Chapter 2

Tubercle bacilli reply on a type VII secretion system for pathogenisis

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CHAPTER 2

TUBERCLE BACILLI RELY ON A TYPE VII ARMY FOR PATHOGENICITY

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ABSTRACT
Mycobacteria, such as the major human pathogen *Mycobacterium tuberculosis*, have a highly unusual and characteristic diderm cell envelope that protects them against harmful conditions. Protein secretion across this hydrophobic barrier requires specialized secretion systems. Recently, a type VII secretion (T7S) pathway has been identified that fulfills this function. Pathogenic mycobacteria have up to five different T7S systems, some of which play a crucial role in virulence. The interactions between secreted substrates and host molecules are only starting to become clear and will help in furthering our understanding of the persistence of these enigmatic pathogens. In this review, we discuss the current knowledge on the role of T7S systems in mycobacterial virulence.
RELEVANCE OF PROTEIN SECRETION IN MYCOBACTERIA

Tuberculosis (TB) is one of the oldest diseases known to man and *Mycobacterium tuberculosis* still infects approximately one-third of mankind, causing over 1.5 million deaths annually (http://www.who.int/tb/publications/global_report/2011). The currently used live attenuated vaccine strain *Mycobacterium bovis* BCG does not effectively protect against pulmonary TB in adults and multi-, extensively- and even totally drug-resistant *M. tuberculosis* strains have been emerging, so there is an urgent need for new TB intervention strategies. A better understanding of host-pathogen interactions underlying mycobacterial pathogenicity would enhance the development of more effective anti-TB therapies. Recently, considerable research effort has focused on mycobacterial protein secretion systems and their substrates. Secreted proteins, either located in outer parts of the cell envelope or secreted into the environment, are well positioned to interact with the host. However, protein secretion in mycobacteria is not straightforward because the transported proteins have to cross the unique mycobacterial cell wall, composed of a cytoplasmic membrane and a second membrane consisting mainly of long-chain fatty acids called mycolic acids. A major pathway by which proteins are transported across this unique hydrophobic barrier is known as ESX or type VII secretion (T7S). Here, we review the recent advances in the field of T7S and focus particularly on the role of T7S in mycobacterial virulence.

IDENTIFICATION OF T7S SYSTEMS IN MYCOBACTERIA

The discovery of T7S systems started with the identification of the highly immunogenic 6-kDa early secreted antigenic target (ESAT-6 or EsxA) protein in culture filtrate of *M. tuberculosis* [1]. Shortly thereafter, comparative genomics showed that a genetic region known as RD1, comprising nine genes including the esxA gene, was absent in all attenuated *M. bovis* BCG vaccine strains [2]. Interestingly, complementation studies demonstrated that, whereas expression of EsxA was restored upon introduction of the esxA operon, secretion was only observed after reintroduction of the entire RD1 region in BCG [3]. This finding, combined with the fact that EsxA lacks a distinguishable N-terminal signal sequence and that the esxA flanking genes are predicted membrane proteins, indicated that this locus encodes a secretion machinery. Indeed, disruption of multiple individual genes flanking esxA abrogated secretion [4, 5]. Since there is no obvious similarity between this new secretion system and other known secretion systems, it is also referred to as type VII secretion (T7S) [6]. Use of the nomenclature for secretion systems in Gram-negative bacteria it was also emphasized that mycobacteria have a diderm cell envelope [7].
**T7S SYSTEM COMPONENTS AND SUBSTRATES**

