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ESX/Type VII Secretion Systems—An Important Way Out for Mycobacterial Proteins

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ABSTRACT The causative agent of human tuberculosis, *Mycobacterium tuberculosis*, has a complex lipid-rich diderm envelope, which acts as a major barrier protecting the bacterium against the hostile environment inside the host cells. For the transfer of diverse molecules across this complex cell envelope, *M. tuberculosis* has a series of general and specialized protein secretion systems, characterized by the SecA general secretion pathway, the twin-arginine translocation pathway, and five specific ESX type VII secretion systems. In this review, we focus on the latter systems, known as ESX-1 to ESX-5, which were first discovered almost 20 years ago during the *in silico* analysis of the genome sequence of *M. tuberculosis* H37Rv. Since then, these systems have been the subject of highly dynamic research due to their involvement in several key biological processes and host-pathogen interactions of the tubercle bacilli.

INTRODUCTION

The different bacterial species within the tree of life (1) possess a range of secretion systems, which play important roles in the transport of proteins across the various types of bacterial cell envelopes. Classically, Gram staining was used for differentiating Gram-positive and Gram-negative bacteria, but classifications on cell envelope architecture might come closer to the biological reality, and thus, bacteria may also be differentiated according to their cell envelopes into diderm-lipopolysaccharide (archetypal Gram-negative), monoderm (archetypal Gram-positive), and diderm-mycolate (archetypal acid-fast) bacteria (2). For Gram-negative bacteria a range of at least eight different secretion systems has been described (types I to VI, VIII, and IX) (3–5). While in monoderm bacteria secretion and export are synonymous, in diderm bacteria the secretion is completed only upon translocation of the substrates across the outer membrane (2). The here-reviewed mycobacterial ESAT-6 secretion (ESX) systems (6, 7), which were also named type VII secretion (T7S) systems (8), represent a particular class of protein export and/or secretion systems, for which at present only the inner-membrane translocation machinery has been explored in more detail (9, 10), whereas it remains unknown how ESX/T7S-exported proteins get transported through the mycobacterial outer membrane into the extracellular environment (11). Indeed, one of the remarkable characteristics of mycobacteria is their complex cell envelope, which is shared to some extent with other members of the *Corynebacterineae*, a suborder of the phylum *Actinobacteria* (1, 12, 13). Mycobacteria are surrounded by a diderm cell envelope, consisting of an inner membrane, a peptidoglycan layer, an arabinogalactan layer, an outer membrane,

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named mycomembrane, which is composed of covalently linked mycolic acids and extractable lipids, and a capsule (14, 15). This unusual cell envelope requires complex secretion systems for the export/secretion of proteins, such as those of the SecA and twin-arginine translocation pathways, as well as the specialized ESX/T7S systems (7, 8, 16), which were first discovered almost 20 years ago during *in silico* analyses of the genome sequence and the proteome of *Mycobacterium tuberculosis* H37Rv (17, 18). Moreover, T7S-like systems that share some core components of mycobacterial ESX/T7S systems exist in various genera of the phylum *Firmicutes*, representing many classical Gram-positive bacterial species (19), which, however, are not the subject of the current review.

M. tuberculosis possesses five ESX/T7S systems (ESX-1 to ESX-5) (7, 8, 11, 16). All five ESX systems share several common features: the presence of small secreted proteins (of about 100 amino acids) with a conserved Trp-X-Gly (WXG) motif (20), an FtsK-SpoIIIE ATPase, several transmembrane proteins, and a subtilisin-like mycosin (MycP) (11, 16) (Fig. 1). These systems, encoded in different sections of the mycobacterial chromosome, seem to have evolved by gene duplication and diversification from simpler systems that were shuffled around in different actinobacterial and mycobacterial species, often mediated by plasmids encoding ESX/T7S elements as well as elements of type IV secretion systems (21–23). ESX/T7S systems play an important role in the biology of *M. tuberculosis*, as well as in the interactions *M. tuberculosis* has with its host. Indeed, a number of secreted effectors, including EsxA (ESAT-6), EsxB (CFP-10), and ESX-1 secretion-associated proteins (Esp), such as EspA or EspC, as well as proteins that carry the characteristic N-terminal motifs Pro-Glu (PE) and Pro-Pro-Glu (PPE), have been suggested to intervene in host cellular and immune signaling pathways (11, 24, 25).

