AMERICAN SOCIETY FOR MICROBIOLOGY | Microbiology and Molecular Biology Reviews®

REVIEW

Modeling Tubercular ESX-1 Secretion Using *Mycobacterium marinum*

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SUMMARY Pathogenic mycobacteria cause chronic and acute diseases ranging from human tuberculosis (TB) to nontubercular infections. *Mycobacterium tuberculosis* causes both acute and chronic human tuberculosis. Environmentally acquired nontubercular mycobacteria (NTM) cause chronic disease in humans and animals. Not surprisingly, NTM and *M. tuberculosis* often use shared molecular mechanisms to survive within the host. The ESX-1 system is a specialized secretion system that is essential for virulence and is functionally conserved between *M. tuberculosis* and *Mycobacterium marinum*. *M. marinum* is an NTM found in both salt water and freshwater that is often used to study mycobacterial virulence. Since the discovery of the secretion system in 2003, the use of both *M. tuberculosis* and *M. marinum* has defined the conserved molecular mechanisms underlying protein secretion and the lytic and regulatory activities of the ESX-1 system. Here, we review the trajectory of the field, including key discoveries regarding the ESX-1 system. We highlight the contributions of *M. marinum* studies and the conserved and unique aspects of the ESX-1 secretion system.

KEYWORDS *Mycobacterium marinum*, ESX-1, mycobacterial pathogenesis, protein secretion, type VII secretion

INTRODUCTION

Chronic and acute mycobacterial infections constitute a significant global health burden. *Mycobacterium tuberculosis* causes human tuberculosis (TB), one of the world's leading causes of death by infectious disease (1). In addition to *M. tuberculosis*,

Citation Chirakos AE, Balaram A, Conrad W, Champion PA. 2020. Modeling tubercular ESX-1 secretion using *Mycobacterium marinum*. Microbiol Mol Biol Rev 84:e00082-19. https://doi.org/10.1128/MMBR.00082-19.

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Published 2 September 2020

many species of nontuberculous mycobacteria (NTM) cause chronic environmentally acquired disease (2, 3). NTM are an emerging public health threat, increasing in incidence as the number of aging and immunocompromised individuals increases (4). The prevalence of NTM infections and their impact on human health are likely underappreciated (5).

Despite differences in host range and transmission, *M. tuberculosis* and NTMs share most molecular pathways that play critical roles in physiology and pathogenesis, including the ESX-1 (ESAT-6 [6-kDa early secreted antigenic target]) secretion system (see Appendix 1 for nomenclature) (6). The ESX-1 system is essential for the virulence of both *M. tuberculosis* and *Mycobacterium marinum* (7–12). *M. marinum* is an NTM pathogen with a broad host range that includes fish and other ectotherms as well as humans (13, 14). *M. marinum* has long been used to study mycobacterial pathogenesis and has provided significant insight into the host immune response to mycobacterial infections (15, 16). Overall, investigating the pathogenicity of *M. marinum* has informed our understanding of *M. tuberculosis* virulence mechanisms and will continue to serve as an informative model for how mycobacteria use ESX-1 secretion for virulence. Within 1 year of the initial publications demonstrating that ESX-1 was a specialized secretion pathway in *M. tuberculosis*, publications followed using *M. marinum*, further expanding our understanding of the ESX-1 system and its role in virulence (see Fig. 1 for a timeline of contributions from *M. marinum* studies). Indeed, it is difficult to discuss the trajectory of the ESX-1 field without considering studies in *M. marinum*. In this review, we highlight how the use of *M. marinum* has informed the molecular mechanisms of substrate secretion, the role of secretion in virulence, and the mechanisms regulating gene expression by the ESX-1 secretion system (type VII secretion).

TYPE VII SECRETION: A CONSERVED, SPECIALIZED SECRETION PATHWAY DISCOVERED IN MYCOBACTERIA

All bacteria transport proteins from the cytoplasm to the cell surface and into the extracellular environment. In addition to the general protein transport pathways (Sec and Tat [recently reviewed by van Winden et al. {17}]), there are several specialized protein transport systems in bacteria, classified as types I through IX (18, 19). ESX secretion was first discovered in mycobacteria and classified as type VII, reflecting its unique nature from the previous six (20, 21). Since then, type VII systems have been further classified as type VIIa to refer to those in *Actinobacteria* and type VIIb to refer to those in *Firmicutes* (22). Type VIIb systems have been widely found in pathogenic and nonpathogenic Gram-positive bacteria (23, 24). Like type VIIa systems, type VIIb systems secrete Esx proteins and include FtsK-SpoIIIE ATPases (22). There has been significant progress in understanding several molecular aspects of protein transport across the Gram-positive cell envelope and the functions of ESX-type secretion in Gram-positive physiology and pathogenesis.

It was well established that mycobacteria secreted ESX-1 proteins into their extracellular environment before the system responsible for transport was identified. In 1999, two of the major ESX-1 substrates, EsxA (ESAT-6) and EsxB (CFP-10 [culture filtrate protein, 10 kDa]), were identified as proteins highly secreted from *M. tuberculosis in vitro* (25) and facilitated the discovery of ESX systems in mycobacteria. There was interest in EsxA because in 1995 and 1996, it was established as a strong T-cell antigen (26, 27), stimulating interferon gamma production in memory effector cells of previously infected mice (27). As such, EsxA was being considered as a potential diagnostic for TB as early as 1997 (28). In 1998, EsxB was discovered as a novel secreted protein that was encoded in the same operon as the *esxA* gene (see Fig. 2 for a schematic of the *esx-1* locus) (29). Neither protein included obvious secretory signals required for secretion through the general Sec or Tat protein transport pathway, indicating that there were additional protein transport pathways in mycobacteria (25, 30).

A major breakthrough occurred in 1995, when ESAT-6 was found in culture filtrates (secreted protein fractions) from *M. tuberculosis*, *M. marinum*, and other pathogenic mycobacterial species but not in culture filtrates from the attenuated *Mycobacterium*

