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Journal

Biochimica et Biophysica Acta, 1843(8)

ISSN

0006-3002

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Publication Date

2014-08-01

DOI

10.1016/j.bbamcr.2013.10.019

Peer reviewed

Published in final edited form as:

Biochim Biophys Acta. 2014 August ; 1843(8): 1674–1686. doi:10.1016/j.bbamcr.2013.10.019.

Selective transport by SecA2: an expanding family of customized motor proteins

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Abstract

The SecA2 proteins are a special class of transport-associated ATPases that are related to the SecA component of the general Sec system, and are found in an increasingly large number of Gram-positive bacterial species. The SecA2 substrates are typically linked to the cell wall, but may be lipid-linked, peptidoglycan-linked, or non-covalently associated S-layer proteins. These substrates can have a significant impact on virulence of pathogenic organisms, but may also aid colonization by commensals. The SecA2 orthologues range from being highly similar to their SecA paralogues, to being distinctly different in apparent structure and function. Two broad classes of SecA2 are evident. One transports multiple substrates, and may interact with the general Sec system, or with an as yet unidentified transmembrane channel. The second type transports a single substrate, and is a component of the accessory Sec system, which includes the SecY paralogue SecY2 along with the accessory Sec proteins Asp1-3. Recent studies indicate that the latter three proteins may have a unique role in coordinating post-translational modification of the substrate with transport by SecA2. Comparative functional and phylogenetic analyses suggest that each SecA2 may be uniquely adapted for a specific type of substrate.

1. Introduction and Overview

The genomes of many Gram-positive bacterial species encode two homologues of the SecA transport-associated ATPase. The first examples of a second SecA, or SecA2 (a SecA homologue different from the core component of the general Sec system), were noted in *Mycobacterium tuberculosis*, *Listeria monocytogenes* and *Streptococcus gordonii* a little more than a decade ago [1–3]. Until very recently, it was thought that only a small group of pathogenic, Gram-positive bacteria expressed two distinctly different SecA homologues. However, as large-scale microbiome projects have made the genome sequences of more understudied organisms available, it has become apparent that a much larger group of bacteria, including commensal and food-grade organisms, also encode SecA2 proteins. The precise number of bacterial species that express SecA2 is difficult to assess, in part because

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there are as yet few defining characteristics for the SecA2 orthologues (interspecies homologues). Thus the SecA2 proteins are often not identified in the databases as such, but rather may be incorrectly annotated as SecA. Although the SecA2 proteins of mycobacteria, listeria and streptococci are all similar to SecA proteins of the general Sec system, it has become apparent that these SecA2s are not closely related to each other, either phylogenetically (Fig. 1) or functionally. The SecA2 proteins have typically been classified into two categories: those that have a corresponding SecY2 and thus belonging to a SecA2/Y2 (or accessory Sec) system, and those that do not. As we will discuss, there is increasing evidence for a much larger spectrum of SecA2 types.

2. General characteristics of the SecA2 proteins

The SecA family of ATPases is characterized by four major domains: two nucleotide binding folds (NBD1 and NBD2), a preprotein binding domain (PPXD) located within NBD1, and a C-terminal region that encompasses several functional or structural domains (the HWD/HSD, IRA1 and CTD; Fig. 2). The HWD/HSD provides a platform to which NBD1 and NBDs are anchored, and interacts with both SecY and preproteins during transport [4–8]. The IRA1 and CTD domains have a significant impact on ATP binding and hydrolysis by the NBD, and preprotein binding by the PPXD, respectively [5, 9]. While it is clear that the ability to hydrolyze ATP is essential for the function of SecA, and that this activity is affected by preprotein binding, the precise means by which SecA couples the energy of ATP hydrolysis with movement of the preprotein through the SecYEG channel is still under intense debate [10–12].

Like SecA, the SecA2 proteins have two NBDs that flank a PPXD. However, they are all shorter than their SecA paralogues (i.e. SecA from the same organism, and sometimes referred to as SecA1 in organisms that have a SecA2), and may contain deletions within one or more of the domains. In contrast to SecA, which is involved in the transmembrane transport of a number of secreted and cell wall proteins, SecA2 is typically involved in the transport of a very limited number of substrates. With a few exceptions, SecA2 is not essential for viability, but instead has a major impact on virulence or colonization. In general, this is because one or more of the SecA2 substrates contributes to survival or the occupation of a specific niche in vivo. So what is the role of SecA2 in transport? The answer may depend on the substrate.

3. Transport by multi-substrate SecA2 proteins

One broad set of SecA2 proteins lack a corresponding SecY2 partner, and facilitate transport in what are sometimes called “SecA2 only” systems. This type of SecA2-mediated transport has been recently reviewed [13, 14], so we will only briefly summarize the composite findings, and present some additional observations and new perspectives. Although the means by which they interact with a transmembrane channel may not be the same in all cases, it is apparent that they all are more selective than SecA, with regard to the substrates they transport. The SecA2-dependent substrates are often, but not always, encoded in the same locus as SecA2. Of note, the substrates for SecA2-dependent transport identified thus far appear to be involved in oxidative stress response, cell wall metabolism or structural

modification of the cell wall. These substrates are not readily distinguishable from general Sec system substrates, and are at least partially transportable by SecA/YEG, although the *secA2* phenotype may be more apparent under certain growth conditions or in vivo.

3.1 Mycobacteria and Corynebacteria (Actinobacterial type)

The first SecA2 homologue was noted upon completion of the *M. tuberculosis* genome in 2000 [2]. It has subsequently become apparent that there are closely related (80–90% identity) SecA2 homologues in all other mycobacterial species. There are also very similar (50–60% identity) SecA2 orthologues in other actinobacterial genera such as Gordinia and Corynebacteria (Table I and Fig. 1), although these differ somewhat from each other and from the mycobacterial SecA2s with regard to deletions or additions within the SecA2 domains. The apparent phylogenetic and structural differences between the Mycobacteria and Corynebacteria SecA2s may reflect a functional difference, since the SecA2 of *Corynebacterium glutamicum* was found to be essential for viability [15], whereas the mycobacterial SecA2s are not.

The most extensively characterized SecA2 proteins are those of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*. Mutational analysis identified two substrates for the *M. tuberculosis* SecA2, KatG and the Fe-dependent SodA [16]. Both of these substrates lack a canonical Sec signal peptide. For *M. smegmatis*, two lipoproteins that are predicted to be involved in sugar uptake have been documented as SecA2 substrates [17]. Although both proteins have a traditional lipoprotein signal peptide, recent evidence indicates that the mature regions confer a dependence on SecA2 for export [18]. That is, rapid folding of the mature region (versus a specific sequence in the mature region) may render the preprotein more dependent on SecA2 for transport.

Consistent with a role as a transport motor, the ATPase activity of mycobacterial SecA2 has been documented by both mutational analyses and by in vitro studies using the purified protein [19]. However, this SecA2 shares just 29% identity (54% similarity) with its SecA paralogue. Most of the similarity between SecA and SecA2 resides within the NBD and PPXD domains (Fig. 2), although SecA2 has a ~35aa deletion between the ATP-binding motifs V and VI of NBD2. This deletion is likely to be functionally relevant, since alterations in NBD2 can affect the rate of ATP hydrolysis by *E. coli* SecA [20–23]. Of note, the affinity of *M. tuberculosis* SecA2 for ATP is ten-fold higher, and the resting or “basal” rate of hydrolysis is five-fold lower, than that of SecA [19]. These differences thus suggest that preprotein binding and ATP hydrolysis by SecA2 may be differently coordinated, as compared with SecA.

