

Type V Protein Secretion: Simplicity Gone Awry?

Mickaël Desvaux, Nicholas J. Parham
and Ian R. Henderson*

Bacterial Pathogenesis and Genomics Unit, Division of Immunity and Infection, The University of Birmingham, The Medical School, Edgbaston, Birmingham B15 2TT, UK

Abstract

Since its discovery in the late 1980's, the family of secreted proteins termed the autotransporters has been expanding continuously to become the largest group of secreted proteins in Gram-negative bacteria. The type V secretion pathway, which includes the autotransporters (type V_a) together with the two-partner secretion system (type V_b) and the Oca family (type V_c), can be defined by secreted proteins that are (i) translocated across the outer membrane via a transmembrane pore formed by a β -barrel and (ii) contain all the information required for translocation through the cell envelope. In the light of new discoveries and controversies in this research field, the secretion process of autotransporters, or the type V_a secretion system, will be discussed here and placed in the context of the more general field of bacterial protein translocation.

Introduction

In contrast, to Gram-positive bacteria which possess only one biological membrane, the cell envelope of Gram-negative bacteria is composed of two asymmetric biological membranes: (i) the inner membrane, also called the cytoplasmic or periplasmic membrane, and (ii) the outer membrane. This double membrane system sandwiches the periplasmic space between them. Thus, proteins which are targeted for secretion must traverse the periplasm and both membranes. To date five major secretion systems, numbered from I to V, which permit the secretion of soluble proteins have been described for Gram-negative bacteria (Salmond and Reeves, 1993; Henderson *et al.*, 2000c). This classification is based on similarities found among the protein components and the mechanism of protein translocation utilised to traverse the outer membrane. Therefore, the secretion pathways in Gram-negative bacteria are categorized primarily by their outer membrane translocation mechanisms which govern translocation of proteins to the extracellular milieu.

For an in-depth understanding of the type I, II, III and IV secretion pathways we refer the reader to other excellent and updated reviews (Stathopoulos *et al.*, 2000; Thanassi and Hultgren, 2000; Christie, 2001; Sandkvist, 2001; Buttner and Bonas, 2002; Sexton and Vogel, 2002; Andersen,

2003; Blocker *et al.*, 2003). The type I secretion system can be exemplified by the HlyA haemolysin of *Escherichia coli*. The secretion pathway involves an oligomeric complex composed of an inner membrane ATP-binding cassette (ABC) exporter (IM-ABC), a membrane fusion protein (MFP) and an outer membrane TolC homologue. The type II secretion system is a two-step process and has been best studied for the pullulanase enzyme, PulaA, from *Klebsiella oxytoca*. It involves the export of proteins across the inner membrane via the Sec system and subsequently the transport of the effector molecule across the outer membrane. A dedicated secretion apparatus called the secreton, which is composed of up to 16 different gene products and at some stage spans the entire cell envelope, mediates this latter step. The type III secretion pathway has been well characterised in *Salmonella* and *Yersinia*. It comprises a complex apparatus of proteins that assembles into a highly regulated channel through both the inner and outer membranes forming a needle-like structure. This apparatus, also called the injectisome or molecular needle, is designed to secrete proteins not only into the extracellular milieu but also directly into a target eukaryotic cell. The type IV secretion system is related to the systems involved in conjugative transfer of DNA and allows the secretion of nucleoprotein complexes e.g. *Agrobacterium* T-DNA, multisubunit toxins such as the pertussis toxin (PT), or monomeric proteins like RecA. Based on sequence homology, this pathway has recently been divided into two subclasses (Christie and Vogel, 2000): (i) the type IV_a corresponds to machinery assembled from VirB homologues of *Agrobacterium tumefaciens* (ii) the type IV_b corresponds to functional secretion system assembled from Tra homologues of IncI ColIb-P9 plasmid of *Shigella flexneri*.

The type V secretion system is one of the most recently described pathways permitting the translocation of proteins into the extracellular milieu. Paradoxically, it possesses the simplest secretion apparatus and represents the largest family of protein-translocating outer membrane porins in Gram-negative bacteria (Yen *et al.*, 2002). This system was first described for the IgA1 protease produced by *Neisseria gonorrhoeae* (Pohlner *et al.*, 1987). By elucidating the relationship between the IgA1 protease gene and its extracellular product, it was shown that the protein precursor contains three functional domains: (i) the N-terminal leader, which is assumed to initiate the inner membrane transport of the precursor, (ii) the mature part of the protein, i.e. the extracellular functional domain, and, (iii) a C-terminal helper domain which is essential for extracellular secretion. Based on their evidence Pohlner *et al.* (Pohlner *et al.*, 1987) proposed a model for secretion in which the helper domain serves as a pore for the secretion of the protease domain through the outer membrane. The protease acquires its active conformation as its extracellular transport proceeds and is further released from the membrane-bound helper by autoprolysis. The soluble proform further matures into

*For correspondence. Email i.r.henderson@bham.ac.uk.

the 106-kDa IgA1 protease and a small stable α -protein. In this model, neither accessory factors for transit from the periplasm to the bacterial surface nor external energy for the secretion across the outer membrane are required. Therefore, this secretion system and the proteins secreted *via* this pathway have been designated autotransporters. Secreted proteins belonging to the type V secretion family (a) contain all the information for translocation through the cell envelope and/or require a single accessory factor and (b) translocate across the outer membrane *via* a transmembrane pore formed by a β -barrel structure (Henderson *et al.*, 1998; Thanassi and Hultgren, 2000). The proteins secreted by this pathway possess unifying characteristics including: (i) an N-terminal signal sequence for inner membrane translocation, (ii) a functional passenger domain that can be surface-exposed or released into the extracellular milieu, (iii) a linker region necessary for translocation of the passenger domain through the outer membrane, (iv) a C-terminal region involved in the formation of a transmembrane pore. Based on these and others criteria, the two-partner system (TPS) has been grouped under the type V secretion umbrella as a distinct subgroup (Henderson *et al.*, 2000a,b; Jacob-Dubuisson *et al.*, 2000). In contrast to the autotransporter pathway where the protein is produced as a single polypeptide, in the TPS the passenger domain (also called the exoprotein) and the pore forming β -domain (also called transporter domain) are translated as two separate proteins, respectively referred as TpsA and TpsB family members (Jacob-Dubuisson *et al.*, 2001). From an evolution point (Danchin, 2003), it is still difficult to discriminate between divergent evolution from a common ancestor or convergent evolution towards a similar solution for secretion of large proteins across the outer membrane (Henderson *et al.*, 2000a,b; Jacob-Dubuisson *et al.*, 2000). Nevertheless, it appears from homology comparisons and the similarities in the gross anatomy of the autotransporter and TPS secretion pathways that the two pathways are related. Thus, in a manner similar to the type IV secretion pathway (Christie and Vogel, 2000), it has been proposed to classify autotransporter protein secretion as type V_a secretion and the TPS in the subgroup of type V_b (Desvaux *et al.*, 2003). More recently, the *Yersinia* adhesin YadA has been presented as the prototype of a novel class of bacterial adhesin belonging to the Oca (Oligomeric coiled coils adhesin) family (Hoiczky *et al.*, 2000; Nummelin *et al.*, 2003; Roggenkamp *et al.*, 2003). These family members possess a particular outer membrane topology and should probably be considered as a subfamily of surface-attached oligomeric autotransporters (Roggenkamp *et al.*, 2003). Thus we propose terming this secretion pathway type V_c . This review will mainly focus on the type V_a secretion pathway, i.e. the autotransporters, for which a considerable amount of research has been completed in the past few years. These recent investigations have challenged, questioned and/or reinforced some aspects of the original model of type V secretion pathway as well as the secretion systems in general. We will first focus on the primary structure of the autotransporters which have been described differently throughout the literature and then summarise the current knowledge regarding the export of autotransporters into the periplasm. Finally, we will describe the translocation of the autotransporter

through the outer membrane discussing the controversies around the folding state of the precursor, the topology of the β -barrel, the energy of translocation and the release effector molecule.

