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Mini Review

Structures and functions of autotransporter proteins in microbial pathogens

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ABSTRACT

Since their discovery more than 20 years ago the autotransporter protein superfamily has been growing continuously and currently represents the largest protein family in (pathogenic) Gram-negative bacteria. Autotransporter proteins (AT) adhere to a common structural principle and are composed of a C-terminal β-barrel-shaped 'translocator' domain and an N-terminal 'passenger' domain. The translocator is anchored in the outer membrane and is indispensable for the N-terminal passenger part to traverse the outer membrane. Most if not all AT harbor a chaperone segment that increases protein stability and may be located in the passenger or translocator domain. The passenger mediates the specific virulence function(s) of the particular AT. Accordingly, passenger domains of AT can be quite variable. Interestingly, AT have been identified as the first glycosylated proteins in Gram-negative bacteria. Despite the considerable efforts invested in the characterization of autotransporter biogenesis, various aspects such as the participation of accessory proteins, the fate of the translocator, or the translocation of glycosylated proteins still remain only poorly understood. In addition, recent evidence indicates that the prefix 'auto' might be slightly exaggerated. Here, we will selectively discuss novel insights at various stages of AT biogenesis.

Autotransporter proteins

Secretion systems in Gram-negative bacteria are numbered for convenience or lack of more creative terms type I–VII (Salmond and Reeves, 1993; Henderson et al., 2000, 2004; Economou et al., 2006; Van Ulsen and Tommassen, 2006). However, even the – at first glance – simplest protein secretion system V consists of several subtypes such as the classic autotransporter system (type Va or AT-1), the two-partner secretion system (Vb), and the Vc system (AT-2). At present, autotransporter proteins (AT) represent the largest protein family in pathogenic Gram-negative bacteria with more than 1000 identified family members (Kajava and Steven, 2006) and have been identified by sequence comparison in virtually all pathogenic Gram-negative bacteria. Although proteins secreted via the autotransporter pathways are similar in their overall organization and primary structure (Fig. 1) (Henderson et al., 2004), they are very heterogeneous concerning their specific functions. All classical AT share a common sequence organization: an often extended signal peptide followed by an N-terminal passenger domain (α-domain) ranging in size from less than 20 to more than 400 kDa and a C-terminal translocator domain (β-domain) of approximately 30 kDa. The functions of the passenger domains can be quite diverse and have frequently been associated with the pathogenesis of the specific bacterium. The C-terminal domain is essential for the translocation of the passenger domain to the bacterial surface and, therefore, translocator domains of different autotransporters share common features. During the process of translocation the passenger protein might be (autocatalytically) cleaved and secreted or might remain covalently bound to the translocator. In some cases, such as the 'adhesin-involved-in-diffuse-adherence (AIDA)' AT, the passenger is cleaved but remains non-covalently associated with the bacterial surface (Suhr et al., 1996; Charbonneau et al., 2009).

The role model for AT secretion is the IgA protease of *Neisseria gonorrhoeae* (Koomey et al., 1982; Halter et al., 1984). Pohlner et al. (1987) proposed a very elegant model of outer membrane (OM) translocation without a requirement for energy coupling or accessory factors. Hence proteins following this pathway of secretion were denoted 'autotransporter'. Since the introduction of this model, literally hundreds of further examples have been reported that share overall similarities in structural organization and mode of translocation across the OM. For a more general description of AT, please refer to recent excellent reviews (Henderson et al., 2004; Desvaux et al., 2004; Wells et al., 2007, 2010; Dautin and Bernstein, 2007; Yen et al., 2008).

In addition to classic autotransporter systems following as single-chain proteins the IgA protease model (such as AIDA-I, TibA, Ag43, BrkA, IcsA, NalP, and the group of SPATEs; see Table 1), trimeric autotransporter adhesins (TAA, AT-2, Vb) and two-partner secretion systems (TPSS, Vc) have been included also in the auto-

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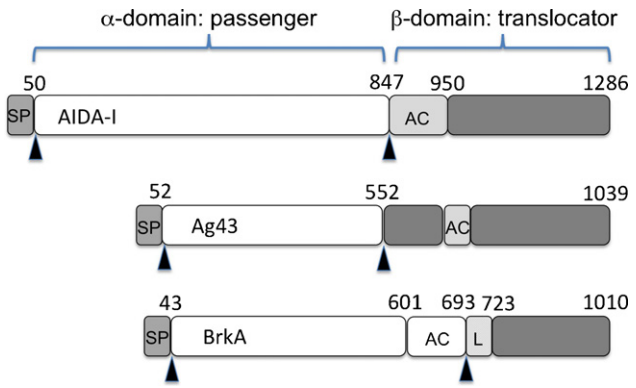

