Type V Secretion Systems in Bacteria

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ABSTRACT Type V secretion denotes a variety of secretion systems that cross the outer membrane in Gram-negative bacteria but that depend on the Sec machinery for transport through the inner membrane. They are possibly the simplest bacterial secretion systems, because they consist only of a single polypeptide chain (or two chains in the case of two-partner secretion). Their seemingly autonomous transport through the outer membrane has led to the term “autotransporters” for various subclasses of type V secretion. In this chapter, we review the structure and function of these transporters and review recent findings on additional factors involved in the secretion process, which have put the term “autotransporter” to debate.

INTRODUCTION
Gram-negative bacteria are surrounded by two membranes, called the outer and inner membranes. The space between these membranes, the periplasm, is spanned by a polymeric glycopeptide network, the peptidoglycan. This net has a closely defined mesh size and is attached to the outer and inner membranes by various proteins in the different Gram-negative organisms, leading to an even distance between the two membranes. Proteins that are to be transported to the outer membrane or the extracellular space thus have to cross various obstacles on their way; this process is mediated by a number of highly specialized secretion systems, mostly classified by Roman numerals from type I to type VII (see other chapters in this book for a comprehensive review of these systems). More secretion systems, or new variations of the ones previously described, are still found on a regular basis, making a comprehensive listing almost impossible. The major systems that are either well described and/or widely present in many Gram-negative species can be classified into two general architectures: those that span both membranes and the periplasm as one large secretion complex and those where the individual membranes are crossed using independent (i.e., not physically connected) secretion machineries. Type V secretion systems are part of the latter systems.

Type V secretion systems come in different “flavors,” currently classified as types Va to Ve (1). They share a common principle that distinguishes them from other secretion systems: in type V secretion, the secretion pore (called the translocation domain) through the outer membrane is typically part of the same polypeptide chain as the payload (called the passenger domain) that is to be secreted to the cell surface (Fig. 1). This makes type V secretion a very simple system in terms of primary structure and its minimalist number of components but raises complicated questions when it comes to the actual mechanism of secretion, as described throughout this chapter. It is this seemingly self-sufficient export mechanism within one polypeptide chain that gave rise to the term “autotransporter” (2)—commonly used at least for the type Va, Vc, and Ve secretion systems.

It is well established that type V–secreted proteins cross the inner membrane through the Sec system, using
cleavable N-terminal signal peptides. Then, the translocation domain forms a pore in the outer membrane through which the passenger domain is secreted. What distinguishes the different subtypes of type V secretion systems is their domain organization (Fig. 1) and the resulting variations in the transport mechanism(s).

Type Va systems are the prototypical (“classical”) autotransporters. The first autotransporter described was IgA1 protease from Neisseria gonorrhoeae (1). In type Va systems, the pore is formed by the C-terminus of the polypeptide, while the N-terminus is exported to the cell surface. In many cases, including IgA1 protease, the passenger domain is cleaved off in an autocatalytic process after translocation is complete, releasing the passenger into the extracellular medium but leaving the translocation domain in the outer membrane. Other type Va systems are not autoprocessed in this way and thus keep the passenger domain anchored to the cell surface via the translocation domain. Typically, the passengers of noncleavable type Va systems function as adhesins, while the cleavable ones have enzymatic (protease or lipase) activities (1).

Type Vb systems, on first sight, seem like a split variant of type Va systems: the passenger domain and translocation domain are two separate protein chains but are expressed from the same operon structure. Type Vb systems are thus also called two-partner secretion (Tps) systems. The translocator proteins of type Vb...
systems are collectively called TpsB proteins, and the transported proteins (corresponding to the passenger domains of type Va autotransporters) are referred to as TpsA proteins. Notably, the TpsB protein that forms the translocation pore contains additional periplasmic domains not present in classical autotransporters. While the splitting of transport and passenger function would in principle allow for multiple passengers being secreted by the same TpsB protein, there are many cases where the TpsA is retained on the surface attached to the TpsB, leading again to a 1:1 transport stoichiometry. It is mostly this observation that has led to the classification of two-partner secretion systems as part of type V secretion (1, 4), although there might be significant differences in the actual transport process compared to classical autotransporters (5).

Type Vc systems are probably the most complex autotransporter systems. They are obligate trimers, and the fact that most if not all of them function as bacterial adhesins has led to their alternative name, trimeric autotransporter adhesins (TAAs) (6). The passenger domains of the type Vc secretion systems are highly diverse and modular, while their translocation domain is highly conserved. Type Vc systems are the only type V systems in which the translocation pore is an oligomeric structure.

Type Vd systems were only recently described in the literature, and only a few exemplars have been studied in detail. Their general setup is very similar to type Va systems, with a C-terminal translocation domain and an N-terminal passenger domain. In contrast to the classical autotransporters, though, these domains are connected via an additional, periplasmic domain homologous to the periplasmic domains of the type Vb translocation pores.

Type Ve systems are also called inverse autotransporters, based on the fact that the domain order is reversed and that it is the C-terminal part that comprises the passenger domain, while the N-terminal part forms the translocation pore (7). The passenger domains are typically Ig-like and lectin-like domains that are not found in other type V secretion systems but that are widespread in adhesins from Gram-positive bacteria and in many other cell surface and periplasmic proteins (8, 9). An additional, small periplasmic domain is present at the N-terminus of the polypeptide chain, but this is not homologous to the periplasmic domains in Vb and Vd systems; instead, it in some cases contains a peptidoglycan-binding motif (10).

It is a matter of continuing debate whether the secretion mechanisms of all type V systems are indeed homologous and thus justify their classification as variations of the “same” theme. The intention of this chapter is to review the data on the different systems, obtained from various computational and experimental methods, to advance this discussion.

IN SILICO ANALYSIS: FROM SEQUENCE TO STRUCTURE

Few of the type V secretion systems have been studied experimentally in detail, and these model systems are typically limited to exemplars from medically important species. In silico analysis shows that a wide variety of type V system genes are also in nonpathogenic organisms and in environmental sequence datasets. Here, we review what information has been obtained on the diversity of type V secretion systems using various bioinformatics tools.

Sequence Alignment and Phylogenetic Analysis

Currently, our knowledge of the diversity of type V secretion systems is mostly based on similarity searches. The most popular in silico sequence analysis algorithm is BLAST, the Basic Local Alignment Search Tool (11–13), which has been widely used for identifying type V systems and other adhesins (1, 6, 7, 14–34). The standard protein-protein BLAST (blastp) has been used routinely for identifying adhesin or autotransporter sequences in genomes and in protein databases. The more sensitive Position-Specific Iterated (PSI)-BLAST has also been used for finding more distantly related type V-secreted proteins from the sequence databases or to identify the new members in a type V secretion system family. The sequences of type V-secreted proteins share high similarities in their respective subgroups due to their domain organization (35, 36) and thus, the cutoffs in the BLAST programs were generally set to the E-value $1 \times 10^{-10}$ to detect as few false-positive hits as possible.

Many sequence analysis methods and applications in bioinformatics (e.g., evolutionary analysis, protein secondary structure predictions, phylogenetic analysis, etc.) rely on multiple sequence alignments as an input. Clustal Omega has been employed in a recent study to identify the prevalence of 18 different type Va autotransporters in 111 publicly available genomes of *Escherichia coli* (36). Clustal Omega uses seeded guide trees and hidden Markov model (HMM) profile-profile techniques to generate alignments between three or more sequences (37, 38).
It is generally accepted that when the pair-wise sequence identity is greater than 40% for continuous, long alignments, the corresponding protein pair will have a similar structure (39). The diverse and multidomain nature of type V secretion systems imposes strong limitations to such inferences: in most type V secretion systems, domains can be small and can occur in different orders. The need to detect individual, small domains with sometimes very high sequence diversity led to the implementation of HMMs for the detection and classification of bacterial type V secretion systems through bioinformatics analysis (19).

