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MINIREVIEW

Esx Systems and the Mycobacterial Cell Envelope: What's the Connection?

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ABSTRACT Mycobacterial 6-kDa early secreted antigenic target (ESAT-6) system (ESX) exporters transport proteins across the cytoplasmic membrane. Many proteins transported by ESX systems are then translocated across the mycobacterial cell envelope and secreted from the cell. Although the mechanism underlying protein transport across the mycolate outer membrane remains elusive, the ESX systems are closely connected with and localize to the cell envelope. Links between ESX-associated proteins, cell wall synthesis, and the maintenance of cell envelope integrity have been reported. Genes encoding the ESX systems and those required for biosynthesis of the mycobacterial envelope are coregulated. Here, we review the interplay between ESX systems and the mycobacterial cell envelope.

KEYWORDS ESX system, cell envelope, lipids, mycobacteria, protein secretion, transport

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Bacterial protein transporters are large molecular machines that assemble within the cytoplasm and bacterial cell envelope, which generally consists of the cytoplasmic membrane (CM) and extracytoplasmic compartments. All bacteria use protein secretion systems to actively transport protein substrates and/or nucleic acids from the bacterial cytoplasm to extracytoplasmic environments (1). In diderm-lipopolysaccharide (LPS) (Gram-negative) bacteria, which have an inner membrane (IM) and an outer membrane (OM) containing LPS, there are nine types of secretion systems (types I to IX) (1). Types I through VI, the most characterized systems, promote the secretion of proteins across the IM and the OM (recently reviewed in reference 2). In monoderm (Gram-positive) bacteria, which have only a CM, there are eight secretion systems that promote protein secretion across the CM (reviewed in reference 1). Based on microscopy studies and lipid analysis, mycobacteria are considered to be diderm bacteria (1, 3–5). However, the mycobacterial cell envelope contains a mycolate-OM (MOM), which differs in lipid content from other diderm OMs (Fig. 1) (reviewed in references 1 and 6 to 8). As such, mycobacteria are classified as diderm-mycolate bacteria (1).

Hundreds of mycobacterial proteins are routinely observed on the cell surface or in the culture medium during *in vitro* growth (9–12). As with other OMs, the MOM is frequently regarded as a permeability barrier (13–15). Yet, it is unknown how any mycobacterial proteins are secreted across the MOM (15). In contrast to secretion, export is the transport of substrates from the cytoplasm across the cytoplasmic membrane. The general secretory pathway (Sec) and the twin-arginine transporter (TAT) are well-characterized examples of protein exporters (1). In mycobacteria and several monoderm bacteria, there is a unique family of protein exporters referred to as 6-kDa early secreted antigenic target (ESAT-6) system/WXG-100 secretion system (ESX/WSS) or type VII secretion systems (16–21). There is no known ESX counterpart in diderm-LPS bacteria. However, proteins with WXG motifs, which are a hallmark of substrates of the ESX/WSS, have been described in diderm-LPS bacteria (22).

As of yet, there is no evidence that the ESX apparatus spans the envelope. Therefore,

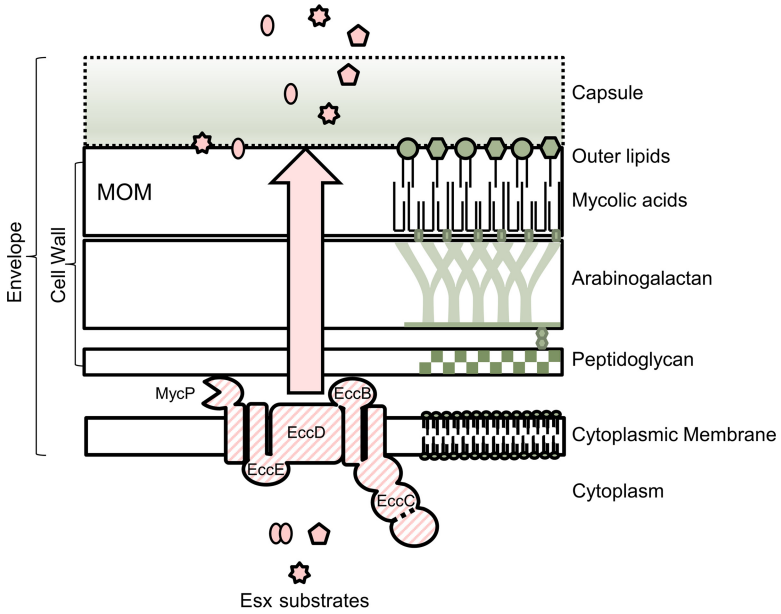

FIG 1 ESX exporters in the mycobacterial cell envelope. The model shows a cartoon representation of the complex mycobacterial cell envelope, spanning from the cytoplasmic membrane through the mycolate outer membrane (MOM) and capsule. Proteins are excluded from the cell envelope, other than ESX proteins, for simplicity. Components of the envelope are not drawn to scale. For a recent review on the mycobacterial cell envelope, please see references 7, 80, and 81. The ESX membrane complex and MycP are indicated in the cytoplasmic membrane (striped). The dotted line in the Ecc protein refers to the fact that specifically in the ESX-1 system, the EccC protein is split into two proteins ($EccCa_1$ and $EccCb_1$). Additional ESX proteins required for transport are excluded for simplicity (Esp and additional Ecc proteins, PE and PPE). For recent reviews on the molecular mechanisms of ESX export, please see references 33 to 36. The cartoon was based on schematics in references 80 and 144. Ecc, ESX-conserved component. Large arrow indicates that proteins traverse the cell wall via an unknown mechanism.

