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Structure and Function of the Mycobacterial Type VII Secretion Systems

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Abstract

Bacteria have evolved intricate secretion machineries for the successful delivery of large molecules across their cell envelopes. Such specialized secretion systems allow a variety of bacteria to thrive in specific host environments. In mycobacteria, type VII secretion systems (T7SSs) are dedicated protein transport machineries that fulfill diverse and crucial roles, ranging from metabolite uptake to immune evasion and subversion to conjugation. Since the discovery of mycobacterial T7SSs about 15 y ago, genetic, structural, and functional studies have provided insight into the roles and functioning of these secretion machineries. Here, we focus on recent advances in the elucidation of the structure and mechanism of mycobacterial T7SSs in protein secretion. As many of these systems are essential for mycobacterial growth or virulence, they provide opportunities for the development of novel therapies to combat a number of relevant mycobacterial diseases.

Contents

1. INTRODUCTION

Mycobacterium tuberculosis was the cause of over 10 million new cases of tuberculosis and 1.5 million deaths in 2018 alone (116). Mycobacteria use type VII secretion systems (T7SSs), also called ESX systems, to transport a diverse repertoire of proteins across their distinctive diderm cell envelope. Since the discovery of mycobacterial T7SSs, approximately 15 y ago (98), evidence has been mounting as to the central roles of these protein transport machineries for the successful establishment of infection, especially of the clinically relevant species, and also for the fitness of mycobacteria (1, 15, 41, 50, 68, 86). With up to five different mycobacterial T7SSs, named ESX-1 to ESX-5, their importance is further emphasized by the lack of the ESX-1 system as the decisive factor in the attenuation of the live attenuated vaccine strain *Mycobacterium bovis* BCG (81, 93). T7SSs are prime candidates for targeted drug discovery, given their overall importance in the life cycle of pathogenic mycobacteria (25, 85). As such, knowledge of the exact structure and function of these systems and their components is crucial in identifying new ways to combat mycobacterial diseases.

T7SSs are not specific for mycobacteria but are well conserved in a wide range of other *Actinobacteria*, including species with a monoderm cell envelope (55). The functionality of T7SSs in other *Actinobacteria* has only been studied in several *Streptomyces* species (3, 43), but their role in protein secretion in these organisms is far from clear. In addition, related T7SSs are functional in several *Firmicutes*, such as *Staphylococcus aureus* and *Bacillus subtilis*, although they share only a limited number of the mycobacterial T7SS components and thereby will have a distinct architecture (15). The homologous system in *S. aureus*, named Ess, has been shown to be required for virulence in different infection models and to be involved in toxin-mediated bacterial competition (20). While the role of the *B. subtilis* system, called the YukE/Yue system, is less clear, specific RNase toxins are predicted to be secreted by this system (52). Predicted T7SS secreted

toxins are actually widespread in *Firmicutes*, indicating a dedicated role of T7SSs in interbacterial antagonism in this phylum (115). In this review, we focus on mycobacterial T7SSs and provide an overview of new findings on their structure and function.

1.1. Build-Up of the Mycobacterial Cell Envelope

The complex cell envelopes of bacteria are pivotal components for their existence in a wide range of environments. For instance, pathogenic mycobacteria strictly depend on their unique cell envelope to withstand conditions faced during their intracellular infection cycle. Small alterations to this layer will generally lead to attenuation. The mycobacterial cell envelope is complex and composed of a cytoplasmic membrane, a cell wall with a specific outer membrane (OM), also known as the mycomembrane, and an outermost capsule-like layer (29, 36). The mycobacterial cell envelope is highly hydrophobic, with up to 40% of its dry weight attributable to lipids, as compared to the 20% lipid content found in gram-negative bacteria (16, 28).

While the mycobacterial cytoplasmic membrane is largely similar to that of gram-positive and gram-negative microorganisms (28), it does contain specific lipids, such as phosphatidylinositol mannosides, lipomannans, and lipoarabinomannans (36). The mycobacterial cell wall is more specific, as it consists of a peptidoglycan layer that is covalently linked to an additional polymer of arabinose and galactose, called the arabinogalactan layer. In addition, this structure is even more extended, as linked to the arabinogalactan layer are long-chain fatty acids (C60 to C90), called mycolic acids, that make up part of the inner leaflet of the mycobacterial outer membrane. The outer leaflet of this second membrane contains free mycolic acids, of which some are linked to trehalose residues to make trehalose monomycolate or trehalose dimycolate, and other specific lipids. The overall low permeability of the mycobacterial cell envelope provides intrinsic resistance to many antibiotics and other antimicrobial (host) factors.

The outermost capsular layer can be observed by cryo–electron microscopy (cryo-EM) as a labile structure that is sensitive to the presence of detergents, which are in fact regularly added to mycobacterial growth media as anticlumping agents (87). This outer layer, which is composed of proteins, glycan-like polysaccharides, and small amounts of lipids, has been implicated as important for the pathogenesis of *M. tuberculosis* (57). Its molecular composition is believed to be different between pathogenic and nonpathogenic species (29, 63, 73). Recent data have outlined an important role for the ESX-5 system and one of its secreted substrates, PPE10, in maintaining the integrity of the capsular layer in the fish pathogen and model species *Mycobacterium marinum* (9).

Mycobacteria use a set of secretion systems to transport proteins across their distinctive diderm cell envelope. The conserved Sec and Tat translocation systems, which exist in all bacteria, mediate protein transport over the inner membrane, as recently reviewed in Reference 110. Substrates of these two pathways contain a characteristic N-terminal signal peptide that is cleaved off upon transport. In addition to this, mycobacteria possess the SecA2 pathway, which exports in concert with the canonical Sec translocon a small subset of proteins (110). While some of the Sec and Tat substrates remain in the putative periplasmic space between the two membranes, other proteins are fully secreted into the extracellular environment by currently unknown secondary mechanisms. For T7SSs, localization of substrates in the periplasm has not been observed so far, indicating that this pathway is dedicated for the transport of proteins across the complete envelope of mycobacteria.

1.2. Roles of ESX Systems in Virulence, Bacterial Physiology, and Conjugation

The initial characterization of T7SSs focused on their critical roles in virulence, such as phagosomal escape and immune modulation. However, in more recent years it has become clear that ESX

systems and their substrates play more diverse roles, including horizontal gene transfer, nutrient and metabolite uptake, and cell physiology (50, 68, 110). While the ESX-2 system has not been characterized, the roles of the other four systems have been studied to various degrees.

ESX-1 is the first discovered and one of the best described mycobacterial T7SSs. It plays an important role in the macrophage infection cycle of pathogenic species. *M. tuberculosis* and *M. marinum* have been shown to be able to rupture the phagosomal membrane and thereby translocate to the cytosol (44, 93). In the cytosol they replicate and induce necrosis-like cell death, resulting in bacterial spread to other macrophages (50). In the absence of an active ESX-1 system, mycobacteria remain in the phagosomal compartment (53, 93). The ability to lyse erythrocytes is also lost in *esx-1* mutants (44, 58). While membrane-lysing activity has been observed for the major ESX-1 substrate, EsxA (34, 58, 94), the role of this substrate in phagosomal rupture has recently been challenged (27; see below).

