From self sufficiency to dependence: mechanisms and factors important for autotransporter biogenesis

Denisse L. Leyton[1,2], Amanda E. Rossiter[1] and Ian R. Henderson[1]*

Abstract | Autotransporters are a superfamily of proteins that use the type V secretion pathway for their delivery to the surface of Gram-negative bacteria. At first glance, autotransporters look to contain all the functional elements required to promote their own secretion: an amino-terminal signal peptide to mediate translocation across the inner membrane, a central passenger domain that is the secreted functional moiety, and a channel-forming carboxyl terminus that facilitates passenger domain translocation across the outer membrane. However, recent discoveries of common structural themes, translocation intermediates and accessory interactions have challenged the perceived simplicity of autotransporter secretion. Here, we discuss how these studies have led to an improved understanding of the mechanisms responsible for autotransporter biogenesis.

Translocation
The movement of proteins from one subcellular location to another (including movement to outside the cell).

Signal peptide
A short sequence found in nascent proteins that directs their transport to a particular subcellular location.

Signal peptidase
An endopeptidase that removes the signal peptide after protein translocation.

[1]*School of Immunity and Infection, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK.*
[2]*Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria 3800, Australia.*
**Present address.*
Correspondence to D.L.L.
e-mail: denisse.leyton@monash.edu
doi:10.1038/nrmicro2733

Proteins destined for secretion from Gram-negative bacteria are confronted with a formidable series of obstacles posed by the cell envelope. Consequently, Gram-negative bacteria have evolved seven secretion pathways (types I–VI and the chaperone–usher pathway)[1] that facilitate navigation through the inner membrane, periplasm and outer membrane. Most of these pathways use specialized machineries that vary greatly in their complexity, structural features and mechanism of protein translocation. The secretion mechanisms that constitute the type V secretion pathway are the simplest and most widely used mechanisms for the delivery of proteins to the surface of Gram-negative bacteria. These pathways comprise proteins secreted by the classical autotransporter (AT) system (also known as the type Va system), the two-partner secretion (TPS) system (also known as the type Vb system) and the trimeric AT adhesin (TAA) system (also known as the type Vc system)[2]. Proteins that exploit these pathways possess sequence and structural similarities and have comparable modes of biogenesis (FIG. 1).

The term AT was initially coined on the supposition that all the functional elements required for secretion were contained in the protein, such that the amino-terminal signal peptide mediates translocation across the inner membrane in a Sec-dependent manner, the central passenger domain is the secreted functional moiety (see Supplementary information S1 (table)) and the carboxyl terminus (the β-domain) forms a β-barrel in the outer membrane that is vital for translocation of the passenger domain to the bacterial cell surface[2,3]. Despite numerous investigations, the mechanism of AT biogenesis remains contentious at the molecular level. In this Review, we examine recent findings that challenge the perceived simplicity of the AT pathway, and discuss the structural themes, translocation intermediates and accessory factors that give new insight into the mechanisms that are important for the biogenesis of classical ATs.

Translocation across the inner membrane

As for all proteins destined for secretion, the genes encoding ATs are transcribed in the cytoplasm. The mechanisms regulating transcription of AT-encoding mRNAs are diverse and sometimes complex (BOX 1). After transcription, the mRNA is translated into a single polypeptide (FIG. 2) that is targeted to the inner-membrane SecYEG translocon, which catalyses their energy-driven export into the periplasm (FIG. 1). Most ATs are synthesized with characteristic Sec-dependent signal peptides, which are typically 20–30 residues in length and possess a tripartite organization comprising a positively charged N domain, a hydrophobic H domain and a C domain with a recognition site for a signal peptidase. The membrane-spanning H domain is thought to insert into the lipid bilayer and, together with the N domain, to function in recognition of the signal peptide by the Sec translocon. Signal peptidases recognize the uncharged residues at positions –1 and –3 relative to the cleavage site[4].

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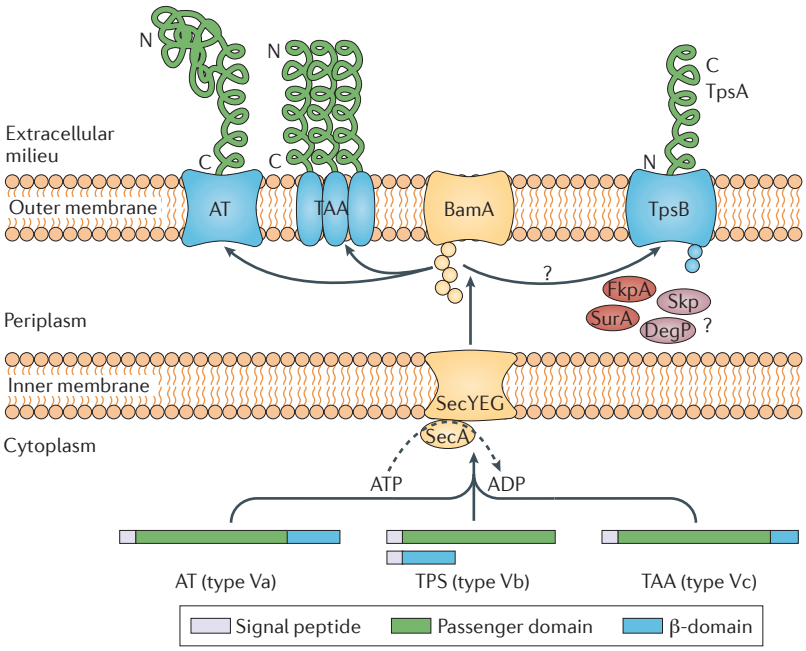

Figure 1 | **Secretion mechanisms that constitute the type V secretion pathway.** These pathways involve proteins that are secreted by the classical autotransporter (AT) system (also known as type Va secretion), the two-partner secretion (TPS) system (also known as type Vb secretion) and the trimeric AT adhesin (TAA) system (also known as type Vc secretion). For each protein, a superficial tripartite organization is shown, and in all cases the amino-terminal signal peptide mediates inner-membrane translocation in a Sec-dependent manner, the central passenger domain is the secreted functional moiety and the carboxyl terminus forms a β-barrel in the outer membrane that is vital for translocation of the passenger domain to the bacterial cell surface. Here, the proteins may or may not undergo further processing. In the TPS system, the passenger domain or exoprotein (the TpsA partner) and the pore-forming β-barrel (the TpsB partner) are translated as two separate proteins that are translocated independently across the inner membrane, a process that is mediated by their respective signal peptides[7]. By contrast, although the TAAs are synthesized as one polypeptide, their carboxyl termini encode only one-third of a β-barrel, so these proteins must trimerize to form a functional β-barrel in the outer membrane for the secretion and subsequent folding of the passenger domain into a trimer[62]. Although β-barrel assembly machinery protein A (BamA) is essential for the insertion of classical ATs and TAAs into the outer membrane, it is not known whether BamA is required for the insertion of TpsB homologues. Furthermore, it is apparent that different ATs require different chaperones (SurA, Skp, DegP and FkpA) as they transit the periplasm, and perhaps some ATs are reliant on one more than others.

Approximately 10% of all ATs, as well as a subset of TpsA (the secreted partners in TPS systems) and TAA proteins, possess unusually long signal peptides (roughly 50–60 residues) that can be separated into five regions termed the N1, H1, N2, H2 and C domains[2,5–7] (FIG. 2). The N2, H2 and C regions resemble a 'classical' Sec-dependent signal peptide and exhibit substantial sequence variability. By contrast, the N1 and H1 domains, which constitute the amino-terminal extended signal peptide region (ESPR), contribute most to the longer lengths of these signal peptides and are remarkably well conserved[8], leading to the suggestion that the ESPR bequeaths additional functional properties on the signal peptide[2,9]. Notably, although the H1 domain is hydrophobic in nature, it is not large enough to fulfil von Heijne's rules for membrane-spanning domains, unlike the H2 domain[10].

SPATE
Serine protease autotransporters from members of the family Enterobacteriaceae.

Lol pathway
A lipoprotein-specific sorting machinery that transfers lipoproteins from the inner membrane to the outer membrane.