The structure of autotransporter proteins

Autotransporter proteins are synthesized as a single polypeptide containing all the information required for their export and secretion (Henderson *et al.*, 1998). The primary structure of these proteins is basically modular and composed of three main domains: the signal peptide, the passenger domain and the translocation unit (Figure 1A). During export through the inner membrane, the signal sequence is cleaved and the autotransporter is released as a protein or pro-protein form (depending on whether or not it is cleaved after secretion through the outer membrane) into the periplasm. Translocation through the outer membrane *via* the translocation unit completes the secretion of the autotransporter from the cytoplasm to the extracellular milieu. Depending on the autotransporter, the passenger domain may be either displayed on the bacterial cell surface or released in the extracellular environment.

At the N-terminus, the signal peptide (also called signal sequence or leader sequence) targets the protein to the inner membrane for its further translocation into the periplasmic space. The signal sequence is composed of three domains (Figure 1A): (i) a positively charged amino terminus or n-domain, (ii) a hydrophobic core region or h-domain, and (iii) a consensus signal peptidase recognition site also called c-domain (Fekkes and Driessen, 1999). However, some autotransporters possess unusual signal sequences (see the following section) (Henderson *et al.*, 1998). A leader peptidase cleaves the signal peptide permitting the release of the pro-protein into the periplasm.

The adjacent domain is the passenger domain (also called α -domain, N-passenger domain or N-domain). This domain corresponds to the effector molecule since it performs the extracellular function of the autotransporter protein. The passenger domains are very diverse in sequence and function, but to date they have always been linked to a role in bacterial virulence. They can (i) display enzymatic activity (protease, peptidase, lipase, esterase), (ii) mediate actin-promoted bacterial motility, or act as (iii) adhesins, (iv) toxins or cytotoxins, (v) immunomodulatory proteins, or as discovered more recently, (vi) permitting maturation of another virulence protein (Henderson *et al.*, 1998; Coutte *et al.*, 2001; Henderson and Nataro, 2001; Yen *et al.*, 2002). Recently, an intramolecular chaperone domain (also called autochaperone domain) has been identified in some autotransporters (Oliver *et al.*, 2003b). This domain, as its name implies, is absolutely necessary for the correct folding of the BrkA passenger domain on the bacterial cell surface. However, at the moment it is not known whether the presence of this intramolecular chaperone is a feature universal to all autotransporters.

At the C-terminus, the translocation unit (also called helper domain, C-domain, transporter domain or, confusingly, autotransporter domain) is the last main domain (Figure 1A). This domain can be divided in two regions: (i) a short linker region having an α -helical

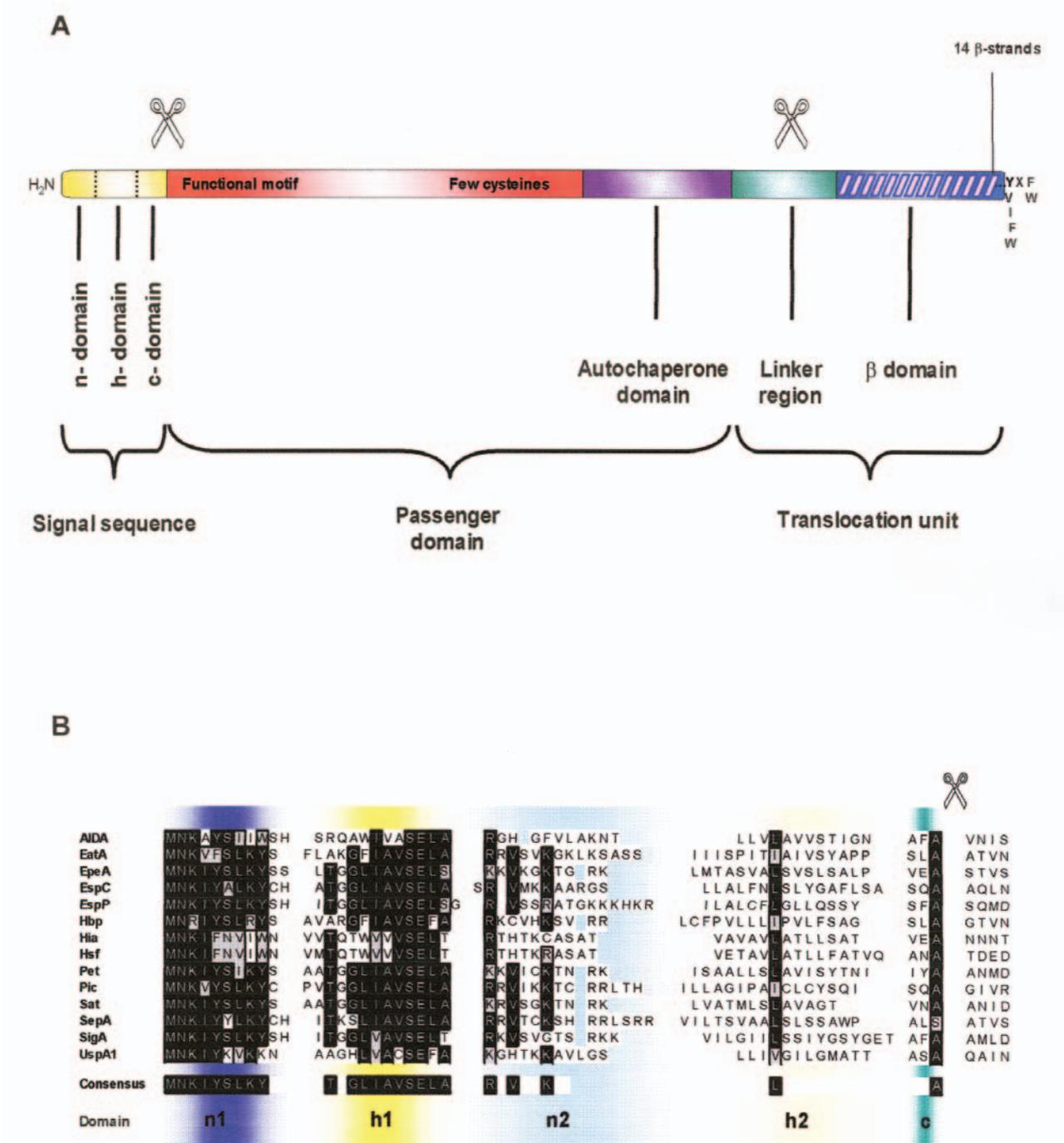


Figure 1. A: Primary structure of an autotransporter. Each of the major domains are depicted, and the scissors symbols denote the points of processing. B: Alignment of the amino-terminal segment of representative autotransporters possessing an extended signal sequence. The blue colour symbolises hydrophilic domains, i.e. the n1- and n2-domains. The yellow colour symbolises hydrophobic domains, i.e. h1- and h2-domains. The green colour symbolises the c-domain. Scissors symbolise the cleavage site.