Fig. 1. Structural domains of processed classical autotransporter proteins of *E. coli* (AIDA-I, Ag43) and *B. pertussis* (BrkA). SP: signal peptide; AC: autochaperone domain essential for stability and folding; L: linker domain; arrowheads: processing sites.

transporter family. However, in this review TAA and TPSS will be touched only very briefly.

Trimeric autotransporters

Trimeric autotransporters (AT-2, Vb) are synthesized just like classic autotransporter molecules via a precursor molecule harboring domains representing passenger and translocator functions (for review see: Cotter et al., 2005; Linke et al., 2006). In AT-2 the domain resembling a translocator is rather short and consists of only 70–76 amino acids (Surana et al., 2004). As a consequence, it is not functional as a monomer. However, as a trimer where each AT pseudo-translocator domain donates four β-sheets, the minimum number of 12 β-sheets to constitute a β-barrel can be assembled for a functional translocator. Thus far the specific mechanism how three passengers might be translocated through a single 12 β-sheet barrel has remained elusive. Examples of trimeric autotransporters are YadA of *Yersinia* spp. (e.g. Heesemann et al., 2006; Skurnik, 2007), Hia (e.g. Meng et al., 2008) or HadA of *Haemophilus influenzae* (Serruto et al., 2009)

Two-partner secretion systems

In the two-partner secretion system (TPSS, Vc), the translocator and passenger functions are contributed by two separate proteins, which are encoded by structural genes frequently located in an operon (Mazar and Cotter, 2007). Fha (e.g. Delattre et al., 2010) and HMW1 (e.g. St Geme et al., 2009) belong to this autotransporter subgroup. Both proteins are transported through the inner membrane in a Sec-dependent way. The translocator consists of an N-terminal periplasmic region and a C-terminal β-barrel that is integrated in the OM (Clantin et al., 2007; Kim et al., 2007; Tommassen, 2007). The periplasmic part carries a polypeptide-transport-associated domain (POTRA), a sequence for interaction with passenger molecules. The passenger carries a corresponding TPSS domain for recognition of the translocator and for initiation of secretion. It appears that the TPSS might serve as a model for protein translocation across membranes giving insights into the function of distantly related eukaryotic proteins found in mitochondria and chloroplasts (Jacob-Dubuisson et al., 2009). Passengers transported via TPSS are as diverse as classical autotransporters. For more detailed information see Mazar and Cotter (2007) and St Geme et al. (2009).

Structural organization and biogenesis of classical autotransporter proteins

Signal peptide and transport through the inner membrane

The signal peptide of most AT consists of about ~20–30 residues to be cleaved by the signal peptidase subsequent to Sec-dependent translocation. Nevertheless, sequence homologies are not very high among AT signal peptides (Dautin and Bernstein, 2007). About 10% of the known AT show an unusual long signal peptide (e.g. SPATE, IcsA, Hbp, AIDA-I) of at least 40 residues that can be organized in five different domains. These domains are based on hydrophobic and charged residue distribution (N1, H1, N2, H2) and are followed by the C-(cleavage) region (Henderson et al., 2004). More recent studies propose a dual-domain organization (Hiss and Schneider, 2009). The C-terminal half of the signal peptide corresponds to a classic signal peptide and appears not to be particularly conserved while the N-terminal extension is conserved. Transport of the precursor proteins through the cytoplasmic membrane is Sec dependent for IcsA (Brandon et al., 2003), while for Hbp the signal-recognition particle (SRP) and SecB pathway is used (Sijbrandi et al., 2003). Recent evidence suggests that besides the requirements for a functional SRP pathway and the Sec translocon, at least for the AT examples EspC and Hbp, the signal peptide interacts with YidC early during biogenesis. YidC is in part associated with the SecYEG machinery acting downstream to facilitate lateral transfer (Jong

Table 1
Exemplary functions of autotransporter passenger domains in pathogenic bacteria.

