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Protein export systems of *Mycobacterium tuberculosis*: novel targets for drug development?

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Abstract

Protein export is essential in all bacteria and many bacterial pathogens depend on specialized protein export systems for virulence. In *Mycobacterium tuberculosis*, the etiological agent of the disease tuberculosis, the conserved general secretion (Sec) and twin-arginine translocation (Tat) pathways perform the bulk of protein export and are both essential. *M. tuberculosis* also has specialized export pathways that transport specific subsets of proteins. One such pathway is the accessory SecA2 system, which is important for *M. tuberculosis* virulence. There are also specialized ESX export systems that function in virulence (ESX-1) or essential physiologic processes (ESX-3). The increasing prevalence of drug-resistant *M. tuberculosis* strains makes the development of novel drugs for tuberculosis an urgent priority. In this article, we discuss our current understanding of the protein export systems of *M. tuberculosis* and consider the potential of these pathways to be novel targets for tuberculosis drugs.

Keywords

drug target; ESX; mycobacteria; protein export; Sec; SecA2; secretion; Tat; tuberculosis

Mycobacterium tuberculosis, the causative agent of tuberculosis, is a serious global health problem accounting for nearly two million deaths per year [1]. With the increasing prevalence of multidrug resistant and extensively drug resistant *M. tuberculosis* there is an urgent need to develop new antimycobacterial drugs. *M. tuberculosis* is spread through aerosols and inhaled bacilli are engulfed by alveolar macrophages within the lungs. Instead of being killed, *M. tuberculosis* survives within the phagosome compartment of the macrophage, blocks phagosome maturation and replicates intracellularly [2]. The ability of *M. tuberculosis* to survive and replicate within macrophages is essential for *M. tuberculosis* pathogenesis. Another significant feature of *M. tuberculosis* is that it can persist long-term in host granulomas and later reactivate to cause disease.

Mycobacterium tuberculosis poses unique challenges to drug development. One challenge is for drugs to reach intracellular *M. tuberculosis* bacilli, which can be within granulomas. An

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additional challenge is presented by the unique structure of the mycobacterial cell envelope (Figure 1) [3]. Mycobacteria have an atypical cell envelope characterized by a cell wall core comprised of peptidoglycan that is covalently linked to arabinogalactan, which is in turn attached to long chain mycolic acids. The mycolic acids, along with intercalating free lipids, form a mycobacterial outer membrane, which was recently visualized for the first time by cryoelectron microscopy [4,5]. Additionally, the mycobacterial outer membrane is surrounded by a thick capsule composed of glycans, glycolipids and proteins [6]. The complex cell envelope of mycobacteria represents a significant barrier to drug delivery.

All bacteria, including mycobacteria, possess protein export systems to transport proteins synthesized in the cytoplasm beyond the cytoplasmic membrane. Exported proteins may remain in the cell envelope or be further secreted beyond the bacterial cell wall. Many exported proteins have essential functions that require an extracytoplasmic location. Consequently, the protein export pathways themselves are commonly essential physiologic processes. In addition to essential protein export pathways, bacterial pathogens commonly have specialized protein export systems that are important for pathogenesis. Such pathways are important to virulence because of their role in exporting effectors that interact with the host.

Mycobacteria possess a functional general secretion (Sec) pathway and a twin-arginine translocation (Tat) pathway for protein export. These conserved protein export pathways are essential in *M. tuberculosis*. Mycobacteria also have specialized protein export systems, dedicated to exporting subsets of proteins. The accessory SecA2 export system is characterized by an additional copy of the SecA cytoplasmic ATPase and SecA2 is required for *M. tuberculosis* virulence. Another set of specialized protein export systems in *M. tuberculosis* are the five ESX pathways. The *M. tuberculosis* ESX pathways are named ESX-1 to ESX-5. ESX-1 is important for virulence and ESX-3 is involved in iron acquisition, an essential function for *in vitro* and *in vivo* growth.

In this article, we first summarize our understanding of Sec and Tat protein export from studies in other bacteria, then review what is currently known about these conserved systems in mycobacteria. We next discuss research on the specialized SecA2 and ESX export systems in mycobacteria. Because all of the known export systems of *M. tuberculosis* are either essential or important to virulence, we propose they be considered as new drug targets. We end the article with discussion of recent progress in targeting protein export in other bacteria and speculation on how these strategies could be extended to *M. tuberculosis* in order to develop novel anti-mycobacterial therapies.

The general Sec secretion pathway

Over 20% of bacterial proteins have functions outside the cytoplasm and are exported to their proper locations by protein export systems [7]. Although best studied in *Escherichia coli*, all bacteria have a general (Sec) pathway, which performs the bulk of protein export (for extensive reviews of Sec export, see references [8,9]). Sec export is an essential cellular process, which makes the pathway a potential drug target.

Sec export is a post-translational process dedicated to exporting unfolded proteins. Central to the Sec pathway is a heterotrimeric protein complex composed of the SecY, SecE and SecG proteins [10]. The SecYEG complex forms a channel in the cytoplasmic membrane through which proteins synthesized in the cytoplasm are transported to the extracytoplasmic environment. Peripherally associated with the SecYEG channel is the cytoplasmic ATPase, SecA, which together with SecYEG comprises the translocase. SecA targets proteins to the SecYEG channel and drives these proteins through the channel with repeated rounds of ATP-binding and hydrolysis [11]. While SecY, SecE and SecA are essential for protein

export, SecG is expendable but increases the efficiency of export. Other auxiliary Sec proteins that contribute to the efficiency of protein export, but are not essential for this process, are SecD, SecF and YajC.

Proteins destined for Sec export are distinguished from the large pool of cytoplasmic proteins by the presence of an N-terminal signal peptide and are called preproteins. Among the proteins exported by the Sec system are lipoproteins, which contain a lipobox motif in the C-terminal region of the signal peptide. The lipobox includes an invariant cysteine that is the site of lipid modification [12]. During or shortly after translocation through the SecYEG channel, the signal peptide is removed. This cleavage event takes place on the periplasmic side of the membrane by one of two possible peptidases: the Type I signal peptidase (LepB) or the lipoprotein Type II signal peptidase (LspA) [13]. After signal peptide cleavage, the protein folds into a mature conformation.

