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Leaving home ain't easy: protein export systems in Gram-positive bacteria

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Abstract

Transport of proteins into or across biological membranes is catalyzed by membrane-bound transport machineries. In Gram-positive bacteria, the vast majority of proteins are exported out of the cytosol by the conserved general secretion (Sec) system or, alternatively, by the twin-arginine translocation (Tat) system, that closely resemble their well-studied counterparts in Gram-negative bacteria. Besides these common major export routes, additional unique protein export systems (such as accessory Sec2 systems and/or type VII/WXG100 secretion systems) exist in some Gram-positive bacteria that are specifically involved in the secretion of limited subsets of proteins.

Keywords: Protein translocation; Sec pathway; Twin-arginine-translocation; Sec2 systems; Type VII secretion; WXG100 secretion

1. Introduction

In contrast to Gram-negative bacteria that are surrounded by two membranes, Gram-positive bacteria have been considered to be less complex in structure, since they were generally thought to lack an outer membrane. As typically described in the textbooks, the cell envelope of Gram-positive bacteria consists of the cytoplasmic membrane followed by a thick peptidoglycan layer to which further compounds such as capsular polysaccharides, cell wall teichoic acids or proteins (either covalently or non-covalently bound) can be attached. Proteins that are covalently bound to the cell wall carry C-terminal sorting signals that are recognized and cleaved by sortase enzymes that subsequently link the respective proteins to the penta-glycine crossbridges of the peptidoglycan. Other cell-wall-associated proteins, the S-layer proteins that form para-crystalline sheets at the surface of some Gram-positive bacteria, possess specific domains, the so-called surface (S)-layer homology domains (SLH), which tether the S-layer proteins to secondary cell wall carbohydrates at the surface of the respective bacteria. For an excellent in-depth overview on the mechanisms involved in the anchoring of exported proteins to the cell wall (including sortase-mediated protein anchoring to peptidoglycan and assembly of S-layer structures), the reader is referred to a recent review article by Schneewind and Missiakas (2012). However, it became clear that some Gram-positive bacteria possess a different cell envelope structure that is reminiscent of the diderm cell envelope structure of Gram-negative bacteria. For example, members of the Corynebacterianeae are classified as Gram-positive due to their behavior in the classical Gram staining procedure (Gram, 1884) but possess an outer membrane made of mycolic acids and other complex glycolipids that is covalently linked to an underlying polymer of arabinogalactan and peptidoglycan (Hoffmann et al., 2008; Marchand et al., 2012; Zuber et al., 2008). Therefore, when considering protein transport issues in Gram-positive bacteria, it has been proposed that the terms monoderm and diderm Gram-positive bacteria should be used in order to refer to the respective cell envelope structures in a more precise and unambiguous way (Desvaux et al., 2009). This review gives an overview on the most important protein export systems of mono- and diderm Gram-positive bacteria with, if applicable, emphasis on notable differences from evolutionary related protein export systems of Gram-negative bacteria.

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2. The general secretion (Sec) protein export pathway

In Gram-positive bacteria, the major route for exporting proteins into or across the cytoplasmic membrane is the general secretion (Sec) pathway (Fig. 1). This pathway has been extensively studied in the Gram-negative model bacterium *Escherichia coli* and, to a somewhat lesser extent, also in *Bacillus subtilis*, the latter being considered as the model organism for the Gram-positive group of bacteria (for recent more extensive overviews see Kudva et al., in press; Yuan et al., 2010).

2.1. Sec signal peptides and signal peptidases

Most Sec substrates that are translocated across the plasma membrane are initially synthesized as precursors with an amino-terminal signal peptide, which is cleaved off from the mature protein during or shortly after membrane translocation. Sec signal peptides possess a tripartite overall structure comprising a positively charged amino-terminal region (n-domain), a hydrophobic core (h-domain) and a polar carboxyl-terminal region (c-domain) that contains the signal peptidase recognition site (Rusch and Kendall, 2007). The average length of Sec signal peptides in Gram-positive bacteria (32 amino acids) is somewhat higher than in Gram-negative bacteria (24 amino acids) (Bendtsen et al., 2004), whereby the extensions do occur in all three of the subdomains (Nielsen et al., 1997; von Heijne and Abrahmsén, 1989). Signal peptides of Sec-dependent lipoproteins possess a so-called lipobox motif [L^{-3}-(A/S)$^{-2}$-(A/G)$^{-1}$↓C^{+1}] including a highly conserved cysteine residue to which the lipid moiety is attached (von Heijne, 1989).