Protein	Species	Function	Reference
Classical autotransporter proteins			
Ag43, antigen 43	*Escherichia coli*	Autoagglutinin, biofilm formation	Sherlock et al. (2006)
AIDA, adhesin involved in diffuse adherence	Enteropathogenic *E. coli*	Adhesin	Benz and Schmidt (1992)
BrkA	*Bordetella pertussis*	Serum resistance	Zhao et al. (2009)
Hbp, hemoglobin protease	Avian pathogenic *E. coli*	Heme binding protein	Otto et al. (2005)
IcsA	*Shigella flexneri*	Intercellular spread	Brandon and Goldberg (2001)
IgA protease	*Neisseria*	Immunoglobulin protease	Pohlner et al. (1987)
Pertactin	*B. pertussis*	Adhesin	Emsley et al. (1996)
SPATE, serine protease autotransporters of *Enterobacteriaceae*	*Enterobacteriaceae*	Protease	Yen et al. (2008)
SSP	*Serratia marcescens*	Protease	Shikata et al. (1993)
TibA	Enterotoxigenic *E. coli*	Adhesin/invasin	Lindenthal and Elsinghorst (1999)
VacA	*Helicobacter pylori*	Cytotoxin	Gangwer et al. (2007)
Trimeric autotransporter adhesins (TAA)			
Hia	*Haemophilus influenzae*	Adhesin	Yeo et al. (2004)
YadA	*Yersinia enterocolitica*	Serum resistance, adhesin	Nummelin et al. (2004)
Two-partner secretion systems (TPSS)			
Fha, filamentous hemagglutinin	*B. pertussis*	Hemagglutinin	Clantin et al. (2004)
HMW1, high molecular weight adhesin	*H. influenzae*	Adhesin	Grass et al. (2003)
ShlB	*S. marcescens*	Cytotoxin	Ondraczek et al. (1992)

et al., 2010). In the case of Hbp loss of YidC results in secretion-incompetent intermediates, which are degraded by the periplasmic protease DegP. These findings might further support earlier studies indicating that – although the N-terminal extension does apparently not play a critical role during transport (Jong and Luirink, 2008) – it is involved in preventing misfolding of the passenger domain in the periplasm by transiently tethering it to the inner membrane (IM) (Szabady et al., 2005). The transport of Pet and EspP (SPATEs) through the IM proceeds in a post-translational manner where the interaction with a cytoplasmic or inner-membrane co-factor prior to protein translocation has been proposed (Desvaux et al., 2007; Peterson et al., 2006). However, recent results suggest that at least in the case of the *Escherichia coli* AT Pet the extended signal sequence is not required for successful secretion and function (Leyton et al., 2010).

Interestingly, IcsA localizes to the 'old' poles of the bacterium and it appears that the targeting occurs already in the cytoplasm. The secretion through the membrane occurs at the pole by the *sec* apparatus but the localization is not SecA dependent. It has been hypothesized that the extension of the signal peptide interacts with a 'polar target' (Brandon et al., 2003). Polar localization was also reported for other autotransporters (AIDA-I, BrkA) in the presence of complete lipopolysaccharide (Jain et al., 2006).

Autotransporter passenger domains: structure and function

The AT passenger domains vary widely in length and sequence exhibiting quite diverse functions. Although no sequence motif common to all known AT passenger domains could be identified, thus far all available structural data and in-silico predictions agree that most AT passenger domains represent variations of a rather limited structural blueprint (Dautin and Bernstein, 2007; Nishimura et al., 2010). About 97% of the passenger domains are predicted to contain an extended right-handed β-helical structure (Junker et al., 2006; Kajava and Steven, 2006). For pertactin (Emsley et al., 1996), Hbp (Otto et al., 2005), VacA (Gangwer et al., 2007), Fha (Clantin et al., 2004), BrkA (Zhao et al., 2009), and *Haemophilus influenzae* IgA1 protease (Johnson et al., 2009) the predicted structure has been verified by X-ray crystallography. In pertactin, the passenger forms a 16-turn parallel β-helix with a V-shaped cross-section and a hydrophobic core. Per turn there are ~25 residues constituting three β-strands that are linked by loops. Comparative sequence analyses have suggested these extended β-helical structures for most classical autotransporter passengers (Kajava and Steven, 2006). It appears that the usually quite rare superhelical or β solenoid-like proteins (Kajava, 2001) are most prevalent in AT passenger structures (Dautin and Bernstein, 2007; Nishimura et al., 2010). AT seem to use these β-helical scaffolds for the functional attachment of various decorations such as glycosylation (see below), loops and other domains important for the specific AT function. Interestingly, the just recently reported structure of the *Pseudomonas aeruginosa* esterase AT EstA exhibits a completely different structure with a globular fold that is dominated by α-helices and loops. It has been suggested that the arrangement of secondary structural elements indicates sequential folding of the AT passenger, thereby providing the driving force for passenger translocation (Van den Berg, 2010). Examples for various functions of the passenger domains are listed in Table 1.