The Sec pathway also participates in inserting integral membrane proteins into the cytoplasmic membrane [14]. Integral membrane proteins are translocated cotranslationally with the help of the signal recognition particle (SRP) and the membrane-bound SRP receptor, FtsY. FtsY transfers nascent preproteins from SRP to the Sec translocase for export. A lateral gate within the SecYEG channel allows the transmembrane domains to embed in the membrane with assistance from the YidC protein [15].

The Sec pathway in mycobacteria

Mycobacteria have homologs of all the Sec export factors reviewed above (Figure 2A). However, detailed studies have focused on only a few components of the mycobacterial Sec pathway. The *M. tuberculosis* LspA removes signal peptides from lipoproteins [16]. An *lspA* mutant of *M. tuberculosis* is attenuated in both macrophages and mice, illustrating the importance of functional lipoproteins to *M. tuberculosis* virulence [16,17]. The other components of the mycobacterial Sec pathway to receive attention are the SecA proteins. Mycobacteria are unusual in having two homologs of SecA: SecA1 and SecA2. SecA1 is the ‘housekeeping’ SecA protein of mycobacteria, responsible for exporting the majority of proteins. SecA2 is an accessory SecA, which is discussed in detail below. As is the case for housekeeping SecA proteins of other bacteria, SecA1 of mycobacteria is essential. The *secA1* gene cannot be deleted from *M. tuberculosis* or the nonpathogenic *Mycobacterium smegmatis* unless an exogenous copy of *secA1* is provided [18–20]. The contribution of SecA1 to protein export in mycobacteria can be examined by conditional silencing of *secA1* in *M. smegmatis* [19]. Under the control of a tetracycline repressor, SecA1 depletion leads to growth inhibition and decreased Sec export as evidenced by reduced export of the cell wall porin, MspA [19,21].

Accessory SecA2 systems

Mycobacteria and some Gram-positive bacteria are unusual in having a second SecA in addition to the canonical SecA1 [18,22]. This accessory SecA, called SecA2, is nonessential in most bacteria with the exception of *Corynebacterium glutamicum* [23]. SecA2 systems can be found in both pathogenic and nonpathogenic bacteria where they export specific subsets of proteins (for an extensive review of accessory SecA2 systems, see [22]). In many cases, SecA2 systems contribute to the virulence of bacterial pathogens [24–28].

Accessory SecA2 systems can be divided into two groups: those that include an accessory SecY2 and those that do not. The emerging theme is that the SecA2/SecY2 systems export large glycosylated proteins that are encoded near the *secA2* and *secY2* genes [24–26,28]. These exported proteins possess cleavable N-terminal signal peptides and are glycosylated prior to export. Experiments in *Streptococcus gordonii* and *Streptococcus parasanguinis*

with their respective SecA2/SecY2-exported proteins show that glycosylation of the mature domains of these proteins prevents export via the canonical SecA1/YEG translocase [29,30]. Thus, it seems the SecA2/SecY2 systems are uniquely capable of accommodating export of post-translationally modified proteins. In addition to the mature domain, features of the signal peptide can be important for specifying SecA2/SecY2 substrates [31].

Mycobacterium tuberculosis and *Listeria monocytogenes* do not contain SecY2 proteins and are examples of bacteria with SecA2-only systems. The exported proteins of SecA2-only systems include examples of proteins with and without N-terminal signal peptides [27,32–35]. The SecA2-only system of mycobacteria is discussed below.

The accessory SecA2 system of mycobacteria

While SecA1 of mycobacteria is essential and the corresponding gene cannot be deleted, the gene encoding SecA2 can be deleted and *secA2* mutants exist in *M. smegmatis*, *M. bovis* Bacille Calmette-Guérin (BCG) and *M. tuberculosis* [18,32,36]. Importantly, a *secA2* mutant of *M. tuberculosis* is attenuated for growth in macrophages and in a mouse model of tuberculosis infection [32,37]. These attenuated phenotypes show that the SecA2 export pathway is important for *M. tuberculosis* virulence. The SecA2 proteins of *M. smegmatis* and *M. tuberculosis* can each function in the opposite species, as shown by interspecies complementation experiments [21]. Because of this functional conservation between SecA2 systems, the nonpathogenic *M. smegmatis* is often used to study the mechanisms of SecA2 export in mycobacteria.

As is true for SecA1 and the canonical *E. coli* SecA, SecA2 is an ATPase and ATP-binding is necessary for SecA2 function in *M. smegmatis* and *M. tuberculosis* [21,38]. However, there are also differences between the two SecA proteins. Importantly, SecA1 and SecA2 are not functionally redundant. SecA2 overexpression cannot replace the requirement for SecA1 in mycobacteria and SecA1 overexpression cannot complement *secA2* mutant phenotypes [18]. Another difference between the two SecAs is revealed by subcellular fractionation experiments. SecA1 is evenly distributed between the cytosolic and membrane fractions, as is the case for the canonical SecA of *E. coli* [21]. The subcellular distribution of SecA2 is notably different, with SecA2 being primarily cytosolic. This observation holds true for both *M. smegmatis* and *M. tuberculosis* and it supports the existence of distinct functions for each SecA protein [21]. Interestingly, an amino acid substitution in the ATP-binding site of SecA2 (K129R) results in a dominant negative SecA2 that is defective in protein export and localizes to the membrane instead of the cytosol [21]. The altered location of SecA2 (K129R) is consistent with a normally transient interaction between SecA2 and a membrane-embedded translocase that is disrupted when SecA2 cannot hydrolyze ATP.