Here we focus on recent updates on the ESX/T7S systems of mycobacteria and summarize new findings on their structure, function, and role in host-pathogen interactions and briefly touch on their significance in translational research.

RECENT INSIGHTS INTO THE STRUCTURAL AND FUNCTIONAL CHARACTERISTICS OF ESX/T7S NANOMACHINES

Five ESX systems are encoded in the genome of *M. tuberculosis* (16, 18), and this number is the highest found in mycobacteria so far; other mycobacterial species show fewer systems (e.g., *Mycobacterium marinum* shows four systems and *Mycobacterium abscessus* shows three systems) (21, 22). While ESX-4, ESX-3, and ESX-1 are present in most fast-growing and slow-growing mycobacteria, ESX-2 and ESX-5 systems are found only in selected slow-growing mycobacteria and thus represent the most recently evolved systems (21, 22). For ESX-2, currently not much is known on its putative function. In contrast, for ESX-1, ESX-3, and ESX-5, recent research has determined that they all contribute to virulence of *M. tuberculosis*, although the insights into the exact molecular functions often remain vague; also, because many studies have been undertaken with different mycobacterial species (*M. tuberculosis*, *M. marinum*, *M. abscessus*, and/or *Mycobacterium smegmatis*), which may show some species-specific differences (reviewed in references 11 and 26 to 28). ESX-3 is important for metal homeostasis, pathogenicity, and immunogenicity (29–31). ESX-5 was suggested to be crucial for nutrient uptake and for the export of members of the PE and PPE protein families (9, 32, 33). These two large protein families have expanded during mycobacterial evolution (34, 35), and they include representative proteins that are associated with ESX/T7S systems and others with highly repetitive sequence motifs that are exported by ESX-5 and impact virulence and immunogenicity (32, 36–39). The ESX-5 nanomachine, which is integrated in the inner mycobacterial membrane, is composed of four proteins, namely, EccB5, EccC5, EccD5, and EccE5 (9, 10), that are organized in a hexameric complex, as recently determined by cryo-electron microscopy and single-particle analysis (10). This organization differs substantially from those of secretion systems of Gram-negative bacteria (10) (Fig. 2). Among the Ecc proteins (ESX conserved components), the structural and functional roles of EccC (FtsK/SpoIIIE ATPase) have been studied in more detail with the thermophilic actinobacterium *Thermomonospora curvata* (40). A certain flexibility of the cytosolic domains of EccC in interaction with effectors was suggested (10, 40), which is different from the cognate ATPase in type IV secretion systems (10).

Another conserved ESX component in mycobacterial ESX systems is the serine protease MycP, although this protein is not directly integrated in the EccBCDE complex (10, 41–43). Moreover, different proteins, such as EspA, EspC, EspD, and EccA1, may be essential to contributing to secretion and stabilizing the core ESX/T7S complex in the case of ESX-1 (44–47). How these related effectors are explicitly recognized and targeted towards the specific system in a single mycobacterial species with different