The specific structure(s) of the passenger domains of AT might harbor a solution to the 'energy problem' As there is neither a significant concentration of ATP in the periplasm nor a proton gradient across the OM, the energy to drive the secretion of the AT across the OM must be supplied by other means (Thanassi et al., 2005). For pertactin, its folding behavior has been analyzed in detail. Pertactin exhibits an unexpectedly slow folding in the isolated state which might help to prevent folding in vivo before secretion through the OM (Junker et al., 2006). This study also revealed a stable core structure encompassing the C-terminal half of the passenger termed 'autochaperone', which was not quite obvious from examining the crystal structure. In most AT the autochaperone has a β-helical structure while in VacA there is additional α-helices (Gangwer et al., 2007). For Hbp aromatic residues in the autochaperone domain are essential for the passenger translocation (Soprova et al., 2010). It has been suggested that vectorial folding of the β-helical structure might contribute to the energy-independent translocation mechanism. A similar stable core structure was identified by folding studies in the Pet AT of *E. coli* (Renn and Clark, 2008) suggesting vectorial folding to be a common trait of AT. This hypothesis was recently tested for the EspP AT (Peterson et al., 2010). This study demonstrated that efficient secretion of the passenger domain of the *E. coli* O157:H7 AT EspP requires the stable folding of the 17-kDa C-terminal autochaperone fragment of the passenger protein. Surprisingly, mutants impaired in the folding of this fragment are not blocked in OM secretion but can no longer translocate the N-terminal passenger domain. Interestingly, experiments with kinetic folding mutants suggest that the 17-kDa C-terminal fragment might be folding extracellularly. Hence, these findings strongly suggest, that OM translocation of AT passengers might be driven by extracellular protein folding.

The autotransporter translocator

The C-terminal domain of AT proteins has been termed β-domain or translocator as this domain is indispensable for OM translocation of the AT passenger (Pohlner et al., 1987; Benz and Schmidt, 1992; Suhr et al., 1996). In contrast to the extensive sequence and length variations found in passenger domains, β-domains are very similar in size belonging to the same protein family that can be identified by search algorithms in the database (PFAM03797) (Dautin and Bernstein, 2007; Yen and Stathopoulos, 2007; Wells et al., 2010). Early on, in-silico predictions suggested a β-barrel structure for the AT translocator domains. Despite rather low sequence similarities all translocators have a high content of β-sheets that are able to form a β-barrel. The β-barrel is build up of at least 12 β-strands as e.g. in the case of EspP. This has been confirmed by the analyses of the crystal structures of currently altogether four translocator domains. The first structure to be elucidated was the *Neisseria meningitidis* NalP translocator in 2004, which even demonstrates the passage of an N-terminal α-helix through the β-barrel hydrophilic pore (Oomen et al., 2004). A homologous structure was identified in *H. influenzae* trimeric Hia which forms a β-barrel with a 1.8-nm diameter hydrophilic pore that is traversed by three N-terminal α-helices that are essential for the stability of the β-barrel (Meng et al., 2006). Likewise, the translocator of *E. coli* EspP exhibits a β-barrel structure traversed by an α-helix. After release of the passenger a small part of this helix remains in the pore, interacting with specific amino acids of the β-barrel. These hydrogen bonds apparently increase the stability of the β-barrel (Barnard et al., 2007; Tian and Bernstein, 2010). Just recently the crystal structure of *Pseudomonas aeruginosa* EstA has been reported – again displaying the traversal of an α-helical strand through the pore of its β-barrel translocator (Van den Berg, 2010). Autotransporter β-domains (translocators) are apparently highly conserved among classical and trimeric AT. Furthermore, despite the lack of pronounced overall sequence homologies there is remarkable homology among the β-domain sequences in AT functional subfamilies. The close evolutionary link between passengers and their specific translocators is further underlined by the emerging functional role of translocators in passenger protein processing and folding (Barnard et al., 2007; Dautin and Bernstein, 2007; Dautin et al., 2007; Yen et al., 2010).

