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Bacterial secretome: the assembly manual and operating instructions (Review)

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Summary

Bacterial protein secretion is a complex multi-stage reaction that is central to membrane and cell wall biosynthesis and essential for cell viability. An impressive array of experimental tools have been used to dissect this reaction into discreet sub-reactions. Synthesis of these data reveals a fascinating cascade of inter- and intra-molecular interactions that select, sort and target secretory polypeptides to the membrane and then spend metabolic energy to bias their vectorial movement across the membrane plane through a lipid-inaccessible proteinaceous environment. Transmembrane crossing is catalyzed by protein translocase, an astonishingly dynamic molecular machine. The unusual molecular features of the Sec pathway components allows a handful of proteins to catalyze the export of hundreds of secretory polypeptide substrates with astonishing fidelity. Knowledge of the molecular details of the secretion pathway allows us to rationally exploit these features in heterologous protein production biotechnologies and in the development of novel antibiotics.

Keywords: Protein translocase, chaperone, signal peptidase, SecA, SecYEG, SecDF, ATPase, motor protein, membrane transporter.

Introduction

Most of cellular chemistry is catalyzed by cytoplasmic proteins separated by the outside world by semi-permeable membranes. However, several cellular polypeptides reside and function in extracytoplasmic locations and in organellar sub-compartments. Some of these are membrane proteins such as channels and pumps and can occupy 20–40% of the coding capacity of a genome (Greenbaum *et al*. 2001). Others, comprising the secretome, like hydrolytic enzymes, adhesins or toxins, are residents of the bacterial periplasm or eucaryotic organelles or are fully secreted in the surrounding milieu and may represent 10–20% of the proteome (Antelmann *et al.* 2001, Greenbaum *et al.* 2001). Understanding how these charged, elongated, heteropolymers with a tendency to fold rapidly are specifically targeted to membranes and how they then cross lipid bilayers has been and remains a major challenge.

A remarkable array of mechanisms that catalyze protein secretion have been invented in the course of evolution. A common theme that is applicable in all cases is that secretory substrates and machineries have co-evolved for optimal recognition. This is achieved mainly through the use of four tools: a. signal or leader peptides on the exported protein. These are conserved primary and/or secondary features on the secretory proteins that act as recognition determinants and allow these proteins to be 'sorted' from the cytoplasmic residents.

(a) Secretory proteins carrying removable signal peptides are referred to as pre- or pro-proteins;
(b) Sophisticated machineries, collectively known as translocases or transolocons, that ferry the proteins to be exported across the membrane plane;
(c) Piloting factors that are on one hand diffusible and on the other capable of bifunctional recognition of both substrates and the membrane transporter; and
(d) Of post-translocation maturation factors that facilitate folding or release or that introduce covalent modifications such as acylation or proteolytic processing of the targeting sequence.

At least six protein export pathways are present in the Bacterial domain of life alone. Some of these mechanisms are only required for pathogenicity. In other cases, the secretion machineries represent adaptations of pre-existing devices as appears to be the case with the Type III system of Gram negative bacteria (Macnab 1999). One system that is ubiquitous (Cavalier-Smith 2002) and essential in the three domains of life is the Sec (for secretion) system. The present review discusses one's current understanding of the bacterial Sec system. Emphasis will be placed mainly on the export of secretory rather than that of membrane proteins (for recent reviews see Dalbey *et al.* (2000) and de Gier and Luirink (2001)). Unless otherwise stated, the data presented largely reflect the fruits of experimental labour using the Gram negative bacterium *Escherichia coli* as a model system. Genomic analyses have demonstrated *a posteriori* that the basic Sec machinery and most of its auxhilliary factors have been conserved in all known bacteria and, therefore, *E.coli* serves as a true paradigm of protein secretion in the Bacteria.

Bacterial Sec studies are currently going through exciting times. The first era of fundamental cataloguing of the genes/proteins necessary for catalyzing Sec protein secretion is over. It has now entered the more demanding (and more intriguing) phase of deciphering the actual molecular mechanism that underlies catalysis. Hence, Sec studies are faced with the challenge of developing novel experimental tools with which to address the structure, catalytic mechanism, kinetics and conformational dynamics of the system.

Protein secretion is a three stage reaction

Cellular processes are complex multistage chemical reactions in which each step biases the degrees of freedom permitted to the subsequent ones. One of the primary aims of genetic and biochemical analyses is to dissect these reactions in defined consecutive sub-reactions amenable to

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reductionist molecular characterization. Using such tools, the Sec pathway can be described in three distinct stages (Economou 1999):

(I) targeting,
(II) transmembrane crossing, and
(III) maturation/release.