secondary structure and, (ii) a β -domain (also called the β -core) that will adopt a β -barrel secondary structure when embedded in the outer membrane (Maurer *et al.*, 1999). The translocation unit is the determinant for the secretion of the passenger domain across the outer membrane and a lot of research has focused on this region in the last few years.

The β -domain of autotransporters are all homologous (Yen *et al.*, 2002). Two integral parameters generally permit classification of polypeptides as β -barrels (Schulz, 2003): (i) n , the number of strands in the β -sheets and, (ii) S , the shear number corresponding to the stagger of the strands in the β -sheet. n and S determine the major geometrical features of β -barrels, such as the mean slope of the strands

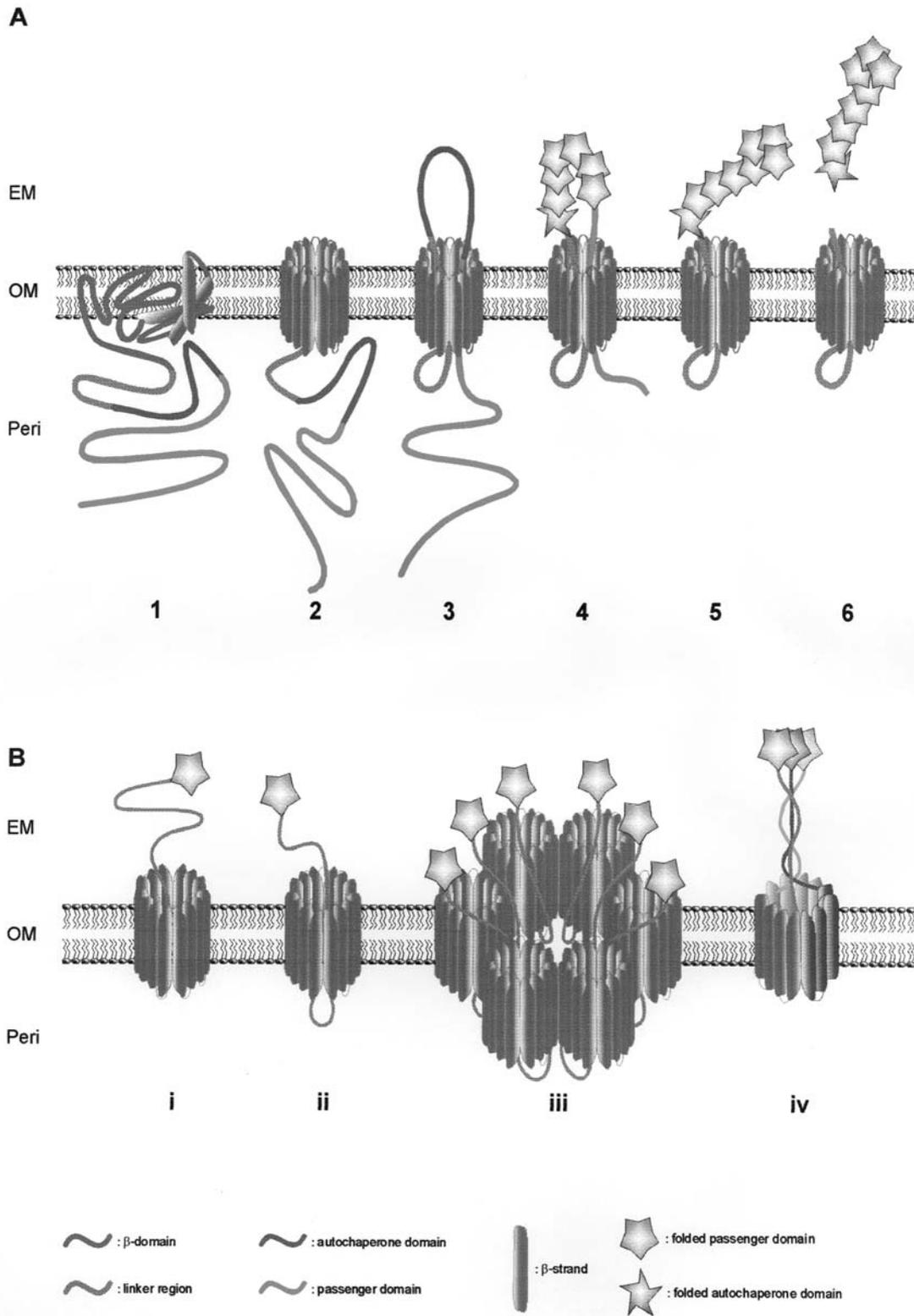


Figure 2. A: Updated model of the passenger domain secretion across the outer membrane. Steps 1 to 2 : insertion of the β -domain into the OM and folding in a β -barrel conformation. Steps 2 to 3 : initiation of secretion directed by the linker region through the pore formed by the monomeric β -barrel. Steps 3 to 4 : secretion and folding of the autochaperone domain which triggers the folding of the passenger domain as it emerges from the pore. Steps 5 to 6 : completion of the secretion and concomitant folding of the passenger domain followed by its release into the extracellular milieu. Peri : periplasm; OM : outer membrane; EM : extracellular milieu. B: Topology of the translocation unit in the outer membrane. i : model of direct folding and display of the passenger domain on the extracellular side of the OM. ii : model of secretion through the monomeric β -barrel. iii : model of oligomerisation of monomeric β -barrels and secretion through the central pore. iv : model of a TolC-like pore formation through trimerisation of translocation units and secretion through the central pore. Peri : periplasm; OM : outer membrane; EM : extracellular milieu.

to the axis of the barrel, α , and the mean radius of the barrel, R (Murzin *et al.*, 1994b, a). Due to the hydrogen-bonding requirements along the whole β -strands, the sheets are also twisted and coiled. β -barrels can have different theoretical topologies, the simplest of those being the all-next-neighbour connection between adjacent strands. Actually, this is the conformation that has been found so far in all integral outer membrane proteins whose structures have been resolved (Delcour, 2002). As these parameters remain unavailable for autotransporters, the exact shape of the autotransporter β -barrels is still elusive and subject to speculation. However, using bioinformatic tools, it has been predicted that the β -domains of most autotransporters exhibit 14 antiparallel amphipathic strands consisting of 9 to 12 residues (Loveless and Saier, 1997; Yen *et al.*, 2002). The β -domains share a consensus amino acid motif at the extreme carboxyl terminus, which represents the final spanning segment (Jose *et al.*, 1995; Loveless and Saier, 1997). The terminal amino acid is always phenylalanine or tryptophan, preceded by alternating hydrophilic (charged or polar) and hydrophobic residues, *i.e.* (Y/V/I/F/W)-X-(F/W).