Early investigations focused on a role for the ESPR in targeting ATs to the inner membrane. Initial studies of EspP (extracellular serine protease, plasmid encoded) and Hbp (haemoglobin-binding protease; also known as Tsh) — two SPATE proteins — suggested that ESPR-containing signal peptides promoted co-translational translocation of ATs via the signal recognition particle (SRP) pathway[11–13]. However, more recent studies have demonstrated that targeting is strictly SRP independent and occurs post-translationally[8,14]. Similar observations were made for filamentous haemagglutinin (FHA), an ESPR-containing TpsA protein from *Bordetella pertussis*[15].

It was subsequently demonstrated that, on deletion of the ESPR, the EspP passenger domain misfolds in the periplasm, substantially impairing its translocation across the outer membrane[16]. However, these observations have not been recapitulated for other proteins that possess ESPR-containing signal peptides. Importantly, replacement of the native signal peptide of serine protease Pet, another ESPR-containing SPATE, with signal peptides representative of the 'classical' Sec- and SRP-targeting pathways and deletion of the Pet ESPR result in secretion of correctly folded and functional toxin, indicating that the ESPR is not essential for secretion, folding or function[17]. Similar observations were made for the AT Hbp and for the TpsA protein FHA[15,18]. Importantly, although a major role for the ESPR in AT biogenesis has been ruled out, it is likely that the ESPR serves subtle functions. Indeed, several studies have shown that the presence of the ESPR delays or slows translocation of the AT to the periplasm[8,14,16,18]. These observations are consistent with reports for FHA[19]. Furthermore, the signal peptide of Hbp interacts with the inner-membrane integrase YidC (also known as OxaA) during inner-membrane translocation, and depletion of YidC leads to the accumulation of secretion-incompetent Hbp intermediates in the periplasm[20]. A model was proposed whereby YidC slows the release of Hbp into the periplasm after inner-membrane translocation (FIG. 3) by hindering access to the signal peptidase or through prolonged interaction with the ESPR, in order to sustain the translocation-competent state of the protein[20]. Thus, it seems that the ESPR has a subtle role, slowing translocation of ATs across the inner membrane to prevent accumulation of misfolded species in the periplasm.

Some ATs possess lipoprotein motifs — L(A/S)(G/A)C — carboxy-terminal to the signal peptide; these are found in, for example, NalP from *Neisseria meningitidis*, the subtilisin-like protease SphB1 from *B. pertussis*, adherence-associated lipoprotein A (AlpA) from *Helicobacter pylori*, capsule biosynthesis protein A (CapA) from *Campylobacter jejuni* and several polymorphic ATs of *Chlamydia* spp.[21–25]. This motif targets the protein to an acyltransferase on the periplasmic side of the inner membrane following Sec-dependent translocation[26] (FIG. 3). Subsequently, the final cysteine residue is targeted for cleavage by a lipoprotein-specific signal peptidase and is modified by attachment of a lipid moiety. The signal sequences are consistent with targeting of lipoproteins to the outer membrane via the Lol pathway[26]; this

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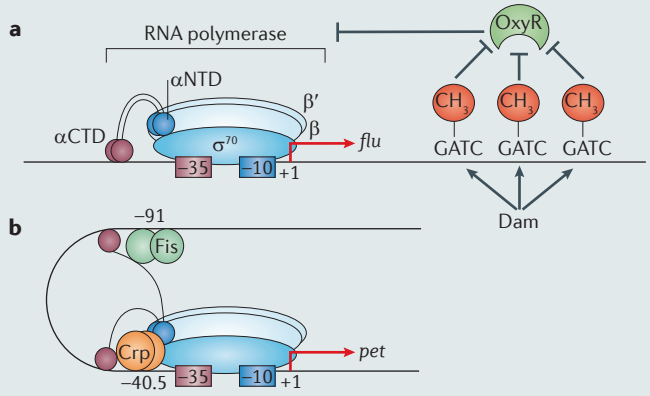
pathway results in incorporation of the amino-terminal lipidated cysteine residue into the inner phospholipid leaflet of the outer membrane. The demonstration that native SphB1 is sequestered in the outer membrane, but that the passenger domain is released from the cell on removal of the cysteine residue[27], supports the notion that these ATs are chaperoned through the periplasm via the Lol pathway. Why such proteins use the Lol system remains enigmatic, and it has yet to be determined whether these proteins also require the chaperones that are normally associated with periplasmic transit of integral outer-membrane proteins (OMPs).

Box 1 | Regulation of autotransporter expression

The micro-niches in which autotransporters (ATs) function are highly variable and, as is the case for many genes, expression of the relevant genes may be controlled by a range of environmental stimuli, including temperature, pH, oxygen tension, media composition and host cell contact[108–116]. Nevertheless, and in contrast to the vast numbers of functional studies that have been carried out, surprisingly little attention has been given to the regulation of ATs. Most studies have been limited to phenomenological investigations. For some ATs, limited mechanistic studies have revealed regulatory components involved in controlling gene expression. For example, expression of BrkA in *Bordetella pertussis* is controlled by BvgS–BvgA, a single-master regulatory two-component system[117]; transcription of the serine protease gene *espC* in enteropathogenic *Escherichia coli* is regulated by quorum sensing through the Per–Ler regulatory pathway[118]; and the expression of *icsA* in *Shigella flexneri* is dependent on a complex mechanism involving both *icsA* mRNA secondary structure and an antisense RNA[119]. The mechanism of regulation has been studied in depth for only a few ATs, the best studied of which are the enterotoxin Pet and the phase-variable outer-membrane protein antigen 43 (Ag43; encoded by *flu*) from *E. coli*. Ag43 expression switches between an on and an off phase through competitive interactions of H_2O_2-inducible genes activator (OxyR) and DNA adenine methylase (Dam) at the *flu* promoter. In addition to the core –35 and –10 promoter elements, the *flu* promoter contains three GATC motifs directly downstream of the +1 transcription start site, and an OxyR-binding site that overlaps the –10 element[120]. Binding of OxyR to its recognition site represses *flu* transcription by blocking RNA polymerase access to the core promoter. Dam-mediated methylation of the adenine residues in the GATC motifs alleviates this repression by abrogating OxyR binding[121,122] (see the figure, part **a**). Furthermore, this methylation, which is independent of OxyR, is necessary for full activation of *flu* through an as-yet-uncharacterized mechanism[120]. It is this interplay between Dam methylation and OxyR binding that sets *flu* expression as on or off, respectively, although the molecular basis of the switching remains unclear. Transcription of *pet*, from enteroaggregative *E. coli* str. 042, is regulated by a novel co-activation mechanism involving the global transcription factors catabolite gene activator (Crp) and Fis[123] (see the figure, part **b**). The architecture of the *pet* promoter has been characterized in great detail, revealing that transcription activation is dependent on both a Crp-binding site positioned at –40.5 and a Fis-binding site positioned at –91. However, the canonical binding site for most Crp-dependent promoters is –41.5. Extensive *in vivo* and *in vitro* experiments have shown that the non-optimal positioning of the Crp-binding site creates a dependence on upstream bound Fis for full activation. Intriguingly, manipulation of the Crp-binding site to the canonical position of –41.5 relieved the Fis dependence of activation. Thus, it is believed that the dependence on both factors restricts *pet* expression to a particular growth phase and is important for the pathogenesis of the strain[123].

Mechanisms of AT regulation remain enigmatic, and focusing attention on this area may provide insights into how ATs manifest their pathogenic function, thereby enhancing our understanding and, ultimately, treatments of bacterial infections.



Passage through the periplasm

On entering the periplasm, OMPs interact with periplasmic chaperones, such as SurA and Skp. They may also interact with the bifunctional periplasmic serine endoprotease DegP, which acts as a chaperone and, paradoxically, as a protease to degrade unfolded proteins. This occurs while they are still in complex with, or as they leave, the Sec translocon, and possibly even before signal peptide cleavage[28]. These chaperones are thought to maintain nascent OMPs in a translocation-competent conformation by preventing their aggregation and misfolding, and/or to have a role in folding OMPs into their characteristic β-barrel structure before insertion into the outer membrane. Periplasmic chaperones may also protect nascent OMPs from degradation by periplasmic proteases such as DegP[29,30]. As ATs possess an integral outer-membrane domain, it was suggested that ATs interact with periplasmic chaperones in a similar manner to the OMPs.