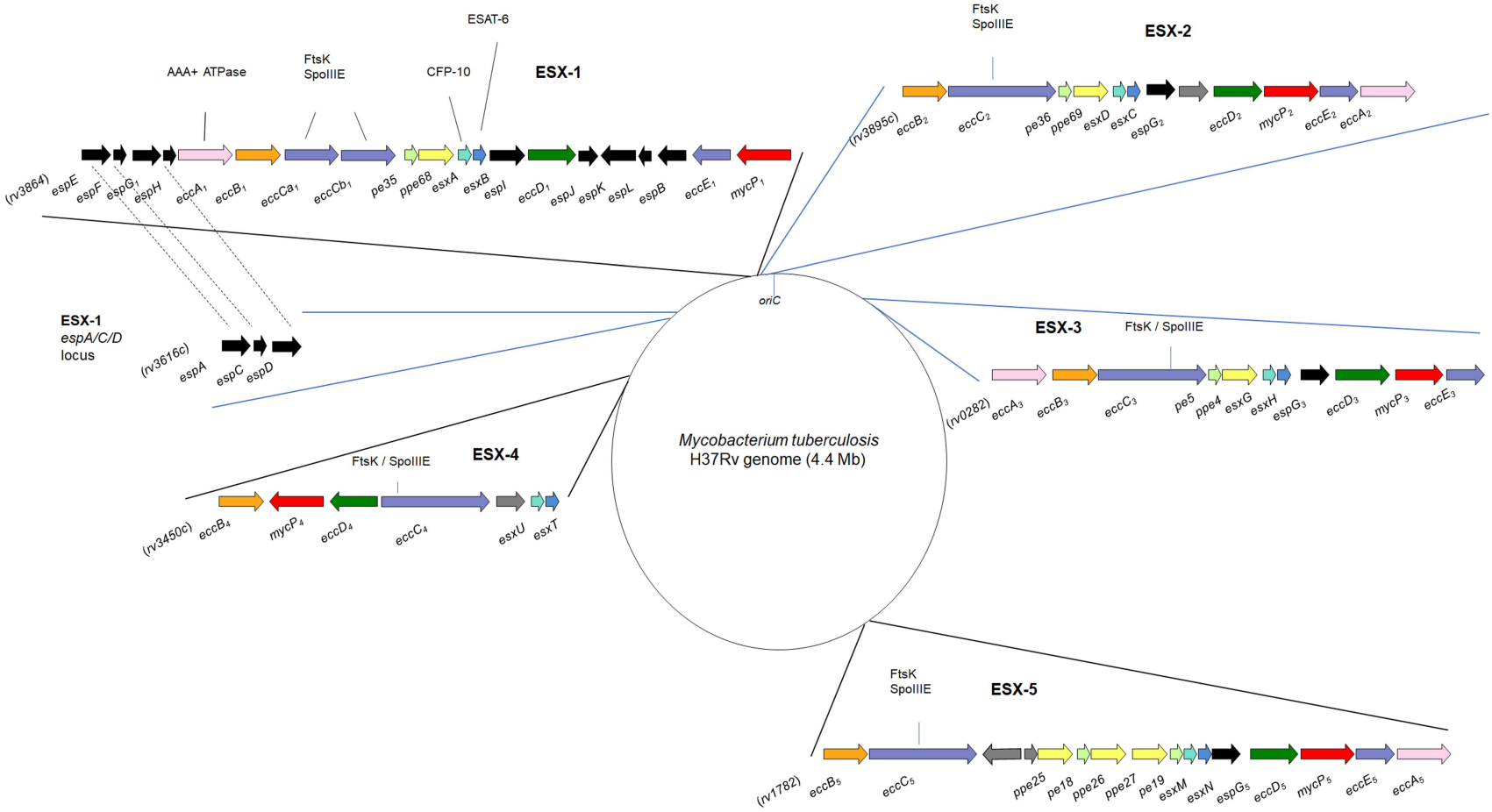


FIGURE 1 Genetic organization of the ESX loci. Shown is a schematic representation of the approximative genomic sites of the ESX-1 to ESX-5 clusters in the *M. tuberculosis* H37Rv genome. Gene nomenclature and gene color scheme were adapted from reference 16.

