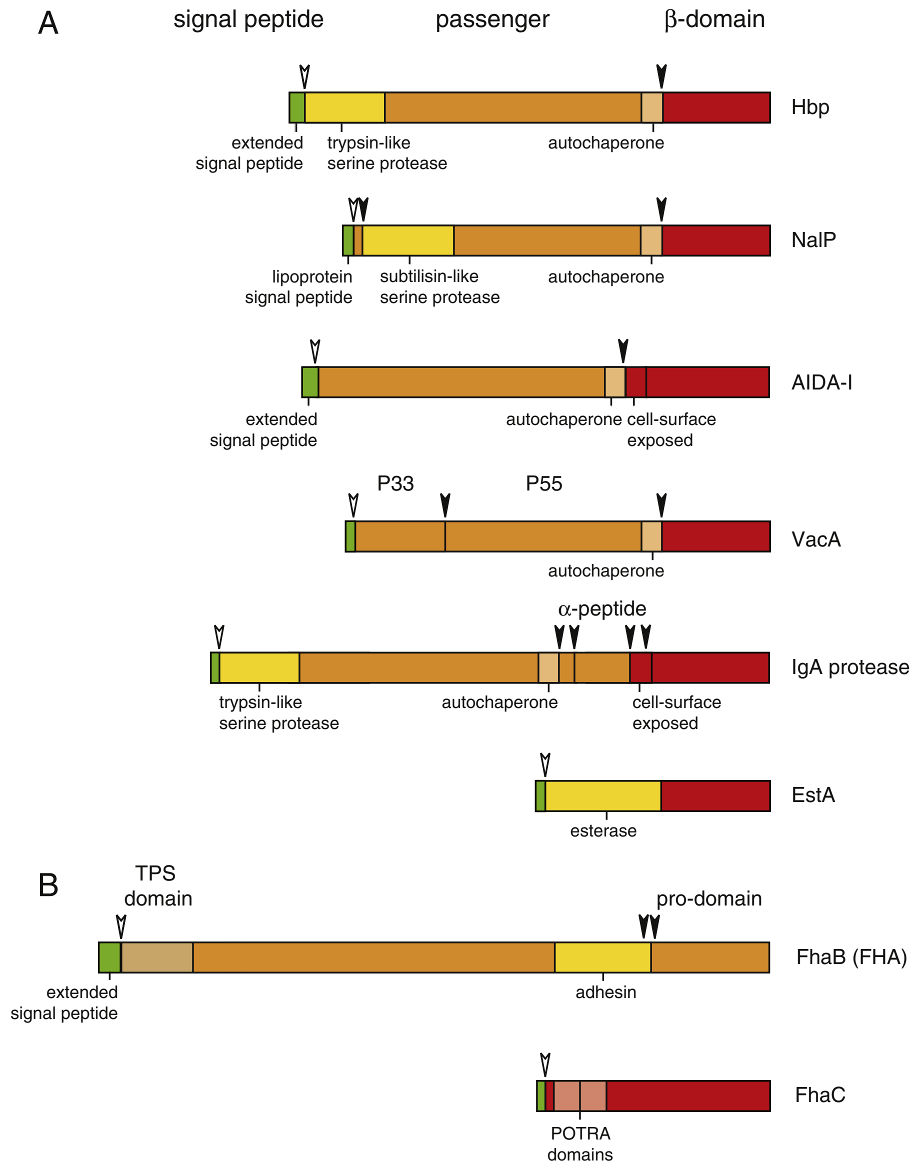

Fig. 1. Schematic representation of the domain organization of autotransporters and the TpsA and TpsB proteins of TPS systems. (A) Autotransporters show a general tripartite domain organization with a signal peptide (green), a passenger (yellow-orange) and a β-domain (red). (B) Domain organization of 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.

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 NalP 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 review. 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 HMW adhesin of *Haemophilus influenza* [20], may add to their functionality or prevent degradation in a hostile environment.