*M. tuberculosis* has five T7S systems, designated ESX-1 to ESX-5 [8, 9]. Phylogenetic analyses established that the mycobacterial ESX clusters evolved through gene duplication and subsequent gene diversification events and that ESX-4 is most ancestral [9]. The ESX loci are centered around a set of *esx* genes, encoding two small EsxA-like proteins. The Esx proteins form tight 1:1 complexes and are the classical T7S substrates [10, 11]. Genes flanking the *esx* genes encode either ESX core components (called Ecc) or ESX secretion-associated proteins (Esp) [12]. All ESX systems contain the core components *eccB*, *eccC*, *eccD* and *mycP*. EccB is a predicted membrane protein; EccC, which in some cases is encoded by two adjacent genes, has an ATPase domain providing energy for protein transport and probably recognizes the secreted substrates; EccD contains multiple transmembrane domains and it has been proposed that it functions as an inner membrane export channel; and the mycosin MycP is a membrane-anchored protein with protease activity that might be involved in processing of some substrates [9]. With the exception of ESX-4, the systems also include the conserved components EccA, a cytosolic ATPase that interacts with a distinct set of substrates, and EccE, another transmembrane protein with unknown function (Fig. 1) [13]. *In silico* analyses suggested that EccE might form the outer membrane channel, which could indicate that in contrast to the other ESX systems, ESX-4 does not function in secretion of proteins across the entire cell envelope [14]. However, currently there is no experimental evidence supporting a one-step secretion mechanism. Therefore, the possibility remains that T7S is in fact a two-step process involving an independent outer membrane translocation machinery. This theory is supported by a study in which secretion of Esx proteins fused to a β-lactamase reporter was analyzed [15].

In addition to the core components, all T7S systems apart from ESX-4, also contain one or several members of the PE and PPE protein family, named after their characteristic N-terminal proline-glutamic acid (PE) and proline-proline-glutamic acid (PPE) motifs [8, 9]. Interestingly, PE and PPE genes are not restricted to T7S systems, but are found scattered throughout the genome [16]. A somewhat similar situation is observed for the *esx* genes, because several tandem repeats of *esx* paralogs are encoded outside ESX loci [9]. The demonstration that extensive amounts of PE and PPE proteins are secreted through ESX-5 indicated that T7S systems have a larger range of substrates than initially anticipated [17]. Finally, some ESX systems have region-specific genes (*esp* genes). It was recently shown that a number of Esp proteins are in fact also T7S substrates, stretching the flexibility of the T7S systems even further [13, 18, 19]. Although the recognized diversity of T7S substrates is increasing, a general picture is emerging according to which substrates either have a recently identified T7S motif (YxxxD/E) in their C terminus or they are secreted as a complex with another protein that has such a motif [20].
Role of T7SS in mycobacterial virulence

MYCOBACTERIAL PATHOGENICITY

A typical characteristic of TB is the formation of granulomas, which consist of extensively clustered immune cells around infection foci. Although granulomas have long been considered host-protective, it has recently become clear that mycobacteria actively promote granuloma formation and use the immunological structures as their replication niche [21]. To induce granuloma formation, mycobacteria first need to enter their primary host cell, the macrophage, where they are faced with an array of antibacterial defense mechanisms. Mycobacteria have developed strategies to circumvent intracellular destruction by interfering with phagosomal maturation and, in later stages, escape from phagolysosomes into the cellular cytosol. After the initial survival phase, infected cells recruit other macrophages and neutrophils to the site of infection, which are exploited by mycobacteria for dissemination. Subsequently, lymphocytes migrate to these cellular clusters and semi-stable granulomas are established. Although granulomas physically contain the infection, they often fail to eradicate the microbes, probably because of extensive immune modulation by the TB bacilli.

Figure 1. Schematic model for the type VII secretion (T7S) system. The four ESX core components present in all characterized mycobacterial systems are depicted in blue and are predicted inner membrane (IM) proteins. EccC contains three nucleotide binding motifs and could therefore energize the secretion process. MycP is a predicted protease probably responsible for processing of certain substrates. The EccA and EccE components, depicted in green, are present in four of the five mycobacterial T7S systems. EccA is a cytosolic ATPase of the AAA family. The red proteins represent T7S substrates, including the small Esx proteins that form heterodimers, Esp proteins, and PE and PPE proteins. The channel facilitating transport across the outer membrane (OM) has not been identified yet.
To survive in the oxygen- and nutrition-scarce environment of the granuloma, mycobacteria adapt their metabolism and switch to a persistent state, which can last for decades. Under specific circumstances, host cells in the center of mature granulomas undergo necrosis, facilitating bacterial replication, granuloma rupture, and bacillus entry into the airways [22]. In the next sections, we discuss the different ESX systems in the order that they probably evolved and their effect on mycobacterial pathogenicity. We will primarily focus on studies of *M. tuberculosis* and of *Mycobacterium marinum*, the causative agent of a TB-like disease in ectotherms such as fish.