The mycobacterial SecA2 also has a 38 amino acid extension of NBD1, but conversely has a 70 amino acid CTD truncation. Perhaps more significantly, this SecA2 has a large deletion in the HWD/HSD. Since the HSD provides a major platform for interacting with SecY [6–8], the omission within this region could be a means to prevent SecA2 from interacting directly with the SecYEG translocon. Functional studies support this possibility [24]. That is, SecA2 is primarily cytosolic, whereas SecA is both cytosolic and membrane-associated, although the expression levels of the two proteins are similar. In addition, SecA depletion studies indicate that SecA2 cannot function independently of SecA. The combined data thus

support the proposed role for SecA2, in which it interacts with the SecA/YEG translocase via interaction with SecA, rather than interacting directly with SecYEG.

3.2 Listeria

The SecA2 of *L. monocytogenes* was fortuitously identified because mutations in *secA2* can have a dramatic effect on colony and cellular morphology [3]. SecA2 mutants display a rough colony phenotype, elongated cells and more extensive chaining. This is at least in part due to impaired export of two enzymes involved in cell wall metabolism, the p60 (CwhA) autolysin and the NamA (MurA) hydrolase. p60 is encoded in the same locus as SecA2 (Fig. 3) whereas NamA is encoded elsewhere in the genome. Each of these substrates has a canonical signal peptide. Deletion of *secA2* markedly reduces (but does not abolish) the export of these two proteins. Accordingly, SecA2 is not essential for viability but instead has a significant effect on virulence [25]. More than a dozen additional SecA2 substrates have been reported in *L. monocytogenes* [25–27]. One of these, an Mn-containing superoxide dismutase (MnSod), lacks an apparent N-terminal signal peptide, so it is unclear what features affect recognition by SecA2. However, MnSod was shown to undergo cytosolic phosphorylation, which renders it inactive, yet only the active, non-phosphorylated form is secreted [27]. Although there is a correlation between the MnSod phosphorylation level and a dependence on SecA2 for secretion, it has not been determined whether SecA2 recognizes the phosphorylated or dephosphorylated form of the preprotein.

The Listeria SecA2 proteins share 40% identity (70% similarity) with their SecA paralogues, with dissimilarities most prevalent in the C-terminal regions. Although they lack a C-terminal CTD, there are otherwise no deletions within SecA2, as compared with the SecA paralogue (Fig. 2). The composite findings thus far do not illuminate whether SecA2 is likely to interact with SecYEG or with a different transmembrane channel. However, a recent report has indicated that the cell division protein DivIVA affects substrate localization to the septal region, whereas SecA2 localizes to the same region independently of DivIVA [28]. The authors suggest that SecA2 may selectively facilitate export of the cell wall hydrolases near the cell septum. In addition to p60, the SecA2 locus encodes YubA, a putative membrane permease that is predicted to have seven transmembrane segments, although a possible role for YubA in transport has not been addressed. A *secA2* locus with a similar gene arrangement is present in non-pathogenic listeria species (Table I), and SecA2 mediates NamA export in at least one of these species (*L. innocua*) [29].

3.3 Bacillus

As was the case in Listeria, the SecA2 of *Bacillus anthracis* was identified and characterized because of its impact on cellular morphology, septation and chain length [30]. The *B. anthracis* SecA2 is encoded within a cluster of genes that are responsible for the formation of the S-layer, a paracrystalline lattice of proteins on the bacterial cell surface (Fig. 3). A similar genetic locus is present in several other Bacillus species (Table I), but is apparently absent from *B. subtilis*. The *B. anthracis* SecA2 is not essential for viability, and deletion of *secA2* results in a decrease, but does not abolish, secretion of Sap and EA1, two major components of the S-layer. The two SecA2 substrates have nearly identical 29 amino acid canonical signal peptides. Sap, but not EA1, is encoded in the *secA2* locus.

Regarding how the *B. anthracis* SecA2 may function, it lacks a C-terminal CTD but otherwise has no deletions compared with SecA (Fig. 2). It shows 75% similarity (45% identity) to its SecA paralogue. The dissimilarities are scattered throughout the protein, but most of the differences are concentrated in the HSD/HWD. A *secY2* is present in the genome, but deletion does not result in decreased Sap or EA1 secretion, indicating that SecA2 does not interact with SecY2 to transport these substrates. Conversely, SecA2 may work in conjunction with the Slayer assembly protein SlaP, which is encoded just downstream of SecA2. Deletion of *slaP* results in a phenotype identical to that resulting from deletion of *secA2*. Topological predictions suggest that SlaP should be cytoplasmic, but cell fractionation studies point to a peripheral membrane location that is independent of SecA2 and the S-layer proteins. Co-purification experiments indicate that the soluble form of SlaP is not tightly associated with any other protein. Other evidence suggests that SecA2 and SlaP affect the uniform distribution of Sap in the cell envelope. That is, in the absence of SecA2 or SlaP, Sap appears in patches deposited throughout the envelope. The even distribution of Sap and EA1 in the envelope appears to be essential for the localization of the cell wall protein BslO at the septal region, where it catalyzes the separation of daughter cells [30, 31]. While it is possible that SecA2/SlaP simply expedite transport through SecYEG, Nguyen-Mau *et al* proposed a more intriguing hypothesis in which SecA2/SlaP may confine the export of Sap and EA1 to discrete subcellular locations, perhaps near folding catalysts.

3.4 Clostridium difficile

The only clostridial SecA2 characterized to date is that of *C. difficile* [32]. As in Bacillus species, *secA2* resides within an S-layer gene cluster. However, the *C. difficile* SecA2 is essential for viability. This may be because one of the SecA2-dependent substrates, SlpA (the main component of the S-layer), is essential for viability. The SecA2-dependent substrates were identified through the combined use of dominant negative variants of SecA2 (NBD mutations) and anti-sense RNA expression (suppression of SecA versus SecA2 expression). In addition to SlpA, an S-layer protein encoded near the *secA2* locus (CwpV) was found to be SecA2-dependent. SlpA and CwpV both have traditional Sec-type signal peptides. Additional putative substrates include Cwp2, Cwp66 and Cwp84, which are also encoded in the *secA2* locus. Cwp84 is a cysteine protease that is responsible for processing SlpA after it is translocated but prior to its incorporation into the S-layer [33].

Unlike most of the other SecA2 orthologues, the *C. difficile* SecA2 is very closely related to its SecA paralogue (77% over-all similarity, 54% identity). Most of the differences are found in the HSD/HWD, but there is a 54 aa deletion in NBD2 between Motif V and Motif VI, and a 54 aa C-terminal truncation (Fig. 2). As in mycobacteria, SecA2 is cytosolic, whereas SecA localizes to the membrane [32]. The data suggest that *C. difficile* SecA2 can function independently of SecA, and thus may interact directly with SecYEG. There do not appear to be SecA2 orthologues in other clostridial species, including the medically relevant *C. tetani*, *C. botulinum*, or *C. perfringens*. Thus, the question of why *C. difficile* employs a SecA2 for Slayer protein transport is intriguing.

3.6 Possible roles in transport

The over-all similarity of the multi-substrate SecA2 proteins to SecA, rather than some other type of chaperone or ATPase, for example, suggests that the coupling of preprotein binding with ATP hydrolysis is important for function. However, nearly all of the multi-substrate SecA2s have a deletion in the NBD2 domain, suggesting they may bind or hydrolyze ATP differently than SecA, and thus may be differently regulated. Indeed, in vitro experiments with the mycobacterial SecA and SecA2 proteins have demonstrated such differences. If the acquisition of SecA2, a specialized motor protein, provides even a small growth advantage (via a slightly more efficient use of ATP), it is likely to persist in the population. A better understanding of SecA2 function, and whether there are significant differences from the SecA paralogues, will help to determine whether they can be selectively targeted for antimicrobial development.

