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The Ins and Outs of *Mycobacterium tuberculosis* Protein Export

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Abstract

Mycobacterium tuberculosis is an important pathogen that infects approximately one third of the world's population and kills almost two million people annually. An important aspect of *M. tuberculosis* physiology and pathogenesis is its ability to export proteins into and across the thick mycobacterial cell envelope, where they are ideally positioned to interact with the host. In addition to the specific proteins that are exported by *M. tuberculosis*, the systems through which these proteins are exported represent potential targets for future drug development. *M. tuberculosis* possesses two well-known and conserved export systems: the housekeeping Sec pathway and the Tat pathway. In addition, *M. tuberculosis* possesses specialized export systems including the accessory SecA2 pathway and five ESX pathways. Here we review the current understanding of each of these export systems, with a focus on *M. tuberculosis*, and discuss the contribution of each system to disease and physiology.

Keywords

secretion; Sec; SecA2; Tat; ESX

Introduction

Mycobacterium tuberculosis is the causative agent of the disease tuberculosis, which the World Health Organization estimates kills almost 2 million people every year [1]. Although tuberculosis is treatable, it requires a long antibiotic regimen that is expensive and difficult, especially for people in the developing world. Furthermore, multidrug resistant (MDR) and extensively drug resistant (XDR) *M. tuberculosis* make treatment even more problematic and novel drugs are desperately needed to combat this disease.

Because of their importance to virulence and bacterial viability, the exported proteins of *M. tuberculosis* and their respective protein export systems can be considered potential drug targets (recently reviewed in [2]). Many exported proteins, which we define as including proteins in the cell envelope of *M. tuberculosis* and proteins secreted by *M. tuberculosis*, interact with the host and are crucial for causing disease. Without its systems for transporting these proteins across the cytoplasmic membrane and to their final destination,

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M. tuberculosis cannot deliver effector proteins that are necessary for virulence. Furthermore, some *M. tuberculosis* protein export systems are essential. Although there are currently no approved drugs that target bacterial protein export pathways, inhibitors of such systems in other bacteria have been identified [3-6].

In *M. tuberculosis*, there are two highly conserved protein export systems that are responsible for the majority of protein export: the Sec and Tat export pathways. *M. tuberculosis* also possesses specialized protein export systems dedicated to the export of a more limited set of proteins: the accessory SecA2 export pathway and ESX pathways. ESX pathways are also referred to as Type VII secretion systems. Interestingly, both of these specialized protein export systems were first identified in *M. tuberculosis* but later found to also exist in some other bacteria. Here we review the current state of research on the protein export systems of mycobacteria with an emphasis on studies performed in *M. tuberculosis*.

Housekeeping Sec Export System

Introduction

The Sec system is highly conserved and present in all bacteria, and it acts as the primary route for exporting proteins to the cytoplasmic membrane and beyond. Because many of the proteins exported by the Sec system perform vital functions that require proper export, the Sec pathway is essential for bacterial viability (Table 1). In addition, many bacterial proteins with roles in virulence are exported by the Sec system [7]. The current understanding of Sec export comes from extensive study in *Escherichia coli* (for recent reviews see [8, 9]). While Sec export functions in essentially the same manner in all bacteria in which it has been studied, only a few aspects of this system have been directly investigated in mycobacteria.

Exported Proteins and Targeting

The Sec system exports proteins across the cytoplasmic membrane post-translationally. Proteins exported by the Sec system, termed preproteins or precursors, are synthesized with an N-terminal signal peptide that is important for targeting the protein for export. Following export, the signal peptide is cleaved to generate the mature protein. Sec signal peptides consist of a positively charged N-terminus, a hydrophobic central domain, and an uncharged polar C-terminus containing the cleavage site [9]. Some Sec-exported proteins are lipoproteins, and in these cases the C-terminal end of the signal peptide contains a lipobox motif with a conserved cysteine that is the site of lipid attachment [10]. Sec signal peptides and lipoprotein signal peptides can be predicted using the bioinformatic programs PSORTb v3.0 [11], SignalP v3.0 [12], and LipoP v1.0 [13].

Proteins must be in an unfolded state to be exported by the Sec system. In many Gram-negative bacteria there is a Sec export chaperone, SecB, that binds preproteins, maintains them in an unfolded state, and delivers them to SecA – a central component of the Sec system discussed below. However, not all preproteins of Gram-negative bacteria require SecB, and Gram-positive bacteria lack a SecB ortholog [14]. In the absence of SecB, it is thought that other proteins fulfill the role of export chaperone. In *M. tuberculosis*, Bordes et al. recently identified Rv1957 as a SecB-like protein [15]. Rv1957 prevents proteolysis and aggregation of *M. tuberculosis* HigA, a presumed cytoplasmic protein that is part of the *M. tuberculosis* HigBA toxin-antitoxin system. These activities are consistent with Rv1957 being a chaperone. However, there is currently no evidence that Rv1957 functions as a chaperone for protein export and it is possible that the function of Rv1957 is restricted to the HigBA system.

System Components and Mechanism

SecA is a multifunctional component of the Sec export system that works with the heterotrimeric SecYEG membrane channel to export proteins across the cytoplasmic membrane. SecA recognizes and binds to the signal peptide, as well as portions of the mature domain, of preproteins [16, 17]. SecA is also an ATPase that harnesses energy from multiple rounds of ATP binding and hydrolysis to "push" preproteins through the SecYEG channel [18, 19]. The proton motive force can also contribute to Sec export, but is not absolutely required [19].

Mycobacteria and some Gram-positive bacteria have two SecA proteins, each with distinct functions. The primary housekeeping SecA, that functions like SecA of *E. coli*, is referred to as SecA1, while the accessory SecA is referred to as SecA2 (see later section for SecA2 discussion). SecA1 is predicted to be essential in *M. tuberculosis* [20, 21] and proven to be essential in the model mycobacterium *M. smegmatis* [22]. Because *secA1* deletion mutants are not viable, conditional silencing has been used to study SecA1 function. As would be expected for a housekeeping SecA, depletion of SecA1 in *M. smegmatis* causes both growth inhibition and reduced export of a test protein with a Sec signal peptide [23, 24]. *M. tuberculosis* SecA1 is also a demonstrated ATPase [25], and the X-ray crystal structure of *M. tuberculosis* SecA1 is similar to other bacterial SecA structures [26].

The SecYEG complex provides the channel through which proteins travel across the cytoplasmic membrane [27] and together with SecA it forms the "translocase," which is the minimum apparatus needed to reconstitute efficient Sec export *in vitro* [28]. SecY is a polytopic membrane protein and the largest component of the SecYEG complex, forming the pore in the membrane through which proteins pass during export. SecE is suggested to act as a "clamp" to stabilize SecY [29]. SecG improves efficiency of protein export [30]. Additional Sec components that improve export efficiency are SecD, SecF, and YajC [31].

