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A new twist on an old pathway – accessory Sec systems

Nathan W. Rigel and **Miriam Braunstein***
Department of Microbiology and Immunology, University of North Carolina, School of Medicine, Chapel Hill, NC 27599-7290

Summary

The export of proteins from their site of synthesis in the cytoplasm across the inner membrane is an important aspect of bacterial physiology. Because the location of extracytoplasmic proteins is ideal for host-pathogen interactions, protein export is also important to bacterial virulence. In bacteria there are conserved protein export systems that are responsible for the majority of protein export: the general secretion (Sec) pathway and the twin-arginine translocation (Tat) pathway. In some bacteria, there are also specialized export systems dedicated to exporting specific subsets of proteins. In this review, we discuss a specialized export system that exists in some Gram-positive bacteria and mycobacteria – the accessory Sec system. The common element to the accessory Sec system is an accessory SecA protein called SecA2. Here we present our current understanding of accessory Sec systems in *Streptococcus gordonii*, *Streptococcus parasanguinis*, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, and *Listeria monocytogenes*, making an effort to highlight apparent similarities and differences between the systems. We also review the data showing that accessory Sec systems can contribute to bacterial virulence.

The conserved general secretion (Sec) pathway

In bacteria, the bulk of protein export across the cytoplasmic membrane is carried out by the general secretion (Sec) pathway (Driessen and Nouwen, 2007; Murphy and Beckwith, 1996). The final destination of Sec exported proteins can be the cell envelope or the extracellular space. Since the Sec pathway exports numerous substrates, many with critical roles in cell physiology, it is not surprising that it is an essential process in all bacteria tested.

The Sec pathway is well-characterized through studies with *Escherichia coli* and *Bacillus subtilis* (for comprehensive recent reviews see Driessen and Nouwen, 2007; Papanikou *et al.*, 2007). At the core of the Sec pathway is a membrane-spanning translocase channel composed of the integral membrane proteins SecY, SecE, and SecG (Brundage *et al.*, 1990). Working closely with SecYEG is SecA, a multi-functional protein that participates in nearly every step of the pathway (Economou, 1998). SecA binds to cytoplasmic precursor proteins destined for export and delivers them to the translocase through its ability to bind both membrane phospholipids and the translocase itself. SecA is also an ATPase that provides energy for translocation. Through repeated cycles of ATP binding and hydrolysis, SecA undergoes conformational changes that drive stepwise export of a precursor protein through the core of the Sec translocon and across the membrane (Economou and Wickner, 1994; Hartl *et al.*, 1990). This transport is post-translational and chaperones play an important role in maintaining precursors in a translocation-competent unfolded state.

Sec-exported proteins are synthesized as precursors with a signal sequence at the amino-terminus. Sec signal sequences have a tripartite domain structure: a positively charged N

*Corresponding author: Phone: 919-966-5051, Fax: 919-962-8103, Email address: braunste@med.unc.edu.

domain, a hydrophobic H domain, and a polar C domain (Driessen and Nouwen, 2007). A signal sequence cleavage site is located at the junction between the C domain and the mature protein. During or immediately after transport across the membrane, the signal sequence is cleaved from the precursor by a signal peptidase (Tokunaga *et al.*, 1984; van Roosmalen *et al.*, 2004; Zwizinski and Wickner, 1980). The resulting mature protein then folds into its final conformation.

Accessory SecAs in bacteria

It was long believed that all bacteria possess a single essential SecA (Economou, 1999). It is now recognized that some, though not all, Gram-positive bacteria and mycobacteria have two SecAs. Some bacteria with extra SecAs are pathogens (*e.g. Streptococcus gordonii*, *S. parasanguinis*, *S. pneumoniae*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Listeria monocytogenes*, and *Bacillus anthracis*) but accessory SecAs are also found in nonpathogenic bacteria (*e.g. Mycobacterium smegmatis*, *Listeria innocua*, and *Corynebacterium glutamicum*) (Figure 1).

In bacteria with two SecA homologues (Table 1), both SecAs are similar across the entire length of the protein to the well-characterized *E. coli* and *B. subtilis* SecA. In all cases, the SecA with the slightly higher degree of sequence similarity to the canonical SecA is named SecA or SecA1. The SecA/SecA1 protein is the presumed essential 'housekeeping' export factor. The other SecA, with a slightly lower degree of similarity to *E. coli* SecA, is named SecA2. With one exception (*Corynebacterium glutamicum*) (Caspers and Freudl, 2008), SecA2 is not essential (Bensing and Sullam, 2002;Braunstein *et al.*, 2001;Braunstein *et al.*, 2003;Chen *et al.*, 2004;Lenz and Portnoy, 2002).

All SecA2 proteins possess conserved domains of SecA proteins: the DEAD-like ATPase motor domain, which includes ATP-binding Walker A and B boxes, the IRA1 (*i*ntramolecular *r*egulator of *A*TP hydrolysis) domain, and the preprotein binding domain (Karamanou *et al.*, 1999; Kimura *et al.*, 1991; Mitchell and Oliver, 1993). A distinction between SecA2 and the canonical SecA is that SecA2 proteins are smaller in size, due in part to a truncation in the C-terminal linker (CTL) (Rigel and Braunstein unpublished). At least in *E. coli*, the CTL functions in binding phospholipids, the SecB chaperone, and Zn^{2+} (Breukink *et al.*, 1995; Dempsey *et al.*, 2004; Fekkes *et al.*, 1997; Matousek and Alexandrescu, 2004).