we refer to ESX-mediated protein transport as export. Yet, ESX substrates are secreted from the mycobacterial cell. Therefore, either the export machinery or the substrates themselves likely intimately interact with the MOM. Several recent studies have reported connections between ESX systems and the cell envelope in different mycobacterial species (23–25), prompting us to revisit the literature linking ESX systems with the mycobacterial cell envelope. Here, we focus on protein transport across the envelope, interaction between ESX proteins and the cell envelope, links between ESX and cell envelope integrity, and coregulation of the ESX systems and components of the cell envelope.

OVERVIEW OF MYCOBACTERIAL ESX SYSTEMS

Mycobacteria have several ESX systems encoded within the genome or on conjugative plasmids (26–29). Up to five ESX systems can be encoded in the genome (ESX-1 to ESX-5). Protein transport has been demonstrated for the ESX-1, ESX-5, and ESX-3 systems but not for the ESX-2 and ESX-4 systems (18, 30–32). ESX systems generally include ESX conserved components (Ecc proteins), a mycosin serine protease (MycP), and a pair of small secreted antigenic proteins with WXG-100 motifs (Esx proteins) (16, 17, 26). Interestingly, outside the conserved components, ESX systems vary in genetic composition and function across diverse mycobacterial species. In this section, we highlight the five ESX systems encoded in the mycobacterial genome and how these systems vary functionally in mycobacteria. The molecular details and functions of the ESX systems have been more comprehensively reviewed elsewhere (18, 33–36).

The genes encoding ESX systems are conserved across diverse mycobacterial species (27, 29). *Mycobacterium tuberculosis* is the causative agent of human tuberculosis (37). Because *M. tuberculosis* grows slowly and must be used in a biosafety level 3 facility, additional mycobacterial species have served as model systems to define

mechanisms and functions of the ESX systems (18, 37–41). Importantly, studying ESX systems in distinct mycobacterial species has provided insight into the function of ESX exporters that would have been otherwise missed by studying a single mycobacterial species.

In *M. tuberculosis* and other mycobacterial pathogens (*M. marinum* and *M. bovis*), the ESX-1 and ESX-5 systems are required for virulence (30, 31, 42–45). The loss of ESX-1 or ESX-5 genes in pathogenic mycobacteria causes attenuation in cellular and animal models of infection (30, 31, 42–51). Pathogenic mycobacteria use the ESX-1 system to damage the phagosomal membrane while residing within phagocytes (52–56). The ESX-5 system, which is restricted to slow-growing mycobacterial species, likely promotes the uptake of nutrients essential for mycobacterial survival in the phagocyte (27, 50, 57, 58). Relative to the ESX-1 system, the ESX-5 system secretes a large number of proline-glutamate/proline-proline-glutamate (PE/PPE) proteins which promote virulence (51, 59–62). ESX-5 is also unique in that it is a modular system; accessory components outside the conserved locus promote the secretion of a specific subset of ESX-5 substrates (36, 63).

In *Mycobacterium smegmatis*, two ESX systems promote atypical conjugation (64–67). Conjugation is a process by which genetic material is transferred directionally from a donor cell to a recipient cell. *M. smegmatis* undergoes distributive conjugal transfer, in which donated DNA is integrated in an unpredictable genome-wide manner. Interestingly, in addition to regulating conjugation, genes encoding ESX-1 confer mating type designations (donor versus recipient) in *M. smegmatis* (66, 68). Recently, Gray et al. (67) found that the ESX-4 system was required for conjugation specifically in recipient strains. Moreover, expression of ESX-4 genes was induced in recipient cells in an ESX-1-dependent manner during donor-recipient coculture experiments. Therefore, in *M. smegmatis*, ESX-1 and ESX-4 systems coordinate communication between mycobacterial cells (67). In support of these findings, a recent study by Boritsch et al. indicated that a similar process of horizontal gene transfer was observed in *Mycobacterium canettii*, a close relative of *M. tuberculosis*. Although the dependence of the observed DNA transfer on the ESX-1 and ESX-4 systems was not tested, this study indicates that horizontal gene transfer is likely widespread in mycobacteria (69).