Once a protein has been exported across the membrane, the signal peptide is removed by one of two signal peptidases – the Type I signal peptidase LepB, or the Type II signal peptidase LspA [32]. LspA acts specifically on lipoprotein signal peptides. The *M. tuberculosis* LspA has been studied and confirmed to function in lipoprotein signal peptide cleavage [33]. Furthermore, an *lspA* mutant of *M. tuberculosis* is attenuated in macrophage and mouse models of infection, demonstrating the importance of correctly exported lipoproteins for *M. tuberculosis* virulence [33].

In addition to the process described above for exporting proteins completely across the cytoplasmic membrane, the SecYEG channel is also used for a significantly different process – co-translational insertion of integral membrane proteins into the cytoplasmic membrane (for a recent review, see [34]). In this process, transmembrane domains of nascent integral membrane proteins are recognized by the signal recognition particle SRP during translation. SRP then delivers the nascent integral membrane protein to the SRP receptor FtsY, which in turn passes the protein to SecYEG for co-translational insertion into the membrane. SecY contains a "lateral gate" which is believed to allow transmembrane domains of integral membrane proteins to pass sideways out of the SecYEG channel and into the membrane, with the help of the YidC protein [35, 36]. While not generally required for integral membrane protein insertion, the SecA protein does assist in the case of integral membrane proteins with large hydrophilic domains [37]. Integral membrane proteins can be predicted using the bioinformatic program TMHMM v2.0 [38], which searches for transmembrane domains.

Contribution to Disease and Physiology

Because the Sec system performs the bulk of protein export, many Sec-exported proteins are important for both *M. tuberculosis* viability and virulence. Several *M. tuberculosis* proteins proven to function in virulence contain Sec signal peptides [39, 40]. Also, as mentioned above, the importance of LspA demonstrates that Sec-exported lipoproteins are important for *M. tuberculosis* virulence [33]. In fact, several lipoproteins with Sec signal peptides are known to be important for *M. tuberculosis* virulence, including LpqH (19kD) [41], LppX [42], and LprG [43]. While SecYEG is homologous to the Sec61 complex found in eukaryotic cells, there is no eukaryotic homolog of SecA. The lack of SecA in mammalian cells, combined with the critical importance of SecA1 for both viability and virulence in bacteria, makes SecA1 a compelling potential drug target (as reviewed in [2]).

Model, Summary, and Future Questions

At this point, the studies of SecA1 and LspA are the only ones to directly investigate housekeeping Sec export in mycobacteria [22-26, 33, 44]. However, the data from these studies, combined with the presence of Sec component orthologues (Table 1) and exported proteins with Sec signal peptides in *M. tuberculosis*, is consistent with the housekeeping Sec system of mycobacteria functioning as described in other bacteria (Figure 1). In this case, preproteins with N-terminal signal peptides are recognized by SecA1, which interacts with the SecYEG channel complex to form the translocase. SecA1 performs repeated cycles of ATP hydrolysis, pushing segments of the preprotein through the SecYEG channel. Signal peptides are removed by a LepB or LspA signal peptidase, and mature exported proteins fold into their final conformations.

In the future it will be important to expand our understanding of the Sec export system in mycobacteria. For example, nothing is currently known about mycobacterial chaperone(s) involved in Sec export. Also, the process of inserting integral membrane proteins into the mycobacterial cytoplasmic membrane is a topic that has so far received no research attention.

Accessory SecA2 Export System

Introduction

All mycobacteria are unusual in having two SecA proteins. In contrast to SecA1, SecA2 is non-essential and *secA2* deletion mutants have been constructed in several mycobacteria, including *M. tuberculosis* [45] and *M. smegmatis* [22]. SecA2 functions in protein export, but its role appears limited to a much smaller subset of proteins than those exported by SecA1. SecA2 is necessary for the full virulence of *M. tuberculosis* [45, 46], suggesting that one or more of the proteins it exports are important in pathogenesis. Accessory SecA2 export has been studied directly in mycobacteria; however, there is still much that is unknown.

Exported Proteins and Targeting

Comparative 2D-PAGE analysis of exported proteins from wild-type and *secA2* deletion mutant strains has been used to identify proteins that require SecA2 for their export [45, 47]. Only a small number of proteins are identified in these studies, and they include examples both with and without signal peptides. In *M. smegmatis*, cell wall proteins were analyzed by 2D-PAGE, identifying Msmeg1704 and Msmeg1712 as proteins exported by the SecA2 system [47]. These proteins share many similarities – both are lipoproteins, contain lipoprotein Sec signal peptides [13], and are predicted sugar-binding proteins. While direct homologues of Msmeg1704 and Msmeg1712 are not found in *M. tuberculosis*, similar sugar-binding lipoproteins are present, although they have yet to be evaluated for SecA2-dependence. The SecA2-dependent proteins with signal peptides are currently indistinguishable from proteins

exported by the housekeeping Sec export system, and experimental evidence shows the signal peptide *is* required for export of Msmeg1712 [47].

In *M. tuberculosis*, proteins exported into the culture media (culture filtrates) were analyzed by 2D-PAGE [45]. Among the very few proteins identified was the antioxidant enzyme SodA (superoxide dismutase). SodA does not have a signal peptide, but its dependence on SecA2 for export is confirmed by western blot and SodA activity assay [45, 48]. Given the role of SodA in detoxifying oxygen radicals, the export of *M. tuberculosis* KatG (catalase), another antioxidant enzyme, was directly evaluated for SecA2-dependence. Like SodA, KatG lacks a signal peptide and is dependent on SecA2 for export [45].

System Components and Mechanism

Accessory SecA2 proteins are found in all mycobacteria and some Gram-positive bacteria (for an extensive review of SecA2 export, see [49]). Several SecA2 export systems include a SecY2 protein. Referred to as SecA2/Y2 systems, these systems seem to be dedicated to exporting a single large protein that is heavily glycosylated prior to export [50-52]. In contrast, there is no SecY2 in mycobacteria and the system is referred to as a SecA2-only system. *Listeria monocytogenes* also has a SecA2-only system. A notable similarity between the *L. monocytogenes* and mycobacterial SecA2 systems is that both are reported to export proteins with signal peptides as well as proteins without signal peptides [53, 54]. In *L. monocytogenes*, this includes export of MnSOD (manganese superoxide dismutase), which lacks a signal peptide [54].

