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ESX/type VII secretion systems of mycobacteria: Insights into evolution, pathogenicity and protection

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S U M M A R Y

Pathogenesis of *Mycobacterium tuberculosis* depends on the secretion of key virulence factors, such as the 6 kDa early secreted antigenic target ESAT-6 (EsxA) and its protein partner, the 10 kDa culture filtrate protein CFP-10 (EsxB), via the ESX-1 secretion system. ESX-1 represents the prototype system of the recently named type VII secretion systems that exist in a range of actinobacteria. The *M. tuberculosis* genome harbours a total of five gene clusters potentially coding for type VII secretion systems, designated ESX-1 - ESX-5, with ESX-4 being the most ancient system from which other ESX systems seem to have evolved by gene duplication and gene insertion events. The five ESX systems show similarity in gene content and gene order but differ in function. ESX-1 and ESX-5 are both crucial virulence determinants of *M. tuberculosis*, but with different mechanisms. While ESX-1 is implicated in the lysis of the host cell phagosomes, ESX-5 is involved in secretion of the mycobacteria specific PE and PPE proteins and cell wall stability. Research on type VII secretion systems has thus become a large and competitive research topic that is tightly linked to studies of host–pathogen interaction of pathogenic mycobacteria. Insights into this matter are of relevance for redrawing the patho-evolution of *M. tuberculosis*, which might help improving current strategies for prevention, diagnostics and therapy of tuberculosis as well as elucidating the virulence mechanisms employed by this important human pathogen.

1. Introduction

Mycobacteria are widely distributed in a variety of environments and represent a single genus regrouped within the actinobacteria. Most mycobacterial species are harmless saprophytes, which is especially true for the so called fast growing mycobacteria that only comprise very few pathogenic variants, such as *Mycobacterium abscessus* [1,2]. In contrast, the slow growing mycobacteria, which form a subcluster in the 16S RNA tree [3], are well known to harbour major human pathogens such as *Mycobacterium leprae* [4] or *Mycobacterium tuberculosis* [5,6]. Mycobacteria are considered as high GC Gram positive bacteria, but in contrast to other Gram positive bacteria such as Staphylococci or Bacillus species, which have no outer membrane, mycobacteria possess a lipid-rich cell envelope that contains a standard inner membrane and a particular outer membrane, named mycomembrane, which is specific to mycobacteria and might fulfil a similar barrier function as the outer membrane of Gram negative bacteria [7-9]. Thus, mycobacteria need efficient secretion systems that can ensure the transport of a range of biomolecules across the complex mycobacterial cell envelope.

2. Secretion systems of *M. tuberculosis*

Tubercle bacilli possess a SecA1 dependent general secretion pathway, an alternative SecA2 dependent system [10], and a twin-arginine translocation (TAT) system [11,12]. Many of the genes involved are essential for the viability of mycobacteria [13,14] and resemble those of comparable bacterial transport systems. Moreover, mycobacteria also contain specific secretion systems that only show weak similarity with systems present in other bacteria [5,15]. These specific secretion systems were named ESX systems [16], referring to one of the first discovered substrates, the 6 kDa early secreted antigenic target ESAT-6 of *M. tuberculosis* [17]. They were later also termed Type VII secretion systems [18]. The ESX-1 system secreting ESAT-6 and its protein partner, the 10 kDa culture filtrate protein CFP-10 [19] was found to be present in the genome of

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M. tuberculosis H37Rv but truncated in several attenuated strains of the *M. tuberculosis* complex [20-22]. A genomic region in the size of 9.5 kb named region of difference 1 (RD1) carrying part of the genes encoding the ESX-1 structural components and substrates is deleted from the genome of *Mycobacterium bovis* BCG ($RD1^{bcg}$), the only currently used attenuated antituberculosis vaccine [20,23]. An $RD1^{BCG}$ overlapping portion ($RD1^{mic}$) is also deleted from *Mycobacterium microti* strains [24], which also have been used as live attenuated vaccines in the 1960s [25,26]. Previous experiments have shown that genomic integration of the extended RD1 locus from *M. tuberculosis* into the genomes of BCG or *M. microti* restored ESAT-6 secretion and partially increased the virulence as well as the protective efficacy of thee resulting recombinant BCG::RD1 and *M. microti*::RD1 strains [27-30]. Similarly, *M. tuberculosis* RD1 deletion/knock-out mutants were found attenuated [31-33], leading to the overall conclusion that the presence or absence of the ESX-1 specialized secretion system has an important impact on *M. tuberculosis* virulence and the attenuation of BCG and *M. microti* based vaccines, respectively.