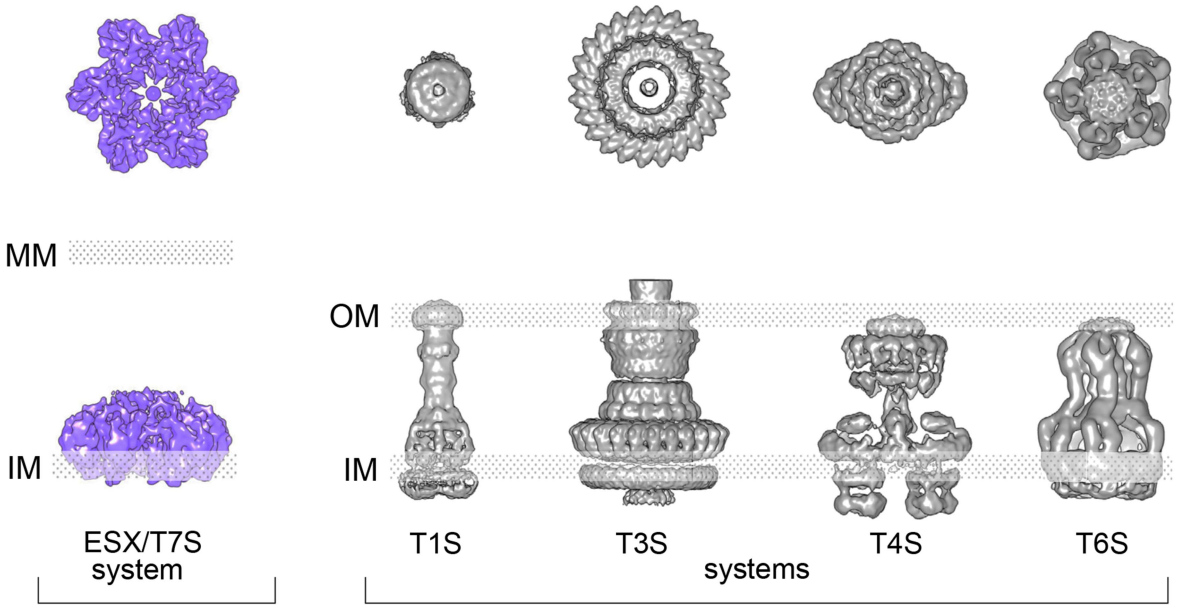


FIGURE 2 Representation of top and side views of the ESX/T7S system based on recent structural data generated by cryo-electron microscopy and single-particle analysis on an ESX-5 system from *Mycobacterium xenopi*, in comparison to selected examples of secretion systems from Gram-negative bacteria. The positions of the inner membrane (IM), outer membrane (OM), and mycomembrane (MM) are indicated. Adapted from reference 10, with permission.

ESX/T7S systems can be a matter of debate. Recently, it has become clear that some of the conserved ESX components could potentially exhibit chaperone-like activity (e.g., EspG or EccA) (48). It was suggested that besides their chaperone activity, these proteins are also involved in determining the secretion system specificity. Indeed, by substituting the binding domain of EspG, the ESX-1-dependent substrate can be rerouted to the ESX-5 system (49). In addition, it was suggested that EspL can have a role as a chaperone and is essential for ESX-1-dependent virulence (50). Therefore, scrutinizing the role of chaperones will certainly help to provide a better understanding of the ESX/T7S functions and mechanisms.