The HMM search strategy resulted in the identification of more than 1,500 putative type Va autotransporter sequences from 1,210 bacterial genomes. Only three broad functional classes (esterases, proteases, and adhesins) of autotransporters are recognized in the literature based on the type of passenger domain, whereas the phylogenetic analysis of autotransporters through HMM search strategies resulted in six major families (Fig. 1) based on the function of the autotransported domains. Further cluster analysis of conserved motifs in the β-barrel domain resulted in 14 distinct groups of type Va autotransporter sequences among the proteobacterial species (19).

A distinct feature of the conventional type Va autotransporter sequences is the size of the β-barrel domain which consists of approximately 300 amino acids that forms the 12 transmembrane β-strand pore with a transmembrane α-helix spanning the pore (40). The sequence composition of the β-barrel domain is different in trimeric or type Vc autotransporter proteins, where it is formed from three polypeptide chains. Each subunit contributes four β-strands that oligomerize to form the transmembrane pore of the type Vc autotransporters. It is self-evident that the sequence alignments of type Va and type Vc autotransporter β-barrel domains will result into two distinct clades in a phylogenetic tree, as shown by Cotter et al. (41). In a study of outer membrane β-barrel proteins in general, Remmert et al. found evidence that that all major groups of outer membrane β-barrels (including all type V secretion systems) from Gram-negative bacteria are homologous and evolved from an ancestral ββ-hairpin sequence (42, 43). Taking this analysis one step further, using the combination of sequence analysis tools and secondary structure prediction tools, an HMM search strategy has been developed for the annotation of various domain regions in the trimeric or type Vc autotransporters (31). The domain annotation of TAs is available as an online server called daTAA (http://toolkit.tuebingen.mpg.de/dataa).

Posttranslational Modification Analysis

As membrane proteins in the outer membrane of Gram-negative bacteria, type V secretion systems generally possess signal peptides for translocation across the inner membrane. The algorithms to identify these secretion signals use specific sequence features present in the short N-terminal signal peptides. The most widely used tool for the identification of signal peptides is SignalP, which is based on a combination of several artificial neural networks (44). According to Dautin and Bernstein, approximately 10% of the known type Va autotransporters possess an unusually long signal sequence of more than 50 amino acid residues (45). Generally, the N-terminal signal sequences in proteins show a tripartite organization of n, h, c, referring to the regions of N-terminal, hydrophobic, and cleavage site (46). Several studies showed variations (n1-h1-n2-h2; n2-h2) of the signal peptide tripartite organization in type V systems (4, 47) and called the region the extended signal peptide region (ESPR), which might have additional functions besides targeting or protein translocation (48–50). It is important to note that using simple sequence analysis tools such as BLAST and ClustalX, Desvaux and his colleagues showed that the ESPR sequence pattern is phylogenetically restricted among the Gram-negative bacterial type V–secreted proteins from the classes of β- and γ-Proteobacteria (47). Later on, several studies analyzing the function of ESPRs of type Vc autotransporters in Gram-negative bacteria showed that the amino acids of the ESPR are required for protein stability and the proper assembly of the monomers to form functional proteins (49, 51, 52). In contrast, the work by Leyton et al. on a serine protease autotransporter (SPATE subgroup of autotransporters of type Va shown in Fig. 1) indicates that ESPR is not essential for the secretion and the function of SPATEs in the Enterobacteriaceae, which makes the role of ESPR in targeting the autotransporter protein contentious or controversial (48, 51, 52).

Glycosylation, one of the most common protein modification processes, plays an important role in the interaction of the bacterium with its environment via secreted or surface-exposed proteins (53). In addition to the proper delivery of the proteins onto the bacterial surface via type V secretion systems, glycosylation is required for proper molecular and cellular adhesion properties of some autotransporters in bacteria (54, 55). A recent study by Lu et al. (56) shows that heptosylation of bacterial autotransporters by autotransporter adhesin heptosyltransferase proteins can modify the adhesion property of type Va transporters to host cells.
The combination of *in vitro* and *in silico* methods has increased our understanding of the complex structure-function mechanism of the autotransporter glycosylation process. Homology modeling of autotransporter adhesin heptosyltransferase protein structure using MODELLER in combination with CHARMM force field-based molecular dynamics simulation helped in gaining insights into the simultaneous hyperglycosylation of six autotransporter protein substrates by autotransporter adhesin heptosyltransferase via dodecamer enzyme complex (56). Glycosylation has also been shown in a type Vc autotransporter, EmaA from *Aggregatibacter actinomycetemcomitans* (57), suggesting that this might be a more widespread mechanism that modulates type V secretion.

**Structural Biology of Type V Secretion Systems**

X-ray crystallography has been the method most widely applied to studying the structure of type V secretion systems (58, 59), and almost all the high-resolution structures currently available have been solved by crystallography. These have confirmed a number of hypotheses concerning autotransport and have been the starting point for new hypotheses. However, crystal structures only give snapshots of the final structures. Furthermore, almost all the available structures are fragments. There is only one complete structure of a classical, type Va autotransporter, the esterase EstA (60), but modeling efforts have produced plausible structures for several type Vc systems (16, 20, 61) and for the type Vb-secreted filamentous hemagglutinin FHA (62). More dynamic data might be obtained using nuclear magnetic resonance (NMR) spectroscopy, but to date NMR has hardly been used to probe autotransporter structures, probably because of the large size, oligomeric nature (for TAAs), and aggregation behavior of these proteins. However, NMR has been used to determine the structure of domains from inverse autotransporters (10, 63), and recently the structure of the translocation domain of the TAA YadA was solved using solid-state NMR (17). Electron microscopy and small-angle X-ray scattering have been used for low-resolution structural analysis, particularly of type Vc systems (64–68).

**Passenger Domains**

The passenger domains of type V secretion systems display a number of common features. Most passenger domains, regardless of type V subclass, are long, fibrous structures, in many cases well over 1,000 residues in length. Although in X-ray structures these fibers appear to be rigid rods, other methods have shown that they contain regions of at least limited flexibility that may be of functional significance (16, 27, 63, 65, 67). Another common theme is the prevalence of β-solenoids in passenger domains (69). β-solenoids are supersecondary structures, where the polypeptide chain winds around a central axis in a helical manner (70). The structure is formed by 2 to 4 parallel β-sheets aligned along the central axis; the corresponding β-strands of each coil contribute to the long β-sheets (Fig. 2). The interior of β-solenoids is usually hydrophobic and tightly packed, which contributes to the overall stability of the structure. β-solenoids are found in the vast majority of type Va, Vb, and Vc proteins (69, 71), though it should be noted that completely different passenger domain folds also exist, as exemplified by the all-α, globular passenger domain of EstA (60) (Fig. 3). In contrast, the passenger domains of type Vc autotransporters typically consist of tandemly repeated immunoglobulin (Ig)-like domains, often capped by a C-type lectin-like domain at the C-terminus of the protein (63, 72, 73) (Fig. 2). To date, there is no experimental structural information on type Vd passenger domains.

The first type Va autotransporter passenger domain structure to be solved was that of pertactin from *Bordetella pertussis*, an important component of whooping cough vaccines (74). The pertactin structure can be considered prototypical of type Va passenger domains. It is a simple, right-handed β-helix with 16 rungs (Fig. 2). β-helices are a subclass of β-solenoids with three axial β-sheets. Though the β-helix of pertactin is continuous in the sense that it is not interrupted by any other kinds of domains, several of the turns connecting adjacent β-strands are extended into loops projecting out of the β-helical core, including the loop with the RGD motif presumed to be important for binding to host receptors (74). In other type Va autotransporters, these loops extend even further and form extrahelical domains bulging out of the β-helical scaffold, as exemplified by the subtilisin-like and chitinase-like domains in the hemoglobin protease Hbp (75). In contrast to the relatively straight β-helices of pertactin and Hpb, a β-helical fragment from the antigen 43 (Ag43) passenger domain adopts a bent form resembling the letter L (76) (Fig. 2). β-helices are also found in the crystal structures of the passenger domains of Hap, EspP, and Pet and the p55 fragment of VacA (77–80).