Two substrates exported by SecA2 in *M. smegmatis* are the cell wall lipoproteins Msmeg1704 and Msmeg1712. These proteins were identified by comparing cell wall proteins from wild-type *M. smegmatis* and the *secA2* mutant using 2D-PAGE analysis [34]. Both of these SecA2 substrates are putative sugar-binding proteins of predicted ATP-binding cassette (ABC) transporters and both proteins contain N-terminal Sec signal peptides with lipobox motifs. In the absence of SecA2, these two lipoproteins are not exported but other *M. smegmatis* lipoproteins are exported normally [34]. *M. tuberculosis* does not have homologs of Msmeg1704 or Msmeg1712, but does contain other predicted sugar-binding lipoproteins, which could be important for virulence. It is unknown if SecA2 is required for export of these *M. tuberculosis* proteins.

Because mycobacteria do not have an accessory SecY2 or obvious alternative translocase, a fundamental question about the mycobacterial SecA2 system is whether it works with the canonical SecA1/SecYEG translocase or an unidentified channel to export its select subset

of proteins. To test these possibilities, the effect of SecA1 depletion on Msmeg1712 export was tested. When SecA1 is depleted, export of Msmeg1712 is significantly reduced and this export defect is equivalent to that seen in a *secA2* mutant [21]. This result reveals a role for both SecA1 and SecA2 in exporting Msmeg1712 and it suggests that SecA2 works in concert with the canonical Sec machinery.

The aforementioned studies support a developing model for SecA2 export in mycobacteria (Figure 2B). SecA1 shuttles evenly between the cytosol and the SecYEG membrane channel to continually deliver and translocate exported proteins. In contrast, SecA2 is primarily cytoplasmic where it may function to recognize or deliver proteins that are normally overlooked by SecA1 to the canonical SecA1/SecYEG translocase for export. Another possibility is that SecA2 is required to energize a specific subset of proteins across the canonical translocase. Alternatively, SecA1 and SecA2 may work together to promote export of proteins through a novel translocase in the membrane other than SecYEG.

In a comparative 2D-PAGE analysis of wild-type *M. tuberculosis* and the *secA2* mutant, SecA2-dependent proteins secreted into the culture media were identified [32]. As observed in the cell wall analysis in *M. smegmatis*, only a small number of proteins were identified as secreted less into culture media by the *secA2* mutant. In this case, all three proteins identified lack recognizable signal peptides. One of these SecA2-dependent *M. tuberculosis* exported proteins is the Fe-superoxide dismutase, SodA [32]. Experiments confirm that secretion of SodA protein and superoxide dismutase activity are both reduced in the *secA2* mutant [32,39]. Interestingly, one of the SecA2-secreted proteins of *L. monocytogenes* is a Mn-superoxide dismutase, MnSOD, which also lacks a signal peptide [35]. This suggests a common mechanism of SecA2 export between pathogenic *M. tuberculosis* and *L. monocytogenes*.

It remains to be reconciled how SecA2 is involved in exporting proteins that lack signal peptides in *M. tuberculosis* and proteins with signal peptides in *M. smegmatis*. One possibility is that the proteins exported by SecA2 are not recognized on the basis of the signal peptide and both signal peptide-containing and signal peptide-lacking proteins can be recognized and exported by SecA2. An alternative is that the role of SecA2 in the secretion of proteins lacking signal peptides is indirect. SecA2 may export a currently unknown protein containing a signal peptide. This unknown protein could itself be part of a specialized secretion apparatus through which proteins such as SodA are secreted.

As previously mentioned, analysis of the *secA2* mutant of *M. tuberculosis* shows that the SecA2 system is important to virulence. Because SodA is an antioxidant, the identification of SodA as a SecA2-dependent protein suggests that the role of the SecA2 system might be to protect *M. tuberculosis* against reactive oxygen intermediates produced by macrophages. However, the *M. tuberculosis secA2* mutant is attenuated for growth in macrophages even if they are derived from *phox*$^{-/-}$ mice, which are unable to elicit an oxidative burst [37]. While these results with *phox*$^{-/-}$ macrophages do not rule out a role for the SecA2 system in resisting oxidative stress, it does reveal another role for SecA2 export beyond detoxification of reactive oxygen intermediates. This result also implies that proteins other than SodA are exported by the SecA2 system of *M. tuberculosis*. Another possible role for SecA2 in *M. tuberculosis* is inhibiting the innate immune response. This possibility is supported by the observation that macrophages infected with the *M. tuberculosis secA2* mutant produce higher levels of proinflammatory cytokines and exhibit more apoptosis than wild-type infected macrophages [37,39]. The *secA2* mutant of *M. tuberculosis* also elicits better protective immunity to *M. tuberculosis* challenge in mice and guinea pigs than vaccination with the *M. bovis* BCG vaccine [39].

In the future, it will be important to identify all the *M. tuberculosis* proteins exported by the SecA2 system. This information will help us better understand the process of SecA2 export and the role of SecA2 in virulence and protective immunity.

The Tat export pathway

Like the general Sec pathway, the Tat pathway exports proteins with N-terminal signal peptides beyond the cytoplasmic membrane (for an extensive review of Tat export, see [8]). The Tat pathway is found in many Gram-positive and Gram-negative bacteria but, unlike the Sec pathway, is not present in all bacteria [40]. Also, the Tat system differs from the Sec system because it only exports proteins that are prefolded in the cytoplasm.

Preproteins destined for Tat export have N-terminal signal peptides similar to Sec signal peptides [41]. However, a distinguishing feature of Tat signal peptides is the presence of a pair of arginine residues (RR) that are contained within the Tat motif, RRXΦΦ, where Φ is a hydrophobic residue. The RR pair is nearly invariant and replacement of both arginines with lysine residues abolishes Tat-dependent export [42].

The Tat export machinery consists of two core components: the TatA and TatC integral membrane proteins. A third protein, TatB, is similar to TatA in amino acid sequence but not all bacteria have a TatB ortholog. The mechanisms of Tat export are less understood than those of Sec export, but there is a growing understanding of the process (Figure 2C) [41]. The current model is that a Tat signal peptide targets a folded preprotein to the TatBC complex in the cytoplasmic membrane. With energy supplied by proton motive force, TatA is then recruited to the TatBC complex and forms a homo-oligomeric translocase channel. There is evidence that the size of the TatA pore can vary, which may explain how the pore can handle folded proteins of different shapes and sizes. The preprotein is then translocated across the cytoplasmic membrane through the TatA channel and the signal peptide is removed by a Type I signal peptidase [43]. Type II signal peptidases may also act on Tat precursors since some Tat signal peptides contain lipobox motifs. For example, in *Haloferax volcanii* a lipoprotein is exported by the Tat pathway [44]. There is also a category of Tat substrates that become integrally embedded in the membrane [45].