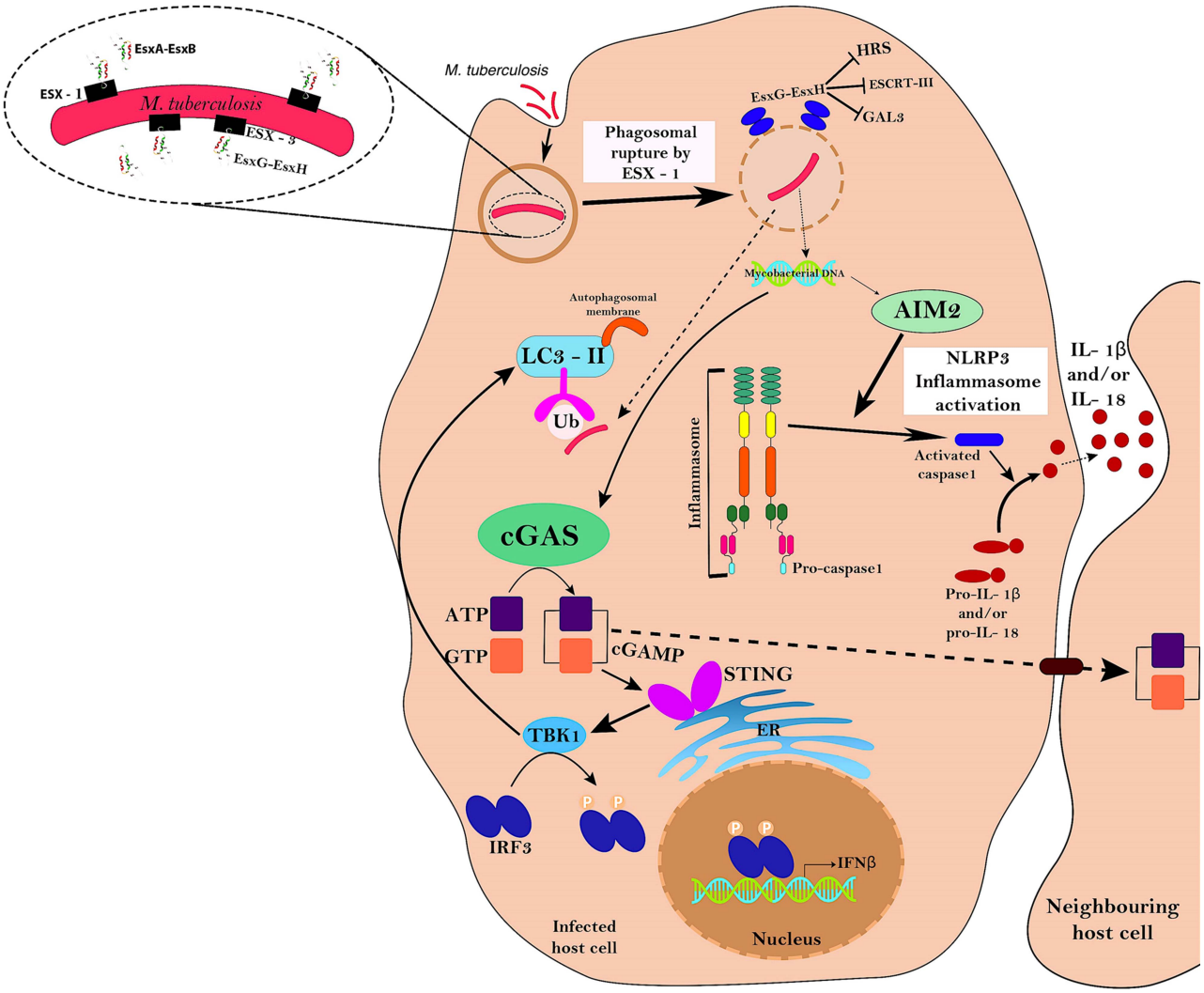
It is also intriguing that certain ESX/T7S systems may have a dual function. For example, the ESX-1 system, which is present in fast- and slow-growing mycobacterial species, is required for distributive conjugal transfer (DCT) of chromosomal DNA from donor into recipient strains of *M. smegmatis* (51, 52). This procedure apparently also involves the ESX-4 system of *M. smegmatis* (53). Moreover, it was shown that SigM, an extracytoplasmic function σ factor, is an activator of ESX-4 expression and necessary for DCT in the recipient strain of *M. smegmatis* (54). Intriguingly, experimental strain-to-strain transfer of chromosomal DNA was also observed in selected *Mycobacterium canettii* strains (55), representing a group of rare tubercle bacilli that are thought to resemble the ancestor of *M. tuberculosis* and have been isolated mainly from tuberculosis patients in the region of the Horn of Africa (East Africa) (56, 57). In contrast to *M. canettii* strains, DNA transfer between *M. tuberculosis* strains was not observed despite numerous trials (55), emphasizing the clonal population structure of *M. tuberculosis* strains (58, 59). While it is predicted that the interstrain DNA transfer between *M. canettii* strains might also involve an ESX-1 system in the recipient strain, in analogy to the situation in *M. smegmatis*, experimental confirmation for this hypothesis has not yet been reported.

ESX SYSTEMS IN HOST-PATHOGEN INTERACTIONS

The potential dual function of the ESX-1 system is best visible by the fact that in slow-growing mycobacteria, in contrast to fast-growing mycobacteria, the ESX-1 system is also involved in the pathogenic potential of the strains. It has been speculated that this phenotype might be associated with horizontal gene transfer of a putative genomic island harboring the ESX-1-associated *espACD* locus (60). *M. tuberculosis* and *M. marinum* mutants with deletion of ESX-1 are attenuated in their respective

hosts (61–64), which is in line with the attenuation of "natural" ESX-1 deletion mutants, such as the *Mycobacterium bovis* BCG (bacillus Calmette-Guérin) vaccine, which has lost ESX-1 functions due to the deletion of the region of difference RD1 (65). The ESX-1 system was shown to be involved in bacterial phagosome-to-cytosol transition of *M. tuberculosis* and host cell death (66–68), an important cell biological process that has numerous consequences for the host cell, such as induction of the cGAS/STING/TBK1/IRF-3/type I interferon signalling axis and NLRP3 inflammasome activation (69–75). However, ESX-1 is not the only factor involved in the process; it has been shown that besides ESX-1, the mycobacterial virulence lipids phthiocerol dimycocerosates also contribute to phagosomal rupture (76–78). Moreover, recent studies have also demonstrated that the endosomal sorting complex required for the transport III (ESCRT-III) system promotes the repair of