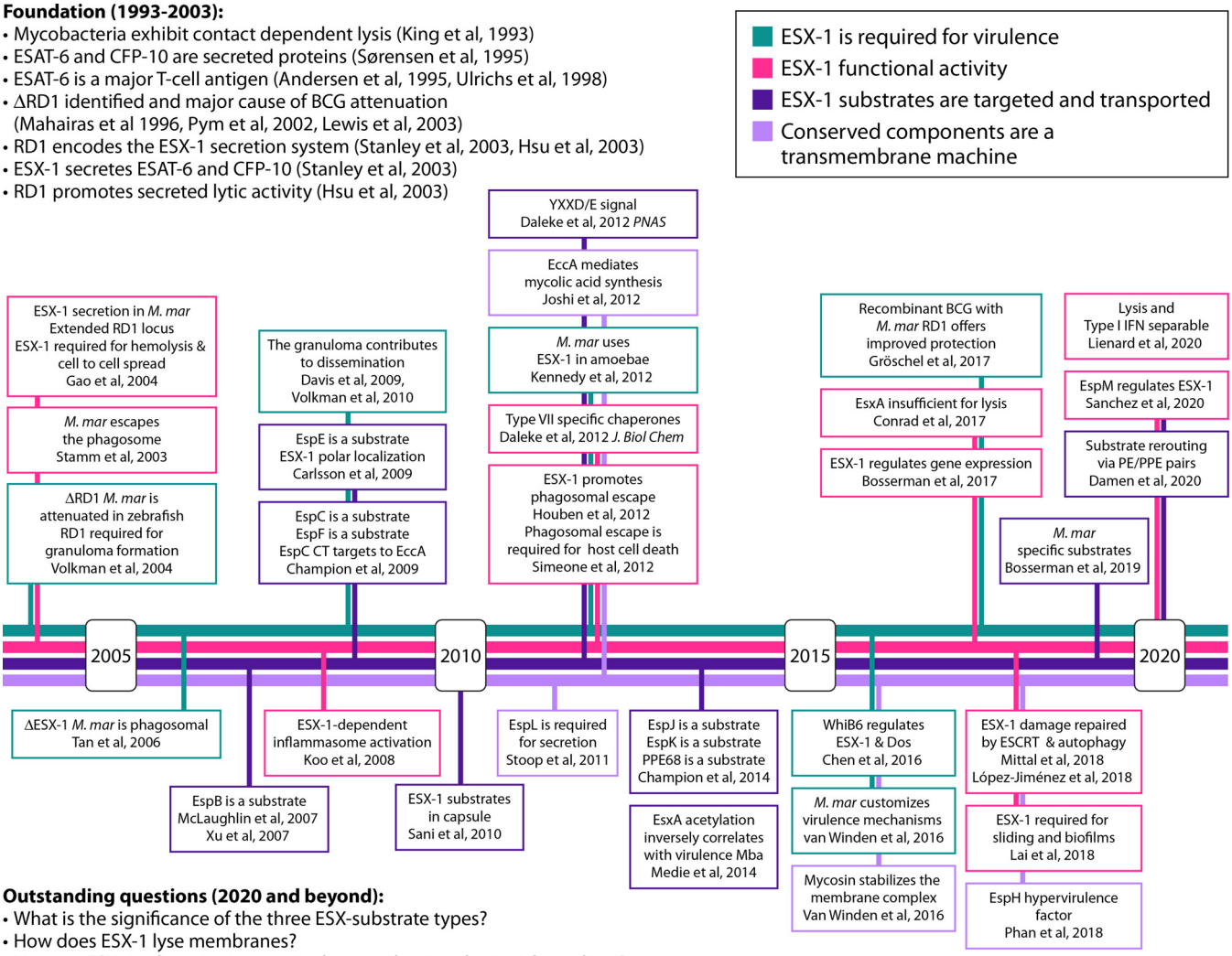

FIG 1 Timeline of ESX-1 studies performed in *M. marinum* (7–12, 26, 27, 31, 44, 47, 48, 63, 73–78, 85, 87, 92, 103, 104, 113, 117, 118, 127, 128, 134, 141, 148, 151, 154, 166, 168, 200–206). The timeline shows major contributions from *M. marinum* (*M. mar*) studies from ~2004 to 2020. The four lines indicate major aspects of the ESX-1 field. The list of foundational studies performed on *M. tuberculosis* and other slow-growing mycobacterial species as well as the highlighted *M. marinum* studies are not comprehensive. IFN, interferon; CT, C terminus.

bovis BCG vaccine strain (26). In 1996, a comparison of the *M. tuberculosis* and *M. bovis* BCG genomes revealed a large region of difference, named region of difference 1 (RD1), which included the *esxBA* operon (Fig. 2) (31, 32). Subsequently, in 1999, it was demonstrated that all BCG strains lacked the RD1 region, which includes the *esxA* gene (33). These studies, coupled with bioinformatic analyses, led to the proposal that the genes at the RD1 locus encoded a specialized secretory apparatus that secreted ESAT-6 and CFP-10 from the mycobacterial cell (23, 25, 34). Sequencing of the *M. tuberculosis* genome in 1998 revealed five paralogous secretion systems (30), which were named ESX-1 through ESX-5 (35). ESX systems can also be encoded on plasmids (36–38). In this review, we focus on ESX-1. However, significant work on the other ESX systems in several mycobacterial species, including *M. marinum*, have greatly advanced our understanding of the conserved features of type VII secretion (17, 39, 40).

Comparisons between the genomes of *Mycobacterium* and several Gram-positive bacteria, including *Bacillus subtilis*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Streptococcus*, demonstrated that the ESX-type systems, now termed type VIIa and type

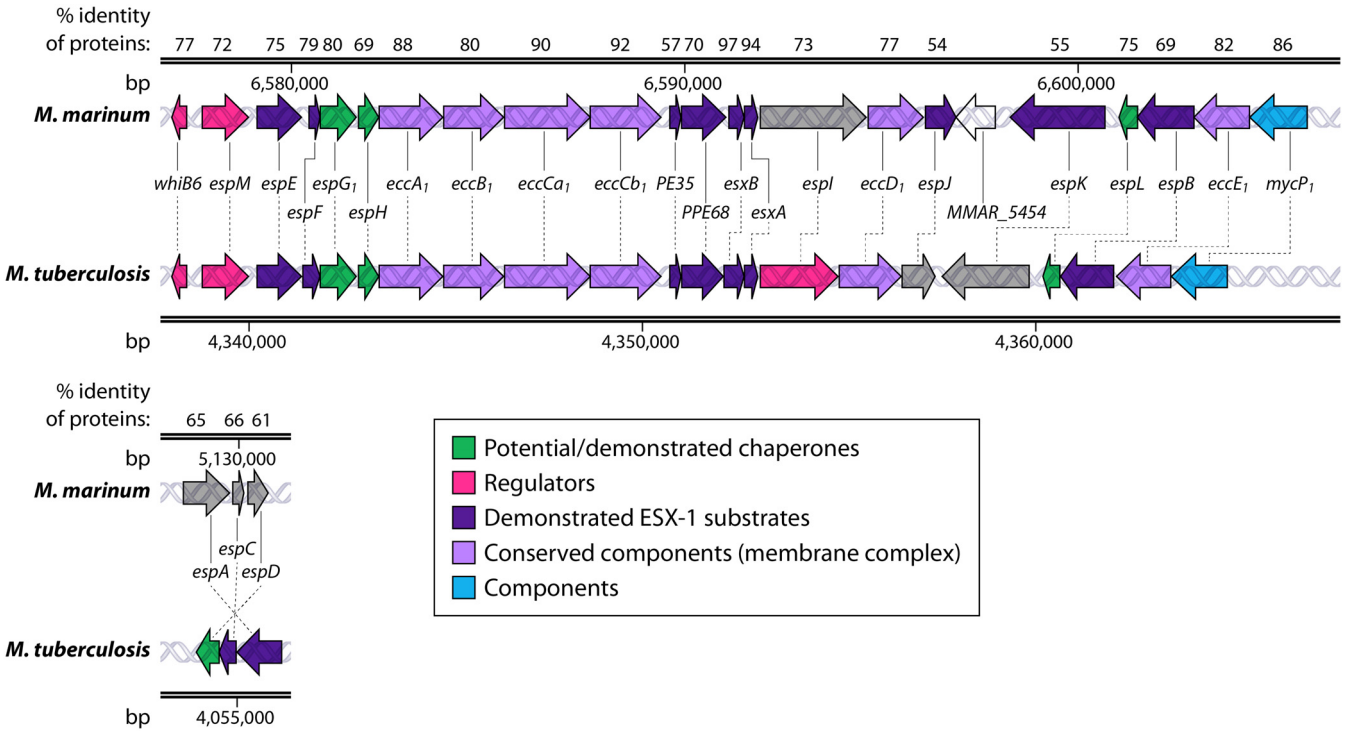

FIG 2 Syntenic *esx-1* loci from *M. marinum* and *M. tuberculosis*. The genes and their locations in each genome as well as the percent amino acid identities of the resulting proteins from the conserved *esx-1* and *espACD* loci in *M. marinum* and *M. tuberculosis* are shown. *MMAR_5454* is a gene found only in *M. marinum*. Gray shading indicates that the gene function has not been defined. Differences in colors between organisms, for example, for PPE68, indicate that PPE68 is a substrate in *M. marinum* but is required for secretion and not yet classified as a substrate in *M. tuberculosis*. The RD1 region is shown (7), as follows. ESX-1 gene products from *M. tuberculosis* (GenBank accession number NC_000962.3) were searched against those from *Mycobacterium marinum* (NCBI taxonomy accession number txid1781) using NCBI BLASTP (169). The percent identities of the top orthologous proteins in *M. marinum* are reported at the top.

VIIb (22), were distinct from previously characterized secretion systems, and they were designated type VII secretion systems (T7SSs), accordingly (21). Further genomic comparisons revealed that T7SSs are widely found in pathogenic and nonpathogenic Gram-positive bacteria (23, 24).

ESX-1 PROTEIN SECRETION IS REQUIRED FOR MYCOBACTERIAL VIRULENCE

Given that the RD1 region was missing from the attenuated *M. bovis* BCG vaccine strain, it was suspected that the secretory apparatus encoded by the genes within the *M. tuberculosis* RD1 region was essential for pathogenesis. The links between the RD1 genomic region, protein secretion, and virulence all occurred rapidly between 2002 and 2004, as detailed below.