In this review 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 [GD**S**GS] 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 [25–27]. Despite significant sequence similarities, the SPATEs display distinct substrate specificities [22–24,28]. 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 pathogenesis.

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. influenza* [34–36]. 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 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

Table 1
Examples of autotransporter functions[a]. [282,283]

Autotransporter	Organism	Function	ref.
SPATEs			
SigA	*Shigella flexneri*	Cytopathic effects on host cells by cleaving intracellular targets	[276]
Pic	*Escherichia coli*	Mucinase-activity, colonization	[31]
Subtilisin-like serine proteases			
AasP	*Actinobacillus pleuropneumoniae*	Release of other proteins from cell surface	[50]
Self-associating adhesins			
TibA	*Escherichia coli*	Adhesin, invasin, bacterial aggregation, biofilm formation	[65,66]
EhaA	*Escherichia coli*	Adhesin, biofilm formation	[277]
GDSL-esterases/lipases			
McaP	*Moraxella catarrhalis*	Esterase/phospholipase B activity, adherence to human epithelial cells	[83]
Other autotransporters			
MapA	*Moraxella catarrhalis*	Acid phosphatase	[278]
PmpD	*Chlamydia trachomatis*	Adhesin, activation of monocytes	[88]
Gingipains	*Porphyromonas gingivalis*	Cysteine proteinases involved in virulence	[279]
AaaA	*Pseudomonas aeruginosa*	Arginine-specific amino peptidase, involved in virulence	[280]
MisL	*Salmonella* Typhimurium	Adhesin, binds fibronectin	[281]
AlpA	*Helicobacter pylori*	Lipoprotein, binds epithelial cells and human laminin	

[a]) Examples listed here are not discussed in the text and are chosen to illustrate the functional diversity of autotransporter passenger.

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 passsenger. Bioinformatic analysis of genome sequences indicated that this group of autotransporters is quite large and diverse, but only few examples have been characterized functionally [7]. The subtilisin-type autotransporters include Ssph1 and h2 of *Serratia marcescens* [48], SphB1 of *B. pertussis* [49], AasP of *Actinobacillus pleuropneumoniae* [50] and NalP of *N. meningitidis* [14]. Subtilisins often function in protein maturation and activation by proteolytic cleavage of the substrate. For example, NalP 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, NalP may regulate the presence of immunogenic proteins at the cell surface. Expression of the *nalP* 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 NalP and SphB1 contain a lipoprotein motif in their signal peptides ([L-A/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 NalP this lipid modification has been shown for the surface-associated form [14]. However, the secreted passenger of NalP 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 NalP 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 [62–64] 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 [59,64,67,69–71].

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-aggegation 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* [74–79] 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* [80]. 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 (phospho)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*. The BrkA autotransporter is involved in serum resistance and thus facilitates the survival of *B. pertussis* in its host [84]. The VacA autotransporter was identified as a 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 cytoxic 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].

Table 2
Functions of TpsA proteins.

TpsA	Organism	Function	Ref.
CDI systems			
BcpA	*Burkhoderia* species	Contact dependent growth inhibition, biofilm formation	[135]
CdiA	*Escherichia coli*	Contact dependent growth inhibition	[134]
Cytotoxins and enzymatic activity			
LepA	*P. aeruginosa*	Secreted protease cleaves human protease-activated receptors	[284]
HpmA	*Proteus mirabilis*	*Ca2+-dependent* cytolysin	[121,122]
EthA	*Edwardsiella tarda*	Cytolysin, mediates cellular invasion in fish	[123,124]
ShlA	*Serratia marcescens*	Cytolysin, haemolysin, pore-forming toxin	[120,285]
Heme-binding proteins			
HxuA	*Haemophilus influenza*	Binding of heme and heme: hemopexin	[99,100]
Adhesins			
HecA	*Erwinia chrysanthemi*	Adhesin to plant tissue, autoaggegation	[286]
HmwA (HM-W)	*Haemophilus influenzae*	Adhesin to epithelial cells	[101]
EtpA	*Escherichia coli*	Adhesin, mediating binding of flagella to host cells	[287]
FhaB (FHA)	*Bordetella pertussis*	Adhesin, binds epithelial cells, biofilm formation, immunomodulation	[108,112,113,288]
HrpA	*Neisseria meningitidis*	Adhesin, intracellular survival, biofilm formation	[289–291]
MhaA	*Moraxella catarrhalis*	Adhesin, binding to epithelial cells	[292]