The composite studies and structural comparisons also suggest that the means by which the motor activity of SecA2 is coupled to active transport may differ from that of SecA, and may further vary amongst the SecA2 orthologues. A direct interaction with SecYEG is most likely for the *C. difficile* SecA2, which is highly similar to SecA. However, other SecA2s differ significantly from their SecA paralogues in the HSD/HWD domain, which is a region known to provide extensive contacts between SecA and SecY during transport [6–8]. It is therefore less likely that the SecA2s interact directly with the SecYEG translocon, or at least not in the same way that SecA does. Instead, a different mode of interaction with SecYEG could be facilitated through another protein docked at SecYEG. In mycobacteria, SecA2 appears to interact with SecYEG via SecA. In this case, SecA2 might have a SecB-like chaperone or targeting role. Of probable functional significance, all SecA2s have a deletion in the CTD (except the *C. glutamicum* SecA2, which has a CTD insertion). In SecA, this domain is thought to help discriminate between true transport substrates (bound to SecB) and hydrophobic regions of cytoplasmic proteins [5].

Another possibility that cannot be excluded is that SecA2 may interact with an entirely different transmembrane channel. The identification of interacting partners for SecA2, and a more thorough assessment of the role of other *secA2* locus components in post-translational modification or transport, will shed light on these possibilities. In addition, since they are related to the newly-discovered SecA2 proteins in plastids of unicellular algae and higher plants [34–36] (Fig. 1), additional studies of these bacterial motor proteins may inform, and be informed by, related studies in eukaryotes [37].

In cases where SecA2 interacts with SecYEG or SecA/YEG, SecA2 may provide “priority boarding” or an express lane to the translocon. Each SecA2 may have different criteria for what merits priority, and the net effect would be to shift the balance of preproteins that are transported. However, the combined findings thus far do not clearly illuminate whether the SecA2 proteins may differ in how they select substrates for transport, whether there are unique features of the preprotein that may be recognized by SecA2, or whether the SecA2 substrates are simply the most abundant preproteins. Targeted mutations within the SecA2 substrates along with an analysis of the effect on SecA2-dependent transport should help to resolve this issue.

4. The accessory Sec system

Many species of streptococci and staphylococci express a specialized transporter known as the accessory Sec (or aSec) system. Along with SecA2, the aSec system invariably includes SecY2 (a paralogue of SecY) and the accessory Sec proteins Asp1-3. The Asps are unlike any other characterized proteins, and appear to be unique to the accessory Sec system. Unlike the general Sec system, which exports a variety of proteins, each aSec system is dedicated to the transport of a large, extensively glycosylated cell wall-anchored protein. These unique substrates, known as serine-rich repeat (SRR) glycoproteins, undergo extensive glycosylation intracellularly, prior to being transported to the bacterial cell surface.

The aSec system has been most extensively studied in *S. gordonii* and *Streptococcus parasanguinis*, two opportunistic pathogens normally present among the oral microbiota, but frequently associated with cardiovascular infections. Of historical note, the aSec system was first identified in a screen of *S. gordonii* mutants for decreased binding to human platelets [1]. The aSec system has since been identified in nine distinct genera and at least 32 different species to date (Table I), and there is a striking conservation of gene organization within the chromosomal loci that encompass this system (Fig. 4). Along with the transport components, the locus also encodes the transport substrate, and two or more glycosyl transferases that are responsible for the post-translational modification of the preprotein with carbohydrate moieties. It is not entirely clear why a dedicated system is necessary for the export of the SRR glycoproteins. One previous notion has been that the aSec system is essential for these unusual substrates because SecA or SecYEG cannot accommodate glycosylated proteins. However, several lines of evidence indicate that this is not entirely true, and suggest a more complex role for the aSec system in SRR glycoprotein expression.

4.1 substrates of the aSec system

The SRR glycoproteins comprise a unique family of adhesins that bind a wide range of ligands, and have a significant impact on biofilm formation and virulence in a diversity of infections. For example, GspB and Hsa (the SRR adhesins of *S. gordonii* strains M99 and DL1, respectively) bind sialylated carbohydrates on both the salivary mucin MG2 (MUC7), and on the platelet membrane protein GPIb [38–43]. This latter interaction appears to be a major factor in the pathogenesis of endocarditis [44, 45]. Fap1 expression by *S. parasanguinis* enhances adherence to saliva and subsequent biofilm formation [46, 47]. The SRR adhesins of *Streptococcus pneumoniae*, *Streptococcus agalactiae*, and *Staphylococcus aureus* have been linked to pneumonia, meningitis and neonatal sepsis, and endocarditis, respectively [48–54]. Although the ligands for the SRR glycoproteins are not known for all species, it is clear that they encompass both carbohydrates (e.g. sialyl T-antigen for GspB), as well as proteins (e.g. human keratin 4 and fibrinogen for the Srr1 adhesin of *S. agalactiae*) [55–57]. This diversity of ligands most likely reflects specific targets for microbial adhesion in different biologic niches.

Although the binding region of these adhesins can differ significantly, the overall domain organization is conserved, with an atypical N-terminal signal peptide followed by a short SRR domain, a ligand binding domain, a long SRR domain, and a C-terminal LPXTG cell

wall anchoring motif (Fig. 5). Carbohydrate moieties are rapidly added to the SRR domains through the combined action of two or more intracellular enzymes. Studies in *S. gordonii* and *S. parasanguinis* have indicated that a heterodimeric complex of GtfA/B (Gtf1/2) catalyzes the proximal linkage of N-acetyl glucosamine (GlcNAc) to the polypeptide backbone [58, 59]. The GlcNAc is presumed to be O-linked to serine residues in the SRR domains, but the precise linkage has not yet been verified, nor has any consensus motif for glycosylation been identified. Other glycosyl transferases may add additional glycan moieties following the initial GlcNAc deposition. The precise structure of the glycan on the mature adhesins is not known, but rough estimates for GspB indicate that approximately 100 monosaccharide residues may be added per each polypeptide backbone [60]. The extensive glycosylation often has a notable effect on solubility, stability, and resistance to proteolysis. In addition, the glycan modifications can have a dramatic impact on the binding properties of the adhesins [53, 61–63].

One reason the aSec systems went undetected for so long may be due to the nature of the substrates, which can be extremely difficult to detect and characterize. With the apparent masses ranging from 200 kDa to much greater than 400 kDa, the SRR glycoproteins are highly refractory to staining with most conventional reagents, and differently glycosylated forms may not be recognized by the same antibodies or lectins. These complications should not be overlooked, since they can make it difficult to distinguish primary effects on glycosylation and transport from secondary effects on these processes (see sections 4.2.2 and 4.3.3).

4.2 Preprotein recognition and trafficking to the aSec system

4.2.1 The SRR signal peptides—To better define the mechanisms of transport, truncated versions of the SRR glycoproteins have been used as model substrates. These have been highly valuable analytical tools since they lack a C-terminal cell wall anchoring domain and therefore are secreted freely into the culture medium, making it easier to quantify changes in export. Moreover, sufficient truncation of the extensive SRR2 region renders the preprotein stable even when not glycosylated, and thus allows examination of export independently of glycosylation. Such studies have revealed some key requirements for transport by the aSec versus canonical Sec system. Surprisingly, glycosylation of the substrate is not necessary for recognition by the aSec system. Instead, a 90 amino acid N-terminal signal sequence is one key portion of the preprotein that is essential for transport [64, 65]. The atypical signal peptide has a tri-partite structure characteristic of general Sec system signal peptides, although the N region is approximately three times longer (Fig. 5). The extended N region invariably includes a “KxYKxGKxW motif”. The functional relevance of this motif is not yet known, but it is predicted to form an amphipathic helix that may aid in targeting of the preprotein to anionic phospholipid patches in the membrane. A targeted deletion within the N region confirmed that it is important for transport, but does not affect the transport route [66].