The TpsA proteins of type Vb systems are also predicted to be rich in β-solenoid structures (62, 70). These predictions were confirmed when the structure...
of the TPS domain of the *B. pertussis* TpsA protein FHA was solved (81). The TPS domain of TpsA proteins is required for targeting to the TpsB protein and secretion; its function could therefore be considered analogous to that of the linker regions of type Va, Vc, and Ve autotransporters (Fig. 1). The FHA TPS domain structure shows a right-handed β-helix with several extrahelical elements protruding from the β-helical core (Fig. 2). The structure of the TPS domain from the *Haemophilus influenzae* adhesin HMW1 is remarkably similar to the FHA TPS domain, though there are some differences at the very N-termini of the structures (82).

**FIGURE 2** Experimental structures of passenger domains from type V secretion systems. Examples from type Va (classical autotransporters), type Vb (TpsA proteins), type Vc (trimeric autotransporter adhesins), and type Ve (inverse autotransporters) are included. Structures are shown in cartoon representation. β-solenoid structures are orange, with extrahelical domains and elements in yellow. β-prism domains are purple and coiled coils are red; connector elements are cyan. For type Vc proteins, each chain is colored with a slightly different hue. Chloride ions within coiled-coil stalks are represented by green spheres. Immunoglobulin-like domains are green, and C-type lectin-like domains blue. The structures representing type Va passengers are the pertactin passenger domain (PDB ID: 1DAB), the Hbp passenger (1WXR), and a fragment of the antigen 43 passenger (4KH3). TpsA proteins are represented by the FHA and HMW1 Tps domains (1RWR and 2ODL, respectively). Trimeric autotransporter adhesin passenger domains are represented by large fragments from EibD (2XOQ) and SadA (2YO1), the Trp ring and GIN domains of the BadA head (3DX9), the head domains of YadA (1P9H), UspA1 (3NTN) and NadA (4CJD), and the region of the YadA stalk transitioning from right-handed (at the top) to left-handed (at the bottom) supercoiling. Fragments from the FdeC (4E9L) and invasin (1CWV) passenger domains represent type Ve passengers. The structures are oriented so that the portion of passenger domains distal to the outer membrane is pointing toward the top of the page. Note that for types Va, Vb, and Vc, the distal end is the N-terminus, whereas for type Ve passengers the C-terminus is distal.
In contrast to both type Va and type Vb, type Vc secreted proteins are obligate homotrimers that form highly intertwined structures. When viewed in electron micrographs, these proteins resemble lollipops, having a globular “head” domain distal from the cell, followed by an elongated “stalk” (27, 64). However, though many type Vc systems follow this relatively simple architecture, it is now clear that they are modular in structure and can have several head domains interspersed by regions of stalk (16, 27) (Fig. 2, Fig. 3). The stalk regions of type Vc autotransporters consist largely of α-helical parallel coiled coils. Though type Vc autotransporters, such as UspA1 (66), contain regions of canonical coiled coil, i.e. a left-handed superhelix, they typically have a significant amount of right-handed coiled coil (20, 83) (Fig. 2, Fig. 3). In contrast to most coiled coils, where the core is hydrophobic, the interior of the coiled coils of type Vc autotransporters can contain a large proportion of hydrophilic residues. In some cases, these residues coordinate the binding of anions such as chloride or nitrate within the core (20, 66, 68, 84). The stalks also often contain large cavities, which have been postulated as sites that would allow deformation of the coiled coil and thus enable bending of the fiber (20). Some connector elements (discussed below) can also confer limited local flexibility (16).

In addition to often lengthy coiled-coil segments, stalks can contain connector regions that mediate transitions from one type of supercoil to another or the transition from head domain to coiled coil or vice versa. Another feature of connectors is to displace the polypeptide chain by 120°, usually in the direction opposite to the handedness of the preceding segment (16). These connectors include necks (31, 85), saddles (20), KG domains (86), and HANS, DALL, and FGG elements (16, 31); the somewhat cryptic names of the domains refer to short sequence motifs typically found in them. It should be noted that transitions of handedness do not necessarily require a connector, as shown by the YadA stalk (83) (Fig. 2).

The type Vc autotransporters also contain β-solenoids, but these structures are limited to the globular head domains. The first such structure to be solved was the head of the Yersinia adhesin YadA. Each monomer of the structure forms a left-handed β-roll (a subclass of β-solenoids with two axially stacked β-sheets). The trimeric structure is stabilized by a shared, tightly packed hydrophobic core and a “lock-nut” structure, where the polypeptide chain from one monomer crosses over to connect with the next monomer at both the N- and C-termini (Fig. 2). At the C-terminus, this crossover is mediated by a neck element, which leads the polypeptide into the right-handed coiled-coil stalk. YadA-like β-roll domains are found in a number of type Vc autotransporters, though not all have the highly stable lock-nut configuration (20, 67, 87) (Fig. 2). Other kinds of head domain also exist, as exemplified by the Trp ring and
GIN domains of Hia and BadA (27, 88). Both domain types are rich in β-structure and are formally variations of a β-prism fold (Fig. 2). Unlike the β-rolls and β-prims of type Vc autotransporters, which form intricately intertwining structures, the very recently solved NadA head structure is formed of three wing-like protrusions from the coiled-coil stalk that neither interconnect nor introduce a 120° twist into the chain (68) (Fig. 2).

**Translocation Domains**

The translocation domains of type V systems formally consist of two components: the transmembrane β-barrel domain, embedded in the outer membrane, and the linker region connecting the β-barrel to the passenger domain. This is the configuration seen for type Va, Vc, and Ve translocation domains (Fig. 4). In contrast, for type Vb, the β-barrel domain and the passenger domain are separate polypeptides, so there is no linker region in the classical sense, though the periplasmic polypeptide transport-associated POTRA domains of TpsB proteins and the TPS domain of TpsAs might play analogous roles to the linker region of the other classes.

In type Va, Vc, and Ve proteins, the translocation domain comprises a 12-stranded β-barrel (Fig. 4). In type Va and type Ve autotransporters, the β-barrel is formed by a single polypeptide chain, whereas in type Vc systems, like the passenger domain, the β-barrel consists of three polypeptide chains. The β-barrels of all three classes are very similar in size, with a pore diameter of approximately 10 Å (17, 89–92).

A remarkable feature of type Va translocation domains is the position of the linker within the β-barrel pore. In the first structure of a translocation domain from a type Va autotransporter, NalP, the linker traverses the pore of the β-barrel from the periplasmic side to the extracellular side and adopts an α-helical conformation (92). Because this structure was obtained from protein refolded from inclusion bodies, the question was raised of whether the position of the α-helical linker within the β-barrel pore might be an artifact of refolding or crystallization. However, other type Va translocation domain structures, including those from EspP and AIDA-I, show the linker positioned similarly in the pore (89, 93) (Fig. 4). The structure of a full-length type Va autotransporter, EstA, with the α-helical linker clearly residing inside the β-barrel, has laid to rest any remaining controversy about the position of the linker (60) (Fig. 3).