The first evidence that ATs might interact with periplasmic proteins came from the demonstration that the periplasmic enzyme DsbA promoted disulphide bond formation between cysteines in a non-native AT passenger domain[31], indicating that ATs form a periplasmic intermediate during biogenesis. Subsequently, AT biogenesis was shown to be impaired in *Shigella flexneri* mutants lacking the periplasmic chaperones Skp, SurA or DegP[32,33]. However, mutation of periplasmic chaperones can have pleiotropic effects, so it was necessary to determine whether these chaperones interact directly with ATs or whether the defective AT biogenesis in these mutants is due to alterations occurring in other protein networks. EspP was the first AT shown to directly interact with the chaperones SurA, Skp and DegP, and Hbp has also been shown to interact with SurA[34–37]. Yeast two-hybrid and surface plasmon resonance studies found that SurA interacts with both the unfolded passenger domain and the β-domain of the AT, whereas DegP interacts with only the passenger domain, and Skp interacts with only the β-domain[34,35]. Immunoprecipitation experiments confirmed the SurA–passenger domain and Skp–β-domain interactions, detected an additional interaction between Skp and the passenger domain, but did not detect an interaction between SurA and the β-domain[36–38]. This may reflect the transient nature of these interactions *in vivo*. Furthermore, the defect in AT biogenesis that is observed after loss of one periplasmic chaperone can be compensated for by overexpression of another, unrelated chaperone[32,34].

These findings suggest that AT biogenesis follows the same rules as normal OMP biogenesis, whereby chaperones mediate transit through the periplasm (FIG. 3) and

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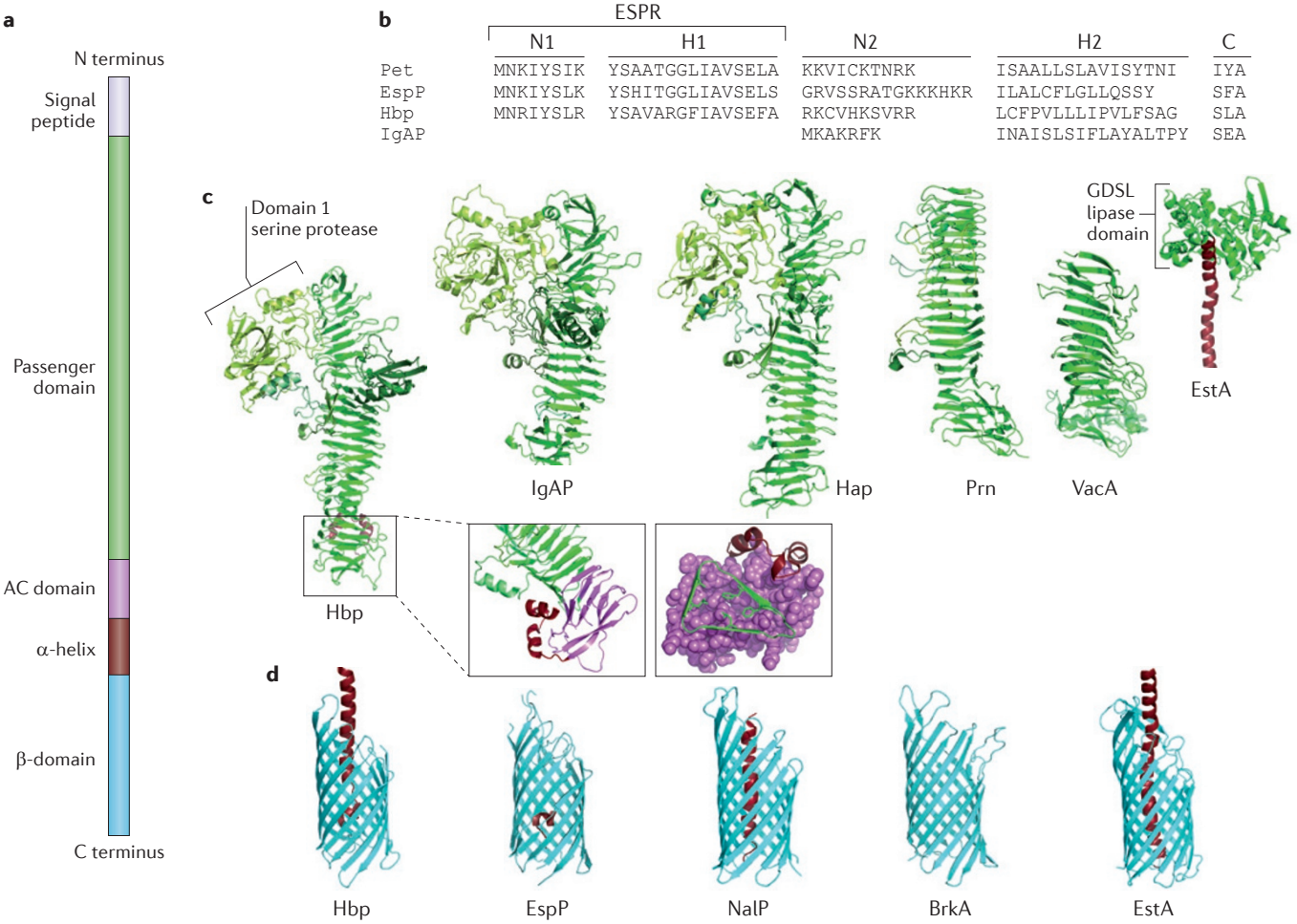

Figure 2 | **Anatomy of the classical autotransporter structure. a** | The domain architecture of a typical classical autotransporter (AT) is depicted, possessing a signal sequence, a passenger domain, an autochaperone (AC) domain, an α-helical linker and a β-domain. **b** | Signal sequences for most ATs adopt a classical tripartite organization with a charged N domain, a hydrophobic H domain and a signal peptidase recognition site, the C domain. Some ATs have an additional amino-terminal extension termed the extended signal peptide region (ESPR). **c** | X-ray crystallography structures of solved AT passenger domains are shown. The AT passenger domains included are haemoglobin-binding protease (Hbp) from *Escherichia coli* (Protein Data Bank (PDB) ID 1WXR)[88], immunoglobulin A1 protease (IgAP) from *Haemophilus influenzae* (PDB ID 3H09)[86], adhesion and penetration protein (Hap), also from *H. influenzae* (PDB ID 3SYJ)[89], pertactin (Prn) from *Bordetella pertussis* (PDB ID 1DAB)[87] and the vacuolating cytotoxin autotransporter (VacA) p55 fragment from *Helicobacter pylori* (PDB ID 2QV3)[85]. These ATs adopt a right-handed β-helical organization with the strands of the β-helix joined by loops of varying length. Sometimes these structures are decorated with additional functional domains (in Hbp, IgAP and Hap, for example). The AC domain has a characteristic β-hairpin fold at the carboxyl terminus and acts as a template for folding of the first β-helical rung. Disruption of the hydrophobic core residues (shown as sticks) abolishes folding of the β-helix. The passenger domain of the *Pseudomonas aeruginosa* AT esterase A (EstA) (PDB ID 3KVN)[48] does not adopt the β-helical configuration but rather adopts a globular fold; it lacks an AC domain. Note that the structure of *E. coli* EspP (extracellular serine protease, plasmid encoded) is not shown. **d** | X-ray crystallography structures of all solved AT β-domains are shown: *E. coli* EspP (PDB ID 2QOM)[47], *Neisseria meningitidis* NalP (PDB ID 1UYN)[50], *P. aeruginosa* EstA (PDB ID 3KVN)[48], uncleaved *E. coli* Hbp (PDB ID 3AEH)[51] and *B. pertussis* BrkA (PDB ID 3QQ2)[49]. All β-domains adopt a 12-stranded configuration, forming a pore in which the α-helix (red) resides. The β-strands are connected by short periplasmic turns and longer extracellular loops. Note that the post-cleavage, but not the pre-cleavage, structure of the EspP β-domain is shown.

are important for the correct insertion of the β-barrel into the outer membrane. Certainly, direct interactions have been observed between SurA and BamA (also known as YaeT) — the essential component of the β-barrel assembly machinery (Bam) complex, which acts as a foldase–insertase for OMPs with an integral β-barrel (BOX 2) — and between BamA and unfolded β-barrel-containing OMPs[35,39]. However, several lines of evidence suggest that chaperones are not required for β-barrel folding and insertion. *In vitro* investigations of AIDA-I (a self-associating AT (SAAT) adhesin from *E. coli*) found that SurA, DegP and Skp do not promote folding of the β-domain[40]. Subsequent studies demonstrated that, although the IcsA passenger domain is less