In *S. gordonii*, the hydrophobic core of the GspB signal peptide (the H region), rather than the extended N region, was found to be a primary determinant of trafficking to the aSec system [66]. In particular, three glycine residues in the H region have a major impact on this

process. Results suggest that the glycines decrease the propensity to form an α -helix, but may also reduce the H region hydrophobicity (both properties are known to affect recognition and transport by the general Sec system). Replacement of one or two glycines with residues that increase the propensity for α -helix formation decreases the stringency of trafficking, such that the preproteins (i.e. truncated variants) are more readily transported by the general Sec system. Replacing all three glycine residues completely abrogates aSec transport, and re-directs the protein to the Sec system. Conversely, substitution of a glycine with proline only partially interferes with aSec transport, and simultaneously prevents Sec transport. Thus, very subtle changes in the hydrophobic core of the signal peptide have a strong influence on the trafficking of the preprotein, and the three glycine residues therefore contribute to a novel type of “Sec avoidance” mechanism.

Nearly all of the SRR glycoproteins identified to date have a similar ~90 residue amino-terminal sequence. One exception is found in the aSec substrates of peptidococci, where the signal peptide has a KxYKxGKxW motif that is immediately followed by the H region (i.e. the signal peptide lacks the intervening portion of the N region; Fig. 5). Another apparent, but not likely valid, exception is in the signal peptides of pneumococcal aSec substrates such as PsrP. The annotated sequences indicate a translational start site at the first methionine codon, which corresponds to codon 38 or 42 of other SRR glycoprotein signal sequences. However, the actual open reading frame is likely to begin at an atypical ATA codon further upstream, although this has not been verified experimentally.

The H regions of the signal peptides display two apparent varieties. In the streptococcal SRR proteins, the H region typically includes three glycine residues arranged in a GxxGG motif, and has a low hydrophobicity. As described above, these combined features may correspond to a unique type of Sec avoidance motif. The staphylococcal substrates have a central pair of glycines, and a slightly higher hydrophobicity in the signal peptide H region (Fig. 5), and may lack a Sec avoidance mechanism. Although this has not been thoroughly assessed, in *S. aureus* some transport of the aSec substrate SraP (also known as SasA) is evident in *secA2* deletion strains [67, 68], which suggests that the substrate may be less stringently trafficked, such that it can be transported by either the Sec or aSec route (Fig. 6C). A recent report indicates that SecY2 expression is up-regulated during the early exponential phase of growth [69]. Thus, trafficking of the SRR glycoprotein in *S. aureus* could be influenced by the relative expression levels of the two transporters.

4.2.2 Impact of glycosylation on trafficking—Although the carbohydrate moieties are not necessary for recognition by the aSec system, glycosylation of the SRR domains can influence trafficking by impeding general Sec transport. However, the extent to which glycosylation affects trafficking may depend on the nature of the signal peptide. For example, in *S. gordonii* and *S. agalactiae*, where the signal peptide of the SRR glycoprotein has an apparently strong Sec avoidance motif, little or no preprotein is transported by the general Sec system as long as all of the glycosyl transferases are intact (Fig. 6A). In *S. parasanguinis*, the Fap1 signal peptide has one less glycine in the H region (Fig. 5), and can readily support transport of a nonglycosylated substrate (e.g. when *gtf1* is deleted) by the general Sec pathway [65]. Accordingly, multiple reports have indicated that, even when the entire *gtf* cluster is intact, a partially glycosylated Fap1 precursor is transported by the

general Sec system (perhaps inefficiently) when aSec transport is impaired (Fig. 6B, and see section 4.3.3). In *S. aureus*, which lacks additional glycosyl transferases and expresses just the core GtfA/B complex, there is also evidence that at least some SRR glycoprotein can be transported by SecA/YEG [67, 68] (Fig. 6C). Thus, in cases where the signal peptide can facilitate transport by the general Sec pathway, partially glycosylated preproteins may be transported by SecA/YEG upon deletion of any aSec component.

4.2.3 The AST domain—In addition to the signal peptide, the aSec substrate must have a specific segment (the accessory Sec transport or AST domain) at the amino terminus of the mature region. Studies in *S. gordonii* and *S. parasanguinis* have demonstrated that deletion of this region abolishes aSec transport [65, 70]. Even single amino acid substitutions within the AST domain can dramatically impair aSec transport. The signal peptide and AST domain together are sufficient for the transport of a heterologous substrate (specifically, a slow-folding variant of the MalE maltose binding protein) by the aSec system. Curiously, the sequence of the AST domain is not highly conserved, and diverges much more rapidly than does the signal peptide. Extensive mutational analysis of the AST domain of GspB, in combination with “pre-gated” variants of the *S. gordonii* SecY2 (see section 4.4 below), indicated that the AST domain affects both targeting to the translocon and opening of the channel. These earlier results therefore suggested that the AST domain might interact with both SecA2 and SecY2.

Subsequent in vivo photo-cross-linking experiments were undertaken to capture any aSec components that interact with the preprotein during transport, and only SecA2 was found to contact the AST domain [71]. This result implies that targeting and gating may both be driven by AST domain interactions with SecA2. Whereas SecA2 can bind the end of the AST domain adjacent to the signal peptide independently of other aSec components, full engagement of the AST domain, as well as transport of the preprotein by SecA2, requires one or more of the Asps (see section 4.5 below). The AST engagement by SecA2 is reminiscent of the high affinity interactions that occur between preprotein mature regions and the SecA/YEG translocon [72]. However, the requirement of a specific segment in the preprotein, along with the involvement of the Asps, is a unique feature of aSec transport that may ensure the passage of just a single substrate via this channel.

4.3 the accessory Sec translocase

4.3.1 SecA2—The SecA2 proteins belonging to the aSec system have several common features. They all have a 45 aa truncation of the CTD, as compared with their SecA paralogues, and typically have a proline residue at the C-terminus. They have 70% similarity (35 to 40% identity) to SecA, and most of the similarity is limited to the nucleotide binding motifs of NBD1 and NBD2 (Fig. 2). Mutations of conserved NBD1 residues known to impair nucleotide binding and transport by *E. coli* SecA abolish transport mediated by *S. gordonii* SecA2, whereas mutations in NBD2 have a less dramatic impact [73]. Compared with SecA, the *S. gordonii* SecA2 has a lower basal rate of ATP hydrolysis, and requires higher magnesium concentrations for activity. The composite data indicate that the streptococcal SecA2 may be more tightly regulated than SecA, and support the possibility

that one or more of the Asps may be required to stimulate ATP binding or hydrolysis, as discussed below (section 4.3.3).

In spite of the similarities amongst the aSec motor proteins, the SecA2 orthologues diverge quite rapidly and are not readily interchangeable. For example, the SecA2 proteins from *Streptococcus sanguinis* and *S. pneumoniae* show 91% and 79% similarity (81% and 61% identity), respectively, to that of *S. gordonii*. Although the *S. sanguinis* SecA2 can partially complement a *secA2* mutation in *S. gordonii*, the *S. pneumoniae* SecA2 is unable to transport GspB [71]. Thus, at least some of the divergence in the SecA2 proteins must reflect differing inter- versus intra-molecular interactions. It is possible that the SecA2 differences may parallel those of the preprotein substrates, but the driving force behind such rapid divergence remains to be determined.

4.3.2 The SecY2 translocon—SecY2 is presumed to form the transmembrane channel through which the SRR glycoproteins exit the cytoplasm. The predicted topology of SecY2 is nearly identical to that of SecY (Fig. 7A). However, the SecY2 orthologues have very low primary sequence similarity to the SecY paralogues (20% identity, 60% similarity), and only a limited number of the SecY residues involved in protein-protein interactions are conserved in SecY2. Importantly, none of the C5 cytosolic loop residues that contact SecA (summarized in Lycklama *et al* [11]) are present in SecY2, which is indicative of a lack of cross-talk between the general Sec and aSec systems. The inactive SecY channel has an hourglass shape (Fig. 7B), and two features help to seal the closed channel: a ring of hydrophobic residues near the central pore (the “pore ring” residues), and a “plug” on the periplasmic or external side comprised of residues in TM2 [74–76]. Upon intercalation of a signal peptide between two of the transmembrane segments, the SecY channel widens, and the plug is displaced [77, 78]. Compared with SecY, the putative pore ring residues of SecY2 include slightly bulkier residues, such as leucine or methionine, versus isoleucines. As yet, there is nothing obviously different about the SecY2 proteins that might account for the ability to transport a glycosylated protein.