The secretion pathway cast: from gene to protein structure

A combination of bacterial genetics and enzyme purification was used to unearth the genes/proteins involved in the Sec pathway. Most likely all of the necessary genes are currently at hand (Economou 2000, Driessen *et al.* 2001). Isolation of the necessary genes premitted their overexpression and the rapid provision of purified components. Mixed with isolated membrane vesicles, these biochemicals led to reconstituted *in vitro* reactions that approximate living cell conditions closely. This achievement was largely made possible through the pioneering work of Tai, Wickner and the late Mizushima and their co-workers (Chen and Tai 1985, Akimaru *et al.* 1991, Wickner *et al.* 1991). These studies have revealed that the three stages of the reaction are catalyzed by specific components and have defined energetic requirements. More recently, high resolution X-ray crystallography (Paetzel *et al.* 2000, Xu *et al.* 2000, Kawaguchi *et al.* 2001, Keenan *et al.* 2001, Weinkauf *et al.* 2001), low resolution electron microscopy (Meyer *et al.* 1999, Manting *et al.* 2000, Collinson *et al.* 2001) and small angle X-ray scattering (Shilton *et al.* 1998, Dempsey *et al.* 2002) analyses have added significant information on the structure of some of the individual components. The currently known enzymes and sub-units of the Sec pathway are presented in figure 1.

Stage I, targeting to the membrane, is achieved through the help of piloting factors. The Signal Recognition Particle (SRP; Keenan *et al.* 2001) and its membrane-associated receptor (FtsY) are important factors that participate in the membrane integration of some hydrophobic proteins and perhaps even some secretory polypeptides (Dalbey *et al.* 2000, Neumann-Haefelin *et al.* 2000, de Gier and Luirink 2001, Millman *et al.* 2001). SRP comprises a 4.5 S RNA species and the Ffh protein (Keenan *et al.* 2001). Ffh contains a GTPase domain that assembles with an amino-terminal membrane binding domain and the M-domain, a four helix pocket, that binds signal peptides or pre-proteins (Keenan *et al.* 2001). While SRP operates through recognition of signal sequences, other soluble factors such as SecB (for a recent review see Driessen 2001) do not recognize the signal peptide (Randall *et al.* 1990) but only the mature domain of the secreted substrate. SecB, a tetrameric chaperone with multiple binding sites (Randall *et al.* 1998, Xu *et al.* 2000), acts in two capacities:

(a) it further prevents aggregation of secretory proteins in the cytoplasm (Lecker *et al.* 1990), and
(b) it contributes to membrane targeting through its affinity for the SecA subunit of the translocase (Hartl *et al.* 1990, Fekkes *et al.* 1997) and for aromatic and basic residues (Knoblauch *et al.* 1999) in the mature region of substrates (Randall *et al.* 1998).

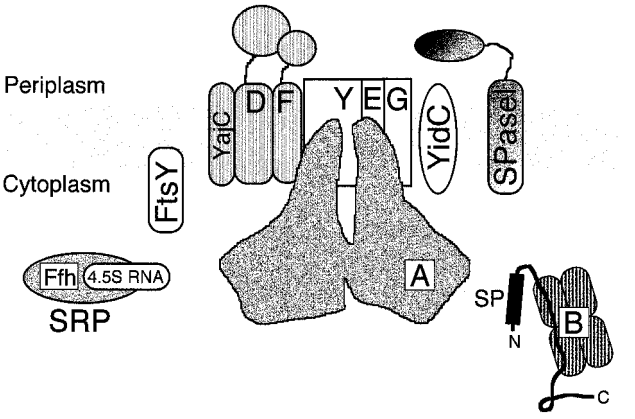

Figure 1. Schematic model of components of the bacterial Sec pathway (see text for details). Secretion proteins are marked with their capital mnemonic letter. SRP (Signal Recognition Particle) is composed of two sub-units: 4.5S RNA species and the GTPase FFh protein and binds to the membrane at FtsY. Signal peptidase I (also known as Leader peptidase) proteolytically processes translocated signal peptides at the *trans* side of the membrane. YidC has been isolated in complex with the translocase core but may also operate as an independent exporter for membrane proteins. The pathway shown is representative of a Gram negative bacterium. In Gram positive bacteria, the tetrameric SecB secretion-specific chaperone shown here is absent and is replaced by other factors such as the CsaA protein. Moreover, Gram positive bacteria have several different Signal peptidases. SP=signal peptide.

SecB is not an essential component (Shimizu *et al.* 1997) and is not omnipresent in the Bacteria. Gram-positive bacteria lack SecB and instead make use of another chaperone, CsaA (Kawaguchi *et al.* 2001) that also binds to SecA (Muller *et al.* 2000) and presumably fulfils a similar targetting/piloting role. Even house-keeping chaperones, such as DnaK and GroEL, have been proposed to have the potential to facilitate protein secretion (Lecker *et al.* 1990, Wild *et al.* 1992).

