Abstract: Pathogenic gram-negative bacteria have evolved several secretion mechanisms to translocate adhesins, enzymes, toxins, and other virulence factors across the inner and outer membranes. Currently, eight different secretion systems, type I–type VIII (T1SS–T8SS) plus the chaperone-usher (CU) pathway, have been identified, which act in one-step or two-step mechanisms to traverse both membrane barriers. The type V secretion system (T5SS) is dependent first on the Sec translocon within the inner membrane. The periplasmic intermediates are then secreted through aqueous pores formed by β-barrels in the outer membrane. Until now, transport across the outer membrane has not been understood on a molecular level. With respect to special characteristics revealed by crystal structure analysis, bioinformatic and biochemical data, five subgroups of T5SS were defined. Here, we compare the transport moieties of members of four subgroups based on X-ray crystal structures. For the fifth subgroup, which was identified only recently, no structures have thus far been reported. We also discuss different models for the translocation process across the outer membrane with respect to recent findings.

Keywords: alternative model; gram-negative; hairpin model; Omp85 model; type V secretion systems.

Introduction

The secretion of proteins is crucial not only for pathogenic gram-negative bacteria, but also a wide variety of enzymes are secreted for utilization of exogenous nutrients. During evolution, different secretion mechanisms developed in order to traverse the inner and outer membrane barriers; they include the type I–VIII secretion systems (T1SS–T8SS) and the chaperone-usher (CU) pathway (Desvaux et al., 2009). Among these, T1SS, T3SS, T4SS, T6SS, and T7SS are composed of protein complexes spanning both the inner and the outer membrane, promoting secretion in a single step (Rego et al., 2010). In contrast, T2SS, T5SS, T8SS, and the CU pathway are dependent on the Sec or the Tat translocon to cross the inner membrane. The final translocation is mediated by specific secretion mechanisms resulting either in the release into the extracellular space or the surface display of effector proteins (Dautin and Bernstein, 2007).

Among the two-step secretion systems, the T5SS is probably the simplest process for the export of adhesins, enzymes, toxins, and other virulence factors with varying sizes and structures (Henderson et al., 2004). Typically, members of T5SS contain a signal peptide at the N-terminus mediating Sec-dependent transport across the inner membrane, and the passenger domain that exerts biological activity in the extracellular space. A linker domain connects the passenger and the β-domain, which forms a β-barrel with a hydrophilic pore in the outer membrane. Different subgroups were identified with respect to special characteristics, such as polypeptide-transport-associated (POTRA) domains, oligomerization state, and domain arrangement.

Subgroup Va is defined as the group of ‘classical’ autotransporters and includes the monomeric autotransporters containing all information required for translocation across both membranes in a single polypeptide chain (Jose et al., 1995; Desvaux et al., 2004). The two-partner-secretion (TPS) system is assigned to subgroup Vb. In this subgroup, the passenger and the transporter are expressed as two different proteins referred to as TpsA and TpsB (Clantin et al., 2007). Here, the transporter TpsB contains two POTRA domains for recognition of its corresponding passenger TpsA. A third subgroup (Vc) covers the trimeric autotransporters, which are composed of three identical
subunits (Cotter et al., 2005). Each subunit contributes four \( \beta \)-strands to the resulting 12-stranded \( \beta \)-barrel and thus deploying three passenger domains in the extracellular space. In 2010, Salacha et al. discovered a new type of autotransporter protein in \textit{Pseudomonas aeruginosa}, termed PlpD. This protein shares the common features of classical autotransporters (Va) and TpsB proteins (Vb) in one polypeptide chain. In PlpD, the passenger domain is connected to the transport domain by only one POTRA domain. Therefore, this protein was assigned to the new subgroup Vd, termed fused TPS systems (Leo et al., 2012).

Recently, the crystal structures of two adhesins, intimin of \textit{Escherichia coli} and invasin of \textit{Yersinia} spp., were described (Fairman et al., 2012). These structures revealed architectures similar to those of classical autotransporters of subgroup Va but in reverse order. Here, the N-terminal part of the protein chain forms a \( \beta \)-barrel domain and the C-terminal part harbors the passenger domain. Consequently, intimin and invasin were assigned to a new subgroup Ve, comprising inverted autotransporters (Leo et al., 2012).

Despite extensive biochemical and structural data, the exact mechanism of the outer membrane transport of T5SS remains unclear. Different pathways have been discussed, including the hairpin model (Henderson et al., 2004) and the BamA/Omp85 model (Voulhoux et al., 2003; Robert et al., 2006), which is based on an evolutionary ancient ensemble of periplasmic and outer membrane proteins, e.g., the Bam complex, found to have homologues even in mitochondria (Tommassen, 2010).

The type V secretion mechanism

In T5SS, the translocation of passenger domains across both membranes of the gram-negative envelope is mediated in a two-step mechanism (Rego et al., 2010). To cross the inner membrane, the signal peptide of the protein chain is recognized by the Sec translocon (Figure 1, step 1) (Desvaux et al., 2004) and translocated therefore in an unfolded form. During translocation, the signal peptidase cleaves off the signal peptide. According to the BamA/Omp85 model (Voulhoux et al., 2003), the autotransporter protein (Figure 1, step 2), devoid of the signal peptide, adopts a stable intermediate in the periplasm by interaction with periplasmic chaperones, such as Skp and SurA, and the outer membrane Omp85/Bam complex (Brandon and Goldberg, 2001; Skillman et al., 2005; Ieva and Bernstein, 2009; Ruiz-Perez et al., 2009; Leyton et al., 2012). The \( \beta \)-domain integrates into the outer membrane in some way, forming a \( \beta \)-barrel with a hydrophilic pore occupied by a linker domain through which the passenger domain is apparently translocated to the cell surface (Figure 1, step 3).