Crossing the periplasmic space

Subsequent to the translocation through the IM via the Sec system, a rather surprising role has been implicated for the extended signal peptides (SP). Due to a relatively slow dissociation from the Sec complex, cleavage by signal peptidases proceeds although the extended SP appear to be involved in maintaining the passenger domain in an OM translocation-compatible conformation. This could be achieved by transiently tethering the passenger to the IM thereby preventing folding of the passenger into a translocation-incompetent conformation (Dautin and Bernstein, 2007). Extended SP have been identified in all SPATE (serine protease autotransporter of *Enterobacteriaceae*) family members as well as in the AIDA-I/Ag43/TibA *E. coli* adhesins. Interestingly, some passenger proteins appear to get by with a certain degree of periplasmic folding such as IcsA of *Shigella flexneri* (Brandon and Goldberg, 2001) that just like an N-terminal CtxB-ΔEspP fusion in *E. coli* (Skillman et al., 2005) also forms disulfide bonds while traversing the periplasm. This possibility has been successfully tested with fusion proteins harboring heterologous passenger proteins such as single-chain Fv fragments (scFv) or variable monodomains derived from camel antibodies (V_{HH}) and the IgA protease translocator domain (Veiga et al., 2004). Furthermore, it has been shown that the β-barrel is at least partially folded in the periplasm and that a polypeptide segment is already incorporated in the β-barrel pore before the passenger is secreted (Ieva et al., 2008).

There has been a long debate whether in addition to the unusually long SP also periplasmic chaperones might play a role in the secretion of AT passengers. Several laboratories could show that the chaperones DegP, Skp, SurA, and DnaK play differential roles in permissive folding of AT passengers, but are apparently not involved in the insertion of the β-domain in the OM (Purdy et al., 2007; Janakiraman et al., 2009; Wagner et al., 2009; Ruiz-Perez et al., 2009). These findings have cast some doubt on whether the secretion process of AT really proceeds autonomously.

Translocation across the outer membrane

Since the discovery of the first autotransporter protein more than 20 years ago (Pohlner et al., 1987) the mechanism of translocation through the OM has remained a subject of intense debate. All currently available crystal structure data support the integration of the translocator as a β-barrel in the OM (see above). As in some translocator β-barrel structures an α-helical part is threaded through the hydrophilic pore this has been taken as support for the so-called 'hairpin' model (Fig. 2). According to this model the passenger domain traverses through the pore of the translocator subsequent to the insertion of the β-barrel in the OM – either directly or via the prior formation of a hairpin. However, N-terminally truncated passengers and chimeric passengers are also translocated across the OM, which indicates that there are no sequence-specific translocation signals targeting a passenger to its cognate β-barrel. Interestingly, it has been found that YaeT (Omp85) is required for the secretion of the *S. flexneri* AT IcsA/VirG and SepA (Jain and Goldberg, 2007). Furthermore, depletion of YaeT (Omp85) results in DegP-dependent instability of unassembled IcsA, lack of integration of the translocator domain in the OM, and lack of surface exposure. Analogous results have been obtained for AIDA-I of *E. coli* and BrkA of *Bordetella pertussis* in a YaeT-minus background. This Omp85 ortholog is necessary for the membrane integration of quite a few β-barrel-structured OM proteins such as e.g. LamB, OmpA, TolC, OmpF/C.

Integration of the β-barrel in the OM should therefore occur in a concerted mechanism that probably involves the participation of additional proteins such as Omp85/YaeT. The β-barrel assembly

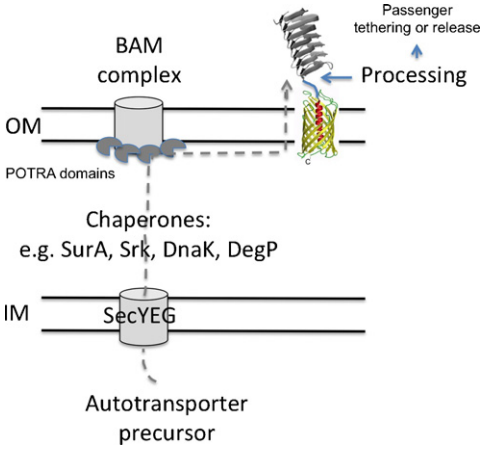

Fig. 2. Scheme of autotransporter translocation across the cell wall of Gram-negative bacteria.

machinery (BAM) appears to be at the core of this complex as almost all known OM proteins require the BAM complex for folding (Wu et al., 2005; Knowles et al., 2009, 2011; Bennion et al., 2010). The only known exception so far is the secretin PulD that inserts into the OM without participation of the BAM complex (Collin et al., 2007).