Irrespective of the precise targeting mode, eventually secretory substrates reach the membrane where they interact with the peripheral SecA sub-unit of the translocase (Stage III). To ensure tight complex formation, the piloting factors themselves have high affinity docking sites at the membrane: the FtsY protein acts as a receptor for SRP (Keenan *et al.* 2001, de Gier and Luirink 2001) and SecA acts as a receptor for SecB (Hartl *et al.* 1990, Fekkes *et al.* 1997, 1998). Formation of these ternary complexes engages substrates with the translocase machine in a specific and reliable manner. Translocase is a complex enzyme with an essential core comprising the SecY, SecE and SecA polypeptides (Akimaru *et al.* 1991, Brundage *et al.* 1990, Douville *et al.* 1995). SecY and SecE are typical polytopic membrane proteins with 10 (Ito 1992) and 1–3 (Schatz *et al.* 1991) transmembrane spanning regions, respectively. Both proteins have been extensively mutagenized, and regions important for function have been identified (Schatz *et al.* 1991, Ito 1992, Mori and Ito 2001). Although one biochemical analysis failed to identify higher order organization in

SecYEG (Yahr and Wickner 2000), all other studies indicate that SecYEG exists in forms ranging from a monomer to a tetramer, with the dimer being the prominent species (Meyer *et al.* 1999, Manting *et al.* 2000, Collinson *et al.* 2001, Bessonneau *et al.* 2002). Electron microscopy studies revealed that SecYEG (Manting *et al.* 2000) and SecYE (Meyer *et al.* 1999) form trimeric or tetrameric ring-like structures with an internal intentation. Recent exciting biochemical data demonstrate that SecYEG trimers assemble dynamically into dimeric structures that represent a minimal translocase core capable of maintaining a preprotein chain in a stable transmembrane state (Bessonneau *et al.* 2002). Higher order SecYEG structures would be necessary in order to envelop the very large SecA dimer (Shilton *et al.* 1998) and shield it from the phospholipid phase (Eichler *et al.* 1997, van Voorst *et al.* 1998). SecA binds to SecYEG (Hartl *et al.* 1990, Matsumoto *et al.* 1997) and leads to stabilization of an apparent $(SecYEG)_4SecA$ holoenzyme form, as seen by STEM analysis (Manting *et al.* 2000). This form was postulated to be the active form of the holoenzyme (Manting *et al.* 2000). Attesting to this, formation of $(SecYEG)_4SecA$ requires the presence either of non-hydrolyzable ATP or of the physiological ligands ATP together with a preprotein intermediate arrested *in statu translocanti* (Manting *et al.* 2000). Additional polytopic membrane polypeptides that optimize catalysis of the translocase machine and provide specialization have been seen to associate under different conditions with SecYE:

(a) SecG, copurifies as a complex with SecYE with an unknown stoicheiometry due to variability in the associated amounts in different experiments (Brundage *et al.* 1990, Joly *et al.* 1994, Duong and Wickner 1997a, Bessonneau *et al.* 2002). SecG is a flexible protein that appears to undergo significant conformational changes during translocation (Nishiyama *et al.* 1996) and to dissociate from SecYE (Joly *et al.* 1994).
(b) SecD and SecF assemble together with YajC, a small polypeptide protein of unknown function (Duong and Wickner 1997a, b). SecD and SecF have large periplasmic domains and have been proposed to participate in later stages of pre-protein release (Matsuyama *et al.* 1993).
(c) YidC, a protein that is essential for the membrane integration of some polytopic membrane proteins (Samuelson *et al.* 2000).

When YidC is overexpressed it forms assemblies with SecYEG (Scotti *et al.* 2000). Since YidC is present in apparent sub-stoichiometric amounts to SecYEG, SecDF are present in supra-stoichiometric amounts to SecYEG, SecG is a 'mobile' component (Joly *et al.* 1994, Nishiyama *et al.* 1996, Duong and Wickner 1997a), SecYE can form higher order forms (Meyer *et al.* 1999, Manting *et al.* 2000, Collinson *et al.* 2001, Bessonneau *et al.* 2002) in a dynamic fashion (Manting *et al.* 2000, Bessonneau *et al.* 2002) and SecA is highly mobile (Economou and Wickner 1994, Economou *et al.* 1995), it is likely that a large number of assembled translocase complexes with a central SecYE core may assemble at any time in the bacterial membranes. Such complexes may specialize in the translocation of sub-sets of substrates. These observations bring to the fore the central distinguishing property of the translocase: its highly dynamic nature. It is this feature that sets this unusual transporter on a class of its own and has evolved as a response to the highly unusual and complex nature of the substrate.

After at least partial transfer of the secretory chain across the membrane has been accomplished (Stage III reactions), the junction between the signal peptide and the mature part of the translocating chain is exposed to the catalytic site of signal peptidase (SPase I) and the signal peptide is severed. The catalytic domain of SPase I resides in the periplasm while being tethered to the membrane through a hydrophobic aminoterminaland anchor and is thought to be apposed to the membrane plane through a hydrophobic interface (Paetzel *et al.* 2000, 2002). A sub-class of secretory proteins undergo fatty acylation on the first residue of their mature domain prior to cleavage and another enzyme (SPase II) has evolved to cleave these signal peptides (Mizushima 1984). In Gram positive bacteria, a bevy of SPases (in some cases at least six) has been selected. In these cells, the various SPases appear to take an active part in the trafficking of secreted enzymes by displaying substrate specificities (Bron *et al.* 1998). Finally, release of translocated proteins and proper subsequent sorting involves additional factors specific for lipoproteins (e.g. Yakushi *et al.* 2000) and non-acylated polypeptides (e.g. Schafer *et al.* 1999, Harms *et al.* 2001, Rizzitello *et al.* 2001).