ESX-3 systems have two independent functional roles in mycobacteria. First, ESX-3 systems promote metal homeostasis (32, 70–74). Although ESX-3 promotes siderophore-mediated iron and zinc acquisition in *M. tuberculosis* (70, 71), in *M. smegmatis*, the ESX-3 system promotes iron homeostasis only (71, 72). Underscoring the importance of metal homeostasis, ESX-3 genes are essential in *M. tuberculosis*; survival in the absence of ESX-3 genes can be restored with metal supplementation (73). Second, the ESX-3 system promotes virulence of *M. tuberculosis* (73, 75). The ESX-3 substrate EsxH directly interacts with host endosomal sorting complexes required for transport (ESCRT) machinery, preventing phagosomal maturation and antigen presentation during macrophage infection with *M. tuberculosis* (75, 76).

Compared to the other ESX systems, little is known about ESX-2. Like ESX-5, ESX-2 systems are restricted to slow-growing mycobacterial species (27). ESX-2 genes are transcriptionally coregulated in *M. tuberculosis* with genes encoding additional ESX systems and were identified in a screen for genes necessary for survival in dendritic cells (77–79).

MYCOBACTERIAL CELL ENVELOPE

After crossing the CM, secreted proteins must transit several layers that form the mycobacterial envelope, including a covalently linked structure of peptidoglycan, arabinogalactan, and mycolic acids (Fig. 1) (7, 80). Because the mycobacterial peptidoglycan structure was recently reviewed by Alderwick et al., we will not discuss it in detail here (81). Covalently attached to the peptidoglycan is arabinogalactan, a macromolecule consisting of a chain of galactan that is modified with 2 to 3 branched arabinan chains (reviewed in references 6 and 80).

The MOM is linked to the arabinogalactan via covalent bonds between mycolic acids

and the arabinan units of arabinogalactan (6). Mycolic acids and extractable lipids and glycolipids form the MOM. Other mycobacterial lipids may include, but are not limited to, trehalose-containing lipids (e.g., sulfolipids, polyacyltrehalose [PAT], diacyltrehalose [DAT], and lipooligosaccharide [LOS]), phthiocerol dimycocerosates (PDIM), and phenolic glycolipids (PGLs) (reviewed in reference 80). The mycobacterial cell envelope also includes a capsule layer (4).

Although many lipid biosynthesis genes are conserved among mycobacterial species, the lipid content of the envelope varies (82). PDIM and PGL biosynthesis genes are conserved in *M. tuberculosis* and in *M. marinum* but not in *M. smegmatis* (83). *M. smegmatis* has glycopeptidolipids (GPLs) on the cell surface (80). *M. tuberculosis* produces sulfolipids, which are not present in *M. marinum* (84, 85). *M. marinum* produces LOS, which are not found in *M. tuberculosis* (86–88). There are even differences in lipid content between isolates of the same species. For example, although the *M. tuberculosis* genome contains genes required for PDIM and PGL synthesis, some isolates of *M. tuberculosis* do not make PGLs (89). The differences in the mycobacterial cell envelope are important because a variety of ESX systems must interact with each unique cell envelope.

The mycobacterial cell envelope functions as a major virulence determinant. Pathogenic mycobacteria with mutations in PDIM biosynthesis genes are attenuated in cellular and animal infection models (90–92). Glycolipids can interact directly with host immune receptors (reviewed in reference 93). Many mycobacterial lipids are antigens that can activate CD1-restricted T cells (94). Intriguingly, it has been suggested that mycobacterial lipids may also insert directly into host cell membranes, altering membrane fluidity and affecting phagocytosis and trafficking (reviewed in references 94 and 95).

CELL WALL SYNTHESIS AND ESX SYSTEMS

ESX systems have been repeatedly linked to envelope biogenesis, primarily at the level of gene expression. Several transcription factors regulate the genes required for both cell wall processes and ESX transport. For example, the MprAB two-component system responds to cell envelope stress and regulates ESX-1 genes (96–99). Sodium chloride stress pathways influence the expression of genes required for cell wall remodeling and of ESX-1 genes (*espACD*) in *M. tuberculosis* CDC1551 (100). EspR is a transcriptional regulator of the ESX-1 system (77, 101). Loss of *espR* expression abrogates ESX-1 secretion and attenuates *M. tuberculosis* (101). In addition to the ESX-1 system, EspR regulates many cell wall genes, including those responsible for PDIM production, PE/PPE genes, and genes at the ESX-2 and ESX-5 loci (reference 77 and reviewed in reference 102). PhoP is part of a two-component system that regulates several virulence pathways in *M. tuberculosis*, including biosynthetic genes of the *M. tuberculosis*-specific lipids, sulfolipid, DAT, and PAT (103). In addition to regulating lipid biosynthesis genes, PhoP also regulates *whiB6* and works directly with EspR, and both regulate ESX-1 gene expression (96, 104, 105). Therefore, under many of the conditions in which cell envelope remodeling may be occurring or where cell wall genes are regulated, ESX genes are also regulated.

Links between the regulation of genes encoding ESX systems and the cell envelope have been extended to cellular models. A 2015 study by Mendum et al. found that lipids (PDIM, TDM, sulfolipids, and PGL), ESX systems (ESX-1, ESX-2, and ESX-4), and ESX-related genes (PPE proteins) were prominent pathways needed for survival in dendritic cells (79). In a macrophage infection model, the expression of PDIM-related genes was downregulated during infection, while the expression of other lipid biosynthesis genes (including those encoding sulfolipids, DAT, and PAT), ESX-1-related genes, and *espR* was induced (106).