Structural and functional diversity of T5SS passengers

Passengers of T5SS are involved in major processes of gram-negative bacteria, such as virulence, bacterial motility, or nutrient acquisition and function as adhesins, enzymes, or toxins (Henderson et al., 2004; Leo et al., 2012; Jacob-Dubuisson et al., 2013). Despite varying in size with a maximum up to 500 kDa, most of the T5aSS and T5bSS passengers form extended \( \beta \)-helices after translocation, such as the passengers of pertactin, EspP, Hbp, VacA, and FHA. One exception is the passenger domain of EstA (Va), which does not contain \( \beta \)-helices (van den Berg, 2010). Each passenger undergoes one of three possible post-translocational treatments: (i) it can either remain covalently linked to the transport unit, (ii) or can be cleaved but remain attached to the cell surface, or (iii)
the passenger is released into the extracellular space after cleavage. Trimeric autotransporters (Vc), e.g., Hia and YadD, contain α-helical passenger domains, which remain covalently linked after translocation across the outer membrane and function as adhesins (Cotter et al., 2005). For PlpD, which is a member of the recently defined subgroup of fused autotransporters (Vd), no structures are available. However, the passenger of the patatin-like protein D (PlpD) was predicted to fold similarly to the potato patatin Pat17 containing α-helices, β-sheets, and connecting loops (Rydel et al., 2003; Salacha et al., 2010). The passengers of T5eSS, e.g., intimin and invasin, are released into the extracellular space and fold into several Big (bacterial immunoglobulin-like) domains and a C-type lectin-like domain at the C-terminus. Intimin and invasin mediate adhesion or the rearrangement of the host cytoskeleton (Hamburger et al., 1999; Batchelor et al., 2000; Luo et al., 2000).

In summary, a structural comparison of only the passenger domains cannot be used to differentiate between the subgroups of T5SS. Thus, it is necessary to consider the characteristics of the whole autotransporter.

The architecture of type V transport units

In contrast to the passengers translocated by T5SS, the transport units share high structural similarity (Henderson et al., 1998; Thanassi and Hultgren, 2000; Meng et al., 2006; Leyton et al., 2012). In recent years, several crystal structures from all T5SS subgroups, except Vd, were solved (Table 1). All structures of NalP (Va), FhaC (Vb), Hia (Vc), and intimin (Ve, Figure 2) compared here (Figures 2 and 3) consist only of the transport unit, except for FhaC (Oomen et al., 2004; Meng et al., 2006; Clantin et al., 2007; Fairman et al., 2012). For NaIP and intimin, only the linker and the β-barrel domains were cloned, expressed, and crystallized. The structure of Hia was solved for a construct where the N-terminal part was deleted.

All type V secretion transport units localize to the outer membrane of gram-negative bacteria. These are composed of a linker and an amphipatic β-barrel domain, which is a common feature of proteins spanning the outer membrane of gram-negative bacteria, mitochondria, and chloroplasts (Schulz, 2002). The amphipatic β-barrel does not appear as a structural component in other proteins, with the peculiar exception of intracellular fatty acid-binding proteins in animal cells (Smathers and Petersen, 2011). The autotransporters β-barrels consist of 12 antiparallel β-strands in the case of NalP, Hia, and intimin (Table 1, Figure 2), and 16 strands for FhaC. The β-strands of the β-barrels are connected by short turns on the periplasmic side and larger extracellular loops. In the case of Hia, the connecting loops have the same length on both sides of the β-barrel (Figure 2). The central pores of the β-barrels are hydrophilic and occupied by the α-helical linker in the case of NalP, FhaC, and Hia. For intimin, the linker domain traverses the pore in an extended conformation (Fairman et al., 2012). The diameters of these pore domains range between 10 and 20 Å, where Hia harbors the widest pore with three α-helical domains in contrast to NalP and FhaC with only one (Figure 2). The surface representation illustrates the dimensions of the linker domain relative to the pore (Figure 3). For NalP, the linker domain is located in the center of the pore, resulting in dense packing of this domain close to the wall of the β-barrel (Figure 3B).