SecA2 has been directly studied in both *M. tuberculosis* and *M. smegmatis*. Importantly, complementation experiments show that SecA2 of *M. tuberculosis* and *M. smegmatis* can substitute for one another to complement *secA2* deletion mutant phenotypes [24]. Because these cross-species complementation experiments demonstrate that the SecA2 proteins of *M. tuberculosis* and *M. smegmatis* can carry out the same functions, *M. smegmatis* has been used as a model for studying the mechanism of mycobacterial SecA2-dependent export.

SecA2 exhibits several similarities to the housekeeping SecA1 protein. SecA1 and SecA2 proteins are present in equivalent amounts in *M. tuberculosis*, and both proteins have proven ATPase activity [25]. Like housekeeping SecA proteins, ATP hydrolysis is also required for SecA2 function in both *M. tuberculosis* [25] and *M. smegmatis* [24].

There are also several differences between SecA1 and SecA2. SecA2 is smaller than SecA1 because it lacks the C-terminal linker region, which in *E. coli* has been implicated in binding to phospholipids, SecB, and zinc [55-57]. SecA2 also differs from SecA1 in how it localizes within the bacterium. Like *E. coli* SecA, SecA1 is evenly distributed between soluble and cell envelope fractions, but SecA2 is found predominantly in the cytosol-containing soluble fraction [24]. Most importantly, it is clear that SecA1 and SecA2 have independent functions. Even when SecA2 is overexpressed, SecA1 cannot be deleted, indicating that SecA2 cannot substitute for SecA1. Similarly, overexpression of SecA1 does not rescue the phenotypes of a *secA2* deletion mutant [22].

Given that there is no accessory SecY or obvious alternate export channel to work with SecA2 in mycobacteria, it seems likely that SecA2 functions with the help of some or all of the housekeeping Sec export system. To address this possibility, export of the SecA2-dependent protein Msmeg1712 was assessed following depletion of SecA1 in *M. smegmatis*. In the absence of SecA1, export of Msmeg1712 was found to be significantly compromised [24]. The simplest interpretation of this result is that the SecA2 export system needs the housekeeping SecA1 to function. However, the possibility that SecA1 depletion has an indirect effect on SecA2 export cannot currently be ruled out.

Contribution to Disease and Physiology

The *M. tuberculosis secA2* mutant is attenuated for growth in both macrophages [46] and the mouse model of tuberculosis infection [45]. These results indicate that the SecA2 system exports proteins important for *M. tuberculosis* virulence. The fact that both SodA and KatG are dependent on SecA2 for export suggests that the SecA2 system may protect *M. tuberculosis* from the oxidative burst of macrophages. Yet, the *secA2* mutant is still attenuated for growth in *phox*$^{-/-}$ macrophages, which cannot produce an oxidative burst [46]. While this result does not exclude a role for SecA2 in resisting oxygen radicals during infection, it does reveal the existence of other roles for the SecA2 system in promoting virulence. During macrophage infection, the *M. tuberculosis secA2* mutant induces increased release of proinflammatory cytokines [46] and increased apoptosis [48], as compared to wild-type *M. tuberculosis*. Thus, the SecA2 system may block innate immune responses to enable *M. tuberculosis* growth in macrophages.

Model, Summary, and Future Questions

More research is needed to elucidate the details of SecA2-dependent export. However the studies conducted so far, particularly those using *M. smegmatis*, help build a model for this system (Figure 1). SecA2 recognizes a small subset of proteins that are normally not compatible with Sec export, and uses its ATPase activity to assist in their export. It seems most likely that SecA2 works with components of the housekeeping Sec export system, either by delivering proteins to SecA1 or by actively participating in exporting proteins through the SecYEG channel. It remains possible, however, that other unknown components are required in addition to or in lieu of the housekeeping Sec components.

Many important questions remain. For the SecA2-dependent proteins with signal peptides, it is not known what distinguishes them from proteins exported by the housekeeping Sec system, or why these SecA2-dependent proteins cannot be exported by the housekeeping Sec system alone. Interestingly, it appears that the signal peptide of *M. smegmatis* SecA2-dependent proteins does *not* impart the requirement for SecA2 in their export (Feltcher, M., Gibbons, H., Ligon, L., Braunstein, M., unpublished results). The fact that some SecA2-dependent proteins do not have signal peptides adds an additional layer of complexity to understanding this system. It is possible that both proteins with signal peptides and proteins without signal peptides are directly exported by the SecA2 export system. Conversely, the SecA2-dependence of proteins without signal peptides may be an indirect effect. For example, it is possible that the SecA2 system exports an unidentified protein containing a signal peptide, which in turn participates in the export of proteins lacking signal peptides via another pathway. Finally, it is likely that more SecA2-dependent proteins exist and remain to be identified, including *M. tuberculosis* proteins containing signal peptides. Important future goals are to identify additional proteins exported by the SecA2 system and understand the role this export system plays in the pathogenesis of *M. tuberculosis*.

Tat Export System

Introduction

Twin-arginine translocase (Tat) export systems exist in both Gram-negative and Gram-positive bacteria, but unlike Sec systems, they are not present in all bacteria [58]. When Tat export systems are found in bacterial pathogens, they are frequently responsible for exporting virulence factors, and thus contribute to pathogenesis [59]. In *M. tuberculosis*, the Tat system plays a role in both virulence and drug resistance, and it is also essential for viability [60]. The current understanding of Tat export comes from studies in *E. coli* (for a recent review see [61]).

Exported Proteins and Targeting

Similar to the Sec system, the Tat system exports proteins across the cytoplasmic membrane in a post-translational manner, and proteins exported by the Tat pathway are synthesized as preproteins with an N-terminal signal peptide. Like Sec signal peptides, Tat signal peptides consist of a positively charged N-terminus, a hydrophobic central domain, and an uncharged polar C-terminus containing a cleavage site. Tat signal peptides are distinguished from Sec signal peptides by the presence of a Tat motif that contains twin-arginine residues at the junction of the charged domain and the hydrophobic domain. The Tat motif is reported as S/T-R-R-X-F-L-K [62], or more generically as R-R-X-Φ-Φ (Φ=hydrophobic). Substitution of the twin-arginines with other residues prevents export of most Tat-dependent proteins, but the twin-arginines are not an absolute requirement, as there are examples of both natural [63-66] and artificial [67, 68] Tat-dependent proteins that have only one arginine.