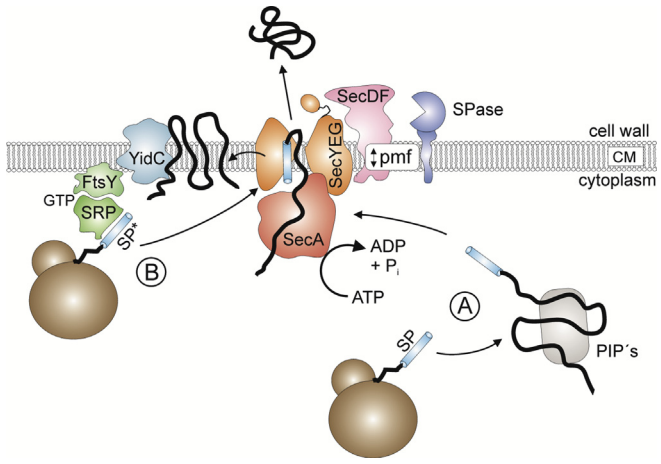

Fig. 1. The general secretion (Sec) protein export pathway in Gram-positive bacteria. (A) In the posttranslational mode, Sec-dependent precursor proteins are kept in an export-competent state by posttranslationally interacting proteins (PIPs) such as the general chaperones GroELS/DnaK-DnaJ-GrpE/trigger factor, the CsaA protein and the soluble form of SecA. The signal peptide (SP) is recognized by the SecA protein which pushes the protein through the SecYEG protein-conducting channel in a stepwise and ATP-dependent manner. In addition, SecDF exerts a proton motive force (pmf)-dependent pulling force on the substrate from the extracytosolic side of the membrane. During or shortly after translocation, the signal peptide is removed by signal peptidase (SPase) and the mature protein is released on the trans-side of the cytoplasmic membrane (CM). (B) Sec substrates possessing highly hydrophobic signal peptides or transmembrane segments (SP*) are recognized at the ribosome by the signal recognition particle (SRP). Subsequently, the ribosome-nascent chain (RNC)–SRP complex docks to the SRP-receptor FtsY and the RNC is then further transferred to the SecYEG translocation pore such that the ribosomal exit site is in close proximity to SecYEG. The energy for translocation in the cotranslational export mode is provided by further elongation of the substrate at the ribosome. The transmembrane segments of integral membrane proteins are released into the membrane through a lateral gate in the SecYEG pore. Membrane proteins that possess large extracytosolic domains require the additional involvement of SecA for translocation of the respective domains across the membrane. Insertion and/or folding of a subset of membrane proteins is assisted by YidC.

Non-lipoprotein Sec precursor proteins are proteolytically processed by a type I signal peptidase (LepB in *E. coli*) (Dalbey et al., 2012). Notably, Gram-positive bacteria frequently contain more than one type I signal peptidase. For example, *B. subtilis* possesses 5 chromosomally encoded type I signal peptidases (SipS, SipT, SipU, SipV and SipW) that cannot fully substitute for each other in vivo and that differ to some extent with respect to their substrate preferences (Dalbey et al., 2012; van Roosmalen et al., 2004). So far, the physiological reason why some bacteria require multiple type I signal peptidases is not clear. In contrast, staphylococci express only one active type I signal peptidase (SpsB), but their genome encodes a variant (SpsA) that lacks the catalytic amino acid residues and seems to have no specific function in protein export (Cregg et al., 1996). As in Gram-negative bacteria, the processing of Sec lipoproteins in Gram-positive bacteria is performed by a type II signal peptidase (Lsp) in a step following the lipid modification of the lipoprotein precursor proteins at their conserved +1 cysteine residue by diacylglyceryl transferase Lgt (Hutchings et al., 2009). A precise sequence of events has been described concerning modifications of lipoproteins in Gram-negative bacteria as follows: (1) addition of diacyl-glycerol by Lgt; (2) cleavage of the signal peptide by type II signal peptidase (Lsp) that recognizes the modified Cys residue within the lipobox; and (3) N-acylation by Lnt (Nakayama et al., 2012). This sequence is less clear in some Gram-positive bacteria. For *Listeria monocytogenes* (Baumgärtner et al., 2007), *Streptococcus agalactiae* (Henneke et al., 2008) and *Mycobacterium smegmatis* (Tschumi et al., 2012), evidence exists that Lsp might also be able to cleave non-lipidated lipoprotein precursors, suggesting that lipoprotein modification might not always occur in a strict sequence in these microorganisms. Since signal peptides have been shown to negatively affect membrane integrity and also can interfere with protein translocation at the level of the translocase, it is crucial for the cell to further degrade them after they have been released from the precursor proteins by signal peptidases. This important task is performed by signal peptide peptidases such RseP in *E. coli* and RasP in *B. subtilis* that attack the h-regions of signal peptides subsequent to processing of precursor proteins by type I or type II signal peptidases (Saito et al., 2011).

2.2. Targeting to the Sec translocase

Sec-dependent protein translocation in bacteria occurs either in a co- or in a posttranslational manner. The cotranslational pathway is mainly used by integral cytoplasmic membrane proteins, whereas the posttranslational pathway is used by proteins that are exported across the membrane. In *E. coli*, either pathway possesses a dedicated system for targeting

of the respective substrates to the translocase. When Sec substrates possessing highly hydrophobic signal peptides or transmembrane segments emerge from the ribosome, a signal recognition particle (SRP) consisting of the Ffh protein and a small cytoplasmic RNA or scRNA (4.5S RNA in *E. coli*) binds tightly to these hydrophobic signals. The SRP–ribosome-nascent chain complex (RNC) subsequently docks to a translocon-bound SRP receptor (FtsY) and, in the next step, the RNC is further transferred to the SecYEG translocation pore (Saraogi and Shan, 2011). As in *E. coli*, SRP and its receptor are also present in all Gram-positive bacteria. However, some peculiarities have been observed in certain species. Notably, besides Ffh and scRNA, the SRP from *B. subtilis* contains an additional component, the histone-like protein HbsU. Like the other SRP components, HbsU is an essential protein for growth of *B. subtilis* and depletion of HbsU results in protein translocation defects and in increased amounts of scRNA within the cell (Yamazaki et al., 1999). Although the latter results suggest an important role of HbsU in Sec-dependent protein translocation in *B. subtilis*, the precise role of this protein is still unclear. In contrast to the situation in other bacteria where SRP and its receptor have been shown to be essential, the genes encoding the components of the SRP targeting system could be deleted in *Streptococcus mutans*. The resulting deletion mutant strains were viable, but showed reduced stress tolerance toward conditions of high salt or low pH. Furthermore, the additional deletion of the gene encoding one of the two YidC proteins (YidC2, for YidC see below) resulted in poorly viable cells and significantly exacerbated the stress sensitivity, suggesting that YidC2 might be involved in cotranslational insertion of membrane proteins and, at least to some degree, is able to compensate for the lack of SRP components in this organism (Hasona et al., 2005).

In contrast to the situation in *E. coli* and other proteobacteria, Gram-positive bacteria generally lack the targeting chaperone SecB that, in the posttranslational export mode, interacts with newly synthesized precursor proteins, maintains their unfolded, translocation-competent state, and targets them to the SecA component of the Sec translocase (Bechtluft et al., 2010). A notable exception is the diderm bacterium *Mycobacterium tuberculosis* for which a SecB-like protein (Rv1957) has recently been described (Bordes et al., 2011). Rv1957 is encoded within the stress-responsive *higB-higA* toxin–antitoxin locus and was found to prevent aggregation and degradation of the HigA antitoxin. Interestingly, Rv1957 could functionally replace the *E. coli* SecB both in vivo and in vitro. Although a direct role of Rv1957 in *Mycobacterium tuberculosis* protein export has not been demonstrated, the authors speculated that it might be required for efficient targeting of mycobacterial outer membrane proteins under certain stress conditions (Bordes et al., 2011).