An exquisite motor for an unusual machine

The SecA sub-unit of protein translocase deserves particular mention due to a number of distinguishing features. SecA:

(a) is an essential component of the reaction and is ubiquitous in the bacterial domain of life,
(b) is physically associated with both membrane and soluble Sec pathway components as well as with substrates and phospholipids,
(c) is the only known energy-converting enzyme involved in the pathway,
(d) shuttles between a peripheral membrane and a soluble cytoplasmic state, and
(e) undergoes measurable conformational changes.

SecA genes can be found in some bacteria in two copies that have distinct roles and specificities (Braunstein *et al.* 2001).

SecA is a dimeric protein of 102 kDa protomer mass that is seen by small angle X-ray scattering as an elongated molecule of 8×12 nm (Shilton *et al.* 1998, Dempsey *et al.* 2002). A high resolution structure of SecA is anticipated (Weinkauf *et al.* 2001). SecA is a multidomain protein, and each protomer is build of two distinct primary structural elements: the aminoterminal ATPase region (N-domain) of 68 kDa and the C-terminal (C-domain) of 34 kDa (Price *et al.* 1996, Karamanou *et al.* 1999). Dimerization is attributed to the C-terminal domain, since this region readily forms dimers in solution (Hirano *et al.* 1996, Karamanou *et al.* 1999). However, even the N-domain alone has a tendency to

assemble at high concentration in tetrameric units (Dempsey *et al.* 2002). The N-domain contains the seven Motifs characteristic Superfamily 2 RNA helicases (Koonin and Gorbalenya 1992, Sianidis *et al.* 2001). Moreover, it is organized in a manner similar to the analogous ATPase region of helicases (Sianidis *et al.* 2001) and will be referred to hereafter as 'DEAD motor'. Intriguingly, the DEAD helicase family motifs of SecA are essential for protein translocation, suggesting conserved mechanistic features between two ATPase motors with different substrate specificities. Nevertheless, the ancestral RNA unwinding activity of SecA has also been maintained (Park *et al.* 1997), although its physiological role remains unclear (Schmidt *et al.* 2001). The C-domain is less well conserved but contains IRA1 (Intramolecular regulator of ATPase), a sequence that is widely conserved in SecA proteins. IRA1 has an essential role in coupling DEAD motor ATP hydrolysis to protein translocation (Karamanou *et al.* 1999).

The domains and sub-domains that build SecA have been physically reconstituted into functional assemblies (Karamanou *et al.* 1999, Sianidis *et al.* 2001). Biocomputing comparisons followed by mutagenesis (Mitchel and Oliver 1993, Mori and Ito 2001, Sianidis *et al.* 2001) and genetic analyses (e.g. Huie and Silhavy 1995, Mori and Ito 2001) have also led to the identification of a number of sites on the enzyme that are important/essential for function. The combination of domain dissection of SecA and the isolation of mutants has permitted detailed catalytic characterization and identification of ligand binding sites.

The number of nucleotide binding sites on SecA has been a matter of debate. Original analysis of ATP binding by cross-linking suggested the presence of three ATP binding sites per SecA protomer (Lill *et al.* 1989) while another study employing equilibrium dialysis later proposed the presence of two sites per protomer (Mitchel and Oliver 1993). One of the proposed ATP sites, termed NBD (Nucleotide Binding Domain), conforms with the well characterized Walker Box sequences that are involved in binding and hydrolysis of ATP in a number of ATPases (Walker *et al.* 1982). A second nucleotide binding sequence was proposed to lie between residues 500–630 of SecA (Mitchell and Oliver 1993). However, this sequence is non-canonical and not shown to be directly involved in nucleotide binding (Sianidis *et al.* 2001). Rather, this region termed IRA2 (Intramolecular Regulator of ATPase) was recently shown to participate in the binding of ATP at the high affinity NBD site and to optimize ADP cycling and ATP hydrolysis (Sianidis *et al.* 2001) in a way analogous to the corresponding mechanism in DEAD helicases.

Pre-proteins (Shinkai *et al.* 1991, van Voorst *et al.* 2000) and signal peptides (Miller *et al.* 1998, Baud *et al.* 2002) bind to SecA in solution and at the membrane (Hartl *et al.* 1990, Roos *et al.* 2001). A specific region of SecA downstream of the NBD site has been implicated in binding pre-protein substrates. This region was termed Substrate Specificity Domain (SSD) and encompasses residues 220–360 that are unique to SecA and absent from other helicases and its borders are accessible by proteolysis (Baud *et al.* 2002). Residues in this region are important for the binding of pre-proteins to SecA:

(a) residues 200–234 are required for high affinity binding of signal peptides (Baud *et al.* 2002),
(b) Tyr326 appears to affect the efficiency of proOmpA binding (Kourtz and Oliver 2000), and
(c) region 267–340 was shown to cross-link to whole pre-proteins (Kimura *et al.* 1991).

Interestingly, binding of pre-proteins to the DEAD motor domain of SecA is sensed (Ding *et al.* 2001) and regulated *in trans* by the IRA1 sequence located in the C-terminal domain of SecA (Tripplett *et al.* 2001, Baud *et al.* 2002).