Several bioinformatic programs are available for predicting Tat signal peptides: TatFind v1.4 [69], TatP v1.0 [70], TIGR01409 [71], and PRED-TAT [72]. However, the list of predicted *M. tuberculosis* Tat signal peptides varies widely between programs [64]. Given the differing predictions of these programs, mycobacterial Tat signal peptides must be identified experimentally. A genetic reporter system utilizing the Tat-exported *M. tuberculosis* β-lactamase (BlaC) is useful for this purpose [63, 64]. β-lactamases must be exported to confer resistance to β-lactam antibiotics, and *M. tuberculosis* BlaC must be exported by the Tat pathway to function. When a truncated BlaC (lacking its endogenous signal peptide) is fused to a protein containing a functional Tat signal peptide, BlaC reports on export of the fusion protein by conferring β-lactam resistance [73]. Using the BlaC reporter, 18 *M. tuberculosis* proteins are shown to have functional Tat signal peptides (Table 2). *M. tuberculosis* likely has additional Tat-exported proteins, and more study is needed to obtain a complete list. Examination of the proven Tat signal peptides shows that *M. tuberculosis* uses a very similar Tat motif (Φ-Σ-R-R-X-Φ-Φ, Σ=small amino acid) to that defined previously.

In addition to a Tat signal peptide, a second requirement for Tat-dependent proteins is that they be folded *prior* to export. Thus, structural features of the mature domain of a protein are also critical in directing a protein for Tat export. Unfolded or misfolded Tat-dependent proteins are generally unacceptable for export, suggesting the Tat export system has a "proofreading" ability that restricts export to properly folded proteins [74, 75].

System Components and Mechanism

As is the case in *E. coli*, mycobacterial Tat export systems consist of three proteins: TatA, TatB, and TatC [60, 73, 76]. TatA and TatB are small homologous proteins, each containing a single transmembrane domain. TatC is a larger protein containing 6 transmembrane domains [77].

TatB and TatC form a complex that contains the binding site for Tat preproteins [78, 79]. After a preprotein binds to TatBC, TatA is recruited to the complex [79]. TatA is generally believed to form the export channel [61] and is found in homo-oligomers of varying size [80, 81], which may give the Tat system the flexibility to export folded proteins of different size and shape. Energy for Tat export is provided by the proton motive force [79, 82]. Following export, Tat preproteins undergo signal peptide cleavage by LepB, the type I signal peptidase [83]. Although it has yet to be proven, it is presumed that the signal peptides of Tat-exported lipoproteins [84] are cleaved by LspA, the type II signal peptidase.

Contribution to Disease and Physiology

In *M. tuberculosis*, the Tat export system is essential. The *tatA*, *tatB*, and *tatC* genes cannot be deleted unless an extra copy of these genes is provided on a plasmid [60]. This essentiality

of the *M. tuberculosis* Tat system is unusual; only one other organism, *Sinorhizobium meliloti*, is reported to have an essential Tat export system [85]. In fact, even the *M. smegmatis* Tat system is not essential, although *tatA*, *tatB*, and *tatC* deletion mutants do grow slowly *in vitro* [73, 76]. The reason for the essentiality of the *M. tuberculosis* Tat pathway is not known.

The Tat export systems of mycobacteria also contribute to drug resistance. Mycobacteria are naturally resistant to β-lactam antibiotics due to the presence of exported β-lactamases. The β-lactamases of *M. tuberculosis* (BlaC) and *M. smegmatis* (BlaS) are both proven Tat-exported proteins that must be exported to protect against β-lactam antibiotics [73].

In addition, *M. tuberculosis* Tat export has a role in pathogenesis, as some of the known Tat-exported proteins of *M. tuberculosis* function in virulence (Table 2). For example, PlcB and PlcA are phospholipase C enzymes that are important for *M. tuberculosis* virulence [86]. Rv2525c is another protein with a proven Tat signal peptide that has a role in *M. tuberculosis* virulence. However, in this case, the protein may act to limit pathogenesis, as an *rv2525c* mutant demonstrates increased growth in macrophages and faster time to death in SCID mice [60]. Tat export systems do not exist in mammalian cells. This fact, combined with the importance of Tat export for viability, drug resistance, and virulence in *M. tuberculosis*, makes the Tat system a promising potential drug target (as reviewed in [2]).

Model, Summary, and Future Questions

While the mechanism of Tat export has not yet been directly investigated in mycobacteria, the requirement for TatA, TatB, and TatC, and the presence of functional Tat signal peptides in Tat-exported mycobacterial proteins suggests that the Tat system of mycobacteria functions like the *E. coli* Tat system (Figure 2). In this case, Tat preproteins contain an N-terminal signal peptide with a twin arginine motif and fold in the cytoplasm prior to export. Tat-dependent preproteins then bind to the TatBC complex in the cytoplasmic membrane. Next, oligomers of TatA are recruited, likely forming the channel complex capable of accommodating folded proteins of varying size. In the presence of the proton motive force, preproteins are exported across the cytoplasmic membrane.

In the future, it will be important to study the mechanism of mycobacterial Tat export, and to confirm that it does indeed function as understood in other bacteria. Some Tat-dependent proteins in *E. coli* require chaperones to assist in their proper folding prior to export [61]; however, it is not known whether chaperones assist with Tat export in mycobacteria. Interestingly, the *M. tuberculosis* PlcB protein is exported by the Tat system of *M. tuberculosis*, but not by *M. smegmatis* [64]. This discrepancy could be explained by the existence of chaperones or other accessory Tat components present in *M. tuberculosis* but absent in *M. smegmatis*. Finally, our ability to predict *M. tuberculosis* Tat-dependent proteins is surprisingly unreliable. As Tat export becomes better understood and more *M. tuberculosis* Tat signal peptides are experimentally identified, Tat signal peptide prediction programs can be refined and more successfully applied to mycobacteria.

ESX Export Systems

Introduction

ESX export systems, which are also referred to as Type VII or WXG100 secretion systems, are specialized protein export systems originally identified in mycobacteria. Some Gram-positive bacteria also possess ESX systems including *Staphylococcus aureus*, where the system is important to virulence [87]. The first ESX system identified was ESX-1 of *M. tuberculosis*, which is responsible for exporting the ESAT-6 protein (early secreted antigenic target 6 kD). The hallmark of ESX systems is that they export ESAT-6-like proteins; hence, the ESX name of these systems refers to ESAT-6. *M. tuberculosis* has five

ESX systems (ESX-1-5) with various functions in mycobacterial physiology and/or virulence (Table 3).