Though the linker is clearly inside the β-barrel pore in all type Va translocation domain structures, there are differences in the size and orientation of the α-helical linker. In NalP, Hbp, AIDA-I, and the full-length EstA, the linker traverses the entire length of the pore and extends some way into the extracellular space (60, 92, 93). In EspP, an autocatalytic reaction within the β-barrel leads to cleavage of the linker and release of the passenger domain into the extracellular medium.

**FIGURE 4** (A) Experimental structures of translocation units of type V secretion systems. Examples from type Va (classical autotransporters), type Vb (TpsB proteins), type Vc (trimeric autotransporter adhesins), and type Ve (inverse autotransporters) are included. Structures are shown in cartoon representation. β-barrel domains are blue, linker regions or intrabarrel α-helices are yellow, significant extracellular loops are brown, periplasmically located domains and extensions are green, coiled-coil stalks are red, and connector elements are cyan. For type Vc proteins, each chain is colored with a slightly different hue. All proteins are oriented such that the extracellular face of the β-barrel is pointing toward the top of the page, and the approximate positioning of the outer membrane is shown in gray. Note that for type Va and type Vc translocation domains, the N-termini of the proteins are extracellular, whereas for types Vb and Ve, the N-termini are periplasmic. Type Va translocation domains are represented by structures from NadP (PDB ID: 1UYN), EspP (2QOM), and AIDA-I (4MEE). TpsB proteins are represented by PhaC (2ODZ), with two POTRA domains. Type Vc translocation domains are exemplified by the YadA (2LME) and Hia (3EMO) structures. The intimin translocation domain (4E1S) and LysM (2MPW, with additional α-helix highlighted) represent type Ve translocation and periplasmic domains. (B) β-barrel proteins involved in the biogenesis of type V-secreted proteins, BamA and TamA. The structures of TamA (4C00) and BamA (4C4V) from *E. coli* are also shown, with the unstable β-strand 16 highlighted (the coloring otherwise corresponds to panel A). TamA has three N-terminal periplasmic POTRA domains. The *E. coli* BamA has five POTRA domains; one is part of the β-barrel structure and the other four have been crystallized separately (2QDF). The approximate span of the outer membrane is shown as a gray bar.
The remaining stub of the linker forms a short α-helix, but this lies perpendicular to the axis of the pore (Fig. 4).

To date, there are only structures from two type Vc translocation domains. These are the crystal structure of the Hia translocation domain and the solid-state NMR structure of the YadA membrane anchor (17, 91). The structures are almost completely superimposable, showing the high degree of structural conservation in the domains. They are also similar in general appearance to the type Va translocation domains, the major difference being the trimeric quaternary structure of the β-barrel and the three chains of the linker passing through the pore of the barrel. Like the linkers of type Va translocation domains, the linkers of type Vc systems also adopt a mostly helical conformation, which forms a canonical left-handed coiled coil when exiting the pore (17, 94). Interestingly, the residues at the transition point into the left-handed coiled coil (termed the ASSA region) show a marked drop in α-helical propensity (17).

In type Va and type Vc autotransporters, the linker is immediately N-terminal to the β-barrel domain. In the type Ve autotransporters intimin and invasin, the linker is C-terminal to the β-barrel, i.e., the topology is inverted (90). In addition, there is a short periplasmically located α-helical stretch between the linker region and the β-barrel. In contrast to type Va and type Vc autotransporters, the linker is not in an α-helical conformation but is an extended chain traversing the β-barrel pore (Fig. 4).

In contrast to the 12-stranded β-barrels of type Va, Vc, and Ve autotransporters, the TpsB proteins of type Vb secretion systems consist of a 16-stranded β-barrel and associated periplasmic POTRA domains. The only TpsB protein structure to have been solved to date is FhaC, the transporter partner of FHA (95) (Fig. 4). In the structure, the pore of the FhaC β-barrel is occluded by the large extracellular loop L6, which reaches inside the pore and extends almost to the periplasmic face of the β-barrel. In addition, an N-terminal α-helix (H1) inserts into the pore from the periplasmic side, thus plugging it. However, H1 is mobile and probably moves out of the pore during FHA secretion (96, 97). FhaC contains two periplasmic POTRA domains, discussed below. The transmembrane domain of type Vd secretion systems is predicted to be a 16-stranded β-barrel, like it is for type Vb systems. In addition, these proteins are predicted to contain a single periplasmic POTRA domain (98); however, no structural verification of these predictions is yet available.

The TpsB proteins are homologous to BamA, the central component of a multiprotein complex, the β-barrel assembly machinery or Bam complex, responsible for the insertion of all transmembrane β-barrel proteins into the outer membrane (99, 100). Because BamA has been implicated in the biogenesis of autotransporter proteins (25, 101–105), we include a short discussion on the structure of this protein. Like FhaC, BamA consists of a 16-stranded β-barrel (106–108) (Fig. 4). Unlike in FhaC, the large L6 loop does not penetrate deep into the pore of the β-barrel but, rather, covers the extracellular side of the pore by forming a dome-like structure with the other extracellular loops. Another notable feature is that β-strand 16 of the barrel is destabilized in two of the four available structures, leading to a shortened hydrophobic sheet on one side of the β-barrel (Fig. 4). This may lead to perturbation of the outer membrane. In addition, the destabilized β-strand may serve as a lateral gate that allows insertion of nascent β-barrel proteins into the outer membrane, possibly involving a hybrid barrel intermediate, but the details of the insertion mechanism remain under debate (106, 109). Enterobacteria encode an additional Bam homologue referred to as TamA, which together with an inner membrane protein TamB forms a complex implicated in the biogenesis of a subset of type Va autotransporters (110). The structure of TamA has been solved, and the 16-stranded β-barrel structure resembles BamA in both the extracellular dome and the potential for lateral opening via a destabilized C-terminal β-strand (111).

Periplasmic Domains

Most type Va and type Vc autotransporters have the translocation unit at their C-terminus; thus, they do not contain any appreciable periplasmic regions. However, some type Vc systems are predicted to have extensions C-terminal to the translocation unit, which would form coiled coils in the periplasm (31). Currently, there are no structures of these putative periplasmic regions. In contrast to type Va and type Vc autotransporters, where periplasmic domains are rare, all type Ve proteins appear to have periplasmic extensions (10, 112). These are usually quite short (<50 residues) and comprise only two predicted α-helices. Some proteins from the Enterobacteriaceae have larger periplasmic regions containing a lysin motif (LysM), a peptidoglycan-binding mini-domain. The structure of the LysM from intimin was recently solved by NMR; it contains two α-helices packed against an antiparallel β-sheet, common to other LysMs, but in addition the intimin LysM has a third...
short α-helix preceding the C-terminal β-strand (10) (Fig. 4).

TPSB proteins and type Vd autotransporters contain periplasmic POTRA domains, as do their homologues BamA and TamA. POTRA domains are small, globular domains of approximately 80 residues (113). The fold consists of a three-stranded β-sheet packed against two α-helices with the topology β-α-α-β-β (Fig. 4). POTRA domains from different proteins and even the same protein share only low sequence similarity, and conserved regions are mostly limited to the hydrophobic core of the fold (114). The number of POTRA domains in different BamA homologues varies; in enterobacteria, BamA has five POTRA domains, but the number can vary from three to seven in other phyla (114). TamA has three POTRA domains, TpsBs such as FhaC have two, and type Vd proteins have a single POTRA domain (95, 98, 111). POTRA domains are often connected by linkers that can confer flexibility to the domains (115). POTRA domains interact with substrate proteins (nascent outer membrane proteins in the case of BamA, TpsAs for TpsBs), possibly through β-augmentation (115, 116), but it is worth noting that the detailed function of the POTRA domains is under debate.

Full-Length Fibers
The only extant experimental structure of a full-length autotransporter is that of EstA (60) (Fig. 3). EstA is an unusually small type Vα autotransporter, and its passenger domain is not a β-solenoid structure, but adopts an all-α fold similar to GDSL lipases.