SecA binds with high affinity to SecY (Hartl *et al.* 1990, Matsumoto *et al.* 1997), but the precise mechanism of SecA interaction with SecY is still unclear. SecY binding is likely to occur at the N-terminal DEAD motor domain (Dapic and Oliver 2000), although binding to the C-terminal domain has also been reported (Snyders *et al.* 1997). Phospholipids appear to bind to at least two sites on SecA (Breukink *et al.* 1993), of which one has been mapped to the extreme C-terminal tail (Breukink *et al.* 1995) that is also the binding site of the SecB chaperone (Breukink *et al.* 1995, Fekkes *et al.* 1997) that delivers some substrates to SecA. This C-terminal tail is found only in some SecA proteins and, as expected, is not essential for *in vitro* function (Breukink *et al.* 1995). The observation that this extreme C-terminal region is required for *in vivo* protein secretion in *E.coli* (Breukink *et al.* 1995) but not in *B.subtilis* (van Wely *et al.* 2000) suggests that it may be involved in optimizing the reaction, presumably by facilitating docking of SecB-preprotein complexes to SecA in Gram-negative bacteria.

Fuelling the translocase machine

Of the three stages of protein secretion, only stages I and II have a proven requirement for metabolic energy. During stage I, the Ffh component of the SRP and its membrane receptor FtsY, both GTPases, use GTP to tightly regulate and coordinate their activities in passing along the substrate to the translocase (Keenan *et al.* 2001, Lu *et al.* 2001, de Gier and Luirink 2001). However, GTP is not required subsequently for actual transmembrane crossing. Other chaperone-like factors such as SecB do not require energy input for substrate binding and for membrane targeting (Hartl *et al.* 1990).

Stage II has an essential requirement for energy input in the form of both ATP (Chen and Tai 1985) and the transmembrane proton gradient (Yamada *et al.* 1989b). The ATP requirement has been solely attributed to SecA-related activities. During translocation the enzyme increases its ATP turnover per NBD site from 3 to 13 min^{1} in a substrate- and SecYEG-dependent manner (Sianidis *et al.* 2001). Binding of ATP and its subsequent hydrolysis drive different conformational states (Economou and Wickner 1994). A stoicheiometry of 20 ATP spent per translocated pre-protein substrate of 70 aminoacyl residues has been determined (Bassilana *et al.* 1992). However, for the larger model pre-protein proOmpA, a substrate of ~280 residues widely used in *in vitro* assays (Chen and Tai 1985, Wickner *et al.* 1991) and that can acquire some secondary and tertiary structure prior to translocation (Lecker *et al.* 1990), a

calculated ~3000 ATP are needed per proOmpA translocated (Schiebel *et al.* 1991). This indicates that in the case of proOmpA either the *in vitro* reaction is largely uncoupled or energy expenditure is required for additional aspects of catalysis, e.g. prevention of folding of the substrate.

Tighter coupling between ATP expenditure and preprotein translocation is seen when membranes are provided with a transmembrane proton gradient (Yamada *et al.* 1989b, Schiebel *et al.* 1991). The proton motive force (pmf) can not initiate the translocation reaction on its own (Yamada *et al.* 1989b, Schiebel *et al.* 1991, Shiozuka *et al.* 1990) since this step depends on SecA and its contribution is substrate-dependent (Yamada *et al.* 1989b). Nevertheless, the pmf provides a four-to-ten fold optimization of translocation yield (Yamada *et al.* 1989a, Driessen and Wickner 1991) and lowers the ATP requirement of the system (Shiozuka *et al.* 1990). This contribution can be made redundant by addition of excess of SecA (Yamada *et al.* 1989a). Importantly, the pmf can complete translocation of chains that have translocated 60–80% of their length (Schiebel *et al.* 1991). An additional role for the pmf is the provision of direction for the vectorial process (Driessen 1992). Although the molecular mechanism of pmf function is completely unknown, one open possibility is that it plays a role in unfolding the substrate (Arkowitz *et al.* 1993). This was shown for the mitochondrial translocase (Huang *et al.* 2002). Two observations indicate that the pmf may operate directly on SecY:

(a) the observation that PrlA4, a mutant SecY protein, is independent of pmf for efficient translocation of a secretory polypeptide (Nouwen *et al.* 1996), and
(b) the pmf can stimulate protein translocation in proteoliposomes containing only SecYEG (Brundage *et al.* 1990). Pmf perhaps acts by altering SecYEG conformation or its oligomerization state.

Other membrane transporters have been suggested to operate through pmf-driven changes (Lolkema *et al.* 1998). A defect in the maintenance of a stable proton gradient was seen in the absence of the SecDF proteins (Arkowitz and Wickner 1994), but this effect was later shown to be indirect (Nouwen *et al.* 2001).