The genetic deletion of the RD1 locus from the *M. tuberculosis* genome led to attenuated virulence in mouse and macrophage models of infection (12). Importantly, complementation of the RD1 region from *M. tuberculosis* increased the virulence of the *M. bovis* BCG vaccine strain (11, 41) and the interferon gamma response (42), indicating that the deletion of the RD1 region is the major cause of attenuation of the vaccine strain (7, 11, 12).

By 2004, it was apparent that *M. marinum* required the RD1 region and the ESX-1 system for virulence, similar to *M. tuberculosis* (10). The genes that make up the RD1 locus, and those surrounding it (the extended RD1 locus), are highly conserved between *M. tuberculosis* and *M. marinum* (6, 9). In 2004, Volkman et al. observed that the *M. tuberculosis* and *M. marinum* RD1 loci are syntenic and that the EsxB and EsxA gene products share 97 and 91% identities at the amino acid level, respectively (Fig. 2) (10). Transposon (Tn) mutagenesis screens for both *M. tuberculosis* and *M. marinum* have consistently identified the RD1 locus as being required for virulence (8, 9, 43–45). The genetic deletion of the orthologous RD1 region in *M. marinum* resulted in attenuated virulence in both adult and larval zebrafish infection models (10).

Because of its optical transparency, the larval zebrafish model for *M. marinum* infection gives unique insight into the early stages of disease progression and the role of ESX-1 in this process. Experiments using the *M. marinum* zebrafish model defined a role for the ESX-1 system in granuloma formation. The granuloma is a characteristic structure of mycobacterial infection, which not only may contain and eradicate the bacterium but also can create optimal conditions for its spread through necrosis and rupture (for a review, see reference 46). Using this model, it was observed that *M. marinum* cells lacking the ESX-1 secretion system were defective for granuloma formation (10) due, at least in part, to reduced matrix metalloprotease 9 (MMP9)-dependent extracellular matrix remodeling at sites of infection (47, 48).

MECHANISM OF ESX-1 SECRETION

ESX-1 substrates are secreted from the mycobacterial cell, transiting from the cytoplasm through the cytoplasmic membrane and the cell envelope to the cell surface and into the extracellular environment (see Fig. 3 for a model of ESX-1 secretion). The step attributed to the ESX-1 system thus far includes crossing the cytoplasmic membrane. It is not known how any protein crosses the mycobacterial envelope beyond the cytoplasmic membrane. Together, studies in *M. tuberculosis* and *M. marinum* have provided complementary insight into the specific mechanisms of how the ESX-1 system recognizes and transports proteins.

Model for Protein Transport

The ESX-1 system is made up of component and substrate proteins. Components are part of the machinery, which include membrane-associated proteins known as ESX conserved components (Eccs), chaperones, and other proteins of unknown function that are not secreted by the ESX-1 system (Appendix 1). ESX-1 substrates are primarily recognized by virtue of their extracellular secretion in an ESX-1-dependent manner *in vitro*. Substrates and other nonconserved components are known as ESX-associated proteins (Esps) (20).

Studies reported in 2003 and 2004 in *M. tuberculosis* and using BCG and *Mycobacterium microti* complementation models demonstrated that the genes within and surrounding the RD1 region encoded a specialized secretion system required for the secretion of EsxA and EsxB and that this system was required for virulence in macrophage and animal models of infection (7, 8, 42, 49). Recombinant *M. bovis* BCG and *M. microti* strains expressing the *M. tuberculosis* genes in the extended RD1 region demonstrated that these genes encoded a system that secretes EsxA and EsxB. Moreover, the reintroduction of EsxA and EsxB secretion by these strains led to increased protection against *M. tuberculosis* infection in animal models (42). At the same time, genetic approaches aimed at understanding *M. tuberculosis* virulence generated attenuated transposon insertion strains (50), which, when characterized, revealed three components ($EccCa_1$, $EccCb_1$, and $EccD_1$ [the subscript "1" indicates that they are part of the ESX-1 system]) and two substrates (EsxA and EsxB) of the ESX-1 system encoded within the RD1 region (Fig. 2) (8). Direct interactions were found between components ($EccCa_1$ and $EccCb_1$), between substrates (EsxA and EsxB), and, importantly, between components and substrates ($EccCb_1$ and EsxB). These data supported a previous study demonstrating a direct interaction between EsxA and EsxB (51). Interestingly, although under the majority of biological conditions, EsxA and EsxB are likely secreted as a tight 1:1 complex, under certain conditions, EsxB was secreted without EsxA from *M. tuberculosis* (52, 53). Likewise, a complementary study demonstrated that the insertion of a transposon into the *esxBA* operon resulted in a loss of secreted lytic activity required for host cell death and mycobacterial tissue invasion (7). Together, these studies linked the ESX-1 system to virulence and resulted in the first models showing that ESX-1 components form a specialized secretion system that interacts with and transports substrates (Fig. 3) (7, 8, 42). Further work demonstrated that individual genes in the RD1 region were required for the secretion of EsxA and EsxB proteins from *M. tuberculosis* (49), strengthening and expanding the initial model.