### Table 1 Overview of crystal structures of type V transport units.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Organism</th>
<th>Type V subgroup</th>
<th>Pdb code</th>
<th>Resolution (Å)</th>
<th>No. of β-strands</th>
<th>Passenger/function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrkA</td>
<td>Bordetella pertussis</td>
<td>a</td>
<td>3QQ2</td>
<td>3.00</td>
<td>12</td>
<td>Serum resistance, adhesion</td>
<td>Zhai et al., 2011</td>
</tr>
<tr>
<td>EspP</td>
<td>Escherichia coli</td>
<td>a</td>
<td>2QOM</td>
<td>2.70</td>
<td>12</td>
<td>Serine protease</td>
<td>Barnard et al., 2007</td>
</tr>
<tr>
<td>EstA</td>
<td>Pseudomonas aeruginosa</td>
<td>a</td>
<td>3KVN</td>
<td>2.50</td>
<td>12</td>
<td>Esterase</td>
<td>van den Berg, 2011</td>
</tr>
<tr>
<td>NaIP</td>
<td>Neisseria meningitides</td>
<td>a</td>
<td>1UYN</td>
<td>2.60</td>
<td>12</td>
<td>Serine protease</td>
<td>Oomen et al., 2004</td>
</tr>
<tr>
<td>FhaC</td>
<td>Bordetella pertussis</td>
<td>b</td>
<td>2QDZ</td>
<td>3.15</td>
<td>16</td>
<td>FHA (filamentous hemagglutinin)</td>
<td>Clantin et al., 2007</td>
</tr>
<tr>
<td>Hia</td>
<td>Haemophilus influenzae</td>
<td>c</td>
<td>3EMO</td>
<td>2.00</td>
<td>12 (3×4)</td>
<td>Adhesion</td>
<td>Meng et al., 2006</td>
</tr>
<tr>
<td>YadA*</td>
<td>Yersinia enterocolitica</td>
<td>c</td>
<td>2LE</td>
<td>1.85</td>
<td>12</td>
<td>Adhesion</td>
<td>Shahid et al., 2012</td>
</tr>
<tr>
<td>Intimin</td>
<td>Escherichia coli</td>
<td>e</td>
<td>4E1S</td>
<td>2.30</td>
<td>12</td>
<td>Rearrangement of host cell cytoskeleton</td>
<td>Fairman et al., 2012</td>
</tr>
<tr>
<td>Invasin</td>
<td>Yersinia pseudotuberculosis</td>
<td>e</td>
<td>4E1T</td>
<td>2.30</td>
<td>12</td>
<td>Adhesion</td>
<td>Fairman et al., 2012</td>
</tr>
</tbody>
</table>

*Structure determination by solid-state NMR spectroscopy.
linker domain of FhaC traverses the pore in a diagonal manner owing to interactions of charged clusters of amino acids in the pore and corresponding amino acids in the linker domain, creating an asymmetric channel (Clantin et al., 2007). The pore of Hia is fully occupied by the three α-helical linker domains (Figure 3). The linker domain of intimin strongly interacts with one side of the pore, which also results in an asymmetric architecture similar to the one observed for FhaC.

As shown by Struyvé et al. (1991), outer membrane proteins of gram-negative bacteria share a common C-terminal consensus sequence containing a phenylalanine or tryptophan at -1 and a hydrophobic residue at positions -3, -5, -7, and -9 (Table 2), which is recognized by Omp85 (Robert et al., 2006). Moreover, it was demonstrated for PhoE, an *E. coli* porin, that the last C-terminal amino acid in the signature sequence (a phenylalanine or tryptophan) is essential for the correct assembly of β-barrels in the outer membrane. Compared with the five conserved amino acids of the consensus signature sequences postulated by Struyvé et al. (1991), NalP, FhaC, and intimin contain all five conserved amino acids in the last β-strand of their β-barrel (Table 2). The same is true for each subunit of the homotrimeric Hia. Loveless and Saier (1997) extended this signature sequence to the last 18 amino acids of the last β-strand by sequence alignment of different autotransporters. For each position, several amino acids were identified, resulting in a considerable variation of the signature sequence except for the terminal phenylalanine or tryptophan (Table 2). The highest agreement with the consensus signature sequence stated by Loveless and Saier (1997) shows the homotrimeric autotransporter Hia, which matches 13 amino acids, followed by FhaC with 12, NalP with 10, and finally intimin with 9 conserved amino acids (Table 2). These results strongly support the thesis that autotransporters are integrated in the outer membrane by a common machinery, which is also responsible for the integration of other outer membrane proteins.

Despite the high similarities of the overall architecture, the transport units contain special features that differ between the subgroups, such as POTRA domains,
Table 2 Comparison of the signature sequence of type V transport units.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Consensus sequence</th>
<th>Consensus sequence</th>
<th>Consensus sequence</th>
<th>Consensus sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NalP</td>
<td>SATQRGH AKLTQNF</td>
<td>SATQRGH AKLTQNF</td>
<td>SATQRGH AKLTQNF</td>
<td>SATQRGH AKLTQNF</td>
</tr>
<tr>
<td>FhaC</td>
<td>SATQRGH AKLTQNF</td>
<td>SATQRGH AKLTQNF</td>
<td>SATQRGH AKLTQNF</td>
<td>SATQRGH AKLTQNF</td>
</tr>
<tr>
<td>Hia</td>
<td>SATQRGH AKLTQNF</td>
<td>SATQRGH AKLTQNF</td>
<td>SATQRGH AKLTQNF</td>
<td>SATQRGH AKLTQNF</td>
</tr>
<tr>
<td>Intimin</td>
<td>SATQRGH AKLTQNF</td>
<td>SATQRGH AKLTQNF</td>
<td>SATQRGH AKLTQNF</td>
<td>SATQRGH AKLTQNF</td>
</tr>
</tbody>
</table>

The numbers correspond to the amino acid position relative to the C-terminal end and number 1 indicates the last amino acid.