Although no experimental structures of other complete autotransporters are available, the existing structures of fragments have allowed modeling of several fibers. This has been particularly successful for the trimmeric autotransporters (type Vc systems), whose modular nature has allowed crystallizing fragments and connecting these later in silico in combination with homology modeling. Using this approach, the group of Andrei Lupas has provided complete models for the relatively short prototypical TAA YadA and for the much longer and architecturally more complex TAA SadA from Salmonella and its two E. coli orthologues, EhaG and UpaG (16, 61). The YadA model shows the simple lollipop-like shape of the molecule, with the globular head projected away from the outer membrane by the coiled-coil stalk. The C-terminal β-barrel domain anchors the protein in the outer membrane (Fig. 3). In contrast, the model for the SadA fiber is much more elaborate, with two head domains connected by coiled-coil regions interrupted by connectors (Fig. 3). This exemplifies the modular make-up of many of the larger type Vc autotransporters.

In the case of TPS proteins, a full-length structure containing both the translocation unit and the passenger domain is not possible, because the two functional domains are separate polypeptides that interact only transiently during export. In this sense, a full-length structure of the TpsB moiety does exist, represented by FhaC. Sequence analysis has allowed modeling of the entire FHA structure, which forms a long, parallel β-helix (62, 117) (Fig. 3).

MOLECULAR BIOLOGY ANALYSIS: POINT MUTATIONS AND DELETIONS OF GENES
Mutagenesis is widely used to decipher parts of genomes and to understand the function of individual genes and proteins. A better understanding of the type V secretion systems has been obtained by introducing insertions, deletions, and point mutations into genes encoding these proteins.

Gene Inactivation and Plasmid Complementation
Reverse genetics is an approach to studying the function of a gene by studying the phenotypic effect of specific known DNA sequences. It has been used to characterize many type V secretion genes in various Gram-negative bacteria. This approach involves inactivation of the putative gene followed by a number of assays to study phenotypic changes. The following paragraphs discuss examples of various type V systems studied by this method, to show the potential of the method to study mainly the function of these systems.

BcaA (type Vα) of Burkholderia pseudomallei was characterized by creating large in-frame deletions in the gene. Plasmid complementation is a method in which a functional copy of a gene is encoded on a plasmid that is transformed into cells where the gene was deleted, to check if the function is fully restored. This is considered the “gold standard” in deletion analysis, because it helps to distinguish direct from indirect effects of the deletion of a gene. In the case of BcaA, plasmid complementation showed that it is required for efficient plaque formation. In vivo studies indicated that BcaA is also required for efficient dissemination and survival of bacteria in spleen (118). In a less targeted approach, transposon mutagenesis with TnphoA was used to identify potential genes encoding surface-exposed virulence factors in enterotoxigenic E. coli. TnphoA is a
derivative of the Tn5 transposon, which also contains a modified alkaline phosphatase gene lacking a promoter and signal sequence. This construct, when inserted in a frame, leads to chimeric proteins, and if the fused protein has a signal sequence, then the alkaline phosphatase activity is restored in the periplasm. Thus, it helps in localizing export signals within a protein and also in identifying secreted proteins or transmembrane proteins (119). Using this approach, the virulence factor EatA belonging to the serine protease family of type Va transporters was identified in E. coli (120). Similarly, random transposon mutagenesis was used to create a mutant library in Fusobacteria species followed by phenotypic screening and identification of virulence traits. Mutants were screened for loss of hemagglutination, and the gene responsible was identified as fap2, an autotransporter protein (121).

The Pdt two-partner secretion system (type Vb) of Pseudomonas aeruginosa was characterized by creating deletion mutants of pdtA and pdtB genes. It was found that the translocation of PdtA across the outer membrane does not occur in the absence of PdtB (122). Similarly, a two-partner secretion system was characterized in Moraxella catarrhalis. MchA1 and MchA2 were found to be secreted into the outer membrane, and some amount was found in the culture medium. Deletion of mchB completely abolished extracellular secretion of both MchA1 and MchA2 (123).

A genomic knockout of the type Vc transporter hemagglutinin of Avibacterium paragallinarum was created to study the function of the gene which is involved in hemagglutination, cell adherence, and biofilm formation (124). To study the mechanism of biofilm formation in Veillonella species, insertional inactivation of eight putative hemagglutinin genes was carried out using a tetracycline cassette. The bag1 gene encodes a YadA-like type Vc transporter that is involved in co-aggregation with other colonizing bacteria and helps in adherence to cells (125).

The product of the eaeA gene of E. coli is also called intimin. It was shown to be membrane-localized and surface-exposed. An intimin-negative mutant still adhered to epithelial cells but failed to form actin pedestals (126). Later studies have shown that intimin belongs to the type Vc class of autotransporters (7).

**Fusions and Exchange of Domains**

Fusion and exchange of domains in autotransporters has been useful in studying the translocation mechanism of autotransporters and also has biotechnological applications, because it can be used for surface display of recombinant proteins (127). The fusion partner size can vary from small peptides to whole proteins. Either the complete autotransporter or just the translocation domain can be used for the fusion, as long as an N-terminal signal peptide is present. Most of such fusion studies have been carried out in type Va autotransporters, though some have also been performed on type Ve (128, 129).

Fusion of the translocation domain of IgA protease (type Va) with cholera toxinB (CtxB) subunit showed outer membrane integration of these fusion proteins as well as surface exposure of ctxB moieties (130). Again using cholera toxin as a fusion partner, AIDA-I-ctxB fusions were designed to identify the minimal unit that would translocate the passenger domain. By stepwise introduction of N-terminal deletions in the passenger domain of AIDA-I, it was found that a minimum linker region between 28 and 48 amino acids is essential for successful translocation (40). Taken together, both studies showed that the N-terminal region of the passenger domain does not play a role in initiating translocation.

The ability of another type Va autotransporter, Pet, to mediate secretion of heterologous proteins was tested by creating fusions with a fluorescent protein (mCherry) and E-SAT6, a diagnostic marker of Mycobacterium tuberculosis. It was shown that the heterologous fusions were expressed and actively translocated to the cell surface. The Pet construct used for expression consisted of the β-barrel, the α-helical linker, and the so-called autochaperone domain, a C-terminal part of the passenger domain essential for proper folding of the complete secreted passenger domain (131). Several type Va autotransporters contain such a folding core in the C-terminal part of the passenger domain, which has been referred to as an autochaperone (132–134). In some instances, the autochaperone can even promote autotransporter folding and secretion when added in trans (132, 135).

The minimum construct length required for translocation was also determined in the Pet study, and it was shown that the autochaperone domain is not required for secretion, but only for folding (131). Following the unfolding of the pertactin passenger domain with circular dichroism spectroscopy revealed a two-step process, where a part of the passenger domain was significantly more stable than the rest of the protein (71). A similar study in the autotransporter Hbp was carried out, again using E-SAT6 as a fusion partner. Substitution of parts of the Hbp passenger domain still gives high-level expression and secretion of the fused protein. It was also found that the Hbp passenger β-helix is not essential for secretion but that its presence improves
secretion and stability of the chimera (136). Likewise, β-lactamase and maltose-binding protein were fused with either the full-length type Va Shigella flexneri transporter, IcsA, or only with its translocation domain. The fused proteins were tested for expression, surface localization, and folding. Fusion of exogenous proteins to full-length IcsA improved expression and surface exposure of the chimeric protein compared to the ones directly fused to IcsA translocation domain, again indirectly suggesting a role of parts of the passenger in proper surface display (137).