In addition to regulatory linkages, ESX-1 proteins have been linked directly to lipid composition and metabolism. In 2012, Joshi et al. (107) demonstrated that $EccA_1$, an ESX-1-associated protein (44, 49), complexes with mycolic acid (Pks13, KasB, KasA, and MmaA4) and PDIM/PGL synthesis (Mas, Pks15/1, PpsD, and PpsE) proteins in *M. mari-*

num. Disruption of the *eccA*$_1$ gene resulted in a 30 to 40% reduction in mycolic acid in *M. marinum*, suggesting that EccA1 promotes mycolic acid synthesis (107).

A recent study examined metabolic changes in the presence and absence of ESX-1 genes in *M. smegmatis* (23). ESX-1-deficient strains showed significantly elevated levels of 22 metabolites compared to the wild-type strains under growth on a variety of carbon sources (23). Several of the identified metabolites were linked to cell envelope biogenesis, including mycolic acid synthesis, peptidoglycan biosynthesis, and arabinogalactan and arabinomannan biogenesis (23). Metabolic changes were not addressed in a complemented strain, leaving some possibility that the metabolic changes were not due to a loss of ESX-1 export. Nevertheless, this study is consistent with the study linking EccA$_1$ to mycolic acid biosynthesis (107).

Mycobacterial species can exhibit a smooth or rough colony morphology when grown on agar. Disruption of some individual genes required for lipid biosynthesis results in changes to colony morphology (from smooth to rough, or rough to smooth) when grown on agar (86, 88, 90, 108). Interestingly, the loss of individual ESX-1 genes in several mycobacterial species also results in changes in colony morphologies. For example, *Mycobacterium bovis* BCG and *Mycobacterium microti* bear natural deletions in ESX-1 genes. The introduction of ESX-1 genes from *M. tuberculosis* into the *M. bovis* BCG Pasteur and *M. microti* strains resulted in the conversion from a smooth to rough colony phenotype (48). In *M. marinum*, strains bearing deletions in ESX-1 or ESX-5 genes also display smooth/shiny colony morphologies (50, 51, 109, 110). *M. tuberculosis* H37Ra is an attenuated laboratory strain. H37Ra is attenuated primarily due to a mutation in the *phoP* gene. Restoration of a wide-type copy of the *phoP* gene was sufficient to restore ESX-1 secretion and virulence and to promote the conversion of the H37Ra strain from a smooth to a rough colony morphology (111). The smooth-colony phenotype observed in the absence of ESX systems may indicate that ESX systems impact the cell envelope composition, either directly or indirectly. However, the mechanism by which the loss of ESX systems impacts colony morphology remains unknown.

Bacterial secretion systems and other large machinery, such as flagella and conjugation systems, frequently require localized cell wall remodeling to insert into the cell envelope (reviewed in references 112 and 113). Mycobacterial cell growth occurs from both cell poles, with faster growth occurring at the old pole (reviewed in reference 114). Proteins involved in peptidoglycan, arabinogalactan, and mycolic acid synthesis and transport localize to polar regions (115, 116). Consistent with a link between ESX systems and cell envelope biogenesis, several protein components of the ESX-1 system have been localized to the mycobacterial cell pole in *M. marinum* and *M. smegmatis* (109, 117). Localization of components of other ESX systems has not been determined. However, the Rv1818c (PE_PGRS33) ESX-5 substrate has been localized to the mycobacterial pole, which may indicate that the ESX-5 system is also polar (61, 118). The polar localization of ESX-1 systems and cell wall biogenesis proteins may indicate that lipid or cell wall processes play a role in ESX-1 localization and assembly.

ESX SYSTEMS AND ENVELOPE PERMEABILITY

The lipid-rich MOM is thought to form a natural permeability barrier to hydrophilic molecules and nutrients, much like the OM of diderm-LPS bacteria (119, 120). However, the cell envelope from diverse mycobacterial species is reportedly 20- to 100-fold less permeable to hydrophilic solutes than the *E. coli* OM (120, 121). Both mycobacteria and diderm-LPS bacteria are intrinsically resistant to several antibiotics, including the large hydrophilic glycopeptide vancomycin (122, 123). Consistent with the idea that mycobacterial lipids provide a permeability barrier, mycobacterial strains lacking PDIM have increased susceptibility to vancomycin (124, 125).

In diderm-LPS bacteria, many outer membrane proteins (OMPs) have β-barrel structures and form channels or porins in the outer membrane that promote nutrient acquisition (126, 127). In mycobacteria, relatively few proteins that function as channels through the MOM have been characterized (128–131). The MspA protein in *M. smeg-*

matis is the best characterized mycobacterial porin but has no orthologue in *M. tuberculosis* (129). More recently, CpnT (Rv3903c) was identified as a mycobacterial toxin and water-filled protein channel that promotes the uptake of glycerol and hydrophilic and hydrophobic antibiotics (131, 132).