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] (Fig. 1B). 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 *B. bronchiseptica* and binds to carbohydrates, heparan sulfates and integrins exposed on ciliated epithelial cells, macrophages and the extracellular matrix in the upper respiratory tract [108–111]. 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 [49,117–119]. 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 *S. 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 [129–131]. 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 the structure (see Section 3.2) were pairing with those in neighboring molecules, which could be instrumental in this conversion [132]. During secretion of ShlA, the conformational change is mediated by the periplasmic POTRA domains of SlhB, since peptide-insertions in this region resulted in the secretion of inactive ShlA 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 [134–137]. The latter protects the producing cell against its cognate CdiA. Bioinformatic analyses 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.

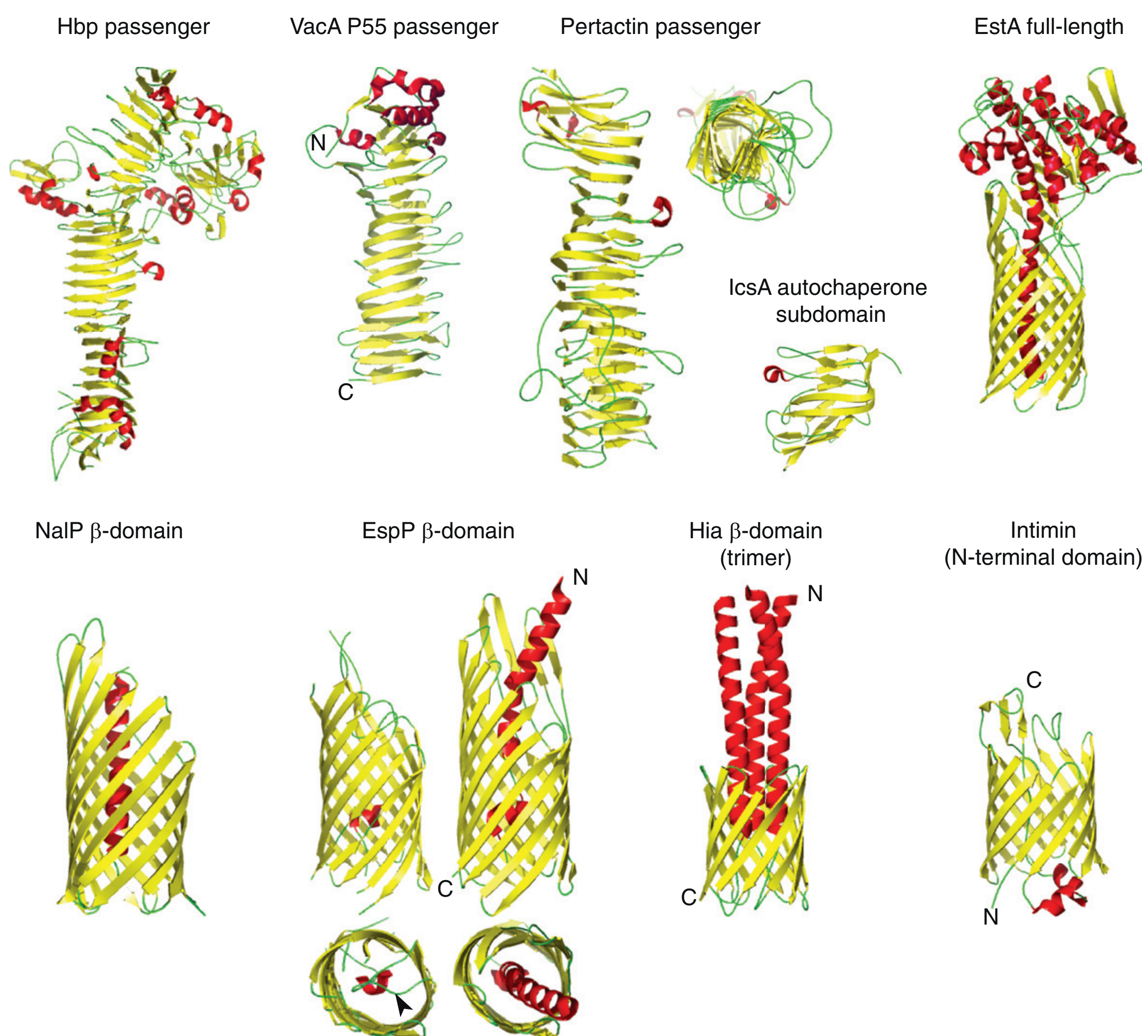

Fig. 2. Crystal structures of autotransporters. Cartoon representations are shown of (from top left to right bottom): the passenger of SPATE Hbp of *E. coli* (PDB code 1WXR) [142], the passenger P55 domain of toxin VacA of *H. pylori* (2QV3) [146], the passenger of the adhesin pertactin of *B. pertussis* (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).