Insertion of a calmodulin moiety into the type Va transporter Hbp completely blocks passenger domain translocation in the presence of calcium because it forms a rigid and stable structure (132), indicating that a more or less flexible passenger domain is required for the translocation to be successful. Similar results were observed when calmodulin replaced part of the intimin passenger domain, a type Ve transporter, where calmodulin could only be exported in the presence of a chelator (129). For type Vc transporters, it was shown that heterologous translocation domains can be used for surface display of other trimeric passengers, using, e.g., the Yersinia YadA membrane anchor for export of passengers from Bartonella (133) or the Haemophilus Hia, Moraxella UspA, or E. coli Eib translocators to export the YadA passenger domain (134, 135). Similarly, the passenger domains can be swapped between the two type Ve transporters intimin and invasin, keeping the binding functions intact (138).

Hybrid constructs for surface display have many possible applications in biotechnology. As an example, a complete protein with a binding function, the lectin moiety of FimH, was surface-displayed and was found to be functionally active after expression. It is worth noting that this domain has two disulfide bridges which did not interfere with the translocation process. (139). Similarly, constructs consisting of the translocation domain of the N. gonorrhoeae type Va transporter IgA and leucine zippers of eukaryotic transcription factors Fos and Jun were expressed in E. coli. The E. coli cells showed a novel adherence trait that resulted in clumping and self-association of cells in liquid media. This and other studies showed that the biotechnological possibilities of autotransporter surface display are not limited to prokaryotic proteins (140). Ag43 of E. coli was used to display immunogenic epitope tags such as CTP3, a conformational loop of CtxB, and Chlam12, a 12-residue epitope of the Chlamydia trachomatis DnaK protein. The site of fusion was within the passenger domain of Ag43. The immunogenic tags were found to be correctly folded and well displayed. In the same way, epitopes but also complete proteins can be displayed on the surface via type Ve secretion systems such as E. coli intimin and Yersinia invasin (7, 128). Thus, type V systems in general are useful for the surface expression of both small peptides and larger proteins.

Insertional Mutagenesis and Point Mutations
To study the contribution of specific amino acids or small subdomains toward the function or structure of a protein, point mutations or short stretches of additional residues can be deliberately introduced in the gene encoding the protein. These approaches have been widely used to study the function of autotransporter passengers but also to study the transport mechanism as such.

An insertion is the addition of nucleotide base pairs into the DNA sequence of a gene and has been useful in studying the structure-function relationship in autotransporters. In a technique called linker scanning mutagenesis the gene is systematically scanned by inserting a linker in various regions (141). A study of the type Va autotransporter, Pet, using a transposon-based in-frame insertion kit which randomly inserts a 19-amino-acid linker into the gene, revealed that linker insertions in the N-terminal half of the passenger domain resulted in lower levels of secretion and aberrantly folded protein, whereas insertions in the C-terminal half of the passenger domain abolished secretion. This study helped in predicting regions of the passenger domain with functions such as protease activity or cell-binding activity but also identified a region essential for the secretion of the passenger domain (142). A penta-peptide linker scanning mutagenesis of another type Va autotransporter, IcsA, showed similar results: insertions in the C-terminal half of the passenger domain led to aberrant production of the protein compared to the wild type. For some mutants, the protein level was restored to wild type when expressed in protease-deficient E. coli UT5600, indicating that the protein may be susceptible to degradation by an endogenous outer membrane protease (143).

Insertions of immunogenic tags can also obstruct the transport process in various type V systems, e.g., in the type Va autotransporter Pet, where a hemagglutinin (HA) tag was used. Although the tag is small in length, the obstruction probably occurs due to the bulky and rigid nature of the tag (144). Similarly, an HA tag introduced after position 453 in the type Ve autotransporter intimin resulted in a stalled translocation intermediate. It was shown that the β-barrel was well inserted into the membrane while the passenger domain was unfolded. The HA tag was accessible by antibodies.
in unpermeabilized cells, whereas the C-terminus of the passenger domain was not. The C-terminus was in turn stained with antibodies only upon permeabilization of cells with detergent. This strongly suggests the presence of a hairpin-like conformation of the stalled translocation intermediate (105).

Point mutations in the C-terminal segment of the EspP passenger domain are predicted to disrupt the packing of the hydrophobic core and obstruct folding and completion of translocation (145), whereas point mutations introduced in the other segments of the EspP passenger domain do not significantly affect the translocation efficiency or structure of the protein, indicating that the C-terminal region of the passenger domain is more important—if not essential—for folding (146), at least in this case, and it has been suggested that this region has an autochaperone function, similar to that in the type Va system Pet mentioned above (131). In another study of Pet, paired cysteine residues were systematically introduced, and the result indicated an inverse relationship between secretion efficiency and size of the disulfide-bonded loop. The longest disulfide bond that did not interfere with secretion consisted of 18 residues, and a loop consisting of 20 or more amino acids stalled the translocation process, indicating that large covalently constrained elements do interfere with the translocation process (144). In the case of the type Va autotransporter Hhp, a disulfide bond between closely spaced cysteine residues also did not interfere with translocation efficiency, whereas distantly placed cysteine residues resulted in a stalled translocation intermediate (132).

A number of residues are conserved in the translocation domain(s) of type V secretion systems. In a study of the type Va transporter Tsh, highly conserved residues among the SPATE family of autotransporters were mutated: nonaromatic hydrophobic residues were changed to tyrosine or alanine. Some of the mutations impaired secretion of the passenger domain into the culture medium and resulted in the accumulation of unprocessed protein in the bacterial outer membrane. It was noticed that the side chains of these residues face the interior of the barrel and therefore might be involved in passenger cleavage or proper positioning of the α-helical linker for cleavage (147). When periplasmic fractions of mutants showing this defective phenotype were screened, only the processed form of Tsh was observed, which according to the authors suggests that the β-barrel already obtains a certain folded conformation before insertion into the outer membrane. Based on these results, a model has been proposed where a proto-β-barrel like structure forms in the periplasm and is stabilized by the formation of α-helical linker in the β-barrel (147). In another study supporting such a proto-β-barrel model, tomato etch virus sites and single cysteine residues were introduced in the junction between the translocation domain and passenger domain of the E. coli type Va autotransporter EspP. Tomato etch virus protease accessibility and accessibility toward a cysteine-specific biotinylation agent were measured, and no differences in accessibility were observed between stalled transport intermediates and fully assembled (wild-type) protein. Cell fractionation experiments suggested that the assembly intermediate forms in the periplasm (148).

Mutation of a conserved glycine residue to larger residues in the type Vc autotransporter YadA resulted in decreased stability of the protein and degradation by DegP protease. When the mutants were grown in a DegP-deficient strain, YadA could reach the cell surface, indicating that the highly conserved glycine is important for efficient transport, probably by providing enough space in the translocation pore (32). In the type Ve transporter invasin and intimin, the same conserved glycine residue was found, and mutations disrupted surface display of the passenger in a similar way, but no significant degradation of the protein was evident (7). Inside the pore of the translocation domain of autotransporters, conserved pairs of aromatic residues and glycine residues are perfectly positioned in neighboring β-strands so that the large aromatic residue can intercalate in the space made available by the small glycine residue. Such motifs are known as mortise-tenon motifs. Mutation of such conserved glycines (mortise motifs) to alanine in the type Va transporter Pet resulted in a slower rate of surface exposure as well as a decreased rate of autocatalytic processing. The results indicate that these motifs allow correct and efficient folding of autotransporters (149). Mutations of hydrophobic residues in the lumen of the translocation domain β-barrel of the type Va transporter BrkA to charged residues such as aspartate or lysine resulted in decreased translocation efficiency without affecting the ability of BrkA to localize in the outer membrane (150). The results of this study support a hairpin model of translocation for a passenger domain, where the presence of hydrophobic patches would provide a suitable environment for anchoring a hairpin-like hydrophobic structural motif.