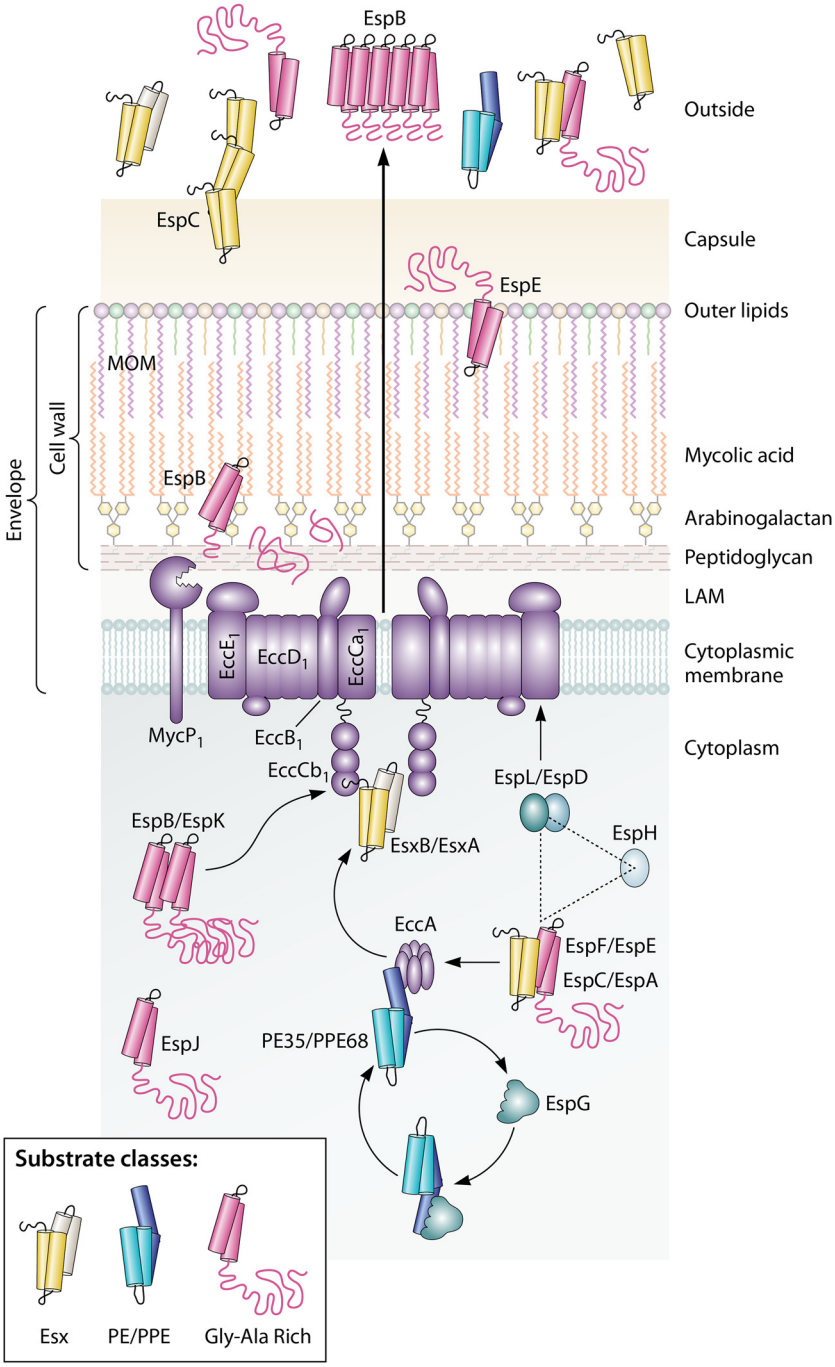

FIG 3 Conserved aspects of ESX-1 protein transport in *M. tuberculosis* and *M. marinum*. The schematic shows that the ESX-1 conserved components EccA to EccE interact with substrates and provide a channel across the cytoplasmic membrane. The three classes of conserved ESX-1 substrates are shown. The $EccCb_1$ ATPase directly interacts with the EsxB and EspK substrates; the EccA ATPase interacts with the PPE68 and EspF substrates. Substrates are often targeted in pairs. The EspG chaperone interacts with PE/PPE substrate pairs and likely brings these substrates to the EccA component before being recycled. It is not known how substrates are transferred from the EccA component to the membrane complex. EspL and EccE interact with EspD. EspL and EspD are required for the stability of EspE, EspF, and EspH. Following transport through the membrane complex, ESX-1 substrates are transported across the envelope via an unknown mechanism. EspB is cleaved by the $MycP_1$ protease. Substrates can be targeted to the MOM (mycolate outer membrane), to the capsule, and out of the cell. Some substrates, including EspC and EspB, form higher-order structures following secretion, which may indicate that they are part of the secretory machinery. LAM, lipoarabinomannan.

Following the initial studies in *M. tuberculosis*, the genes required for ESX-1 secretion were rapidly expanded using other mycobacterial species, demonstrating the conserved nature of ESX-1 secretion. In 2004, a genetic screen for nonhemolytic *M. marinum* revealed that several additional genes in the extended RD1 region were required for EsxA secretion and cell-to-cell spread (9). *Mycobacterium smegmatis* is a nonpathogenic fast-growing mycobacterial species that is widely used as a model to study mycobacterial physiology (54–56). In 2005, the genetic deletion of genes within a region orthologous to RD1 demonstrated that the ESX-1 system was functional in *M. smegmatis* and that many of the genes required for secretion were conserved (57). *M. microti* has a natural genetic deletion that partially overlaps the RD1 region (58). In 2006, complementation of the *M. microti* deletion with genes from *M. tuberculosis* revealed several additional genes important for EsxA secretion and further linked the secretion system to virulence and immunogenicity (59). Based on the genes collectively identified by these studies, in 2009, a yeast two-hybrid analysis of the gene products encoded from within the extended RD1 locus from *M. tuberculosis* added further interactions between components and substrates (60). Together, studies in *M. tuberculosis*, *M. marinum*, *M. microti*, and *M. smegmatis* led to the proposal that several conserved membrane components formed a channel across the cytoplasmic membrane, allowing the secretion of EsxA and EsxB through the cytoplasmic membrane and into the extracellular environment (Fig. 3).

The ESX-1 Membrane Complex

Building on the initial characterization of the extended RD1 locus as a novel class of secretory systems, there remains a great focus on understanding the mechanisms of protein transport across the mycobacterial cell envelope (61). The studies described above revealed that there were five membrane-associated ESX-1 conserved components ($EccB_1$, $EccCa_1$, $EccCb_1$, $EccD_1$, and $EccE_1$) and one cytoplasmic Ecc ($EccA_1$) that together make up the machinery that forms a channel across the cytoplasmic membrane and provides energy for transport and substrate selection through several conserved AAA ATPase domains (Fig. 3). In addition to these components, a conserved membrane-bound mycosin protease, $MycP_1$, processes specific ESX-1 substrates (62) and stabilizes the core membrane complex (63).

All of the known conserved ESX-1 components were identified in Tn insertion studies in both *M. tuberculosis* and *M. marinum* in 2003 and 2004 (8, 9, 49). In 2012, a study performed using both *M. marinum* and *M. bovis* BCG contributed the first biochemical isolation of the conserved core ESX-5 membrane complex, showing direct interactions between membrane components (64). Because components of the membrane complex are conserved between ESX systems and across species, these findings are considered generalizable to additional ESX systems, including ESX-1 (64).

Studies using proteins from several mycobacterial species and related actinobacteria provided insight into specific domains of individual ESX-1 core components. From 2013 to 2020, structures were reported for the soluble domains of $EccB_1$ and $EccD_1$ from *M. tuberculosis* (65, 66) and *M. smegmatis* (65). The study of the individual domains suggested that $EccB_1$ anchors the ESX-1 membrane complex into the periplasm and that $EccD_1$ forms a dimer with a ubiquitin-like fold (65, 66). The N-terminal domain of $EccA_1$ from *M. tuberculosis* included six tetratricopeptide repeats that likely mediate direct interactions with ESX-1 substrates (67). The structures of EccC (the ortholog of $EccCa_1$ and $EccCb_1$) from *Thermomonospora curvata* (52) and the C terminus of $EccCb_1$ from *M. tuberculosis* (68) revealed a hexameric structure with three interlocking AAA ATPase domains, the third of which directly binds ESX-1 substrates. The structures of the mycosins from *M. smegmatis* (69) and *M. tuberculosis* (70) uncovered mechanistic insight into how the membrane-anchored protease interacts with and cleaves the EspB substrate (62). Between 2017 and 2019, substantial work yielded structural insight into the intact membrane components of ESX-type transporters. Based on the structures of the ESX-3 systems from *M. smegmatis* (71, 72) and ESX-5 systems from *Mycobacterium xenopi*, ESX systems have a conserved multimeric architecture that spans the cytoplas-

mic membrane and protrudes into the periplasm and cytoplasm that is not shared by other types of secretion systems. These findings should be applicable to the *M. tuberculosis* ESX-1 system because the components are conserved.

The ESX-1-Secreted Substrates

Identification of conserved ESX-1 substrates. While the components of the ESX-1 system were being elucidated, beginning in 2005, studies in both *M. tuberculosis* and *M. marinum* contributed to our current understanding of which substrates were secreted by the ESX-1 system. Importantly, *M. marinum* provided complementary and unique insight into the molecular mechanisms of protein secretion to those discovered in *M. tuberculosis*. In particular, *M. marinum* has been and continues to be used to identify and study new ESX-1 substrates (73–77), some of which have been confirmed in *M. tuberculosis*. Moreover, the expression of *M. tuberculosis* genes in *M. marinum* has provided a means to broaden our understanding of functional conservation between ESX-1 proteins.

There are several general types of ESX-1 substrates (Fig. 3), including small 100-amino-acid (aa) proteins (similar to EsxA and EsxB [73]), PE/PPE proteins (PE is proline-glutamic acid and PPE is proline-proline-glutamic acid) (45, 74, 75, 78), and alanine-rich proteins (74, 76, 77). It is unclear how the three general groups of substrates contribute to ESX-1 secretion and virulence. However, all three substrate classes are secreted by both *M. tuberculosis* and *M. marinum*.

Although most of the initial studies in the early 2000s focused on the RD1 region and the secretion of EsxA and EsxB, it was clear by as early as 2005 that there were additional ESX-1 substrates, some of which were also encoded at unlinked genomic loci. In some cases, substrate genes were identified as being important for ESX-1 secretion and virulence before the resulting proteins were classified as secreted substrates. In 2005, the first substrates outside the RD1 locus were identified at the *espACD* locus (Fig. 2), which is required for *M. tuberculosis* virulence and ESX-1 secretion (79, 80). The *espACD* locus encodes two ESX-1 substrates in *M. tuberculosis*, EspA and EspC (73, 79, 80). EspA is an alanine- and glycine-rich 392-aa protein (81). EspC is a 103-aa protein (81). EspD, also encoded at the *espACD* locus, is required for the stability of EspA and EspC but is not itself strictly secreted as an ESX-1 substrate (82). Work by Fortune et al. revealed that EspA exhibited mutually dependent secretion with EsxA and EsxB in *M. tuberculosis*. The deletion of the *espA* gene resulted in the loss of EsxA and EsxB secretion, and the deletion of the *esxA* gene resulted in the loss of EspA secretion (80). It has not yet been determined if EspA is secreted from *M. marinum* or if it is required for *M. marinum* virulence.