Generally, however, it is not clear which proteins functionally substitute for SecB in Gram-positive bacteria. The CsaA protein of *B. subtilis* was identified as a suppressor of a temperature-sensitive *E. coli secA* mutant strain (Müller et al., 1992). Since CsaA can bind to the mature part of certain Sec-dependent precursor proteins and was shown to possess an affinity for SecA, it has been proposed that CsaA might fulfill a SecB-like function in *B. subtilis* for a subset of exported proteins (Müller et al., 2000). In addition, the general chaperones GroEL-GroES/DnaK-DnaJ-GrpE/trigger factor (Lyon et al., 1998; Wu et al., 1998) and the soluble form of SecA (Herbort et al., 1999) have also been suggested to be involved in maintaining Sec-dependent precursors export-competent in Gram-positive bacteria.

2.3. The Sec translocase: core components and accessory factors

The central part of the *E. coli* Sec translocase is the SecYEG translocation pore. Besides providing a path for proteins that are translocated to the trans-side of the membrane, SecYEG also mediates the insertion of integral membrane proteins containing hydrophobic transmembrane segments. In the cotranslational mode of export (used mainly by integral proteins of the cytoplasmic membrane, but also by some exported proteins with highly hydrophobic signal peptides), a ribosome-RNC complex is targeted to SecYEG via the SRP/FtsY targeting system in a way that positions the ribosomal exit site in close proximity to the translocation pore. The energy for the actual translocation in the cotranslational pathway is thought to be provided by the further elongation of the substrate at the ribosome (du Plessis et al., 2011). During biogenesis of integral membrane proteins, the hydrophobic α-helical transmembrane domains are first inserted into SecYEG and subsequently moved into the lipid bilayer through a lateral gate of the translocation pore (Dalbey et al., 2011). In the posttranslational mode of export (used by exported proteins with less hydrophobic signal sequences), translocation is driven by the motor protein SecA. The translocation ATPase SecA interacts with the SecYEG pore and, after delivery of a respective substrate protein by the cytosolic targeting factor SecB to the SecA–SecYEG complex, the substrate protein is pushed by SecA through the SecYEG pore in a stepwise and ATP-dependent manner (Lycklama a Nijeholt and Driessen, 2012). Furthermore, a complex of the accessory factors SecD and SecF has recently been found to exert a proton-motive-force(pmf)-dependent pulling force on the substrate from the periplasmic side of the membrane (Tsukazaki et al., 2011).

The core components of the Sec translocon (i.e. SecA and SecYEG) are present in all Gram-positive bacteria. In contrast, SecD and SecF are present in most, but not all Gram-positive bacteria, whereby in some species (such as *B. subtilis* and *Staphylococcus aureus*), SecD and SecF are present in the form of a SecDF "siamese twin" fusion protein that most likely has arisen by a natural fusion of the *secD* and *secF* genes (Bolhuis et al., 1998; Sibbald et al., 2006). *Lactococcus lactis* lacks SecD and SecF but, interestingly, it has been shown that expression of the *B. subtilis* SecDF protein in this organism resulted in the improved secretion of an otherwise poorly secreted heterologous model protein (Nouaille et al., 2006).

In *E. coli*, the essential YidC protein, a member of the YidC/Oxa1/Alb3 family of membrane proteins, has been shown to be involved (alone or in conjunction with the Sec translocase) in the insertion of a subset of integral membrane proteins (Kol et al., 2008). YidC is also present in all Gram-positive bacteria and, interestingly, in many species, even

more than one YidC protein is present. For *B. subtilis*, it has been shown that the two YidC homologs SpoIIIJ and YqjG possess partially overlapping substrate specificities and can functionally substitute for each other, at least to some extent (Yuan et al., 2010). As noted in the previous chapter, in the case of *S. mutants*, one of the two YidC proteins that are present in this organism (YidC2, but not YidC1) could at least partially substitute for a functional SRP targeting system. It has been suggested that YidC2 may bind directly to ribosomes, thereby allowing a cotranslational insertion of inner membrane proteins (Funes et al., 2009). Despite these mentioned peculiarities that are observed in some Gram-positive species, the generally accepted view is that the basic molecular mechanism of protein translocation by the general Sec system is essentially conserved in all eubacteria.

3. Accessory Sec systems

Recently, it became clear that some (mostly pathogenic) Gram-positive bacteria possess a second SecA and, in some cases, also a second SecY protein, termed SecA2 and SecY2, respectively (Feltcher and Braunstein, 2012; Rigel and Braunstein, 2008). In contrast to the classical housekeeping SecA and SecY proteins (termed SecA1 and SecY1) that are essential for viability and responsible for exporting the vast majority of all extracytoplasmic proteins, the Sec2 components are involved in the export of a few substrates only that, in the case of pathogenic species, often are important virulence factors. With two exceptions, *Corynebacterium glutamicum* (Caspers and Freudl, 2008) and *Clostridium difficile* (Fagan and Fairweather, 2011), the accessory Sec2 components were found to be dispensable for the viability of all bacteria analyzed so far, at least under the growth conditions tested (Bensing and Sullam, 2002; Braunstein et al., 2001; Chen et al., 2004; Gibbons et al., 2007; Lenz and Portnoy, 2002). Depending on the presence or absence of a SecY2 protein, the accessory Sec systems can be divided into two groups, the SecA2-only and the SecA2/SecY2 systems (Fig. 2).