In nonpathogenic *Mycobacterium smegmatis*, ESX-1 seems to have a very different role, as in this species the system has been shown to be involved, together with the more archaic ESX-4 system, in a special form of horizontal gene transfer called distributive conjugal transfer (DCT) (48). This process is unidirectional and allows for the transfer of large fragments of chromosomal DNA (49). Interestingly, the recipient and donor strains have different genetic requirements for DCT to take place. While the recipient strain requires both an ESX-1 system and an ESX-4 system for DNA uptake, for the donor strain crucial mutations in the ESX-1 system result in hyperconjugation, and ESX-4 is dispensable for DCT (48). In addition, the ESX-4 transcription regulator SigM is upregulated in the recipient strain upon contact with the donor cell, suggesting that ESX-4 is induced upon cell-cell contact (24). Another ESX system plays a role in a more classical form of conjugation, namely, the ESX-P1 system encoded by the pRAW plasmid in *M. marinum* (105). Conjugation of pRAW is in fact dependent on two separate plasmid-encoded gene clusters, one coding for ESX-P1 and the second encoding for a type IV–like secretion system. Type IV secretion systems are required and sufficient for plasmid conjugation in other bacteria (23). More pRAW-like plasmids have been identified in different mycobacterial species with the same gene clusters, leading to the hypothesis that these plasmids could also be conjugative and important for the evolution and distribution of *esx* gene clusters (71).

ESX-4 is considered to be the most archaic ESX system, but its function is still ill characterized. In most species it is lacking a number of genes that are present in the other ESX systems, i.e., genes encoding for PE and PPE substrates and the conserved system components EspG and EccE (12, 26; see below). Besides its role in DCT in *M. smegmatis*, ESX-4 has been described to be required for full virulence in *Mycobacterium abscessus* (62). More specifically, a functional ESX-4 system was shown to be required for phagosomal rupture and bacterial escape to the cytosol (62). Of note is that in *M. abscessus*, in contrast to the case of most ESX-4 systems, a copy of $EccE_4$ is present. Furthermore, *M. abscessus* lacks an ESX-1 system, suggesting that ESX-4 takes over the role of ESX-1 in this species.

While ESX-1, ESX-2, and ESX-4 are dispensable for growth of *M. tuberculosis*, both the ESX-3 and the ESX-5 systems are essential for viability, probably due to their role in the uptake of metabolites and nutrients, respectively (8, 88). In addition, specific ESX-3 and ESX-5 substrates have been implicated to play a role in immune modulation (6, 103). The ESX-3 system is upregulated under low levels of iron (67, 83) and is involved in the uptake of iron (92), and in the case of *M. tuberculosis* also of zinc (89). Its role in iron uptake is further supported by the fact that addition of high levels of hemin in the growth medium allows for mutations in the *esx-3* locus of *M. tuberculosis* (89).

ESX-5 is the most recently evolved T7SS (71) and is only present in slow-growing mycobacteria, which include most pathogenic species, such as *M. tuberculosis*, *M. leprae*, *M. ulcerans*, and

M. marinum (46). The role of ESX-5 in nutrient uptake is supported by the observation that this system is involved in uptake and utilization of fatty acids in *M. marinum* (8) and that in *M. tuberculosis* ESX-5 is upregulated under low phosphate conditions by the Pst-SenX3-RegX3 system (39). In addition, the essentiality of ESX-5 can be alleviated in *M. marinum* by increasing the OM permeability, either by mutations that block synthesis of phthiocerol dimycocerosates and phenolic glycolipids or by heterologous expression of the MspA porin, which is exclusively found in fast-growing mycobacterial species (8, 99). This suggests that ESX-5 or ESX-5 substrates play a role in nutrient transfer across the mycobacterial OM. Recently, it has been shown that, in *M. tuberculosis*, specific PE/PPEs might indeed act as selective channels to take up nutrients across the impermeable mycomembrane (61, 114).

2. T7SS SUBSTRATES

2.1. Esx Substrates

T7SS substrates are among the most abundantly secreted proteins in mycobacteria. An unusual feature of these proteins is that many of them are secreted as (partially) folded heterodimers. The first group of substrates, called Esx proteins, belongs to the family of WxG100 proteins, which are characterized by a length of approximately 100 amino acids and a conserved WxG motif in between two α helices. One partner protein of Esx heterodimers has a C-terminal conserved sequence YxxxD/E, which is required for secretion (78). In mycobacteria, the two genes that encode for these heterodimers are always paired in an operon. In contrast, for the T7SS-like systems of certain *Firmicutes* species, the Esx proteins are produced from monocistronic transcripts and are secreted as homodimers (10, 90, 102). Each of the five mycobacterial T7SS loci contains a pair of *esx* genes. Furthermore, additional *esx* gene pairs that are highly homologous to the loci-associated genes are scattered throughout the mycobacterial genome (12, 26).

The first-discovered and best-studied Esx heterodimer, the EsxB/EsxA proteins (also called CFP10/ESAT-6), are secreted via the ESX-1 system. The two proteins interact by their helix-turn-helix structures in an antiparallel manner, forming a four-helix bundle, which is a conserved feature for all T7SS substrates (4, 56) (**Figure 1*a***). The structure of the EsxB/EsxA complex also reveals that the N- and C-terminal tails of both proteins are flexible (**Figure 1*a***). This has important implications, as the C terminus of EsxB, which also contains the YxxxD/E secretion signal, is recognized by the ESX-1 secretion machinery (22, 84). This YxxxD/E motif is structurally in close proximity to the WxG motif of the partner protein EsxA, likely forming a composite secretion signal (82).

EsxA has been shown to have pore-forming abilities after dissociation from EsxB postsecretion, suggesting this substrate is responsible for phagosomal rupture upon macrophage infection (93, 106). As EsxB does not show any membrane-lysing competence, this protein is hypothesized to be a chaperone, keeping EsxA in a translocation-competent state (34, 94). The role of EsxA in phagosomal rupture, however, is still debatable, as newer studies assigned the pore-forming activities to the detergents present in the protein preparations (27).