The same genetic screen that identified specific genes in the ESX-1 locus as being important for *M. tuberculosis* virulence in mice (50) also identified a Tn insertion in the *rv3615c* (*espC*) gene, which was reported in 2005 (79). Based on the similarity between the *espE* (*rv3864*), *espF* (*rv3865*), and *espH* (*rv3867*) genes within the extended RD1 locus, it was hypothesized that the *espACD* genes were also required for ESX-1 secretion (79). A disruption of the *espC* gene resulted in an inability of *M. tuberculosis* to suppress the proinflammatory response in macrophages, similar to a disruption in the $eccD_1$ gene, which encodes a conserved membrane component of the ESX-1 system. Finally, using yeast two-hybrid analysis, this study demonstrated an interaction between EspD (Rv3614c) and the $EccE_1$ membrane component (79), further linking the *espACD* locus to the ESX-1 machinery. In 2009, the first demonstration that EspC from *M. tuberculosis* was secreted came from the expression of EspC from *M. tuberculosis* ($EspC_{MT}$) in *M. marinum* strains in the presence and absence of a functional ESX-1 system (73). Using this model, in addition to the ESX-1-dependent secretion of $EspC_{MT}$ by *M. marinum*, a direct interaction between the C terminus of $EspC_{MT}$ and the EccA component was defined (73). Recent studies in *M. tuberculosis* in 2017 suggested that EspC forms a filament, which may mean that EspC functions as a "needle" for the ESX-1 system (Fig. 3) (83). However, the role of EspC or its requirement for secretion has not yet been investigated in *M. marinum*. It is also important to note that despite the conservation

between the EspA, EspC, and EspD proteins, the *espACD* loci are found at different locations and in opposite orientations in the *M. tuberculosis* and *M. marinum* genomes (84).

Within the extended RD1 locus, there are 8 ESX-1 substrate genes, which, in addition to EsxA and EsxB, encode EspB, EspE, EspF, PE35, PPE68, EspJ, and EspK (Fig. 3). The EspB protein is an ~460-aa alanine- and glycine-rich protein (81) that was identified as a secreted substrate in both *M. marinum* and *M. tuberculosis* in 2007 (76, 85). EspB is secreted independently of EspA and EspC in *M. tuberculosis* (86). The structure of the EspB protein from *M. tuberculosis* was solved and revealed an N-terminal PE/PPE with an unstructured C terminus (87, 88). The PE/PPE domain oligomerizes (87, 88) and forms rings (89). The EspF protein was first identified as a substrate in *M. marinum* in 2009 (73) and is also secreted from *M. tuberculosis* (90). EspF is ~103 aa long, similar to the EspC protein (81). It was previously demonstrated that the *espF* gene was important for virulence in the *M. microti-M. tuberculosis* complementation model (59), which was later confirmed in *M. tuberculosis* (91). EspE was identified as a cell-associated substrate of the ESX-1 system in *M. marinum* in 2009 (77) and is also secreted from *M. tuberculosis* (90). The EspE protein is an ~402-aa protein, similar to the EspA protein (81). The requirement of the *espE* gene in virulence is still unresolved, as the *espE* gene has been considered essential for virulence in some studies (43, 77, 92) and dispensable in others (59). Both EspJ and EspK were identified as ESX-1 substrates in *M. marinum* through proteomic profiling in 2014 (74). EspJ and EspK are both alanine- and glycine-rich proteins of 280 aa and 729 aa, respectively (81). EspK was previously linked to the ESX-1 system in *M. marinum* as a potential targeting partner for EspB; it was demonstrated biochemically that EspK interacts directly with the $EccCb_1$ component and the EspB substrate (76). Neither EspK nor EspJ has been studied as the substrate in *M. tuberculosis*. PPE68 and PE35 constitute a PE/PPE pair at the ESX-1 locus. The PE/PPE proteins have a wide variety of roles, including but not limited to immunomodulation and host cell death (93), maintaining mycobacterial cell wall and capsule integrity (94, 95), antivirulence (96), and nutrient transport across the mycobacterial outer membrane (97, 98). PPE68 was first shown to be an immunogenic protein encoded by the RD1 locus in *M. tuberculosis* (99). In 2014 and 2019, PPE68 and PE35 were identified as ESX-1 substrates in *M. marinum* (74, 100) but have not yet been confirmed as substrates in *M. tuberculosis*.

ESX-1 substrates unique to *M. marinum*. Although the earliest identified ESX-1 substrates at the *esx-1* and *espACD* loci are highly conserved between *M. tuberculosis* and *M. marinum* (see above), several additional substrates have recently been identified in *M. marinum*, which are absent from *M. tuberculosis*. In part, diverse substrates between *M. marinum* and *M. tuberculosis* could indicate that *M. marinum* customizes its virulence to survive an expanded host range relative to that of *M. tuberculosis* (Appendix 2) (45). Accordingly, consistent with its more restrictive niche, it is interesting to note that there have not been ESX-1 substrates reported thus far that are specific to *M. tuberculosis* and absent from *M. marinum*.

The *M. marinum* genome includes 281 *pe/ppe* genes (6). Many PE/PPE proteins are known to be secreted by the paralogous ESX-5 system in *M. marinum* (94, 95, 101). However, there is evidence that there are PE/PPE proteins secreted by the ESX-1 system whose genes are located at unlinked chromosomal loci in the *M. marinum* genome. In 2018 and 2019, MMAR_2894 was identified as an ESX-1 substrate through proteomic screens (78) and verified experimentally (75). MMAR_2894 is required for the optimal secretion of the additional conserved ESX-1 substrates (75). Importantly, the secretion of ESX-1 substrates in the absence of *MMAR_2894* was reduced relative to the wild-type (WT) strain (75). However, MMAR_2894, although required for the hemolytic activity of *M. marinum*, was dispensable for the lysis of host cells in a macrophage model of infection (75). The study of this unique *M. marinum* substrate has potentially revealed basic mechanistic differences between hemolysis and host cell lysis. Alternatively, the levels of ESX-1 secretion from strains lacking *MMAR_2894* were sufficient to promote phagosomal escape and host cell death but were insufficient for hemolysis.

Additional PE/PPE proteins that are unique to *M. marinum* are secreted by ESX-1. For example, *M. marinum* has a partial ESX locus, known as ESX-6. In the ESX-6 locus, there are genetic duplications of several genes at the ESX-1 locus, including *pe35_1* and *ppe68_1* as well as *esxB* and *esxA*. EsxB_1, EsxA_3, PE35_1, and PPE68_1 are all secreted by the ESX-1 system in *M. marinum* (102–105). The ESX-6 locus, although containing several genes that appear to be duplications of ESX-1 genes, is dispensable for *M. marinum* virulence (105). Likewise, the EsxB_1 and EsxA_3 proteins encoded at this locus can be secreted by the ESX-1 system (104, 105) but must also have alternative routes out of the cell, as they are detected at high levels in secreted protein fractions from *M. marinum* strains lacking functional ESX-1 secretion systems (73, 74, 105, 106).

Substrate recognition by the ESX-1 system. ESX-1 substrates are recognized through direct interactions with membrane components and chaperones of the ESX-1 system. Work in both *M. tuberculosis* and *M. marinum* resulted in our current understanding of substrate targeting. Importantly, as indicated by early studies of EsxA and EsxB (8, 24, 29, 51, 107, 108), some ESX-1 substrates are likely targeted and secreted in pairs. Moreover, there are substrate-specific and general secretory signals required for targeting proteins for secretion. Overall, the data suggest that different types of ESX-1 substrates have different targeting rules.