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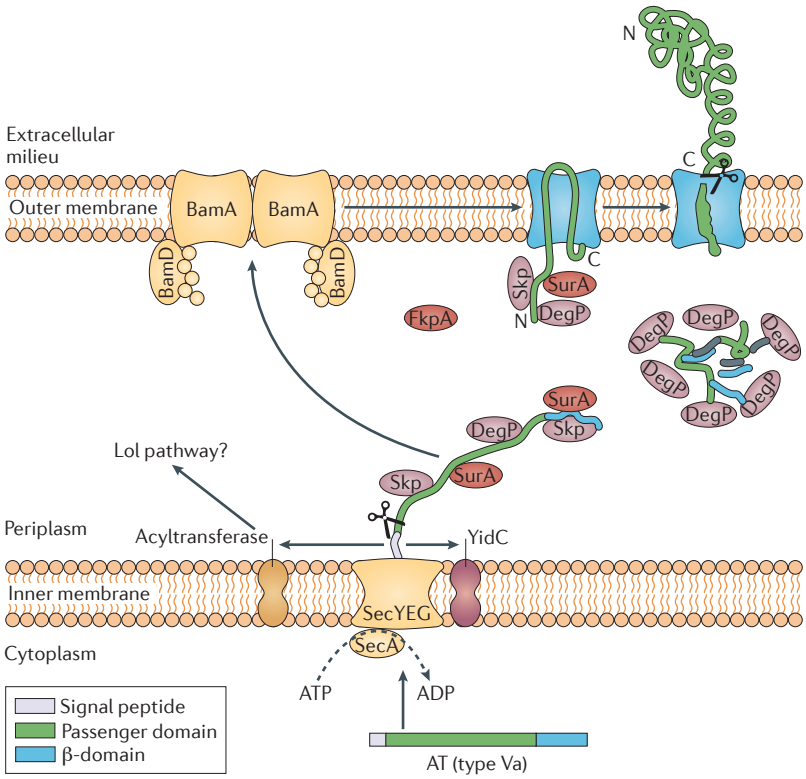

Figure 3 | **Classical autotransporter biogenesis.** The autotransporter (AT) is synthesized in the cytoplasm and then targeted to the inner-membrane SecYEG translocon, which catalyses energy-driven export into the periplasm. ATs such as NalP, which harbour a lipobox that is carboxy-terminal to their signal peptide, are targeted to an acyltransferase after inner-membrane translocation, and this enzyme lipidates the ATs, after which they are cleaved by a lipoprotein-specific signal peptidase and may be trafficked by the Lol pathway. The signal peptide of other ATs, including Hbp (haemoglobin-binding protease) and possibly EspP (extracellular serine protease, plasmid encoded) interacts with the integrase YidC during inner-membrane translocation, which may slow the release of these proteins into the periplasm. There is evidence to suggest that, following AT entry into the periplasm, the chaperones Skp and SurA bind the AT passenger domain and β-domain, whereas the bifunctional periplasmic serine endoprotease DegP binds only the passenger domain[34,38]. In addition to its chaperone-related function, DegP may act as a quality control mechanism to remove aggregated and/or misfolded proteins (grey regions) at any point during periplasm transit. It is not known whether these periplasmic chaperones bind the AT passenger domain and β-domain while they are still in complex with or as they leave the Sec translocon. Nevertheless, chaperone-bound ATs are targeted to the β-barrel assembly machinery (Bam) complex, where the BamA oligomer may bind nascent barrels, fold them completely and finally insert the folded species into the outer membrane. This process also requires BamD but not BamB, BamC or BamE[37,44] and occurs without the input of energy[125]. However, redundant roles for BamB, BamC and BamE in AT biogenesis have not been ruled out. Passenger domain translocation may be triggered on BamA oligomer dissociation and is sustained until the extreme amino terminus of the passenger domain traverses the pore. When the passenger domain is folded on the bacterial cell surface, it may or may not undergo further processing.

efficiently translocated to the bacterial cell surface in *S. flexneri surA*, *skp* and *degP* mutants, the protein nonetheless still localizes to the outer membrane and is heat modifiable, and the β-barrel is therefore presumably folded in its native conformation[32,33]. Similarly, although reduced secretion of the EspP passenger domain is observed in *E. coli surA*, *skp* and *degP* mutants, the levels of the β-barrel domain in outer-membrane fractions remain constant[34], and most of the Hbp expressed in an *E. coli surA* mutant remains unprocessed[37]. Such results suggest that periplasmic chaperones function specifically to retain passenger domains in an extended, translocation-competent conformation in addition to protecting them from degradation by periplasmic proteases. Notably, these studies remain inconclusive, as the AIDA-I studies were conducted in the absence of components of the essential Bam complex[40], and it was not determined whether the EspP and Hbp β-barrels were stably incorporated into the outer membrane as folded species[34,37]. Whereas the accumulated evidence supports a definite role for chaperones in AT biogenesis in *E. coli* (FIG. 3), the scenario for *N. meningitidis* is starkly different. None of the neisserial periplasmic chaperones studied to date has a significant role in AT biogenesis[41]. This may reflect the differences in OMP biogenesis between the two organisms. For example, loss of SurA has no substantial impact on OMP accumulation in the neisserial outer membrane but does have an effect for the *E. coli* outer membrane[29,41]. Furthermore, in *E. coli* the simultaneous deletion of *surA* and *skp* leads to synthetic lethality, but the same mutations in *N. meningitidis* do not produce a synthetically lethal phenotype[29,41].

Insertion of β-domains into the outer membrane

Several investigations have demonstrated the requirement for the Bam complex in the biogenesis of IcsA, AIDA-I, Pet and Hbp, the *S. flexneri* SPATE SepA, the *B. pertussis* serum resistance protein BrkA and *N. meningitidis* immunoglobulin A1 (IgA1) protease (IgAP)[42–44]. Several groups have specifically demonstrated that ATs interact with BamA during the course of biogenesis[34,36,37,45] and, importantly, depletion of BamA abrogates secretion of mature AT passenger domains to the exterior of the cell[42–44]. Based on evidence from *E. coli* that BamA interacts directly with SurA, it has been proposed that BamA receives the nascent AT β-barrel from the chaperone before folding of the β-barrel (FIG. 3). A recent study also found that BamD is required for biogenesis of the AT Pet[44], consistent with the essential nature of this Bam protein. This is further supported by *in vivo* photo-crosslinking experiments that demonstrated a direct interaction between BamD and EspP[38]. Interestingly, this investigation revealed that the EspP–BamD interaction lasted longer than the EspP–BamA interaction, suggesting that BamD mediates the final step in the incorporation of the folded AT β-barrel into the outer membrane. A similar observation was made for the AT Pet[45]. Although interactions between BamB and EspP have also been demonstrated[38], BamB is not required for insertion of the Pet or Hbp β-barrels into the outer membrane, or for passenger domain translocation or folding[37,44]. Furthermore, BamB is lacking in *Neisseria* spp., in which ATs are readily assembled[41]. Thus, the EspP–BamB and Hbp–BamB interactions observed *in vivo*[36,37] may be indirect and a consequence of BamB independently interacting with BamA. Notably, null mutations in *bamC* and *bamE* had no significant effect on insertion of the Pet β-barrel into the outer membrane, or on passenger domain translocation or

Crosslink
To form a covalent bond between adjacent chains of a molecule.