2.2. PE/PPE Substrates

The largest group of T7SS substrates is the highly polymorphic PE and PPE proteins that, with 169 *pe* and *ppe* genes, cover ∼10% of the coding capacity of *M. tuberculosis* (26). Both the PE and PPE protein families are named after their conserved proline (P) and glutamic acid (E) motifs, present at their N termini. *esx* clusters, except for *esx-4*, contain *pe* and *ppe* genes, but most PE and PPE substrates are not encoded by *esx* loci (12, 26, 46). PE and PPE proteins contain conserved

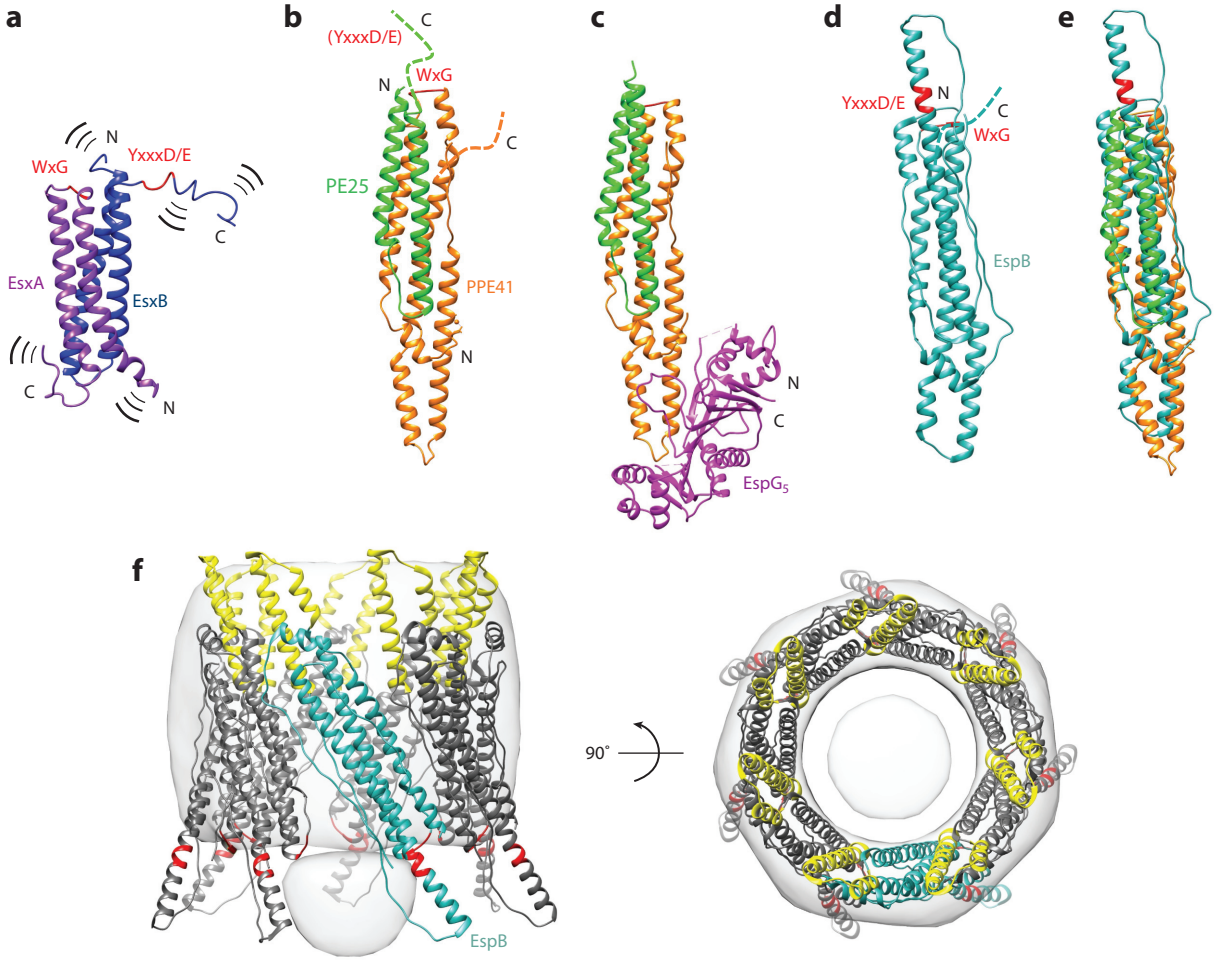

Figure 1

Structures of type VII secretion system substrates. (*a*) Ribbon representations of the NMR structure of EsxA/EsxB (PDB 1WA8) with the flexibility of the N and C termini indicated by motion lines, (*b*) crystal structures of PE25/PPE41 (PDB 2G38), (*c*) PE25/PPE41/$EspG_5$ (PDB 4KXR), and (*d*) EspB (PDB: 4WJI), and (*e*) superposition of EspB with PE25/PPE41 (PDB 4WJI and 2G38). The YxxxD/E secretion signal motif and WxG motif are shown in red. Dotted lines represent missing structural features. (*f*) Side and top views of a negative-stain model of an EspB multimer (EMD 6120) with a heptameric EspB model (PDB 3J83) fit into the EM density. The hydrophobic rim proposed to be able to penetrate membranes is depicted in yellow. N and C denote the N termini and C termini. Abbreviations: EM, electron microscopy; EMD, Electron Microscopy Data; NMR, nuclear magnetic resonance; PDB, Protein Data Bank.

N-terminal so-called PE and PPE domains of ~110 and 180 amino acids, respectively (6, 46), while their C termini can be highly variable in sequence and size (46).

The conserved PE and PPE domains that have been examined show a characteristic helix-turn-helix structure, akin to the Esx substrates (100) (**Figure 1*b***). For PE proteins, these two α helices almost completely cover the 110–amino acid PE domain, which also contains the conserved YxxxD/E secretion signal at the C terminus (30, 84, 96). The slightly longer PPE domain contains, besides a helix-turn-helix structure with the conserved WxG motif in the turn, three additional α helices (**Figure 1*b***). This extended domain, not present in PE and Esx proteins, binds the EspG chaperone (see below).

Several *pe* genes are paired with a *ppe* gene in an operon, and these paired genes produce PE/PPE heterodimers (6, 46, 86). Dimerization is mediated by the two helices of the PE domain together with the helix-turn-helix structure of the PPE domain, forming an antiparallel four-helix bundle, similar to Esx heterodimers. Also in these heterodimers, the YxxxD/E motif in the C terminus of the PE protein is placed in proximity to the conserved WxG motif of the PPE protein (38, 59, 100).

The highly variable C-terminal domains of PE and PPE proteins are much less characterized, as only a few PE and PPE proteins have (predicted) functional domains in this region (19, 35, 103). Nevertheless, several conserved motifs can be distinguished in these C-terminal domains, which have been used to subclassify *pe* and *ppe* genes. The major subgroup of the PE protein family is the PE_PGRS proteins, named after the polymorphic GC-rich repetitive sequence motifs in their genes. While some studies show that PE_PGRS proteins are required for virulence (14, 41), another study indicated a role in virulence attenuation (7). PPE proteins can be subdivided into three distinct subclasses, i.e., the PPE-PPW proteins that contain a PxxPxxW motif close to their C terminus, the PPE-SVP proteins that contain an SVP amino acid sequence at their C terminus, and the most recently evolved group of PPE-MPTR proteins, named after the presence of major polymorphic tandem repeat sequences that vary around a NxGxGNxG motif (6, 46).

As the genetic analysis of PE/PPE substrates is complicated by their large number of repeats and high sequence identity due to gene duplications, a clear function has not been assigned for most of them. Additionally, detection by mass spectrometry is hindered by the low number of trypsin digestion sites.