SecA2/SecY2 systems

The accessory SecA systems can be divided into two groups: those that include an accessory SecY2 and those that do not (Figure 1). In some bacteria with an accessory SecA2 and SecY2, (*e.g. S. gordonii*, *S. parasanguinis*, *S. pneumoniae*, and *S. aureus*) the genomic locus contains conserved genes with a similar organization (Figure 2). As discussed later, genes in this locus have roles in export and glycosylation of SecA2-dependent substrates and encode an exported substrate. Some *Bacillus sp.*, most notably *B. anthracis*, also contain genes predicted to encode an accessory SecA2 and SecY2. However, *B. anthracis* does not exhibit similar genomic organization to the SecA2/SecY2 systems studied in *Streptococcus sp.*

SecA2-only systems

For bacteria with a SecA2 but lacking SecY2, the genomic region is not conserved (Figure 2). In mycobacteria, the gene immediately downstream of *secA2* is *pgsA2*, which encodes a phosphatidylglycerolphosphate synthase homologue predicted to function in production of acidic phospholipids, which have multiple cellular functions including a role in Sec export (de Vrije *et al.*, 1988;Jackson *et al.*, 2000). Three genes further downstream encode small

putative membrane proteins of unknown function. In the SecA2 system of *L. monocytogenes*, *secA2* is adjacent to a gene encoding the p60 protein. These two genes appear to be divergently transcribed. The p60 protein is exported by the *L. monocytogenes* SecA2 system (Lenz and Portnoy, 2002). Downstream of *secA2* is *yubA*, which encodes a predicted membrane protein with no informative homology.

Evolution of accessory SecA systems in bacteria

For the SecA2/SecY2 systems of *Streptococcus sp.* and *Staphylococcus sp.*, the high level of genomic similarity in the locus suggests a common source. However, not all *Streptococcus sp.* and *Staphylococcus sp.* contain an accessory *sec* locus. This could be indicative of recent horizontal gene transfer. In *M. tuberculosis* and *L. monocytogenes*, which have the SecA2-only systems, the genomic region is not conserved. We propose that these SecA2-only systems are not directly related to each other or to the SecA2/SecY2 systems, and they evolved independently.

The basic questions of how the SecA2 systems operate are just beginning to be asked. What proteins interact and work with SecA2 in export? What proteins are exported by SecA2 and what features define them? What roles do accessory Sec systems play in virulence? Below, we summarize our current understanding of accessory Sec systems of bacteria.

SecA2/SecY2 systems

S. gordonii and S. parasanguinis

The SecA2/SecY2 systems of the oral pathogens *S. gordonii* and *S. parasanguinis* have many similarities. In *S. gordonii* strain M99, the SecA2 system is responsible for exporting GspB, a large serine-rich glycoprotein to the cell surface (Bensing and Sullam, 2002). Exported GspB promotes *S. gordonii* binding to platelets. Therefore, the SecA2 system is likely to contribute to pathogenesis, as platelet binding is believed to be important for *S. gordonii* attachment to damaged cardiac tissue and subsequent infective endocarditis. In *S. parasanguinis*, the SecA2 system exports a similarly large serine-rich glycoprotein Fap1 (Chen *et al.*, 2004). Adherence of *S. parsanguinis* to saliva-coated hydroxylapatite, a model for the tooth surface, requires long fimbriae built from Fap1 subunits (Wu *et al.*, 1998). Because export of Fap1 is required for optimal adherence of *S. parasanguinis*, the accessory Sec pathway in this bacterium is believed to be important to dental plaque formation, which is linked to caries and periodontal disease.

1) Discovery of the accessory SecAs—In both *S. gordonii* and *S. parasanguinis*, the accessory *sec* locus was initially discovered in genetic screens. In *S. gordonii*, a deletion of the putative operon that includes *secA2* and *secY2* was identified in a mutant defective in platelet binding (Bensing and Sullam, 2002). It was hypothesized that the mutant phenotype reflects a failure to export a platelet binding protein. In an elegant experimental approach using immune serum enriched for antibodies to SecA2-dependent proteins, GspB was identified as a protein exported to the cell wall and released into culture media in a SecA2-dependent manner (Bensing and Sullam, 2002). Later, GspB was recognized as being encoded by a gene in the accessory *sec* locus. GspB is a large (286 kDa) heavily glycosylated protein (Bensing *et al.*, 2004b).

In *S. parasanguinis*, transposon insertions at the 3′ end of *secA2* were identified in a screen for mutants that fail to glycosylate Fap1 (Chen *et al.*, 2004). It was later shown that SecA2 is required for export of Fap1 to the cell wall and for its release into culture media. In addition to these extracytoplasmic locations, some Fap1 is also detected in cytosolic and membrane

fractions of wild-type cells (Chen *et al.*, 2004). Like GspB, the large (263 kDa) glycosylated Fap1 protein is also encoded in the accessory *sec* locus.

2) Proteins that function in export and glycosylation of SecA2-dependent proteins—From mutation analysis of the genes in the accessory *sec* loci of *S. gordonii* and *S. parasanguinis* (Figure 2), genes can be placed in one of two groups: 1) genes required for export and 2) genes required for glycosylation. As detailed below, the current data reveal many similarities but also some differences between the *S. gordonii* and *S. parasanguinis* systems.

Export Factors: Disruption of *secA2* or *secY2* individually in *S. gordonii* prevents GspB export to the cell wall and release into culture media (Bensing and Sullam, 2002). In the absence of export, GspB protein accumulates on the cytosolic face of the membrane where it is protected from attack by externally added protease (Bensing and Sullam, 2002; Takamatsu *et al.*, 2004a). Similarly, Fap1 is not exported in a *secA2* deletion mutant of *S. parasanguinis* and non-exported Fap1 accumulates primarily in the membrane fraction (Chen *et al.*, 2004). One of the differences between the SecA2/SecY2 systems is that in *S. parasanguinis* a *secY2* mutation does not eliminate Fap1 export (Wu *et al.*, 2007a).