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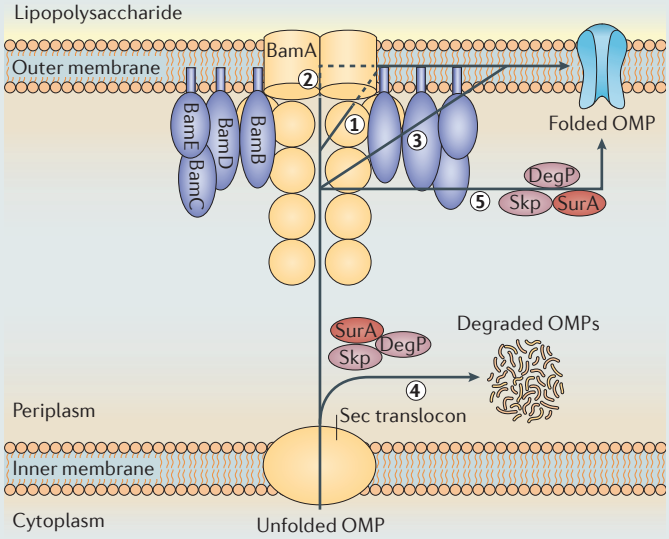
Box 2 | **The Bam complex**



Most integral outer-membrane proteins (OMPs) adopt a β-barrel conformation[124] and require the recently described β-barrel assembly machinery (Bam) complex to be correctly inserted into the outer membrane as a folded species, a process that occurs without the input of energy[28,125]. In *Escherichia coli*, this hetero-oligomeric complex consists of an integral β-barrel protein, BamA, and four peripheral lipoproteins, BamB, BamC, BamD and BamE, that localize to the inner leaflet of the outer membrane[28]. BamA contains a carboxy-terminal domain that forms an OMP with an integral β-barrel, and an amino-terminal periplasmic region with five structural repeats, termed POTRA domains. POTRA 1 and POTRA 2 have been shown to bind peptides of proteins destined for the outer membrane[126], and POTRA 1 was shown to be the site of docking for the periplasmic chaperone SurA[39]. BamD makes a direct contact with BamA via POTRA 5, and deletion of POTRA 5 results in disassembly of the Bam complex[127], indicating that BamD functions as a scaffold for BamC and BamE. By contrast, BamB directly interacts with BamA in a manner that is independent of the other components[127–130]. Although only BamA and BamD are vital for cell viability and OMP biogenesis[128], deletion of *bamB*, *bamC* or *bamE* results in membrane barrier defects and changes in the ability to incorporate stably folded β-barrel proteins into the outer membrane[129,131–134]. Interestingly, a new role for BamE has been proposed whereby it functions to indirectly control the conformation of BamA through direct interactions with BamD[135].

Although it is still unclear how the Bam complex facilitates β-barrel incorporation into the outer membrane as a folded species, a number of models have been proposed. In the pore-folding model, nascent OMPs are threaded through the BamA β-barrel pore by the POTRA domains and/or accessory periplasmic components of the Bam complex, through weak interactions that permit processive sliding[126] (see the figure, arrow 1). In this model, BamA pore activity could be modulated by the conserved extracellular region, L6 — which, if functionally analogous to L6 of the two-partner secretion translocator (TpsB) protein filamentous haemagglutinin transporter (FhaC), may vacate the β-barrel to accommodate the substrate[56,136]. L6 of FhaC is folded as a hairpin that spans the barrel interior, with its tip extending into the periplasm[56]. Interestingly, this tip contains a motif, VRG(Y/F), that is highly conserved in sequence and location in other TpsB proteins and in the BamA superfamily of foldases–insertases[136]. Although mutations in this motif have no effect on substrate recognition, they almost completely abolish filamentous haemagglutinin (FHA) translocation and affect FhaC stability[136]. However, FhaC forms a pore that mediates translocation of substrates across the outer membrane rather than into the outer membrane, and it is unlikely that the network of hydrogen bonds in the BamA β-barrel would break to permit lateral passage of the OMPs out of the pore and into the bilayer, because of the energetically unfavourable structural destabilization required[28]. According to the complex pore-folding model, BamA oligomerizes to form a central core that serves as the site of insertion into the lipid bilayer[28] (see the figure, arrow 2). In this model, the peripheral lipoproteins help fold and deposit the OMP directly into the distorted bilayer, and then release of the folded OMP could occur on dissociation of the multimeric Bam complex. In the chaperone-folding model, DegP and/or other chaperones fold the protein in the periplasm, and the sole role of the Bam complex is to insert the protein into the outer membrane (see the figure, arrow 3). In support of this model, X-ray crystallography and electron microscopy demonstrated that DegP can adopt 12-mer or 24-mer cage-like structures with an inner cavity containing a folded OMP cargo, suggesting that DegP sequesters unfolded and folded proteins in the periplasm and either degrades them or stabilizes them to allow their insertion into the outer membrane[137] (see the figure, arrow 4). In another model, the Bam complex has an accessory function, folding nascent OMPs, whereas their delivery and insertion into the outer membrane are mediated by the chaperones SurA, Skp and/or DegP (see the figure, arrow 5). In support of this model, the DegP 24-mer cage is capable of interacting with lipid membranes[137].

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folding[44]. It is possible that BamB, BamC and BamE have redundant roles, but current evidence suggests that only BamA and BamD are required for AT biogenesis.

How BamA and BamD facilitate the incorporation of the AT β-domain into the outer membrane, and indeed the integration of other β-barrel-containing OMPs into the lipid bilayer, remains unknown. Nevertheless, several models have been proposed that might explain this process[28] (BOX 2). It is conceivable that ATs are delivered to BamA by periplasmic chaperones, and BamA and BamD work in concert to ensure stable incorporation of the AT β-domain into the outer membrane as a folded β-barrel species (FIG. 3). Certainly, it has been suggested that there are two distinct pathways for OMP assembly in *E. coli*, both of which converge at the core of the Bam complex (comprising BamA and BamD)[44]:

POTRA domains
(Polypeptide transport-associated domains). Protein domains that are found in β-barrel assembly machinery protein A (BamA) family members.

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one that is dependent on BamB, BamC and BamE and for which known substrates include porins, and another that is independent of these factors and for which known substrates include ATs and the trimeric OMP TolC.

β-domain structure

When integrated into the outer membrane, the AT carboxyl terminus adopts a β-barrel conformation reminiscent of most other integral OMPs[46–52]. Early bioinformatic predictions suggested that this β-domain is evolutionarily conserved and always adopts a similar conformation[53]. Indeed, despite their diverse bacterial origins, almost perfectly superimposable crystal structures have been reported for the β-domains of five classical ATs: EspP, BrkA, Hbp, NalP and esterase A (EstA; from *Pseudomonas aeruginosa*)[47–52]. All these structures revealed a 12-stranded β-barrel connected by extracellular loops and periplasmic turns of varying length, with a narrow (~1 × 1.25 nm) hydrophilic pore (FIG. 2). An additional feature that is conserved among all these β-barrels is a single α-helix residing in the barrel lumen and connected by a periplasmic loop. This helix, which is present in the primary structure of all proteins that exploit the AT pathway, is derived from a portion of the protein that sits immediately amino-terminally to the segment that codes for the β-barrel. An obvious variation is observed in classical ATs for the length of the α-helix in the barrel lumen, reflecting the position of passenger domain cleavage. Thus, ATs that remain uncleaved, and those that are cleaved extracellularly, possess α-helices that span the length of the hydrophilic pore (for example, 27 residues in NalP and 43 residues in EstA)[48,50]. By contrast, those that undergo intra-barrel cleavage have a much shorter α-helix remaining associated with the barrel. In the case of the SPATE proteins, the 14 amino acid α-helical region is cleaved in a manner that leaves a five amino acid helical segment associated with the folded and outer-membrane-incorporated β-barrel[47,51,52].

All pore-forming OMPs must be gated in some manner to maintain the integrity of the selective permeability function of the outer membrane[46]. Electrophysiological experiments, antibiotic sensitivity studies and molecular dynamics simulations have illustrated the importance of the α-helix in plugging the pore as well as maintaining the width of the β-barrel[50,54–56]. Certainly, in the case of Hbp and EspP, the short α-helix is orientated perpendicular to the barrel axis in both pre-cleavage and post-cleavage structures[47,51,52]. In addition to the α-helix, the long extracellular loop (L5 of SPATEs) is folded into the β-barrel pore, making extensive contact with residues in β-strands that are closer to the surface of the barrel, and closing the channel from the extracellular side[47].