3.1. The accessory SecA2-only export pathway

The first SecA2-only system was discovered by Braunstein et al. (2001) in *M. tuberculosis*. SecA2-only systems can also be found in all other *Mycobacterium* species, even in non-pathogenic *M. smegmatis*. Although both mycobacterial SecA proteins are quite similar over their entire length to the SecA proteins of *E. coli* and *B. subtilis*, it was found that the two SecA proteins were functionally not redundant and that SecA2 could not take over the function of the housekeeping SecA1 protein and vice-versa (Braunstein et al., 2001). A proteomic study of the cell envelope fraction of *M. smegmatis* revealed that the amounts of two lipoproteins (Msmeg 1704 and 1712) were strongly reduced in the corresponding fraction of a *secA2* deletion mutant, indicating that both proteins are substrates of the SecA2-only pathway (Gibbons et al., 2007). In contrast, export of other lipoproteins was not affected by the absence of SecA2. Both Msmeg 1704 and Msmeg 1712 possess an amino-terminal signal peptide with a classical lipobox motif, and processing of their signal peptides was inhibited by globomycin, a known inhibitor of type II signal peptidases. Furthermore, for Msmg1712, it was shown that its signal peptide was strictly required for SecA2-dependent export (Gibbons et al., 2007). Remarkably, in this respect, is the finding that, in *M. tuberculosis*, superoxide dismutase (SodA) and catalase–peroxidase (KatG) that both lack an obvious signal peptide were identified as SecA2-dependent substrates (Braunstein et al., 2003). Furthermore, SecA2-dependent substrates either possessing a signal peptide (e.g. the autolysin p60 (Lenz et al., 2003)) or lacking a signal peptide (e.g. superoxide dismutase MnSod (Archambaud et al., 2006); fibronectin-binding protein FbpA

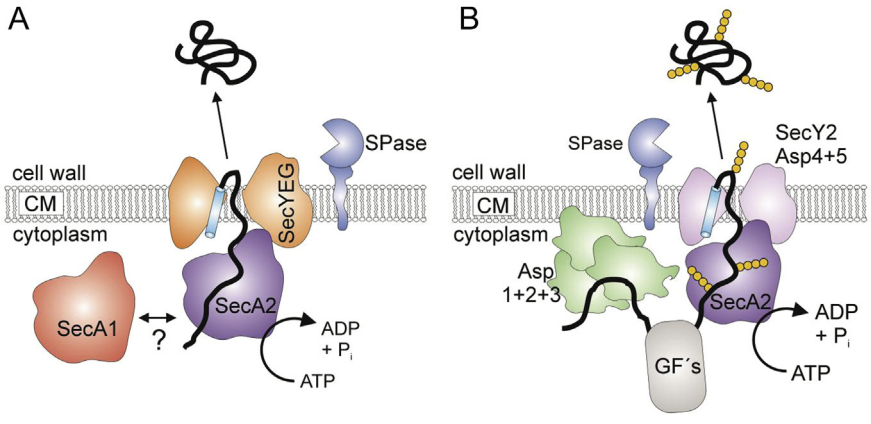

Fig. 2. Accessory Sec systems. (A) SecA2-only systems. SecA2-only substrates with (as shown here) or without an obvious signal peptide are recognized by SecA2 and are translocated most likely through the same SecYEG pore used by the housekeeping Sec1 export pathway. The energy for substrate translocation might be provided by the ATPase activity of SecA2. Thus far, it is unclear as to whether the SecA1 protein plays a direct role in translocation of substrates of the SecA2-only export pathway. CM: cytoplasmic membrane; SPase: signal peptidase. (B) SecA2/SecY2 systems. The serine-rich substrate of the SecA2/SecY2 export pathway is targeted to the SecA2/SecY2 export machinery by virtue of its signal peptide and the adjacent AST domain. A complex of Asp1, Asp2, and Asp3 might be involved in keeping the nascent SecA2/SecY2 substrate competent for its glycosylation (yellow dots) by various glycosylation factors (GFs) and possibly also in targeting of the substrate to the SecA2 protein. Translocation of the glycosylated substrates is powered by the translocation ATPase activity of SecA2 and proceeds through a protein-conducting channel that is formed by SecY2 and, most likely, additional proteins such as Asp4 and Asp5. CM: cytoplasmic membrane; SPase: signal peptidase.

(Dramsi et al., 2004)) have been identified in *L. monocytogenes*, a human pathogen that likewise possesses a SecA2-only-type secretion system. So far it is not clear (1) what the features are that distinguish SecA1 substrates from SecA2-only substrates, (2) how SecA2-only substrates are specifically recognized by SecA2, especially those substrates that do not possess a signal peptide, and (3) why the housekeeping SecA1-driven Sec1 system is not sufficient to efficiently mediate the membrane translocation of SecA2-only substrates. An interesting observation in this respect is that export of SecA2-only substrates is not completely blocked in the absence of SecA2 (Braunstein et al., 2001, 2003; Lenz and Portnoy, 2002), indicating that, in principle, these substrates can make use of the housekeeping Sec1 system and, consequently, do not possess an intrinsic export incompatibility that inheritably would preclude their membrane translocation via the Sec1 machinery. Although a completely novel translocation pathway independent of the general SecYEG pore for SecA2-only substrates cannot be totally excluded at the moment, recent experimental evidence strongly suggests that SecA2 mediates and/or stimulates membrane translocation of its respective substrates, either alone or in cooperation with SecA1, through the same SecYEG pore that is used by Sec1 substrates (Fig. 2A) (Rigel et al., 2009).