A folded conformation prior to export is not only a characteristic of Tat substrates, but is actually a requirement for Tat export. Proteins are only exported by the Tat system when conditions are favorable for cytoplasmic folding [46]. Therefore, in addition to the Tat signal peptide there are features of the mature domain of Tat substrates that promote folding and thereby dictate Tat export [47].

The Tat pathway is present and linked to virulence in a number of bacterial pathogens (reviewed in [48]). In *Pseudomonas aeruginosa*, the Tat pathway exports multiple virulence factors and a mutant defective in Tat export is attenuated in the rat model of infection [49]. Two of the Tat-dependent virulence factors in *P. aeruginosa* are secreted phospholipase C enzymes [49]. In *Legionella pneumophila*, Tat export is required for replication in the amoeba host as well as in macrophages [50,51]. Furthermore, the *L. pneumophila* phospholipase C enzyme also requires the Tat pathway for export [51].

The mycobacterial Tat pathway

The Tat pathway is functional in both *M. tuberculosis* and *M. smegmatis*. Both species contain genes encoding TatA, TatB and TatC. Tat export is essential for growth of *M. tuberculosis*, at least under standard laboratory conditions, as shown by the inability to delete *tatA*, *tatB*, or *tatC* unless exogenous copies of the *tat* genes are provided [52]. However, deletion mutants of *tatA*, *tatB* and *tatC* can be made in *M. smegmatis.* These

mutants have growth defects *in vitro*; nonetheless, *M. smegmatis tat* mutants can be utilized to study Tat export in mycobacteria [53,54].

Another phenotype of *M. smegmatis tat* mutants is increased sensitivity to β-lactam antibiotics [53,54]. Because β-lactamases need to be exported to the cell wall in order to degrade β-lactams, the hypersensitivity of these *tat* mutants can be attributed to reduced export of the β-lactamase, BlaS. *M. smegmatis* BlaS has a predicted Tat signal peptide and BlaS is not exported by a *tat* mutant [53,54]. By expressing the *M. tuberculosis* β-lactamase (BlaC) in wild-type *M. smegmatis* and *tat* mutants, the Tat dependence of BlaC is also established [53]. Furthermore, when the RR dipeptide of the BlaC signal peptide is changed to KK, BlaC export in *M. smegmatis* is abolished, indicating that the twin-arginine motif is required for Tat export in mycobacteria, as expected [53].

In silico analysis of the *M. tuberculosis* genome using several Tat prediction programs predicts a total of 108 proteins with Tat signal peptides [55,56]. Some of these predicted Tat substrates have demonstrated or suggested roles in *M. tuberculosis* pathogenesis or essential physiologic processes. However, relying exclusively on Tat prediction programs to identify Tat substrates is risky. The current Tat prediction programs are built on Tat consensus sequences defined in bacteria other than mycobacteria. There is also little overlap in the predictions of the currently available Tat prediction programs [56]. Furthermore, there is an increasing list of unusual Tat exported proteins that lack a cleavable signal peptide with a twin arginine motif and are missed by the current programs [57].

To overcome some of the bias of Tat prediction programs, a genetic reporter approach can be used to identify Tat-exported proteins of *M. tuberculosis* [56]. This approach utilizes a BlaC reporter lacking its endogenous Tat signal peptide and a β-lactam-sensitive *blaC* or *blaS* mutant mycobacteria background. When the signal peptide from a Tat substrate is fused to BlaC, the resulting fusion protein can be exported and confer β-lactam resistance, reporting on Tat export. Importantly, the BlaC reporter only works when exported by the Tat pathway and not by the Sec pathway [53]. Using the BlaC reporter, 17 *M. tuberculosis* proteins are shown to have functional Tat signal peptides [55,56]. The list of proteins with proven Tat signal peptides includes two phospholipase C proteins, PlcA and PlcB. Phospholipase C is necessary for the full virulence of *M. tuberculosis* in mice, providing strong evidence that the Tat pathway contributes to *M. tuberculosis* pathogenesis [58]. Another protein identified as having a functional Tat signal peptide is Rv2525c, which is suggested to have a role in infection by the demonstration of increased virulence of a *M. tuberculosis rv2525c* mutant in macrophages and mice [52,56].

ESX export pathways in mycobacteria

Mycobacterium tuberculosis also has five specialized ESX export systems (ESX-1 to ESX-5). The ESX systems are named for the first known secreted substrate of any ESX pathway, the 6kDa early secreted antigenic target (ESAT-6) of *M. tuberculosis*. The hallmark of the ESX systems is that they secrete small proteins with homology to ESAT-6. These ESAT-6-like proteins (Esx) lack Sec or Tat signal peptides and rely on ESX systems for secretion. Although the ESX systems were first discovered in *M. tuberculosis* they also exist in a small subset of Gram-positive bacteria. More recently, ESX systems have been referred to as Type VII secretion systems (for an extensive review of ESX systems, see [59]).

Each of the five ESX loci has a pair of genes encoding secreted Esx proteins as well as a suite of genes encoding the secretion machinery. The first ESX system identified was ESX-1 and it is the best described of the systems. At the center of the ESX-1 locus (spanning genes *rv3864–rv3883c*) are the *esxA* and *esxB* genes, which encode ESAT-6 (EsxA) and culture

filtrate protein 10 kDa (CFP-10; EsxB) (Figure 3B) [59]. CFP-10 is an ESAT-6-like protein that is co-secreted with ESAT-6. Other genes in ESX-1 encode predicted membrane proteins and ATPases, many of which are required for secretion of ESAT-6 and CFP-10 [60–64]. An additional locus involved in ESX-1 secretion is located at a distal site (*rv3616c–3614c*) [65,66]. Interestingly, ESAT-6 and CFP-10 are not produced by the attenuated *M. bovis* BCG vaccine strain. This is because the genomic region of difference 1 (RD1), which is deleted in BCG but present in the genomes of *M. tuberculosis* and of *M. bovis*, includes part of the ESX-1 locus [67].