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 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 *Ricketssia* [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

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. influenza* 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 a 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 *P. 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 extra-cellular 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

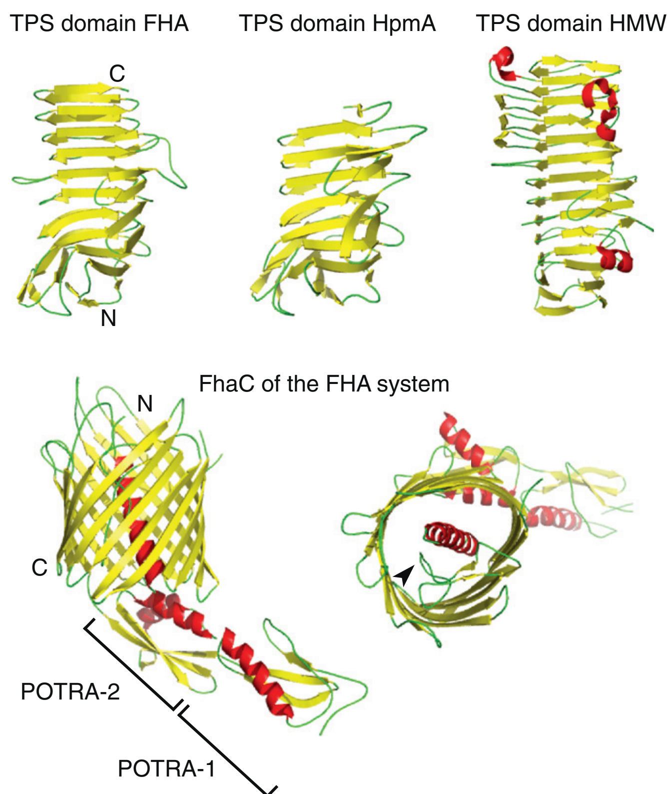

Fig. 3. Crystal structures of TPS systems. Shown are cartoon representations of TPS domains of FHA OF *B. pertussis* (1WXR) [164], HmpA of *P. mirabilis* (3FY3) [132] and HMW of *H. influenzae* (2ODL) [165], and the FhaC transporter of FHA (2QDZ) [171]. The TPS domains show a β-helical stem and a β-sheet attached to that stem (in HMW including an α-helix). Indicated are the two POTRA domains of FhaC which interact with the TPS domain in the periplasm. The top view shows that the channel of the sixteen-stranded β-barrel of FhaC is occupied by a α-helix that is located N-terminal of the POTRAs. Furthermore, loop 6 is a very long extracellular loop that folds inwards and traverses to the periplasm the channel (arrowhead).