Oligomerization state, and domain arrangement. For example, the TpsB protein FhaC (Vb) contains two POTRA domains (Figure 2, blue subdomains), which are responsible for the recognition of their dedicated passenger protein, filamentous hemagglutinin (FHA) (Clantin et al., 2007). Each POTRA domain consists of three β-strands and one short α-helix. Intimin (Vc) contains a periplasmic α-helix, which is located between the linker and the β-barrel domain (Figure 2, orange subdomains) (Fairman et al., 2012). The exchange of this helix for a short glycine-glycine linker had no influence on the heat modifiability and proteinase K sensitivity, indicating a properly folded β-barrel and an unaffected translocation of the passenger (Fairman et al., 2012). This helix could possibly function as a plug of the pore from the periplasmic side that avoids leakage of the cells. In contrast to members of all other subgroups, Hia (Vc) translocates its high identical passenger domains within only one β-barrel (Figure 2).

Next, we were interested if it is possible to distinguish between transport units of different subgroups of T5SS by a structural comparison. Therefore, the global root mean square deviation (RMSD) was calculated, which is a relative measurement for describing overall structural similarity. For that, the transport unit structures of each subgroup were compared to a reference structure of the same subgroup (Table 3). Among the monomeric autotransporters, EspP, a serine protease autotransporter (SPATE) from E. coli, showed the highest (RMSD of 1.20 Å) and BrkA from Bordetella pertussis the lowest (RMSD of 2.57 Å) structural similarity to NalP with respect to the Cα atoms. Regarding the function of EspP, which like NalP is assigned to the SPATEs, the high structural similarity is not surprising. The structures of YadA and Hia, which both function as adhesins, are very similar over a length of 273 Cα atoms (RMSD of 1.81 Å). The global RMSD for invasin compared with intimin is very low (0.69 Å), indicating a nearly identical structure over the whole length of the protein. Comparing structures of different subgroups, Hia and intimin to NalP, the global RMSDs of intimin (2.25 Å) and Hia (2.63 Å) are in the same range as for BrkA (Va) (2.57 Å). For FhaC, the structural identity is significantly lower (RMSD of 3.63 Å) owing to the different numbers of β-strands.

In consequence, the structural comparison of the transport units is not sufficient to differentiate between the subgroups of T5SS. For example, NalP and intimin share a higher structural similarity compared with NalP and BrkA, but belong to different subgroups, Va and Ve, respectively. Thus, a comprehensive analysis of the whole protein is required for the correct classification of each autotransporter.
Table 3  Global RMSD calculations of $C_\alpha$ atoms of type V transport units.

<table>
<thead>
<tr>
<th>Protein</th>
<th>No. of amino acids</th>
<th>No. of $C_\alpha$ atoms compared</th>
<th>RMSD (Å)</th>
<th>Reference structure</th>
<th>No. of amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrkA</td>
<td>266</td>
<td>223</td>
<td>2.57</td>
<td>NalP</td>
<td>308</td>
</tr>
<tr>
<td>EspP</td>
<td>285</td>
<td>227</td>
<td>1.20</td>
<td>NalP</td>
<td>308</td>
</tr>
<tr>
<td>EstA</td>
<td>276</td>
<td>269</td>
<td>1.84</td>
<td>NalP</td>
<td>308</td>
</tr>
<tr>
<td>YadA</td>
<td>$3 \times 105$</td>
<td>273</td>
<td>1.81</td>
<td>Hia</td>
<td>$3 \times 162$</td>
</tr>
<tr>
<td>Invasin</td>
<td>245</td>
<td>236</td>
<td>0.69</td>
<td>Intimin</td>
<td>242</td>
</tr>
<tr>
<td>FhaC</td>
<td>554</td>
<td>191</td>
<td>3.63</td>
<td>NalP</td>
<td>308</td>
</tr>
<tr>
<td>Hia</td>
<td>$3 \times 162$</td>
<td>186</td>
<td>2.63</td>
<td>NalP</td>
<td>308</td>
</tr>
<tr>
<td>Intimin</td>
<td>242</td>
<td>164</td>
<td>2.25</td>
<td>NalP</td>
<td>308</td>
</tr>
</tbody>
</table>

The smaller the RMSD, the higher the structural similarity is. For example, intimin and invasin have a high structural similarity (RMSD 0.69 Å); FhaC and NalP (3.63 Å) share a low structural similarity.

In recent years, considerable experimental data were obtained in terms of the biogenesis of the monomeric autotransporter EspP from *E. coli* (Ieva and Bernstein, 2009; Ieva et al., 2011; Pavlova et al., 2013). In these studies, several conserved amino acids were analyzed by mutational and crosslinking experiments and by bioinformatic comparison to other autotransporters. To analyze if other autotransporters contain identical amino acids at the same position in the spatial arrangement as in EspP, a structural alignment of EspP with NalP, FhaC, Hia, and intimin was performed. Indeed, four of these already investigated amino acids of EspP (Pavlova et al., 2013) were also present in NalP and FhaC, including the C-terminal phenylalanine of the conserved signature sequence (see above; Figure 4, highlighted in blue). As depicted in Figure 4, $G_1$ and $G_2$ correspond to the glycine residues at the left and the right side of each β-barrel, to which the distance measurements listed in Table 4 refer. For EspP, heat modifiability tests revealed that mutation of G2(1066) or G2(1081) slightly interferes with the stability of the β-barrel (Pavlova et al., 2013). In consequence, the exposure of the passenger domain on the cell surface and the integration of the β-barrel into the outer membrane were delayed, as shown by crosslinking and protease accessibility tests. However, the release of the passenger domain was unaffected. The midbarrel residue Y(1125) was shown to interact with the periplasmic chaperone Skp and lipopolysaccharides from the outer membrane (Ieva et al., 2011). The role of G1(1207) in the biogenesis of EspP has not yet been determined.