2.3. Esp Substrates

While each ESX system secretes its own set of Esx, PE, and PPE proteins, the final group of substrates, i.e., the Esp (ESX secretion associated protein) proteins, are specific for the ESX-1 system (21, 70, 77). Structural information is only available for EspB, which is secreted as a monomer and cleaved by the conserved component $MycP_1$ upon export (72) (**Figure 1*d***; see below). Although monomeric, the N-terminal part of this protein shows a structure that is highly similar to that of the PE/PPE heterodimer (96) (**Figure 1*e***). Interestingly, this N-terminal domain of EspB has been shown to multimerize into doughnut-shaped heptamers, hypothesized to be able to insert into host cell membranes via a hydrophobic rim (60, 96) (**Figure 1*f***). A number of Esp proteins, especially EspA, EspC, and EspD, which are encoded by the same operon, have been shown to be involved in ESX-1 functioning, as deletion of the corresponding genes results in a general ESX-1 secretion defect (42, 66). As the *espACD* operon has been found only in slow-growing and thereby pathogenic mycobacteria and it is transcriptionally regulated by multiple regulatory systems (50), it has been suggested to be involved in virulence. Three Esp proteins that are encoded by the *esx-1* locus, i.e., EspE, EspF, and EspH, are homologous to EspA, EspC, and EspD, respectively (77). EspC and EspF have, similar to Esx proteins, a size of ~100 amino acids and contain the conserved YxxxD/E motif at their C terminus. In addition, these proteins interact with EspA and EspE, respectively, suggesting these Esp proteins are also secreted as heterodimers (64, 77). EspH, which is not secreted, is required for the secretion of the EspE/EspF pair and interacts with EspE in the cytosol, suggesting this protein acts as a specific chaperone for the secreted Esp pair (77). As two other Esp proteins, i.e., EspD and EspL, show (structural) homology to EspH, these proteins might constitute a group of specific chaperones for the Esp substrate family (75).

3. T7SS CYTOSOLIC COMPONENTS

3.1. EspG and Other Specific Chaperones

EspG proteins are conserved T7SS chaperones that are exclusively located in the cytosol. They are present in all mycobacterial *esx* loci that encode PE/PPE substrates (75). Due to their shared low identity, they were originally not considered an Ecc (ESX conserved component) and therefore mistakenly named Esp (12). EspG chaperones specifically interact with their cognate PE/PPE heterodimers in the cytosol (31), and this interaction is essential for secretion. For instance, $EspG_5$, i.e., EspG of the ESX-5 system, is important for the secretion of several ESX-5 substrates (2), whereas the absence of $EspG_1$ results in a defect of the ESX-1 substrate PPE68 (14).

Although EspG components from different *esx* loci show relatively little sequence conservation, their overall structure is highly similar, with a quasi-twofold symmetry, where each half contains five β strands and four helices (38, 59, 104). Heterotrimeric $EspG_5$-PE25-PPE41 crystal structures reveal that $EspG_5$ interacts at the helical tip of the PPE protein through multiple hydrophobic interactions, hydrogen bonds, and salt bridges (31, 38, 59) (**Figure 1*c***). The tip structure, consisting of the loop between helices four and five of the PPE protein, is inserted in a hydrophobic groove of the EspG chaperone, which protects the PE/PPE dimer from self-aggregation (38, 59) (**Figure 1*c***). The tip region is well conserved among the ESX-1, ESX-3, and ESX-5 secreted PPE proteins, and so far, no specific amino acids have been found to define the specificity for EspG binding (38, 59). Importantly, the EspG-binding domain is involved in determining system specificity, as exchanging this domain between PPE proteins of different ESX systems, i.e., ESX-1 and ESX-5, leads to their rerouting via the new system in *M. marinum* (76). As discussed above, similar specific chaperones might be required for the secretion of Esp proteins, namely EspH and its homologs.

As also already mentioned, from the observation that at least several substrates are secreted as heterodimers, it has been suggested that one partner protein of these heterodimers might act as a (second) chaperone of the other partner protein (1). However, it is highly unusual that these potential chaperones are cosecreted with their substrates, suggesting that they might also have a role during translocation over the mycobacterial cell envelope.

3.2. EccA

EccA is a T7SS cytosolic conserved component that belongs to the family of AAA+ (ATPases associated with diverse cellular activities) proteins and potentially has chaperone-like activity. Similar to the case of *pe*, *ppe*, and *espG* genes, *eccA* is present in all *esx* loci, except *esx-4* (12, 26). EccA is composed of two structural domains, the C-terminal AAA+ ATPase domain and an N-terminal domain that contains six tandem tetratrico-peptide-repeat (TPR) motifs, known to be involved in protein-protein interactions (45, 65, 113). Only the structure of the TPR domain has been solved, showing 12 antiparallel helices arranged into the six TPR motifs (113). In vitro expression of only the ATPase domain shows more robust activity compared to the full-length protein, suggesting that the N terminus might play a regulatory role. Although not solved structurally, the C-terminal ATPase domain can be modeled based on available structures of other AAA+ proteins (65). These models show that the ATPase domain drives oligomerization into hexamers, a general feature of AAA+ proteins (65).

Although EccA is a conserved component, its role in secretion remains unclear. While several reports have documented the requirement of $EccA_1$ and $EccA_5$ for secretion of ESX-1 and ESX-5 substrates, respectively, other studies have shown that they are dispensable for secretion (75). This variation can be explained by a more recent observation where the importance of $EccA_1$ in the secretion of ESX-1 substrates in *M. marinum* varied between different growth media (77).

As EccA homologs are present only in systems that encode for PE/PPE substrates, and an AAA+ ATPase is involved in the disassembly of the type VI secretion apparatus (13), EccA has been proposed to have a role in the dissociation of the EspG chaperone from the PE/PPE substrate heterodimers upon targeting to the membrane complex for secretion (38). However, evidence for such a role is still lacking.

4. T7SS MEMBRANE COMPLEX

4.1. Membrane Components

The mycobacterial ESX systems contain a set of five conserved membrane proteins called EccB, EccC, EccD, EccE, and MycP. All systems code for these five membrane components, besides ESX-4, which usually does not have a copy of EccE, the exception being *M. abscessus*. Structures of several soluble domains of these membrane components have been solved by X-ray crystallography (84, 95, 111, 112, 117) (**Figure 2*a–d***). In addition, structures of ESX-5 and ESX-3 complexes, composed of the four Ecc components, have recently been solved by negative stain and cryo-EM (11, 40, 79) (**Figures 2*d*** and **3*a***).

