

OPINION

Type VII secretion — mycobacteria show the way

Abdallah M. Abdallah, Nicolaas C. Gey van Pittius, Patricia A. DiGiuseppe Champion, Jeffery Cox, Joen Luirink, Christina M. J. E. Vandenbroucke-Grauls, Ben J. Appelmek and Wilbert Bitter

Abstract | Recent evidence shows that mycobacteria have developed novel and specialized secretion systems for the transport of extracellular proteins across their hydrophobic, and highly impermeable, cell wall. Strikingly, mycobacterial genomes encode up to five of these transport systems. Two of these systems, ESX-1 and ESX-5, are involved in virulence — they both affect the cell-to-cell migration of pathogenic mycobacteria. Here, we discuss this novel secretion pathway and consider variants that are present in various Gram-positive bacteria. Given the unique composition of this secretion system, and its general importance, we propose that, in line with the accepted nomenclature, it should be called type VII secretion.

Bacterial pathogenicity depends on the ability to secrete virulence factors, which can be displayed on the bacterial cell surface, secreted into the extracellular milieu or injected directly into a host cell¹. Historically, the mechanisms of protein secretion have been most extensively investigated in Gram-negative bacteria, which has resulted in the identification of different specialized secretion systems that have been designated type I–VI (BOX 1). Protein secretion in Gram-negative bacteria is particularly complex because these bacteria are surrounded by two membranes that the secreted proteins must pass through to enter the extracellular environment or a host cell.

In contrast to Gram-negative bacteria, Gram-positive bacteria are generally regarded as being simpler in structure because they lack a second membrane; consequently, secretory proteins of Gram-positive bacteria only need to traverse the cytoplasmic membrane and peptidoglycan layer to enter the extracellular environment². However, recent studies have provided evidence that there is an alternative protein-secretion system in Gram-positive bacteria^{3–6}. Perhaps unsurprisingly, this specialized secretion system was identified in *Mycobacterium tuberculosis*,

a Gram-positive bacterium with a highly complex cell envelope. Phylogenetically, this bacterium belongs to the Actinobacteria (high G+C Gram-positive bacteria). Within this class they are placed in a distinct taxon of organisms, the Mycolata, that are characterized by the presence of large hydroxylated branched-chain fatty acids called mycolic acids^{7,8}. The mycolic acids are covalently linked to the cell-wall matrix and form a second hydrophobic barrier, called the mycomembrane⁹ (FIG. 1). Proteins that are secreted across the mycomembrane probably require a specialized secretion system.

The identification of this specialized secretion system began with the isolation of the tuberculosis vaccine strain *Mycobacterium bovis* Bacille Calmette–Guérin (BCG) at the Pasteur Institute (Lille, France) in 1921. This strain was isolated following prolonged serial passages of a virulent strain of *M. bovis*, during which time attenuating genetic alterations occurred¹⁰. This weakened strain protected animals when they were challenged with a lethal dose of virulent tubercle bacilli and has since been used as a vaccine to prevent human tuberculosis, although its efficacy for preventing tuberculosis in adults is questionable¹¹.

During continuous *in vitro* passage, BCG lost 38 open reading frames^{10,12}. The deleted regions included region of difference 1 (RD1), which is crucial for the attenuated virulence of BCG^{13–15}.

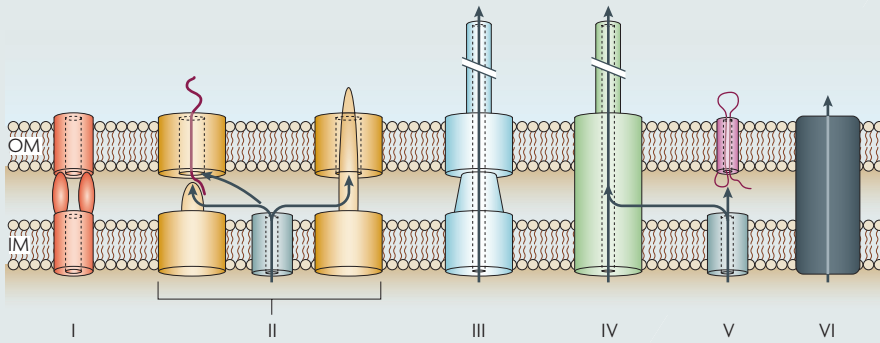
RD1 (FIG. 2) is 9.5 kilobases (kb) in length and comprises 9 genes, including the genes that encode the secreted proteins ESAT-6 (early secreted antigenic target of 6 kDa) and CFP-10 (culture filtrate protein of 10 kDa). Both of these proteins are important T-cell antigenic targets and are essential for the virulence of *M. tuberculosis* (discussed below). However, both lack a distinguishable Sec-signal sequence, which suggests the existence of a specialized secretion pathway. Several independent studies have demonstrated that the genes that surround the ESAT-6- and CFP-10-encoding genes are involved in the production of such a specialized secretion system. Perhaps even more intriguingly, *M. tuberculosis* contains four additional gene clusters that are homologous to the RD1 secretion locus^{16–18}.

In this Opinion, we first discuss the secretion system that is encoded by RD1, the so-called ESX-1 system, and propose that this system is an example of type VII secretion, named in line with the conventional nomenclature. Next, we describe the additional type-VII secretion systems (T7SSs) in mycobacteria and related secretion systems in other Gram-positive bacteria. Last, we discuss the role of these secretion systems in virulence.

The ESX-1 system

The existence of the ESX-1 secretion system had been predicted by several *in silico* analyses prior to its identification^{16–18}. These predictions were based on the clustering of the genes that encode ESAT-6 and CFP-10 with genes that encode membrane-associated proteins and putative ATPases. The first experimental evidence for such a system was obtained when the BCG vaccine strain was complemented with the RD1 locus. Secretion of ESAT-6 was only restored when a complete RD1 region was introduced³. In a separate approach, individual genes in the RD1 locus were identified as virulence factors for *M. tuberculosis*^{4–6}. The disruption of

Box 1 | Type I–VI secretion systems



Secretion in Gram-negative bacteria involves transport across a multipart cell envelope that consists of two membranes (the inner membrane (IM) and the outer membrane (OM)) and the periplasm in between. Gram-negative bacteria, and pathogenic species in particular, have developed strategies to get substrates into the extracellular milieu or directly into a host cell. Generally, secretion involves either a one-step mechanism, in which the cell envelope is crossed in one go, or a two-step mechanism, in which the OM is crossed using a specific machinery (see the figure).

In the one-step type I secretion pathway, proteins are secreted by a simple machinery that spans the entire cell envelope⁶⁸. The translocon consists of an IM ATPase-binding cassette (ABC) transporter, a membrane-fusion protein and an OM pore. Substrates of this pathway, such as α -haemolysin, possess an uncleaved carboxy-terminal signal sequence.

Substrates of the two-step type II system contain a normal amino-terminal signal sequence to mediate translocation across the IM by the general Sec- or Tat-translocons⁶⁹. The proteins fold in the periplasm before translocation across the OM, which is mediated by a complex structure known as the secretion. The secretion consists of a conserved OM pore (the secretin) and a pilus-like structure in the IM that might act as a piston to push substrates through the secretin. The biogenesis of the type II system is closely related to that of type IV pili.

The type III system is characterized by the injectisome, which is a needle-like structure that forms a channel that crosses the entire cell envelope and extends to contact host cells⁷⁰. The architecture of the structure allows the direct injection of virulence factors from the bacterial cytoplasm into a host cell.

Like the type III system, the type IV secretion system transports substrates directly into host cells through complex trans-envelope structures that culminate in a pilus structure at the bacterial cell surface³⁴. This versatile system can transport DNA and protein by a one-step or a two-step mechanism.

The type V system seems to use a simple two-step mechanism in which the Sec-translocon is used for translocation across the IM⁷¹. A β -barrel translocator domain that is either contiguous with the secreted protein (the autotransporter variant) or expressed as a separate entity (the two-partner secretion pathway) is needed for translocation across the OM. The translocator might act as a cognate pore, but recent evidence has questioned this model⁷².

The recently discovered type VI system^{73,74} has not yet been studied in detail. The system is required for the secretion of certain virulence factors in *Vibrio cholerae*⁷⁴ and *Pseudomonas aeruginosa*⁷³. The substrates are synthesized without an amino-terminal Sec-type signal sequence, which suggests that the trans-envelope translocation machinery is independent of the Sec or Tat pathway. The term type VI might also describe the secretion of cell material by the budding of vesicles from the OM. Periplasmic cargo proteins may be included in this process, but the specificity of recruitment remains elusive and the typing is therefore premature.