ESX-1

The ESX-1 pathway is required for full virulence in several pathogenic mycobacteria [59]. Mutations throughout the ESX-1 locus in *M. tuberculosis*, *M. bovis* and *M. marinum* lead to attenuated phenotypes in macrophages and mice [60–65,68–70]. *M. marinum* is a fish pathogen that has a ESX-1 pathway homologous to *M. tuberculosis*. There are many reported effects of the ESX-1 system on the host; however, additional research is required to determine which of these effects account for the ESX-1 function in virulence. During macrophage infection with *M. tuberculosis* or *M. marinum*, ESX-1 contributes to the process of blocking phagosome maturation [71,72]. ESX-1 also limits the production of several proinflammatory cytokines that are important for controlling *M. tuberculosis* [64]. The ability to disrupt membranes is a property of ESAT-6; therefore, another role for the ESX-1 system may be to lyse host cell membranes [62,73–75]. A function in membrane disruption could explain the data showing that ESX-1 promotes host cell necrosis, bacterial spread to other cells and even phagosomal escape of *M. tuberculosis* and *M. marinum* in infected host cells [61,62,70,75,76]. Finally, experiments with *M. marinum* in a zebrafish model show ESX-1 promotes recruitment of uninfected macrophages to nascent granulomas, which appears to aid in proliferation and spread of the bacteria [77].

ESX-3

The ESX-3 pathway is essential in *M. tuberculosis* as shown by analysis of an *esx-3* conditional mutant [78,79]. The growth defect of *esx-3* mutants is attributed to defects in siderophore-dependent iron acquisition. The growth phenotype of *esx-3* mutants can be transcomplemented by co-culture with a wild-type strain, suggesting one or more ESX-3 secreted factors are important for ESX-3 mediated iron acquisition.

ESX-2 & -4

The ESX-2 and ESX-4 systems have not been directly studied in mycobacteria; however, whole genome mutagenesis studies do not predict a requirement for ESX-2 or ESX-4 for *in vitro* growth or virulence [80,81].

ESX-5

There are no reported studies of ESX-5 in *M. tuberculosis* and the locus is not predicted to be important for *in vitro* growth or virulence [80,81]. However, in *M. marinum*, ESX-5 is required for export of many PE/PPE proteins [82,83]. PE/PPE proteins are a highly abundant but poorly understood family of proteins restricted to mycobacteria. PE and PPE proteins contain N-terminal domains rich in Pro–Glu (PE), or Pro–Pro–Glu (PPE) repeats connected to a variable C-terminal domain giving them their name. The best characterized of these ESX-5-secreted PPE proteins is PPE41, which lacks a Sec or Tat signal peptide [82].

ESX secreted proteins & machinery

All five ESX loci in mycobacteria are characterized by a pair of *esx* genes, encoding homologs of ESAT-6 and CFP-10 and genes encoding the secretion machinery [59,84]. Our

understanding of how ESX systems work comes almost exclusively from research on ESX-1 and is described below.

ESX secreted proteins—The best-studied ESX secreted proteins, ESAT-6 and CFP-10, are secreted as a heterodimer [85]. ESAT-6/CFP-10 complex formation is required for stabilizing each protein. The complex is also important for secretion because CFP-10, but not ESAT-6, has a C-terminal signal peptide that targets the protein complex to the export machinery [86]. Other proteins secreted by the ESX-1 system are EspR, EspA, EspB and EspC, [65,69,87,88]. These proteins are not homologous to ESAT-6 and they are larger than ESAT-6. However, like ESAT-6 and CFP-10, these Esp secreted proteins lack N-terminal Sec or Tat signal peptides.

EspR is a particularly interesting protein secreted by the ESX-1 system. EspR is a transcriptional activator that regulates ESX-1 secretion. More specifically, EspR regulates expression of the *espACD* genes, which are required for ESAT-6/CFP-10 export. When the ESX-1 pathway is active, EspR is secreted, resulting in reduced expression of *espACD.* Conversely, when the ESX-1 pathway is inactive, the cytosolic levels of EspR increase, which results in increased transcription of *espACD* [87].

ESAT-6 and CFP-10 are secreted as a complex in a co-dependent fashion. Co-dependent secretion is also observed for other ESX-1 secreted proteins [65,88]. For example, EspA depends on ESAT-6/CFP-10 for its secretion and, conversely, ESAT-6/CFP-10 depends on EspA for secretion [65]. These co-dependent relationships suggest an interaction prior to secretion and it raises the possibility that the secreted proteins known so far are actually part of the ESX-1 machinery as opposed to being virulence factors that act on the host. Thus, it is possible that the ESX-1 secreted proteins with direct roles in virulence remain to be identified.

ESX machinery—Each ESX locus contains genes encoding conserved secretion machinery components termed EccABCDE and MycP. All of these core components are required for ESAT-6/CFP-10 secretion [60,61,63–65,70].

ESX-associated AAA ATPases (EccA1 & EccCb1)—Both EccA1 and EccCb1 are predicted cytoplasmic AAA ATPases, suggesting that these proteins supply energy for the secretion process. For EccA1, *in vitro* ATPase activity has been demonstrated [89]. Each of these ATPases is involved in targeting proteins for ESX-1 secretion. EccCb1 binds a seven amino acid C-terminal signal peptide of CFP-10, which is required for secretion of the ESAT-6/CFP-10 complex [86]. Similarly, EccA1 binds a C-terminal region of EspC that is required for secretion [88].