3.2. The accessory SecA2/SecY2 export pathway

SecA2/SecY2 systems (Fig. 2B) are highly specialized export systems that occur in some pathogenic Gram-positive bacteria (i.e. *Streptococcus* sp., *Staphylococcus* sp., *Bacillus* sp.). These Sec2 systems are specifically involved in the export of virulence factors that are members of a highly conserved family of large, serine-rich glycoproteins which, due to their glycosylation, cannot be translocated via the housekeeping Sec1 translocase. The first SecA2/SecY2 systems were discovered and characterized in the oral pathogens *Streptococcus gordonii* (Bensing and Sullam, 2002) and *Streptococcus parasanguis* (Chen et al., 2004). In both organisms, the gene encoding the Sec2 substrate is part of an accessory *sec2* locus that encodes various proteins that are required either for substrate glycosylation or for substrate export. In *S. gordonii*, the accessory *sec2* locus encodes five accessory secretory proteins (Asp1–5) that, besides SecA2 and SecY2, are required for export of the platelet-binding glycoprotein GspB. Interestingly, the predicted membrane proteins Asp4 and Asp5 show some similarities to SecE and SecG, respectively (Takamatsu et al., 2005), suggesting that in *S. gordonii* the components SecA2/SecY2–Asp4–Asp5 might constitute a second Sec preprotein translocase whose action is completely independent of the housekeeping Sec1 translocase components. However, genes encoding homologs of Asp4 and Asp5 are not always present in bacteria with SecA2/SecY2 export systems. Some species such as *S. agalactiae* lack Asp5, whereas others such as *S. aureus* and *Staphylococcus epidermidis* lack both Asp4 and Asp5, indicating that the requirements for Asp4 and Asp5 and, as a consequence, the partner subunits that together with SecY2 form the protein conducting channel for SecA2/SecY2 substrates, might differ between the respective organisms (Siboo et al., 2008; Takamatsu et al., 2005). In contrast to Asps4–5, the Asps1–3 are highly conserved components of SecA2/SecY2 systems. Recent results indicate that Asps1–3 interact with each other and, via Asp3, also with the SecA2 protein, suggesting that these Asps might be involved in protein targeting of Sec2 substrates to the Sec2 translocase (Seepersaud et al., 2010). Furthermore, Asp2 and Asp3 have been shown to directly interact with the serine-rich repeat domains of the Sec2 substrate prior to its full glycosylation (Yen et al., 2011), suggesting that Asps1–3 form a chaperone complex that keeps the nascent Sec2 substrate competent for glycosylation and membrane translocation and, in addition, is involved in the targeting of the Sec2 substrate protein to the Sec2 translocase.

Substrates of the SecA2/SecY2 pathway possess unusually long signal peptides that show a tripartite structure consisting of an atypically long n-region followed by a h- and a c-region. In the h-region, two or more glycine residues are present that act as Sec2 pathway specificity determinants and prevent export via the housekeeping Sec1 translocase (Bensing et al., 2007). The signal peptide is essential (Bensing et al., 2005) but not sufficient for Sec2 transport, since it has been shown for the Sec2 substrate GspB of *S. gordonii* that, in addition, a specific domain of approximately 20 amino acids (the so-called accessory Sec transport or AST domain) adjacent to the signal peptide is required (Bensing and Sullam, 2010). Using a site-specific in vivo cross-linking approach, Bensing et al. (2012) demonstrated that the N-terminal end of the AST domain cross-linked to SecA2 in the presence or absence of Asps1–3. In contrast, cross-linking of the C-terminal end of the AST domain to SecA2 required the presence of these Asps, suggesting that a full engagement of the precursor by SecA2 and the initiation of Sec2-mediated transport is somehow influenced by Asps1–3. Based on these findings, it has been proposed that rather than being involved in preprotein targeting, Asps1–3 might be required for keeping the preprotein in a conformation in which the AST domain is accessible for binding by SecA2 or, alternatively, that the Asps may directly alter SecA2 such that its AST binding site is more exposed (Bensing et al., 2012). Despite these significant advances that have been made in the understanding of SecA2/SecY2 systems, it is still unknown so far what the mechanistic tricks are which enable these systems to translocate glycosylated proteins across the cytoplasmic membrane.

4. The twin-arginine translocation (Tat) export pathway

In many (but not all) bacteria, a protein translocation pathway exists that, in contrast to the Sec pathway, has the remarkable ability to export completely folded proteins across the cytoplasmic membrane. This pathway has been designated twin-arginine translocation (Tat) pathway due to the fact that a characteristic amino acid motif, including two consecutive arginine residues, can be identified in the signal peptides of the corresponding precursor proteins. Many of the Tat substrates are proteins that have to recruit a cofactor in the cytosol and, as a prerequisite, have to acquire a folded status prior to

export. In addition, also cofactor-less proteins are exported via the Tat route, presumably because their rapid folding kinetics precludes transport via the Sec pathway. The Tat system has been most extensively characterized in *E. coli* and, to a lesser extent, also in the Gram-positive model bacterium *B. subtilis* (for recent more extensive overviews see Kudva et al., in press; Palmer and Berks, 2012; Robinson et al., 2011). Although some noteworthy differences have been identified, the Tat systems of Gram-negative and Gram-positive bacteria likely function in a similar way.

4.1. Twin-arginine signal peptides

Both Sec and Tat signal peptides possess a similar tripartite overall structure, consisting of an amino-terminal n-domain, a central hydrophobic h-domain and a carboxyl-terminal c-domain. However, Tat signal peptides possess a conserved consensus motif (S/T^{-1}-**R-R**-X^{+1}-F^{+2}-L^{+3}-K^{+4}; whereby X stands for any amino acid) that is located at the boundary between the n-domain and the hydrophobic h-domain (Berks, 1996). In addition, the h-region of Tat signal peptides is usually less hydrophobic than those of Sec signal peptides (Cristobal et al., 1999). Furthermore, basic amino acid residues are sometimes found in the c-region of Tat signal peptides that are involved in preventing a faulty interaction of the respective precursor proteins with the Sec machinery ("Sec-avoidance") (Blaudeck et al., 2003; Cristobal et al., 1999). The amino acids of the Tat consensus motif are required for the productive recognition and binding of the Tat precursor protein by the Tat translocase and their importance for the membrane translocation of Tat-dependent precursor proteins has been analyzed in numerous mutagenesis studies in *E. coli* and *B. subtilis* (Berks et al., 2003; Lausberg et al., 2012; Mendel et al., 2008). For Tat signal peptides in both organisms, three major determinants (i.e. the twin-arginine residues, the serine/threonine residue at the −1 position, and a hydrophobic amino acid residue located at position +2 or +3) were identified to be especially critical within the Tat consensus motif. Although the importance of the respective determinants varies somewhat between different signal peptides (also within a given organism), the mode of substrate recognition via the Tat consensus motif by the respective Tat translocases seems to be highly conserved in Gram-positive and Gram-negative bacteria (Robinson et al., 2011).