The grey channel in the IM represents the signal-sequence-dependent transport of proteins through the Sec and/or Tat system.

different mycobacterial species (FIG. 2). By examining the components that seem to be unambiguously involved in ESAT-6 secretion, in at least one *Mycobacterium* species, a number of proteins with known functional domains can be identified: *Rv3868*, a putative cytoplasmic chaperone with an AAA+ATPase domain; *Rv3883c* (MycP1), a subtilisin-like serine protease; and *Rv3870* and *Rv3871*, which together probably form an FtsK/SpoIIIE-like ATPase. The other proteins that are involved in ESAT-6 secretion (such as *Rv3869*, *Rv3877*, *Rv3881c* and *Rv3882*) have no homology with proteins of known functions, but most are predicted to be located in the cytoplasmic membrane (FIG. 2). For example, *Rv3877* is a multi-transmembrane-spanning protein that could be part of the translocation pore in the cytosolic membrane. As discussed below, a second gene cluster, the *Rv3614c–Rv3616c* locus, is also involved in ESAT-6 secretion^{22,23} (FIG. 2).

How does type VII secretion work?

The proteins of the ESX-1 system are dissimilar to those of other secretion systems (BOX 1), and the secretion process itself has some unusual and novel characteristics (discussed below) that would justify a special name for this system. Now that additional ESX-1-like secretion systems have been identified, both within the mycobacteria²⁴ and in other Gram-positive species²⁵, there is a pressing need for a more universal nomenclature. Therefore, we propose to call this system a T7SS.

Secretion of ESAT-6 and other substrates by ESX-1.

The ESX-1 components probably form a multisubunit cell-envelope-spanning structure that is similar to those of the type I–IV secretion systems (BOX 1), although structural data are lacking. However, protein–protein interaction studies have provided some insight into the working of ESX-1. First, the secreted proteins ESAT-6 and CFP-10 are dependent on each other for stability and form a tight dimer^{4,26,27}. The solution structure of the ESAT-6–CFP-10 pair revealed that each protein forms a 2-helix hairpin and that they are held together by extensive hydrophobic interactions (FIG. 3). The carboxy-terminal tail of CFP-10, which is unstructured, does not participate in dimer formation²⁶. Both proteins are also dependent on each other for secretion, although these data are more difficult to interpret owing to the interdependent expression and stability of the proteins.

In yeast two-hybrid experiments, it was shown that *Rv3870* probably interacts with *Rv3871* (REF. 4). Furthermore, *Rv3870* and

these individual genes (specifically, *Rv3870*, *Rv3871* and *Rv3877* — for consistency we will use the *M. tuberculosis* H37Rv gene nomenclature throughout this Opinion) also prevented the secretion of ESAT-6 and CFP-10. This system, which is now called ESX-1, was subsequently analysed in more detail in *M. tuberculosis*, the fish pathogen *Mycobacterium marinum*¹⁹ and the non-pathogenic species *Mycobacterium smegmatis*²⁰. ESX-1 is involved in virulence

and haemolysis in *M. marinum*¹⁹ and in conjugation in *M. smegmatis*²¹.

Together, these experiments have shown that, in addition to *Rv3870*, *Rv3871* and *Rv3877*, the RD1 gene cluster contains many other components — possibly more than 14 — that are essential for the functioning of the ESX-1 secretion system. However, the exact number of components that are involved in ESX-1 secretion is still debated and seems to vary between

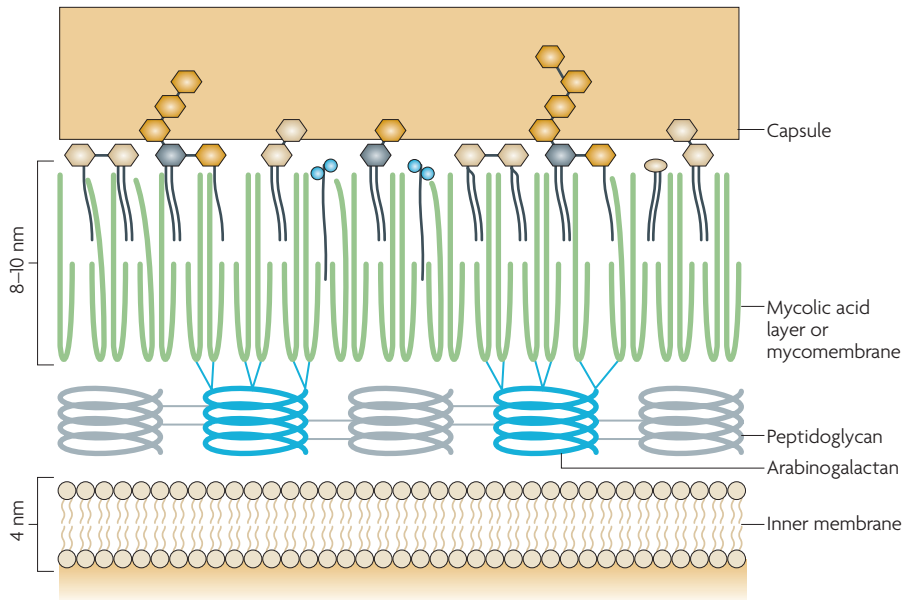


Figure 1 | Schematic representation of the cell envelope of *Mycobacterium tuberculosis*. Depicted here is one of the current views of the mycobacterial cell wall. The cell wall is mainly composed of a large cell-wall core or complex that contains three different covalently linked structures (peptidoglycan (grey), arabinogalactan (blue) and mycolic acids (green)). The covalent linkage of mycolic acids results in a hydrophobic layer of extremely low fluidity. This layer is also referred to as the mycomembrane. The outer part of the mycomembrane contains various free lipids, such as phenolic glycolipids, phthiocerol dimycocerosates, cord factor or dimycolyltrehalose, sulpholipids and phosphatidylinositol mannosides, that are intercalated with the mycolic acids. Most of these lipids are specific for mycobacteria. The outer layer, which is generally called the capsule, mainly contains polysaccharides (glucan and arabinomannan).

Rv3871 homologues in other T7SSs are fused and form a single gene (discussed below)^{16–18}. Because the results of yeast two-hybrid experiments also indicated that *Rv3871* interacts with CFP-10, it was proposed that *Rv3871* recognizes the CFP-10–ESAT-6 substrate pair, and delivers it in an ATP-dependent manner to *Rv3870* and thereby to the secretion machinery at the cell membrane⁴. The interaction of *Rv3871* with CFP-10 in yeast two-hybrid assays was used to identify the interacting domain in CFP-10 (REF. 28). By making single amino-acid changes to the carboxy-terminal 7 amino acids of CFP-10, these 7 amino acids were shown to be crucial for the interaction with *Rv3871* and for the secretion of both ESAT-6 and CFP-10 (REF. 28). As mentioned above, this region of CFP-10 is unstructured and is not required for the interaction with ESAT-6. Therefore, *Rv3871* can readily bind this region and thereby target the ESAT-6–CFP-10 dimer for secretion. Importantly, this ESX-1 signal sequence is portable: the last 7 amino acids of CFP-10 are sufficient for an unrelated soluble protein to be secreted by ESX-1 (REF. 28).

The situation became more complex with the realization that a second locus is

involved in ESX-1 secretion (the *Rv3614c–Rv3616c* locus)^{22,23}. First, *Rv3616c* (also called *EspA*) was shown to be secreted into the culture filtrate in an ESX-1-dependent manner²³. *EspA* probably forms an operon together with *Rv3614c* and *Rv3615c* and, as *Rv3614c–Rv3616c* are homologous to *Rv3864–Rv3867* from RD1 (FIG. 2), this locus is probably a product of a gene-duplication event. Another protein that is encoded by the *espA* operon, *Rv3615c*, is also a secreted protein (J.A. MacGurn and J.C., unpublished observations). Furthermore, like CFP-10, the carboxyl terminus of *Rv3615c* is required for secretion (P.A. Champion and J.C., unpublished observations). Interestingly, because *EspA* seems to lack a secretion signal, it is possible that *EspA*, like ESAT-6, ‘piggybacks’ on another protein for targeting.

The most surprising finding from these studies was that the ESAT-6–CFP-10 dimer is produced efficiently, but is retained in the bacterial cell in the absence of *EspA* or *Rv3615c*^{22,23}. This means that all ESX-1 substrates (*EspA*, *Rv3615c*, ESAT-6 and CFP-10) are dependent on each other for secretion^{22,23}. Understanding the as-yet-unidentified molecular basis for this phenomenon will be crucial for understanding the mechanism

of substrate recognition and secretion by ESX-1. For example, it may indicate that these type VII substrates are only secreted as multimeric complexes. Alternatively, these four proteins may actually be components of the secretion machine and form some sort of pilus or extracellular structure²⁹. This surface structure might occasionally be lost by shearing and end up in the supernatant. This line of reasoning suggests that the true substrates of ESX-1 have not yet been detected. This hypothesis is strengthened by a recent report that showed that, although ESX-1 is required to arrest phagosome maturation, the known ESX-1 substrates are not involved in this process³⁰. A candidate for a new ESX-1 substrate is *Rv3881c* (also known as MTB48), which is encoded by RD1 and is a secreted antigen³¹.