**ESX-4**

As mentioned previously, ESX-4 is the most archaic T7S system in mycobacteria and homologs are also found in other GC-rich Gram-positive Actinobacteria such as Corynebacterium diphtheriae [9]. The ESX-4 locus is the smallest ESX locus and contains the minimal set of T7S genes. Proteomic studies did not reveal secretion of EsxU and EsxT under laboratory conditions, putting the functionality of the system into question [23]. ESX-4 is conserved in most mycobacteria, but is, with the exception of esxT, absent in *Mycobacterium leprae* [9].

Functional data on ESX-4 are lacking, but multiple *M. tuberculosis* genetic screens have shown that ESX-4 genes are not essential [24, 25]. More is known about the regulation of this secretion system. Several ESX-4 genes, including esxU and esxT, are regulated by the mycobacterial sigma factor SigM. However, a *M. tuberculosis* sigM mutant was not attenuated for growth in murine macrophages or for progressive infection in mice [26]. Accordingly, an important role for this T7S system in the early stages of mycobacterial virulence seems unlikely, although the involvement of ESX-4 in persistent infection remains to be established.

**ESX-1**

**System components, substrates, and species specificity**

The ESX-1 locus has evolved as the first of the larger T7S systems and is specific for mycobacteria, although a more distant homolog is present in *Nocardiia farcinica* [12]. It is the most extensively studied ESX system and encodes all the basic core components. After its duplication from ESX-4, the eccA and eccE core components and PE and PPE genes were inserted into this region [9]. In addition, the region has an extensive number of esp genes. The number of identified substrates secreted by the ESX-1 system has been increasing recently. Besides EsxA and EsxB (ESAT-6 and its partner the 10-kDa culture filtrate protein CFP-10), ESX-1-dependent secretion has been reported for EspB, EspE, EspF, EspK, and PPE68, although there are some species-specific differences [13, 18, 19, 27]. The ESX-1 system is conserved in a large number of mycobacterial species, including both pathogens and non-pathogens. By contrast, the genomes of a number of (opportunist) pathogens, such as *Mycobacterium avium* and *Mycobacterium ulcerans* show (partial) deletion of this system, indicating that these pathogens have an infection cycle independent
of ESX-1 [9, 28]. A genetic cluster not linked to the ESX-1 locus encodes EspA, EspC, and EspD. These proteins, homologs of EspE, EspF, and EspH, respectively, are also secreted in an ESX-1-dependent manner [29-31]. Interestingly, the espACD locus is exclusively conserved in pathogenic mycobacteria including M. marinum and M. leprae, suggesting a virulence-related role. Furthermore, espA is extensively duplicated in M. marinum; 18 paralogs are present and 15 of these espA-like genes are located in a M. marinum-specific region directly upstream of the ESX-1 locus [32]. Although it is not known whether these additional Esp homologs are secreted, a transposon insertion in the extended ESX-1 region reduced the capacity of M. marinum to initiate granuloma formation [33].

Mechanism of secretion
Numerous genetic studies have identified ESX-1 or ESX-1-related genes crucial for EsxA/B secretion (Table 1). Studies in M. tuberculosis, M. marinum or Mycobacterium smegmatis demonstrated that disruption of most of the conserved core components (eccB-E and mycP) abrogates secretion of EsxA and most other substrates [4, 5, 18, 19, 29, 33-38]. The role of the conserved component EccA is somewhat less clear because it is required for EsxA secretion in M. tuberculosis and M. marinum whereas conflicting data on its role in EsxA/B secretion in M. smegmatis have been reported [4, 36, 37, 39]. EsxA/B secretion also depends on the presence of a number of Esp proteins (Table 1) [4, 5, 18, 29, 31, 33, 36, 37, 39-42]. Intriguingly, EspA, EspC, and EspD are essential for EsxA secretion and vice versa [29-31, 42]. This mutual dependence could also be true for some of the other Esp proteins and complicates the identification of unique functions for individual ESX-1 substrates in pathogenicity. The interdependence also suggests that complex formation of secreted substrates occurs during or after secretion and is required for ESX-1 system activity.