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 [106,120,175–180]. 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 [(reviewed in Section 2)]. 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 [175–178,186,188] 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.

4.3. 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 applied [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 Section 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 *S. 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]. Simultaneous with the folding of the β-barrel folding, 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 thus facilitating interaction with chaperones such as Skp and preventing premature folding [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 [210–212]. 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-domains 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 [224–226]. 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 [201,228–230] 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 β-strands in the β-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 β-domain that bound efficiently to the Bam complex but showed impaired passenger translocation supported the notion that the β-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 β-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 a. *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 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 α-helix is already positioned inside a proto-form of the β-barrel and protected from external cleavage [196]. It is important to note that the β-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 β-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 [150,203,231,238–240]. In particular, mutation of the aromatic phenylalanine located at the C-terminal end of the β-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,

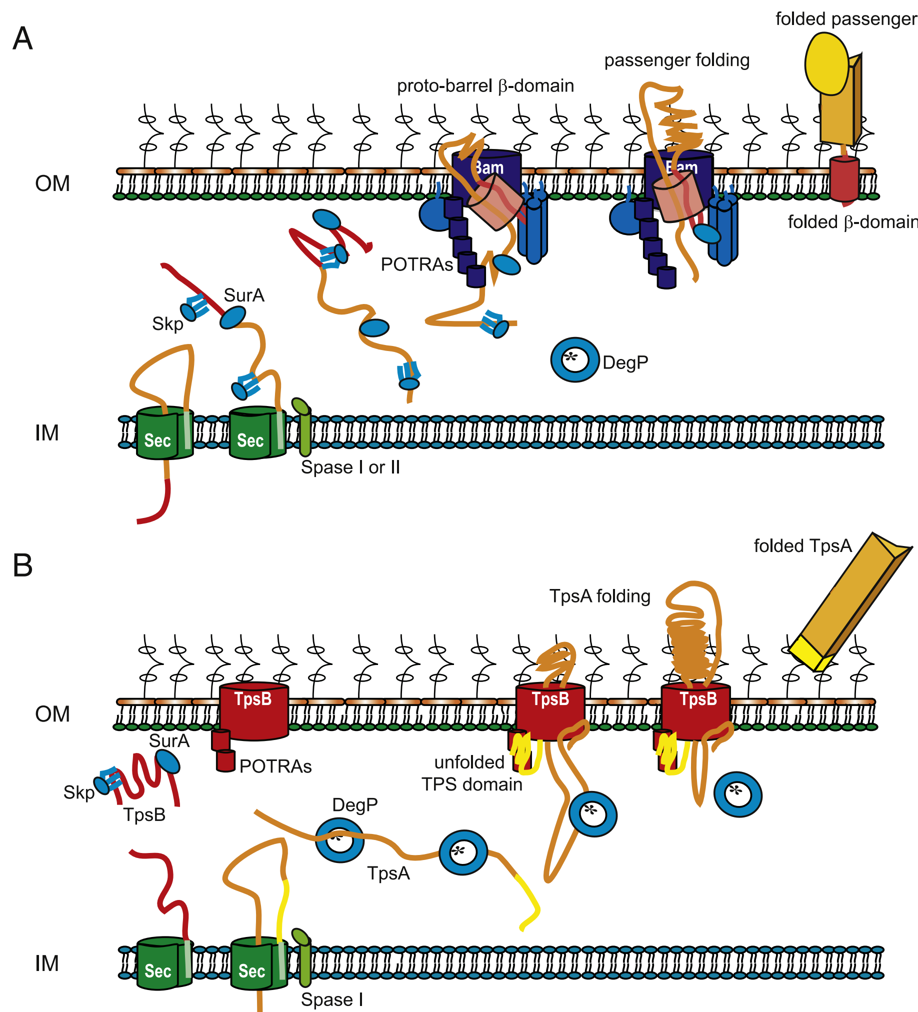

Fig. 4. Schematic models of autotransporter (A) and TPS (B) secretion across the inner (IM) and outer (OM) membranes of Gram-negative bacteria. (A) Model of autotransporter secretion. Autotransporters are transported to the periplasm by Sec in the inner membrane. The signal peptide (pale green) is cleaved by signal peptidase I or II (Spase) and the protein interacts with periplasmic chaperones Skp and SurA. The β-domain (red) interacts with the components of the Bam complex (blue) and folds into a proto-barrel structure that contains the C terminus of the passenger (yellow) in a hairpin configuration. This hairpin is detectable at the cell surface and from this hairpin the vectorial folding of the β-helical passenger proceeds, resulting in translocation of the passenger. During translocation the passenger interacts with components of the Bam complex and periplasmic chaperones while the β-domain remains in proto-barrel conformation and the Bam complex completes the folding and membrane insertion of the β-domain after translocation. (B) Model of TPS secretion. The TpsB (red) and TpsA (yellow) proteins are transported to the periplasm by Sec in the inner membrane. The signal peptides are cleaved and the proteins may interact with periplasmic chaperones, as shown for TpsA and DegP. The TpsB protein inserts into the outer membrane as a 16-stranded β-barrel with two POTRA domains that protrude into the periplasm. The N-terminal TPS domain (bright yellow) of the TpsA interacts with the POTRA domains in an unfolded form and also the C-terminal end of the TpsA interacts with the TpsB, while initially a hairpin of the TpsA protein extends through tpsB to the cell surface. From this hairpin the vectorial folding of the β-helical TpsA protein proceeds, resulting in its translocation to the cell surface.

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 α-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 [242–245]. 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 activiy 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 POTRAs 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 mileu 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