The translocase machine at work

With all the available data amassed by reductionist approaches, one can now piece together a stepwise, sequential model of protein trafficking through the bacterial Sec pathway:

(a) *'Pre-assembly' step.* Cytoplasmic SecA rapidly hydrolyzes ATP (Karamanou *et al.* 1999, Sianidis *et al.* 2001) and remains in the thermodynamically stable and catalytically inactive ADP state (den Blaauwen *et al.* 1996, Sianidis *et al.* 2001). The ADP state of SecA is more compact (den Blauwen *et al.* 1996, Karamanou *et al.* 1999), although this is not reflected in any gross alteration in its shape (Shilton *et al.* 1998). Substrates (Lecker *et al.* 1990, Shinkai *et al.* 1991, van Voorst *et al.* 2000, Roos *et al.* 2001) and SecB (Fekkes *et al.* 1997) can bind to cytoplasmic SecA but with very low affinity (den Blaauwen *et al.* 1997, Baud *et al.* 2002).
(b) *'Assembly' step*. SecA is targeted to the membrane by virtue of the high affinity of its DEAD motor for SecYEG (Dapic and Oliver 2000). This affinity is four-fold enhanced by the presence of the C-terminal IRA1 switch (Vrontou and Economou unpublished results).
(c) *'Priming' step*. SecA binds to SecYEG with nanomolar affinity (Hartl *et al.* 1990) through its DEAD motor domain (Dapic and Oliver 2000). In the assembled holenzyme, the IRA1 switch is partially inactivated, thus enhancing nucleotide cycling (Karamanou *et al.* 1999) and signal peptide binding (Baud *et al.* 2002). This 'primed' translocase displays enhanced affinity for isolated signal peptides (Baud *et al.* 2002) and nanomolar affinities for substrate/SecB complexes (Hartl *et al.* 1990, den Blaauwen *et al.* 1997) and as a result stable SecA/substrate ternary assemblies are formed.
(d) *'Triggering' step*. Binding of substrates activates SecA translocation ATPase (Lill *et al.* 1990). Although the precise mechanism of this activation is unknown, it is anticipated that pre-proteins lose IRA2-NBD association and thus stimulate nucleotide exhange (Shinkai *et al.* 1991). Signal peptides (Miller *et al.* 1998) and mature domains (Bassilana *et al.* 1992) may have distinct roles ATPase stimulation upon transfer onto SecA (Fekkes *et al.* 1998).
(e) *'SecA-preprotein co-insertion' step*. The binding energy of ATP stabilizes a more 'loose' SecA conformation (den Blaauwen *et al.* 1996) and drives a conformational change in SecYEG-bound SecA that renders SecA more membrane integral (Economou and Wickner 1994, Kim *et al.* 1994, Eichler and Wickner 1997). This reaction has been termed membrane 'insertion' and occurs at SecYEG (Economou and Wickner 1994). During SecA membrane insertion, extensive regions of SecA become excluded from the lipid phase (Eichler *et al.* 1997, van Voorst *et al.* 1998) and others become exposed to the *trans* periplasmic side (Kim *et al.* 1994, Ramamurthy and Oliver 1997). Different studies revealed that regions both present on the DEAD motor and on the C-domain become membrane-inserted (Economou and Wickner 1994, Eichler and Wickner 1997, Chen *et al.* 1998). Membrane integration of the various SecA domains has never been studied with non-invasive biophysical tools and, therefore, the extent of the conformational changes that they undergo are still a matter of debate. SecB may at this stage facilitate nucleotide SecA interactions (Miller *et al.* 2002) but after SecA membrane insertion has taken place it is no longer necessary and is expelled (Fekkes *et al.* 1998). Binding of signal peptide may stimulate the SecA insertion reaction by preventing the hydrolysis of ATP (Baud *et al.* 2002). Concomitant with SecA insertion, short preprotein segments of 20–30 aminoacyl residues are also moved into the membrane plane during this subreaction (Schiebel *et al.* 1991, Joly and Wickner 1993, Economou and Wickner 1994, van der Wolk *et al.* 1997). Auxhilliary contribution from SecDF (Economou *et al.* 1995, Duong and Wickner 1997b) and SecG (Nishiyama

et al. 1996, Duong and Wickner 1997a) stabilize the inserted form of SecA by an unknown mechanism. Exactly how SecA and SecY recognize and move hundreds of different substrates across the membrane remains unknown.

(f) *'Pre-protein dissociation from SecA' step*. Using a model liposome system it was proposed that ATP hydrolysis causes dissociation of pre-proteins from SecA (Schiebel *et al.* 1991). A similar reaction may take place during translocation, although the extent of the release of the aminoacyl polymer from the enzyme and the exact mechanism remain a matter of conjecture. However, it is clear that at advanced stages of translocation, secretion intermediates can be stably trapped *in statu translocanti* after removal of SecA (Schiebel *et al.* 1991, Joly and Wickner 1993).

(g) *'SecA deinsertion' step*. A single round of ATP hydrolysis reverts SecA to the 'closed' ADP state (Karamanou *et al.* 1999, Sianidis *et al.* 2002). The DEAD motor domain of SecA acquires the 'compact' ADP state (den Blaauwen *et al.* 1996), 'tightens' and associates more closely with the IRA1 switch. The binding energy of ADP is sufficient to drive deinsertion, since D209N-SecA, a mutant which is unable to acquire the ADP-conformation, can insert in the membrane but can not deinsert (Economou *et al.* 1995). SecA, therefore, reverts to a more 'peripheral' asssociation with the membrane, presumably in such a way that it can be reloaded with the next segment of the pre-protein polymer. A hand-over-hand mechanism may be at play and can explain the processivity of the enzyme (Economou 1998, 1999). The ability of SecG alter its membrane topology has been proposed to facilitate SecA cycling by an unknown mechanism (Nishiyama *et al.* 1996).