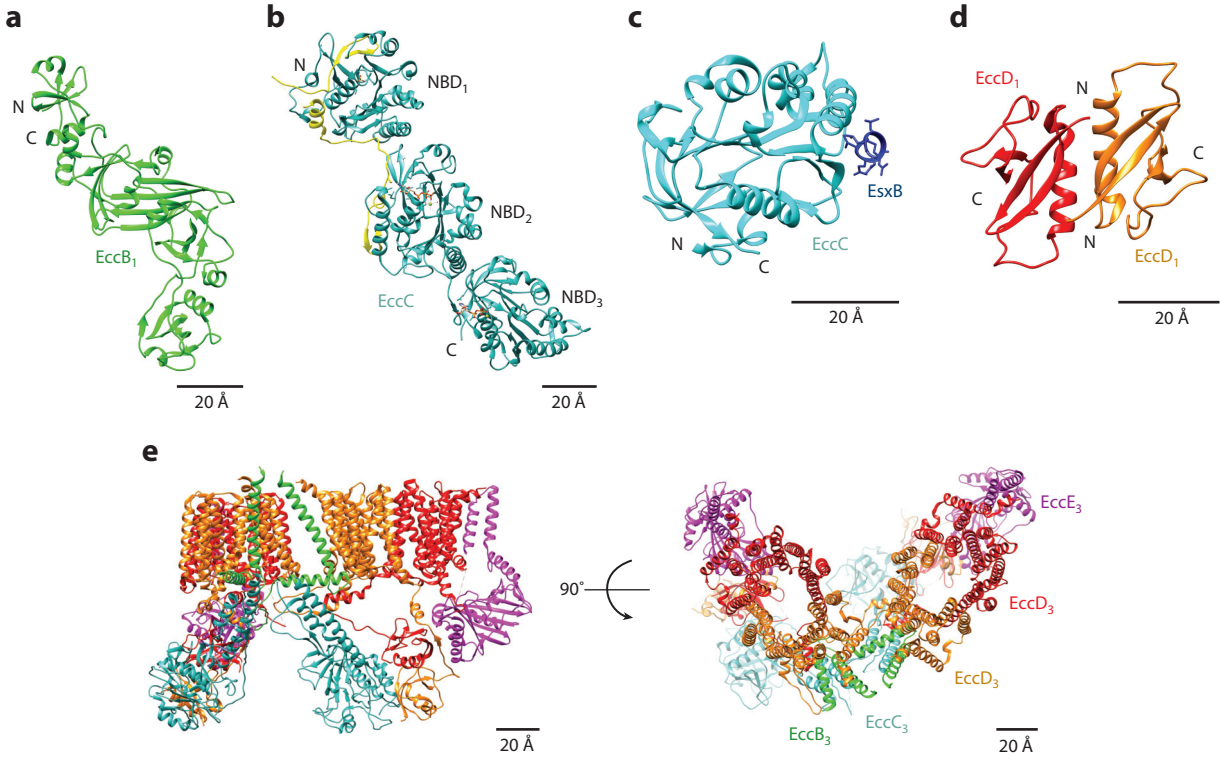

Figure 2

Membrane components of mycobacterial type VII secretion systems. Crystal structures of (*a*) soluble domain of $EccB_1$ from *Mycobacterium tuberculosis* (PDB 4KK7), (*b*) the three NBDs of EccC from *Thermomonospora curvata* (PDB 4NH0), with linker 2 connecting the first and second NBD in yellow, (*c*) the third NBD of EccC from *T. curvata* (*light blue*) in complex with a peptide mimicking the C terminus of EsxB (*dark blue*; PDB 4N1A), and (*d*) dimeric soluble domain of $EccD_1$ from *M. tuberculosis* (PDB 4KV2). (*e*) Front and top views of a cryo–electron microscopy structure of the dimeric ESX-3 membrane complex from *M. smegmatis* (PDB 6UMM). N and C denote the N termini and C termini. Abbreviations: NBD, nucleotide-binding domain; PDB, Protein Data Bank.

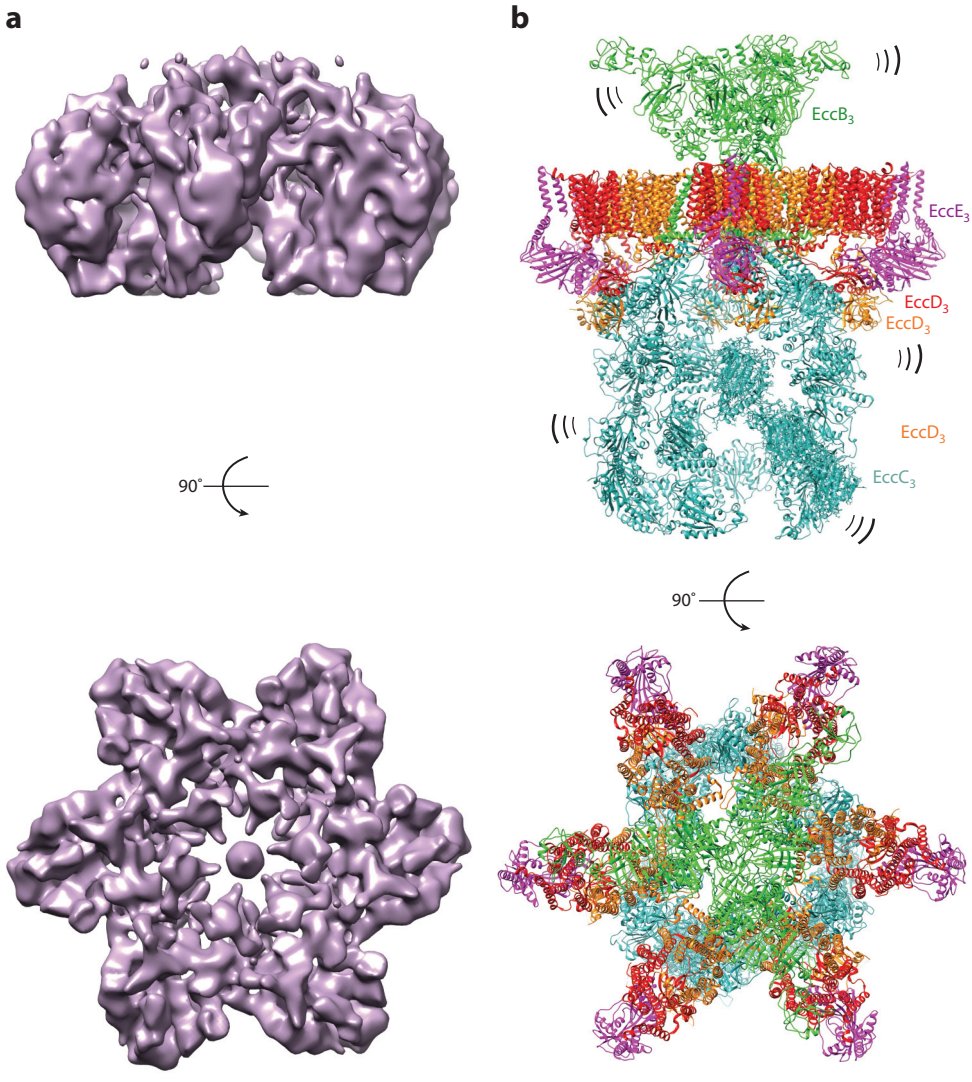

Figure 3

Hexameric view of the membrane complex. (*a*) Side and top views of a negative-stain electron microscopy structure of the ESX-5 membrane complex from *Mycobacterium xenopi* (EMD 3596). (*b*) Side and top views of a hexameric model of the ESX-3 membrane complex based on the dimeric structure from **Figure 2*e*** (PDB 6UMM), dimeric EccB (PDB 6SGY), and a structural model of $EccC_3$ generated with Phyre2 using the structure of EccC from *Thermomonospora curvata* (PDB 4NH0) as a template. The flexibility of the soluble domains of the $EccB_3$ and $EccC_3$ subunits is indicated by motion lines. Abbreviations: EMD, Electron Microscopy Data; PDB, Protein Data Bank.