Several lines of evidence suggest that the barrels of ATs are not simply passive pores but, instead, are Bam-targeting devices that actively participate in their own assembly and in translocation of their cognate passenger domains[57]. Further studies have also challenged the passive role of these structures in AT biogenesis. Mutations in a hydrophobic cavity in the BrkA β-barrel, comprising the hydrophobic patches on loop L4 and β-strands 5 and 6, resulted in substantially decreased passenger domain translocation despite the β-barrel being correctly targeted to the outer membrane[49]. Comparisons with other available classical β-barrel structures revealed that the hydrophobic patch is conserved, so it has been suggested[49] that the hydrophobic cavity has a role in passenger domain translocation by sequestering a portion of the passenger domain to initiate the translocation event (see below). Furthermore, a large mutagenesis study of the conserved residues in SPATE β-domains demonstrated that mutation of Tyr1227 in β-strand 6 of the Hbp β-barrel results in reduced passenger domain translocation[58]. Importantly, this residue seems to be the equivalent of the Tyr859 located in the hydrophobic cavity (in β-strand 6) of the BrkA β-barrel. This last study also found roles for certain other conserved residues in assembly, targeting and insertion of the Hbp β-barrel into the outer membrane and in passenger domain processing. Moreover, several groups have demonstrated additional roles for the α-helix, including β-barrel folding and stability, and passenger domain processing[47,59–63].

Translocation and folding of passenger domains

After insertion of the β-barrel into the outer membrane, the passenger domain is translocated to the exterior of the cell and folds into a characteristic β-helix (see below). Several putative mechanisms of passenger translocation have been described. The amino-terminal threading model and the oligomeric model have now been largely discounted by a substantial body of evidence and are not discussed further here. The remaining models of AT passenger domain translocation, for which there is a large amount of supporting evidence, are the hairpin model and the BamA model (FIG. 4).

The hairpin model. Translocation through the β-barrel was thought to commence by the formation of a hairpin structure at the most carboxy-terminal portion of the passenger domain, and it was thought that this structure was sustained until the extreme amino terminus of the passenger domain traversed the pore. Such a translocation model implies that a static strand is sequestered in the lumen of the β-barrel and that a sliding strand moves through the pore. Given the size constraints of the β-barrel pore, such strands have to be maintained in an unfolded conformation during the translocation event. Consistent with this model, it has now been confirmed that the carboxyl terminus of the passenger protein is presented on the bacterial cell first and folds into a stable, protease-resistant structure[64–67]. Indeed, translocation-incompetent intermediates have been detected that display the carboxy-terminal portion of the passenger domain on the cell surface, where it is accessible to protease or antibodies, while the amino-terminal portion of the molecule remains in the periplasm[45,64,68,69]. It has recently been observed that crystal packing of the BrkA β-domain results in a β-strand hairpin-like structure (formed by extracellular loop four) inserted into the adjacent monomer, and a hydrophobic cavity has been identified in the AT barrels (see above), together indicating that such a hairpin motif can be accommodated in the central pore[49]. Interestingly, at the extreme

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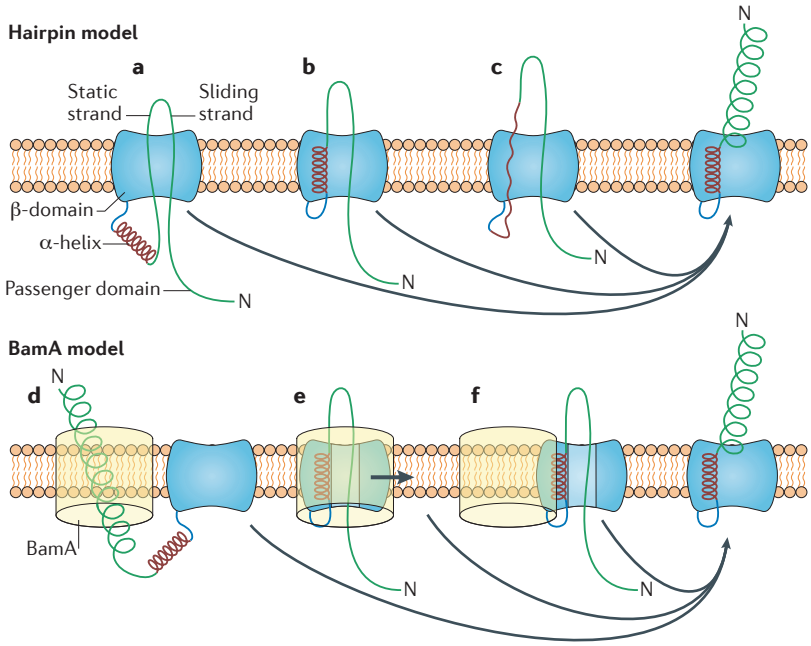

Figure 4 | **Models of passenger domain translocation across the outer membrane.** Two basic models currently exist for translocation of autotransporter (AT) passenger domains: the hairpin model and the β-barrel assembly machinery protein A (BamA) model. **a**–**c** | In the case of the hairpin model, translocation of the passenger domain occurs in the absence of accessory factors such as BamA. Either the α-helix is located periplasmically during secretion (part **a**) and inserts into the β-barrel pore of the AT when translocation is complete, or the α-helix occupies the pore in a folded (part **b**) or unfolded (part **c**) state during passenger domain translocation. **d**–**f** | In the case of the BamA models, one suggestion is that the passenger domain moves through the pore of the BamA β-barrel, with the AT β-barrel being inserted into the outer membrane afterwards (part **d**). An alternative suggestion is that the AT β-barrel enters the BamA pore and is maintained in a semi-folded state (denoted by dotted lines), and when translocation (via a hairpin mechanism) is complete BamA releases the protein into the outer membrane (part **e**). A recent hypothesis suggests the AT β-barrel interacts with the exterior of BamA, which maintains the β-barrel in a semi-folded state until translocation is complete; then, a fully folded AT is released into the outer membrane (part **f**).

carboxyl terminus of most AT passenger domains there is a β-strand hairpin structure that forms a portion of the autochaperone (AC) domain (FIG. 2); this domain is essential for folding of β-helical passenger domains (see below). Thus, the hydrophobic cavity might interact with the AC to promote secretion and folding after the β-barrel has been inserted into the outer membrane by the Bam complex[49]. This is an attractive model, as it is the AC domain that emerges first onto the bacterial cell surface.

If a hairpin mode of translocation occurs, there are three possible conformations that could be adopted. One possibility is that the α-helix is located outside the barrel lumen during translocation, in the periplasmic space, with amino-proximal portions of the passenger domain forming the hairpin (FIG. 4a). Only after translocation of the passenger domain has occurred will the α-helix insert into the pore. Such a model is consistent with the proposed sequestration of the AC β-strands[49]. Furthermore, in support of this model, *in vitro* pore activities have been demonstrated for NalP, consistent with repeated insertion and extraction of the α-helix from the barrel lumen[50]. For the SPATE proteins, this model would prevent cleavage until translocation was fully complete (see below). However, at least one study has indicated that the α-helix is sequestered in the pore before insertion of the already partially folded barrel into the outer membrane[70], and others have shown that without the α-helix, the β-barrel is unable to form a stable hydrophilic channel in the outer membrane for subsequent protein translocation[47,59,61]. Thus, an alternative model is that the α-helix occupies the pore during the translocation event (FIG. 4b), although this would suggest that the interaction between the α-helix and the β-barrel is a prerequisite for translocation of the passenger domain across the outer membrane. However, it is difficult to envisage passenger domain translocation occurring after this bonding pattern has been established, mainly because the crystal structures of AT β-barrels revealed that the channel is almost completely occupied by the α-helix and would be unable to accommodate an unfolded protein as well. Alternatively, the α-helix occupies the barrel lumen but is maintained in an unfolded state until translocation is complete (FIG. 4c).

Despite the striking simplicity of the hairpin model, there are several excellent studies which provide data that are conceptually difficult to reconcile with the model. For example, there is substantial evidence for the translocation of disulphide-bonded segments of chimeric passenger domains[61,71,72] and for the translocation of glycosylated passenger domains across the outer membrane[73–75]. On the basis of the dimensions of the AT β-barrels, such a modified passenger domain could not pass through the lumen of the barrel even if it was occupied by an unfolded α-helix. By contrast, the efficient secretion of disulphide-bonded segments within naturally occurring passenger domains can be explained by recent data demonstrating that, when present, endogenously paired cysteine residues are intrinsically closely spaced to avert blockage of the translocation pore by large disulphide-bonded regions[45].