There are conflicting data linking the ESX-1 system and membrane permeability. Garces et al. reported that *M. tuberculosis* ESX-1-deficient strains (specifically lacking the EspA substrate) are more sensitive to SDS treatment and other envelope stresses, indicating increased permeability (133). In contrast, Chen et al. found that loss of the *espA* gene or deletion of other ESX-1 genes did not impact cell wall integrity. They found no differences between the wild-type and *espA* mutant strains in Nile red or ethidium bromide (EtBr) uptake or in sensitivity to SDS, which are common measures of permeability (134). We observed no differences in EtBr uptake between ESX-1-deficient and wild-type *M. marinum* strains (135). Differences in strain background could account for the divergent conclusions.

In contrast to ESX-1, the ESX-5 system is clearly involved in OM permeability. The ESX-5 system is essential for *in vitro* growth of *M. tuberculosis*, *M. bovis*, and *M. marinum* (58, 136). The essential nature of a subset of ESX-5 genes, primarily encoding components, has been reported by several groups (31, 51, 136–138). Interestingly, essentiality could be bypassed under certain conditions where the MOM was permeabilized. Individual ESX-5 genes could be deleted from the *M. marinum* genome when PDIM/PGL biosynthesis genes were mutated or by expressing the MspA porin from *M. smegmatis* to permeabilize the envelope. From these findings, the ESX-5 system may include the uptake of essential nutrients (58). While the essential nutrient capable of passing through the MspA porin was unidentified, Ates et al. demonstrated that ESX-5 promotes the uptake of fatty acids (58). The ESX-5 system was recently shown to be induced by phosphate limitation (139, 140). However, it has not been tested whether ESX-5 or ESX-5 substrates can promote the uptake of phosphate.

Ates et al. proposed that the activity of ESX-5 substrates, rather than the ESX-5 system itself, is essential (58). Although the precise substrates have yet to be identified, overexpression of the PE19 substrate in *M. tuberculosis* leads to increased membrane permeability (139, 141).

The fact that deletions of essential ESX-5 genes could be generated when the envelope was permeabilized raises the possibility that spontaneous mutations in lipid biogenesis genes are present in strains with defective ESX-5 systems. In support of this idea, strains with transposon insertions in the $eccC_5$ and $eccD_5$ genes, which are essential in *M. tuberculosis* (136), were isolated in the clinical *M. tuberculosis* strain CDC1551 (59). Although both strains exhibited increased membrane permeability, the permeability phenotype was genetically unlinked from the ESX-5 genes (58, 59).

A new study has linked ESX-5 to a drug resistance phenotype. Resistance to ofloxacin and other fluoroquinolones is generally established through mutation of the genes encoding DNA gyrase (142). Interestingly, ofloxacin-monoresistant *M. tuberculosis* clinical isolates lacking DNA gyrase mutations were reported to have mutations in ESX-5 genes (143). The $eccC_5$ V762G mutation found in the clinical strains was sufficient to promote ofloxacin resistance when recapitulated in the *M. tuberculosis* H37Rv laboratory strain (143). While the mechanism linking ESX-5 to ofloxacin resistance is unknown (143), based on the studies presented above (58, 141), mutation of an ESX-5 component could cause increased ofloxacin resistance by decreasing OM permeability and reducing the uptake of ofloxacin.

Not all ESX-5 genes are essential. Transposon insertions in the $eccA_5$ and $espG_5$ genes have been previously described (31, 51, 86). Disruption of $espG_5$ and the substrate-containing gene *ppe10* in *M. marinum* or the $espG_5$ gene in *M. tuberculosis* impacted the appearance and composition of the capsule, indicating a role for ESX-5 in maintaining capsule integrity (50).

CROSSING THE ENVELOPE

The mechanism of protein secretion across the envelope and the MOM is perhaps one of the most prominent unanswered questions in the field. Because ESX substrates are secreted from the mycobacterial cell, there are several options to consider for transport across the mycobacterial envelope. If the mycobacterial envelope acts as an impermeability barrier, as discussed above, there must be at least one apparatus that spans the envelope. Each ESX system could have independent means to cross the MOM, making the ESX systems true secretion systems. Alternatively, there could be a shared mechanism used by several exporters (ESX, Sec, or Tat) by which proteins cross the envelope. If the mycobacterial envelope is permeable, no apparatus may be needed to promote substrate transit across the envelope. Instead, properties intrinsic to secreted proteins could promote transit across the envelope. Unlike other secretion systems, the structure of an assembled ESX apparatus remains unknown (1). However, consideration of the localization of ESX-associated proteins in the envelope may inform potential mechanisms of transport.