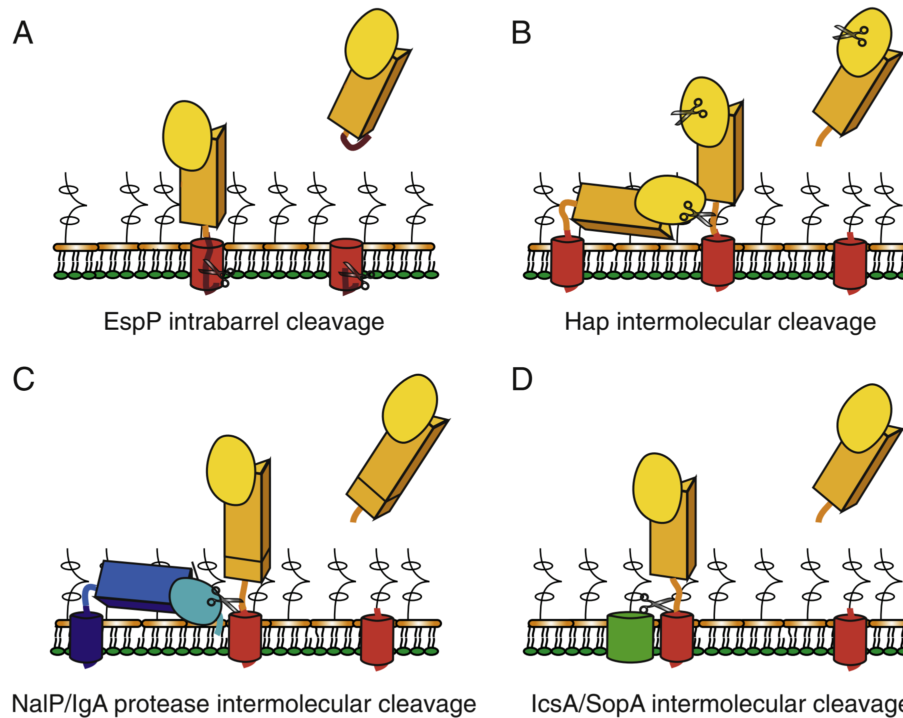

Fig. 5. Schematic models of the release of autotransporter passengers from the β-domain at the cell surface. Examples are mentioned below the panels. The catalytic protease site involved is depicted by the scissors. (A) Release via proteolytic cleavage in the β-domain as shown for SPATES and pertactin of *B. pertussis* [25]. A catalytic site in the β-barrel wall results in cleavage of a linker region once it is inserted into the β-barrel channel. The AIDA-I autotransporter of *E. coli* is also cleaved by a catalytic site in the β-domain, but this site is not located in the barrel wall, but at the cell surface [249]. (B) Release by the activity of the serine protease domain of the passenger, as shown for Hap of *H. influenzae* [250]. (C) Release by the activity of the serine protease domain of another autotransporter as shown for the release of the IgA protease passenger by NalP [14]. (D) Release by an outer membrane-based protease exemplified by the release of the passenger of IcsA of *S. flexneri* by SopA [251].

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 Section 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 [254–256] and it is this part that emerges first from the cell surface during translocation of the outer membrane [10].

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 [15,16,259]. 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 as well [260–263]. Similar to

Table 3
Examples of heterologous proteins fused to autotransporters.

Autotransporter	Heterologous protein	Function	Expression host	Display/ secretion	Application	Ref.
Fusions directly to autotransporter β-domains						
IgA protease	β-subunit of cholera toxin (CTB)	Host receptor binding	*Escherichia coli*, *Salmonella* Typhimurium	Display and secretion	Translocation studies	[218]
AIDA-I	β-subunit of cholera toxin (CTB)	Host receptor binding	*Escherichia coli*	Display	Translocation studies	[274]
IgA protease	Single chain Fv fragments (scFv)	Virus-neutralizing immunoglobulin (Ig) domains	*Escherichia coli*	Display	Viral intervention	[271,293]
AIDA-I	Adrenoxin (Adx)	Steroid biosynthesis	*Escherichia coli*	Display	Whole-cell biocatalysis	[294,295]
AIDA-I	P15 peptide of C-reactive protein	Screening cathepsin G inhibitors	*Escherichia coli*	Display	Library screening	[296]
IgA protease	Metallothionein	Heavy metal adsorption	*Cupriavidus metallidurans*	Display	Bioremediation	[297,298]
Fusions to (part of) the autotransporter passenger domains						