In the general Sec system, two small proteins combine with SecY to form the translocon. SecE is essential for transport and helps stabilize the open form of the channel [78, 79]. SecG enhances the translocation efficiency and was recently shown to contact SecA during transport [7]. In some streptococcal species, the aSec system includes one or two additional small proteins (Asp4 and Asp5) that may be structural components of the transmembrane channel [80]. Asp4 and Asp5 have one or two predicted transmembrane segments, respectively, and thus display some topological similarity to SecE and SecG. It is suspected that they may interact with SecY2, but this has not been verified experimentally. Functional studies in *S. gordonii* indicate that Asp4 is partially dispensable for the export of truncated or non-glycosylated GspB variants via the aSec route [81]. This is consistent with a role of Asp4 in stabilizing the open state of the transmembrane channel, rather than a role in the initiation of translocation. In *S. aureus*, there is evidence for interaction between SecY2 and SecG [82]. In other organisms that lack Asp4 and Asp5, it is possible that the SRR signal peptide could facilitate or stabilize the open SecY2 channel, and therefore preclude the need for these Asps.

Several lines of experimental evidence suggest that SecA2 interacts directly with SecY2 to facilitate SRR glycoprotein transport. First, deletion of *secY2* results in the same phenotype as deletion of *secA2* [1, 67]. Second, certain alterations in SecY2 mimic the effect of *prl* (protein localization) mutations in SecY and compensate for defects in the GspB signal peptide and some AST mutations [70]. For example, the SecY2 substitution I382N mirrors the I408N (*prlA4*) mutation in SecY [83], a substitution of one of the pore ring residues that destabilizes the closed channel and is thought to override a gating mechanism that is usually facilitated by signal peptide intercalation between the second and seventh transmembrane segments of SecY [77] (Fig. 7B). It is therefore highly likely that SecY2 is a structural component of the SecA2 translocase.

4.3.3 additional accessory Sec system components—Although Asps1-3 are invariable components of the aSec system, their precise roles in SRR glycoprotein expression are just beginning to be understood. In *S. gordonii* these proteins clearly have a direct role specifically related to aSec transport, and are essential for this process even when glycosylation of the preprotein is blocked by deletion of the core glycosyl transferase gene *gtfA* [81]. Since the Asps have no primary sequence similarity to any proteins of known function, a variety of genetic and biochemical techniques have been used to assess how and where they function. Asp2 and Asp3 have been shown to have a range of interactions. Both proteins directly bind the SRR regions of the GspB preprotein [84]. Rather than binding to specific motifs, Asps2 and 3 appear to recognize the unstructured or non-folded sections of the preprotein. Although these Asps bind GspB directly, they do not appear to function as conventional chaperones, since they are not required for GspB stability or targeting to SecA2. As described above, evidence indicates that they are important for the full engagement of the AST domain with SecA2, and subsequent GspB transport. Experiments also indicate that Asp2 and Asp3 both directly bind SecA2 [81]. Asp2, in particular, appears to have a high affinity for SecA2, since it localizes at the inner membrane in the same punctate pattern as SecA2, when co-expressed with this protein in *E. coli* [85]. Thus, either or both Asps may directly alter the conformation of SecA2 to facilitate the full engagement of the preprotein AST domain, or to stimulate ATP binding or hydrolysis.

Subcellular localization studies of Asp1, Asp2 and Asp3 indicate that all three of these proteins fractionate to both the membrane and cytosol compartments, and their location is at least partially affected by other aSec components. Thus, all three of the Asps may transit between the cytosol and the cytoplasmic membrane. There is also evidence that Asps1-3 form a complex that is soluble and cytosolic, but will partially localize to the membrane when co-expressed with SecA2 [85]. What is as yet unresolved is whether these proteins transit individually or collectively between the two compartments.

The Asp homologues of *S. parasanguinis* have been designated as glycosylation-associated proteins Gap1-3, in part because they are thought to have a primary affect on glycosylation rather than transport, and in part because a portion of Gap1 resembles some glycosyl transferases [86]. In addition, results of earlier studies suggested different roles for SecA2 (i.e. a clear role in transport) versus SecY2, Gap1 and Gap3 (an impact on glycosylation) [87–89]. However, later reports indicate that deletion of *gap1*, *gap2* or *gap3* results in a phenotype identical to that of a *secY2* deletion, which is the loss of correctly glycosylated or

“mature” Fap1 on the cell surface, and a loss of binding to saliva-coated hydroxylapatite [90, 91]. Rather than abolishing transport, deletion of any of these components results in the expression of a partially glycosylated Fap1 precursor on the cell surface, but possibly in reduced amounts. This is most likely due to the absence of a strong Sec avoidance motif in the Fap1 signal sequence, and less stringent trafficking of the glycosylated preprotein (see section 4.2.2). Thus, if the Gaps primarily affect glycosylation and not transport, it is unclear why the Fap1 precursor is not efficiently transported by the aSec system, as happens upon deletion of other glycosyl transferases such as Gtf1 or GalT2 [88, 92]. As with the Asps, a number of interactions amongst the Gap proteins, and between the Gaps and SecA2, have been characterized [90, 93, 94]. However, unlike the Asps, the stability of the Gaps is interdependent. That is, Gap1 and Gap2 affect the stability of Gap3, and are thus thought to serve as chaperones for Gap3. The importance of these interactions towards either the glycosylation or transport of Fap1 as yet has not been defined.

4.3.4. Asp1-3 (Gap1-3) as bi-functional components of the aSec system—Based on the combined results of studies in *S. parasanguinis*, *S. gordonii* and *S. salivarius*, Zhou and Wu [95] suggested that the processes of transport and glycosylation are coupled, and proposed a two-step model for the glycosylation of Fap1 in which SecA2, SecY2, Gap1 and Gap3 convert a partially glycosylated precursor to a mature glycoform. Recent studies on the function of Asp2 in *S. gordonii* demonstrated that this component of the aSec system does indeed affect the carbohydrate content of GspB [63]. Importantly, the specific effect on glycosylation was not apparent upon deletion of *asp2*, but only by making targeted substitutions in the protein. Tertiary structure predictions indicate that a region of Asp2 resembles the catalytic region of esterases and hydrolases. Alignment of the Asp2 sequence with that of the esterase catalytic domains led to the identification of a Ser-Glu-His catalytic triad. Somewhat surprisingly, mutation of these residues did not impair transport, but did result in changes in the glycan composition of GspB, as indicated by decreased electrophoretic mobility, altered affinity for several lectins, and increased incorporation of GlcNAc. Moreover, the altered glycoform of GspB was unable to facilitate binding of *S. gordonii* to human platelets. Thus, Asp2 is clearly involved in both the transport and glycosylation processes. However, no catalytic activity was evident when assessing the isolated, purified protein. It was therefore postulated that the Asp2 catalytic activity might be dependent upon the formation of a multimeric Asp complex.