Besides ESX-1, there are four paralogous ESX loci present in the *M. tuberculosis* genome, named ESX-2 – ESX-5 [5,34,35] (Figure 1). Although ESX-1 can be considered as the paradigm of Type VII secretion systems due to its early discovery and numerous follow-up studies, new mechanistic data became recently available for the ESX-5 systems of *Mycobacterium marinum* and *M. tuberculosis* [36,37] as well as for the ESX-3 systems of *Mycobacterium smegmatis* and *M. tuberculosis*, which play an important role in metal ion homeostasis [38]. Each ESX secretion apparatus consists of a multi-protein complex, built by ESX conserved components (Ecc) and ESX-secretion–associated proteins (Esp) as well as Esx and PE/PPE proteins [39] (Figure 1). The main components are cytosolic and membrane-anchored ATP-binding proteins (EccA and EccC, respectively), and other proteins containing several transmembrane domains (EccB, EccD, EccE) that are supposed to mediate the ATP-dependent export of ESX substrates across the cytoplasmic membrane (inner membrane) [30,39]. In contrast, little is known on the mechanisms, which might enable translocation of ESX substrates across the outer membrane, the mycomembrane that is composed of different lipids than usual phosholipid-based biomembranes. It has been suggested that one of the components of the membrane-anchored complex, namely EccE or EccC, might span both the inner and outer membrane, but no experimental demonstration of this hypothesis is yet available [36]. Further components of the ESX systems include membrane-bound mycobacteria-specific subtilisin-like serine proteases named mycosins ($MycP_1$-$MycP_5$) [40,41], which might impact ESX activity via the proteolytic digestion of the ESX substrates (e.g. EspB) [42].

3. Evolution of ESX systems

Phylogenetic analyses and comparative genomics revealed that the ESX-4 cluster is the smallest ESX locus, and apparently also the most ancestral ESX system in the genus *Mycobacterium* [35]. The other ESX loci seem to have evolved from ESX-4 like systems by gene duplication events and insertion of additional genes [35]. As one example, the insertion of *pe* and *ppe* genes, which are absent from the ESX-4 locus, might have occurred during and after the ESX-1 diversification. These *pe* and *ppe* genes in the ESX-1 locus appear to be ancestral members of the *pe/ppe* gene families that have heavily expanded in slowly growing, pathogenic mycobacteria to occupy almost 8% of the coding capacity of *M. tuberculosis* [5,34,43]. PE and PPE proteins encoded in the ESX regions have been shown to be recognized by the host's immune system, thus constituting an important repertoire of immunogens [44,45]. ESX-1 is highly conserved in *M. tuberculosis* as well as in the early branching, *Mycobacterium canettii* strains [46], indicating that the ESX-1 system is an important requirement for a diverse range of tuberculosis-causing mycobacteria. Furthermore, an additional locus, the

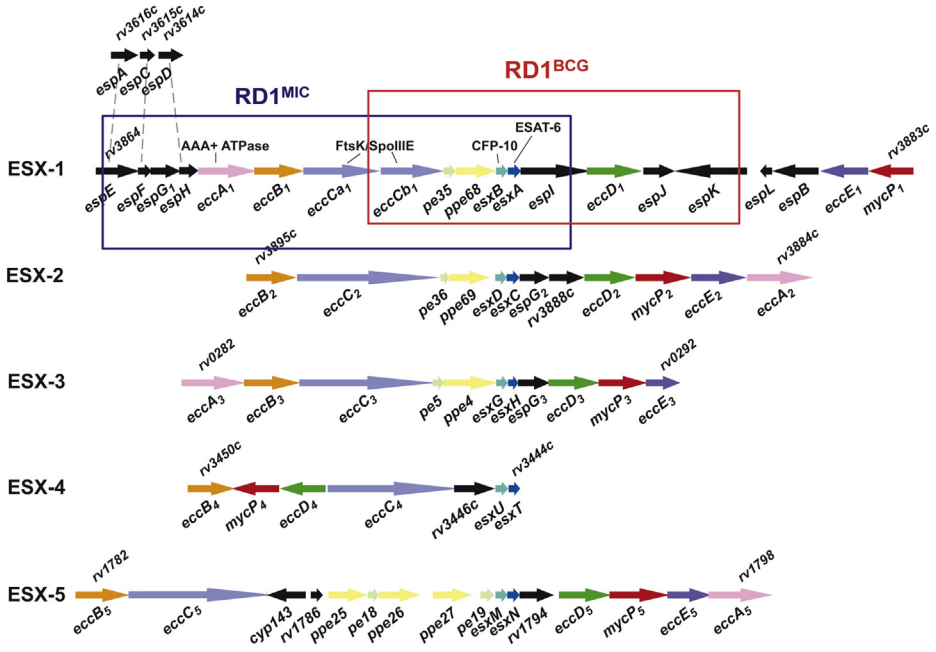

Figure 1. Genetic organization of genes encoding different components of ESX secretion systems at the five ESX loci and the *espACD* operon in *M. tuberculosis* H37Rv (after Bitter et al. [39] and Di Luca et al. [73]). Coloured arrows represent the various ESX genes encoding proteins belonging to different protein families: pink, AAA + ATPase; orange, amino-terminal transmembrane protein; violet, amino-terminal transmembrane ATPase; yellow, PPE family; light green, PE family; light blue, CFP-10 (EsxB) and homologs; blue, ESAT-6 (EsxA) and homologs; green, integral membrane protein; red, mycosin (subtilisin-like serine protease); black, Esp protein. Grey arrows represent region-associated genes coding for proteins not predicted to be involved in the ESX secretion machineries. The red and blue square corresponds to the deleted regions of difference in BCG and *Mycobacterium microti*, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)