The BamA model. The evolutionary similarity of BamA and the TpsB protein FhaC, which forms a pore that translocates its TpsA partner (TpsA proteins are structurally analogous to most AT passenger domains[56]), implies that the AT passenger domain, even when partially folded, could be transported through a wider channel formed by BamA, whereas the β-domain is integrated into the outer membrane by another mechanism (FIG. 4d). Certainly, liposome-swelling assays have demonstrated that *Neisseria gonorrhoeae* BamA can form pores approximately 2.5 nm in diameter[76], which may be big enough to accommodate partially folded AT passenger domains. However, the fact that ATs are synthesized as a single polypeptide excludes any model in which the passenger passes through an alternative channel without its β-domain, as opening of BamA for lateral transfer of the passenger domain is biophysically unfavourable. More likely scenarios are derived from a combination of both the hairpin model and the BamA model. In one such alternative model, the β-domain inserts into the pore of BamA, and the β-barrel is held 'open' by the BamA

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monomer, allowing the passenger domain to be translocated to the exterior of the cell[70] (FIG. 4e). However, this is difficult to envisage, as the BamA pore is certainly not large enough to accommodate the AT β-barrel, and lateral passage of both domains would be required for final partitioning of the AT β-barrel into the lipid bilayer. In another possibility, the AT β-barrel docks with BamA and is held 'open' in a loosely folded conformation while the passenger domain is translocated through it, after which the Bam complex disassembles, triggering sealing of the AT β-barrel and its folding into a stable barrel[37,38] (FIG. 4f). Although it is clear that ATs associate closely with BamA during outer-membrane translocation, the precise role of the Bam complex in passenger domain translocation remains speculative. The reversibility of stalled secretion, as observed for Hbp and pertactin (an AT adhesin of *B. pertussis*)[64,68], suggests that BamA is involved in the insertion of β-domains into the outer membrane rather than in passenger domain translocation, as a stalled passenger domain intermediate would be expected to sequester BamA in a non-functional state, leading to cell death.

Interestingly, the conundrum of passenger domain translocation through the β-barrel may be explained by recent observations for the biogenesis of type 1 fimbriae. The 24-stranded usher FimD, a β-barrel OMP, undergoes a conformational rearrangement from an oval-shaped pore (5.2 nm x 2.8 nm) to a near circular pore (4.4 nm x 3.6 nm) to allow translocation of the folded pilus subunit FimH[77]. This is unprecedented in β-barrel proteins, which were until now considered rigid structures, and similar conformational contortions may allow passenger domains with secondary structures or glycosyl modification to pass through the AT β-barrel pore without the intervention of accessory factors such as BamA.

Driving force for passenger domain transport. Integral to the mechanism of passenger domain translocation is the driving force that moves the passenger domain from the periplasm to the exterior of the cell, and it has been proposed that folding is the driving force behind efficient passenger domain transfer[2,67]. The AC domain is essential for folding of the β-helical structure that is common to most ATs (FIG. 2). Evidence of the requirement for the AC domain in folding is derived from mutagenesis studies of BrkA, Ssp (extracellular serine protease of *Serratia marcescens*), AIDA-I, IcsA, Pet and Hbp[78–83], which demonstrated that folded passenger domains do not accumulate extracellularly when the AC domain is substantially perturbed. In the case of BrkA and Ssp, the stability of the surface-exposed AC domain mutants was rescued by providing the AC domain in *trans* through surface expression of the AT carboxyl terminus, demonstrating that passenger domain translocation occurs before folding and that folding does not occur in the periplasm[80,81]. Further evidence that the AC domain serves as a template for folding of the complete passenger domain is provided by the demonstration that the carboxyl termini of the pertactin and Pet β-helices are considerably more stable than the amino-terminal rungs[65,67]. This anisotropic dispersal of stability within AT passenger domains may facilitate vectorial secretion by a Brownian ratchet mechanism that is independent of ATP hydrolysis and driven by correct folding of the stable C-terminal core[84]. Folding of the passenger domain on the exterior of the cell may then prevent the protein from sliding back through the barrel pore[83]. However, the secretion of AC domain-mutant passenger domains that are misfolded and susceptible to digestion by OmpT in the outer membrane suggests that passenger domain folding and outer-membrane translocation are not mutually exclusive[81].

Brownian ratchet
Directionally biased diffusion.

Passenger domain structure

Despite their diversity in length, sequence and function, the passenger domains of most classical ATs fold into characteristic right-handed β-helical stalk-like structures adjoined by loops of varying length and structure[65,67,85–90] (FIG. 2). Pertactin was the first AT passenger domain for which a structure was solved[87]. Pertactin consists of a uniform β-helix comprising 16 turns, each composed of three parallel β-sheets, connected by several protruding loops with sequence motifs that facilitate protein function. Subsequently, the structures for the ATs Hbp, IgAP, EspP, vacuolating cytotoxin autotransporter (VacA; from *H. pylori*), and adhesion and penetration protein (Hap; a SAAT adhesin from *Haemophilus influenzae*) were solved, revealing β-helices studded with elaborate surface structures. In the case of Hbp and IgAP, these include a large globular domain (domain 1) that has a trypsin-like fold and is rigidly fixed to the amino-terminus of the β-helical stem, a more flexible domain (domain 2) that folds independently of the β-helix and is capable of independent movement, and additional loops (domains 3 and 4) that are inserted between the β-strands of the β-helix and extend away from the β-helical spine[86,88,91]. Although the general fold of the EspP passenger domain is highly similar to the fold of the passenger domains of Hbp and IgAP, architectural differences include the absence of domain 2, and a much bulkier domain 3 comprising a disordered loop that contains two closely spaced cysteine residues[90]. In addition, recent work has demonstrated that the cleaved Hap passenger domain is structurally similar to those of Hbp and IgA, with domains 1–4 protruding away from and returning to the β-helical stem at the same positions[89]. Architectural differences include a smaller domain 2 in the Hap passenger domain and a triangular prism-like structure (the SAAT domain) at the carboxyl terminus of the β-helix, both of which potentiate high-order intermolecular oligomerization, resulting in bacterial cell–cell interactions[89]. Importantly, this intercellular oligomerization mechanism may mediate bacterial aggregation and biofilm formation in other SAATs such as AIDA-I and Ag43 (REF. 89). Notably, there are subtle structural differences in domain 1 of Hbp, IgAp, EspP and Hap that are likely to account for their differences in substrate specificity. Interestingly, after secretion the mature 88 kDa VacA toxin is naturally processed into fragments of 33 kDa and 55 kDa (designated p33 and p55, respectively), which remain associated after cleavage and adopt a range of star-shaped single-layered

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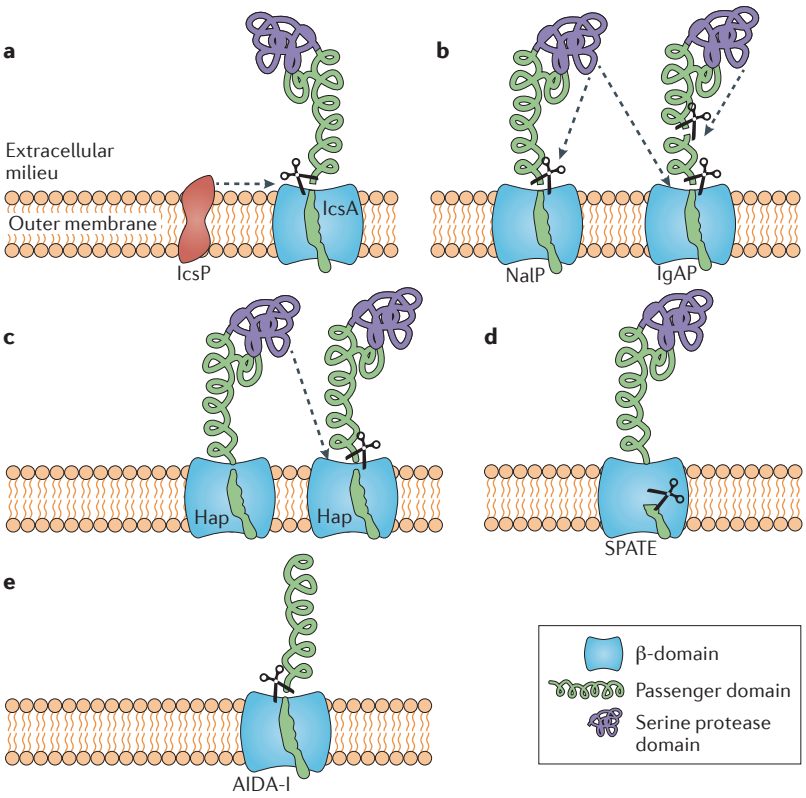