In *S. gordonii*, the three open reading frames that separate *secY2* and *secA2* are named *asp1–3* (for *a*ccessory *s*ecretory *p*rotein) (Figure 2). Mutations in these *asp* genes result in a GspB export defect. Asp2 contains a predicted transmembrane domain and could function as part of a channel in the cytoplasmic membrane. Asp1 and Asp3 do not have signal sequences or transmembrane domains, suggesting intracellular functions that are not readily predicted from the protein sequence. Two additional genes in the *secA2/secY2* locus, *asp4* and *asp5*, are required for GspB export (Takamatsu *et al.*, 2005). Asp4 and Asp5 share sequence homology with *B. subtilis* SecE (52% similar) and SecG (55% similar). Thus, Asp4 and Asp5 might function as components of a membrane translocase as is the case for SecE and SecG, making the accessory Sec system completely independent of the canonical Sec system components. This possibility is worthy of further investigation.

A different result is obtained with an *asp3* mutation in *S. parasanguinis* (Peng *et al.*, 2008; Wu *et al.*, 2007b). Like a *secY2 S. parasanguinis* mutant, an *asp3* (renamed *gap3* for *g*lycosylation *a*ssociated *p*rotein 3*)* mutant still exports Fap1, but in both of these mutants the protein runs at a higher apparent molecular weight in an SDS-PAGE gel and is not correctly glycosylated (Peng *et al.*, 2008; Wu *et al.*, 2007b). It is highly unlikely that SecY2 and Gap3 are directly involved in Fap1 glycosylation. Rather, SecY2 and Gap3 may be indirectly involved. For example, full glycosylation of Fap1 might be coupled to its normal route of export through a SecY2-containing translocase.

Glycosylation factors: Two genes downstream of *secA2*, *gtfA*, and *gtfB* (for *g*lycosyl*t*rans*f*erase) in *S. gordonii* or *gtf1* and *gtf2* in *S. parasanguinis* function in GspB and Fap1 glycosylation, respectively. In *gtf* mutants, glycosylation of a GST-GspB fusion protein or Fap1 protein does not occur (Bu *et al.*, 2008; Takamatsu *et al.*, 2004b; Wu *et al.*, 2007a). Further, glycosylation of these proteins can be achieved when they are co-expressed in *E. coli* with GtfA and GtfB or Gtf1 and Gtf2 (Bu *et al.*, 2008; Takamatsu *et al.*, 2004b). For GspB, glycosylation is shown to influence protein stability and/or solubilization (Bensing *et al.*, 2005; Takamatsu *et al.*, 2004b).

Also found in the accessory *sec* loci of both *S. gordonii* and *S. parasanguinis* are additional genes encoding proteins that share amino acid homology with glycosyltransferases and nucleotide sugar synthetases. Examples in *S. gordonii* are the *gly* and *nss* genes. Disruption of either of these genes does not prevent glycosylation or export of GspB, but it does alter

the lectin binding properties and carbohydrate composition of GspB (Takamatsu *et al.*, 2004a). In *S. parasanguinis*, there are *gly*, *galT1*, *galT2*, and *nss* genes. Of these genes, *galT2*, which does not have a direct homologue in *S. gordonii*, is directly tested. A *S. parasanguinis galT2* mutant exhibits a partial defect in Fap1 glycosylation (Wu *et al.*, 2007b). Thus, it seems these additional glycosylation genes function in defining the carbohydrate composition of the glycan on GspB and Fap1.

Interestingly, glycosylation of GspB and Fap1 seems to occur prior to export. When GspB is not exported in a *secA2* or *secY2* mutant or when the signal sequence of GspB is removed, the protein retained in the cytosol is still glycosylated (Bensing *et al.*, 2004a). Fap1 protein detected in the cytosol also appears to be glycosylated, as shown with antibodies specific for a Fap1 glycan (Chen *et al.*, 2004). In eukaryotes, protein glycosylation occurs only when proteins are exported into the endoplasmic reticulum (Chavan and Lennarz, 2006). In *M. tuberculosis* and *Campylobacter jejuni*, where glycosylation has been studied, glycosylation is similarly linked to export across the cytoplasmic membrane (Nita-Lazar *et al.*, 2005; VanderVen *et al.*, 2005). Glycosylation of GspB and Fap1 in the cytosol and in the absence of export is unusual and suggests that the SecA2 system might be adapted to exporting proteins that are post-translationally modified.

3) Features of SecA2-dependent proteins GspB and Fap1 that target them for export—GspB and Fap1 are comprised of similar multi-domain structures (Figure 3) (Chen *et al.*, 2007;Takamatsu *et al.*, 2004b). Both GspB and Fap1 have an abnormally long amino-terminal signal sequence (90 and 68 amino acids, respectively). The signal sequence is followed by short and long serine rich regions that are the sites of glycosylation. In GspB, the serine rich domains are named SRR1 and SRR2 and in Fap1, they are named RI and RII. In GspB, the SRR domains are separated by a region rich in basic residues (BR). In Fap1, there are two interspersed non-repetitive regions named NRI and NRII. At the C-terminus of both GspB and Fap1 is a cell wall anchoring (CWA) domain containing a LPXTG motif that is required for attachment to the cell wall. The CWA domain is consistent with the cell wall localization of these proteins. Consequently, the GspB and Fap1 proteins additionally seen released into culture media may be a result of cell wall remodelling.

Native GspB and Fap1 are not in the cell wall or released into culture media by a *secA2* mutant; rather, they accumulate in the cytosol (Bensing and Sullam, 2002). This leads to two questions. What features of SecA2-exported proteins target them for export by the SecA2 pathway and what features prevent them from being exported by the canonical Sec pathway? In short, it appears that glycosylation in the mature domain and elements of the signal sequence are both important for determining export by SecA2 and preventing export by the canonical Sec pathway.