Using *M. tuberculosis*, it was demonstrated in 2006 that the C terminus of the EsxB substrate mediates a direct interaction with the C-terminal half of the $EccCb_1$ protein (8, 107). The terminal 7 amino acids of EsxB (LSSQMGF) were shown to be sufficient for the interaction with $EccCb_1$ and for the targeting of EsxB and EsxA substrates for secretion to the ESX-1 system. The sufficiency of the direct interaction between the terminal 7 aa of EsxB and $EccCb_1$ has since been confirmed by structural studies in both *T. curvata* in 2015 (52) and *M. tuberculosis* in 2020 (68). Importantly, it was demonstrated that the third AAA ATPase domain of $EccCb_1$ directly interacts with the EsxB C-terminal 7 amino acids (52, 68). This interaction promotes the oligomerization of $EccCb_1$, which may link the energy required for transport to substrate recognition (52).

M. marinum has been widely used to further define how specific ESX substrates are selected by and targeted to ESX systems. Similar to EsxB, it was shown in 2009 that the C terminus of the EspC substrate from *M. tuberculosis* directly interacts with the EccA component from *M. marinum* (73). EccA is a cytosolic ATPase that interacts with several ESX-1 substrates, including EspF (73) and PPE68 (60), which may function to remove substrates from the $EspG_1$ chaperone (see below) prior to secretion (67, 109, 110).

Using *M. marinum*, additional regions of ESX-1 substrates have since been recognized to be required for substrate targeting, including the conserved YXXXD/E motif, which was reported in 2012 (87). Accordingly, mutation of this motif prevents substrate secretion. However, the YXXXD/E motif is insufficient to confer secretion through an ESX system (87) and does not confer specificity for ESX secretion (102).

The PE/PPE substrate pairs from both *M. tuberculosis* and *M. marinum* directly interact with EspG proteins, which are chaperones specific to each ESX system (103, 109–111). Chaperones escort substrates to the secretory apparatus but are not secreted from the cell. A comprehensive review of ESX chaperones was recently published (110). The EspG-substrate interaction has been defined structurally and genetically (103, 109–112). Interestingly, it was demonstrated in 2017 that the interaction between PE/PPE substrate pairs and the EspG chaperone dictates which ESX system secretes these proteins (Fig. 3) (102). Moreover, in 2020, it was demonstrated that the targeting of the PE/PPE heterodimers can also impact the targeting of Esx-type substrates (104). Therefore, the targeting of individual substrate pairs can be influenced by other substrates. Together, these studies in both *M. tuberculosis* and *M. marinum* suggest that substrate selection is complex, relying on the recognition of multiple signals present on substrates by both chaperones and secretory components through direct interactions.

ESX-1 LYSES HOST MEMBRANES

One long-standing and well-accepted hypothesis is that ESX-1 is essential for virulence because it lyses host membranes (7). In 1993, King and others observed that the virulent H37Rv strain of *M. tuberculosis* lysed red blood cells in a contact-dependent manner, while the avirulent strain H37Ra and the *M. bovis* BCG vaccine strain were defective for contact-dependent lysis (113). Although this study identified specific genetic loci required for lysis, it did not identify the ESX-1 system, in part because this study was reported prior to the observation that both *M. tuberculosis* H37Ra (114) and *M. bovis* BCG lack ESX-1 secretion and prior to the sequencing of the *M. tuberculosis* genome (30). Contact-dependent hemolysis of *M. tuberculosis* has been observed in numerous clinical isolates in addition to laboratory strain H37Rv (115). The King laboratory also discovered that *M. tuberculosis* exhibits contact-dependent lysis of pneumocytes *in vitro* (116). Using this lysis assay, it was discovered in 2003 that the insertion of a transposon into the *esxBA* operon resulted in a loss of cytolysis of pneumocytes and macrophages and reduced tissue invasiveness by *M. tuberculosis* (7). All activities were restored upon genetic complementation, linking the secretion of EsxA and EsxB to host cell lysis for the first time.

ESX-1-mediated lysis of host membranes is hypothesized to promote the rupture of the phagosomal membrane and to be required for intracellular growth (117). The first mycobacterial species shown to lyse the phagosome was *M. marinum* (118, 119). In 2003, *M. marinum* was shown to robustly enter the cytosol and polymerize host actin for motility (118), reminiscent of other intracellular pathogens, including *Listeria monocytogenes* (120). Consistent with early studies in *M. marinum*, *M. tuberculosis* was first observed to disrupt phagosomal membranes in macrophages by electron microscopy in 2007 (121). Phagosomal permeabilization was subsequently quantified using fluorescence resonance energy transfer (FRET). ESX-1-competent bacteria (*M. tuberculosis*, ESX-1-complemented BCG, and *M. marinum*) accessed the cytosol, whereas ESX-1-defective mycobacteria (BCG and *M. tuberculosis* ΔRD1) did not. Quantification of phagosome permeabilization by electron microscopy confirmed these data (117). It is important to note that although both *M. tuberculosis* and *M. marinum* access the cytoplasm, *M. marinum* is distinct in its use of actin-based motility downstream of phagosomal lysis (118, 122).

Mechanisms of Host Membrane Lysis

The mechanism of ESX-1-mediated host membrane lysis remains elusive and controversial. Since 2003, it was hypothesized that EsxA was necessary and sufficient for membrane lysis (7, 9, 123–126), stemming from the initial demonstration in *M. tuberculosis* that the ESX-1 system promoted host cell lysis (7). The requirement of EsxA for membrane lysis stemmed from genetic studies, while the sufficiency of EsxA for membrane lysis stemmed from biochemical approaches. The deletion or disruption of the *esxA* gene resulted in a loss of lytic activity in *M. tuberculosis* (7, 8), hemolysis in *M. marinum* (9), and phagosomal lysis and the translocation of mycobacteria to the cytosol (117, 121, 127). Together, these studies demonstrated that that EsxA was required for the lytic activity of the ESX-1 system. However, the interpretation of the results of these genetic experiments is complicated by the fact that EsxA is required for the secretion of the additional ESX-1 substrates (74, 76, 78, 80, 90) and may be a secreted component of the secretory apparatus (74, 78, 128–130). Therefore, the role of EsxA in the lytic activity of the ESX-1 system may be indirect.

Biochemical studies demonstrated that purified EsxA exhibited lytic activity *in vitro* (7, 124, 125, 131–133), suggesting sufficiency. However, subsequent work in 2015 demonstrated that EsxA exhibited lytic activity only when treated with detergent (133). Moreover, in 2017, it was demonstrated that contaminating detergent was the source of lytic activity from widely used protocols and preparations of the EsxA protein (134). Therefore, either EsxA demonstrates lytic activity under conditions other than those tested in the two previous studies or additional ESX-1 substrates or other virulence factors promote phagosomal lysis. Over the past several years, it has become clear that

mycobacterial virulence lipids, including phthiocerol dimycocerosates (PDIMs), work in concert with the ESX-1 system to promote optimal membrane lysis (135–137).

Investigation of *M. marinum* ESX-1-mediated membrane lysis has revealed that this lysis is pH independent. Moreover, as observed by electron microscopy, ESX-1-competent mycobacteria cause gross membrane disruptions at sites of direct contact with host membranes (134). These gross disruptions were observed in an extracellular hemolysis assay conducted at neutral pH. Furthermore, ESX-1-competent *M. marinum* disrupts macrophage phagosomal membranes independent of pH. Inhibiting phagosomal acidification using bafilomycin did not affect ESX-1-dependent cytosolic access as observed by a cytosolic CCF4 assay, in which bacterial contact with the host cytoplasm results in CCF4 cleavage and the loss of FRET (134).

Although the precise mechanism of membrane lysis remains elusive, it is clear that the downstream consequences of phagosomal rupture promote the host response to infection and the survival of mycobacterial pathogens. The consequences of phagosomal lysis include the release of nucleic acid (138–140) and secreted proteins (141, 142) into the cytosol, the activation of the host immune response (8, 143), inflammasome activation, autophagy, host cell death (117, 127, 144–146), and cell-to-cell spread (147). This list is far from comprehensive, and the downstream consequences of phagosomal lysis continue to be the subject of great interest to the field. (For an extensive review of the downstream impact of phagosomal lysis, see reference 142.)

EspH is required for the secretion of the known virulence factors EspE and EspF in *M. marinum* (78). EspH was found to be essential for infection of RAW macrophages, but *espH* mutants were hypervirulent in *M. marinum* zebrafish embryo infections (78). Because *M. marinum* encounters a greater diversity of environments than the obligate human pathogen, its transcriptional and posttranscriptional regulatory pathways may vary slightly to adapt to this organism's changing needs.