All ESAT-6-like proteins are small (~100 amino acids), possess a WXG amino acid motif, and notably lack a Sec or Tat signal peptide. In *M. tuberculosis*, each ESX system is encoded by a genomic locus of 7-18 genes that includes genes encoding core components of the ESX system and a pair of genes encoding exported ESAT-6-like proteins [88]. Progress in understanding ESX systems has come from studies in *M. tuberculosis*, *M. smegmatis*, and *M. marinum*. In our description below, we use the nomenclature recently introduced for ESX system components and exported proteins [89]. The ESX-2 and ESX-4 systems have not yet been studied; therefore, we limit our discussion below to the ESX-1, ESX-3, and ESX-5 systems.

ESX-1

Introduction—ESX-1 is the most thoroughly studied and best understood ESX system and it provides the model for how other systems likely function (for recent reviews of ESX-1 export, see [90, 91]). Extensive mutant analysis has identified individual genes (both in and outside of the *esx-1* locus) that are required for ESX-1 export [92-96]. Studies of *M. tuberculosis* mutants lacking a functional ESX-1 system also reveal the importance of this system to virulence [92, 94, 96-98]. Interestingly, the *esx-1* genomic locus is located in the region of difference (RD1) that is present in *M. tuberculosis* but absent in the attenuated *M. bovis* BCG vaccine strain. The lack of ESX-1 in BCG explains the failure of BCG to express and secrete ESAT-6 and it also helps explain the attenuated virulence of BCG [94, 97, 99].

Exported Proteins and Targeting—Two ESAT-6-like proteins are exported by the ESX-1 system: the prototypical ESAT-6 protein and the ESAT-6-like protein CFP-10 (culture filtrate protein 10kD). Both of these proteins are found exported to the cell wall of *M. tuberculosis* as well as secreted by *M. tuberculosis* [99, 100]. ESAT-6 and CFP-10 are exported together as a 1:1 complex and each protein depends on the other for its export [98, 101]. Additionally, neither protein has a Sec or Tat signal peptide. Instead, the CFP-10 protein has a seven amino acid targeting element at its C-terminus that directs export of the ESAT-6/CFP-10 complex via the ESX-1 system [102].

In addition to small ESAT-6-like proteins, other larger proteins lacking classical N-terminal signal peptides are exported by ESX-1. One such protein is EspA. As with the co-dependence of ESAT-6 and CFP-10 for export, EspA export depends on the ESAT-6/CFP-10 complex and, likewise, ESAT-6 and CFP-10 require EspA for their export [92, 95]. These co-dependent relationships suggest the need for these proteins to interact prior to export, and it raises the possibility that these exported proteins function in the export process itself. Other proteins exported by the *M. tuberculosis* ESX-1 system are EspC [95], EspB [103], and EspR [104].

EspR is a particularly interesting ESX-1 exported protein because it is a transcriptional regulator of the *espACD* genes, which are required for ESAT-6 export. High cytoplasmic levels of EspR increase expression of *espACD* and, therefore, ESX-1 export. This results in more exported EspR and less EspR in the cytoplasm, which in turn reduces *espACD* expression [104]. In this way, EspR is part of a feedback loop for regulating the ESX-1 system.

System Components and Mechanism—From analyses of mutants lacking individual *esx-1* genes, proteins necessary for ESX-1 export have been identified including predicted ATPases (EccA1and EccCb1) and membrane proteins (EccB1, EccCa1, EccD1, EccE1, MycP1).

EccA1 and EccCb1 are predicted cytosolic AAA ATPases that could provide energy for export through ATP hydrolysis. For EccA1, ATPase activity is demonstrated *in vitro* [105], but for EccCb1 there is currently no demonstration of ATPase activity. Interestingly, both EccA1 and EccCb1 recognize C-terminal targeting sequences of ESX-1 exported proteins. EccA1 binds to the C-terminus of EspC that is required for EspC export [106]. EccCb1 recognizes the C-terminal targeting element of CFP-10 that is required for CFP-10/ESAT-6 export [102].

Because of their probable cytoplasmic membrane location, the predicted membrane proteins required for ESX-1 export are candidates for being components of a channel or proteins that regulate or work with a channel. Of these proteins, EccD1 is a large protein with 10-11 predicted transmembrane domains and a compelling candidate for being a translocation channel component. Another predicted cytoplasmic membrane protein, EccCa1 interacts with the EccCb1 ATPase [96]. This protein interaction implies a role for EccCa1 in connecting protein targeting to the ESX-1 system (as carried out by EccCb1 recognition of the CFP-10 targeting element) with translocation across the cytoplasmic membrane. MycP1 is a predicted membrane protein with a serine protease domain located on the cell wall side of the membrane. While the absence of MycP1 abrogates ESX-1 export [107], a mutant form of MycP1 with amino acid substitutions in the proteolytic active site actually has the opposite effect of increasing ESX-1 export [108]. Thus, it appears that MycP1 has multiple roles in ESX-1 export which remain to be resolved.

In the *M. marinum* ESX-1 system, immunofluoresence microscopy demonstrates that the EccCa1 homolog Mh3870 is localized at the cell pole, most often at the new pole [109]. Since EccCa1 is an important membrane component of the ESX-1 system (discussed above), this finding suggests that ESX-1 export occurs at new poles of the bacillus. Further studies are needed to discern if the ESX-1 system is similarly localized in *M. tuberculosis* and whether polar localization is necessary for ESX-1 secretion.

Contribution to Disease and Physiology—Research conducted in numerous laboratories clearly establishes a role for ESX-1 in *M. tuberculosis* virulence and, more specifically, a role promoting growth of *M. tuberculosis* in macrophages [93, 96, 97, 108]. ESX-1 export is implicated in numerous *M. tuberculosis* processes with potential importance to pathogenesis. However, it remains to be clarified which ESX-1 effects are responsible for the role of this system in *M. tuberculosis* virulence. During macrophage infection, ESX-1 is important for the pathogen's ability to block phagosome maturation [110, 111], limit host cytokine production [96], and promote apoptosis of infected cells [112]. There is also data indicating the ESX-1 system promotes phagosome escape by *M. tuberculosis* [113] and host cell lysis that facilitates dissemination [93, 94]. ESAT-6 can disrupt membranes, which may explain some of these effects [112, 114, 115]. Finally, in *M. marinum* the ESX-1 system recruits macrophages to nascent granulomas, promoting maturation of the granuloma and bacterial growth [116].

Model, Summary, and Future Questions—While several components of the ESX-1 system are now known, the mechanism of ESX-1 export is not yet fully understood. A working model for the system is as follows (Figure 3). A C-terminal targeting element directs proteins, such as CFP-10, for ESX-1 export. Other proteins, such as ESAT-6, lack a targeting element and can form a complex with a protein like CFP-10 for targeting. By way of this C-terminal targeting element, proteins reach AAA ATPases EccA1 or EccCb1. These ATPases then interact with membrane components that may comprise or impact a membrane channel. EccD1 may be a component of the cytoplasmic membrane channel and ATP hydrolysis by the AAA ATPases may provide energy for the targeting and/or translocation events.