5. New model and key unanswered questions

Based on the most recent composite data, we propose a new model for aSec transport, and a new mechanistic role for the Asps in SRR glycoprotein biogenesis (Figure 8). In this new model, the Asps are bifunctional proteins involved in both transport and glycosylation. The Asps have individual roles in transport that are not dependent on the glycosylation process. In addition, however, they have a collective and direct role in glycosylation that is dependent on transport via SecA2/Y2. More specifically, one or more of the Asps facilitate the full engagement of the preprotein AST domain by SecA2 and the initiation of transport. A complex of the Asps may then modify the glycan composition of the SRR glycoprotein as it undergoes transport by SecA2/Y2. By functioning as the nexus of these two processes, the Asps serve as a control point for the correct biogenesis of the SRR proteins. Glycosylation is

essential for stability, but must be done with high precision for the SRR protein to have optimal binding properties. Thus, the specialized aSec transporter may have evolved not merely to accommodate a glycoprotein, but to couple the modulation of glycan composition with transport. Determining exactly how this coupling occurs is fundamental to understanding SRR glycoprotein expression. This model is more consistent with data that indicate at least some of the post-translational modifications are compatible with SecA/YEG transport (as is the case for *S. aureus* and *S. parasanguinis*), but the precise glycan composition of the substrate is affected by the Asps associated with the SecA2/SecY2 translocon. The model also raises an interesting question concerning the role of SecA2 proteins in general: do all SecA2 proteins facilitate the coupling of post-translational (or post-translocational) modifications with transport? In cases where they are not essential for viability, further study of SecA2 transporters may provide significant insights and understanding of general Sec system transport in Gram-positive bacteria.

Acknowledgments

This work was supported by the Department of Veterans Affairs and the VA Merit Review program; the Northern California Institute for Research and Education; grant R01-AI41513 from the NIH (P.M.S.); and a Fellowship Award from the American Heart Association (AHA), Western Affiliate (YY).

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Highlights

- SecA2 proteins comprise a selective class of transport-associated ATPases.
- SecA2 is found in an increasingly large number of Gram-positive bacterial species.
- One type of SecA2 transports multiple substrates, and may interact with the general Sec system.
- A second type transports a single substrate, and is a component of the accessory Sec system.
- The accessory Sec system couples post-translational modifications with transport.

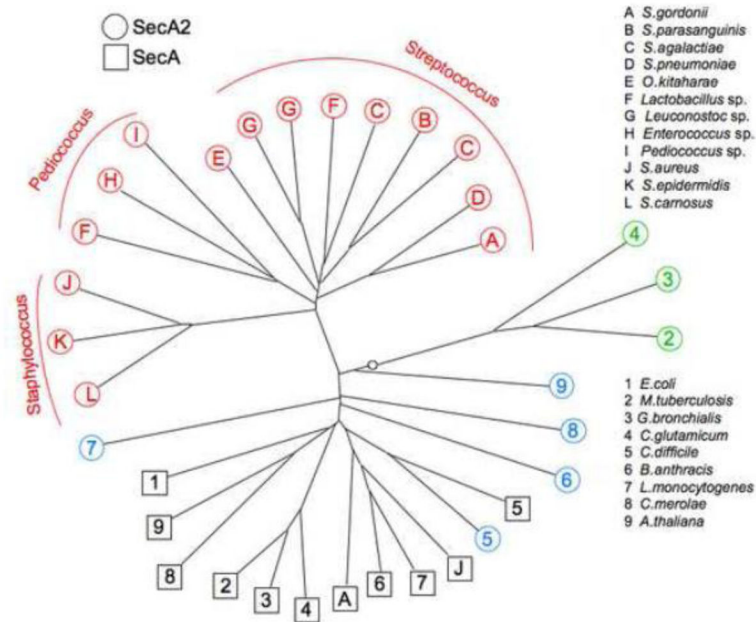


Figure 1. Phylogenetic relatedness of the SecA and SecA2 proteins

Phylogenetic analysis of the SecA and SecA2 proteins from selected Gram-positive bacterial species was performed using DARWIN [96]. Species are presented more than once if there are strains that encode significantly different SecA2 orthologues. The unrooted tree shows three main branches of SecA2 divergence: 1) SecA2s of the accessory Sec system (red), 2) SecA2s of the Actinobacteria species (green), and 3) other SecA2s (cyan) and SecA proteins of the general Sec system (black), with *E. coli* SecA included as a point of reference. SecA2 homologues from *Arabidopsis thaliana* and the unicellular red alga *Cyanidioschyzon merolae* are included for comparison.

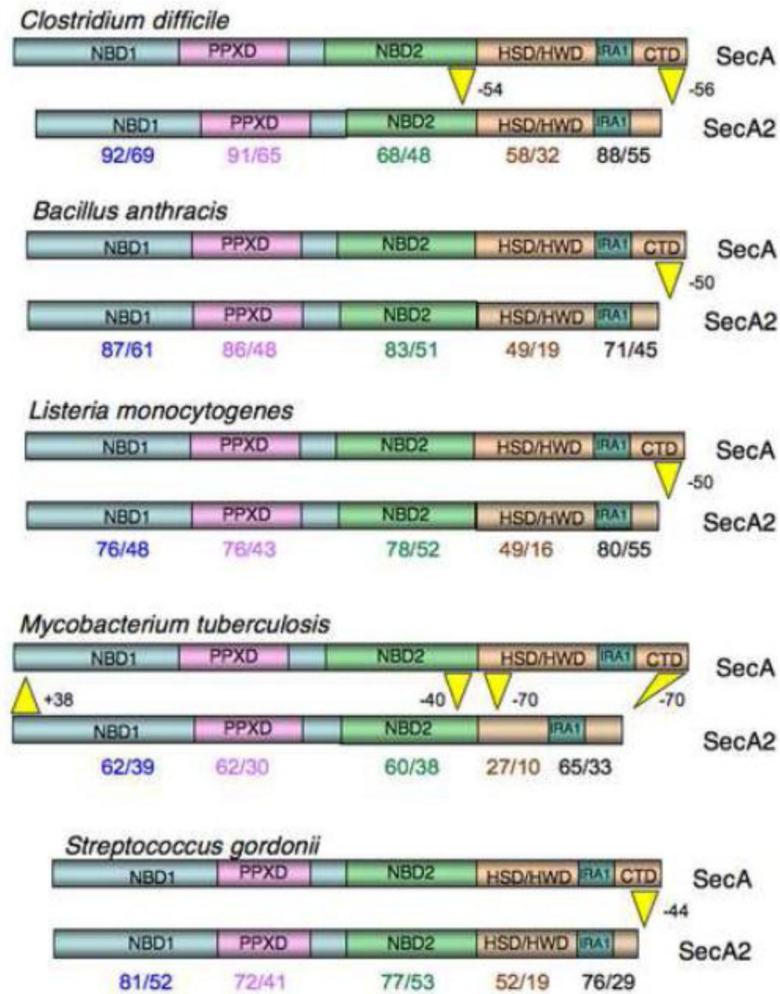


Figure 2. Comparative domain organization among SecA2 proteins

SecA2 domains were identified by alignment with *E. coli* SecA. Domain boundaries are based on those determined by Papanikolaou et al [4]. NBD, nucleotide binding domain; PPXD, preprotein cross-linking domain; HWD, helical wing domain; HSD, helical scaffold domain; IRA1, intramolecular regulator of ATPase activity; CTD, C-terminal domain. IRA1 is a functional domain that overlaps with the two-helix finger structural domain [6, 97]. Numbers shown below the SecA2 proteins indicate the percent similarity/identity with the corresponding domain of the SecA paralogue.