Building a working model of type VII secretion. Although there are no sequence homologies with other secretion systems, there are some interesting functional parallels between ESX-1 secretion and type IV secretion systems (T4SSs). Like CFP-10, type IV substrates are targeted for secretion by an unstructured carboxy-terminal signal sequence^{32–34}. In type IV secretion, this signal sequence is recognized by coupling proteins, which are integral membrane proteins with two transmembrane domains and a large cytoplasmic domain. These coupling proteins are also members of the FtsK/SpoIIIE protein family, which is an obvious parallel with *Rv3870* and *Rv3871* (REF. 28).

On the basis of protein–protein interaction data (FIG. 4a), a working model for type VII secretion can be generated (FIG. 4b). First, the ESAT-6–CFP-10 dimer is targeted for secretion through the recognition of the carboxy-terminal signal sequence by the cytoplasmic protein *Rv3871*. *Rv3871* then interacts with *Rv3870* at the cell membrane to form an active ATPase. The *Rv3871–Rv3870* complex could, by analogy to ATPases that are involved in type II or type IV secretion, form a hexameric ring structure with a central cavity that propels ESX-1 substrates through the secretion channel. One of the major candidates that might constitute, at least in part, the inner-membrane secretion channel is the multi-transmembrane protein *Rv3877*.

Other ESX-1 components. The functions of the other ESX-1 components are more difficult to predict, and the available data on their protein–protein interactions are difficult to interpret. For example, *Rv3614c*, which is predicted to be a cytosolic protein, interacts in

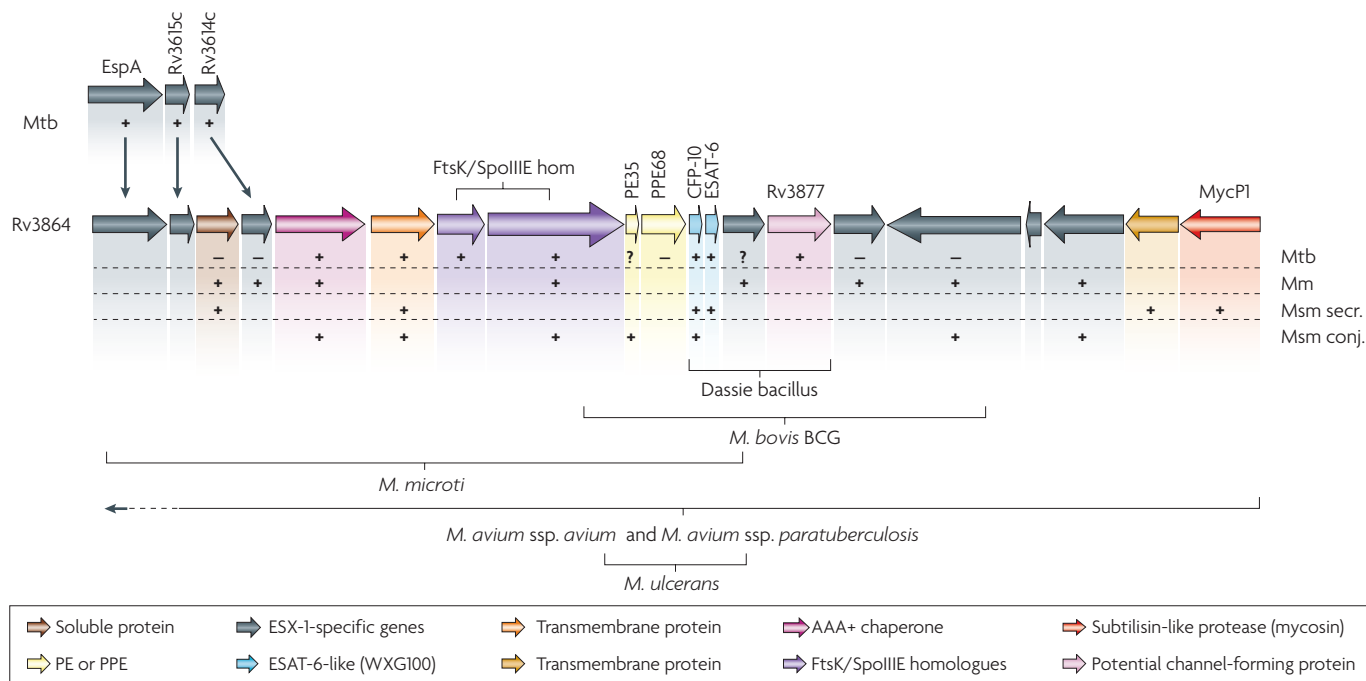


Figure 2 | Genes involved in the ESX-1 secretion system. The ESX-1 secretion system is encoded by two different loci, the ESX-1 locus and the EspA operon (shown in the top left corner). Homologies between these two loci are indicated by arrows. Plus or minus signs below the coding sequences show the involvement of each gene in: ESAT-6 secretion in species belonging to the *Mycobacterium tuberculosis* complex (Mtb)^{4–6,22,23,78}; haemolysis and ESAT-6 secretion in *Mycobacterium marinum* (Mm)^{19,79}; ESAT-6 secretion in *Mycobacterium smegmatis* (Msm secr.)²⁰; and conjugation in *M. smegmatis* (Msm conj.)²¹. A question mark indicates conflicting results and a blank means

that no data is available. Gene families that are also present in other ESX gene clusters are shown in different colours, whereas ESX-1-specific genes are shown in dark grey. Different deletions in the ESX-1 region that affect ESAT-6 secretion in the vaccine strain *Mycobacterium bovis* Bacille Calmette–Guérin (BCG) — the RD1 deletion — and in the natural mycobacterial species *Mycobacterium microti*, *Mycobacterium ulcerans*, *Mycobacterium avium* and the Dassie bacillus⁸⁰ are also indicated. All undeleted genes of the ESX region in *M. ulcerans* are pseudogenes. The arrows represent the different coding sequences and the direction of their transcription.

yeast two-hybrid experiments with Rv3882c, a membrane protein that is predicted to face the periplasm²². If these proteins indeed interact, either one of the predictions is wrong or one of these proteins has a dynamic topology.

It was recently shown using a new protein–protein interaction assay that CFP-10 interacts with Rv0686 (REF. 35). Because Rv0686 is a member of the signal-recognition-particle (SRP) family of GTP-binding proteins, it has been suggested that ESAT-6 secretion might be linked to the Sec system³⁵. However, these results must be treated carefully because Rv2916c, not Rv0686, is the most probable SRP protein of *M. tuberculosis*, and there is no other evidence that the Sec system is directly involved in the translocation of ESAT-6 or CFP-10.

Clearly, many questions about the working mechanism of ESX-1 remain unanswered (BOX 2). For example, what is the function of the subtilisin-like MycP1 protease in ESX-1 secretion? Although this protein is essential for the function of ESX-1 (REF. 20), secreted substrates that have been processed by MycP1 have not yet been identified³⁶. Another major question is: how are ESX-1 substrates

exported across the mycomembrane? This layer is exceptionally thick (9–10 nm) and no components of the ESX-1 system are predicted to be located in it. However, knowledge of mycomembrane proteins is limited. In fact, structural information is only available for one transmycomembrane protein of *M. smegmatis*, MspA^{37,38}, for which there are no homologues in *M. tuberculosis*. It is possible that one of the identified ESX-1 components forms the channel across the mycomembrane. Alternatively, as the genetic screens that have been used have not been comprehensive and some components might be essential (and therefore cannot be mutated), the mycomembrane component (or components) might not yet have been detected.

Type VII secretion in mycobacteria

Although the identification of a specialized secretion system in *M. tuberculosis* was expected because of the nature of the cell wall^{7,8}, it was unexpected that *M. tuberculosis* would encode five such secretion systems. The *M. tuberculosis* genome contains 11 or 12 loci (depending on the strain) that comprise tandem

pairs of genes that encode ESAT-6 family members^{39,40} (Supplementary information S1 (figure)). Four of these gene clusters are part of larger loci, which contain more homologues of the ESX-1 cluster; these clusters have been named ESX-2–5.

A comparison of the different ESX systems resulted in the identification of a set of six genes that are present in all of these clusters and, therefore, probably encode the core components of the mycobacterial T7SSs^{16,17}. These proteins are: two members of the ESAT-6 family; a member of the FtsK/SpoIIIE family (although this is sometimes encoded by two genes, for example, Rv3870 and Rv3871 of ESX-1); a subtilisin-like protease (MycP1 in ESX-1); an integral membrane protein with 10–11 transmembrane domains (Rv3877 in ESX-1); and a member of another membrane-protein family (Rv3869 in ESX-1). In addition, some genes are shared by most, but not all, ESX systems, such as the PE and PPE genes (discussed below). Finally, some ESX clusters have genes that are unique for their system (the dark-grey arrows in FIGS 2.5). These genes could encode secreted substrates or specific components.