Inner membrane translocation pathways

The issue of inner membrane targeting and translocation of the autotransporter proteins must in fact be placed in the context of the more general issue of protein export which to date is still not fully understood. In bacteria two inner membrane translocation systems have been described which allow protein export into the periplasmic space. On the one hand, the most recently discovered twin-arginine translocation (Tat) pathway is composed of the TatABCE translocon (Berks *et al.*, 2000), while on the other the Sec (Secretion) system involves the SecYEG translocon (a protein conducting channel) and the cytoplasmic ATPase, SecA, which provides the energy for translocation (Driessen *et al.*, 1998). However, two distinct protein targeting pathways converge at the Sec translocon: the SRP (Signal Recognition Particle)-dependent and the SecB-dependent pathways (Valent *et al.*, 1998). It is worth mentioning that YidC, the *Escherichia coli* homologue of the yeast mitochondrial inner membrane protein (IMP) Oxa1p, is involved in both the Sec-translocase dependent and Sec-translocase-independent IMP assembly (de Gier and Luirink, 2001). This led some authors to classify the YidC pathway as a third inner membrane translocation system (Yen *et al.*, 2002), yet to date YidC has not been implicated in the translocation of proteins across the inner membrane with their further release into the periplasmic space (Samuelson *et al.*, 2000; Froderberg *et al.*, 2003). A subject of controversy is the involvement of other molecular chaperones as targeting factors instead of SecB. While the possible involvement of other molecular chaperones has long been questioned (Mori and Ito, 2001; Muller *et al.*, 2001) and some have even claimed that they cannot substitute for SecB function (Fekkes and Driessen, 1999), it is now clear that some proteins are routed to the Sec system *via* SecB- and SRP-independent pathways (Qi *et al.*, 2002; Beha *et al.*, 2003). Furthermore, SecB is not an essential cell component and should be considered as only one of the plethora of molecular chaperones available

to a newly synthesized polypeptide before its export (Economou, 2002; Randall and Hardy, 2002). However, for the alternative chaperones, like GroEL, GroES or DnaK, the recognition mechanism permitting the targeting of the exported protein to the Sec translocon remains elusive. Nevertheless, taken all together these data show that the inner membrane translocation is more flexible and versatile than originally thought (Pugsley, 1993).

Thus, the first step in autotransporter protein secretion is the recognition and targeting of the cytoplasmic pre-protein, with the help of a cleavable N-terminal signal sequence, to one of the inner membrane transport systems. Using SignalP (Nielsen *et al.*, 1999), bioinformatic analyses revealed that the N-terminal signal sequences of most autotransporters function as prototypical signal sequences of the SecB-dependent pathway (Henderson *et al.*, 1998). In the SecB-dependent pathway, SecB binds to the mature part of the pre-protein. Actually, it has long been held that SecB functions primarily as a specific signal recognition factor by binding to the signal sequence of exported proteins (Watanabe and Blobel, 1989, 1995). However, further investigations have shown definitively that although SecB binds to the mature part of the pre-protein, the signal sequence provides no positive contribution to the binding energy or affinity of the interaction but it only slows the folding of the mature part (Randall *et al.*, 1998). Thus the question arises of how SecB discriminates between cytoplasmic proteins and those to be exported. While the precise mechanism of this discrimination is still not fully understood, it is known that in addition to proteins exported *via* the Sec translocon, SecB acts as a molecular chaperone binding to non secreted proteins and some proteins secreted *via* the Type I protein system (Sapriel *et al.*, 2003). Therefore, some kind of selectivity must govern the SecB targeting towards the Sec system. The slow folding rate of secretory proteins has long been assumed to be the basis for this discrimination, allowing the binding by the secretory chaperone, SecB, and subsequent targeting to the translocase (Fekkes and Driessen, 1999). However, recent investigations suggest that while slow folding may be a necessary criterion for export, it is not sufficient and secreted proteins must contain information, in addition to the signal sequence and slow folding characteristics, that target them to the Sec pathway (Mallik *et al.*, 2002). Nevertheless, it is now clearly established that SecB functions both as a targeting factor and as a molecular chaperone, keeping the precursor in a loosely folded conformation and conferring export competence.

Some autotransporters possess signal peptides containing around 50 amino acid residues which is unusually long compared to other SecB-dependent exported proteins (Figure 1B), but of a similar size to signal sequences of the Tat family. It is worth mentioning that aside from the autotransporter proteins such unusual signal sequences have only been observed in the TpsA and Oca family members of the type V secretion system (Henderson *et al.*, 1998). All of these extended signal sequences consist of a MNKIYSLKY(S/C/H) motif containing hydrophilic and positively charged residues, followed by a conserved motif with a succession of aromatic and hydrophobic residues mainly, *i.e.* GLIAVSELAR, immediately preceding the core n-domain. This sequence extension can either be

described as an extended n-domain (Henderson *et al.*, 1998; Sijbrandi *et al.*, 2003) or perhaps more appropriately as a second signal sequence fused to the first one since two separate n- and h-domains are present (Figure 1B). Thus, these extended signal sequences can be divided into five regions, which from the N-terminus can be successively termed n1-, h1-, n2-, h2- and c-domains. The n2-domain is unusual in the number of basic amino acid residues that it contains, i.e. lysine, arginine and histidine (Brunner *et al.*, 1997). For most of those autotransporter precursors, the h2-domain contains helix-breaking residues. While the extension constituted by the n1- and h1-domains is highly conserved, the sequence of the n2-domain is variable and the following h2-domain even more so. It has been proposed that these conserved unusual features may impose additional properties such as recruiting other accessory proteins like SRP or by driving secretion through the inner membrane in a Sec-independent manner e.g. Tat (Fernandez and Weiss, 1994; Henderson *et al.*, 1998).

While it has long been assumed that the Tat signal peptide motif was highly specific and conserved, recent investigations tend to temper such an assertion. In fact, it has been shown that substitution of one or both arginine residues by lysine could still permit targeting and translocation of the protein through the Tat translocon (Ize *et al.*, 2002). Moreover, some proteins harbouring very distantly related twin arginine motifs, for example the penicillin amidase of *Escherichia coli*, which possess a signal sequence bearing two arginine residues separated by an asparagine residue, can nevertheless be routed towards the Tat pathway (Ignatova *et al.*, 2002). Thus, involvement of the Tat pathway in the autotransporter secretion cannot be completely ruled out.