The effect of glycosylation: Glycosylation is not required for GspB export by *secA2/secY2*, but it does have a role in blocking GspB export by the canonical Sec pathway. Since full-length non-glycosylated GspB forms aggregates or is unstable, a truncated version of GspB known as $GspB_{736}$-FLAG is used in many of the experiments that address this phenomenon (Figure 3). $GspB_{736}$-FLAG is exported even in a *gtfA* mutant, which fails to glycosylate GspB (Bensing *et al.*, 2005). Further, unglycosylated GspB can be exported by the SecA2 pathway as shown by reduced $GspB_{736}$-FLAG export in a *gtfA secA2* double mutant. However, the level of $GspB_{736}$-FLAG export by *gtfA secA2* double mutant is actually higher than that in the single *secA2* mutant, which is totally deficient. Treatment with azide, a well-established inhibitor of *E. coli* SecA, diminishes the residual export of $GspB_{736}$-FLAG in the double mutant. This argues that the canonical SecA is also able to export GspB in the absence of glycosylation. Because the canonical Sec pathway does not have an obvious role in exporting glycosylated GspB, this implies that glycosylation of GspB blocks recognition

by the canonical pathway. One possibility is that the size or shape of glycosylated GspB is incompatible with the canonical SecYEG translocon. It also suggests that the accessory Sec pathway is adapted to export the modified protein.

With Fap1, a similar finding is that the glycosylated R1 and RII regions of the protein prevent its export by the canonical SecA pathway. Simultaneous deletion of RI and RII, or RII alone, enables Fap1 export in the absence of SecA2 (Chen *et al.*, 2007). This export is sensitive to azide, implying these Fap1 deletion variants are exported by the canonical SecA. Thus, it seems that glycosylated regions of GspB and Fap1 have a similar effect of inhibiting export via the canonical SecA.

The signal sequence: The signal sequences of GspB and Fap1 are atypical. All elements of a standard Sec signal sequence are present, but the positively charged N domain is longer than normal. For GspB, deletions in the signal sequence eliminate export (Bensing *et al.*, 2005; Bensing *et al.*, 2007). In addition, N-terminal sequencing of exported GspB shows the signal sequence is cleaved at a site between residues 90 and 91. Thus, the signal sequence appears functional.

The signal sequence is a good candidate for specifying SecA2-dependent export. GspB variants with alterations in the signal sequence reveal an important role for three glycines in the hydrophobic (H) domain in directing GspB export (Bensing *et al.*, 2007). Substitution of these three glycines (G3) with α-helix promoting amino acids in full-length glycosylated GspB completely blocks export in wild-type *S. gordonii*. This indicates that these glycines are essential to SecA2-dependent export.

When the same (G3) substitution is introduced into a $GspB_{736}$-FLAG protein, a slightly different result is observed. Export of $GspB_{736}$ (G3)-FLAG, which is mostly unglycosylated, is only slightly reduced in a wild-type strain. When expressed in a *secA2* mutant, the level of $GspB_{736}$ (G3)-FLAG is further reduced but some protein is still exported. Results of azide treatment of the *secA2* mutant expressing $GspB_{736}$ (G3)-FLAG suggest the residual export seen is by the canonical Sec pathway (Bensing *et al.*, 2007). The different results obtained with (G3) mutations in native GspB and the $GspB_{736}$-FLAG protein are likely due to the added influence of glycosylation preventing canonical Sec export. Full-length GspB is larger and more glycosylated than the truncated $GspB_{736}$-FLAG (Figure 3). Interestingly, when tested in the absence of glycosylation and absence of SecA2 (in a *gtfA secA2* double mutant) the $GspB_{736}$ (G3)-FLAG is more efficiently exported than the non-mutated $GspB_{736}$-FLAG. These results indicate that, in addition to being required for SecA2-dependent export, the (G3) residues in the GspB signal sequence function with glycosylation of the mature domain of GspB to inhibit export by the canonical SecA.

In light of the above results, it seemed possible that the GspB signal sequence alone might be sufficient to direct a protein to the accessory Sec pathway. On the contrary, the GspB signal sequence is unable to drive export of GspA, a heterologous surface protein with a consensus signal sequence (Bensing *et al.*, 2005). This indicates that, in addition to the signal sequence, specific features of the mature protein are also required for compatibility with the accessory Sec pathway.

The Fap1 signal sequence was also tested for its ability to drive export of a heterologous protein. In this case, the Fap1 signal sequence is able to promote some export of the green fluorescent protein (GFP), although it was not determined if the exported GFP is functional (Chen *et al.*, 2007). However, the export is independent of SecA2. This provides further indication that elements of the mature protein are important for defining SecA2-specificity.

For Fap1 it is suggested that the signal sequence, NRI, and NRII are the minimal elements required for export by the SecA2-pathway (Chen *et al.*, 2007).

4) Other SecA2-dependent proteins—In *S. parasanguinis*, the FimA adhesin is another protein reported to be exported in a SecA2-dependent manner (Chen *et al.*, 2004). There are many differences between Fap1 and FimA. FimA has a classical signal sequence of normal length, it does not share homology with Fap1, and is not likely to be glycosylated. FimA is a predicted lipoprotein with a lipobox motif (Fenno *et al.*, 1995). Unlike Fap1, FimA is still exported, though to a reduced extent, in a *secA2* mutant (Chen *et al.*, 2004). The residual export is presumably through the action of the canonical SecA. Thus, it seems that FimA differs from GspB and Fap1 in not being exclusively dependent on SecA2. Rather, it would appear that FimA is compatible with both the accessory and canonical Sec pathways. However, there has only been minimal study of FimA and additional work is needed to better define the relationship between FimA and SecA2.

SecA2-only systems

Mycobacterium smegmatis and Mycobacterium tuberculosis

M. smegmatis is a fast-growing non-pathogenic mycobacterium that is commonly used as a model to study the general physiology of mycobacteria. *M. tuberculosis* is a slow-growing intracellular pathogen responsible for tuberculosis disease. Mycobacteria are sometimes classified as Gram-positive bacteria; however, mycobacteria are distinguished by acid-fast staining, a property imparted by the highly impermeable mycobacterial cell wall (Goren *et al.*, 1978). The SecA2-exported proteins identified in *M. smegmatis* and *M. tuberculosis* include examples with and examples without signal sequences. While nothing is yet known about factors that work with mycobacterial SecA2 to recognize or translocate substrates, it is clear that the SecA2 system of *M. tuberculosis* is important to virulence and protective immunity in the host.