Phylogenetic analyses and comparative genomics have revealed that the five different ESX systems in the genus *Mycobacterium* probably evolved by gene duplication, in the order ESX-4, ESX-1, ESX-3, ESX-2 and, most recently, ESX-5 (REF. 17) (Supplementary information S2 (figure)). ESX-4 is, therefore, the most archaic T7SS in the mycobacteria¹⁷. It is also the smallest (9,870 base pairs in *M. tuberculosis* compared with 14–22 kb of other ESX loci) and has the fewest genes (7 genes in *M. tuberculosis* compared with the 11–18 genes of other ESX loci). Interestingly, the most recently evolved ESX system, ESX-5, separates two groups of mycobacterial species. The genus *Mycobacterium* can be roughly divided into fast-growing (colonies that form within 7 days) and slow-growing species, and the duplication of ESX-5 seems to coincide with the emergence of the slow-growing species⁴¹ (Supplementary information S2 (figure) and S3 (table)).

All the mycobacterial ESX clusters contain genes that encode ESAT-6 family members and these proteins are, of course, the major substrate candidates for the various putative secretion systems. Different studies have shown that (in addition to ESAT-6 and CFP-10) the ESAT-6 family members encoded by ESX-3 and ESX-5 as well as the ESAT-6 family members that are highly homologous to the ESX-3 and ESX-5 clusters (Supplementary information S1 (figure)) are indeed present in the culture supernatant of *M. tuberculosis*^{22,28,42,43}. Furthermore, an intact ESX-5 region is essential for the secretion of the ESX-5-encoded ESAT-6-family member EsxN by *M. marinum* (A.M.A. and W. B., unpublished observations). Together, these data indicate that ESX-3 and ESX-5 are indeed functional secretion systems. By contrast, the ESAT-6 homologues of ESX-2 and ESX-4 have not yet been detected extracellularly.

PE and PPE proteins in the ESX systems.

PE and PPE are two gene families that are unique to mycobacteria, and they are significantly expanded in slow-growing pathogenic mycobacteria; almost 9% of the coding capacity of *M. tuberculosis* is dedicated to these gene families³⁹. The most archaic PE and PPE genes were probably inserted into the first duplication of the ESX system (ESX-1) and were subsequently co-duplicated with the ESX gene clusters, until their recent expansion⁴¹. This co-evolution suggests that these proteins are functionally associated with the ESX secretion systems.

PE and PPE proteins also share a number of characteristics with ESAT-6 and CFP-10: they are secreted proteins that do not have a classical secretion signal; the ancestral PE- and PPE-encoding genes often form gene pairs (and are adjacent to ESAT-6–CFP-10 gene pairs)¹⁷; and they form a tight 1:1 complex⁴⁴. Evidence for the association between PE–PPE proteins and ESX secretion systems has been accumulating. First, the RD1-encoded PPE protein Rv3873 probably interacts with CFP-10 and/or ESAT-6, as shown by overlay experiments and yeast two-hybrid experiments^{45,46}. This could mean that Rv3873 is secreted together with the ESAT-6–CFP-10 complex. Second, a transposon insertion of the *M. smegmatis* ESX-1 locus in the PE gene showed the same phenotype as other ESX-1 mutations — increased conjugation frequency²¹. Third, in a recent study that investigated *M. marinum*, it was shown that PPE41 is secreted both in culture and in infected macrophages. It was also determined that this secretion is dependent both on the presence of PE25, which forms a complex with PPE41 (REF. 44), and on an intact ESX-5 cluster. The reconstitution of the entire ESX-5 cluster in the fast growing *M. smegmatis*, which does not contain an endogenous copy of ESX-5, enabled *M. smegmatis* to secrete heterologously expressed PPE41 (REF. 24). Thus, the ESX-5 locus is both necessary and sufficient to produce a functional secretion machinery for PPE41. To what extent the PE and PPE genes are functionally linked to the ESX clusters remains unknown, but recent data suggest that many more PE and PPE proteins are secreted by ESX-5 (A.M.A. and W.B., unpublished observations). The analysis of ESX-5 mutants might shed some light on the function of these mysterious PE and PPE proteins.

ESX systems do not complement one another.

The presence of five T7SSs raises the question of why several secretion systems exist and why they cannot complement each other. This is highlighted by the inability of the four ESX systems (ESX-2–5) to complement the loss of virulence that is caused by deletions in ESX-1. This is especially intriguing for ESX-5, as both ESX-5 and ESX-1 seem to be involved in macrophage escape and cell-to-cell spread^{6,19,24}. This might mean that these two secretion systems have independent roles in the successive steps of the cellular infection cycle.

The inability of the various ESX systems to complement each other could be due to the divergent evolution of their secretion

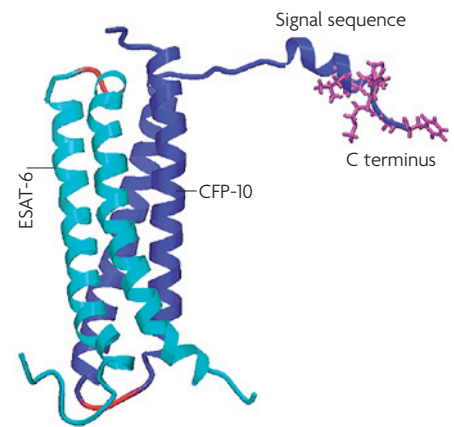
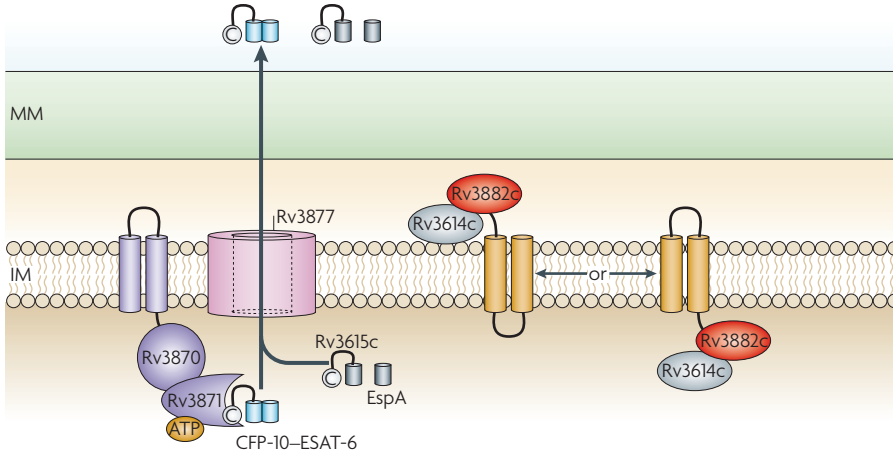


Figure 3 | Structure of the ESAT-6–CFP-10 dimer. ESAT-6 (light blue) and CFP-10 (dark blue) form a tight 1:1 complex²⁶. The subunit interface mainly contains hydrophobic residues. The carboxy (C)-terminal tail of CFP-10 is indicated and the 7 amino acids that are involved in the secretion signal are shown in pink. The location of the tryptophan-variable-glycine (WXG) motif is indicated for both proteins in red. This figure is modified, with permission, from REF. 26 © (2005) Macmillan Publishers Ltd.

signals²⁸ and also their differential regulation. For example, ESX-1 genes are downregulated upon starvation, whereas genes from ESX-2 are upregulated under these conditions⁴⁷. Furthermore, ESX-3 is regulated by the availability of iron and zinc, as part of the *ideR* and *Fur* (also known as *Zur*) regulons^{48,49}, whereas ESX-4 is regulated by the alternative sigma factor σ^M ⁵⁰. Apart from the differences in regulation, there are also some other apparent differences. For example, high-density transposon mutagenesis studies have shown that whereas genes from ESX-1, ESX-2 and ESX-4 can all be disrupted, most genes from ESX-3 and some from ESX-5 cannot be disrupted^{51,52}. This suggests that these systems are essential for growth in culture. This hypothesis has been proven for ESX-3 in *M. tuberculosis* (K. Lawrence and W.R. Jacobs, personal communication), although ESX-3 is not essential for the viability of *M. smegmatis*. The fact that knockouts of specialized secretion systems are usually not essential for growth in culture medium probably means that either ESX-3 secretes components that are involved in an essential function (or functions) or that the cytoplasmic accumulation of substrates is toxic for the cell.