Controversy exists over whether the SRP-dependent pathway could be involved in the export of secreted proteins. While some results obtained from experiments with fusion proteins suggest that secretory proteins require SRP for export (Kim *et al.*, 2001), it is generally assumed that secretory proteins of Gram-negative bacteria are not substrates of SRP. Certain polytopic membrane proteins of *Escherichia coli* are specifically and exclusively recognized by SRP for inner membrane integration (Koch *et al.*, 1999; de Gier and Luirink, 2001). Therefore, SRP seems specific to IMPs and not involved in the export of secretory proteins (Beha *et al.*, 2003). Interestingly, it has been reported that for some IMPs, while SRP was absolutely required for their translocation, the proteins were not delivered to the Sec translocase (Cristobal *et al.*, 1999). While it has long been assumed that the insertion of IMPs into the inner membrane occurs spontaneously, it now appears that they all require the assistance of protein factors (Froderberg *et al.*, 2003). IMP assembly can be (i) SRP dependent which does not rule out their SecB dependence and *vice versa*, (ii) SRP dependent but independent of the Sec translocase, i.e. SecAYEG, (iii) Sec translocase dependent but differing in their specific Sec-translocase requirements, suggesting a highly flexible translocation mechanism (Samuelson *et al.*, 2000). Indeed, such flexibility is supported by a recent investigation of the Hbp autotransporter of *Escherichia coli*, which possesses the unusual extended signal sequence, which demonstrated that contrary to current opinion the targeting and translocation of this protein through the

inner membrane involved the SRP-dependent pathway including the Sec translocon (Sijbrandi *et al.*, 2003). The Tat translocon did not seem to be involved in secretion and SecB was apparently not required for targeting of Hbp to the Sec translocon, but it could compensate to some extent for the absence of functional SRP. In the SRP-dependent pathway, which in *Escherichia coli* involves the SRP homologue Ffh and the SRP receptor FtsY, as the signal peptide emerges from the ribosome it is first recognised by the targeting factor SRP, presumably by its NG-domain (Cleverley and Gierasch, 2002). As suggested by crystal structure analysis of the SRP, where an unusual RNA-protein interface has been described (Batey *et al.*, 2000), it was speculated that directly or indirectly the extended part of the autotransporter signal sequence, and/or the h2-domain, was important for the recognition by SRP. Once the SRP-nascent polypeptide complex is formed, SRP arrests translation, ensures that the nascent protein complex is delivered to the Sec translocon *via* interaction with the SRP-receptor and is subsequently released allowing the remainder of the protein to be translated such that translation and export are temporally coupled (von Heijne, 1998; Valent, 2001; Koch *et al.*, 2003). In contrast to the SecB-dependent pathway, which permits posttranslational translocation of the protein, the co-translational export of the SRP pathway offers the advantage of preventing premature degradation or folding of the autotransporter protein in the cytoplasm. This is a particularly important feature when one considers that autotransporters range in size from 60-500 kDa. The secretion of the autotransporter Hbp represents the first ever example of secretion of an extracellular protein *via* an SRP-dependent pathway (Sijbrandi *et al.*, 2003).

In contrast, another recent investigation with an autotransporter displaying the unusual extended signal sequence, i.e. EspP of enterohaemorrhagic *Escherichia coli*, revealed that contrary to Hbp, only the SecB dependent pathway seemed to be involved in the secretion (Szabady and Bernstein, 2003). A similar conclusion was drawn from the study of the autotransporter IcsA of from *Shigella flexneri* (Brandon *et al.*, 2003). For EspP, it appeared that the N-terminal extension, i.e. the n1- and h1-domains, could not act as a signal peptide by itself. When it was removed, the core signal peptide facilitated translocation across the inner membrane but seemed to prevent the further processing into the mature cleaved EspP, although it is generally assumed that the signal sequence has no further role after the initiation of the translocation event (Manting and Driessen, 2000). It is of general acceptance that once it has been cleaved by the peptidase, the fate of the signal sequence is to be digested into free amino acids by various peptidases. However, it has been speculated that a second function, distinct from the role of targeting, could be endorsed by release of this peptide (Martoglio and Dobberstein, 1998; von Heijne, 1998). In another recent study by Bernstein's group, it was shown that the fusion of Δ Esp, i.e. the truncated EspP signal sequence containing the n2- and h2- domains only, to proteins normally targeted to the Sec system by SecB leads to the rerouting of these proteins into the SRP pathway (Peterson *et al.*, 2003). It was suggested that the basic amino acids in Δ Esp contribute to eliminating the SecB requirement and, more

generally, that basic residues promote the binding of SRP to only a subset of signal peptides whose hydrophobicity falls slightly below a critical level. Such results seem apparently contradictory with previous finding (Szabady and Bernstein, 2003), however, in the latter study only Δ Esp was used and no data are available about the influence of the original signal sequence on the targeting of EspP or other proteins fused to it. In fact, it seems that the length, the net positive charge and number of hydrophobic amino acids in a targeting signal permit an exquisite degree of specificity and effectiveness for SRP recognition (Peterson *et al.*, 2003). Further insight into the function of the unusual signal peptides in its full length is needed, particularly concerning the intriguing result suggesting the direct or indirect involvement of the signal peptide in the further processing of the mature autotransporter.

Once the pre-pro-protein has been targeted to a specific inner membrane translocon the signal sequence must be removed to release the autotransporter pro-protein into the periplasm. Two types of leader peptidase are known to cleave the signal peptide: the type I serving ordinary pre-proteins and the type II for lipoproteins. The type I peptidase recognises the motif S_nXS_n , where S_n stands for Small neutral side chain residue and X is highly variable. The S_n residues are located at position -3 and -1 relative to the start of the mature protein and are either helix breaking amino acids, such as proline or glycine, or uncharged and/or short lateral chain residues, such as alanine, serine, threonine or valine. The motif recognised by the type II peptidase is L_hXS_nC where L_h stands for large hydrophobic residue and C for cysteine. The amino acid in position +1 is always a cysteine while the L_h residue in position -3 is preferentially a leucine. Several autotransporter lipoproteins, such as AspA from *Nesseira meningitidis*, AlpA from *Helicobacter pylori* or SphB1 from *Bordetella pertussis*, have been identified and characterised (Odenbreit *et al.*, 1999; Coutte *et al.*, 2001; Turner *et al.*, 2002; Coutte *et al.*, 2003; Massignani *et al.*, 2003). However, while the involvement of a type II signal peptidase for the cleavage of those signal sequences can be assumed, to date no direct evidence is readily available for autotransporter lipoproteins.

Outer membrane pore formation

It has been suggested that after its export through the inner membrane, the autotransporter protein, or pro-protein, interacts with the hydrophobic environment of the outer membrane, which promotes its spontaneous insertion into the outer membrane and folds in a thermodynamically favoured β -barrel conformation before passenger domain translocation (Figure 2A) (Henderson *et al.*, 1998). This model was originally based on the mechanism of porin biogenesis (Tamm *et al.*, 2001). The insertion model proposes that as the unfolded protein approaches the outer membrane in an unstructured form, it forms a "molten-disc" intermediate which locates at the interface of the leaflets of the lipid bilayer and subsequently adopts an inside-out "molten-globule" form penetrating more deeply into the phospholipid bilayer to finally attain a well-defined native transmembrane structure (Tamm *et al.*, 2001). These successive intermediates should not be considered as

defined structures but rather as a large ensemble of structures characterized by common properties.