Recent evidence suggests that the BAM complex might be involved in autotransporter secretion beyond its role in the OM insertion of the β-barrel translocator. Photocrosslinking studies utilizing a translocation intermediate of EspP indicated that distinct subunits of the BAM complex interact with and facilitate the translocation of passenger domains. It was found that several residues interacted with the BAM subunit BamA and that residues closer to the N-terminal part interact with the chaperones SurA and Skp. However, these interactions were found to be short-lived and only detectable during stalled translocation. Based on these findings Ieva and Bernstein (2009) suggested that molecular chaperones such as SurA and Skp prevent periplasmic misfolding of the passenger domain and that the BAM complex catalyzes not only the folding and insertion of the β-barrel by its POTRA domains but also facilitates the translocation of the passenger domain across the OM. Similar results have been obtained for Hbp (Sauri et al., 2009). Furthermore, yeast two-hybrid experiments showed a direct interaction between BamA and YadA of *Yersinia enterocolitica* and also in this case the C-terminal nine residues were found to be essential for stability and the BamA interactions (Lehr et al., 2010). Interestingly, there are many structural and functional relationships between the mitochondrial OM TOB complex and the bacterial BAM as these can be substituted for one another (Walther et al., 2009; Endo et al., 2011). This functional comparability indicates an evolutionary relationship.

These novel findings greatly enhanced our insight into the insertion pathway of OM proteins including the β-barrel translocator of AT. Nevertheless, the detailed molecular mechanism of traversal of the passenger domain across the OM remains enigmatic. There appears to be general consensus that for most single-chain autotransporter passengers translocation proceeds through the pore of its own cognate β-barrel. This model is supported by the structural data available for translocators of monomeric and trimeric AT. In an alternative model proposed for the IgA protease of *N. gonorrhoea* by Veiga and coworkers translocation should occur through the inner pore formed by a hexameric complex of six single β-barrels when expressed in *E. coli* (Veiga et al., 2002). However, more recent experiments clearly favor the translocation of the autotransporter passenger domain through the pore of the monomeric translocator, most probably following a hairpin model as has been proposed for

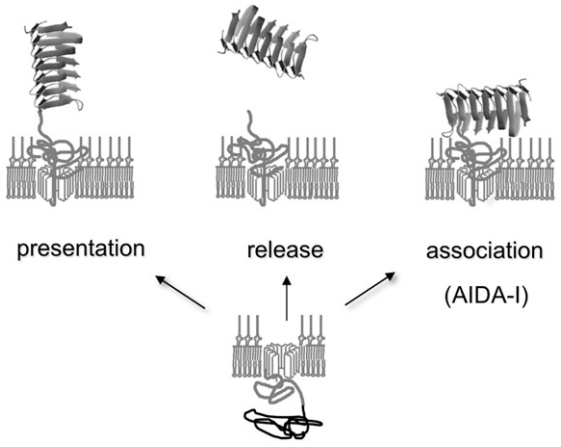

Fig. 3. Scheme of various options in extracellular presentation and/or release via autotransporter translocators.

NalP. Due to the pore size of ~10 Å × 12 Å the translocating protein should be mostly unfolded (Oomen et al., 2004).

For pertactin of *B. pertussis* it has been shown that the C-terminal part of the α-domain crosses the OM first (Junker et al., 2009). Independent of the complete translocation of the α-domain the passenger domain begins to fold at the surface of the bacterium. In the case of *E. coli* Hbp, for which the complete structure of the autotransporter passenger domain is known, it has been shown that Hbp can fold in the periplasm but must retain a certain degree of flexibility and/or modest width to allow translocation across the OM (Jong et al., 2007). In this process, the C-terminus of the passenger – the so-called autochaperone domain – appears to play an important role as mutations in this region affect secretion and processive folding of the β-helix at the cell surface (Soprova et al., 2010). In AIDA-I an analogous domain with stabilizing functions for the β-barrel has been identified at the N-terminus of the β-domain (β_1-domain) (Konieczny et al., 2001).