In the future, it will be particularly important to investigate the process of ESX-1 export of proteins across the mycobacterial outer membrane. The model so far only addresses transport across the cytoplasmic membrane and the final steps of secretion remain a mystery. The issue of whether known ESX-1 exported proteins are part of the export system and/or have effector functions in virulence also requires attention. Finally, it is unlikely that all proteins exported by the ESX-1 pathway are known at this point. Discovering new substrates and elucidating their functions will help researchers better understand the ESX-1 system and its role in virulence. Since the ESX-1 system plays a role in *M. tuberculosis* virulence and is not found in eukaryotic cells, it has potential utility as a novel target for anti-tuberculosis drug development (reviewed in [2]).

ESX-3

Introduction—ESX-3 is essential in *M. tuberculosis* and appears important for iron and zinc uptake or homeostasis [117, 118]. However, in comparison to ESX-1, ESX-3 has received less research attention and little is known about the mechanism of ESX-3 export.

Exported Proteins and Targeting / System Components and Mechanism—As is the case for all ESX systems, the ESX-3 locus encodes a pair of ESAT-6-like proteins. One of these proteins, EsxH, is shown to be secreted by the ESX-3 system of *M. smegmatis*, and its export is increased in low iron conditions [118]. At this point, no other ESX-3 components have been characterized.

Contribution to Disease and Physiology—The first clues for understanding the essential function of ESX-3 in *M. tuberculosis* came from the identification of the *esx-3* gene locus as being regulated by the iron-dependent regulator IdeR [119] and the zinc uptake regulator Zur [120]. Subsequently, it was shown that addition of extra iron or zinc can rescue the phenotype of *esx-3* depletion in *M. tuberculosis*. Culture supernatant from wild-type *M. tuberculosis* can also rescue the *esx-3* depletion phenotype. Taken together, these results suggest that ESX-3 secretes a product that is necessary for iron and zinc homeostasis or uptake [117, 118].

A completely different set of studies suggests a role for ESX-3 in eliciting protective immunity against *M. tuberculosis*. An *M. smegmatis esx-3* mutant that is engineered to express the *M. tuberculosis esx-3* genes, named IKEPLUS, elicits a robust immune response and better protective immunity against *M. tuberculosis* challenge than vaccination with *M. bovis* BCG in mice [121]. However, additional studies are required to understand the role of ESX-3 in this novel potential vaccine strain.

Model, Summary, and Future Questions—Our current understanding of the ESX-3 export system of mycobacteria is limited. The ESX-1 system may serve as a model for ESX-3 export, but this possibility remains to be evaluated. While the available data suggests that ESX-3 is responsible for iron and zinc homeostasis or uptake, the identification of ESX-3-exported proteins will be critical for understanding the role of this specialized export system in *M. tuberculosis*.

ESX-5

Introduction—After ESX-1, the next best-studied ESX system is ESX-5 (for a recent review see [122]). The distinguishing feature of ESX-5 is that it exports PE/PPE proteins that contain N-terminal Pro-Glu or Pro-Pro-Glu repeats [123]. The PE/PPE protein family is unique to mycobacteria. Some PE/PPE proteins are localized to the cell surface and are implicated in virulence and/or elicitation of host immune responses (for a review of PE/PPE proteins see [124]). At this point, ESX-5 has only been directly investigated in *M. marinum* and there

are no published studies of *M. tuberculosis* mutants lacking *esx-5*. There are, however, reports of *M. tuberculosis* PE/PPE proteins that are exported in an ESX-5-dependent manner when expressed in *M. marinum* [123, 125, 126]. Thus, the potential exists for there being an equivalent ESX-5 system in *M. tuberculosis*.

Exported Proteins and Targeting—As expected for an ESX system, there is an ESAT-6-like protein secreted by the *M. marinum* ESX-5 system although the exact identity of this protein is not yet known [123]. The more notable feature of ESX-5 is that it secretes PE/PPE proteins that lack Sec or Tat signal peptides. *M. tuberculosis* and other slow-growing mycobacteria possess a large number of these PE/PPE proteins, with almost 10% of the *M. tuberculosis* genome encoding for members of this protein family [127]. Comparative 2D-PAGE analysis of proteins secreted by an *esx-5* mutant or wild-type *M. marinum* identified eight PE/PPE proteins that depend on ESX-5 for export.

LipY is one of the PE/PPE proteins known to be exported by the ESX-5 system of *M. marinum* [126]. *M. tuberculosis* LipY has a PE domain at its N-terminus while *M. marinum* LipY has a PPE domain at its N-terminus. LipY has a lipase domain at its C-terminus and is a demonstrated triacylglycerol hydrolase making it the only PE/PPE protein with a confirmed function [128]. Interestingly, in one study the PE/PPE domain of LipY is cleaved upon export and this domain is necessary for LipY export, suggesting it may function as a targeting element for the ESX-5 system [126]. However, this data is in conflict with another study that shows the PE/PPE domain of LipY is not needed for export when the protein is expressed in *M. bovis* BCG [129].

As mentioned above, an *esx-5* mutant of *M. tuberculosis* has yet to be investigated. However, select *M. tuberculosis* PE/PPE proteins (PPE13, PPE41, PPE10, PE_PGRS45, LipY) are exported to the bacterial cell surface or fully secreted in an ESX-5 dependent fashion when they are expressed in *M. marinum* [123, 125, 126].

System Components and Mechanism—Because *M. smegmatis* lacks an ESX-5 system, it can be used as a mycobacterial host for reconstituting ESX-5 export. When the entire *M. marinum esx-5* locus is introduced into *M. smegmatis* it functions to secrete the *M. tuberculosis* PPE41 protein that is co-expressed in these experiments [125]. At this point, there has been no study of the requirement or function of individual ESX-5 components, but this *M. smegmatis* system should prove useful for such analysis.

Contribution to Disease and Physiology—In *M. marinum*, the ESX-5 system is implicated in modulating innate immune responses of macrophages and in facilitating *M. marinum* dissemination from infected macrophages [125, 130]. In *M. tuberculosis*, however, there are no studies linking ESX-5 to virulence and the system is not predicted by whole genome mutagenesis studies to be important for virulence [131, 132].