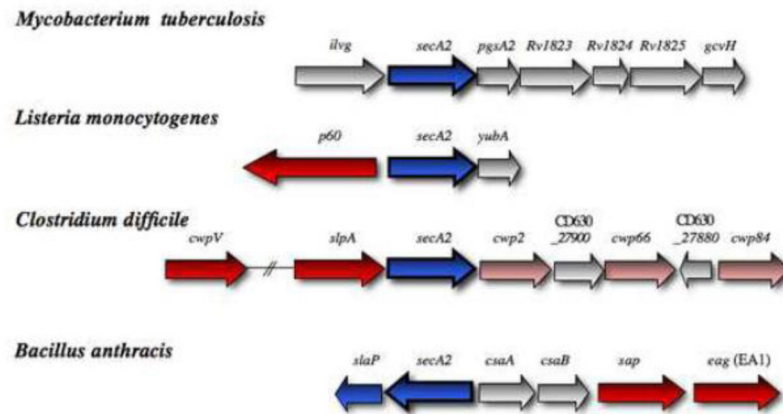


Figure 3. Genetic loci encoding the multi-substrate SecA2 proteins

The chromosomal regions flanking *secA2* (blue) in *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Clostridium difficile* and *Bacillus anthracis* are shown. A component required for SecA2 transport in *B. anthracis* is also in blue. Genes encoding defined SecA2 substrates are shown in red, and presumed substrates are indicated in pink. Substrates encoded elsewhere in the chromosome are not shown.

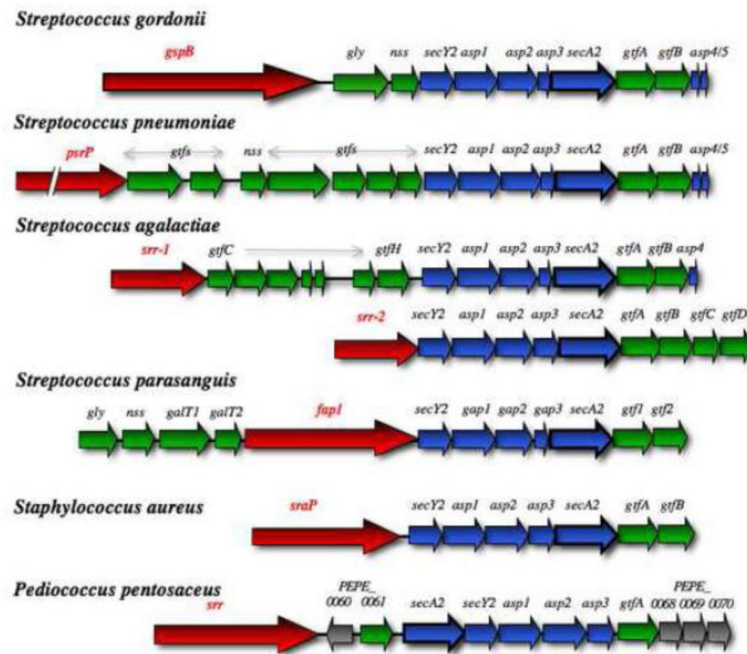


Figure 4. Gene organization within the accessory Sec loci

Comparison of the aSec locus in selected Gram-positive bacterial species. Genes encoding the aSec system components are colored blue, and the export substrate is shown in red. Glycosyl transferases and other enzymes involved solely in carbohydrate modifications are shown in green. Genes of unknown function are shown in grey, while genes encoding potential regulatory proteins are depicted in black. Two different variations found in *Streptococcus agalactiae* strains are shown. The *Staphylococcus* locus is representative of most *Staphylococcus* species. A slightly different arrangement, in which *secA2* is immediately upstream of *secY2* and the *asp123* genes, is found in *Pediococcus*, *Enterococcus* and some *Lactobacillus* species.

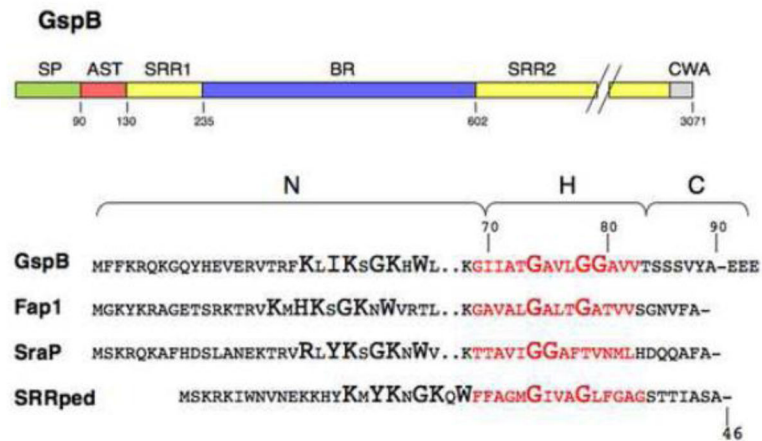


Figure 5. SRR glycoprotein domain organization and conserved signal peptide features
 Upper diagram: The domains identified in GspB are characteristic of the SRR glycoprotein family. SP, signal peptide; AST, accessory Sec transport domain; SRR1 and SRR2, serine-rich repeat regions 1 and 2, respectively; BR, ligand binding region; CWA, cell wall anchoring domain (including an LPxTG motif).
 Lower diagram: Amino-terminal sequence of the SRR glycoproteins from *S. gordonii* (GspB), *S. parasanguinis* (Fap1), *S. aureus* (SraP), and *P. acidilactici* (SRRpp).

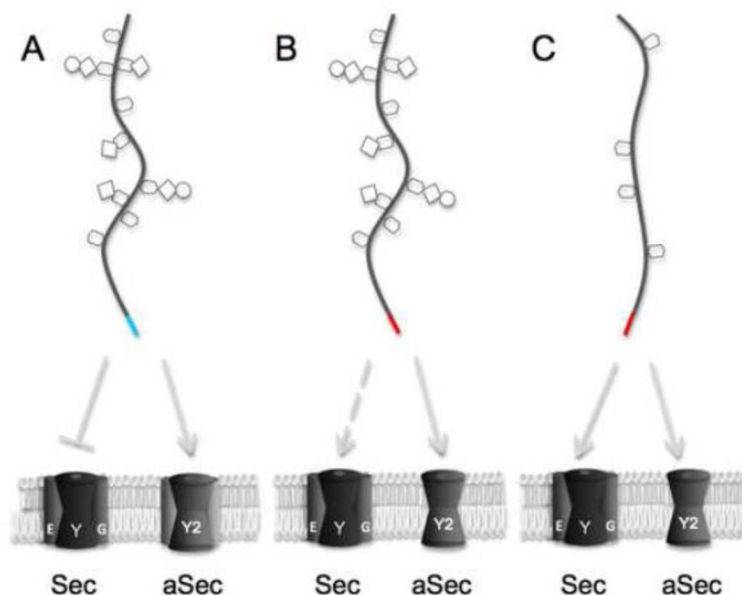


Figure 6. Impact of signal peptides and post-translational modifications on trafficking through the general Sec or aSec system

A signal sequence with a relatively strong Sec avoidance motif is indicated in cyan, and signal sequences that can support transport by the general Sec system are indicated in red. A: In most streptococci, the aSec locus encodes four or more putative glycosyl transferases, and the SRR glycoprotein signal peptide is not optimal for general Sec transport. B: GspB variants with one or more substitutions of critical glycine residues in the signal peptide H region, or wild-type Fap1, may be inefficiently transported by the general Sec system (dashed line indicates transport of partially or incorrectly glycosylated substrates). C: In staphylococci, the aSec locus includes just the core GtfA/B glycosyl transferase, and the SRR glycoprotein signal peptide can facilitate transport via either the Sec or aSec pathway.