Figure 5 | **Mechanisms of passenger domain cleavage. a** | The IcsA passenger domain is specifically cleaved by host outer-membrane protease IcsP. **b** | The passenger domain of immunoglobulin A1 protease (IgAP) (depicted), adhesion and penetration protein (App) and the peptidase AusI is cleaved by the serine protease domain of NalP. Some of these autotransporters, including NalP itself (which is also cleaved by an unknown protease), are then further processed through endogenous serine protease activity. **c** | Adhesion and penetration protein (Hap) (depicted) and App passenger domain processing is mediated by intermolecular cleavage. **d** | Passenger domain cleavage of SPATEs (serine protease autotransporters from members of the family Enterobacteriaceae) such as EspP (extracellular serine protease, plasmid encoded) and Hbp (haemoglobin-binding protease) is intramolecular and autocatalytic, involves residues that are part of the α-helix and β-barrel, and occurs inside the barrel lumen. **e** | Intramolecular cleavage of the AIDA-I passenger domain transpires through autoproteolysis and, unlike cleavage of the SPATEs, occurs at the bacterial cell surface.

structures with six- or seven-fold symmetry that are well suited to promoting the formation of channels in a range of biological membranes[92,93].

Notable exceptions to the characteristic β-helix structure exist: EstA is a full-length AT with a passenger domain comprising a globular fold rich in α-helices and loops, with no β-helix and no AC domain[48]. The esterase active-site catalytic triad residues (Ser14, Asp286 and His289) reside at the entrance of a large hydrophobic pocket, at the apical surface of the passenger domain.

Passenger domain cleavage

Following translocation across the outer membrane, some ATs remain intact (for example, EstA), whereas the passenger domains of others are cleaved from their β-domains and secreted into the extracellular milieu (for example, the SPATEs). For some ATs, processing serves to release the passenger domain for downstream effector functions such as cytotoxicity (for example, VacA)[93]. Intriguingly, some ATs are processed but remain strongly associated with their cognate β-domains. This cleavage event is perplexing, as uncleaved mutants of AIDA-I[94] maintain the same *in vitro* functions as the wild-type protein with regard to cell–cell aggregation and biofilm formation. However, the advantages of this processing event may be subtle and evident only during *in vivo* or niche-specific growth.

Although the mechanisms of passenger domain proteolysis have been elucidated for only a few ATs, they are diverse (FIG. 5). The passenger domains of some ATs are cleaved by exogenous host proteases, exemplified by *S. flexneri* IcsP, an outer-membrane protease that is homologous to *E. coli* OmpT and is responsible for processing of the IcsA passenger domain[95,96]. Similarly, the serine protease domain found in the passenger domain of NalP is responsible for proteolytic processing of several *N. meningitidis* ATs, including IgAP, adhesion and penetration protein (App) and the peptidase AusI (also known as MspA)[21,97,98]. This cleavage is believed to occur before and then in competition with autoproteolytic cleavage of IgAP and App passenger domains[21]. ATs such as IgAP, App, NalP, Hap and SphB1 may also undergo autoproteolysis through endogenous serine protease activity[3,22,99–101], which in Hap and SphB1 is mediated by intermolecular cleavage on the bacterial cell surface[27,102]. By contrast, AIDA-I does not possess a serine protease domain, but intramolecular cleavage of the passenger domain occurs through autoproteolysis that instead requires two acidic residues (Asp878 and Glu897) that reside in the passenger domain[94].

Although the SPATEs harbour a characteristic serine protease motif in domain 1, many studies have shown that cleavage occurs immediately after passenger domain translocation and is not dependent on the proteolytic activity of domain 1 (REFS 16,65,72,103–106). Instead, the SPATE β-barrel mediates cleavage of the passenger domains in an autocatalytic reaction involving residues that are part of the α-helix and β-barrel lumen[47,51,52,107]. Earlier biochemical data suggest that EspP autoproteolysis commences when a carboxyl group from Asp1120, which faces the pore of the barrel, is used to activate an Asn residue (Asn1023) N-terminal to the cleavage site for nucleophilic attack on its own polypeptide backbone, a mechanism that is used by some viral capsid proteins for maturation[107]. The residues important for autoproteolysis are conserved in all SPATEs, suggesting that this cleavage mechanism is universal among all members of this subfamily. Certainly, mutation of the putative catalytic residues Asp1197 and Asn1100 in the Hbp β-barrel abolishes passenger domain cleavage[58,60]. Furthermore, mutation of Asp733 and Asp833 in the pertactin and BrkA β-barrels, respectively, both of which correspond to Asp1120 in EspP, also abolishes processing[107], suggesting that the same intramolecular autoproteolytic mechanism is conserved in these non-SPATE ATs. However, a recent crystal structure of an uncleaved Hbp β-domain has demonstrated that Asn1100 is activated for nucleophilic attack by a different and perhaps conserved mechanism that does not involve the Asp residue (Asp1197) that is equivalent

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to Asp1120 of EspP[51] — specifically, through deprotonation of Asn1100 by a catalytic water molecule that is positioned near the side chain of this active-site residue by Tyr1227 and Glu1249. A modified version of the cleavage mechanism has now been reported[52], whereby a catalytic water molecule is positioned near one or a combination of three conserved acidic residues (Asp1120, Glu1157 and Glu1172 in EspP) to increase the nucleophilicity of the active-site Asn.

Concluding remarks

Although AT passenger domains are structurally conserved, they are functionally diverse and exhibit considerable sequence variation (for example, the presence or absence of the ESPR, cysteine residues and various functional motifs) and distinct post-translational modifications (for example, lipidation, glycosylation, cleavage and oligomerization). Thus, the discrepancies in the data obtained between different studies are perhaps not surprising and are suggestive of variations in the mechanisms responsible for AT biogenesis. However, important advances in the field of AT biogenesis have transpired over the past few years. Structural biology has identified a β-helical architecture that is common to most classical ATs and that is used as scaffolding on which to append distinct loops and/or domains that seem to confer functionality. Structural biology and mutagenesis studies have also demonstrated a remarkable conservation of β-domain architecture and provided evidence that β-barrels actively participate in several aspects of AT biogenesis, including their own assembly, targeting and insertion into the outer membrane, and passenger domain translocation and processing. Biophysical and biochemical studies have shown that vectorial folding and translocation of the β-helix are driven by folding of the AC domain as it emerges from the bacterial cell surface. Furthermore, studies demonstrating that passenger domain secretion requires the aid of accessory factors have challenged the theory that ATs are self-contained secretion systems.

However, many questions remain. What are the exact mechanisms by which AT expression is regulated? Is there some advantage or benefit to containing the ESPR when a bacterium lives in its natural environment? What are the functions of domains 2–4 in SPATEs, IgAP and Hap? Why are some passenger domains cleaved from their β-domains only to stay strongly associated with their carboxyl termini? What is the process of β-barrel insertion in the outer membrane? Have AT passenger domains co-evolved with their cognate β-barrels to optimize the translocation process, or are β-barrels generic and interchangeable? Do passenger domains pass through their own β-barrel and, if so, do these β-barrels undergo a conformational change during active translocation? Do accessory factors aid in passenger domain translocation? Are ATs 'substrates' to be secreted rather than autonomous secretion systems? In the coming years, it is probable that the structures of stalled translocation intermediates will unravel the mechanism of passenger domain translocation. Further insights into AT biogenesis may emerge as the mechanisms responsible for OMP trafficking and folding into the outer membrane are deciphered. Further characterization of the AT secretion pathway will be particularly useful in the development of AT-based systems for the secretion of unrelated target proteins to the bacterial envelope and beyond for biotechnical and biomedical applications.

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Acknowledgements
We thank R. E. Fitzpatrick for helpful discussion and critical reading of the manuscript. This work was supported by the UK Medical Research Council and UK Biotechnology and Biological Sciences Research Council grants to I.R.H.

Competing interests statement
The authors declare no competing financial interests.

FURTHER INFORMATION
Ian R. Henderson's homepage: http://www.birmingham.ac.uk/staff/profiles/iandi/henderson-ian.aspx
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