Although all five ESX secretion systems seem to function independently, there may be a certain level of cross-talk. For example, the deletion of the *cfp-10–esat-6* operon resulted in the increased secretion of the ESX-5 substrate PPE41 (REF. 24), whereas the

a Model with proven protein–protein interactions



b Model with all proteins

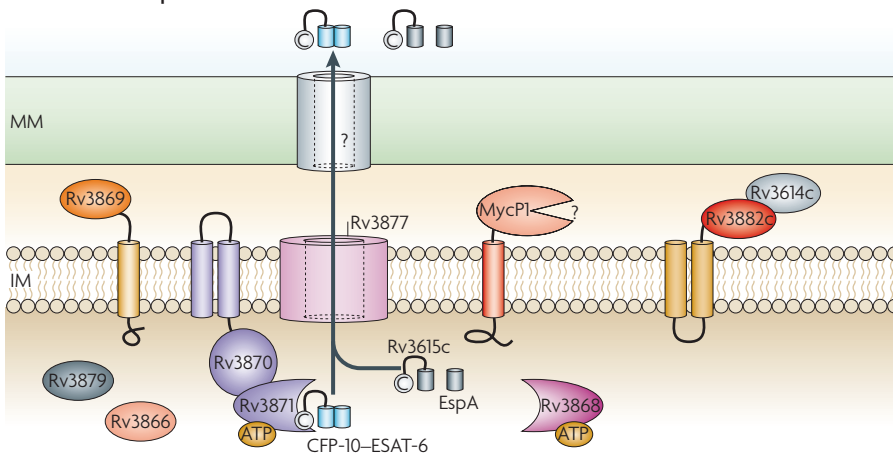


Figure 4 | Working model for the ESX-1 secretion system. Known interactions (a) and predicted localizations (b) of the ESAT-6–CFP-10 heterodimeric complex. The secretion of Rv3616c (also known as EspA) is co-dependent on the presence of the ESAT-6–CFP-10 complex. However, there is no formal evidence that these proteins form a larger complex. The ESAT-6–CFP-10 complex is recognized by the FtsK/SpoIIIE-like protein Rv3871, which binds the carboxy (C)-terminal tail of CFP-10. Rv3871 itself is associated with the inner membrane (IM) by its interaction with Rv3870. The translocation channel in the IM is probably formed by Rv3877, which has many transmembrane domains, although it is unknown which protein (or proteins) forms the channel in the mycomembrane (MM). The AAA+ chaperone-like protein Rv3868 could be involved in the biogenesis of the secretion machinery. The function of the subtilisin-like protease MycP1 is essential, but it is not known why, as no protein has been identified that is cleaved upon secretion by ESX-1. Gene families that are also present in other ESX gene clusters are shown in colours, whereas ESX-1-specific genes are shown in dark grey. A question mark indicates that the mycomembrane channel has not yet been identified.

ESX-5 mutant itself showed an increase in the secretion of certain ESX-1 substrates (A.M.A. and colleagues, unpublished observations). Partial complementation between ESX secretion systems could also explain why different mycobacterial species require different ESX-1 components for protein secretion (FIG. 2).

Other Gram-positive T7SSs

T7SSs seem to be involved in the transport of proteins across the mycobacterial cell envelope, which includes the mycomembrane. Therefore, it is unsurprising that

similar secretion systems have been identified in other mycolata species, such as *Corynebacterium* (FIG. 5) and *Nocardia*¹⁷. However, it is surprising that some high G+C Gram-positive bacteria that do not have a mycomembrane, such as *Streptomyces* spp., also contain a putative T7SS (FIG. 5). As T7SSs in high G+C Gram-positive bacteria are most related to the archaic ESX-4 system, this might indicate that ESX-4 was not designed to transport proteins across the mycomembrane and that this function was only introduced upon the evolution of ESX-1.

The WXG100 motif. Initially, no T7SSs were identified other than in the high G+C Gram-positive bacteria. However, when the different homologues of the ESAT-6 family were systematically compared¹⁸, the sequence similarity of these proteins was found to be low and the members were characterized by a central tryptophan-variable-glycine (WXG) motif. This WXG motif is located in the loop that connects the two main α -helices of ESAT-6 and CFP-10 (REF. 26) (FIG. 3). Replacing the conserved tryptophan residue of ESAT-6 with arginine did not affect ESAT-6 secretion, but did result in severe attenuation²⁷. As well as the conserved WXG motif, all family members identified were composed of ~100 amino acids, and so these family members are also called WXG100 proteins. Subsequently, other members of the ESAT-6/WXG100 family that shared the same characteristics were identified using PSI-BLAST, which resulted in the identification of dozens of distantly related members¹⁸.

Surprisingly, these proteins were not restricted to the Actinobacteria, but were also found in members of the Firmicutes (low G+C Gram-positive bacteria) such as *Bacillus* and *Clostridium* spp., *Staphylococcus aureus*, *Streptococcus agalactiae* and *Listeria monocytogenes*¹⁸ (FIG. 5). An analysis of genomic loci from these species showed that, in addition to the ESAT-6/WXG100 family members, they also contain a gene encoding an FtsK/SpoIIIE family protein that is homologous to the one found in T7SSs¹⁸. However, other genes that are homologous to those of the T7SSs of the high G+C Gram-positive bacteria are missing (FIG. 5). Instead, these loci contain a variable number (between 3 and 10) of additional genes, 3 of which are present in all loci in the Firmicutes (FIG. 5), but not in the T7SSs of the Actinobacteria. Together, these data strongly suggest that the T7SSs of Firmicutes belong to a specific and distant subfamily, which we propose should be designated type VIIb secretion systems. The existence of a subfamily is comparable to the T4SSs, which are subdivided into three different groups³⁴.

***S. aureus* type VII secretion.** Recently, Burts and colleagues²⁵ showed that the human pathogen *S. aureus* secretes ESAT-6/WXG100 family members, which is the first report of a functional T7SS outside the mycobacteria. The secreted proteins *EsxA* and *EsxB* are encoded by genes of the *ess* locus. This locus contains 12 coding sequences (CDSs) (FIG. 5), including the FtsK/SpoIIIE family member *essC*, which is

Box 2 | Important questions

- Are type VII secretion systems (T7SSs) only involved in protein transport or are they also involved in DNA, glycolipid and carbohydrate transport?
- Do T7SSs form injectosomes that can directly inject secreted proteins into eukaryotic host cells, similarly to type III secretion systems (T3SSs)?
- Is secretion through T7SSs inducible, as is the case for T3SSs? If it is, this could mean that we have missed a large number of substrates.
- Are ESX substrates secreted across the cell envelope in a one-step mechanism?
- Why are T7SS substrates binary complexes of proteins (for example, ESAT-6–CFP-10 and PE–PPE)? Is this finding merely coincidental?
- Does the interdependence of EspA, Rv3515c, ESAT-6 and CFP-10 mean that multi-component protein complexes are the real units of secretion?
- Which protein (or proteins) forms the channel in the mycomembrane?
- Are the recently identified mycobacterial pili⁷⁵ functionally linked to one of the ESX secretion systems?
- Why is the ESX-1 locus deleted in a number of mycobacterial pathogens?
- Is ESAT-6 an effector protein, a structural protein or both?
- Why is ESX-3 essential for *Mycobacterium tuberculosis* viability?
- Does ESX-5 secrete all the recently evolved PE and PPE proteins?
- Why are there so many PE and PPE proteins, and why are they not redundant^{76,77}?

Bacillus species type VII secretion. Various *Bacillus* species also contain a gene cluster that is homologous to the *S. aureus* *ess* cluster. In *Bacillus subtilis* this cluster is known as the *yuk* locus^{18,53}. The *yuk* locus contains five CDSs (*yukAB*, *yukC*, *yukD*, *yukE* and *yueB*), of which *yukE* encodes an ESAT-6/WXG100 family protein. At present it is not clear whether *YukE* is secreted, but an ESAT-6/WXG100 family member of *Bacillus anthracis* is present in the culture supernatant (as shown by secretome analysis)⁵⁴. An unexpected finding was that one protein encoded by the *Yuk* regulon of *B. subtilis*, *YueB*, is a phage receptor^{55,55}. Homologues of this phage receptor are also present in the *S. aureus* *ess* cluster and in other type VIIb clusters.

Overview of type VII secretion in Gram-positive bacteria. The fact that putative T7SSs are present in several non-pathogenic and non-related bacterial species indicates that this system is not designed to act as a secretion system for virulence *per se*, and the virulence function was probably acquired through the evolution of function. For example, the putative T7SS of *L. monocytogenes* does not seem to be involved in virulence⁵⁶.

essential for the secretion of *EsxA–EsxB*²⁵. Furthermore, two other CDSs (specifically, *essA* and *essB*) that encode transmembrane proteins are also involved in secretion (FIG. 5). Finally, the secretion of *EsxA* is dependent on the presence of *EsxB* and vice

versa. This co-dependence is similar to that of ESAT-6 and CFP-10 in *M. tuberculosis*. Interestingly, *S. aureus* mutants that fail to secrete *EsxA* and *EsxB* display significantly reduced virulence and are defective in dissemination and colonization²⁵.