**Additional Factors Involved in Autotransporter Assembly**

Type V secretion systems, and especially the classical type Va autotransporters, were previously believed to be...
self-sufficient for their secretion and insertion into the outer membrane. Later studies showed the involvement of a number of proteins which aid in the assembly of autotransporters, many of them periplasmic chaperones. Double (deletion) mutants of the periplasmic chaperone factors skp, degP, and surA can be created in all possible combinations, but a triple mutant resulted in a synthetic lethal phenotype (153). Depletion of SurA resulted in reduced levels of the outer membrane proteins LamB and OmpA, whereas depletion of Skp and DegP did not cause these effects, suggesting that SurA plays a major role in the assembly of outer membrane proteins, whereas Skp and DegP act in a rescue pathway for proteins that did not assemble normally (152).

The _S. flexneri_ type Va transporter IcsA was found to be less accessible on the surface when expressed in a _degP_ mutant, although the amount of IcsA in the outer membrane was comparable to the wild-type levels, indicating a defect in proper insertion of the protein in the outer membrane (153). Yeast two-hybrid analysis and surface plasmon resonance analysis have shown SurA, Skp, and DegP to directly interact with the type Va autotransporter EspP (102, 154). However, _in vitro_ studies indicate that chaperones are not always required for type Va autotransporter biogenesis. In the case of _E. coli_ AIDA-I, refolding of the translocation domain was not enhanced upon transfer from urea to refolding conditions with chaperones such as Skp, DegP, and SurA (155). Note also that although the IcsA passenger domain was less efficiently translocated in _surA, skp_, and _degP_ mutants, the protein still localized to the outer membrane (153, 156). Similarly, the secretion of the EspP passenger domain was reduced in _surA, degP_, and _skp_ mutants, but the translocation domain levels in the outer membrane were comparable to the wild type (154). Involvement of the same periplasmic factors has to some extent also been shown for type Vc and type Ve autotransporters. The type Vc transporter YadA is degraded by the chaperone/protease DegP under over-expression conditions when the passenger translocation is impaired (32), and YadA interacts with periplasmic chaperones as well as with the Bam complex during its biogenesis (23). The same is true to some extent for the type Ve systems invasin and intimin (7, 105). The type Vc transporter SadA from _Salmonella_ requires an additional, specific export factor, SadB, for its biogenesis. The genes _sadA_ and _sadB_ are organized in an operon structure. SadB is a trimeric, inner membrane lipoprotein, suggesting that it helps in trimerization of the type Vc system before translocation of the passenger (15).

**IN VITRO ASSAYS TO STUDY MEMBRANE TRANSLOCATION**

The _in vitro_ reconstitution of protein translocation pathways is a powerful tool to identify the components required for a specific translocation mechanism and to study the details of the translocation process. In principle, four distinct types of model membrane systems exist for such reconstitution assays: lipid monolayers, supported lipid bilayers, liposomes, and lipid nanodiscs (157). Until today, only the type Va and Vb secretion pathways have been reconstituted successfully using liposomes (Fig. 5), solid-supported membranes, and nanodiscs (104, 158–160), while the reconstitution of type Vc, Vd, and Ve pathways has not been reported yet.

The C-terminal translocation domain of type Va secretion systems was originally believed to be the sole mediator of outer membrane translocation of its N-terminal passenger domain, which led to the term “autotransporter.” However, this concept was challenged by the findings that only 12 β-strands form the β-barrel of the translocation domain, and the diameter of the formed pore is only ≈10 Å according to the available crystal structures (89, 92). It has been suggested that this pore is too narrow to accommodate type Va-secreted, folded domains (132, 161, 162). Further investigations showed that BamA depletion in _S. flexneri_ interferes with the secretion of the passenger domains of type Va systems (101), suggesting that the Bam complex is involved in the biogenesis of type Va systems, a concept that was further supported by the observation that Bam complex components could be photo-cross-linked to type Va transporters _in vivo_ (102). However, it remains unknown if the secretion defect caused by BamA depletion is a direct or an indirect effect.

To complicate the matter, a recently identified translocation and assembly module (TAM or Tam complex) seems to be involved in the secretion of some type Va proteins in proteobacteria (110, 160). It is still unclear if the Tam and Bam complexes function individually or have to cooperate with each other to mediate the secretion of all or only a subset of autotransporters, but _in vitro_ biochemical reconstitution assays have shed some light on the matter. With the help of reconstituted liposomes containing the complete, purified _E. coli_ Bam complex, it was demonstrated that two type Va systems, EspP and Ag43, depend on the presence of Bam to be successfully translocated through the membrane _in vitro_ (104, 159). Furthermore, though the exact function of each Bam component during type Va translocation is unknown, at least BamB and BamC are not essential
for the process, because the lack of both components in the reconstituted liposomes merely resulted in a less efficient transport of EspP (104). Further analysis using a nanodisc-based strategy demonstrated that a single copy of the Bam complex can mediate the biogenesis of EspP and that the translocation domain assembly seems to be the rate-limiting step in the whole process (104).

In a similar approach, liposomes were used to analyze the translocation mechanism of type Vb secretion systems, using the filamentous hemagglutinin FHA of *B. pertussis* as a model system. When the type Vb translocation component of FHA (FhaC) was reconstituted into liposomes to perform swelling assays, it formed a channel. The swelling rate correlated positively with the amount of reconstituted FhaC but negatively with the size of the sugars, suggesting that the reconstituted pore is at least large enough to transport sugar molecules (163). In a similar study, the calculated pore diameter of the type Vb translocator protein HMW1B *H. influenzae* was ~1.9 nm (164). In single-channel conductance measurements using planar lipid bilayers to determine the electrophysiological properties of the channel, a comparable channel conductance in 1 M KCl was observed both for FhaC (1,200 pS) and HMW1B (1,400 pS) (96, 165). In agreement with liposome swelling analysis, these electrophysiological measurements show that the translocator proteins of type Vb are able to form conductive pores, but with low open probability. It is interesting to mention that for purified translocator domains of type Va proteins, including Hbp (166), NalP (92), and IgA (167), liposome swelling assay and electrophysiological measurements suggest that these translocator domains have pore activity as well. Assuming these domains form perfect cylinders, their pore diameters can be calculated to be ~0.8 nm or ~2.1 nm depending on the method used, in line with the diameter of regular β-barrel outer membrane proteins (166, 168).

In a cell-free *in vitro* system, in which purified FhaC was reconstituted into liposomes and combined with

**FIGURE 5** Overview of the liposome-based *in vitro* system for type Va and Vb analysis. The test substrates are prepared as urea-denatured, purified proteins or by using spheroplasts. Translocation may need additional factors added to the proteoliposome mix, such as chaperone(s) for urea-denatured substrates. The success of the translocation is then monitored either by protease accessibility assays or by checking heat-modifiability (a unique feature of bacterial β-barrels).
a fragment of the FHA passenger that was previously produced in and secreted from spheroplasts, FHA was translocated without the addition of any other protein component. Even fragments of FHA that contained all the necessary information required for outer membrane translocation in vivo (81) were successfully translocated into the lumen of liposomes by reconstituted FhaC (159). Likewise, it was shown that for a well-studied heterologous yet complementary type Vb pair—a passenger protein called HpmA (a hemolysin from Proteus mirabilis) and a translocator protein called ShlB from Serratia marcescens—full translocation could be reproduced using this liposome strategy (159). Taken together, these in vitro reconstitution assays of type Vb secretion systems clearly demonstrate that a single TpsB outer membrane protein is sufficient to enable the outer membrane translocation of its cognate TpsA substrates.