Several recent papers have been discussing the regulation of the ESX-1 secretion system. The first study showed that attenuation of the H37Ra laboratory strain of M. tuberculosis was due to a phoP mutation, impairing transcription of the espACD operon and thereby disrupting EsxA secretion and M. tuberculosis growth in macrophages and mice [43]. A second study showed that EspR functions as an ESX-1 regulator through activation of the same espACD operon. The same study suggested that EspR is an ESX-1 substrate itself and that active EspR secretion would negatively regulate ESX-1 activity [44]. However, another group recently found that EspR is not a secreted protein and functions as a more general regulatory nucleoid-associated protein [45].

Role in virulence
Although comprehensive knowledge of the process of ESX-1 secretion is lacking, it is clear that ESX-1 is required for mycobacterial pathogenicity. Reintroduction of the deleted RD1 fragment in M. bovis BCG enhanced virulence; conversely, deletion of RD1 from M. tuberculosis resulted in attenuation of virulence [3, 35]. ESX-1-related phenotypes have been widely investigated (Table 1) and several
Table 1. Reported effects of ESX-1 components.

<table>
<thead>
<tr>
<th>Component</th>
<th>EsxA/B secretion</th>
<th>Intracellular growth</th>
<th>Phagosomal maturation</th>
<th>Translocation</th>
<th>Lysis</th>
<th>Immunomodulation</th>
<th>DNA transfer</th>
<th>Virulence</th>
</tr>
</thead>
<tbody>
<tr>
<td>espE</td>
<td>No [37]</td>
<td>No [5]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Yes [59]; No [37]</td>
</tr>
<tr>
<td>espF</td>
<td>Yes [39]; No [37, 47]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Yes [37, 47]</td>
</tr>
<tr>
<td>espH</td>
<td>Yes [4, 18, 40]; No [37]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No [37]</td>
</tr>
<tr>
<td>eccB</td>
<td>Yes [36, 37, 39]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Yes [39, 62]</td>
</tr>
<tr>
<td>eccCa</td>
<td>Yes [5, 34, 36, 37]</td>
<td>Yes [4, 34]</td>
<td>Yes [50]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Yes [5, 37, 59]</td>
</tr>
<tr>
<td>eccCb</td>
<td>Yes [3, 5, 33-37, 39, 48]</td>
<td>Yes [33, 48, 50]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Yes [33, 37, 59]</td>
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<tr>
<td>pe35</td>
<td>Yes [37, 39]; No [35]</td>
<td>Yes [3, 34, 48]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Yes [39, 62]</td>
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<tr>
<td>ppe68</td>
<td>No [35, 37]</td>
<td></td>
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<td></td>
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<td></td>
<td>Yes [59]; No [35, 37]</td>
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<tr>
<td>espI</td>
<td>Yes [3, 34-37]</td>
<td>Yes [34]</td>
<td>Yes [50]</td>
<td>Yes [41]</td>
<td>Yes [34]</td>
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<tr>
<td>eccD</td>
<td>Yes [4]; No [18, 37]</td>
<td>Yes [52]</td>
<td>Yes [4]</td>
<td>No [37]</td>
<td></td>
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<tr>
<td>espL</td>
<td>Yes [33]; No [37]</td>
<td>Yes[33, 49]</td>
<td>Yes [49]</td>
<td>Yes [33]</td>
<td></td>
<td></td>
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<tr>
<td>mycP</td>
<td>Yes [36, 38, 39]; No [37, 39]</td>
<td>Yes [38]</td>
<td>Yes [41]</td>
<td>Yes [4]</td>
<td>Yes [39]; Yes [38]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>espC</td>
<td>Yes [30, 42]; No [31]</td>
<td>Yes [50]</td>
<td>Yes [50]</td>
<td>Yes [42]</td>
<td>Yes [59]</td>
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<tr>
<td>espD</td>
<td>Yes [31]</td>
<td>Yes [31]</td>
<td>Yes [59]</td>
<td>Yes [59]</td>
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</table>