4.2. The Tat translocase

In *E. coli* and other Gram-negative bacteria, the functional unit of a Tat translocase consists of three integral membrane proteins, i.e. TatA, TatB, and TatC. Another Tat protein, TatE, is a paralogue of TatA that is present in some, but not all, Gram-negative bacteria (Sargent et al., 1998) and, at least in *E. coli*, the *tatE* gene is expressed at a level 50–200 times lower than *tatA* (Jack et al., 2001). Therefore, *tatE* is commonly regarded as a cryptic gene duplication of *tatA*. A complex of TatB and TatC (the TatBC receptor complex) recognizes and binds the signal peptides of Tat substrates by virtue of their Tat consensus motifs (Alami et al., 2003; Lausberg et al., 2012). Subsequent to this binding event, homo-oligomeric complexes of TatA are recruited to the TatBC–substrate complex in a proton motive force (pmf)-dependent manner (Mori and Cline, 2002). Some models of Tat translocation propose that differently sized TatA complexes form protein-conducting channels that are fitted to the sizes and shapes of the various Tat substrates and through which the folded proteins are transferred across the membrane (Gohlke et al., 2005). In contrast, an alternative model proposes that association of TatA multimers with the TatBC–substrate complex might lead to a punctual weakening of the membrane, thereby allowing a TatC-driven translocation of the Tat substrate across the membrane without the need of a translocation pore (Brüser and Sanders, 2003). Following this still very poorly understood translocation step, the Tat signal peptides are proteolytically removed from the Tat precursor proteins by type I signal peptidases (e.g. LepB in *E. coli*) and the mature substrates are released on the trans-side of the membrane (Lüke et al., 2009).

Similar TatABC-type Tat translocases are present in Gram-positive bacteria with high-GC-content genomes, such as members of the phylum Actinobacteria (Fig. 3A). In contrast, Gram-positive bacteria with low-GC-content genomes (including *B. subtilis*) do not possess TatB and, in these organisms, Tat translocation is mediated by TatAC-type minimal translocases in which the TatB function is thought to be carried out by a bifunctional TatA subunit (Fig. 3B). Such a view was experimentally supported by the identification of bifunctional *E. coli* TatA mutant proteins that allowed significant membrane translocation of Tat substrates in the absence of TatB (Blaudeck et al., 2005) and, furthermore, by heterologous complementation experiments which showed that a TatA protein ($TatA_d$, see below) from *B. subtilis* is able to functionally replace both TatA and TatB in *E. coli* (Barnett et al., 2008).

Remarkably, and in contrast to most other bacteria, *B. subtilis* possesses two TatAC-type translocases that have been termed $TatA_dC_d$ and $TatA_yC_y$, respectively, which show different, but overlapping substrate specificities and whose usage strongly depends on the growth conditions (van der Ploeg et al., 2011). The $TatA_yC_y$ translocase is expressed under all conditions tested and was shown so far to mediate the translocation of a Dyp-type peroxidase (YwbN) and a metal-dependent phosphoesterase (YkuE) (Monteferrante et al., 2012a). In contrast, the $TatA_dC_d$ translocase and its substrate PhoD (a phosphodiesterase) are specifically expressed under phosphate starvation conditions (Jongbloed et al., 2000). In addition to $TatA_d$ and $TatA_y$, a third TatA subunit ($TatA_c$) is found in *B. subtilis* that can form active Tat translocases with either $TatC_d$ or $TatC_y$ and it has been speculated that $TatA_cC_d$ and $TatA_cC_y$ translocases could be involved in the specific export of as-yet-unidentified Tat substrates in *B. subtilis* (Monteferrante et al., 2012b). From a mechanistic point of view, it is completely unclear as to why *B. subtilis* requires two or even up to four Tat translocases for exporting such a very limited set of proteins and why, as in other bacteria, a single Tat translocase is not enough to do the job.

The number of Tat substrates varies substantially between different Gram-positive bacteria. Whereas only a few proteins are exported in a Tat-dependent manner in *B. subtilis* and other