ESX membrane proteins (EccCa1, EccB1, EccD1, EccE1 & MycP1)—EccD1 has 10–11 predicted transmembrane domains with short periplasmic and cytoplasmic loops, suggesting a possible role as a channel used for protein translocation across the cytoplasmic membrane. EccCa1 is a predicted integral membrane protein that interacts with the ATPase EccCb1, discussed above [64]. EccB1 and EccE1 are predicted transmembrane proteins with domains in the periplasm [90]. EccE1 is shown to interact with EspD, a cytoplasmic protein of unknown function that is required for ESAT-6/CFP-10 secretion [66].

MycP1 has a single predicted transmembrane domain at the C-terminus and the protein localizes to the cytoplasmic membrane and cell wall of *M. tuberculosis* [91]. MycP1 is a serine protease that cleaves EspB after export [63]. As with all the core components, deletion of *mycP1* eliminates ESX-1 secretion. Surprisingly, point mutations inactivating the protease activity of MycP1 do not eliminate secretion but result in higher than normal levels

of ESAT-6 secretion. Together, these results suggest two roles for MycP1 in ESX-1 secretion. First, MycP1 has an undefined role in ESX-1 secretion that is independent of its protease activity. Second, the protease activity of MycP1 has a negative regulatory effect on ESX-1 export. A smaller, possibly processed form of MycP1 is found in the culture supernatant and in macrophages infected with *M. tuberculosis*; however, the significance of this cleaved product is unknown [91].

Model of ESX-1 transport

While conserved core components are defined in the ESX systems, the specific roles of these core proteins are still being clarified. Nevertheless, with the existing data it is possible to start building a model describing ESX secretion (Figure 3). Substrates containing a C-terminal signal peptide (i.e., CFP-10 or EspC) or secreted proteins bound to such proteins (i.e., ESAT-6) are targeted to a cytoplasmic ATPase, such as EccA1 or EccCb1. In turn these ATPases interact with membrane proteins that are likely to assemble into a multiprotein translocation complex. EccD1 is the most attractive candidate for the translocation channel and the energy to drive the export process may be supplied by ATP hydrolysis. It is important to point out that this model only accounts for how exported proteins traverse the cytoplasmic membrane. How ESX-1 secreted proteins cross the mycobacterial outer membrane is an important question that remains to be addressed.

Protein export systems as targets for new drug development

The increasing prevalence of drug-resistant strains of *M. tuberculosis* makes the development of novel drugs for tuberculosis an urgent priority. When exploring options for new drug targets of *M. tuberculosis*, there are several criteria that should be considered [92]. First, the drug target must be essential to bacterial viability, virulence or the persistence of *M. tuberculosis* in granulomas. We include virulence pathways as potential targets because antivirulence therapies are a new and promising approach to developing antimicrobial agents [93]. Second, targeting a novel pathway not inhibited by existing drugs may reduce the chance of cross-resistance with current drug-resistant strains. Third, targets not conserved in humans may reduce the likelihood of off-target effects. Fourth, the target should be amenable to high-throughput assays to screen for inhibitors. Fifth, the target must be accessible to inhibitors, which is particularly important for penetrating the unique and highly impermeable cell envelope of *M. tuberculosis*. In general, targets that are positioned outside the cytoplasmic membrane will be more accessible to drugs. Finally, a target with a known function is advantageous for drug optimization. The concept of developing drugs that inhibit bacterial protein export systems is not new and there are reports of inhibitors of both conserved and specialized export systems (discussed below) [94,95]. Despite this, there are no approved drugs that target protein export.

The protein export pathways of *M. tuberculosis* satisfy nearly all of the above criteria for new drug targets. Many of the export pathways of *M. tuberculosis* are essential for *in vitro* growth or are required for virulence. Protein export is not exploited by existing *M. tuberculosis* drugs and many components of the mycobacterial protein export systems are not conserved in humans. Finally, some components of *M. tuberculosis* protein export systems are themselves secreted or have active domains positioned outside of the cytoplasm.

Below, we review efforts to target protein export systems in other bacteria. Because no such efforts have been applied to *M. tuberculosis* yet, we end with speculation on how inhibitors against components of the *M. tuberculosis* export systems could be identified.

Identification of Sec export inhibitors

The Sec export system is highly conserved and essential in all bacteria, making this pathway an attractive broad-spectrum drug target. One approach used to identify inhibitors of Sec export is whole-cell screening using an *E. coli* strain carrying a *secA–lacZ* reporter fusion [96]. This screening strategy relies on the fact that SecA expression increases when Sec export is blocked. Inhibitors of Sec export are identified as inducers of *secA–lacZ* expression. This screening method identified compounds that are bactericidal against *Staphylococcus aureus* and reduce export of the toxic shock syndrome toxin-1. However, these compounds also cause membrane damage in both *S. aureus* and eukaryotic cells, eliminating the possibility of therapeutic use. Nevertheless, this type of whole-cell screen may be useful in the future if it can be optimized to avoid selection of membrane-damaging agents.

An alternate strategy to target the Sec pathway is to search for inhibitors of specific Sec components: the SecYEG membrane channel, SecA ATPase or the signal peptidases. With the exception of SecG, all of these proteins are essential for Sec export and would cause cell death if targeted. The eukaryotic endoplasmic reticulum contains proteins homologous to SecY (Sec61α) and SecE (Sec61γ) making these members of the translocase unattractive targets. However, this does not eliminate the possibility of finding novel inhibitors that indirectly target SecY or SecE. Some currently used antibiotics indirectly target SecY for degradation by stalling protein translation and jamming the SecYEG translocase [97].

Unlike SecY and SecE, there is no eukaryotic homolog of SecA, making it a promising target for inhibitors of Sec export. Inhibitors of *in vitro* SecA ATPase activity were identified using the crystal structure of *E. coli* SecA and structure-based virtual screening of compounds [98]. With optimization, two of these inhibitors became effective against SecA in the micromolar range in a biochemical ATPase assay [99]. However, when tested on whole cells these compounds only elicit growth inhibition of an *E. coli* strain with increased membrane permeability, indicating that further optimization is needed in order to access SecA in wild-type strains. In another approach, SecA inhibitors were identified by screening a *S. aureus* strain expressing antisense RNA to *secA*. This antisense strain has increased sensitivity to inhibitors of SecA, which makes them easier to identify [100]. One of these compounds identified is pannomycin, which is structurally similar to a demonstrated SecA inhibitor identified independently in another study [100,101]. While pannomycin has low antibacterial activity against a panel of bacteria tested, there is potential in optimizing its activity [99,100].