Figure 4  Conserved amino acids among the transport units of EspP, NalP, and FhaC.
Green: transport units in ribbon presentation; blue: conserved amino acids in stick presentation. For a better overview, the POTRA domains of FhaC are hidden. The conserved amino acids are labeled with one-letter code and position number. $G_1$ and $G_2$ correspond to the distance measurements in Table 4.
Table 4  Analysis of conserved amino acids between EspP, NalP, and FhaC.

<table>
<thead>
<tr>
<th>Distances (Å)</th>
<th>EspP</th>
<th>NalP</th>
<th>FhaC</th>
</tr>
</thead>
<tbody>
<tr>
<td>G₁-G₂</td>
<td>26.24</td>
<td>24.07</td>
<td>24.11</td>
</tr>
<tr>
<td>G₁-Y</td>
<td>29.70</td>
<td>28.90</td>
<td>29.70</td>
</tr>
<tr>
<td>G₁-F</td>
<td>32.48</td>
<td>31.76</td>
<td>38.32</td>
</tr>
<tr>
<td>Y-F</td>
<td>27.64</td>
<td>28.29</td>
<td>27.64</td>
</tr>
<tr>
<td>Y-G₂</td>
<td>19.26</td>
<td>19.74</td>
<td>16.88</td>
</tr>
<tr>
<td>G₂-F</td>
<td>9.23</td>
<td>9.94</td>
<td>16.92</td>
</tr>
</tbody>
</table>

G₁(1066) was used for distance measurements in EspP.

but this residue seems to be important for the general translocation mechanism as it is highly conserved among autotransporters (see superfamily entry pfam03797 in the Conserved Domain Database, http://www.ncbi.nlm.nih.gov/cdd).

Regarding the spatial arrangement, the distances between the conserved residues in EspP and NalP are very similar to each other (Table 4). Owing to the 16-stranded β-barrel of FhaC, the distances of G₂(249) and G₁(359) to F(554) and Y(287) to G₂(249) differ from those observed in EspP and NalP, which are composed of 12-stranded β-barrels. Nevertheless, all of these amino acid side chains in each structure are exposed to the environment and thus are accessible for interactions in a comparable spatial arrangement (Figure 4). Regardless of the subgroup, these conserved amino acids could presumably play the same role in the biogenesis of T5SS.

Translocation across the outer membrane

In the past decade, different models for translocation of passenger domains across the outer membrane of gram-negative bacteria were postulated (Veiga et al., 2002; Oomen et al., 2004; Kostakioti and Stathopoulos, 2006). Most biochemical and structural data obtained by numerous investigations support two of these models: (i) the hairpin model and (ii) the Omp85 model (Figure 5).

The hairpin model

The hairpin model was developed on the basis of data obtained for the prototype of the type V secretion pathway, the IgA1 protease from Neisseria gonorrhoea (Pohlner et al., 1987). In principle, this model relied on the discovery of a β-barrel structure at the C-terminus of the precursor. In addition to that observation, transport of IgA1 protease to the cell surface was observed in other gram-negative species such as E. coli or Salmonella by simply transferring the corresponding gene, indicating that translocation of these proteins are self-sufficient. This observation was later confirmed for other proteins in different species, leading to the introduction of the name ‘autotransporters’ (Jose et al., 1995), first for the family of IgA1 protease-like proteins (Va). According to this model, Sec-mediated translocation of the protein across the inner membrane is followed by the C-terminal part of the linker domain, forming a hairpin-like structure with the β-barrel during the assembly of the latter in the outer membrane (Figure 5A, step 1). This insertion of the linker is followed by the N-terminus of the protein to be transported, the so-called passenger. This results in the appearance of the passenger C-terminus at the cell surface before its N-terminus. By initial folding of the extracellular loop of this hairpin, the unfolded passenger domain is pulled through the pore of the β-barrel and subsequently folds at the cell surface (Figure 5A, step 2). This folding process would prevent backsliding of the passenger domain through the pore and provides an ATP-independent driving force for the translocation process (Klauser et al., 1993; Renn et al., 2012). Finally, the fully folded passenger is presented at the cell surface (Figure 5A, step 3). The hairpin model took into consideration that the periplasm and the cell surface is devoid of ATP and the only alternative, threading of the N-terminal end of the periplasmic intermediate into the pore formed by the β-barrel (Dautin and Bernstein, 2007), appeared to be thermodynamically unlikely.