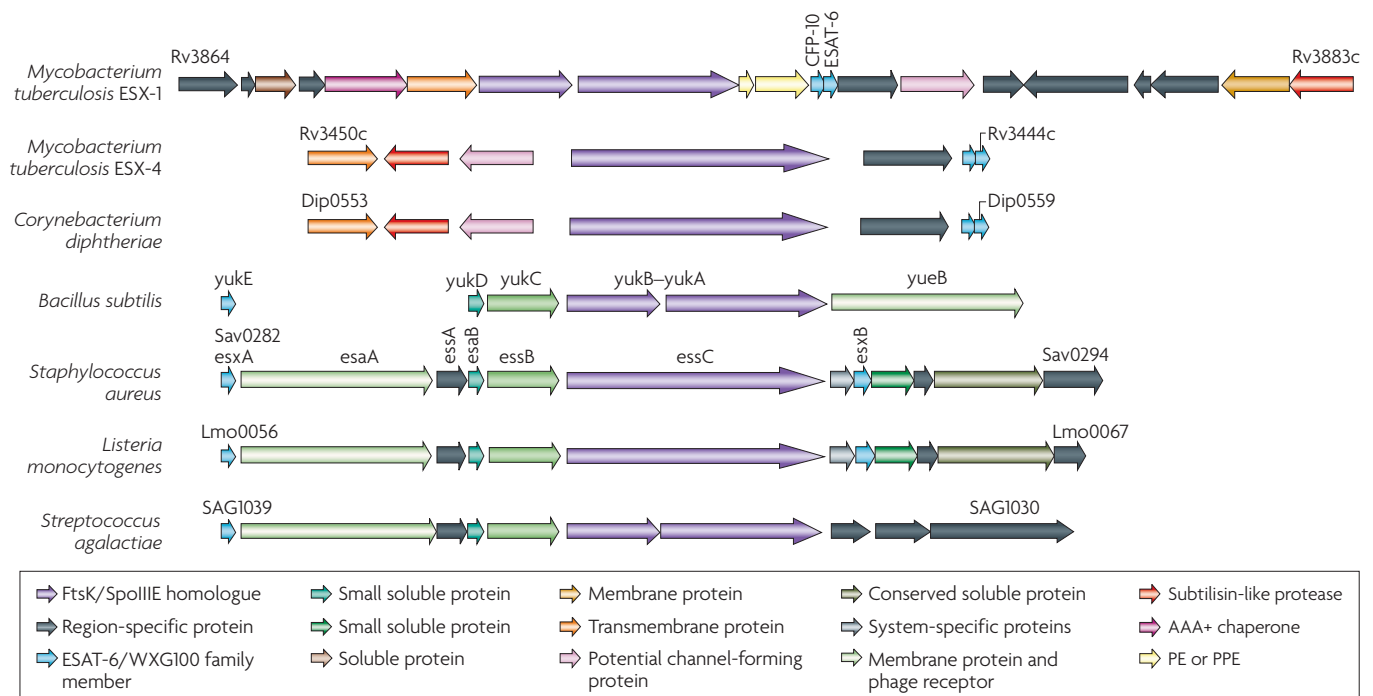


Figure 5 | Comparison of different gene clusters that encode type VII or type VIIb secretion systems. The colour coding for the figure is presented in the key. Type VIIb secretion systems are indicated and all the region-specific genes are shown in dark grey. Members of the FtsK/SpoIIIE family and the ESAT-6/WXG100 family, which are both present in

all of the different type VII secretion systems, are shown in purple and blue, respectively. The physical gaps between the different coding sequences do not reflect the relative length of these gaps. They have been chosen to clearly show the conservation between the different regions.

It remains to be elucidated why Gram-positive species without a mycolic acid layer need special secretion systems. Perhaps T7SSs in these bacteria are involved in the biosynthesis of specialized surface structures or appendages. Investigations of the function of the archetypal T7SS ESX-4 and the *Bacillus* Yuk system are key to understanding the evolution, as well as the original function, of these systems.

Type VII secretion in virulence

Although T7SSs are not virulence systems *per se* (as discussed above), it is clear that some T7SSs have important roles in virulence: the ESX-5 system of *M. marinum* seems to be crucial for cell-to-cell spread²⁴ and the *S. aureus* Ess–Ess system is involved in dissemination and colonization²⁵.

The role of the ESX-1 system in pathogenic mycobacteria has been studied in most detail. The lack of virulence of *M. bovis* BCG as compared with *M. bovis* and *M. tuberculosis* indicated that ESX-1 is essential for virulence^{10,12}. This notion was confirmed by the finding that if *M. tuberculosis* lacks a functional ESX-1 system it is reduced in virulence^{5,13}, whereas if *M. bovis* BCG is complemented with an intact ESX-1 locus it partially regains virulence^{14,15}. *Mycobacterium leprae*, which has a strongly degenerated genome owing to reductive evolution, also seems to have an active ESX-1 secretion system, as T cells of patients with leprosy react strongly to *M. leprae* ESAT-6 (REF. 57). However, it should be realized that a functional ESX-1 system is not unique to pathogenic species (Supplementary information S3 (table)). Furthermore, among the few mycobacterial species that lack part of the ESX-1 region, and therefore lack ESAT-6 secretion, are some pathogens, including *Mycobacterium ulcerans*⁵⁸, *Mycobacterium microti*⁵⁹ and *Mycobacterium avium*¹⁷ (FIG. 2). So, what are the roles of the secreted proteins in virulence, and why does the ESX-1 secretion system only contribute to virulence in certain species? Two possibilities can be envisaged: the ESAT-6 and/or CFP-10 of pathogenic mycobacteria have an additional function compared with non-pathogenic species, or additional proteins are secreted through ESX-1 by mycobacterial pathogens. Therefore, the key questions are: which secreted proteins are crucial for virulence and with which host components do they interact? At present, the available knowledge is mostly phenomenological and fragmentary. This

is illustrated in Supplementary information S4 (table), which summarizes the literature on the role of ESX-1 and its substrates in mycobacterial virulence. A complicating factor is the co-dependence of the various ESX-1 substrates for secretion, which makes it difficult to pinpoint the exact component that is responsible for virulence.

Interestingly, the lack of *in vivo* persistence of mycobacteria with a defective ESX-1 system seems to run in parallel with their delay in forming granulomas⁶⁰. Recent data provide an important clue to how this defect might arise: both *M. tuberculosis* and *M. marinum* can, at some stage in the macrophage-infection cycle, escape the phagolysosome compartment and be translocated into the cytoplasm^{61,62}. A functional ESX-1 system seems to be crucial for this transition⁶². Apparently, the presence of mycobacteria in the cytosol of infected macrophages is needed for the attraction of other macrophages. Which of the ESX-1-secreted polypeptides is causing this lytic effect and how lysis occurs is not known, but the principal suspect for this function is ESAT-6, which has been found to cause apoptosis⁶³ and membrane perturbation^{5,64}. The detailed characterization of ESAT-6 point mutations that do not affect secretion but do lead to an attenuated phenotype²⁷ might prove this hypothesis.

Conclusion

The mycobacterial ESX secretion systems form the paradigm for a new secretion pathway — type VII secretion. This pathway is regarded as novel because: the T7SS is composed of a unique set of proteins; the main secreted proteins all belong to the same unique protein family (the ESAT-6/WXG100 family); and this pathway is mechanistically unlike any other secretion system that has been detected, as all the secreted proteins seem to be co-dependent on each other for secretion. The T7SS is also different from type I–VI secretion as, unlike these other systems, T7SSs seems to be present only in Gram-positive bacteria. Therefore, it could be argued that this system should not form part of the sequential secretion-system nomenclature system. However, this is a Gram-negative-centred view, and type I–VI secretion systems are also present outside of the Gram-negative bacteria. For example, flagellar biosynthesis in archaea and type IV pili of clostridia belong to the class of type II secretion^{27,65}, and genes that encode T4SSs have been identified on plasmids from different Gram-positive bacteria^{66,67}.

T7SSs are probably complicated machineries that are involved in the secretion of protein complexes and possibly in the assembly of extracellular structures. The most important questions on T7SSs that must be answered are listed in BOX 2. Answering these questions and understanding T7SSs will change our way of thinking about the virulence of *M. tuberculosis* and hopefully give us new opportunities to combat this old foe.

Abdallah M. Abdallah, Christina M. J. E. Vandembroucke-Grauls, Ben J. Appelmeik and Wilbert Bitter are at the Department of Medical Microbiology, VU medical centre, Amsterdam 1081BT, The Netherlands.

Patricia A. DiGiuseppe Champion and Jeffery Cox are at the Department of Microbiology and Immunology, University of California, San Francisco, California 4143-2200, USA.

Nicolaas C. Gey van Pittius is at the DST/NRF Centre of Excellence in Biomedical Tuberculosis Research, US/MRC Centre for Molecular and Cellular Biology, Division of Molecular Biology and Human Genetics, Department of Biomedical Sciences, Stellenbosch University, Tygerberg 7505, South Africa.

Joan Luirink is at the Department of Molecular Microbiology, Vrije Universiteit, Amsterdam 1081HV, The Netherlands.