Model, Summary, and Future Questions—At least for *M. marinum*, it seems that ESX-5 is required for exporting a number of PE/PPE proteins. While the mechanistic details of ESX-5 export remain to be studied, our current knowledge of the ESX-1 system may be a useful starting point for understanding this system. It should be noted, however, that there is no evidence for ESX-1 exporting PE/PPE proteins which makes it possible that significant differences exist between systems. A major question to be resolved is whether the PE/PPE domain is required for export and serves as a targeting signal for the ESX-5 system [126, 129]. In the future, it will also be important to study the ESX-5 system directly in *M. tuberculosis* and determine if it is functional and important for virulence.

Conclusion

M. tuberculosis has multiple ways to export proteins. In addition to the well known Sec and Tat systems, *M. tuberculosis* possesses specialized SecA2 and ESX protein export systems, and likely other systems as well. Continued study of mycobacterial protein export will be important to fully understand the mechanisms of export and the roles each system plays in pathogenesis. All of the currently known *M. tuberculosis* protein export systems are important in some way to bacterial viability and/or virulence. As such, any of these systems could prove useful as targets for future drug development to combat tuberculosis.

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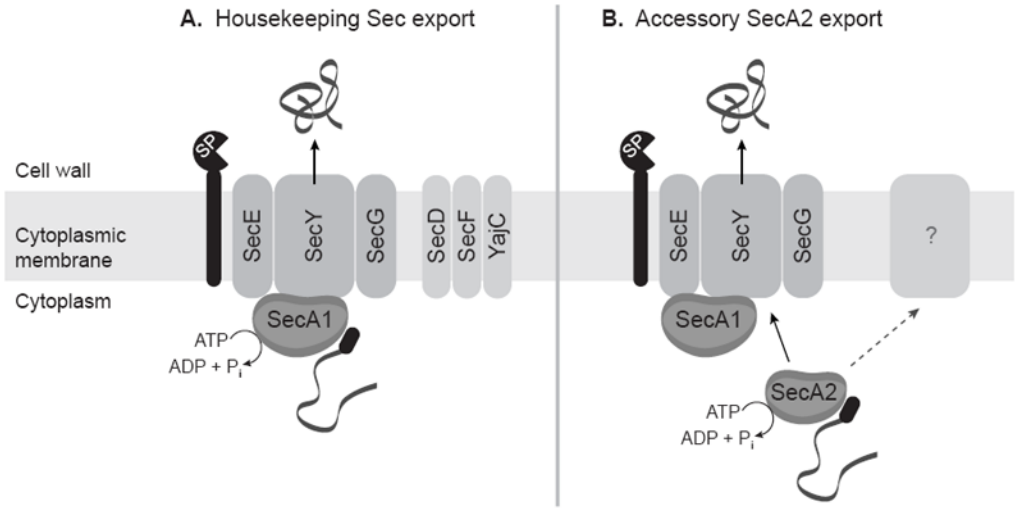

Figure 1. A model of the housekeeping Sec and accessory SecA2 export systems

(A) Preproteins with N-terminal signal peptides (black oval) are recognized by SecA1, which interacts with the SecYEG channel complex to form the translocase. SecA1 performs repeated cycles of ATP hydrolysis, pushing the unfolded preprotein through the SecYEG channel. SecD, SecF, and YajC increase efficiency of protein export. Signal peptides are removed by a LepB or LspA signal peptidase (SP), and mature proteins fold into their final conformations. **(B)** SecA2 recognizes a small subset of proteins and uses its ATPase activity to assist in their export. In the most likely scenario, SecA2 works with components of the housekeeping Sec export system and exports proteins across the cytoplasmic membrane through the SecYEG channel complex. However, it remains possible that other unknown components are required in addition to or in lieu of the housekeeping Sec components. The role of SecA2 in the export of proteins lacking signal peptides (not shown) is currently not understood.

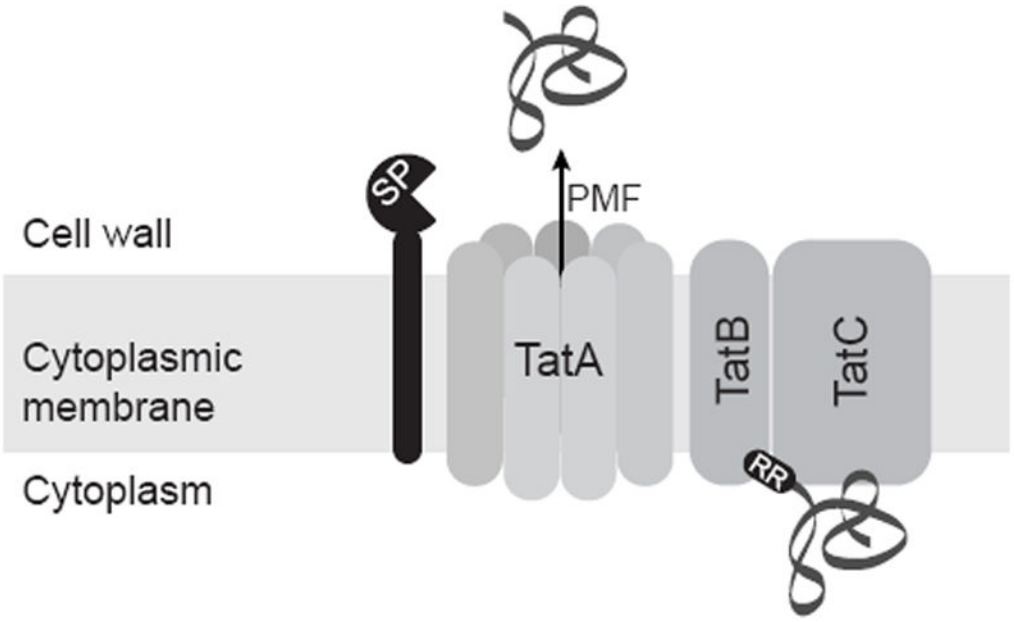

Figure 2. A model of the Tat export system

Tat preproteins contain an N-terminal signal peptide (black oval) with a twin arginine (RR) motif and fold in the cytoplasm prior to export. Tat-dependent preproteins then bind to the TatBC complex in the cytoplasmic membrane. Next, oligomers of TatA are recruited, likely forming the channel complex. In the presence of the proton motive force (PMF), preproteins are exported across the cytoplasmic membrane. Signal peptides are removed by a LepB or LspA signal peptidase (SP).