In vitro reconstitution systems not only provide the opportunity for in-depth analysis of the molecular details of the processes of β-barrel assembly and the passenger domain translocation of type V secretion pathways, but also make it possible to address the functions of chaperones and other more peripheral components in type V secretion. The assembly of urea-denatured EspP from E. coli can only be successfully reconstructed in vitro in the presence of the periplasmic chaperone SurA, while another chaperone, Skp, seems to inhibit the biogenesis of EspP under the reported experimental conditions (104). This is somewhat in contrast to earlier reports that Skp prevents misfolding, while SurA promotes the folding of β-barrel outer membrane proteins (169, 170).

**DISCUSSION**

**What Drives the Transport Process?**

The outer membrane of Gram-negative bacteria has no direct access to cytosolic energy sources such as ATP, and it lacks an electrochemical gradient of protons or other ions, simply because it is full of pore proteins that allow free diffusion of small substrates into and out of the periplasm. The most probable driving force for type V secretion is thus the free energy gained by folding the passenger domain on the cell surface, which probably also includes entropic effects that are based on molecular crowding in the periplasm. It has been shown that passenger domains of various type V systems are slow-folding entities (171), presumably to avoid premature folding in the periplasm. At the same time, they do not seem to aggregate easily when unfolded, which would be an advantage for slow-folding proteins and would give enough time for a complex transport process. The overall negatively charged nature of passenger domains has been implicated as an additional factor (172).

**Is Type V Secretion a Uniform Mechanism that Just Comes in Different Flavors (from Type Va to Type Vb)?**

To be able to answer this question, it is important to understand the hairpin model for the transport process (Fig. 6). When looking at monomeric autotransporters of type Va, there are in principle two ways of pushing an attached polypeptide chain through the pore: (i) the attached chain can go into the pore “head-first,” with the advantage that only one unfolded polypeptide chain is in the translocation pore at any given time but with the disadvantage that finding the entrance to the pore is probably difficult for a passenger that can be hundreds—or even thousands—of residues long. (ii) The attached chain can enter the translocation pore as a hairpin (“tail-first”): in this case, two unfolded polypeptide chains are in the translocation pore at any given time until the transport process is concluded and the head reaches the cell surface, leading to potential problems with space in the very small pore but solving the problem of how the passenger finds the entrance to the translocation domain in the first place. Both of these models work under the assumption that the translocation pore is formed first and that transport of the passenger proceeds after this step.

The hairpin model is strongly favored based on several lines of evidence. The passenger domains in type Va systems have their folding core at their C-terminus, strongly suggesting that folding proceeds from the C- to N-terminus. This was demonstrated for various exemplars of type Va systems, including pertactin (71), Hbp (173), and EspP (145). Moreover, the so-called autochaperone domain of many type Va autotransporters at the very C-terminus of the passenger domain catalyzes (or at least initiates) the folding of the rest of the passenger (174, 175), again speaking for a stepwise process that starts at the C-terminus. Cysteine scanning mutations leading to stalled autotransport intermediates showed that the C-terminal part of the passenger domain passes the autotransporter pore first (161). On top of this, the unusually long signal peptides observed in many type V-secreted proteins function as a retention signal at the Sec machinery, presumably to allow the passenger domain to initiate translocation—and possibly to initiate folding on the cell surface—prior to
FIGURE 6 Models of outer membrane insertion and passenger secretion in type Va autotransporters. (A) Models of membrane insertion. Membrane insertion of autotransporters (and other outer membrane β-barrel proteins) depends on BamA (depicted in light gray). The autotransporter itself is shown in dark gray. Currently, three models are envisaged to explain membrane insertion and initiation of autotransport. In model 1, BamA catalyzes the insertion of the β-barrel domain of the autotransporter, after which hairpin formation and passenger domain secretion proceed autonomously. In model 2, membrane insertion and hairpin formation happen concomitantly. A third model (model 3) has been proposed, where a semifolded “protobarrel” already containing the hairpin is formed in the periplasm. This is then inserted into the outer membrane by BamA. All three models lead to the autotransporter β-barrel being folded and inserted into the outer membrane and passenger secretion proceeding via a hairpin from the C- to the N-terminus (model 4). Note that BamA may also be involved in passenger secretion (see panel B). Small black arrows depict the direction of secretion; large light arrows depict the flow of events. The N-terminus of the autotransporter is denoted by an N for clarity.

(B) Models of passenger secretion. Two models exist where passenger secretion is autonomous, i.e., genuine autotransport (models 1 and 2). In the threading model (model 1), the passenger is secreted through the pore of the autotransporter β-barrel (in dark gray) N-terminus first. The hairpin model (model 2) is preferred over this because it is more in line with current biochemical evidence (see text); here, the C-terminus of the passenger domain (the linker region) forms a hairpin within the pore of the autotransporter β-barrel. Secretion then proceeds C to N. In the third model of passenger secretion, a secondary protein (BamA or TamA, in light gray) assists in passenger secretion, possibly by forming a hybrid barrel with the autotransporter as depicted here. Small gray arrows depict the direction of secretion; the N-terminus of the autotransporter is denoted by an N for clarity.
cleavage of the signal and release from the inner membrane (52). Taken together, all of this biochemical evidence strongly favors the hairpin over the threading model for type Va autotransport (Fig. 6).

Based on the fact that all type V translocation domains are homologous to the larger family of outer membrane β-barrel proteins (42), it is safe to assume that they follow the same route of outer membrane insertion, by interacting with the Bam complex. The question is whether the translocation pore—after membrane insertion—can autonomously transport the passenger or whether this process is also mediated by BamA (or TamA). While the hairpin model described above might still be valid (and seems proven at least for type Va systems), the direct interaction of autotransporters with different parts of the Bam complex has thus added a different level of complexity to the system. Current models of transport range from autotransport to the insertion of a hairpin intermediate by BamA to a complete process run by BamA (or its paralogue TamA), where a larger, fused pore is formed as an intermediate (Fig. 6).

In this context, it is interesting to look at type V systems other than just the well-studied type Va ones. The membrane insertion of the type Vb translocator protein requires the Bam complex (159), but at the same time, translocation of the passenger is independent of the Bam complex when the translocation pore is already present (158). On the one hand, this could suggest that the only requirement for type V secretion is a membrane-inserted pore, in line with a unifying model for autotransport where the Bam complex inserts the pore, but the passenger translocation is an autonomous process. On the other hand, type Vb translocation pores themselves are very similar to BamA: they include POTRA domains, and they are 16-stranded barrels and thus have larger pores compared to the 12-stranded barrels of type Va or Ve systems. Some models suggest that the Bam complex creates a fused pore with the Va and Ve translocation domains to achieve a larger pore. But space is even more scarce in type Vc translocation domains, where three polypeptide chains need to be transported in parallel through the 12-stranded barrel. In these highly intertwined structures, it is hard to see how one or, alternatively, three Bam complexes could assemble the type Vc trimeric barrel after individual passenger transport of three such fused pores is completed.

The physical connection of the passenger domain with the translocation domain in Va systems might relieve the requirements for a molecular recognition event such as by the POTRA domains, which is essential in type Vb systems. But if this is true, what is the function of the POTRA domain present in type Vd secretion systems, which are in principle a fusion of a 16-stranded TpsB (the translocation pore), a POTRA linker, and a TpsA (passenger) domain?

There might be a unifying model that explains type V secretion. There seems to be no doubt that all systems (with the possible exception of type Vb or at least some of its variants [5]) use a hairpin intermediate to initiate translocation of the passenger domain and that the Bam complex (or its paralogue, the Tam complex) plays a role in membrane insertion of the translocation pore. An open question in the field of type V secretion is whether there are significant differences between the involvement of Bam or Tam in the biogenesis of the various subclasses and whether Bam or Tam are also directly involved in the later steps of passenger secretion.

REFERENCES