(h) *'Pmf-driven' step*. At advanced stages of translocation and whenever the translocating chain is not engaged with SecA, forward movement can be driven by the pmf (Schiebel *et al.* 1991, Driessen 1992). On the other hand, the pmf was shown to promote SecA cycling (Nishiyama *et al.* 1999). The pmf may, therefore, both promote SecA deinsertion and drive forward pre-protein movement.

(i) *'Maturation' step*. Signal peptides get cleaved soon after translocation has initiated (Paetzel *et al.* 2000). Henceforth, the mature chain is allowed to move freely within the translocase 'channel' (Driessen 1992).

(j) *'Multiple catalytic turnovers'*. The aminoacyl polymeric substrate is never released from the grasp of the translocase until the stepwise reaction (Schiebel *et al.* 1991, Economou *et al.* 1995, van der Wolk *et al.* 1997) is complete and, therefore, the enzyme is processive by necessity (Economou 1998). During its transmembrane crossing, the pre-protein is shielded from phospholipid and remains in the viscinity of both SecA and SecY (Joly and Wickner 1993). SecA removal is maintained stably within a SecYEG dimer (Schiebel *et al.* 1991, Driessen 1992, Bessonneau *et al.* 2002). Despite being fully engulfed, sterically trapped translocation intermediates that no longer contain signal peptides must associate loosely with the translocase, since they can be made to rapidly move forward or backward (Schiebel *et al.* 1991, Driessen 1992, Joly and Wickner 1993). To complete translocation of the elongated polymeric substrate that, if extended, is several times as long as the membrane is wide, translocase was proposed to perform several turnovers (Schiebel *et al.* 1991, Economou and Wickner 1994, Economou *et al.* 1995). Adding to the challenge, since translocase can translocate hundreds of different substrates it should not be recognizing side chains on the substrates but rather the α-carbon backbone. Understanding the molecular mechanism of translocase processivity remains an exciting future challenge.

(k) *'Release' step*. Clearly, as pre-protein chains are pumped out from the *trans* side of the inner membrane, folding may be initiated. Although protein translocation *in vitro* in purified systems does not appear to require any periplasmic factors, the folded state of the translocated material has never been thoroughly examined in these systems. Sec components such as SecDF (Matsuyama *et al.* 1993) or additional periplasmic chaperone-like proteins may enhance the rate of the folding and/release reaction *in vivo* (Rizzitello *et al.* 2001). It is possible that events in the *trans* side actively facilitate/enahance the rate of the translocation reaction itself. This could be analogous to the role of Hsp70 and Bip in mitochondria (Matouschek *et al.* 2000) and the endoplasmic reticulum (Johnson and van Waes 1999). The completely translocated mature chain is fully released into the periplasmic space of Gram negative bacteria, where it may become resident or then targeted to the outer membrane, or, in the case of Gram positive bacteria, the surrounding milieu.

The availability of new biophysical assays and of more high resolution structures are expected to allow thorough testing of the above model.

Exploiting the secretome: protein secretion biotechnology

The significant strides made in understanding of the basic machinery in protein secretion in living cells has opened up multiple opportunities for biotechnological exploitation (Gumpert and Hoischen 1998, Baneyx 1999, Braun *et al.* 1999, Cornelis 2000, Swartz 2001) and these will be briefly discussed here. One avenue that has been exploited is further optimization of production of natively secreted or surface-exposed enzymes. This was particularly important in the case of industrial enzymes of the type used in the food, garment, paper, detergent industries (lipases, proteases, amylases, etc.) and in the development of vaccines. In these efforts there is a departure from the basic biology workhorse (*E.coli*) and a venture in bacteria with more fascinating properties and adaptations. Key players in this effort are the Gram-positive bacteria. These cells miss an outer membrane and can, therefore, secrete proteins directly into the medium where they can be easily harvested. Moreover, these industrial microorganisms have been a main source of natively produced industrial enzymes, and so optimization is easier than starting *de novo* with genetic engineering of

heterologous systems. Finally, Gram positive bacteria are frequently hyper-secretors at least of some enzymes, suggesting that they may have secretion pathways that have been optimized through evolution.

Another effort in protein secretion biotechnology has focused on the 'forcing' of heterologous, sometimes even cytoplasmic, proteins through the secretory pathway. This approach would be of great value for heterologous biotechnology products including biopharamaceuticals, antibodies, etc. In essence, heterologous protein production of any type tries to have a foreign polypeptide efficiently synthesized in a bacterial (or other) host and, subsequently, establishes methodologies to fish out the foreign product from within a mixture of $\sim 2000-4000$ polypeptides produced natively by the microbial host. Having achieved remarkable progress in highly-efficient bacterial gene expression technologies (Makrides 1996, Baneyx 1999), one of the most important remaining rate limiting steps for the cost-efficient production of heterologous polypeptides is the solubility of the produced proteins, the down-stream processing effort involved and issues of product quality. Expressing heterologous proteins in such a way that they are secreted from the bacterial host aims at solving a number of potential problems:

(a) Expression in the periplasm or even better in the surrounding medium has the potential of reducing downstream-processing since the number of host proteins is low (at best 200 polypeptides in *B.subtilis*; Antelmann *et al.* 2001).
(b) Secretion of some proteins prevents their accumulation in the cytoplasm in inclusion bodies (Lilie *et al.* 1998).
(c) Secretion may alleviate the toxicity of the heterologous protein to the secreting host.