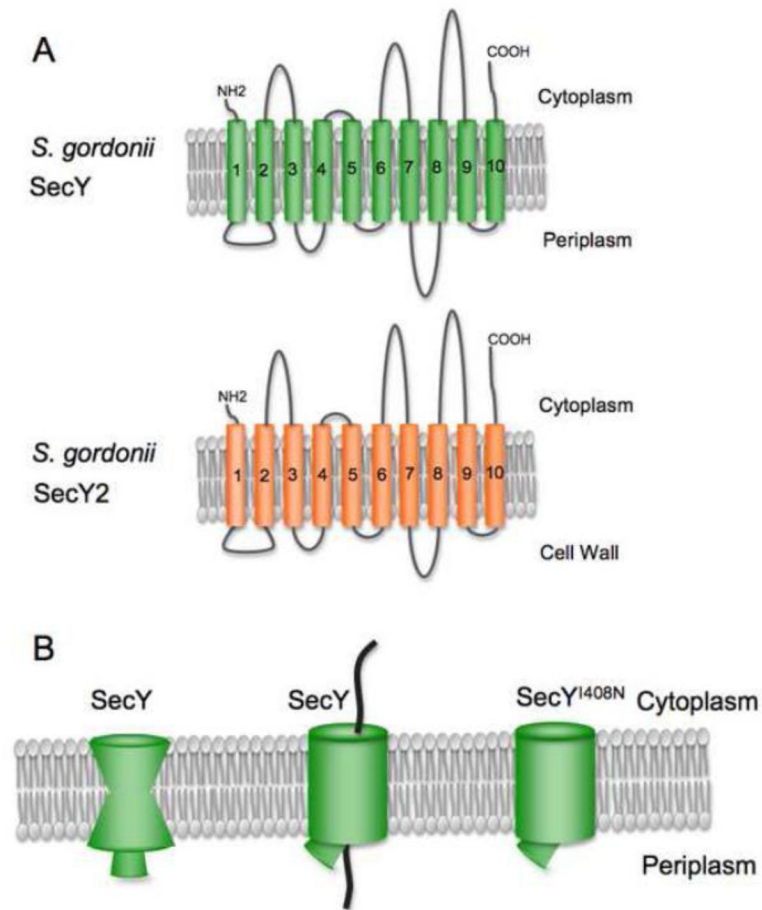


Figure 7. SecY versus SecY2 topology and structural features

A: The predicted topology of SecY2 is very similar to that of SecY. B: The inactive SecY channel is impermeable to small molecules, due to the constriction by a ring of hydrophobic residues near the center of the channel along with a plug formed by residues of TM2. In the active channel, the central pore widens and the plug is displaced. Some *prl* mutations in *E. coli* SecY result in a partially or more readily opened channel.

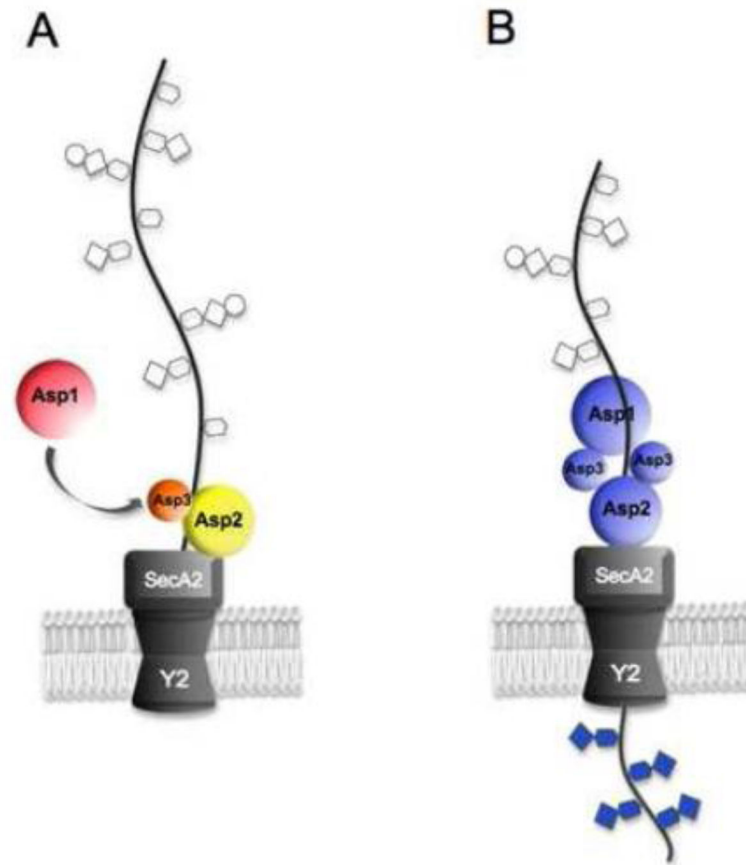


Figure 8. Model for SRR glycoprotein biogenesis

A: A partially glycosylated preprotein arrives at the translocon, and one or more of the Asps facilitate an interaction between SecA2 and the preprotein AST domain that trigger opening of the SecY2 channel. B: An Asp123 complex further modifies the glycan composition as translocation of the SRR glycoprotein proceeds through SecA2/Y2. The Asp123 modifications could include trimming, replacement, or addition of carbohydrate moieties. SecA2 and SecY2 are thought not to have a direct enzymatic role in glycan modification, but instead facilitate the interaction between the Asp complex and the substrates.

Table 1

Gram-positive bacterial species that encode SecA2.^a

Actinobacteria	Listeria	Bacillus^b	Streptococcus^c	Staphylococcus	Pediococcus^d
<i>Mycobacteria</i>	<i>Listeria</i>	<i>Bacillus</i>	<i>Streptococcus</i>	<i>Staphylococcus</i>	<i>Pediococcus</i>
<i>M. tuberculosis</i>	<i>L. monocytogenes</i>	<i>B. anthracis</i>	<i>S. pneumoniae</i>	<i>S. aureus</i>	<i>P. pentosaceus</i>
<i>M. smegmatis</i>	<i>L. innocua</i>	<i>B. cereus</i>	<i>S. mitis</i>	<i>S. epidermidis</i>	<i>P. acidilactici</i>
<i>M. leprae</i>	<i>L. seeligeri</i>	<i>B. thuringiensis</i>	<i>S. oralis</i>	<i>S. warneri</i>	<i>P. lolii</i>
<i>M. bovis</i>	<i>L. marthii</i>	<i>B. smithii</i>	<i>S. sanguinis</i>	<i>S. carnosus</i>	<i>P. clausenii</i>
<i>M. marinum</i>	<i>L. vanovii</i>	<i>B. methanolicus</i>	<i>S. salivarius</i>	<i>S. intermedius</i>	
<i>M. canetti</i>	<i>L. welshimeri</i>		<i>S. suis</i>		<i>Enterococcus</i>
<i>M. avium</i>	<i>L. grayi</i>		<i>S. infantae</i>		<i>E. saccharolyticus</i>
<i>M. abscessus</i>			<i>S. porcinus</i>		<i>E. raffinosus</i>
			<i>S. australis</i>		<i>E. avium</i>
<i>Gordonia</i>			<i>S. vestibularis</i>		<i>E. sulfureus</i>
<i>G. otitidis</i>			<i>S. cristatus</i>		<i>E. pallens</i>
<i>G. rhizosphaera</i>					
<i>G. bronchialis</i>			<i>Lactobacillus</i>		<i>Lactobacillus</i>
<i>G. alkanivorans</i>			<i>L. salivarius</i>		<i>L. paracasei</i>
<i>G. hirsuta</i>			<i>L. johnsonii</i>		<i>L. suebicus</i>
<i>G. effusa</i>			<i>L. murinus</i>		<i>L. fructivorans</i>
<i>G. sputi</i>			<i>L. gastricus</i>		<i>L. rhamnosus</i>
					<i>L. zeae</i>
<i>Corynebacterium</i>			<i>Leuconostoc</i>		
<i>C. glutamicum</i>			<i>L. gasicomitatum</i>		
<i>C. diphtheriae</i>			<i>L. gelidium</i>		
<i>C. durum</i>			<i>L. kimchii</i>		

^a grouped according to the major and minor branch types in Fig. 1 (not all-inclusive)^b absent from *B. subtilis*^c absent from *S. pyogenes* and *S. mutans*^d absent from *E. faecalis* and *E. faecium*