FIGURE 3 Interplay of ESX-1 and ESX-3 in host-pathogen interactions. ESX-1 is essential for the bacterial phagosome-to-cytosol transition by involving a cGAS/STING/TBK1/IRF-3/type I interferon signalling axis and AIM2 and NLRP3 inflammasome activities. In an ESX-1-dependent manner, the ESCRT machinery is recruited to phagosomes, while ESX-3 effectors (EsxG-EsxH) antagonize the host damage response by blocking the recruitment of HRS, ESCRT-III, and GAL3. The scheme is adapted from reference 11, with some additions from reference 81, with permission.



small perforations in the endolysosomal membrane (79). Intriguingly, certain ESX-3-secreted effectors can block ESCRT-dependent receptor trafficking to the lysosome (80). It was shown that effectors of ESX systems differentially respond to the ESCRT endomembrane damage response. In an ESX-1-dependent manner, the ESCRT machinery is recruited to phagosomes, while ESX-3 effectors (EsxG-EsxH) antagonize the host damage response by blocking the recruitment of HRS, ESCRT-III, and GAL3 (81) (Fig. 3).

ESX-4 is one of the least well-characterized ESX systems, although it is considered the most ancestral *esx* locus in mycobacteria (21, 82). The ESX-4 loci usually lack *pe/ppe* and *espG* genes, which may be involved in host-pathogen interactions (34), as well as the *eccE* gene. However, the ESX-4 locus of *M. abscessus* is different from that of other species, as it does contain EccE4 (21). In a recent study, by using an *M. abscessus* genome-scale *Himar mariner* transposon library, it was shown that an intact ESX-4 system is needed for full virulence in this fast-growing mycobacterium and emerging human pathogen, whereby the ESX-4 function in infection was associated with phagosomal rupture and transition of bacteria to the cytosol of amoebae and human macrophages (83). As *M. abscessus* does not possess an ESX-1 system, in this particular case, ESX-4 might be considered a surrogate of ESX-1.

Because of extensive sequence similarities and immune cross-reactions among Esx and PE/PPE proteins secreted by the ESX/T7S systems, investigation of the secretion and regulation of these effectors is challenging. Recently, a technology termed multiplexed analysis of substrate secretion by transduced T cell hybridomas (MASSTT) was developed to explore the intra-host cell secretion profiles of various mycobacterial strains via fluorescence-mediated detection of specific *M. tuberculosis* major histocompatibility complex class II (MHC-II) epitopes by highly discriminative T cell hybridomas (84). This method thus allows investigators to follow the intracellular secretion profiles of selected mycobacterial ESX proteins, such as EsxA or EspC, as well as other secreted proteins, such as the members of the Ag85 complex. The secretion of the latter proteins (e.g., Ag85B) is regulated by the PhoP/PhoR two-component regulatory system and the small RNA Mcr7 (85). Interestingly, strains of different phylogenetic lineages showed distinct secretion levels of Ag85B proteins in a preliminary set of *M. tuberculosis* strains by the MASSTT assay (84), information that needs to be confirmed with a larger strain collection.

It is clear from the few examples mentioned here that ESX systems have a strong impact on mycobacterial host-pathogen interaction, although more work is needed to elucidate the various molecular mechanisms by which the effects are generated. New insights into these phenomena are also of interest for translational implications, as is shown by the example of attenuated whole-cell vaccines against tuberculosis. Loss of ESX-1 is one of the main reasons for the attenuation of the BCG vaccine (65), while ESX-1 effectors are important antigens in immune responses (6). Due to the absence of ESX-1, BCG does not gain access to the host cell cytosol and thus lacks the induction of certain immune signaling pathways (74, 86). Several recombinant BCG vaccine candidates have been constructed to overcome these limitations of BCG (75, 87–90). Alternatively, rationally attenuated *M. tuberculosis* vaccine candidates may also secrete particular ESX antigens that are absent from BCG (36, 91–93) and thereby may induce improved protection.

CONCLUDING COMMENTS AND PERSPECTIVES

In summary, we have presented here a few examples showing that mycobacterial ESX/T7S systems represent dynamic molecular machines which play important roles in various aspects of the biology of mycobacteria and the interaction with their hosts. Advances in structural biology together with the use of new approaches (e.g., MASSTT) will be very helpful for better understanding the functional details that are linked to their biological activities and for exploiting this knowledge for improved intervention strategies against tuberculosis.

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