Currently available evidence suggests a basic summary of autotransporter biogenesis: after traversal of the IM via the Sec complex ATs are stabilized by various periplasmic chaperones and appear to be passed along to and by POTRA domains of the BAM complex subunits which in turn facilitate insertion of the C-terminal β-barrel translocator in the OM. Subsequently, the passenger domain utilizes the pore of the β-barrel for traversal of the OM (Fig. 2).

Passenger processing

Following their traversal across the OM passenger proteins either remain covalently bound to the translocator (e.g. TibA: Lindenthal and Elsinghorst, 1999), others are proteolytically processed, however, remain tethered to the bacterial surface (e.g. AIDA-I: Suhr et al., 1996), or are released to the environment (e.g. IgA1 protease: Pohlner et al., 1987; SPATE: Dautin et al., 2007) (Fig. 3).

Some AT with protease activity in the passenger domain are able to perform their own processing and are autoproteolytically cleaved. This is the case for Hap (*H. influenzae*), SphB1 (*B. pertussis*), NalP, App and IgA protease (all *N. meningitidis*) and AIDA-I (*E. coli*) (Suhr et al., 1996; Charbonneau et al., 2009). The serine protease activity of NalP is additionally involved in partial processing of IgA protease, App, AusI, and MspA of *N. meningitidis* (Van Ulsen et al., 2003, 2006; Turner et al., 2006). Cleavage occurs either extracellularly (AIDA-I) resulting in the retention of a small fragment of the passenger domain on the cell surface, or inside the β-barrel (e.g. EspP) (Dautin et al., 2007).

Interestingly, EspP – a member of the SPATE family – is not processed by the catalytic domain of the passenger domain but by an additional proteolytic centre in the translocator domain. In the catalytic reaction an aspartate and a conserved asparagine residue are involved and form an unusual catalytic dyad that mediates self-cleavage through the cyclization of the asparagine (Dautin et al., 2007). Because of their high sequence homology it is likely that other members of the SPATE family are proteolytically processed by the same or a similar mechanism. Likewise, autocatalytic processing has been shown for pertactin, BrkA of *B. pertussis*, and Hbp of *E. coli* (Tajima et al., 2010). From X-ray crystallography of the post-cleavage state of the β-domain of EspP we know that the processing occurs halfway in the lumen of the β-barrel. After proteolytic cleavage 15 amino acids are still inserted in the lumen of the barrel from the periplasmic site. Following proteolytic cleavage of the passenger from the translocator, a conformational change in the translocator occurs. There is a repositioning of the remaining α-helical domain (perpendicular to the barrel axis) leading to an increase in the stability of the barrel. In a second conformational change an extracellular loop of the β-barrel is folded into the lumen of the barrel pore, closing the pore from the extracellular site (Barnard et al., 2007). These data support a model in which a single β-barrel facilitates the translocation of a single passenger domain to the extracellular surface. Hence, an oligomeric translocation model can be excluded.

Processing of AIDA-I is independent of the *E. coli* proteases DegP, OmpT, and OmpP, takes place also in *Shigella* and *Salmonella*, and was therefore proposed to occur in an autocatalytic way (Suhr et al., 1996). Recently, Charbonneau et al. (2009) identified several amino acid residues in the junction region of the autotransporter that were shown to be essential for intramolecular processing.

Following cleavage and release of the passenger proteins the translocator β-barrels remain in the OM. Therefore, the observed conformational changes and the ~30 residues remaining in the translocator pore after complete translocation and processing might serve an additional purpose by plugging the translocator channel (Konieczny et al., 2001; Oomen et al., 2004; Skillman et al., 2005). Thus far, the fate of the translocator in the OM following cleavage of the passenger is not known.