1) Discovery of the accessory SecA—The accessory *sec* locus of mycobacteria was discovered during evaluation of the *M. tuberculosis* genome sequence (Braunstein *et al.*, 2001). The presence of two *secA*s appears to be a property shared by all *Mycobacterium sp.* including non-pathogenic *M. smegmatis.*

In mycobacteria, there is substantial evidence that SecA1 is the ‘housekeeping’ SecA that exports the majority of proteins. Like *E. coli* SecA, mycobacterial SecA1 is essential, as shown by the inability to delete the *M. smegmatis secA1* gene unless *secA1* is expressed from a plasmid (Braunstein *et al.*, 2001). Furthermore, SecA1 depletion in *M. smegmatis* eliminates export of the Sec signal sequence-containing *M. smegmatis* porin (MspA) and is associated with loss of viability (Guo *et al.*, 2007). *secA1* also appears to be essential in *M. tuberculosis* (Sassetti *et al.*, 2003). In contrast, the *secA2* gene is not essential and *secA2* mutants have been constructed in *M. smegmatis*, *M. tuberculosis*, and *M. bovis* BCG (Braunstein *et al.*, 2001; Braunstein *et al.*, 2003; Braunstein, unpublished). As described below, mycobacterial SecA2 functions in the export of a subset of proteins.

Although both mycobacterial SecAs function in protein export, the two SecAs are not functionally redundant or interchangeable. SecA2 cannot fulfil the essential role of SecA1 since, even when *secA2* is overexpressed, the chromosomal copy of *secA1* cannot be deleted (Braunstein *et al.*, 2001). The converse is also true; SecA1 cannot fulfil the role of SecA2, since overexpression of *secA1* does not rescue *in vitro* phenotypes of the *M. smegmatis secA2* mutant. (Braunstein *et al.*, 2001; Rigel and Braunstein, unpublished).

2) SecA2-exported proteins of *M. smegmatis*: Msmeg1704 and Msmeg1712 lipoproteins—By comparing cell envelope fractions of *M. smegmatis* using 2D-PAGE, two *M. smegmatis* SecA2 dependent proteins were identified as Msmeg1704 and Msmeg1712 (YtfQ) (Gibbons *et al.*, 2007). In the absence of SecA2, these proteins are not exported to the cell wall and they accumulate in cytosolic and membrane fractions as slightly larger species that are presumed to be unprocessed precursors. Of note is that the export defect in the *secA2* mutant is not complete (Gibbons *et al.*, 2007).

Msmeg1704 and Msmeg1712 are encoded by genes in an apparent operon. Both proteins are homologous to periplasmic sugar binding proteins and possess amino-terminal signal sequences with a lipobox motif. Both proteins appear to be genuine lipoproteins, as shown by Triton X-114 extraction, a fractionation method that enriches for lipoproteins, and sensitivity to globomycin, an inhibitor of the lipoprotein signal peptidase LspA (Gibbons *et al.*, 2007). The role of SecA2 in the export of these two lipoproteins is specific; other mycobacterial lipoproteins are not affected. There are no direct homologues of Msmeg1704 and Msmeg1712 in *M. tuberculosis*. However, when expressed in *M. tuberculosis*, Msmeg1704 is also influenced by SecA2. This indicates some degree of conservation of the accessory Sec systems of these mycobacteria.

It is interesting that both of these SecA2-dependent proteins are lipoproteins. Because glycosylation plays a role in defining SecA2 substrates of other systems, specific post-translational lipid modification of Msmeg1704 and Msmeg1712 might make them dependent on SecA2 for export and/or block effective export by the canonical SecA1.

3) SecA2-exported proteins of *M. tuberculosis*: SodA—A comparative proteomic analysis of *M. tuberculosis* secreted proteins also identified SecA2-dependent proteins (Braunstein *et al.*, 2003). Only a small number of secreted proteins exhibit differences between wild-type and *secA2* mutant *M. tuberculosis* on 2D-PAGE. Three protein spots were less abundant in culture filtrates of a *M. tuberculosis secA2* mutant: SodA (Iron superoxide dismutase), Acr (Alpha-crystallin, HspX), and Rv0390 (protein of unknown function). All of these proteins lack signal sequences.

Of these proteins, only SodA was studied further. In the absence of SecA2, the amount of SodA protein and superoxide dismutase activity secreted into culture media by *M. tuberculosis* is reduced, as measured by immunoblot analysis and enzymatic assays (Braunstein *et al.*, 2003; Hinchey *et al.*, 2007). Furthermore, in the *secA2* mutant SodA is observed to accumulate in the cell pellet. Secreted antioxidants like SodA could protect intracellular *M. tuberculosis* from the oxidative burst of macrophages and be important to virulence. Catalase-peroxidase (KatG) is another antioxidant detected in culture filtrates of *M. tuberculosis*. Immunoblot analysis with anti-KatG antibodies show its secretion is also dependent on SecA2 (Braunstein *et al.*, 2003). Like SodA, KatG lacks a signal sequence.

These studies in *M. smegmatis* and *M. tuberculosis* show that SecA2 functions in the export of a subset of proteins. It remains to be clarified how proteins with and proteins without signal sequences can both be exported in a SecA2-dependent manner by mycobacteria. There might be dual roles for SecA2: one in the export of signal sequence-containing proteins and another in the export of substrates lacking signal sequences. Alternatively, the role of SecA2 in exporting proteins like SodA might be indirect. There may be unidentified signal sequence-containing proteins exported by SecA2, that are themselves components of a specialized export machinery, responsible for secreting unconventional proteins such as SodA. This latter possibility is worth serious consideration. There are examples of exported lipoproteins synthesized with signal sequences (*i.e.* YscJ) that function as components of

specialized Sec-independent Type III secretion systems of didermic bacterial pathogens (Silva-Herzog *et al.*, 2008).