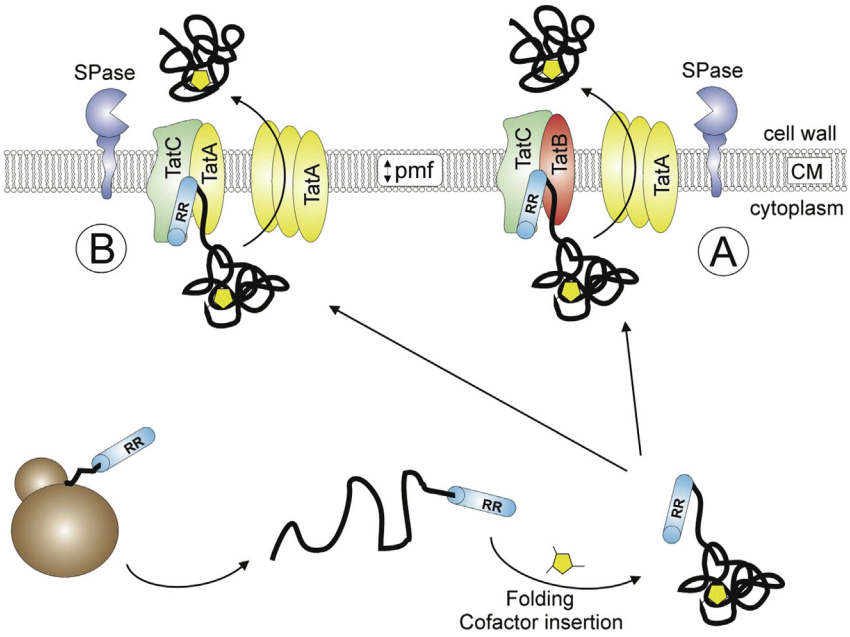

Fig. 3. The twin-arginine translocation (Tat) export pathway of Gram-positive bacteria. After folding and, if required (as shown here), cofactor insertion, pre-proteins containing a signal peptide with a twin-arginine motif are recognized by a receptor complex consisting of (A) TatC and TatB (in Gram-positive bacteria with high-GC-content genomes), or (B) TatC and a bifunctional TatA protein that, besides the TatA function, also carries out the TatB function (in Gram-positive bacteria with low-GC-content genomes). Subsequently, homo-oligomeric complexes of TatA are recruited to the substrate-loaded receptor complex in a proton-motive-force(pmf)-dependent manner, followed by translocation of the substrate across the cytoplasmic membrane (CM) in a step that is still very poorly understood. Most current models propose that TatA multimers form a substrate-fitted protein-conducting channel. In an alternative view, TatA multimers might lead to a punctual weakening of the membrane and thereby allow TatC-driven translocation of the substrate without the need for a translocation pore. Following substrate translocation, the signal peptide is removed by signal peptidase (SPase) and the mature protein is released on the trans-side of the membrane.

firmicutes, some Gram-positive bacteria, such as various *Streptomyces* sp., make more extensive use of this export pathway. For example, of the approximately 800 predicted exported proteins of *Streptomyces coelicolor*, more than 100 of these were predicted and/or experimentally verified to be Tat-dependent, indicating that the Tat pathway represents a major protein export route in this class of Gram-positive bacteria (Widdick et al., 2006).

5. Type VII/WXG100 secretion systems

Some high-GC diderm pathogenic Gram-positive bacteria (such as, e.g., *M. tuberculosis* and *Mycobacterium leprae*) possess specialized protein export systems (termed type VII systems in the style of the nomenclature of extracellular secretion systems in Gram-negative bacteria (Abdallah et al., 2007)) that mediate the secretion of virulence factors belonging to the WXG100 or to the PE/PPE families of proteins. WXG100 proteins share a size of approximately 100 amino acids, a helical structure and a characteristic hairpin bend that contains a highly conserved Trp-Xaa-Gly (WXG) motif (Pallen, 2002). PE/PPE proteins are unique to mycobacteria and are characterized by the presence of N-terminal Pro-Glu or Pro-Pro-Glu repeats (Abdallah et al., 2009).

The best studied type VII system is the *M. tuberculosis* ESX-1 system (Fig. 4A), one of a total of five ESX systems present in this organism. ESX-1, which generally is considered the paradigm for type VII export systems, secretes the WXG100 substrates ESAT-6 (early secreted antigen target 6 kDa, also named EsxA) and CFP-10 (culture filtrate protein 10 kDa, also named as EsxB) into the cell wall and culture supernatant of *M. tuberculosis*. ESAT-6 and CFP-10 are exported as a 1:1 complex and channeling of this complex into the ESX-1 export pathway is mediated by a seven amino acid secretion signal at the C-terminus of CFP-10 that contains a conserved YxxxD/E motif (Daleke et al., 2012; DiGiuseppe Champion et al., 2006). The genes for ESAT-6 and CFP-10 are part of the *esx-1* locus that also contains the genes encoding the so-called Esx conserved components EccA1 through E1 and the protease MycP that, together, are thought to constitute the ESX-1 export machinery that is involved in ESAT-6/CFP-10 secretion. The ESAT-6/CFP-10 substrate complex is recognized by virtue of its C-terminal targeting sequence by cytosolic ATPase EccCb1 that, together with the integral membrane protein EccCa1, forms an FtsK/SpoIIIE-type ATPase (DiGiuseppe Champion et al., 2006). The ATPase activity of EccCb1/EccCa1 and/or those of the cytosolic AAA-type ATPase EccA1 (Luthra et al., 2008) could provide the energy for the actual substrate translocation that is thought to be mediated by a complex consisting of the membrane proteins EccB1, EccCa1, EccD1, EccE1, and MycP. And, in fact, a recent study of another type VII system (ESX-5 of *Mycobacterium marinum*) that is involved in export of PE/PPE-type proteins has shown that four Ecc membrane components of this system, i.e. EccB5, EccC5, EccD5, and EccE5, form a stable complex in the mycobacterial cell envelope (Houben et al., 2012). Based on this result, the authors proposed two