Until very recently it was still believed that even if chaperone proteins bind to unfolded outer membrane proteins to prevent their misfolding, the membrane insertion and concomitant folding and/or assembly reactions were a spontaneous thermodynamically-driven process which did not require any accessory protein (Tamm *et al.*, 2001). This evidence is mostly derived from *in vitro* refolding experiments using the monomeric outer membrane protein OmpA as a model of β -barrel outer membrane proteins (OMP). Recently, this view has been changed by the first description of an assisted folding pathway for integral membrane proteins (Bulieris *et al.*, 2003). Previous investigations performed with other transmembrane-forming β -barrels have suggested that lipopolysaccharide (LPS) and periplasmic chaperone(s), such as Skp or SurA, might be important for the proper OMP assembly into the outer membrane (Freudl *et al.*, 1986; Lazar and Kolter, 1996; de Cock *et al.*, 1999). Using OmpA as an example Bulieris *et al.* (2003) recently proposed a model in which a small number of molecules of the periplasmic chaperone Skp bind to the OMP, maintaining it in a soluble unfolded state. This intermediate periplasmic complex then interacts with a small number of LPS molecules to form a folding competent intermediate. Finally, the folding competent OmpA inserts and folds into the lipid bilayer. Skp and LPS function as co-chaperones and seem to be involved in a general folding pathway of outer membrane proteins as suggested by (i) the binding of Skp to several OMPs, (ii) the lower expression levels of several OMPs following the deletion of the *skp* gene (Chen and Henning, 1996) and (iii) the report of specific LPS binding motifs in several OMPs (Ferguson *et al.*, 2000). However, since deletion of the *skp* gene does not entirely eliminate the presence of OMPs in the outer membrane, but only decreases their concentration, it was also pointed out this Skp-LPS assisted folding pathway was certainly not the only mechanism by which OMPs insert and fold into the outer membrane. This assertion proved to be predictive as in the following months an even more decisive discovery was made, involving the surface antigen omp85, which radically challenged the idea of a spontaneous assembly of OMPs in the outer membrane (Voulhoux *et al.*, 2003). Omp85 is a highly conserved protein since homologues are present in all the complete genome sequences of Gram-negative bacteria. Interestingly, the gene is located in close proximity to the *skp* gene and to the *lpxA* and *lpxB* genes involved in lipopolysaccharide biogenesis. This protein is essential for cell viability and for OMP assembly. Following Omp85 depletion it has been shown that unassembled forms of various outer membrane proteins, including autotransporters, accumulated. Thus Omp85 could possess a general function in the assembly of OMPs though the molecular mechanisms underlying this function remain to be elucidated. More recently, however, Genevrois *et al.* challenged this view by demonstrating that Omp85 was in fact involved in lipid export from the inner to the outer membrane (Genevrois *et al.*, 2003). In this study, the localization of some OMPs was not impaired upon Omp85 depletion but degradation products were observed. Moreover, the isolation of an *omp85* knockout mutant in a LPS-deficient mutant, which would have

definitively demonstrated that Omp85 was only involved in the LPS transport, proved to be nearly impossible to obtain suggesting additional function(s) for Omp85. Also, a concerted mechanism where Omp85 permits phospholipid and LPS transport to and assembly in the outer membrane, as well as the assembly of OMPs, cannot be ruled out completely and necessitates further investigations.

In the case of PhoE, an *Escherichia coli* porin, substitution or deletion of the C-terminal phenylalanine has a drastic effect on protein folding and stabilization of the monomer resulting in an ineffective trimerization and outer membrane localization of the protein (Struyve *et al.*, 1991; de Cock *et al.*, 1997; Jansen *et al.*, 2000). This observation has also been extended to the autotransporter proteins with the example of the autotransporter Hap from *Haemophilus influenzae* where deletion, but not mutation, of the three terminal amino acid residues has been shown to abolish outer membrane localization of the protein (Hendrixson *et al.*, 1997). In addition, interaction with the periplasmic chaperone, Skp, has so far never been reported for autotransporters. The status of the autotransporter proteins in the periplasm is highly controversial, especially when considering how an unfolded or partially folded protein could be maintained and resist degradation by proteases present in the periplasmic space. It has been suggested that general or autotransporter specific chaperones could be involved, but few have been identified so far. However, in the case of the autotransporter IcsA, it has been shown that the periplasmic chaperone activity of DegP was involved (Purdy *et al.*, 2002). For this autotransporter the periplasmic intermediate was also described as being transient suggesting that the translocation unit inserts rapidly into the outer membrane (Brandon and Goldberg, 2001).

Secretion through the outer membrane

The original model of outer membrane secretion predicts that the first of the β -strands passes from the periplasmic space to the external surface thus leaving the passenger domain temporarily extending into the periplasm (Henderson *et al.*, 1998). The first and last β -sheets spontaneously form hydrogen bonds in an antiparallel fashion to close the ring conformation, permitting the establishment of a molecular pore. The alternating hydrophobic side chains of amino acids are embedded within the hydrophobic lipid bilayer while the hydrophilic side chains project into an aqueous environment in the centre of the barrel. Several topologies and associated secretion processes have been proposed throughout the literature (Figure 2B). The simplest hypothesis to explain the secretion of the passenger domain was the direct folding of the β -barrel in a conformation where the passenger domain was displayed in the extracellular milieu. In this model secretion would occur concomitantly with the folding of the β -domain (Figure 2B).

Originally, it was shown that outer membrane translocation was a two step process involving first the insertion of the C-terminal β -domain into the outer membrane and then the translocation of the passenger domain (Klauser *et al.*, 1990, 1992; Klauser *et al.*, 1993a; Klauser *et al.*, 1993b) (Figure 2B). In this model, the

passenger domain is secreted through the hydrophilic channel formed *anteriori* by the monomeric β -barrel. From the seminal studies on the secretion of IgA1 protease, it appears that translocation across the outer membrane takes place in an unfolded or translocation competent conformation. It was thought that a globular conformation was not compatible with the translocation of the protein through the pore formed by the monomeric β -barrel. This view was corroborated by the fact that the formation of disulfide bonds in the passenger domain, effected by the major periplasmic disulfide bond forming enzyme DsbA, reduced the efficiency of secretion of the passenger domain (Jose *et al.*, 1996). This also suggested that the pro-protein (or protein) was accessible to periplasmic enzymes and that at least partial folding of the autotransporter arises in the periplasm. However, the paucity of cysteine residues is also a unifying characteristic of autotransporter passenger domain (Jose *et al.*, 1995). It was then predicted that some mechanisms exist to maintain the protein in a translocation compliant state and to promote proper folding of the passenger domain on the surface of the bacterium. This point stressed that the issues of the folding of the passenger domain, the topology of the β -barrel and the secretion of the passenger domain are intimately linked and cannot easily be dissociated. The existence of an ion channel was demonstrated for the first time with the C-terminal domain of the autotransporter BrkA of *Bordetella pertussis* which displayed an average conductance of 3.0 nS in 1M KCl (Shannon and Fernandez, 1999). It was recently argued that in this study, the channel could not have been present in a native state since the β -barrel of BrkA was refolded *in vitro* after its solubilisation from inclusion bodies (Veiga *et al.*, 2002). Further investigations challenged the view of an unfolded conformation being required for passenger domain secretion. By using a single-chain antibody as a reporter passenger domain, it was concluded that the folding of the passenger domain takes place in the periplasm before, or at least simultaneously, with its own translocation through the outer membrane (Veiga *et al.*, 1999). Moreover, using IcsA as an example, it was demonstrated that the presence or absence of DsbA does not make any difference in the stability and targeting of the passenger domain to the outer membrane, even if reduction of the disulfide bridges allowed a more efficient secretion of the passenger domain (Valls *et al.*, 2000; Brandon and Goldberg, 2001).