4) Role of SecA2 in virulence and immunity—Evaluation of the *M. tuberculosis secA2* mutant in models of tuberculosis clearly demonstrates a role for SecA2 in virulence. In mice, the *secA2* mutant exhibits a growth defect early in infection and mice infected with the mutant survive longer than wild-type infected animals (Braunstein *et al.*, 2003; Kurtz *et al.*, 2006). The *secA2* mutant is also defective in its ability to grow in cultured macrophages (Kurtz *et al.*, 2006). Because the *secA2* mutant is defective in the export of the antioxidants SodA and KatG, the role of SecA2 in macrophages could be to protect against the oxidative burst. However, there must be additional roles for SecA2-dependent export in *M. tuberculosis* because the *secA2* mutant is still defective for growth in macrophages that do not generate an oxidative burst. Macrophages infected with the *secA2* mutant can exhibit more apoptosis than macrophages infected with wild-type *M. tuberculosis*, a phenotype that appears to reflect the SodA defect of the *secA2* mutant (Hinchey *et al.*, 2007). However, this effect on macrophage apoptosis does not account for the mutant phenotype in macrophages (Kurtz, McCann, and Braunstein unpublished). Macrophages infected with the *secA2* mutant also release more proinflammatory cytokines, so another role for SecA2-dependent export might be to limit the host cell response (Kurtz *et al.*, 2006).

Finally, the *secA2* mutant of *M. tuberculosis* elicits better protective immunity in mice and guinea pigs to *M. tuberculosis* challenge than the current live attenuated *M. bovis* BCG vaccine (Hinchey *et al.*, 2007). The increased protection is attributed to generation of an enhanced $CD8^+$ T cell response in animals infected with the *secA2* mutant. This could reflect the increased apoptosis phenotype mentioned above. These results reveal a role for *M. tuberculosis* SecA2 in evading protective immunity.

Listeria monocytogenes

L. monocytogenes is a Gram-positive intracellular pathogen responsible for the food-borne illness listeriosis. A larger collection of proteins is reported as exported by SecA2 of *L. monocytogenes* than in the above cases. This list includes examples of proteins with and without signal sequences. Like *M. tuberculosis*, SecA2 of *L. monocytogenes* is important to virulence and protective immunity.

1) Discovery of the accessory SecA—The accessory *sec* locus of *L. monocytogenes* was discovered during the study of mutants with rough colony morphologies (Lenz and Portnoy, 2002). In one such mutant, a transposon is inserted in *secA2* while other rough mutants have point mutations in *secA2*. Complementation experiments demonstrate that mutations in *secA2* cause the rough colony phenotype (Lenz and Portnoy, 2002; Machata *et al.*, 2005).

2) SecA2-dependent proteins of *L. monocytogenes*—Comparison of surface and secreted proteins of wild-type and *secA2* mutant *L. monocytogenes* by 1D gel analysis reveals that 8 out of 25 secreted proteins and 19 of 49 surface extractable proteins are either completely absent or significantly reduced in the fractions prepared from the *secA2* mutant (Lenz and Portnoy, 2002; Lenz *et al.*, 2003). Of these proteins, 17 have been identified (Lenz *et al.*, 2003). An additional SecA2-dependent secreted protein was identified in a separate study (Archambaud *et al.*, 2006). As is the case with mycobacteria, some of the SecA2-dependent proteins have signal sequences and some do not.

Proteins with signal sequences: The list of SecA2-dependent proteins with signal sequences includes the p60 autolysin, two predicted lipoproteins, two predicted transporters,

and a penicillin binding protein (Lenz *et al.*, 2003). Only p60 was studied further. The p60 protein (also known as CwhA and Iap) is a major secreted protein of *L. monocytogenes*. It is a cell wall hydrolase with bacteriolytic activity that can cleave peptidoglycan (Lenz *et al.*, 2003; Machata *et al.*, 2005; Wuenscher *et al.*, 1993). p60 is abundant in cell wall and secreted protein fractions of wild-type *L. monocytogenes* (Lenz and Portnoy, 2002; Machata *et al.*, 2005). Mutants lacking *secA2* produce p60 but exhibit a 50% reduction in its secretion (Lenz *et al.*, 2003; Machata *et al.*, 2005). Thus, p60 is a protein that can be exported in a SecA2-dependent or SecA2-independent manner. In the *secA2* mutant, p60 accumulates in membrane and cytosolic fractions (Machata *et al.*, 2005). The gene encoding p60 is located upstream of *secA2* in a divergent transcriptional unit.

Proteins without signal sequences: Like p60, MurA/NamA (Muramidase) is a cell wall hydrolase able to cleave peptidoglycan (Carroll *et al.*, 2003). Based on our sequence analysis, MurA does not possess a canonical signal sequence. In a *secA2* mutant MurA is not detected in surface-extractable or cell wall fractions (Lenz *et al.*, 2003; Machata *et al.*, 2005). It is striking that similar peptidoglycan hydrolases are SecA2-dependent. However, in a *secA2* mutant MurA is not only absent from the cell wall but is also absent in all subcellular fractions (Machata *et al.*, 2005). While this could reflect intracellular degradation of MurA when it is not being exported, other possibilities such as altered expression in a *secA2* mutant are not yet ruled out.

Like SodA of *M. tuberculosis*, the manganese superoxide dismutase (MnSOD) of *L. monocytogenes* lacks a signal sequence and depends on SecA2 for export (Archambaud *et al.*, 2006). Proteins like superoxide dismutase that lack signal sequences and have well-described cytosolic functions are not commonly thought of as being exported, but there are examples of such unconventional exported proteins in bacteria (Pancholi and Fischetti, 1992, 1998). Ten additional SecA2-dependent surface proteins lacking signal sequences and with predicted cytosolic functions are identified by 1D gel analysis and peptide mass fingerprinting of *L. monocytogenes* (Lenz *et al.*, 2003).