CM. Four ESX component proteins (EccB, EccC, EccD, and EccE) form a complex in the CM (59, 144). The ESX-5 membrane complex from *Mycobacterium xenopi* was recently resolved to 13 Å by electron microscopy and revealed a novel oligomeric complex with 6-fold symmetry. The complex resides exclusively within the cytoplasmic membrane and hints at a novel mechanism of protein translocation (145). Higher-resolution structures have been solved for individual components in the membrane complex (146–150). Importantly, the membrane complex is restricted to the CM and does not span the envelope. The mycosin proteases may function to stabilize the membrane complex (144). In addition to having a structural role, the mycosin proteases cleave ESX substrates. The ESX-1-secreted protein EspB is cleaved by $MycP_1$ upon secretion (151). The mature processed form of EspB binds phospholipids (phosphatidic acid and phosphatidyl serine), suggesting that EspB may interact with either the cytoplasmic membrane or with phospholipids in the host cell (152).

Cell wall. For bacterial protein secretion systems and other molecular machinery, the apparatus is inserted into the bacterial cell envelope, where it interacts directly with the peptidoglycan. Peptidoglycan-binding proteins promote pilus assembly and anchoring in the cell envelope. In the *Neisseria gonorrhoeae* type IVa pilus system, a peptidoglycan-binding protein anchors the apparatus in the membrane (153). In the *Pseudomonas aeruginosa* type IV pilus system FimV, an inner membrane protein that binds peptidoglycan is required for multimerization of PilQ (outer membrane component of the type VI pilus [154]).

The interaction of any ESX component with peptidoglycan/arabinogalactan has yet to be demonstrated in mycobacteria. Based upon the structure of the $EccB_1$ component (subscripted "1" indicates that this protein is part of ESX-1 [17]), part of $EccB_1$ may extend into the periplasm and interact with peptidoglycan (148). The peptidoglycan-binding properties of $EccB_1$ have not been directly tested.

The characterization of MOM proteins may provide insight into their secretory mechanism. It is possible that known proteins integral to the MOM promote the transit of proteins across the envelope. For example, *in silico* analyses have suggested that Mce1 family proteins, which have a role in lipid homeostasis and transport, may promote ESX-1-mediated translocation across the MOM (130, 155, 156). The proposed link between the Mce1 proteins and ESX-1 is based on phylogenetic profiles, predicted protein-protein relationships, and analyses suggesting the Mce proteins may be MOM pore-forming proteins (155). A direct role for Mce1 family proteins in protein transport has not been demonstrated.

Alternatively, the ESX substrates may direct their own transit across the MOM, by either forming the apparatus spanning the MOM or by transiting the MOM directly (48, 104, 157–159). Notably, the EspC substrate of the ESX-1 system in *M. tuberculosis* (160) was recently reported by Lou et al. to self-assemble into filaments *in vitro*. EspC filaments were localized to the membrane fraction and were visualized on the surface/

capsule of *M. tuberculosis* (157). The assembly of EspC into filaments led to several intriguing ideas, including one that EspC is a component of the MOM channel or a "needle" for the ESX-1 system (157, 161). EspC is encoded with the EspA and EspD substrates by the *espACD* operon (160, 162, 163). There are no recognized *espACD* counterparts for the ESX-2 through ESX-5 systems. The EspACD proteins may fulfill a function that is specific to the ESX-1 system, for example by promoting interaction with the host cell. Alternatively, additional systems may have genes which are functionally redundant with the *espACD* genes.

ESX substrates have been localized to the mycobacterial cell surface, extrinsically associated with the MOM (4, 31, 58, 60, 109, 164–167). Surface-localized ESX-1 substrates include EsxA and EspE. The EsxA substrate has been visualized and quantified on the mycobacterial cell surface (164, 166, 167). Several PE_PGRS proteins, a subfamily of PE proteins with polymorphic GC-rich sequences, are surface-localized ESX-5 substrates (51). The mechanism determining whether ESX substrates are targeted to the cell surface or the extracellular environment and the relationship between the surface-localized and secreted populations is unknown. However, a link between PE_PGRS secretion and LOS biosynthesis genes was reported in *M. marinum* (86). Interestingly, in strains lacking LOS, PE_PGRS proteins and the ESX-1 substrate EspE were more strongly attached to the cell surface, possibly linking LOS to the localization of ESX substrates on the cell surface (86).

Extracellular locations. *In vitro* mycobacterial growth conditions preclude retention of the mycobacterial capsule. Specifically, mycobacterial strains are grown *in vitro* in the presence of detergent, usually either Tween 80 or tyloxapol, to reduce bacterial clumping during growth. Growth in detergent promotes the release of the mycobacterial capsule into the growth medium (4, 168). When mycobacteria are grown without detergent, to promote the retention of the capsule, several ESX-1 and ESX-5 substrates are localized to the capsule layer (4, 165, 169). Mycobacterial protein secretion is largely studied by the presence or absence of proteins in spent medium during *in vitro* growth. Indeed, a long list of proteins found in spent medium are dependent on ESX systems (30–32, 42–44, 51, 57, 59, 65, 73, 74, 101, 110, 162, 163, 165, 169–172).