An alternative strategy to inhibit Sec export is to target the signal peptidases that cleave signal peptides from preproteins. The active sites of signal peptidases are located on the periplasmic side of the membrane, which bypasses the need to penetrate the membrane with inhibitors [102]. Type I signal peptidases are essential in all bacteria tested, making them good drug targets [103,104]. Furthermore, the catalytic domain of bacterial Type I signal peptidases is highly conserved among Gram-positive and -negative bacteria, which could lead to broad-spectrum inhibitors [13]. Even though eukaryotic cells have signal peptidases, their catalytic domains differ from those of bacterial Type I signal peptidases making off-target effects of inhibitors unlikely [13]. Penem compounds inhibit the *E. coli* Type I signal peptidase by mimicking the peptide bond of natural substrates [105–107]. Some of these compounds work with both *E. coli* and *S. aureus* in cell-based assays [107]. There is also a class of lipopeptides called arylomycins, which act against Type I signal peptidases and are bactericidal against Gram-positive, but not Gram-negative, bacteria [108,109]. The bacterial Type II signal peptidase represents another possible drug target. There are no eukaryotic homologs of Type II signal peptidases and the antibiotic globomycin inhibits these enzymes

[110]. While antibacterial globomycin derivatives that work on Gram-negative and Gram-positive bacteria exist, globomycin is not used therapeutically [111].

Identification of Type III secretion Inhibitors

Type III secretion systems (T3SSs) are specialized protein export systems used by Gram-negative pathogens. T3SSs employ a needle-like multiprotein complex to directly inject bacterial virulence factors into host cells. Many Gram-negative pathogens rely on T3SS effectors to infect host cells or evade host responses [112]. There is recent success with cell-based screening to identify inhibitors of T3SSs. While mycobacteria do not have T3SSs, identification of T3SS inhibitors in Gram-negative pathogens highlights the potential of developing similar screens to find inhibitors of specialized export systems in *M. tuberculosis*.

In *Yersinia pseudotuberculosis*, the T3SS secretes Yop proteins, including YopE. YopE is highly expressed when the T3SS is active and its expression is repressed by the LcrQ regulator, which is itself secreted by the T3SS. When T3SS secretion is not active, LcrQ accumulates in the cytosol and blocks YopE expression. Inhibitors of the T3SS were identified by screening a library of compounds against a *Y. pseudotuberculosis* strain containing a *pyopE–luxAB* reporter fusion. The most potent T3SS inhibitors identified in this screen are acylated hydrazones of various salicylaldehydes [113,114]. These compounds reduce *Y. pseudotuberculosis* and *Chlamydia pneumoniae* replication in HeLa cells, which validates the concept of targeting specialized secretion systems for drug development [114,115]. Using a screening strategy very similar to that described for *Y. pseudotuberculosis*, inhibitors of the *Pseudomonas aeruginosa* T3SS were also identified. The most promising of these T3SS inhibitors is a phenoxyacetamide that also works on the T3SS in other Gram-negative pathogens [116].

Future perspective: identification of inhibitors of mycobacterial protein export

Mycobacterial protein export systems satisfy many criteria of good drug targets but they have yet to be exploited as such. Below we discuss the potential of targeting *M. tuberculosis* protein export systems and speculate on experimental approaches that could be used to identify inhibitors.

Targeting mycobacterial Sec export

As in other bacteria, the canonical Sec pathway of *M. tuberculosis* is essential and the SecA1 ATPase a good target for inhibiting Sec export. The crystal structure of *M. tuberculosis* SecA1 is very similar to the SecAs of *E. coli* and *Bacillus subtilis*, suggesting similar mechanisms of action and highlighting the possibility of optimizing SecA inhibitors discussed above for use in mycobacteria [117,118]. Because the functional domains of *M. tuberculosis* SecA1 are conserved in SecA2 and both SecAs are demonstrated ATPases, it may even be possible to develop inhibitors that target both SecA proteins [21]. Targeting both SecA proteins simultaneously would inhibit both *M. tuberculosis* growth and virulence.

It is also possible to search for inhibitors of the *M. tuberculosis* Sec pathway directly with cell-based screening, which will ensure the inhibitors penetrate the mycobacterial cell envelope. One possible method for finding SecA inhibitors in mycobacteria is to employ a whole-cell antisense-based screening method, as was done in *S. aureus* [100]. A second approach is to generally screen for inhibitors of Sec export using a mycobacterial strain engineered to export a reporter protein via the Sec pathway. One possible strategy is to express the TEM-1 β-lactamase, which is an established reporter of Sec export in *M.*

tuberculosis, in either a *blaC* mutant of *M. tuberculosis* or *blaS* mutant of *M. smegmatis* [119]. Compounds could be screened for inhibition of secreted β-lactamase activity in culture supernatants of the reporter strains, using the chromogenic β-lactam nitrocefin [116,119,120]. Export of the TEM-1 β-lactamase reporter also protects the β-lactam sensitive mycobacterial *bla* mutants from β-lactam antibiotics. Therefore, inhibitors could also be screened for loss of β-lactam resistance in this system.

Mycobacterium tuberculosis signal peptidases are other components of the Sec export machinery that could be targeted directly. The Type I signal peptidase of *M. tuberculosis*, LepB, is predicted to be essential [80]. The progress in developing inhibitors of Type I signal peptidase in other bacteria is encouraging and could be extended for use against *M. tuberculosis*. The *M. tuberculosis* Type II signal peptidase, LspA, could also be targeted. An *lspA* deletion mutant of *M. tuberculosis* is attenuated in mice, which suggests inhibitors of Type II signal peptidases could have therapeutic value [16]. Globomycin is an available Type II signal peptidase inhibitor. Interestingly, globomycin kills *M. tuberculosis* but the bactericidal effect is independent of LspA and remains to be elucidated [121].