For these examples, it is important to recognize that additional investigation is needed to determine if SecA2 is involved directly or indirectly in the export of these proteins or if the effect of SecA2 on these proteins is unrelated to export. As a starting point, experiments to demonstrate intracellular accumulation of unexported protein in a *secA2* mutant should be conducted.

3) Role of SecA2 in virulence and in eliciting an immune response—SecA2 of *L. monocytogenes* clearly plays a role in virulence. The LD_{50} of a *secA2* mutant is higher than that of the wild-type strain (Lenz and Portnoy, 2002) and the *secA2* mutant is cleared from mice faster than wild-type bacteria (Lenz *et al.*, 2003; Muraille *et al.*, 2007).

Secretion of p60 might be one way SecA2 contributes to virulence, since a *p60* mutant is also attenuated in mice (Lenz *et al.*, 2003). The *secA2* mutant is defective in cell to cell spread (Lenz *et al.*, 2003) and, in at least one study, p60 appears to promote cell-cell spread (Pilgrim *et al.*, 2003). Another possible explanation is that the muramyl peptides released by the p60 hydrolase limit protective host immune responses and thereby promote virulence (Lenz 2003). Because the attenuated phenotype of a *p60* mutant is not as dramatic as that of a *secA2* mutant, additional proteins must be involved. MurA and MnSOD are other SecA2-dependent proteins that could act in virulence (Lenz *et al.*, 2003; Muraille *et al.*, 2007).

On the other end of the host pathogen spectrum, SecA2 promotes production of protective immunity to *L. monocytogenes* challenge. Unlike wild-type *L. monocytogenes*, the *secA2* mutant fails to produce protective $CD8^+$ T cells (Muraille *et al.*, 2007). It is striking that

opposite effects of SecA2 on the establishment of protective immunity are seen with *L. monocytogenes* and *M. tuberculosis*. In *M. tuberculosis*, SecA2 limits priming of antigen specific $CD8^+$ T cells and protection *in vivo*, while in *L. monocytogenes* SecA2 promotes these effects.

Conclusion and future investigations

We now know of bacteria that possess accessory Sec systems dedicated to exporting a subset of proteins. In pathogens, the accessory Sec systems are linked to virulence. However, our understanding of SecA2-mediated export and how it compares among different bacteria is incomplete and many basic questions need to be answered.

The biochemical activities of SecA2 await investigation. SecA2 might function in a similar manner to the canonical SecA by delivering precursor proteins to a translocase and energizing export across the membrane. At least in mycobacteria, the two SecA proteins are not interchangeable (Braunstein *et al.*, 2001). Furthermore, subcellular fractionation and immunoblot analysis shows that SecA/SecA1 partitions equally between cell envelope and cytosolic fractions, while SecA2 is found predominantly in cell envelope fractions in *S. parasanguinis* (Chen *et al.*, 2006). These data reveal differences between SecA proteins and it emphasizes the need of direct study of SecA2 functions.

SecA2-interacting proteins need to be identified. This will greatly help understand the process of SecA2-mediated export. In two basic models, SecA2 either works with a novel translocase composed of dedicated accessory components or it works with the canonical SecA1/SecYEG translocase (Figure 4). While it is tempting to assume that the SecA2/SecY2 system works with a novel translocon and SecA2-only systems work with the canonical Sec translocase, these basic assumptions need to be experimentally addressed. Regarding the latter model, the role of SecA2 could be to recognize substrates that are normally overlooked for export and deliver them to the general SecYEG translocase. *E. coli* and *B. subtilis* SecA are known to form dimers (Ding *et al.*, 2003;Jilaveanu *et al.*, 2005), although whether or not the dimer is the translocation-active form of the protein remains controversial (de Keyzer *et al.*, 2005;Jilaveanu *et al.*, 2005;Or *et al.*, 2005). One interesting possibility is that SecA2 forms heterodimers with SecA1 and/or interacts with other translocase components to promote export.

The studies of GspB and Fap1 provide a framework for understanding features that make a protein dependent on SecA2 for export. Two types of defining elements exist: signals that direct proteins to SecA2 and signals that block export by SecA/SecA1. In addition, there are some SecA2-dependent proteins that are still exported, albeit at a reduced level, in the absence of SecA2. We propose that exported proteins span a continuum from those with features that make them completely independent of SecA2 and exclusively reliant on SecA1 to those completely dependent on SecA2 and unaffected by SecA1. Future experiments are needed to understand how SecA1 and SecA2 substrates differ. Finally, for the SecA2-only systems it will be important to determine if the role of SecA2 in exporting proteins without signal sequences is direct or indirect.

Now that the existence and biological significance of accessory Sec pathways is well established, we anticipate that future studies will reveal the details of the export process itself. Given the sophisticated level of understanding of the general Sec pathway, we are optimistic rapid progress can be made in characterizing similarities and differences in the mechanism of accessory Sec export.