How can heterologous proteins be forced through the Sec pathway? The observation that signal peptides are only temporary aminoterminal add-ons that fulfill a piloting function and that similar signal peptides guide the export of hundreds of dissimilar native polypeptides raised the possibility that the signal peptide can be used as a covalent tag that can be added to heterologous polypeptides. Many heterologous proteins expressed in this way are guided to the secretion pathway (Lammertyn and Anne 1998, Braun *et al.* 1999, Cornelis, 2000, Pozidis *et al.* 2001). The Sec components are of course oblivious of the heterologous chain's genetic history and the system, being as tolerant for side-chain information as it is, operates on the heterologous substrate as if it were a native protein. Several examples of this approach have been demonstrated. Optimized signal peptides have been used as tools to enhance the secretion capacity of different strains (Berges *et al.* 1996, Lammertyn and Anne 1998, Braun *et al.* 1999). Putting this idea to practice has revealed aspects of the methodology that need to be addressed such as optimal translation levels (Lammertyn *et al.* 1996, Simmons and Yansura 1996), co-expression with cytoplasmic chaperones (Hayhurst and Harris 1999) and proper folding in the periplasm (Swartz 2001) in order for optimal yields to be obtained. In general, protein secretion biotechnology is not a panacea for all production evils but has enormous room for development and has proven very powerful in several cases (for excellent reviews see Baneyx 1999, Cornelis 2000, Swartz 2001).

Exploiting the secretome: novel antibiotics

One of the prominent features revealed by basic studies of the Sec pathway was that several of the Sec components are essential for bacterial life through their requirement for the assembly and maintenance of the cell envelope and/or pathogenicity through the delivery of toxins, adhesins, etc. Importantly, some of the Sec components are unique to bacteria and absent from humans. These observations raised the possibility that the bacterial Sec pathway could become a target of novel antibiotics. Similar past efforts have had only moderate success. Nevertheless, the recent basic biology insights, the availability of novel bioassays and near-atomic resolution structures have renewed hope for such a possibility. Since these ideas have been presented in detail elsewhere (Economou 2001), only two of the most prominent potential targets, Signal peptidase I and the SecA motor, will be briefly discussed here.

Maturation of translocated polypeptides after proteolytic removal of the signal peptide is essential for membrane biogenesis (see above). The availability of simple *in vitro* assays and the fact that the catalytic domain of the processing enzymes SPase I and II are exposed to the periplasmic space prompted early HTS efforts. Several inhibitors have been identified for both bacterial peptidases (Black and Bruton 1998). Recently, an atomic resolution structure of the periplasmically exposed catalytic domain of SPase I in an apoprotein form (Paetzel *et al.* 2002) and in complex with a beta-lactam inhibitor has become available (Paetzel *et al.* 2000). Availability of SPase I structures will be a major step towards the optimization and development of available lead compounds.

Its modular architecture and several enzymatic activities render SecA a highly attractive candidate for antibiotic targeting. Several inhibitors affecting distinct SecA sub-reactions could be potentially identified. One such target could be the SecA ATPase activity that is essential for translocase to work (Lill *et al.* 1990, Economou *et al.* 1995). A particularly attractive feature of this activity is that it appears to be controlled by several regions of the molecule (Karamanou *et al.* 1999, Sianidis *et al.* 2001). High throughput screening efforts by a number of companies employing mainly the *in vivo* assays have identified inhibitors of the SecA ATPase activity (Alksne *et al.* 2000, Sugie *et al.* 2002). However, these small-size inhibitors were of limited value in the former case since they were shown to be general inhibitors of many other ATPases. The availability of several defined *in vitro* assays that follow SecA sub-reactions and the anticipated three-dimensional structure of SecA (Weinkauf *et al.* 2001) should contribute significantly to this effort.

Conclusions

The bacterial Sec pathway ushers cytoplasmically synthesized polypeptides to the outside of a cell. A complex cascade of sequential protein–protein interactions that occur with affinities regulated by nucleotides or pre-protein ligands

results in vectorial unidirectional transfer of proteins through the pathway. Recent studies have gradually led the field to increasing maturity. Structural biology and biophysical tools and quantitative enzymology used in concert with traditional *in vitro* and *in vivo* assays now open the way to a true understanding of translocase catalysis and regulation. These features can now be exploited in rational approaches to optimize biotechnological production of secreted enzymes and biopharmaceuticals that require little down stream-processing. Moreover, in depth knowledge of atomic resolution structure and catalysis of the targetable components of the pathway permits the rational design of novel antibiotics.

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