ESX-1 REGULATES GENE EXPRESSION

In addition to its well-established role in protein transport, it was demonstrated that the ESX-1 system regulates gene expression in *M. marinum* in 2017 (148) and in *M. tuberculosis* in 2019 (100). RNA sequencing of *M. marinum* and *M. tuberculosis* strains in the presence or absence of the ESX-1 system revealed widespread changes in gene expression, indicating a second, recently appreciated role for this protein transport system (100, 148), as discussed below. *M. marinum* studies have contributed the majority of our understanding of this new topic of research.

Feedback Control by the ESX-1 System

It was long established for several mycobacterial species that in the absence of a functional ESX-1 secretion system, ESX-1 substrates failed to accumulate within the mycobacterial cell (8, 9, 57, 59, 74, 77). This observation implied that the expression of ESX-1 substrates was regulated. In 2017, studies in *M. marinum* demonstrated that the ESX-1 system is subject to feedback regulation in which the levels of ESX-1 substrate production are linked to the assembly of the ESX-1 apparatus (148). In the absence of the ESX-1 membrane complex, the expression of the *whiB6* gene was reduced 50-fold (148). WhiB6 is an iron-sulfur cluster transcription factor (149) that positively regulates the expression of many genes encoding ESX-1 substrates (148, 150, 151). Reduced *whiB6* gene expression caused a corresponding decrease in the expression of the ESX-1 substrate genes at the *esx-1* locus (148). ESX-1-dependent changes in *whiB6* gene expression were also reported in *M. tuberculosis* albeit with a lower degree of repression (~5-fold) in the absence of ESX-1 secretion (90, 100).

The precise signals underlying feedback control of the ESX-1 system remain an open question. In *M. marinum*, at least one signal is the assembly of the ESX-1 membrane complex (148). It is impossible to separate the loss of the membrane complex from the loss of secretion. The deletion of individual genes encoding ESX-1 components in both *M. tuberculosis* and *M. marinum* destabilizes the entire complex (63, 64, 148). However, the expression of $EccCb_1$ alleles that result in a stable nonfunctional ESX-1 membrane

complex (52, 152) failed to repress *whiB6* gene expression in *M. marinum* (148). These data are consistent with the possibility that the loss of the membrane complex was the signal resulting in the loss of *whiB6* expression in *M. marinum*. It is also possible that ESX-1-associated mycosin also contributes to the signal for feedback control of ESX-1 because mycosins stabilize ESX membrane complexes in *M. marinum* (63), although this remains to be tested. The assembly of the secretory apparatus as a signal is reminiscent of the feedback control mechanism of flagella in *Campylobacter jejuni* (153). It remains possible that additional signals may also contribute to feedback control of the ESX-1 system.

In 2020, further studies in *M. marinum* demonstrated that the repression of *whiB6* gene expression in the absence of the ESX-1 system was at least partly dependent on a conserved transcription factor named EspM (154). The EspM protein directly binds the *whiB6-espM* intergenic region, repressing *whiB6* gene expression. An *M. marinum* strain lacking the *espM* gene revealed elevated levels of WhiB6, which were restored or repressed by the expression of the *espM* gene from *M. marinum*, *M. tuberculosis*, and *M. smegmatis*. Cross-species complementation of the *espM* gene confirms the functional conservation of the EspM protein across all three species (154). Interestingly, the *espM* gene is divergently transcribed from the *whiB6* gene and is immediately upstream of the start of the *esx-1* locus, expanding the extended ESX-1 locus (Fig. 2). However, the *espM* gene may be essential in *M. tuberculosis*, revealing a potential difference in regulation between *M. marinum* and *M. tuberculosis* (154).

In addition to this pathway, two-component systems, including PhoPR, control ESX-1 secretion by regulating *whiB6* and *espR* gene expression in *M. tuberculosis* (114, 150, 155, 156). Regulation of the *whiB6* gene by PhoPR in *M. marinum* has not been reported. The EspR transcription factor positively regulates the expression of ESX-1 substrates at the *espACD* locus in *M. tuberculosis* (157). The deletion of the *espR* gene in *M. tuberculosis* resulted in the loss of ESX-1 secretion. The loss of secretion was caused by the reduced expression of the *espACD* operon, which is required for secretion in *M. tuberculosis* (157). All of the studies characterizing the role and mechanisms of EspR in regulating gene expression have been performed in *M. tuberculosis* (157–162). However, a screen aimed at understanding *M. marinum* pathogenesis in cell lines derived from different hosts and amoebae indicated that EspR was disadvantageous for *M. marinum* infection of fish-derived cells but not human cells (45). This study demonstrates that regulators, including EspR, may be differentially required for *M. marinum* virulence, depending on the host (45). Together, the studies focused on feedback control of the ESX-1 system demonstrate that fine-tuning ESX-1 is an important part of virulence.

Regulation of Gene Expression by the ESX-1 System

It was demonstrated in 2017 and 2018 that the deletion of the *eccCb*$_1$ gene resulted in widespread changes in gene expression in both *M. marinum* and *M. tuberculosis* (100, 148). Based on studies in *M. marinum*, the WhiB6 and EspM transcription factors regulate a subset of genes affected by the loss of the *eccCb*$_1$ gene, indicating that there are likely additional transcription factors contributing to the control of gene expression by the ESX-1 system (148, 154). It is unclear why the ESX-1 system controls genes that are not obviously linked to the secretion system in both *M. marinum* and *M. tuberculosis*. However, functional analyses from *M. marinum* studies reveal that ESX-1-regulated genes include those involved in cellular metabolism, lipid synthesis, and stress response pathways, suggesting a potential link to adaptation to cytoplasmic exposure following the lysis of the phagosome by the ESX-1 system (154). In this way, work in *M. marinum* not only revealed novel roles for the ESX-1 system but also shed light on potential pathways required to respond to phagosomal lysis.

Posttranscriptional Regulation of the ESX-1 System

In addition to these transcriptional mechanisms, it has been widely established that specific ESX-1-associated proteins regulate protein secretion posttranscriptionally on

several levels. Based on studies in both *M. tuberculosis* and *M. marinum*, ESX-1 substrates are regulated at the level of protein stability through direct interactions. For example, the interaction between EsxA and EsxB is required for the stability of both proteins (8, 51, 108, 163). Several ESX-1-associated proteins stabilize substrate proteins and may act as substrate-specific chaperones. For example, studies in *M. tuberculosis* demonstrated that the EspD protein is required for the stability of the EspC and EspA substrates (82). Likewise, the EspL protein (which interacts with EspD) is required for the stability of the EspE and EspF substrates and the EspH component in *M. tuberculosis* (90). Studies in *M. marinum* demonstrated that EspH is required for the secretion of EspE and EspF (78). It is unclear whether EspL or EspH is the chaperone or if there is a difference in how these proteins function in *M. marinum* and *M. tuberculosis*.

In 2015, it was reported that EspI negatively regulates ESX-1 secretion in response to ATP levels in *M. tuberculosis* (164). The depletion of cellular ATP levels blocked ESX-1 secretion in an EspI-dependent manner. EspI is an ATP-binding protein that is highly conserved between *M. tuberculosis* and *M. marinum*. However, EspI has not been studied in *M. marinum*. EspI is predicted to have an FlhG domain, which is involved in the negative regulation of flagellar biosynthesis in *Vibrio cholerae* (165). The precise mechanism used by EspI to regulate the ESX-1 system is not known.

In *M. tuberculosis*, it was shown in 2010 that $MycP_1$ regulates secretion by post-translationally cleaving EspB. $MycP_1$ is required for infection in mice, and its deletion results in increased secretion and hyperactivation of innate immune pathways (62). Based on the conservation of the membrane complex between *M. tuberculosis* and *M. marinum*, it is likely that the role of the mycosin proteases is conserved.