The contradictory results of these previous investigations with the original model raised the question of how a folded polypeptide could be translocated through the somewhat narrow hydrophilic channel of a monomeric β -barrel (Nikaido, 1992, 1994). On the basis of a reinvestigation of the IgA1 protease secretion, an alternative secretion model has recently been proposed. This model suggests that instead of secretion through a pore formed by one monomeric β -barrel, secretion would occur through an oligomeric ring-shaped structure consisting of a minimum of 6 β -barrels forming a central pore of about 2 nm diameter (Veiga *et al.*, 2002) (Figure 2B). This structure seemed similar to that of outer membrane complexes found in some other secretion systems, e.g. secretin or fimbrial ushers (Thanassi, 2002). This pore was supposed to be large enough to tolerate the passage of protein in a folded state. Still, the exact

structure of this oligomeric ring remains speculative since it was proposed that the oligomeric ring could also consist of 8-10 monomers. This study contradicts earlier findings, made with the *Escherichia coli* autotransporter Antigen 43, in which passenger-domain/passenger-domain and passenger-domain/ β -domain interactions could be detected by cross-linking experiments, but in which no β -domain/ β -domain interactions were detected (Owen *et al.*, 1996). It is worth mentioning here that the initial characterisation of this oligomeric ring-shaped structure was performed *in vitro* using the C-terminal part of the truncated IgA1 β -domain. IgA1 is the only autotransporter for which such quaternary structural organisation of the β -barrels has been proposed so far. This model raises several questions, notably how the pore formed by this oligomeric ring could be hydrophilic if we consider that the outside of the individual β -barrels are predicted to be mostly hydrophobic and, if so, how could it permit the efficient translocation of a hydrophilic passenger domain. A channel of this size, however, cannot permit the secretion of large folded passenger domains such as a non-reduced single chain antibody (Veiga *et al.*, 1999; Oliver *et al.*, 2003b). Experimental evidence of passenger domain secretion through the common oligomeric ring-shaped channel is still lacking. Nevertheless, such an oligomeric organisation remains compatible with either the secretion of the passenger domain in an unfolded or partially folded state or a secretion process occurring through individual monomeric β -barrels as originally proposed. It is therefore too early to establish this structure and secretion model as a general feature of all autotransporters. The controversy around the topology of the β -barrel of autotransporters should hopefully be resolved in the near future through efforts being made by several research teams to crystallize the translocation unit.

In addition to the β -domain, a very important and essential domain for secretion of the passenger domain is the linker region found upstream of the β -core, which together form the translocation unit (Miyazaki *et al.*, 1989; Maurer *et al.*, 1999; Oliver *et al.*, 2003a,b). While the size of the translocation unit can be quite variable among autotransporters (between 30 and 60 kDa) the size of the β -core is remarkably similar (Oliver *et al.*, 2003a). It has been proposed that the linker region, consisting of a 21 to 39-amino acid α -helical region preceding the β -core, is involved in forming a hairpin structure that leads secretion of the passenger domain through the channel formed by the β -barrel (Henderson *et al.*, 1998).

From the study of the autotransporter PrtS from *Serratia marcescens*, it was demonstrated that the folding of the passenger domain only occurs on the bacterial cell surface and implied a putative intramolecular chaperone region (Ohnishi *et al.*, 1994). A recent investigation has definitively revealed the presence of an intramolecular chaperone, the PD002475 domain, in several autotransporters including BrkA, IgA1 and IcsA (Oliver *et al.*, 2003b). This well conserved domain is found upstream of the translocation unit. It was demonstrated that this domain is important for the folding of the BrkA passenger domain, probably by triggering or initiating correct folding of the passenger domain. In BrkA, this domain could act as an intramolecular building block by scaffolding the folding of any protein linked to it (Ma *et al.*, 2000). This autochaperone domain

is cleaved from the passenger domain of PrtS but not from BrkA suggesting that the folding mechanism(s) may differ depending on the autotransporter (Oliver *et al.*, 2003b). In PrtS, however, a PD002475 domain was not found suggesting that the junction domain probably corresponds to a distinct type of endochaperone. From this study, the model proposed for transport of the autotransporter to the surface was that the passenger domain remains unfolded, or partially folded, as it transits through the channel. Folding is triggered by the autochaperone domain and begins vectorially in a C-terminal direction on the bacterial surface as the passenger domain emerges from the β -domain channel (Figure 2A). The complete folding of the protein then occurs on the surface of the bacteria, suggesting that previous findings were probably biased by the study of a passenger domain unrelated to the autotransporters (Veiga *et al.*, 1999).

Concerning the energy used to translocate proteins across the bacterial outer membrane it has long been speculated that translocation across both membranes could be coupled and the free energy of folding might be the driving force for translocation. In fact, since ATP or GTP hydrolysis has never been reported to occur in the periplasm and that there is no evidence for the existence of a proton motive force (Δp) across the outer membrane, those possibilities have never been considered as a driving force for translocation of the passenger domain through the outer membrane. However, from the study of Veiga *et al.* (1999) it appeared unlikely that the energy required for translocation through the outer membrane would be provided by the self-folding of the passenger domain in the extracellular environment. But since this set of experiments used a non-native passenger domain lacking the intramolecular chaperone domain, a common sorting mechanism assisting the assembly and export of autotransporter proteins that could involve the autochaperone domain cannot be ruled out.

Cleavage between the passenger domain and the translocation unit of the pro-protein can occur on the bacterial surface either well upstream of the linker region or within the predicted α -helical region (Oliver *et al.*, 2003a). In some cases, the passenger domain remains steadfastly associated with the bacterial surface *via* a noncovalent interaction with the β -domain and possibly with the linker region which could act as an anchor for the passenger domain (Li *et al.*, 1991; Benz and Schmidt, 1992; Owen *et al.*, 1996). The passenger domain is not necessarily cleaved but can also remain intact as a large protein with a membrane-bound carboxyl-terminal domain and an amino-terminal domain extending into the extracellular milieu (St Geme and Cutter, 2000). In those cases where cleavage occurs, controversy still exists over whether the cleavage is a result of a membrane bound protease or an autoproteolytic event. For some autotransporters, it has been demonstrated that cleavage was the result of an autocatalytic event involving a serine protease active site present in the passenger domain of the autotransporter (Serruto *et al.*, 2003). The passenger domains may also be processed at several sites and possibly by a variety of proteases (Hendrixson *et al.*, 1997). Some autotransporters have been shown to undergo processing even after deletion of their serine protease motif suggesting the action of

alternative proteases (Benjelloun-Touimi *et al.*, 1995). For *lcsA*, it has been demonstrated that cleavage was the result of another membrane protease called *lcsP* (Shere *et al.*, 1997).