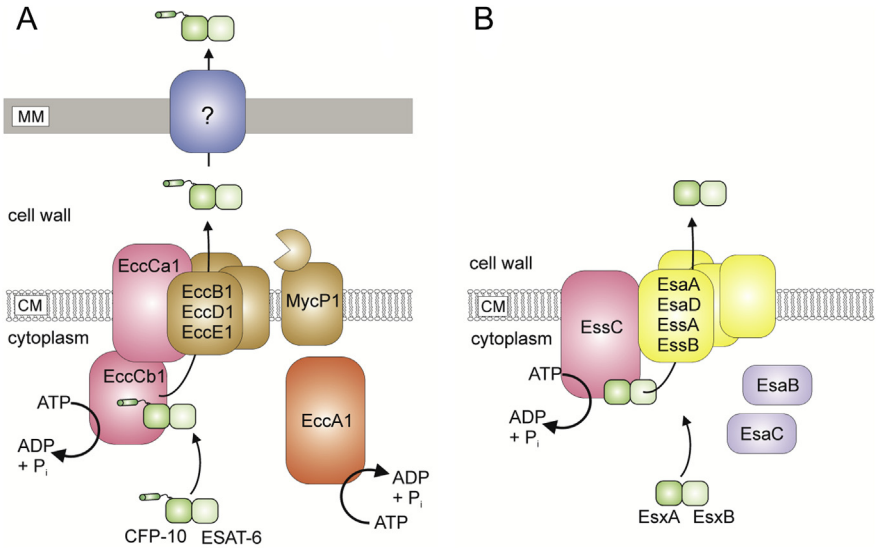

Fig. 4. Type VII/WXG100 secretion systems. (A) Type VII secretion in diderm Gram-positive bacteria. The paradigm of type VII protein export systems is the ESX-1 system of *M. tuberculosis* (shown here) that secretes the WXG100 substrates ESAT-6 and CFP-10 into the culture supernatant. ESAT-6 and CFP-10 are exported as a 1:1 complex that is recognized by the FtsK/SpoIIIE-type ATPase EccCb1/EccCa1 by virtue of a secretion signal at the C-terminus of CFP-10. Translocation across the cytoplasmic membrane (CM) most likely occurs through a protein-conducting path that is formed by a complex of EccB1, EccCa1, EccD1, EccE1 and the protease MycP. The energy for substrate translocation across the CM might be provided by ATPase activities of the FtsK/SpoIIIE-type ATPase EccCb1/EccCa1 and/or the AAA-type ATPase EccA1. Thus far, it is completely unknown as to how the substrate crosses the outer mycolic-acid-containing membrane (MM) and no components corresponding to a protein-conducting channel in the MM have been identified thus far. (B) WXG100 secretion in monoderm Gram-positive bacteria. An example of WXG100 secretion systems present in some low-GC Gram-positive bacteria is the Ess system of *S. aureus* (shown here), that secretes the WXG100 proteins EsxA and EsxB into the culture supernatant. EsxA and EsxB are dependent on each other for export and most likely are translocated across the cytoplasmic membrane (CM) through a protein-conducting path that is formed by the integral membrane proteins EssA, EssB, EssC, EsaA, and EsaD. The energy for substrate translocation might be provided by FtsK/SpoIIIE-type ATPase EssC. The functions of cytosolic proteins EsaB and EsaC are unknown.

alternative models for type VII protein secretion in diderm Gram-positive bacteria. In their two-step model, the four core membrane components are proposed to form a complex solely in the inner membrane that builds a large protein conducting channel through which type VII substrates are translocated to the intermembrane space. Subsequent to this step, the periplasmic intermediate must then be transported across the outer mycolic acid-containing membrane in a second step by an uncharacterized mechanism for which no involved components have been identified so far. In their alternative model, the authors propose that the identified membrane protein complex might alternatively span both mycobacterial membranes and that substrate translocation might occur in one step over both the inner membrane and the mycomembrane.

Genes encoding WXG100 substrates and their dedicated secretion proteins have also been identified in the genome of some low-GC monoderm Gram-positive bacteria belonging to the firmicutes (e.g. *S. aureus*, *Bacillus anthracis*, or *L. monocytogenes*) (Abdallah et al., 2007). For example, the *S. aureus* WXG100 proteins EsxA and EsxB are secreted by the Ess secretion system (Fig. 4B) into the culture supernatant and strictly depend on each other for their secretion (Burts et al., 2005), consistent with what has been described for the *M. tuberculosis* ESX-1 system. The genes encoding the secreted proteins EsxA and EsxB are part of the *S. aureus ess* gene locus that contains genes required for their synthesis and/or secretion. The proteins encoded by these genes are predicted cytosolic proteins (EsaB, EsaC) and membrane proteins (EsaA, EsaD, EssA, EssB, EssC), including a gene that encodes an FtsK/SpoIIIE-type ATPase (EssC) (Anderson et al., 2011; Burts et al., 2005). Besides EssC, no other components homologous to the components of the type VII systems of diderm high-GC Gram-positive bacteria can be identified. Due to this fact and due to the lack of an outer membrane in the respective microorganisms, it has been proposed that the corresponding secretion systems should be named WXG100 systems to differentiate them from true type VII secretion systems (Bitter et al., 2009). Thus far, the mechanism by which WXG100 systems operate and the reason why Gram-positive bacteria without a mycomembrane require such dedicated export systems are completely unknown.

6. Conclusion

Although significant progress has been made in the past few years with respect to the characterization of the various protein export pathways present in Gram-positive bacteria, many important questions remain to be answered. In particular, the export systems that have no counterparts in Gram-negative bacteria (i.e. accessory Sec systems and type VII/WGX100 systems) are poorly understood. In some cases (SecA2-only systems; WXG100 systems of monoderm Gram-positive bacteria), it is not even clear why these additional export systems are specifically needed for membrane translocation of their respective substrate proteins and why the "classical" export systems (Sec or Tat) are not sufficient to fulfill this task. Furthermore, one of the most intriguing open questions, i.e. how proteins are transported across the highly efficient outer membrane-like permeability barrier (i.e.

the actinobacterial mycomembrane) of diderm Gram-positive bacteria, urgently needs to be answered. Clearly, there is still a long way to go before we fully understand how these fascinating molecular nanomachines help their passengers to "leave their homes" and deliver them to their final destinations.

Protein export systems of pathogenic bacteria are often involved in the secretion of proteins that contribute to the virulence of the respective microorganisms. For example, all of the known protein export pathways of *M. tuberculosis* are either essential for viability or important for virulence, and therefore might be considered as potential novel tuberculosis drug targets (Feltcher et al., 2010). And, in fact, inhibition of signal peptidase I (LepB) of *M. tuberculosis* by the substance MD3 (a beta-aminoketone) resulted in rapid killing of persistent and replicating forms of this bacterium (Ollinger et al., 2012). Besides drugs that block type-I signal peptidases (such as MD3), other protein export inhibitors have been described that target the canonical Sec pathway via SecA (Chen et al., 2010) or SecY (van Stelten et al., 2009). Furthermore, in a recent study, inhibitors of the bacterial Tat translocase have also been described (Vasil et al., 2012). Since there is increasing need for novel therapies to combat infections caused by multidrug-resistant bacteria, research on protein export systems of Gram-positive bacteria such as accessory Sec systems or type-VII/WXG100 systems is therefore useful not only for fundamental microbiology, but also for paving the way to development of new antibacterial drugs.

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