ESX-1, A MEMBRANOLYTIC SYSTEM

The ESX-1 system has long been known to promote membrane lysis. In mycobacterial pathogens, the ESX-1 system damages the phagosomal membrane, promoting interaction between the bacteria and cytoplasm of host macrophages (46, 52, 55, 56, 173–175). Cytosolic signaling is required for mycobacterial virulence (173, 174, 175). Therefore, mycobacterial strains lacking ESX-1 genes are attenuated likely because they are retained in the phagosome and cannot trigger cytosolic signaling. Indeed, the expression of a secreted lysin from *Listeria monocytogenes* (LLO) in the absence of a functional ESX-1 exporter was sufficient to bypass the need for ESX-1 export in macrophage infections (173).

ESX-1 substrates have been considered prime candidates for mycobacterial membrane lysins. In 2003, it was proposed that EsxA, a major ESX-1 substrate, was the major membrane lysin secreted by the ESX-1 system. Genetic analysis of the function of EsxA is complicated. EsxA and its binding partner, EsxB, are encoded from the *esxBA* operon (176). Deletion of the *esxBA* operon, or of the *esxA* gene, abrogates ESX-1 export in pathogenic mycobacteria (30, 43). Because the loss of EsxA and EsxB secretion from pathogenic mycobacteria prevents the secretion of all of the other known ESX-1 substrates (165), it is difficult to ascribe a function to individual ESX-1 substrates using genetics alone. As such, the proposed membranolytic activity of EsxA has been supported by biochemical and biophysical analyses conducted by several research groups.

EsxA was first proposed to be a membrane lysin by Hsu et al. in a landmark paper which linked the loss of the region of deletion 1 (RD1) to attenuation of the BCG vaccine strain (42). The authors conducted a screen designed to identify genes required for cytolysis of lung epithelial cells, and one of the strains they identified was an *M.*

tuberculosis strain bearing a transposon insertion in the *esxB* gene, which was polar on *esxA* expression. The authors then demonstrated that purified EsxA, in the presence or absence of EsxB, was sufficient to cause membrane destruction, as determined by measuring changes in conductance across an artificial membrane bilayer. These initial biophysical data were extended by several independent reports, including those demonstrating EsxA-dependent lysis of physiologically relevant liposome membranes (177), characterizing pore formation in sheep red blood cell (sRBC) membranes by EsxA (53), and the lysis of type 1 and type 2 pneumocytes by either purified EsxA or EsxA applied to the surface of ESX-1-deficient mycobacterial strains (167). Several more recent studies have focused on the mechanism of EsxA membrane lysis by investigating changes to membrane lysis as a function of pH (177), by comparing the activities of EsxA proteins from pathogenic and nonpathogenic mycobacterial species (178, 179), and by generating specific point mutations which disrupt the membranolytic activity of EsxA (180). For a recent comprehensive review of the evidence of EsxA pore-forming activity, please see the study by Peng and Sun (181).

Despite the aforementioned studies, the ability of EsxA to lyse membranes independently has been recently challenged. Conrad et al. found undeniably that EsxA is not sufficient to promote membrane lysis (182). Recombinant EsxA is widely acquired directly from the BEI repository or produced using a nondenaturing protocol, which includes the addition of detergent (183). In a series of well-controlled and clearly interpretable experiments, Conrad et al. demonstrated that the detergent present in the EsxA preparations, and not the EsxA protein itself, was responsible for the observed membranolytic activity of EsxA. Treatment of recombinant preparations of EsxA or *Staphylococcus aureus* α-hemolysin or *Streptococcus pneumoniae* pneumolysin (Hla and PLY, respectively, established bacterial lysins) with proteinase K abrogated Hla and PLY activity but did not alter the lytic activity of the EsxA preparation. Although the authors observed pH-dependent lytic activity of recombinant EsxA prepared without detergent, they show that changes in pH are not required to mediate ESX-1-mediated phagosomal lysis in a cellular model of infection. Thus, either EsxA is not required for phagosomal lysis, or additional ESX-1-associated proteins are required (182).

Although EsxA appears to be insufficient to promote ESX-1-mediated membrane lysis, the study by Conrad et al. definitively showed that the *M. marinum* ESX-1 system functions to lyse membranes in a contact-dependent manner (182). Contact-dependent membrane lysis by mycobacteria has been suggested but not directly demonstrated in several earlier studies (44, 167, 184). Coupled with evidence that ESX-1 substrates are present in the capsule and on the cell surface, discovered by our group and several others, direct translocation of ESX-1 substrates into the host macrophage may not occur. Rather, the presence of ESX-1 substrates on the cell surface may be sufficient for promoting phagosomal lysis (134, 166–185). Alternatively, contact with membranes may induce ESX-1 secretion through an unknown signal transduction mechanism.