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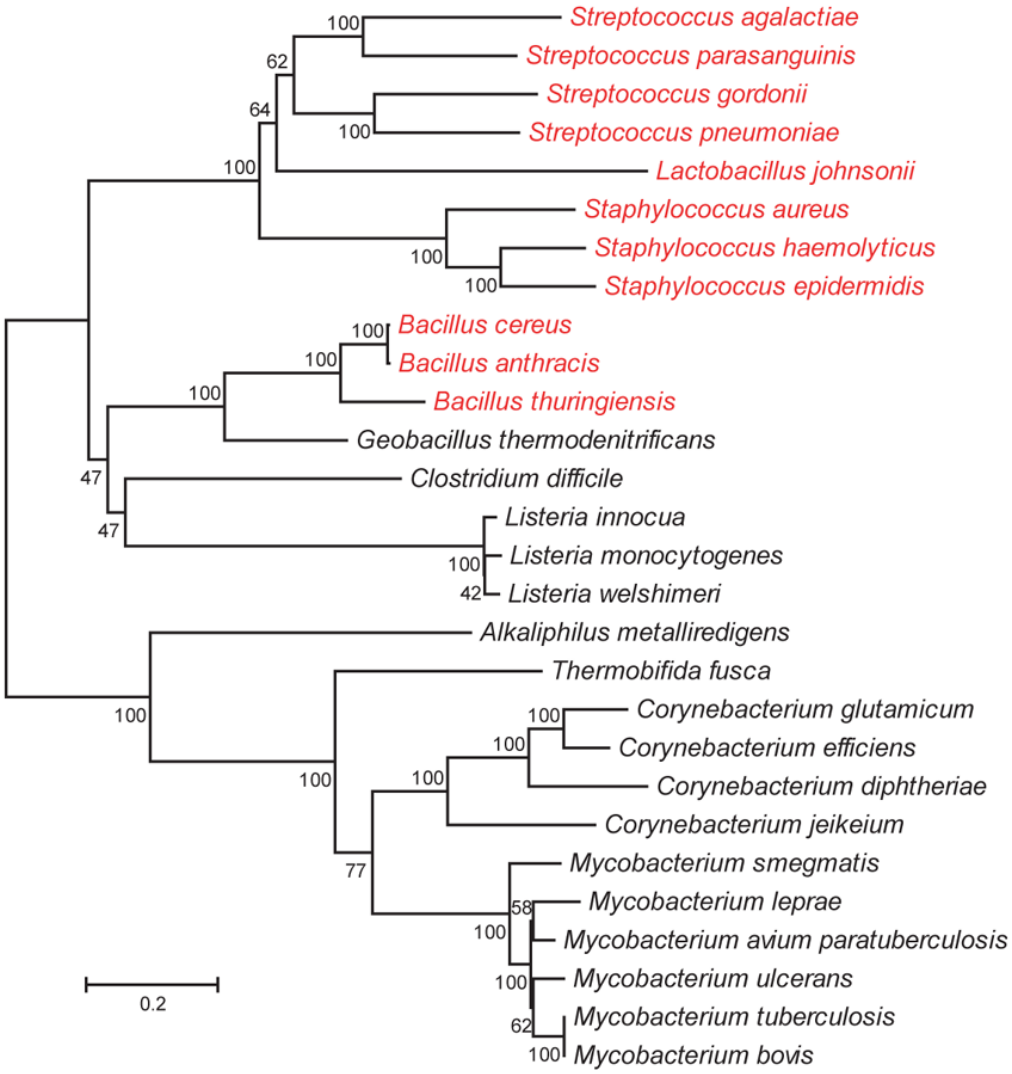

Fig 1. Evolutionary relationships of SecA2 proteins

The phylogenetic tree was generated in MEGA4 using the Neighbor-Joining method. The length of the branches reflects the number of amino acid changes between different SecA2s, as indicated by the bar. Bootstrap values are shown at the junctions. SecA2 sequences were obtained by searching all bacterial genome and protein databases available from NCBI and TIGR (as of April 2008) for the term SecA. For organisms with two SecA sequences, the one least like *B. subtilis* SecA was defined as SecA2 and used to build the tree. For simplicity, only one species of *Lactobacillus* with a SecA2 is included on the tree. Organisms in red possess two SecY homologs.

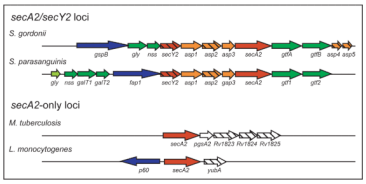

Fig 2. Organization of accessory *sec* loci

Similar colouring indicates encoded proteins with homology or similar properties. Genes encoding SecA2-dependent substrates are in blue, glycosyltransferases in green, accessory Sec proteins in red, accessory SecA2-dependent secretion factors in orange. Any gene that does not fit in the above categories is white. Genes encoding proteins with predicted transmembrane domains are hatched.

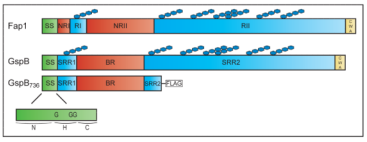

Fig. 3. Domain organization of SecA2-dependent serine rich glycoproteins

Domains in full-length Fap1 and GspB are shown, with similar domains colour coded. Truncated $GspB_{736}$-FLAG is also depicted, and the GspB signal sequence is shown, including the location of critical glycine (G3) residues.

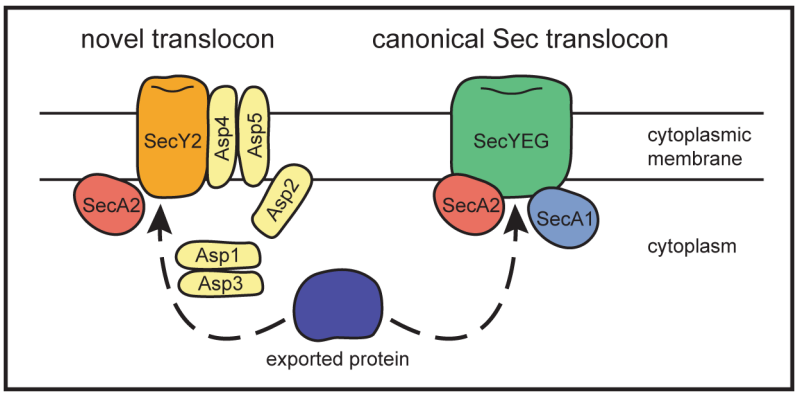

Fig. 4. Models for SecA2-dependent export

In the various accessory Sec systems, a SecA2-exported protein (shown in blue) might be exported either through a novel translocon or the canonical SecA1/SecYEG translocon with the assistance of SecA2. The example of a novel translocon is modelled on the SecA2/SecY2 system of *S. gordonii*, which is a candidate for this type of pathway.

Table 1

Percent amino acid similarity to *B. subtilis* SecA

	SecA/SecA1	SecA2
B. subtilis	100	N/A
S. gordonii	74	59
S. parasanguinis	73	60
M. tuberculosis	63	52
L. monocytogenes	81	65