EccB is a single-pass transmembrane protein that mainly localizes to the periplasm with an elongated domain in the shape of a distorted propeller showing a quasi-twofold symmetry (111, 117) (**Figure 2*a***). The structure contains a hydrophobic core domain that is stabilized by a disulfide bridge between the central β strands and is flanked by two repeat domains on each side (111). Although it has no obvious structure similarity, it shows weak homology to the gram-positive

phage lysin protein PlycB, suggesting it might be able to bind to the cell wall (111), which, given its localization, seems likely. The observation that EccB would have ATPase activity is rather unusual, as the compartment where the main portion of this protein is located, the periplasm, is devoid of ATP (117). While predominantly periplasmic, the high-resolution cryo-EM structure of an ESX-3 membrane complex shows that the small N terminus of $EccB_3$ reaches into the cytoplasm, where it interacts with $EccC_3$ (40, 79). This suggests that potential conformational changes that take place upon substrate binding and/or nucleotide hydrolysis by EccC (see below) could induce conformational changes of the periplasmic domain of EccB (11, 40; see below).

EccD is, with 11 transmembrane domains, the most hydrophobic membrane component. The ~110–amino acid structure of the soluble N-terminal domain of EccD shows a ubiquitin-like fold and is dimeric in solution (111) (**Figure 2*d***). In the context of the membrane complex, EccD forms crucial interactions with both EccC and EccE, revealing why previous characterizations of $eccD_5$ knockouts in *M. tuberculosis* showed a strong negative effect on the functionality and assembly of the ESX-5 membrane complex (54).

EccE has two transmembrane domains and a C-terminal cytosolic domain that shows weak structural homology to glycosyl transferase proteins, although it lacks binding pockets or catalytic sites (11, 79). EccE is localized at the exterior of the membrane complex (11, 40, 79).

The EccC ATPase contains two transmembrane domains at its N terminus and is, together with the Esx dimers, the only protein that is conserved in the related T7SSs in *Firmicutes* and therefore a central component of T7SSs (12, 74). Protein predictions and subsequent structural studies show that the protein has three consecutive FtsK/SpoIIIE-like nucleotide-binding domains (NBDs) (12, 84) (**Figure 2*b***). Members of the FtsK/SpoIIIE ATPase family form hexamers, which is generally the state in which they show ATP hydrolysis capacities (5, 37), and in certain cases activity/hexamerization is regulated by substrates (69). Of the three domains, the ATPase activity of only the first one is critical for system functionality (8, 84). The ATPase activities of the two most C-terminal domains are only required for optimal protein secretion and probably serve a regulatory role within T7SSs (8, 84). Critical for EccC ATPase activity seems to be an uncoupling event where the detachment of the so-called linker 2 domain from the first NBD is required for NBD_1 activation (84). From structural data on the ESX-3 complex, it appears that the previously characterized DUF domain, which links the transmembrane domains and the three NBDs (84), actually exhibits an NTPase-like fold and might be an additional ATPase domain (40). This DUF domain interacts with the cytoplasmic domain of EccD and the N-terminal tail of EccB in the membrane complex (40, 79). An interesting feature of the EccC ATPase is its inherent flexibility (11, 40, 79, 84). This has been observed both for the isolated soluble domain of EccC (11, 84) and for full-length EccC in the context of the membrane complex (11, 40, 79).

The fifth membrane component, mycosin or MycP, is a membrane-bound, subtilisin-like serine protease that is not part of the core membrane complex (11, 54, 107) but is highly conserved within actinobacterial T7SSs (55). Crystal structures of $MycP_1$ and $MycP_3$ show a typical subtilisin-like α/β structure with seven β sheets surrounded by eight α helices and an active site consisting of an aspartic acid, histidine, and serine residues (95, 112). While subtilisin-like proteases are usually produced with an N-terminal extension, called propeptide, that blocks the active site and is degrading upon protease maturation, mycosins contain a rather unusual N-terminal extension that does not block the active site and is not processed (112). Instead, this domain is tightly wrapped around the protease domain and is most likely involved in protein stability (101, 109). Mycosins are probably involved in processing of substrates, as $MycP_1$ has been shown to cleave the ESX-1 substrate EspB (72, 107). Intriguingly, while MycP is essential for ESX secretion, its protease activity is not (72, 107). This essentiality of MycP is probably owed to its role in the stabilization of the T7SS core membrane complex through a relatively loose or transient interaction (107, 108).

4.2. Assembly of the Core Complex

All five membrane components have been shown to be essential for secretion in the ESX systems that have been functionally analyzed so far, i.e., ESX-1 (17, 72, 98), ESX-3 (91), and ESX-5 (8, 14, 54). Additionally, deletions of EccB, EccC, or EccD majorly affect the assembly of the membrane complex (54), while in the absence of EccE or MycP, the membrane complex becomes unstable (11, 107). The recent EM structures provide much needed information on the composition and architecture of the T7SS membrane complex (11, 40, 79) (**Figures 2*e*** and **3*a***) and represent a milestone in mycobacterial research.

Initial blue native PAGE (polyacrylamide gel electrophoresis) analysis has shown that the four Ecc components of ESX-1 and ESX-5 from *M. marinum* and *M. bovis* form similar full-length membrane complexes of >1.5 MDa (54, 107). Subsequently, a 13-Å resolution structure of an ESX-5 system, solved by negative-stain EM, described a large and relatively unstable hexameric complex (11) (**Figure 3*a***). Based on the size and stoichiometry determination by mass spectrometry, it was estimated that the complex is composed of six copies of each component. Although the use of staining agents limits the overall acquired detail of the protein complex, a large central pore is visible with a diameter of ∼50 Å, which should be able to accommodate folded heterodimeric substrates. In addition, six copies of EccB could fit in the center of the periplasmic side of the complex, while immunogold-labeling positioned EccE at the periphery of the membrane complex. Although visible on the single-particle level as randomly oriented long extensions stemming from the core structure, EccC is not resolved in the 3D reconstructed image due to its high flexibility (11).