Although strains bearing mutations in genes required for PDIM production are still competent to secrete ESX-1 substrates *in vitro*, recent studies have suggested that PDIM works synergistically with ESX-1 to promote phagosomal damage (24, 25). A recent study by Quigley et al. linked the levels of PDIM production with the ability of *M. tuberculosis* to promote phagosomal damage and downstream events in the macrophage (25). MmpL7 transports PDIM from the cytoplasm, where it is generated, to the MOM. In the absence of Mmpl7, PDIM accumulates in the mycobacterial cytoplasm (90). Quigley et al. demonstrated that *M. tuberculosis* strains bearing a transposon insertion in the *mmpL7* gene damage the phagosomal membrane of THP-1 cells significantly less than the wild type (WT) or the complemented strain. As such, the *mmpL7*::Tn strain promoted less autophagy and host cell necrosis, events that are downstream of phagosomal damage. Importantly, a clean deletion of the *mmpL7* gene did not impact the secretion of EsxA by *M. tuberculosis in vitro* (25). In another recent study, Augenstreich et al. demonstrated that *M. tuberculosis* strains lacking genes required for PDIM production led to reduced phagosomal damage in human monocyte-derived macrophages. The same study investigated if the mycobacterial lipid PDIM enhances phago-

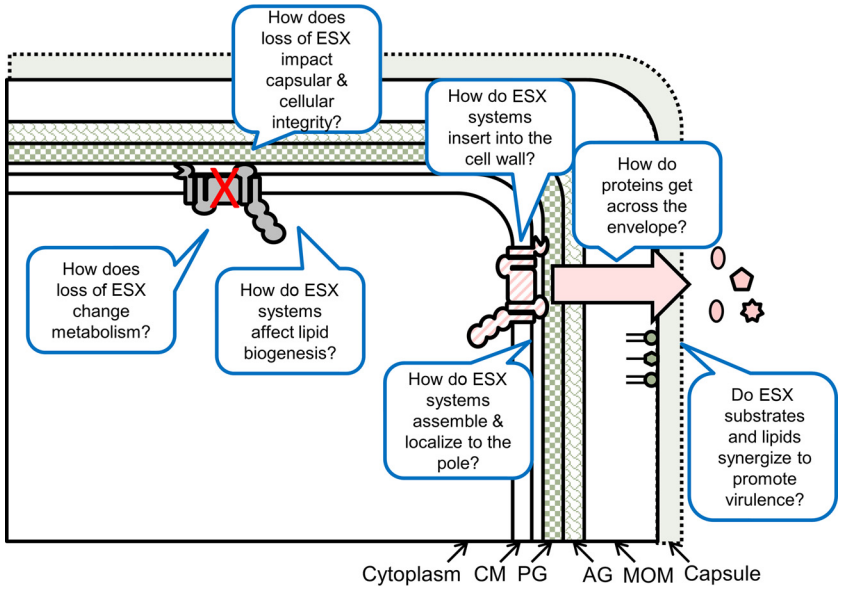

FIG 2 Unanswered questions regarding the interactions between ESX systems and the mycobacterial cell envelope. CM, cytoplasmic membrane; PG, peptidoglycan; AG, arabinogalactan; MOM, mycolate outer membrane.

somal lysis by the ESX-1 system by incubating EsxA with vesicles with and without PDIM. Membrane lysis was enhanced in the PDIM-containing vesicles (24). In light of the paper by Conrad et al. (182), the mechanism linking PDIM to ESX-1 lysis is unlikely directly through EsxA. Yet, both studies concluded that PDIM aids in ESX-1-mediated membrane damage by likely inserting into and changing the biophysical properties of the phagosomal membrane, making it more susceptible to lysis by the ESX-1 system (24, 25).

We also recently observed a connection between PDIM biosynthesis and ESX-1 function (135). We found a spontaneous ochre mutation in the *eccCb*$_1$ gene, which encodes a component of the ESX-1 system (30, 43, 44). The ochre mutation in *eccCb*$_1$ resulted in a loss of ESX-1 function in *M. marinum*. Interestingly, we identified several suppressor strains which allowed the production of EccCb$_1$ and restoration of ESX-1 function. In our efforts to map the suppressor of the ochre mutation, we found that the suppressor strains all had the same 13-bp insertion in the *ppsC* gene. *ppsC* is part of the *pps* operon, which is required for the production of PDIM (90, 186). While we do not yet understand the mechanism of suppression, our findings genetically link the ESX-1 system to PDIM production.

Additional links between the ESX-1 system and mycobacterial lipids have been reported. For example, pathogenic mycobacteria prevent the maturation of the phagosome within the host macrophage. In a screen designed to elucidate genes required for phagosome maturation arrest (PMA), lipid biosynthesis genes and genes encoding ESX-1 components were both identified (187). The mechanisms connecting ESX-1 genes and lipid biosynthetic genes in PMA are unknown.

CONCLUSIONS

In summary, the relationship between ESX systems with the mycobacterial envelope is a complex and evolving story. Many details and unanswered questions remain (Fig. 2). How do secreted proteins cross the mycolate outer membrane? Do ESX substrates directly interact with the envelope, or are the ESX systems true secretion machines with components that span the envelope? How do ESX systems impact lipid biogenesis, and what are the downstream consequences? Do ESX systems provide additional functions for the mycobacterial cell, for example, by maintaining lipid biogenesis or envelope integrity in addition to roles in protein secretion? And finally, do these two complex

systems, the mycobacterial cell envelope and the ESX exporters, work together to promote virulence within the host? It is clear that the answers to these questions will provide many exciting and revealing findings in the future.

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