The Omp85 model

Recent studies have shown that the outer membrane factor, Omp85, YaeT, or BamA (Voulhoux et al., 2003; Kostakioti and Stathopoulos, 2006; Bernstein, 2007), a conserved component of the Bam complex, is required for the integration of autotransporters as well as for the assembly of outer membrane proteins in general in gram-negative bacteria, mitochondria, and chloroplasts by recognition of their C-terminal consensus signature sequence (Loveless and Saier, 1997; Robert et al., 2006; Tommassen, 2010). After translocation across the inner membrane, the autotransporter partially folds in the periplasm while interacting with periplasmic chaperones, such as Skp and SurA, and the assembly factor Omp85 (Figure 5B, step 1) (Ieva and Bernstein, 2009). In the model, this
Figure 5  Postulated mechanisms of type V passenger translocation across the outer membrane. Green: β-barrel; blue: passenger domain; red: linker domain; orange: Omp85 complex; violet: periplasmic chaperones. According to the hairpin model (A), the linker domain forms a hairpin-like structure in the pore of the β-barrel (step A1) and the passenger domain is pulled through this pore (step A2). Finally, the passenger domain adopts its fully folded conformation at the cell surface (step A3). In the Omp85 model (B), the autotransporter interacts with BamA (step B1) and is subsequently integrated into the outer membrane in an ‘open’ conformation (step B2), allowing transport of partially folded passengers. After translocation, across the outer membrane, the passenger domain obtains its active conformation in the extracellular space (step B3).

Evidence and difficulties of the hairpin model

Despite some evidence for the Omp85 model, considerable experimental data were also produced supporting the hairpin model. When the β-subunit of cholera toxin (CTB), which contains intramolecular disulfide bonds, was fused to the transport unit of IgA1 protease, surface translocation was not possible in E. coli. In contrast, surface translocation was possible after mutation of the two cysteines to leucine and glycine, or when the gene encoding DsbA, which promotes formation of disulfide bonds in the periplasm of E. coli, was deleted (Jose et al., 1996). Both findings indicate that disulfide bond formation in the passenger domain is only allowed after the translocation of the IgA1 transport unit. Similar results were obtained when aprotinin, a rapid-folding Kunitz-domain-like protease inhibitor, with several disulfide bonds, was fused to the autotransporter domain of the subgroup Va autotransporter AIDA-I (Jose and Zangen, 2005). Surface translocation was only detectable when a strong reducing cell environment was provided by the addition of 1 mM 2-mercaptoethanol. Moreover, when the genes encoding the periplasmic proteases DegP or DeqQ were deleted, the aprotinin-AIDA-I fusion without the addition of 2-mercaptoethanol accumulated in the periplasm (Jose and Zangen, 2005). These results indicated that aprotinin containing disulfide bonds represented a
translocation-incompatible structure that is degraded by periplasmic proteases before it could fold into its active conformation and inhibit these proteases. Recent studies with slow-folding mutants of the wild-type autotransporter EspP in *E. coli* provided experimental evidence for the formation of a hairpin structure within the passenger domain during transport (Leva et al., 2008; Leva and Bernstein, 2009). In addition, after transport of the cytochrome P450 enzyme CYP106 to the cell surface by fusion to the transport unit of AIDA-I, the enzyme was found to be devoid of its prosthetic group, porphyrin. This is normally incorporated into the passenger in the periplasm of *E. coli*. As the heme group is bound to the enzyme through non-covalent interactions, it is unlikely that it is co-transported with an unfolded CYP106 enzyme, as required in the hairpin model. Porphyrin was, however, incorporated subsequently at the cell surface even without adding it to the cultures (Schumacher et al., 2012). Recent findings showed that porphyrin groups can be exported by the outer membrane channel TolC (Tatsumi and Wachi, 2008). The strongly reduced CYP106 enzyme activity in TolC mutants clearly indicates the dependency of heme export mediated by TolC. However, after addition of heme to the growth medium, the CYP106 enzyme activity could be restored (Schumacher et al., 2012). These results were a hint that surface translocation of the CYP enzyme and the export of porphyrin are independent secretion processes. Moreover, it is likely that CYP enzymes are translocated in an unfolded state across the outer membrane, thus being consistent with the hairpin model. Also in line with this model are several studies that showed that the C-terminal end of the passenger domain reaches the extracellular space before the N-terminus, driving the translocation of the passenger domain (Junker et al., 2009; Peterson et al., 2010; Soprova et al., 2010). Thus, Junker et al. (2009), working with the monomeric autotransporter pertactin from *B. pertussis*, inserted reversible cysteine pairs at different positions in the passenger domain, which stalled the secretion process at different stages. Proteinase K treatment and detection of a stable, proteinase K-resistant 28-kDa core led to the conclusion that the C-terminal end of the passenger domain reaches the extracellular space before the N-terminus and that the secretion process is vectorial. The second study (Peterson et al., 2010) was performed with the monomeric autotransporter EspP from *E. coli*. Here, the folding rate of a β-helix in the extracellular space was reduced by introduction of different point mutations in the C-terminal end of the passenger domain. Proteinase K treatments and immunodetection experiments revealed that this part of the passenger is translocated across the outer membrane before the rest of the passenger domain is detected in the extracellular space. Thereby, the reduced folding rate of the β-helix did not abolish the translocation of the rest of the passenger domain, while in contrast deletion of this C-terminal part blocked translocation (Peterson et al., 2010). In the third study (Soprova et al., 2010), the folding behavior of the monomeric autotransporter hemoglobin protease (Hbp) from *E. coli* was analyzed. For this purpose, different mutations were introduced into the conserved C-terminal stretch of the passenger domain, which was identified as an autochaperone (AC) domain by deletion and mutagenesis experiments as well as by bioinformatic comparison to homologous autotransporters, such as BrkA (Oliver et al., 2003). Proteinase K treatments, crosslinking experiments, and immunodetection analysis showed that the amino acid tryptophan at the C-terminal end of the AC domain is crucial for the translocation of the passenger domain of Hbp (Soprova et al., 2010).