The core components of the system are depicted in bold. ‘Yes’ indicates that an effect was found; ‘no’ indicates that no effect was observed.
independent studies have shown that a functional ESX-1 system is involved in the early stages of infection by promoting intracellular growth of *M. tuberculosis*, *M. bovis* BCG, and *M. marinum* in macrophages [4, 5, 33-35, 38, 40, 42, 46-49]. The molecular basis of the intracellular attenuation of ESX-1 mutants is still a matter of debate; it has been demonstrated that ESX-1 of both *M. tuberculosis* and *M. marinum* is involved in phagosomal maturation arrest [40, 48-50], whereas other studies showed that mycobacteria circumvent eradication in phago(lyso)somes by translocating to the cytosol of their host cell in an ESX-1-dependent manner [41, 51, 52]. In line with these data, it has been reported that ESX-1 secretion is associated with membranolytic activity and that EsxA functions as a pore-forming toxin [4, 35, 41, 52, 53]. However, it should be mentioned that these studies were performed with monomeric EsxA without its natural partner EsxB. Houben et al. recently analyzed the subcellular localization of a range of mycobacterial species and found that cytosolic translocation was restricted to the pathogenic species, suggesting that phago(lyso)somal escape is a mycobacterial virulence determinant and that EsxA secretion is required for this process [54]. Although all these data indeed seem to indicate that EsxA is involved in phago(lyso)somal rupture, it is puzzling that the non-pathogenic *M. smegmatis* secretes EsxA, but is unable to translocate to the cytosol. Differences in EsxA sequences between *M. tuberculosis* and *M. smegmatis* may account for this different function. Alternatively, because *M. smegmatis* is not able to replicate intracellularly owing to its non-pathogenic nature, it is possible that cell division and active metabolism are required for EsxA secretion and phago(lyso)somal escape. As a third possibility, EsxA of pathogenic mycobacteria might facilitate the export of another (unidentified) effector protein required for translocation. EspA is a primary candidate for this, considering its exclusive conservation in pathogenic mycobacteria.

It has been reported that phago(lyso)somal escape of pathogenic mycobacteria enhances bacterial replication and precedes an atypical form of host cell death [51, 52, 55]. Mycobacteria most probably exploit this host cell death to escape innate host immune responses and to initiate dissemination and subsequent granuloma formation. Indeed, the ESX-1 system has been associated with inflammasome activation and manipulation of cytokine responses of infected macrophages in order to secure mycobacterial intracellular survival and/or to elicit an increased inflammatory response favoring bacterial spread [34, 42, 56, 57]. However, it is currently unknown whether this effect is directly mediated by ESX-1 substrates or is a consequence of ESX-1-mediated phago(lyso)somal escape. Consistent with a role for ESX-1 in dissemination of infection, *M. marinum* induced ESX-1-dependent formation of an actin-based structure called the ejectosome in the amoeba *Dictyostelium*. This structure is required for bacterial escape from these host cells and subsequent intercellular spread [58].

In animal models of infection, major differences are observed between ESX-1 mutants and their parental strains. Infection with mutants in genes encoding core components resulted in increased survival of infected animals, reduced bacterial replication, and mild inflammatory responses with lower numbers of granulomas.
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[4, 5, 33-35, 37, 38, 59-61]. Mutants of many of the ESX-1 substrates also seem to be attenuated, although variable results were obtained for some substrates, depending on the mycobacterial species or the infection model used (Table 1) [4, 5, 29, 33, 37, 47, 59].

Although the role of ESX-1 in virulence of pathogenic mycobacteria is clear, nonetheless it has a completely different function in the environmental species M. smegmatis. ESX-1 is involved in conjugal DNA transfer in this bacterium: it is required for conjugation by the donor strain, whereas it impairs conjugation in the receptor strain [39, 62]. The link between these very different ESX-1 functions is not understood, but could be related to substrate variation.

ESX-3

The ESX-3 locus encodes the full set of core components, a PE/PPE pair, and a single Esp protein, i.e. EspG. Similar to EsxA/B, EsxG and EsxH form a tight heterodimeric complex and have been detected in culture filtrates of M. tuberculosis [11, 23]. Apart from these two proteins, no other substrates of ESX-3 have yet been identified. This T7S system is conserved in all available mycobacterial genomes, suggesting an important role. Indeed, genes of the ESX-3 system are essential for M. tuberculosis but not for M. smegmatis [24, 25, 63, 64].