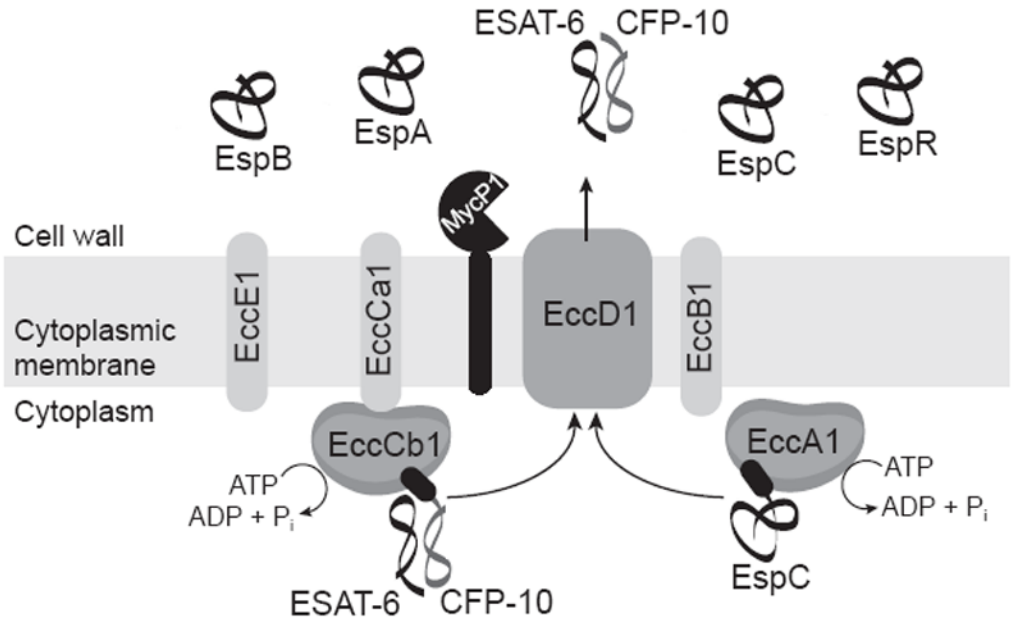

Figure 3. A model of the core components of the ESX-1 export system

ESX-1 exported proteins CFP-10 and EspC have a targeting element at their C-terminus (black ovals) that directs them to interact with cytosolic AAA ATPases EccCb1 and EccA1, respectively. Through interactions with cytoplasmic membrane proteins, these AAA ATPases are proposed to deliver proteins to an ESX-1 export channel. EccD1 is a leading candidate for being a component of this channel. ESAT-6 lacks a C-terminal targeting element and must form a complex with CFP-10 in order to be exported. The functions of EccE1, EccCa1, and EccB1 are unknown, but all are predicted cytoplasmic membrane proteins and core components of the ESX-1 pathway. MycP1 is a membrane protein with protease activity. Other proteins reported to be exported by the ESX-1 system and pictured here are EspA, EspB, and EspR.

Table 1

M. tuberculosis homologs of conserved export system components.

Export system components	Required for *E. coli* growth[a]	*M. tuberculosis* homolog	Required for *M. tuberculosis* growth[b]
SecA	yes	Rv3240c (SecA1)	yes
		Rv1821 (SecA2)	no*† [45]
SecY	yes	Rv0732	yes
SecE	yes	Rv0638	yes
SecG	no	Rv1440	no
SecD	no	Rv2587c	yes
SecF	no	Rv2586c	yes
YajC	no	Rv2588c	no
SecB	no	Rv1957‡	no
Ffh (SRP)	yes	Rv2916c	yes
FtsY	yes	Rv2921c	yes
YidC	yes	Rv3921c	yes
LepB	yes	Rv2903c	yes
LspA	yes	Rv1539	no*† [33]
TatA	no	Rv2094c	yes* [60]
TatB	no	Rv1224	yes* [60]
TatC	no	Rv2093c	yes* [60]

[a] information compiled from references [133-141]

[b] unless marked by *, requirement for growth is predicted by deep sequencing of transposon libraries (Tn-seq) [21]

* experimentally demonstrated

† experimentally demonstrated required for *M. tuberculosis* virulence

‡ see discussion of Rv1957 in text

Table 2

M. tuberculosis proteins containing functional Tat signal peptides.

Protein	Description	Required for *M. tuberculosis* growth[d]	Required for *M. tuberculosis* virulence[e]
Rv0063[a]	Possible oxidoreductase	no	no
Rv0125[b] (PepA)	Possible serine protease	no	no
Rv0129c[b] (Ag85C)	Mycolyl transferase; fibronectin-binding protein C	no* [142]	yes
Rv0132c[c] (Fgd2)	Possible F420-dependent glucose-6-phosphate dehydrogenase	no	no
Rv0315[a]	Possible β-1,3-glucanase precursor	no	no
Rv0483[a] (LprQ)	Possible conserved lipoprotein	no	no
Rv0519c[a]	Possible lipase	no	no
Rv0774c[a]	Possible lipase; short-chain alcohol dehydrogenase family	no	no
Rv0846[a]	Possible multicopper oxidase	no	no
Rv1860[b] (Apa)	Glycosylated fibronectin-binding protein	no	no
Rv2041c[a]	Possible sugar-binding lipoprotein	no	no
Rv2068c[a] (BlaC)	Class A β-lactamase	no* [143]	no
Rv2350c[a] (PlcB)	Phospholipase C	no* [86]	yes* [86]
Rv2351c[a] (PlcA)	Phospholipase C	no* [86]	yes* [86]
Rv2525c[a]	Conserved hypothetical protein	no* [60]	yes*‡ [60]
Rv2833c[a] (UgpB)	Possible glycerol-3-phosphate-binding lipoprotein	no	no
Rv2843[a]	Possible conserved transmembrane alanine-rich protein	no	no
Rv3804c[b] (Ag85A)	Mycolyl transferase; fibronectin-binding protein A	no* [144]	yes* [144, 145]

[a] identified in [64]

[b] identified in [63]

[c] Perkowski, E., Bashiri, G., Baker, E., Braunstein, M., unpublished data

[d] unless marked by *, requirement for growth is predicted by deep sequencing of transposon libraries (Tn-seq) [21]

[e] unless marked by *, requirement for virulence is predicted by Transposon Site Hybridization (TraSH) [131, 132] or Designer Arrays for Defined Mutant Analysis (DeADMAn) [146]

* experimentally demonstrated

‡ see discussion of Rv2525c in text

Table 3

Functions of ESX export systems in mycobacteria.

System	Function
ESX-1	virulence (*M. tuberculosis* [92, 94, 96-98]) (*M. marinum* [147])
	conjugation (*M. smegmatis* [148, 149])
ESX-2	not studied
ESX-3	essential for viability (*M. tuberculosis* [117]) iron and zinc uptake or homeostasis (*M. tuberculosis* [117, 118])
ESX-4	not studied
ESX-5	immune modulation (*M. marinum* [125, 130])