Nevertheless, the hairpin model cannot explain how some passenger domains containing disulfide bonds or glycosylated amino acids are successfully translocated across the outer membrane (Lindenthal and Elsinghorst, 1999; Skillman et al., 2005). In one study, the CTB passenger, known to fold and undergo disulfide formation in the periplasm, was fused to the transport unit of EspP (Skillman et al., 2005). Pulse-chase labeling experiments, proteinase K treatment, and immunodetection analysis revealed that the already folded passenger domains containing disulfide bonds were secreted efficiently across the outer membrane (Skillman et al., 2005). In addition, Lindenthal and Elsinghorst (1999) showed that with TibA, a monomeric autotransporter from *E. coli*, the passenger domain is translocated across the outer membrane containing glycosylated amino acids, detected by digoxigenin and proteinase K treatment of whole cells. Regarding the size constraints of the β-barrel pores (diameter of ~20 Å for monomeric and ~10 Å for trimeric autotransporters, Figure 3), only two protein chains without any modifications could be accommodated within a fully folded β-barrel if they are present in an extended conformation (Kostakioti and Stathopoulos, 2006; Bernstein, 2007). This fact excludes the formation of an α-helical linker before or during the translocation event. In contrast, Leva et al. (2008) assumed that such an α-helical structure is achieved in the pore of EspP from *E. coli* before insertion of the β-barrel in the outer membrane. Accessibility tests with tobacco etch virus protease or proteinase K, cell fractionation analysis, and pulse-chase labeling experiments revealed that a 30- to 35-amino-acid segment of the linker is embedded in the β-barrel pore before passenger domain secretion.
Evidence and difficulties of the Omp85 model

Today, it is known that most if not all gram-negative bacteria contain a set of periplasmic chaperones, e.g., Skp and SurA, and the Bam complex that facilitate partial folding and assembly of β-barrels into the outer membrane (Tommassen, 2007; Grijpstra et al., 2013). Clearly, autotransporter proteins use this machinery for the transport of their passengers to the cell surface, dependent on a specific recognition motif at the C-terminus of the β-barrel, as mentioned above (Robert et al., 2006). In support of the Omp85 model, Ieva and Bernstein (2009) revealed the interaction of EspP, a monomeric autotransporter, with Omp85 and periplasmic chaperones. In this study, a small linker domain was inserted into the passenger domain, creating a periplasmic intermediate by transient stalling of the translocation process. Crosslinking experiments revealed that the C-terminal part of the passenger domain and the β-barrel interact with Omp85 (Ieva and Bernstein, 2009). The N-terminal part of the passenger domain was shown to interact with SurA and Skp, which was later confirmed by the same group (Ieva et al., 2011).

As depicted in the model in Figure 5B, Omp85 forms a pore in which the transport unit is integrated and through which the passenger domain is transported. With liposome-swelling experiments and planar lipid bilayers, Robert et al. (2006) showed that Omp85 is able to form voltage-activated pores with a diameter of 2.5 nm. However, this pore would be too narrow to accommodate the (partially) folded transport unit and the passenger domain. Consequently, the proposed Omp85 model cannot provide an explanation of how the translocation of folded passenger domains across the outer membrane is managed.

As mentioned above, the ensemble of chaperons and the Bam complex involved in type V secretion is an ancient machinery, existing before phylogenetic separation of the bacteria occurred. Therefore, divergent evolution of the system in different bacteria, especially of the Bam complex (Webb et al., 2012), should be considered. The heterologous expression of an autotransporter can lead to differences in transport not least owing to the lack of a ‘compatible’ β-barrel and a C-terminal recognition sequence. Finally, when recombinant passenger proteins are fused to a heterologous transport domain, this brings a third variable into play, which can also influence transport and folding. At this point, it appears important to consider that the contradictory results, e.g., the transport of passengers containing disulfide bonds (Jose et al., 1996; Skillman et al., 2005), were not obtained with identical transporter proteins in identical gram-negative bacteria. Therefore, only data obtained with constructs with identical transporter, passenger, and host gram-negative bacterium can be compared and considered valid (Jose and Meyer, 2007). Notably, data obtained from studies comparing identical systems give consistent results.

Figure 6  Alternative model mechanism of type V passenger translocation across the outer membrane. Green: β-barrel; blue: passenger domain; red: linker domain; light orange: Omp85; yellow: BamB; dark orange: BamD; violet: Skp; purple: SurA; light gray: TamA; dark gray: TamB; P: POTRA domains. The autotransporter interacts with the periplasmic chaperone Skp and forms a stable intermediate with the linker domain incorporated in the pore of the prefolded β-barrel (step 1). This intermediate is targeted to the outer membrane interacting with TAM and the Bam complex (step 2). The prefolded β-barrel is integrated in the outer membrane while the passenger domain interacts with SurA and the POTRA domains of BamA (step 3), resulting in a stepwise transfer across the outer membrane (step 4). Finally, the passenger domain adopts its fully folded conformation at the cell surface (step 5).
Alternative model

Because neither the hairpin model nor the Omp85 model can describe a translocation mechanism consistent with all data derived from structural and biochemical analyses (Bernstein, 2007; Leyton et al., 2012), a synopsis of both appears to be worth looking at and has been proposed before (Benz and Schmidt, 2011; Ieva et al., 2011; Jose et al., 2012; Grijpstra et al., 2013; Pavlova et al., 2013).