IcsA	β-lactamase (Bla)	Antibiotic hydrolysis	*Escherichia coli*	Display	Translocation studies	[275]
EstA	Lipases of several species	Esterolytic activity	*Escherichia coli*	Display	Whole-cell biocatalysis	[299]
Hbp	ESAT-6	*Mycobacterium tuberculosis* antigen	*Escherichia coli*, *Salmonella* Typhimurium	Display and secretion	Live vaccine development	[266]
Pet	ESAT-6 mCherry	*Mycobacterium tuberculosis* antigen Red fluorescent protein	*Escherichia coli*	Display and secretion	Live vaccine development, translocation studies	[267]

autotransporters, secretion of bulky passengers by intimins appears limited [262]. 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 [263]. 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 [259]). 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 [264–267] (see Table 3 for examples). A variety of fusion partners have been explored, ranging from small peptides to relatively large complete proteins [15,16,259]. 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 [15,16,259]. 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 [268]. 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 [266].

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 [219,267–270] (Table 3), similar constructs failed to be secreted in other studies [209,218,271–273]. 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 [10,209,218,267,271]. In addition, co-expression of periplasmic chaperones might help to maintain the translocation competent state of fusion constructs during periplasmic transit [16,264].

9.4. Selecting an autotransporter secretion system

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 [15,16,259]. 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 [274]. 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 [272].

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 [15,16,259]. In a comparative study, a range of heterologous proteins were fused to either the complete EstA autotransporter of *P. stutzeri* or its β-domain [272], 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 [275].

A slightly different strategy has been applied to introduce recombinant proteins in the passenger of the SPATEs Hbp and Pet [16,266,267]. 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 [266]. 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.

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Competing interests

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

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