Correspondence to W.B. e-mail: w.bitter@vumc.nl

doi:10.1038/nrmicro1773

Published online 8 October 2007

1. Finlay, B. B. & Falkow, S. Common themes in microbial pathogenicity revisited. *Microbiol. Mol. Biol. Rev.* **61**, 136–169 (1997).
2. van Wely, K. H., Swaving, J., Freudl, R. & Driessen, A. J. Translocation of proteins across the cell envelope of Gram-positive bacteria. *FEBS Microbiol. Rev.* **25**, 437–454 (2001).
3. Pym, A. S. *et al.* Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. *Nature Med.* **9**, 533–539 (2003).
4. Stanley, S. A., Raghavan, S., Hwang, W. W. & Cox, J. S. Acute infection and macrophage subversion by *Mycobacterium tuberculosis* require a specialized secretion system. *Proc. Natl Acad. Sci. USA* **100**, 13001–13006 (2003).
5. Hsu, T. *et al.* The primary mechanism of attenuation of bacillus Calmette–Guerin is a loss of secreted lytic function required for invasion of lung interstitial tissue. *Proc. Natl Acad. Sci. USA* **100**, 12420–12425 (2003).
6. Guinn, K. M. *et al.* Individual RD1-region genes are required for export of ESAT-6/CFP-10 and for virulence of *Mycobacterium tuberculosis*. *Mol. Microbiol.* **51**, 359–370 (2004).
7. Sutcliffe, I. C. Cell envelope composition and organisation in the genus *Rhodococcus*. *Antonie Van Leeuwenhoek* **74**, 49–58 (1998).
8. Minnikin, D. E., Kremer, L., Dover, L. G. & Besra, G. S. The methyl-branched fortifications of *Mycobacterium tuberculosis*. *Chem. Biol.* **9**, 545–553 (2002).
9. Bayan, N., Houssin, C., Chami, M. & Leblon, G. Mycomembrane and S-layer: two important structures of *Corynebacterium glutamicum* cell envelope with promising biotechnology applications. *J. Biotechnol.* **104**, 55–67 (2003).
10. Mahairas, G. G., Sabo, P. J., Hickey, M. J., Singh, D. C. & Stover, C. K. Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. *J. Bacteriol.* **178**, 1274–1282 (1996).
11. Andersen, P. & Doherty, T. M. The success and failure of BCG — implications for a novel tuberculosis vaccine. *Nature Rev. Microbiol.* **3**, 656–662 (2005).
12. Gordon, S. V. *et al.* Identification of variable regions in the genomes of tubercle bacilli using bacterial artificial chromosome arrays. *Mol. Microbiol.* **32**, 643–655 (1999).