The alternative model is a combination of both the hairpin and the Omp85 model (Figure 6), supported by recently obtained evidence from crosslinking and mutagenesis experiments, heat modifiability analysis, and protease accessibility tests with the monomeric autotransporter EspP (Ieva et al., 2011; Pavlova et al., 2013). As described below, we extend this model by the fact that a recently discovered translocation and assembly module (TAM) is involved in the translocation of autotransporters (Selkrig et al., 2012).

After translocation across the inner membrane, the autotransporter partially folds in the periplasm, interacting with the periplasmic chaperone Skp (Figure 6, step 1). At this stage, the linker domain is incorporated inside an incompletely folded β-barrel, forming a hairpin-like structure (Ieva et al., 2008). Subsequently, the prefolded autotransporter is targeted to the outer membrane interacting with the Bam complex in a unique orientation (Figure 6, step 2, highlighted in yellow/orange) (Ieva et al., 2011; Pavlova et al., 2013). As the midbarrel residues are accessible to crosslinking and the hairpin formed by the linker domain is not detectable at the cell surface at this stage, the β-barrel does not seem to be fully integrated into the outer membrane (Pavlova et al., 2013). Recently, another secretion complex, the TAM, was discovered in different proteobacteria (Selkrig et al., 2012). This module consists of TamA, an Omp85-family protein located in the outer membrane, which interacts with TamB in the inner membrane, presumably through the POTRA domains of TamA. It was shown for Citrobacter rodentium that mutants lacking TamA or TamB do not express the putative autotransporter protein p1121. Furthermore, double mutants of E. coli lacking both TamA and TamB were not able to secrete the recombinant autotransporter adhesins Ag43 and EhaA. Therefore, cell-cell aggregation was abolished. Additionally, the precursor form of Ag43 accumulated in the periplasm (Selkrig et al., 2012). Moreover, crosslinking experiments with EspP in E. coli revealed that during the transient delay of passenger translocation, the passenger domain of EspP was crosslinked to an unidentified cellular protein with a molecular weight of 30–40 kDa (Ieva et al., 2011), which might correspond to TamA. Consequently, the TAM is assumed to interact with the passenger domain in the periplasm, providing an essential step in the translocation process presumably by stabilizing the periplasmic intermediate (Figure 6, step 2, highlighted gray). Probably, owing to a conformational change or repositioning of this intermediate, the β-barrel integration into the outer membrane is completed and the hairpin is exposed at the cell surface, leading to the initiation of passenger domain translocation (Figure 6, step 3).

In this way, the TAM would dissociate from the passenger domain, while a stretch of 80 amino acids of the passenger domain, as shown by crosslinking experiments, interacts with several POTRA domains of BamA/Omp85 and the chaperone SurA in the periplasm (Pavlova et al., 2013). Thus, in the alternative model, the passenger is transferred in a stepwise process through a pore composed of the autotransporter in an ‘open conformation’ mediated by the Bam complex. During another repositioning of the β-barrel (Figure 6, step 4), the passenger domain is fully translocated and the assembly of the β-barrel is completed, resulting in the dissociation of the Bam complex and the surface exposure of the passenger domain (Figure 6, step 5).

Concluding remarks

In the last decade, more and more insight into the T5SS has been gained by structural, bioinformatic, and biochemical experiments. Comparing the crystal structures of transport units, which represent the state after translocation, the overall architecture of the type V transport units share high similarity also between the different subgroups, indicating a common translocation mechanism through the outer membrane. Different model mechanisms were discussed. Among those, the hairpin and the Omp85 model are supported by some but not all experimental results and clearly show the dependency of autotransporter proteins on additional factors, such as the Bam complex. Thus, the term ‘autotransporter’ is now redundant.

An alternative model was proposed, which is a combination of both the hairpin and the Omp85 model. Here, the fact that passengers contain disulfide bonds or modified amino acids, the interaction of the autotransporter with periplasmic chaperones and the Bam complex, and the formation of an α-helical structure in the pore before insertion of the β-barrel in the outer membrane are taken into account. We extended this model by a novel TAM, which was discovered recently and shown to be involved...
in the translocation of autotransporters across the outer membrane (Selkirk et al., 2012). To determine the exact function of the TAM and its interaction partner, further investigations are obviously required. Nevertheless, our understanding of the type V secretion mechanism improved during the last decade, resulting in a more and more complete picture.

Acknowledgments: We thank Dr. Astrid Höppner, Crystal Farm and X-ray facility, Heinrich-Heine University Düsseldorf, for assistance and helpful comments. We also gratefully acknowledge the support (and training) from the International NRW Research School BioStruct, granted by the Ministry of Innovation, Science and Research of the State North Rhine-Westphalia, the Heinrich-Heine University Düsseldorf, and the Entrepreneur Foundation at the Heinrich-Heine-University of Düsseldorf.

References


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