CONCLUDING REMARKS

As we highlight above, it is impossible to fully appreciate the state of the ESX-1 field by considering work in *M. tuberculosis* or *M. marinum* alone. Studies using *M. marinum* complement the work in *M. tuberculosis*. *M. marinum* continues to provide key insight into the conserved genes required for secretion and the functional relationships between ESX-1 components and substrates. Studies in *M. marinum* continue to reveal ESX-1 substrates. One of the major contributions of the *M. marinum* system in recent years has been defining new conserved roles of the ESX-1 system in pathogenesis and physiology. In addition to what is covered here regarding cell lysis and regulation, there have interesting publications on *M. marinum* linking the ESX-1 system to additional functions, including motility and biofilm formation (166) and downregulating microRNAs (miRNAs) (167). Moreover, several aspects of *M. marinum* physiology and pathogenesis, from its robust hemolytic activity to its unique interaction with zebrafish, make this species a powerful tool to study ESX-1-mediated secretion and virulence. Importantly, understanding the conserved aspects between the ESX-1 systems in *M. tuberculosis* and *M. marinum* is likely as important as understanding how these systems differ (Fig. 2, Appendix 2, and Table 1). It is clear that *M. marinum* customizes both the arsenal of ESX-1 substrates as well as regulation by the ESX-1 system to promote virulence in a variety of natural hosts.

As we as a field continue to study the molecular mechanisms and consequences of ESX-1 secretion, there are several outstanding questions that will likely be facilitated by the continued use of *M. marinum* in concert with *M. tuberculosis* and other mycobacterial species. How are ESX-1 substrates and other secreted proteins transported across the mycobacterial envelope beyond the cytoplasmic membrane? How conserved is the regulation of and by the ESX-1 system between *M. marinum* and *M. tuberculosis*? Do differences in the regulation of and by the ESX-1 system between *M. marinum* and *M. tuberculosis* explain differences in the degree of phagosomal lysis? How does the ESX-1 system promote membrane lysis, and are these mechanisms conserved between *M. tuberculosis* and *M. marinum*? Finally, can *M. marinum* be exploited to enhance protection against or treatment of *M. tuberculosis* infection? A recent study exploited the *M. marinum* RD1 region to increase the efficacy of the BCG vaccine (168), indicating that studies in *M. marinum* may have more translational applications. In the end, it is clear

TABLE 1 Comparison between *M. tuberculosis* and *M. marinum*

Property	Description (reference[s])[a]	
	M. tuberculosis	***M. marinum***
Disease burden	1.5 million people worldwide in 2018 (1) Top 10 cause of mortality and leading cause from a single infectious agent (1)	Pulmonary NTM now surpass *M. tuberculosis* infections in the US, Canada, and western Europe (3); for *M. marinum*, the incidence rate in Denmark from 2010–2016 was 0.05–0.13 per 100,000 person-years (170)
Host range	Humans are the only reservoir for TB (171), with some sublineage specificity (172)	Amphibians, fish, and humans (173) Suspected free-living amoebae (174)
Aerobe?	Obligate aerobe (175)	Suspected obligate aerobe (176)
Cell wall properties	Acid fast (177) Contains mycolic acids, PDIM, TDM, PGL, and SL1 (178)	Acid fast (15) Similar lipid composition; lacks the nonvirulent SL1 (178); unique LOS (179)
Generation time (h)	17–18 (180)	4–6 (181)
Optimal temp (°C)	37 (182)	30 (181)
Biosafety level	3 (183)	2 (183)
Role of phagolysosome?	Resisted (184)	Similar to *M. tuberculosis* (185)
Role of TNF-α?	Increased susceptibility by anti-TNF treatment (186) Increased susceptibility in TNF KO (187) Excessive TNF-α increases susceptibility (188)	Higher incidence than TB during anti-TNF therapy (189) Increased susceptibility in TNF receptor knockdown (190) Excessive TNF-α increases susceptibility (191)
Susceptibility to adaptive immunity?	Controlled by polyfunctional $CD4^+$ and $CD8^+$ T cells (192) Unknown what T-cell responses protect against infection (192)	*Rag1* KO zebrafish are hypersusceptible (193) IL-10 mutant zebrafish resist *M. marinum* infection via interferon gamma (194)
Granuloma formation	Forms necrotic granulomas (46)	Forms necrotic granulomas (46)
Presence of ESX-1 secretion system?	Yes (7, 8, 31, 33)	Yes (6, 9)
Susceptibility to first-line antibiotics, by MIC (μg/ml)	Isoniazid, 0.1 Rifampicin, 0.5 Ethambutol, 4 (195) Pyrazinamide, 25 (196)	Isoniazid, 16 Rifampicin, 1.0 Ethambutol, 1.0 (197, 198) Pyrazinamide, intrinsically resistant (4)

[a]LOS, lipooligosaccharide; TNF-α, tumor necrosis factor alpha; KO, knockout; IL-10, interleukin-10; TDM, trehalose-6-6-dimycolate; PGL, phenolic glycolipids.

that *M. marinum* will remain an important organism for revealing the mechanism, regulation, and role of ESX-1 secretion.

APPENDIX 1

The glossary below serves as a reference to tie these diverse names and nomenclature referring to type VII/ESX secretion together.

CFP-10 Ten-kilodalton culture filtrate antigen, also known as EsxB (26).

Ecc proteins The secretory machinery is made up of ESX-1 conserved components, or Ecc proteins, which span the cytoplasmic membrane and reside in the cytoplasm (Fig. 2 and 3). Subscripts in the Ecc or Esp names refer to the ESX system with which the protein is associated. For example, $EccD_1$ is the EccD protein associated with the ESX-1 system. (For further nomenclature information, see reference 20.)

ESAT-6 Six-kilodalton early secreted antigenic target, also known as EsxA (26).

Esp proteins Esx secretion-associated proteins that can be secreted substrates or components of the secretion system, such as EspB (76) or EspL (90). These proteins are not conserved across ESX systems.

ESX-1 The name reflects that these specialized systems secrete proteins similar to EsxA, which was originally named ESAT-6, or 6-kDa early secreted antigenic target.

Esx proteins Small ~100-aa substrates of ESX systems, which include a WXG domain (24), including EsxA and EsxB.

gene identifiers Genes identified from sequencing the laboratory strain *M. tuberculosis* H37Rv are labeled in descending order of genome locus, with a "c" if the gene is present on the complementary strand. For instance, the *espJ* gene has the identifier *rv3878* and is found on the forward strand. In comparison, the neighboring gene *espK* has the gene identifier *rv3879c* and is found on the complementary strand.

PE or PPE proteins Proline-glutamic acid or proline-proline-glutamic acid proteins secreted by ESX systems, such as PPE68 (99, 199).

RD1 locus The region of difference 1 (RD1) locus is a genomic region lost in the vaccine strain *M. bovis* BCG (31, 33).

Snm proteins Esx-1 proteins were initially referred to as Snm proteins, which stands for "secretion in mycobacteria." One example is Snm4, which is synonymous with EccD1 (8).

type VII secretion The ESX secretion systems were discovered to be distinct from the six other classes of secretion systems and were categorized as type VII secretion systems (T7SSs) (21).

APPENDIX 2

What is different about *M. marinum*? Despite the conservation between the ESX-1 systems, there are important differences between *M. marinum* and *M. tuberculosis*. *M. marinum* has an expanded host range relative to *M. tuberculosis*, which likely impacts how it uses conserved virulence pathways, including ESX-1 (45). Accordingly, *M. marinum* has a significantly larger genome than *M. tuberculosis* (6). *M. marinum* has plasmids that are absent in *M. tuberculosis* (6), and some strains of *M. marinum* carry an additional ESX system on a conjugative plasmid that is absent from the M strain (36). *M. marinum* has an expanded ESX-1 substrate repertoire that is not conserved in *M. tuberculosis*. *M. marinum* has an ESX-6 locus that includes a duplication of several genes from the ESX-1 locus, including the genes encoding demonstrated substrates, PE35_1, PPE68_1, EsxB_1, and potentially EsxA_3 (78, 103–105). Likewise, additional substrates, similar to MMAR_2894 (75, 78), are likely to be identified. Importantly, following phagosomal lysis, *M. marinum* uses actin-based motility (118), which has not been observed for *M. tuberculosis*. For additional differences, see Table 1.

ACKNOWLEDGMENTS

The Champion laboratory is supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under award numbers AI149235 and AI142127 to P.A.C. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. A.B. and W.C. are supported by Lake Forest College.

We thank Micah Ferrell and Lalita Ramakrishnan for the critical reading of the manuscript.

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