A function for ESX-3 in metal homeostasis has been suggested, because it was demonstrated that the ESX-3 region of M. tuberculosis is regulated by iron and zinc availability [65, 66]. Interestingly, expression of the M. smegmatis ESX-3 locus depends solely on iron [64, 67]. These findings could reflect the different habitats of both species, because iron is limiting in most environments, whereas zinc-deficient conditions are predominantly encountered in the host. The in vitro growth deficiency of (conditional) ESX-3 mutants could be rescued by the addition of iron or zinc or the culture supernatant from wild-type mycobacteria, implying the involvement of one or more secreted substrates in metal homeostasis [63, 64]. One study suggested that ESX-3 is required for the uptake of iron via the siderophore mycobactin [64]. This would explain why the ESX-3 mutation in M. smegmatis is not lethal, because this species produces a second siderophore, exochelin. However, mycobactin-dependent iron uptake via ESX-3 cannot explain the situation in M. leprae, which has ESX-3 but is unable to produce mycobactin [68]. Therefore, ESX-3 could be involved in an alternative mycobacterial iron uptake mechanism. Taken together, these studies clearly show that the ESX-3 system is involved in metal uptake, although the actual mechanism is still unclear.

A recent study using an artificial murine infection model with a high dose of M. smegmatis demonstrated a specific role for the ESX-3 locus in mycobacterial pathogenesis [69]. In contrast to wild-type bacteria, virulence of a M. smegmatis ESX-3 mutant was highly attenuated. This attenuation was not due to a decreased viability, since infection of myd88-/- mice was comparable between mutant and parental bacteria. This led the authors to conclude that ESX-3 of M. smegmatis is involved in evasion of Myd88-dependent innate immunity. Intriguingly,
introduction of the *M. tuberculosis* ESX-3 locus in the *M. smegmatis* ESX-3 mutant elicited high levels of protective immunity against *M. tuberculosis* in mice. At present it is not completely clear if this protective effect is due to the *M. tuberculosis* ESX-3 region, because the introduced cosmid also contained a dozen other genes. Further studies are therefore required to unravel the involvement of ESX-3 in virulence and granuloma formation.

**ESX-2**

The ESX-2 region is immediately adjacent to the ESX-1 locus and harbors genes encoding the complete set of core components, a PE and PPE protein couple, and two Esp proteins. Similar to the ESX-4 system, neither the candidate substrates of this system, EsxC and EsxD, nor PE36, PPE69, or the associated Esp proteins have been detected extracellularly. Furthermore, several pathogenic and non-pathogenic mycobacteria do not have an orthologous system.

Transposon mutagenesis screens in *M. tuberculosis* showed that mutants for all ESX-2 genes are viable [24, 25], but none of these mutants has yet been described phenotypically. Therefore, although a function of the ESX-2 system in *M. tuberculosis* remains to be elucidated, a relation with the early stages of infection seems unlikely.

**ESX-5**

**System components, substrates, and species specificity**

The ESX-5 system is the most recently evolved mycobacterial T7S system and contains, besides the core components, three region-specific genes and multiple copies of *PE* and *PPE* genes. Interestingly, the expansion of *PE* and *PPE* genes in mycobacterial genomes seems to have occurred after the ESX duplication event that resulted in the emergence of ESX-5. Therefore, PE and PPE proteins were thought to be functionally linked to this secretion system [16]. Multiple studies have now demonstrated that, besides EsxN/M, several PE and PPE proteins are secreted by ESX-5 in both *M. tuberculosis* and *M. marinum* [17, 70-73]. Furthermore, it has been hypothesized that in fact most PE and PPE proteins are secreted through ESX-5, which would make this the most versatile T7S system in mycobacteria [17]. This theory was supported by a recent study demonstrating that the presence of T cells specific for PE and PPE peptides, whether encoded by ESX-5 or not, depends on the presence of ESX-5 [73]. Interestingly, ESX-5 is exclusively present in the so-called slow-growing mycobacterial species and seems to be highly conserved in these species, even in *M. leprae*, although only the core components are preserved in this species.