13. Lewis, K. N. *et al.* Deletion of RD1 from *Mycobacterium tuberculosis* mimics bacille Calmette–Guerin attenuation. *J. Infect. Dis.* **187**, 117–123 (2003).
14. Majlessi, L. *et al.* Influence of ESAT-6 secretion system 1 (RD1) of *Mycobacterium tuberculosis* on the interaction between mycobacteria and the host immune system. *J. Immunol.* **174**, 3570–3579 (2005).
15. Pym, A. S., Brodin, P., Brosch, R., Huerre, M. & Cole, S. T. Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines *Mycobacterium bovis* BCG and *Mycobacterium microti*. *Mol. Microbiol.* **46**, 709–717 (2002).
16. Tekaiia, F. *et al.* Analysis of the proteome of *Mycobacterium tuberculosis* in silico. *Tuber. Lung Dis.* **79**, 329–342 (1999).
17. Gey Van Pittius, N. C. *et al.* The ESAT-6 gene cluster of *Mycobacterium tuberculosis* and other high G+C Gram-positive bacteria. *Genome Biol.* **2**, research0044.1–0044.18 (2001).
18. Pallen, M. J. The ESAT-6/WXG100 superfamily — and a new Gram-positive secretion system? *Trends Microbiol.* **10**, 209–212 (2002).
19. Gao, L. Y. *et al.* A mycobacterial virulence gene cluster extending RD1 is required for cytolysis, bacterial spreading and ESAT-6 secretion. *Mol. Microbiol.* **53**, 1677–1693 (2004).
20. Converse, S. E. & Cox, J. S. A protein secretion pathway critical for *Mycobacterium tuberculosis* virulence is conserved and functional in *Mycobacterium smegmatis*. *J. Bacteriol.* **187**, 1238–1245 (2005).
21. Flint, J. L., Kowalski, J. C., Karnati, P. K. & Derbyshire, K. M. The RD1 virulence locus of *Mycobacterium tuberculosis* regulates DNA transfer in *Mycobacterium smegmatis*. *Proc. Natl Acad. Sci. USA* **101**, 12598–12603 (2004).
22. MacGurn, J. A., Raghavan, S., Stanley, S. A. & Cox, J. S. A non-RD1 gene cluster is required for Snm secretion in *Mycobacterium tuberculosis*. *Mol. Microbiol.* **57**, 1653–1663 (2005).
23. Fortune, S. M. *et al.* Mutually dependent secretion of proteins required for mycobacterial virulence. *Proc. Natl Acad. Sci. USA* **102**, 10676–10681 (2005).
24. Abdallah, A. M. *et al.* A specific secretion system mediates PPE41 transport in pathogenic mycobacteria. *Mol. Microbiol.* **62**, 667–679 (2006).
25. Burts, M. L., Williams, W. A., DeBord, K. & Missiakas, D. M. EsxA and EsxB are secreted by an ESAT-6-like system that is required for the pathogenesis of *Staphylococcus aureus* infections. *Proc. Natl Acad. Sci. USA* **102**, 1169–1174 (2005).
26. Renshaw, P. S. *et al.* Structure and function of the complex formed by the tuberculosis virulence factors CFP-10 and ESAT-6. *EMBO J.* **24**, 2491–2498 (2005).
27. Brodin, P. *et al.* Functional analysis of early secreted antigenic target-6, the dominant T-cell antigen of *Mycobacterium tuberculosis*, reveals key residues involved in secretion, complex formation, virulence, and immunogenicity. *J. Biol. Chem.* **280**, 33953–33959 (2005).
28. Champion, P. A., Stanley, S. A., Champion, M. M., Brown, E. J. & Cox, J. S. C-terminal signal sequence promotes virulence factor secretion in *Mycobacterium tuberculosis*. *Science* **313**, 1632–1636 (2006).
29. Ize, B. & Palmer, T. Microbiology. Mycobacteria's export strategy. *Science* **313**, 1583–1584 (2006).
30. MacGurn, J. A. & Cox, J. S. A genetic screen for *Mycobacterium tuberculosis* mutants defective for phagosome maturation arrest identifies components of the ESX-1 secretion system. *Infect. Immun.* **75**, 2668–2678 (2007).
31. Lodes, M. J. *et al.* Serological expression cloning and immunological evaluation of MTB48, a novel *Mycobacterium tuberculosis* antigen. *J. Clin. Microbiol.* **39**, 2485–2493 (2001).
32. Nagai, H. *et al.* A C-terminal translocation signal required for Dot/Icm-dependent delivery of the *Legionella* RalF protein to host cells. *Proc. Natl Acad. Sci. USA* **102**, 826–831 (2005).
33. Vergunst, A. C. *et al.* Positive charge is an important feature of the C-terminal transport signal of the VirB/D4-translocated proteins of *Agrobacterium*. *Proc. Natl Acad. Sci. USA* **102**, 832–837 (2005).
34. Christie, P. J., Atmakuri, K., Krishnamoorthy, V., Jakubowski, S. & Cascales, E. Biogenesis, architecture, and function of bacterial type IV secretion systems. *Annu. Rev. Microbiol.* **59**, 451–485 (2005).
35. Singh, A., Mai, D., Kumar, A. & Steyn, A. J. Dissecting virulence pathways of *Mycobacterium tuberculosis* through protein–protein association. *Proc. Natl Acad. Sci. USA* **103**, 11346–11351 (2006).
36. Dave, J. A., Gey Van Pittius, N. C., Beyers, A. D., Ehlers, M. R. & Brown, G. D. Mycosin-1, a subtilisin-like serine protease of *Mycobacterium tuberculosis*, is cell wall-associated and expressed during infection of macrophages. *BMC Microbiol.* **2**, 30 (2002).
37. Faller, M., Niederweis, M. & Schulz, G. E. The structure of a mycobacterial outer-membrane channel. *Science* **303**, 1189–1192 (2004).
38. Niederweis, M. Mycobacterial porins — new channel proteins in unique outer membranes. *Mol. Microbiol.* **49**, 1167–1177 (2003).
39. Cole, S. T. *et al.* Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**, 537–544 (1998).
40. Brodin, P., Rosenkrands, I., Andersen, P., Cole, S. T. & Brosch, R. ESAT-6 proteins: protective antigens and virulence factors? *Trends Microbiol.* **12**, 500–508 (2004).
41. Gey Van Pittius, N. C. *et al.* Evolution and expansion of the *Mycobacterium tuberculosis* PE and PPE multigene families and their association with the duplication of the ESAT-6 (*esx*) gene cluster regions. *BMC Evol. Biol.* **6**, 95 (2006).
42. Skjot, R. L. V. *et al.* Comparative evaluation of low-molecular-mass proteins from *Mycobacterium tuberculosis* identifies members of the ESAT-6 family as immunodominant T-cell antigens. *Infect. Immun.* **68**, 214–220 (2000).
43. Alderson, M. R. *et al.* Expression cloning of an immunodominant family of *Mycobacterium tuberculosis* antigens using human CD4⁺ T cells. *J. Exp. Med.* **191**, 551–559 (2000).
44. Strong, M. *et al.* Toward the structural genomics of complexes: crystal structure of a PE/PPE protein complex from *Mycobacterium tuberculosis*. *Proc. Natl Acad. Sci. USA* **103**, 8060–8065 (2006).
45. Okkels, L. M. & Andersen, P. Protein–protein interactions of proteins from the ESAT-6 family of *Mycobacterium tuberculosis*. *J. Bacteriol.* **186**, 2487–2491 (2004).
46. Teutschbein, J. *et al.* A protein linkage map of the ESAT-6 secretion system 1 (ESX-1) of *Mycobacterium tuberculosis*. *Microbiol. Res.* 11 Apr 2007 (doi:10.1016/j.micres.2006.11.016).
47. Betts, J. C., Lukey, P. T., Robb, L. C., Mcadam, R. A. & Duncan, K. Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. *Mol. Microbiol.* **43**, 717–731 (2002).
48. Maciag, A. *et al.* Global analysis of the *Mycobacterium tuberculosis* Zur (FurB) regulon. *J. Bacteriol.* **189**, 730–740 (2007).
49. Rodriguez, G. M. & Smith, I. Mechanisms of iron regulation in mycobacteria: role in physiology and virulence. *Mol. Microbiol.* **47**, 1485–1494 (2003).
50. Agarwal, N., Woolwine, S. C., Tyagi, S. & Bishai, W. R. Characterization of the *Mycobacterium tuberculosis* sigma factor SigM by assessment of virulence and identification of SigM-dependent genes. *Infect. Immun.* **75**, 452–461 (2007).
51. Lamichhane, G. *et al.* A postgenomic method for predicting essential genes at subsaturation levels of mutagenesis: application to *Mycobacterium tuberculosis*. *Proc. Natl Acad. Sci. USA* **100**, 7213–7218 (2003).
52. Sassetti, C. M., Boyd, D. H. & Rubin, E. J. Genes required for mycobacterial growth defined by high density mutagenesis. *Mol. Microbiol.* **48**, 77–84 (2003).
53. Sao-Jose, C., Baptista, C. & Santos, M. A. *Bacillus subtilis* operon encoding a membrane receptor for bacteriophage SPP1. *J. Bacteriol.* **186**, 8337–8346 (2004).
54. Francis, A. W. *et al.* Proteomic analysis of *Bacillus anthracis* Sterne vegetative cells. *Biochim. Biophys. Acta* **1748**, 191–200 (2005).
55. Sao-Jose, C. *et al.* The ectodomain of the viral receptor YueB forms a fiber that triggers ejection of bacteriophage SPP1 DNA. *J. Biol. Chem.* **281**, 11464–11470 (2006).
56. Way, S. S. & Wilson, C. B. The *Mycobacterium tuberculosis* ESAT-6 homologue in *Listeria monocytogenes* is dispensable for growth *in vitro* and *in vivo*. *Infect. Immun.* **73**, 6151–6153 (2005).
57. Geluk, A. *et al.* Identification and characterization of the ESAT-6 homologue of *Mycobacterium leprae* and T-cell cross-reactivity with *Mycobacterium tuberculosis*. *Infect. Immun.* **70**, 2544–2548 (2002).
58. Stinear, T. P. *et al.* Reductive evolution and niche adaptation inferred from the genome of *Mycobacterium ulcerans*, the causative agent of Buruli ulcer. *Genome Res.* **17**, 192–200 (2007).
59. Brodin, P. *et al.* Bacterial artificial chromosome-based comparative genomic analysis identifies *Mycobacterium microti* as a natural ESAT-6 deletion mutant. *Infect. Immun.* **70**, 5568–5578 (2002).
60. Volkman, H. E. *et al.* Tuberculous granuloma formation is enhanced by a *Mycobacterium tuberculosis* virulence determinant. *PLoS Biol.* **2**, 1946–1956 (2004).
61. Stamm, L. M. *et al.* *Mycobacterium marinum* escapes from phagosomes and is propelled by actin-based motility. *J. Exp. Med.* **198**, 1361–1368 (2003).
62. van der Wel, N. *et al.* *M. tuberculosis* and *M. leprae* translocate from the phagolysosome to the cytosol in myeloid cells. *Cell* **129**, 1287–1298 (2007).
63. Derrick, S. C. & Morris, S. L. The ESAT6 protein of *Mycobacterium tuberculosis* induces apoptosis of macrophages by activating caspase expression. *Cell. Microbiol.* **9**, 1547–1555 (2007).
64. de Jonge, M. I. *et al.* ESAT-6 from *Mycobacterium tuberculosis* dissociates from its putative chaperone CFP-10 under acidic conditions and exhibits membrane-lysing activity. *J. Bacteriol.* **189**, 6028–6034 (2007).
65. Varga, J. J. *et al.* Type IV pili-dependent gliding motility in the Gram-positive pathogen *Clostridium perfringens* and other *Clostridia*. *Mol. Microbiol.* **62**, 680–694 (2006).
66. Van der Auwera, G. A., Andrup, L. & Mahillon, J. Conjugative plasmid pAW63 brings new insights into the genesis of the *Bacillus anthracis* virulence plasmid pXO2 and of the *Bacillus thuringiensis* plasmid pBT9727. *BMC Genomics* **6**, 103 (2005).
67. Abajay, M. Y. *et al.* A type IV secretion-like system is required for conjugative DNA transport of broad-host-range plasmid pIP501 in gram-positive bacteria. *J. Bacteriol.* **189**, 2487–2496 (2007).
68. Holland, I. B., Schmitt, L. & Young, J. Type I protein secretion in bacteria, the ABC-transporter dependent pathway (Review). *Mol. Membr. Biol.* **22**, 29–39 (2005).
69. Johnson, T. L., Abendroth, J., Hol, W. G. & Sandkvist, M. Type II secretion: from structure to function. *FEMS Microbiol. Lett.* **255**, 175–186 (2006).
70. Cornelis, G. R. The type III secretion injectisome. *Nature Rev. Microbiol.* **4**, 811–825 (2006).
71. Henderson, I. R., Navarro-Garcia, F., Desvaux, M., Fernandez, R. C. & Ala'Aldeen, D. Type V protein secretion pathway: the autotransporter story. *Microbiol. Mol. Biol. Rev.* **68**, 692–744 (2004).
72. Oomen, C. J. *et al.* Structure of the translocator domain of a bacterial autotransporter. *EMBO J.* **23**, 1257–1266 (2004).
73. Mougous, J. D. *et al.* A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science* **312**, 1526–1530 (2006).
74. Pukatzki, S. *et al.* Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. *Proc. Natl Acad. Sci. USA* **103**, 1528–1533 (2006).
75. Alteri, C. J. *et al.* *Mycobacterium tuberculosis* produces pili during human infection. *Proc. Natl Acad. Sci. USA* **104**, 5145–5150 (2007).
76. Li, Y., Miltner, E., Wu, M., Petrofsky, M. & Bermudez, L. E. A *Mycobacterium avium* PPE gene is associated with the ability of the bacterium to grow in macrophages and virulence in mice. *Cell. Microbiol.* **7**, 539–548 (2005).
77. Ramakrishnan, L., Federspiel, N. A. & Falkow, S. Granuloma-specific expression of *Mycobacterium tuberculosis* virulence proteins from the glycine-rich PE-PCR family. *Science* **288**, 1436–1439 (2000).
78. Brodin, P. *et al.* Dissection of ESAT-6 system 1 of *Mycobacterium tuberculosis* and impact on immunogenicity and virulence. *Infect. Immun.* **74**, 88–98 (2006).
79. Tan, T., Lee, W. L., Alexander, D. C., Grinstein, S. & Liu, J. The ESAT-6/CFP-10 secretion system of *Mycobacterium marinum* modulates phagosome maturation. *Cell. Microbiol.* **8**, 1417–1429 (2006).
80. Mostowy, S., Cousins, D. & Behr, M. A. Genomic interrogation of the *dassie* bacillus reveals it as a unique RD1 mutant within the *Mycobacterium tuberculosis* complex. *J. Bacteriol.* **186**, 104–109 (2004).

DATABASES

Entrez Genome Project: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj>
[Bacillus anthracis](#) | [Bacillus subtilis](#) | [Listeria monocytogenes](#) | [Mycobacterium bovis](#) | [Mycobacterium leprae](#) | [Mycobacterium marinum](#) | [Mycobacterium smegmatis](#) | [Mycobacterium tuberculosis](#) | [Staphylococcus aureus](#) | [Streptococcus agalactiae](#)
 Entrez Protein: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=protein>
 CFP-10 | ESAT-6 | EsxA | EsxB | Rv3616c | Rv3868 | Rv3869 | Rv3870 | Rv3871 | Rv3877 | Rv3881c | Rv3883c

SUPPLEMENTARY INFORMATION

See online article: S1 (figure) | S2 (figure) | S3 (table) | S4 (table)

ALL LINKS ARE ACTIVE IN THE ONLINE PDF