Glycosylation of autotransporter passenger proteins

The identification of glycosylated proteins in bacteria and particularly in *E. coli* around ten years ago came as a big surprise (Lindenthal and Elsinghorst, 1999; Benz and Schmidt, 2001, 2002; Schmidt et al., 2003; Zhou and Wu, 2009). The TibA autotransporter adhesion/invasion protein derived from enterotoxigenic *E. coli* (ETEC) strain H10407 has been suggested to be glycosylated on the basis of a general carbohydrate detection reaction (Lindenthal and Elsinghorst, 1999). The first autotransporter protein and in fact the first *E. coli* protein truly identified to be glycosylated in molecular terms is AIDA-I (Benz and Schmidt, 2001). AIDA-I is modified by AAH (autotransporter adhesin heptosyl transferase) by adding heptose moieties to serine residues of the AIDA-I passenger protein. AAH uses ADP-glycero-manno-heptose as activated substrate that is apparently pirated from the LPS biosynthesis pathway. Glycosylation occurs already in the cytoplasm (Benz and Schmidt, 2001; Charbonneau et al., 2007) and has been shown to be required for stability but not for the adherence function of the protein. The ETEC AT TibA is modified by the TibC protein. It has been proposed that TibA is also modified by the addition of heptose moieties because of the sequence homology between AAH and TibC and the fact that AIDA-I can by functionally modified by TibC (Moormann et al., 2002). The autoagglutinin Ag43 can also be glycosylated by AAH and TibC (Sherlock et al., 2006; Reidl et al., 2009). Due to glycosylation there is an increase in stability of the protein and reduced

cellular aggregation (Knudsen et al., 2008). However, in most *E. coli strains* there seems to be no specific heptosyltransferase mediating the glycosylation of Ag43 with the exception of the uropathogenic strain 536 (Sherlock et al., 2006).

A different type of glycosylation was found for the passenger domain of HMW1. The adhesin HMW1 of *H. influenzae* is part of a TPSS where for the synthesis of a functional adhesin not only the translocator HMW1B is necessary but also the putative glycosyltransferase HMW1C. Glycosylated HMW1 is more resistant against degradation than the non-glycosylated form, and glycosylation seems to be required for optimal tethering of HMW1 to the bacterial surface (Grass et al., 2003). Recently, it could be shown that asparagine residues are the acceptor sites for the carbohydrate moieties. Mono- or di-hexoses are connected to sites identical to the conventional consensus sequence of N-linked glycans (Gross et al., 2008). In summary, glycosylation of autotransporter proteins appears to play a role in protein stability and folding. As glycosylation proceeds in the cytoplasm these modifications add another level of complexity to the traversal of AT across the OM that has remained unresolved.

Autotransporter-mediated surface presentation

The large size of most autotransporter passenger proteins and the possibility to express also heterologous proteins functionally on the bacterial surface generated tremendous interest to employ AT for applied purposes. In most examples of AT utilized for surface presentation, extracellular production of proteins, or surface (auto)display the authentic passenger domains have been replaced by the particular molecule of interest. Specifically the AIDA-I and IgA protease systems have been shown to be able to accommodate a wide variety of heterologous passenger proteins (e.g. Konieczny et al., 2000; Rutherford and Mourez, 2006; Jose, 2006; Jose and Meyer, 2007; Buddenborg et al., 2008; Wouter et al., 2010; Van Bloois et al., 2010). Due to its versatility and for being an authentic *E. coli* autotransporter, for many laboratories AIDA-I became the system of choice for expression of functional passengers in an *E. coli* background. Peptides and proteins that have been surface-expressed via AT translocator constructs are functionally very divergent and include various enzymes (e.g. Li et al., 2008; Kaeßler et al., 2011), immunogenic and functional T cell epitopes (e.g. Konieczny et al., 2000; Westendorf et al., 2005; Buddenborg et al., 2008; Petermann et al., 2010), and bacterial toxin subunits (e.g. Maurer et al., 1997; Konieczny et al., 2000). In these examples the cognate SP of the authentic passenger protein had been mostly exchanged for either the SP of the specific passenger or for an unrelated Sec-targeting sequence. In light of the recent advances in our understanding of the molecular secretion pathway and the participating compounds, however, it might be advisable to maintain the authentic passenger SP.

Conclusion

Among the increasing number of secretion systems in Gram-negative bacteria the autotransporter pathway still maintains its charm as the most versatile and also still most simple system. However, it is already quite obvious that autotransporter-mediated secretion pathways are apparently not as straightforward as they had appeared previously and thus the term 'autotransporter' might be slightly exaggerated. Further elucidation of the molecular mechanisms guiding the AT emerging from the Sec system of the IM across the periplasm and finally into and across the OM will certainly help in optimizing AT systems for the extracellular presentation of heterologous passengers. One possible option might be to increase the expression of the BAM complex to subsequently also enhance the incorporation of β-barrels in the OM. However, one should not forget that AT systems are stoichiometric and not catalytic so there will be a limit of the number of β-barrels able to incorporate in the OM.

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