High-resolution structural information was recently obtained by two groups who analyzed an intermediate, more stable, dimeric structure of the ESX-3 complex from *M. smegmatis* (40, 79) (**Figure 2*e***). Although higher oligomeric states were observed, these were not analyzed in detail (40, 79). The 3.7-Å structures show two protomers, linked through EccB-EccB interactions mainly through their periplasmic domains, with an EccB/C/D/E stoichiometry of 1:1:2:1. The difference in stoichiometry is probably due to the hydrophobic nature of EccD, which is prone to aggregation upon detergent extraction (E.N.G. Houben, C.M. Bunduc, personal observation). Each EccD monomer forms crucial interactions with either the cytoplasmic domain of the peripheral protein EccE, or with EccC, explaining why EccD is crucial for complex stability (40, 79). It has been suggested that the observed protomer, with a lipid-containing cavity between the EccD dimers (**Figure 2*e***), might function as secretion machinery. However, based on multiple observations of high-molecular-weight oligomeric complexes in mycobacteria (11, 40, 54, 107), but also on the likelihood that EccC functions as a hexamer, similar to other FtsK/SpoIIIE ATPases (47), the functional T7SS unit most likely is an ∼2-MDa hexameric complex (**Figure 3*b***). Intriguingly, the hexameric complex is relatively unstable upon extraction from the mycobacterial cell envelope, resulting in the appearance of distinctive subcomplexes, all of which contain the four Ecc membrane components (11, 40, 54). As such, multiple intermediate states are visible upon membrane extraction, similar to pie slices, where each slice, i.e., the protomer, still contains all four components. This suggests that, while the single or dimeric protomer is a more stable unit (11, 40), the hexameric complex requires certain stabilization techniques for its successful (structural) analysis upon extraction from their native environment (11, 40). A possible explanation for this high instability could be that the MycP protease, which is a stabilizing factor for the core membrane complex, is not copurified with the membrane complex (107). In addition, lipids of the inner membrane might be crucial for complex stability (36), as lipids are known to alter the oligomeric state of membrane proteins (51, 80). What is clear from the structural analysis is that the dimensions of the complex dictate that it only span the mycobacterial inner membrane.

This means that the mechanism of substrate translocation across the mycobacterial OM remains unknown (see below).

5. MECHANISM OF SUBSTRATE RECOGNITION AND TRANSPORT

5.1. Substrate Recognition by the EccC ATPase and Mechanism of Inner Membrane Translocation

Although the mechanism of substrate recognition and transport through the diderm mycobacterial cell envelope is still little understood, recent studies have provided crucial insight into the mode of translocation across the inner membrane.

The EccC ATPase is a key component for substrate export from the cytoplasmic compartment. Biochemical and structural analysis using the soluble domains of $EccC_1$ of *M. tuberculosis* and EccC of the actinobacterium *Thermomonospora curvata*, respectively, have shown that the most C-terminal seven amino acids of EsxB, C-terminal from the YxxxD motif, bind to a pocket on the third NBD of EccC (84) (**Figure 2*c***). This interaction should potentially reduce the flexibility of EccC, thereby triggering the multimerization of the cytoplasmic ATPase domains (84). This binding event might induce multimerization of the flexible domains of the membrane-bound ATPase, potentially into a triple-stacked (or quadruple-stacked when including the DUF domain) ATPase hexamer (11, 84) (**Figure 4**). EccC multimerization could lead to ATPase activation of the crucial NBD_1 (11, 40, 84). It should be noted that EsxB is able to trigger multimerization of the soluble domain of EccC only when it is not in complex with EsxA, as this protein has an antagonizing effect on the EccC-EsxB interaction (84). Considering that EsxB and EsxA form highly stable heterodimers and are cosecreted, the role of these substrates in EccC multimerization remains unclear. In addition, as the YxxxD/E secretion motif is not directly involved in binding to EccC, the role of this conserved motif in substrate recognition remains to be clarified.

An additional question that remains is how the other substrate classes, i.e., the PE, PPE, and Esp proteins, are recognized by the secretion complex. Interestingly, a specific region in the linker 2 region of $EccC_5$ has been shown to be responsible for the species-specific secretion of the large ESX-5 substrate subfamily of PE_PGRS proteins (18). This region in the linker 2 domain is missing in the reported crystal structure of EccC of *T. curvata* (84) and shows structural variability between the two protomers of the dimeric membrane complex, suggesting flexibility (79). Although a direct interaction between this region and the substrates has not been shown, it does indicate that this domain is involved in the recognition of PE_PGRS substrate, and therefore perhaps also of other PE and PPE substrates. As EspG is involved in determining the system specificity of PE/PPE substrates, this chaperone might also play a role in the binding of PE/PPE substrates to the linker 2 domain. Dissociation of EspG from the substrate pair, potentially mediated by EccA, most likely occurs before or during the EccC activation step.

Additionally, the crystal structure of the dimeric soluble domain of $EccD_1$ shows a different arrangement compared to the two equivalent domains in the ESX-3 membrane protomer (**Figure 2*d*,*e***). As such, it seems likely that conformational changes of these two EccD domains can release or allow movement of EccC, which in turn will lead to rearrangement of the cytoplasmic ATPase domains into a secretion-competent state upon substrate binding (see below). Although it is becoming clear that EccC plays a central role in substrate recognition, the mechanism of substrate transport through the membrane complex remains ill characterized.

5.2. Substrate Codependence for Secretion

While it is obvious that the two partner proteins that form a heterodimer are interdependent for secretion, this phenomenon of codependence is observed beyond dimeric partners, especially