**Role in virulence**

Given the abundance of PE and PPE proteins in slow-growing pathogenic mycobacteria and the reported localization of these proteins at the mycobacterial
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cell surface, the hypothesis was raised that ESX-5 has a role in manipulating host immune responses [74]. In vitro studies have revealed that, in contrast to ESX-1, ESX-5 is not required for escape into the cytosol [75, 76]. Apparently, ESX-5 substrates are involved in later steps of the macrophage infection cycle. In M. marinum, it was shown that ESX-5 manipulates macrophage immune responses by skewing cytokine responses towards a more anti-inflammatory state and by the induction of host cell death, presumably promoting bacterial dissemination [75, 76]. These results are supported by the observations that a M. tuberculosis ESX-5 mutant has a reduced ability to replicate both in vitro and in vivo and that early granuloma formation in zebrafish embryos was somewhat attenuated for a M. marinum ESX-5 mutant [72, 77]. Unexpectedly, and in stark contrast to results for M. tuberculosis, this M. marinum ESX-5 mutant was hypervirulent during infection in adult zebrafish and induced an enhanced pro-inflammatory response resulting in rapid granuloma formation, elevated bacterial burdens, and high mortality [77]. This suggests that ESX-5 substrates might be important in downregulation of the host immune response in order to establish a moderate and long-lasting infection and might imply that the process of mycobacterial granuloma formation is tightly regulated. Because many different substrates are potentially secreted via ESX-5, it is difficult to pinpoint which substrate is crucial for immune modulation. However, a number of substrates seem to stand out, one of which is LipY, one of the few PE or PPE proteins with a known function. This surface-located protein is a highly active lipase that is upregulated and secreted upon macrophage infection [71, 78]. Interestingly, overexpression of lipY in M. bovis BCG mitigates its protective effect, although the molecular mechanism for this is currently unclear [79]. Another putative important ESX-5 substrate is PE_PGRS30. This protein is upregulated during the chronic stage of infection and is involved in phagosomal maturation arrest, replication in macrophages, and in vivo persistence during the chronic phase of M. tuberculosis infection in mice [80, 81].

CONCLUDING REMARKS

In recent years, T7S and its role in mycobacterial pathogenicity have become a popular research topics. Despite substantial progress in our understanding of T7S, no data are currently available on ESX-4 and ESX-2. More is known about the ESX-3 system, which has been implicated in iron and zinc acquisition. Its presumptive role in metal homeostasis explains the role of this T7S in mycobacterial viability. The intensively studied ESX-1 system is clearly involved in mycobacterial virulence and granuloma formation, whereas the mechanism of secretion and its effector molecules have not been fully elucidated yet. This T7S system is required for the initial steps of infection and most probably directs mycobacteria to a favorable intracellular niche from where they can manipulate their host. Recent studies offer new insights into the implication of the ESX-5 system in mycobacterial virulence. Although many aspects of ESX-5 secretion remain to be defined, current knowledge points to a role in the manipulation of
host immune responses affecting the complex host-pathogen interactions required for successful persistence and efficient granuloma formation. As summarized in Figure 2, T7S systems play important roles in distinct steps of the mycobacterial infection cycle. Further studies are necessary to unravel the numerous undefined aspects of T7S and their exact role in mycobacterial pathogenicity, which might possibly lead to the identification of future anti-TB targets.

Figure 2. Representation of the Mycobacterium tuberculosis infection process and the role of type VII secretion (T7S) systems. In the extracellular (and possibly intracellular) milieu, mycobacteria depend on the ESX-3 system for metal homeostasis and therefore viability. In the host, ESX-5 secreted substrates manipulate the host immune system. On internalization in macrophages, ESX-1 secreted factors facilitate translocation from the phago(lyso)some into the cytosol. The cytosolic localization promotes mycobacterial replication and ESX-5 secreted proteins further modulate host immune responses, resulting in inflammasome activation, cell host death, and macrophage recruitment. Finally, additional macrophages, neutrophils, and lymphocytes cluster around infection foci, resulting in a granuloma.
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