Interestingly, *lcsA* has the unusual characteristic of being localized at a single pole of the bacillus (Goldberg *et al.*, 1993). The current model suggests that, upon translocation into the outer membrane at one pole of the bacillus, *lcsA*, which is anchored in the membrane by its β -domain, diffuses laterally within the outer membrane towards the septum (Steinhauer *et al.*, 1999). *lcsP*, which is localized in the outer membrane, slowly cleaves *lcsA* at all sites on the bacterial surface, but as the insertion of *lcsA* is occurring exclusively at one pole it results in an unipolar distribution of *lcsA* (Steinhauer *et al.*, 1999). Since the localization of *lcsA* is independent of the signal sequence, it has been also proposed that *lcsA* targeting and secretion across the inner membrane occurs only at one pole (Charles *et al.*, 2001). It was suggested that the N-terminal region of *lcsA*, including the signal sequence, could interact with an unknown structure at the pole (Brandon *et al.*, 2003). These models have, however, been revised recently since this apparent polar localisation was partly due to the masking of *lcsA* by lipopolysaccharide (LPS) O-antigen side chains (Oag) (Morona and Van Den Bosch, 2003). Although *lcsA* is predominantly exported to one cell pole export also occurs at the lateral region of the cell but is not detected due to the presence LPS Oag chains.

Outer membrane translocation in related systems

In the bacterial two-partner system (TPS) or type V_b secretion pathway (Desvaux *et al.*, 2003), the passenger domain *TpsA* also inserts into an outer membrane pore formed by a β -barrel of *TpsB* (Jacob-Dubuisson *et al.*, 2001). Compared to the autotransporter proteins, the β -barrel topology is different as indicated by the prediction of 19 amphipathic β -strands in *TpsB*, instead of 14 for the autotransporter β -domain (Guedin *et al.*, 2000). Furthermore, these systems produce different conductance values in 1M KCl (Jacob-Dubuisson *et al.*, 1999). In addition, TPS possesses additional levels of complexity, as suggested by the involvement of *TpsB* in the maturation of the passenger domain into its active form (Jacob-Dubuisson *et al.*, 2001) or by the presence of C-terminal extensions in *TpsA* (Renauld-Mongenien *et al.*, 1996). The TPS implies specific recognition events between the passenger domain and the transporter domain that involves, beyond the signal peptide, a secretion domain harbouring a conserved motif (Schonherr *et al.*, 1993; Jacob-Dubuisson *et al.*, 1997; Grass and St Geme, 2000). The TPS has an apparent lack of versatility since each *TpsB* transporter appears to be devoted essentially to the secretion of one exoprotein (Jacob-Dubuisson *et al.*, 2001). It is suggested that the exoprotein transits the periplasm in an unfolded conformation and folds progressively at the cell surface after it is translocated through the transporter domain (Guedin *et al.*, 1998). Thus, translocation across both membranes seems coupled and the free energy of folding might be the driving force for outer membrane translocation (Jacob-Dubuisson *et al.*, 2001). Several

passenger domains undergo further proteolytic processing concomitantly with secretion. The efficient extracellular release of the mature filamentous haemagglutinin (FHA) of *Bordetella pertussis* depends on the presence of a C-terminal domain in *FhaB*, which is proteolytically removed in the course of secretion. As observed in some autotransporters, this domain acts as an intramolecular chaperone to assist secretion by preventing premature folding (Renauld-Mongenien *et al.*, 1996). Interestingly, it has been demonstrated that the *Bordetella pertussis* autotransporter *SphB1* acted as a specific protease responsible for the bacterial surface maturation of *FhaB*, the filamentous haemagglutinin (FHA) secreted by the TPS (Coutte *et al.*, 2001; Coutte *et al.*, 2003).

More recently, the members of the *Oca* family have been described as a subfamily of surface-attached oligomeric autotransporters, with the example of *YadA* as a prototype (Roggenkamp *et al.*, 2003). *YadA* possesses the three main domains of autotransporter, i.e. N-terminal signal sequence, passenger domain and translocation unit, but with the peculiarity that the C-terminal region consists of only four β -strands. Deletion of the C-terminal domain abolished membrane insertion of *YadA* (Tamm *et al.*, 1993) while the deletion of the linker region resulted in the degradation of the whole protein (Roggenkamp *et al.*, 2003). This region forms a β -barrel pore consisting of 12 β -strands after trimerisation and confers an overall lollipop-like structure on the proteins displayed on the cell surface (Hoiczyc *et al.*, 2000). *TolC* possesses an analogous β -barrel structure, though it also possesses α -helical regions which extend into the periplasmic space (Koronakis *et al.*, 2000). This conformation suggests an additional putative topology for the type V secretion systems (Figure 2).

Concluding remarks

The type V secretion pathway could be briefly defined as the secretion of proteins that are translocated across the outer membrane *via* a transmembrane pore, formed by a β -barrel, and that contain all the information required for translocation of an effector molecule through the cell envelope (Henderson *et al.*, 1998). Three different subgroups of secretion system fulfilling this definition have been described in the literature: the autotransporters, the TPS and the *Oca* family, also called respectively type V_a , V_b and V_c secretion system (Desvaux *et al.*, 2003). The autotransporters are most certainly the subgroup which have attracted most interest in the last decade. Although the original secretion model proposed by Pohlner *et al.* (1987) at first appears quite simplistic, an in-depth analysis of the secretion processes reveals that much remains to be learned about the translocation steps through the inner and outer membranes. These questions have to be placed in the context of more general issues of protein translocation in bacteria. Future discoveries in the secretion process of the autotransporters, the TPS and the *Oca* family will certainly bring mutual benefits and a better understanding of these respective pathways of type V secretion.

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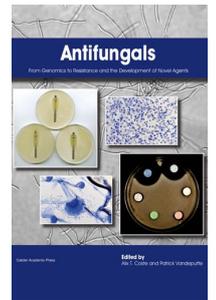
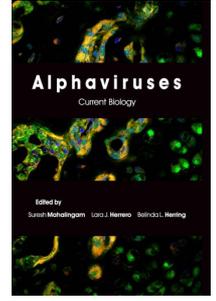
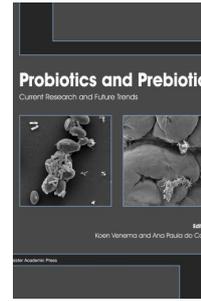
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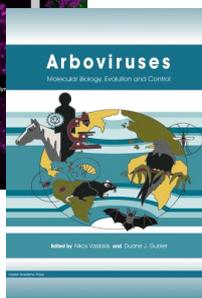
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