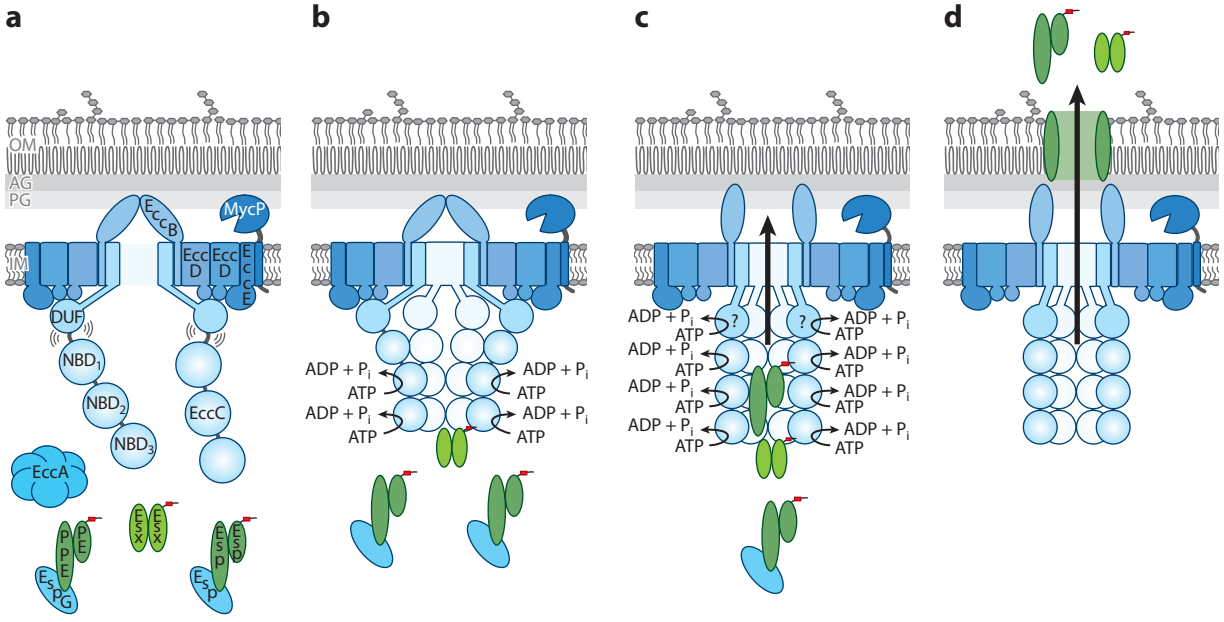

Figure 4

Proposed model for activation of and secretion by mycobacterial T7SSs. (*a*) Esx, PE/PPE, and Esp substrates form heterodimers with the conserved YxxxD/E secretion motif in the C terminus of one of the partner proteins (*red box*). EspG and Esp chaperones keep PE/PPE and Esp heterodimers, respectively, soluble in the cytoplasm and possibly target them to the secretion machinery in the mycobacterial cell envelope. The cytosolic domains of EccC in the hexameric membrane complex are highly flexible, while the secretion pore is closed by the periplasmic domains of the EccB subunits. (*b*) Binding of Esx substrates on the C-terminal NBD_3 reduces EccC flexibility and arranges the ATPase domains into a hexameric assembly, required for the R-finger mechanism of ATPase activation. (*c*) Protein export is possibly triggered by the binding of a PE/PPE heterodimer to the linker 2 domain connecting NBD_1 and NBD_2, activating the crucial NBD_1. Concurrently, changes in the conformation of the EccD cytoplasmic domains release or allow movement of the DUF domain of EccC. These changes in EccC trigger EccB rearrangements through the observed EccC-EccB interaction, resulting in opening of the membrane channel. (*d*) In our model, secretion through the OM is mediated by pores created by specific T7SS substrates. Abbreviations: AG, arabinogalactan; IM, inner membrane; NBD, nucleotide-binding domain; OM, outer membrane; PG, peptidoglycan; T7SS, type VII secretion system.

for ESX-1 substrates. For instance, secretion of EspC/EspA is dependent on the presence of EsxB/EsxA and vice versa (42, 64). Similarly, secretion of EspB and secretion of EsxB/EsxA are also codependent (70). In addition, this substrate interdependence has also been observed for ESX-5; for instance, the ESX-5 substrate PPE38 has been shown to be required for secretion of the PE_PGRS and PPE-MPTR protein subfamilies (7).

While the mechanism of substrate interdependence remains unknown, recent data show that in *M. marinum*, secretion of an ectopically expressed ESX-1-dependent Esx heterodimer requires the coexpression of a PE/PPE pair that is encoded by the same operon (32). Intriguingly, rerouting of this PE/PPE pair to another system, by changing the EspG-binding domain on the PPE protein, results also in the co-redirection of the Esx pair (32). This suggests that the PE/PPE pair determines the system specificity of the Esx pair and indicates that the binding of Esx substrates to EccC is not system specific.

The observed codependence of Esx and PE/PPE substrates can be explained by a dual substrate binding model for EccC (84). In this model, the cytosolic domains of the six EccC subunits of the membrane complex are flexible in the nonactive state. Upon binding of Esx substrates to the third NBD, these domains become less flexible and assemble into a staked three-NBD hexamer

(**Figure 4**). A second binding event to the linker 2 domain by substrates belonging to the PE/PPE family and/or their interacting chaperones, relieves the inhibitory effect of this linker domain on NBD_1, thereby activating the membrane complex (**Figures 2*b*** and **4**). This second binding event primes NBD_1 for nucleotide binding and subsequent hydrolysis. Additionally, this step could trigger conformational changes in EccB through the observed EccB-EccC interactions, allowing protein transfer through the membrane complex.

5.3. Possible Mechanisms of Outer Membrane Translocation

The size of the T7SS core membrane complex dictates that it is not large enough to span the complete diderm cell envelope of mycobacteria. As such, translocation over the mycobacterial OM still remains a mystery.

As all T7SS membrane components are present throughout the *Actinobacteria*, including those that lack an OM, it suggests that they do not play a role in transport across this second membrane. Consequently, translocation over the OM is most likely mediated by additional components. The observation that no such components are copurified with the core complex (11, 40, 54, 79) suggests that there is no stable interaction between the inner membrane complex and the putative OM component(s), which hints to secretion as a two-step process. However, a one-step secretion mechanism cannot be ruled out yet, as putative transient interactions can be lost during the purification procedure (107) and no periplasmic accumulation of T7S substrates has been identified.

Finding the OM component of T7SSs is complicated by the scarce information available on mycobacterial OM proteins. MspA and its homologs MspB, MspC, and MspD are essential in mediating uptake of glycerol and phosphate over the OM in fast-growing species by forming β barrel–like porins (99). However, these porins are all absent in *M. tuberculosis* and other slow-growing species. In *M. tuberculosis*, CpnT and SpmT, and more recently also specific PE/PPE proteins, have been found to mediate uptake of nutrients (33, 61, 97, 114). However, structural information on the membrane-spanning domains of these proteins is still lacking (33, 97). Additionally, neither of these proteins has been associated with protein transport, making it unlikely that these are involved in T7SS.

Based on the observed substrate interdependence for secretion, it has been postulated that specific T7SS substrates might mediate protein transport over the mycobacterial OM, although there is no hard evidence for this yet. Two indications for this are the observations that two ESX-1-secreted substrates, EspB and EspC, have the tendency to multimerize into heptameric tubular-shaped particles and filaments, respectively, structures that were both hypothesized to be able to penetrate membranes (60, 64, 96). As EspB shows a similar fold as PE/PPE dimers, this suggests that PE/PPE substrates might also oligomerize upon secretion and that EspG binding is required to prevent premature multimerization (96). In line with this hypothesis, several PE and PPE proteins have been shown to be firmly associated with the mycobacterial cell envelope (41, 61, 114). A final support for the role of specific PE/PPE proteins in OM translocation comes from *M. smegmatis* reconstitution experiments. Introduction of the entire *Mycobacterium xenopi esx-5* locus in *M. smegmatis* results in a functionally reconstituted ESX-5 system (11). As *M. smegmatis* naturally lacks an ESX-5 system, functional reconstitution, including OM translocation, should probably be driven entirely by the plasmid-encoded components. As the genes coding for the membrane components can probably be ruled out, the plasmid-encoded substrates and especially the PPE proteins are the major candidates. However, how these substrates are inserted into the mycolic acid–containing layer and how subsequent transport takes place across this layer remain uncertain. Alternatively, the OM translocation step could be achieved by a component already present in *M. smegmatis*, which would imply that this protein is able to function as an OM channel for different ESX systems from different species.

6. CONCLUSIONS AND OUTLOOK

Since the discovery of the ESX-1 secretion system in 2003 (98), substantial progress has been made in the molecular, functional, and structural characterization of T7SSs. Nevertheless, we are only starting to get real mechanistic insight into substrate recognition by and transport through T7SSs, as studies to address this are complicated due to the intricate interplay between various substrates and the localization of the T7SS membrane complex in the relatively inaccessible mycobacterial cell envelope. A high-resolution structure of the full hexameric membrane complex, together with its accessory proteins, such as MycP, will undoubtedly help in the further understanding of the mechanism of transport. Visualization of the complex within the mycobacterial cell envelope by cryo–electron tomography could also help in addressing how substrates are translocated across the outer membrane. Finally, more genetic and biochemical work is required to understand the mechanism of the observed codependence of substrates for secretion. Considering the importance and diverse roles of T7SSs in the life cycle of *M. tuberculosis*, elucidating their mechanism and functions would provide opportunities for understanding the ancient disease it causes and combating this pathogen.

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