Targeting mycobacterial Tat export

There are many features of the Tat export pathway that make it an attractive drug target [122]. Most importantly, Tat export is essential in *M. tuberculosis* [52]. Furthermore, Tat inhibitors are unlikely to cause off-target effects on the host as no mammalian homologs of Tat machinery exist. There are also extracytoplasmic domains of the membrane-embedded Tat proteins to help drug accessibility. The endogenous *M. tuberculosis* β-lactamase (BlaC) is a Tat substrate and can be used as a reporter for Tat-export [53]. In a similar strategy as that proposed for finding inhibitors of Sec export in mycobacteria, inhibitors of Tat export could be identified by screening a library of chemical compounds for reduction in BlaC export.

Targeting mycobacterial ESX export

The specialized ESX export systems represent other potential targets for new anti-tuberculosis drugs. Of the five *M. tuberculosis* ESX pathways, ESX-1 and ESX-3 are known to be essential for virulence and growth of *M. tuberculosis*, respectively. An inhibitor that targets a conserved core component of the ESX pathways has the potential to disrupt all ESX systems simultaneously, which could reduce the potential for drug resistance to arise.

The ESX systems meet many of the desired criteria for new drug targets. There are no known homologs to ESX systems in eukaryotic organisms. The ESX export pathways have a relatively high number of core components with predicted periplasmic domains (EccB, EccD, EccE and MycP). There are also secreted proteins of the ESX system that may function in the ESX secretion process (EspA and ESAT-6/CFP-10) that could be accessible to inhibition.

Unlike the Sec and Tat pathways, ESX systems do not have established reporters to test ESX activity. However, EspR has potential to be exploited as a reporter of ESX-1 activity. Similar to the transcriptional regulator LcrQ of *Y. pseudotuberculosis* T3SS, the EspR transcriptional activator is itself secreted by ESX-1. When ESX-1 is not active, EspR accumulates in the cytosol and induces *espACD* expression [87]. Using mycobacteria carrying a transcriptional fusion of the *espACD* promoter to luciferase, high-throughput screens could be conducted for compounds that inhibit ESX-1 secretion. An inhibitor of the ESX-1 system would lead to increased cytosolic EspR and induced expression of the reporter construct.

Conclusion

Protein export is essential for *M. tuberculosis* growth and virulence. It is our belief that the protein export systems of *M. tuberculosis* represent novel pathways that could be targeted for drug development. While the basic aspects of the protein export systems of *M. tuberculosis* are known, there remains much to be learned. In particular, we do not fully understand how the accessory SecA2 and specialized ESX systems operate or contribute to pathogenesis. A wealth of potential drug targets may exist within these and other protein export pathways of *M. tuberculosis*.

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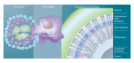

Figure 1. Barriers to effective drug delivery for *Mycobacterium tuberculosis* infection

M. tuberculosis is an intracellular pathogen that can reside long-term within macrophages in granulomas. Additionally, *M. tuberculosis* has an unusual and complex cell wall that is a physical barrier for drug delivery. The outermost layer of the cell wall is composed of a glycan- and glycolipid-based capsule, followed by the mycobacterial outer membrane. The mycobacterial outer membrane is composed of free lipids intercalated with mycolic acids that are covalently linked to the arabinogalactan component of the cell wall. The arabinogalactan is, in turn, covalently linked to peptidoglycan. The cytoplasmic membrane is the final permeability barrier before the cytosol.

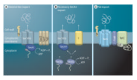

Figure 2. The Sec, SecA2 and Tat protein export systems of mycobacteria

(A) The conserved Sec pathway exports unfolded preproteins that are synthesized with N-terminal signal peptides (hatched marks). The SecA ATPase recognizes and drives preproteins through the SecYEG membrane channel using repeated rounds of ATP-binding and hydrolysis. The SecD, SecF and YajC proteins increase efficiency of Sec protein export. During or shortly after translocation, the signal peptide is removed by a membrane-bound SP. The exported protein folds into a mature conformation. **(B)** The accessory SecA2 system of mycobacteria is characterized by a functionally distinct ATPase called SecA2, which is required for the export of a subset of proteins. Current data suggests that SecA2 uses the canonical SecA1/SecYEG translocase for exporting proteins, but the possibility of a requirement for a distinct channel within the membrane has not yet been eliminated. Some SecA2-dependent exported proteins have signal peptides and some do not. The role of SecA2 in the export of proteins lacking signal peptides is unresolved. **(C)** The conserved Tat pathway exports folded preproteins containing N-terminal signal peptides with a twin-arginine (RR) motif. Tat preproteins are recognized by the TatBC complex in the membrane and then translocated across a homo-oligomeric TatA channel. As with Sec export, the signal peptide is removed by a membrane-bound SP.

RR: Arginine residues; Sec: Secretion; SP: Signal peptidase; tat: Twin arginine translocation.

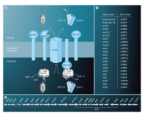

Figure 3. The ESX-1 system of *Mycobacterium tuberculosis*

(A) The core components of the ESX-1 pathway are EccA1, EccB1, EccCa1, EccCb1, EccD1, EccE1 and MycP1. In the developing model of ESX export, C-terminal signal peptides of ESX-1 substrates (EspC and CFP-10) are recognized by cytoplasmic AAA ATPases (EccA1 and EccCb1). Through interactions with membrane components of the ESX-1 system, the ATPases are proposed to deliver these proteins to the translocation channel. EccD1 is the putative membrane channel through which the proteins are translocated. **(B)** The genomic ESX-1 locus of *M. tuberculosis* is shown along with the distally located *espACD* and esp*R* genes. The RD1 deleted in BCG is indicated. **(C)** There is a recently introduced systematic nomenclature for genes of the ESX-1 locus [84]. The new gene names and previously used nomenclature, derived from the *M. tuberculosis* genome annotation, are shown.

BCG: Bacille Calmette-Guérin; CFP: Culture filtrate protein; ESAT: Early secreted antigenic target; RD1: Region of difference 1.