

Type VII Secretion Systems in Gram-Positive Bacteria

Daria Bottai, Matthias I. Gröschel and Roland Brosch

Abstract Bacterial secretion systems are sophisticated molecular machines that fulfil a wide range of important functions, which reach from export/secretion of essential proteins or virulence factors to the implication in conjugation processes. In contrast to the widely distributed Sec and Twin Arginine Translocation (TAT) systems, the recently identified ESX/type VII systems show a more restricted distribution and are typical for mycobacteria and other high-GC *Actinobacteria*. Similarly, type VII-like secretion systems have been described in low-GC Gram-positive bacteria belonging to the phylum *Firmicutes*. While the most complex organization of type VII secretion systems currently known is found in slow-growing mycobacteria, which harbour up to 5 chromosomal-encoded systems (ESX-1 to ESX-5), much simpler organization is reported for type VII-like systems in *Firmicutes*. In this chapter, we describe common and divergent features of type VII- and type VII-like secretion pathways and also comment on their biological key roles, many of which are related to species-/genus-specific host-pathogen interactions and/or virulence mechanisms.

Contents

1	Introduction	236
2	The Discovery of Type VII Secretion Systems	237
3	Gene Clusters Encoding Type VII Secretion Systems	239

D. Bottai

Dipartimento di Ricerca Traslazionale e delle Nuove Tecnologie
in Medicina e Chirurgia, University of Pisa, Pisa, Italy

M.I. Gröschel · R. Brosch (✉)

Institut Pasteur, Unit for Integrated Mycobacterial Pathogenomics,
Paris, France

e-mail: roland.brosch@pasteur.fr

M.I. Gröschel

Department of Pulmonary Diseases and Tuberculosis, University Medical
Center Groningen, University of Groningen, Groningen, The Netherlands

Current Topics in Microbiology and Immunology (2017) 404:235–265

DOI 10.1007/82_2015_5015

© Springer International Publishing Switzerland 2015

Published Online: 05 January 2016

3.1	The Mycobacterial ESX Loci	239
3.2	Type VII-Like Gene Clusters in Actinobacteria and Firmicutes	241
4	Type VII Secretion Machineries	243
4.1	Mycobacterial ESX Secretion Machineries: The Paradigm of Type VII Secretion Systems	243
4.2	ESX Substrates.....	245
4.3	Regulation of ESX Secretion in Mycobacteria	247
4.4	Type VII-like Secretion Machineries in Non-mycobacterial Species.....	248
5	Type VII Secretion Systems in Bacterial Virulence and Pathogenicity.....	251
5.1	The Mycobacterial ESX-1, ESX-3 and ESX-5 Secretion Systems	251
5.2	Ess Secretion System in Virulence of <i>S. Aureus</i>	254
5.3	The ESAT-like Secretion System in Virulence of Bacillus Anthracis	255
6	Concluding Comments	255
	References	256

1 Introduction

Bacteria have evolved a multitude of secretion pathways for the export of a wide range of substrates (Costa et al. 2015), some of which may also help the bacteria to shape and/or adapt to an intracellular environment (Stanley and Cox 2013; Majlessi et al. 2015). The effector molecules transported by the secretion systems often play crucial roles in different physiologic processes, in horizontal gene transfer and/or in host–pathogen interactions (adhesion, pathogenicity and survival). In addition to the widely distributed Sec and Twin Arginine Translocation (TAT) systems, which are responsible for substrate translocation across the cytoplasmic membrane, both in Gram-positive and Gram-negative bacteria, other less broadly conserved secretion systems fulfil more specialized functions. These latter systems include the different specialized secretion systems of Gram-negative bacteria, known as type I, type II, type III, type IV, type V and type VI secretion pathways, which are responsible for secretion of substrates across the typical cell envelop of Gram-negative bacteria that is constituted by a plasma membrane and an outer membrane (Costa et al. 2015). In contrast, few specialized secretion systems are known in Gram-positive bacteria. One exception constitute the so-called type VII secretion systems, which have been first described in *Mycobacteria*, where they serve as specialized secretion machineries devoted to the export of peculiar subsets of protein substrates across the complex and highly hydrophobic mycobacterial cell envelope (Mahairas et al. 1996; Pym et al. 2002; Abdallah et al. 2007; Brodin et al. 2004b). However, type VII-like systems have also been identified by in silico analyses in other closely related high-C+G bacterial species (*Actinobacteria*), as well as in more distantly related Gram-positive bacteria belonging to the low-C+G group (*Firmicutes*) of Gram-positive bacteria (Gey Van Pittius et al. 2001; Pallen 2002; Houben et al. 2014).

In this book chapter, we focus on type VII secretion systems in *Actinobacteria* and also describe the type VII-like secretion systems in other Gram-positive

bacteria, with focus on the most relevant features of the Ess system of *Staphylococcus aureus* and the Yuk/Yue system of *Bacillus subtilis*, for which a functional secretion activity has recently been demonstrated.

2 The Discovery of Type VII Secretion Systems

The first type VII substrate to be identified was the 6-kDa early-secreted antigenic target ESAT-6 (EsxA) of *Mycobacterium tuberculosis*. This small protein, which is lacking a classical N-terminal signal sequence, is present in large amounts in short-term culture filtrate of *M. tuberculosis* and was shown to act as an immunodominant T-cell antigen (Sorensen et al. 1995). During comparative and functional genomic analyses, it was found that certain attenuated strains (e.g. *Mycobacterium bovis* BCG or *Mycobacterium microti*) lacked the gene *esxA* encoding ESAT-6, as well as the genomic region up- and downstream of *esxA* (Mahairas et al. 1996; Pym et al. 2002; Brodin et al. 2002), suggesting a key role of this region in mycobacterial virulence, which was later confirmed by complementation (Pym et al. 2002, 2003; Brodin et al. 2004a) and gene knockout studies (Lewis et al. 2003; Stanley et al. 2003; Hsu et al. 2003). These studies also provided evidence of an ESAT-6 specialized secretion system, independently referred to as ESAT-6 system (ESX-1) (Brodin et al. 2004b), Snm system (Converse and Cox 2005; MacGurn et al. 2005; Stanley et al. 2003), or type VII secretion system (Abdallah et al. 2007; Bitter et al. 2009a, b). This classification is in line with the diderm structure of the mycobacterial cell envelope (Zuber et al. 2008; Hoffmann et al. 2008), which in addition to the cytosolic inner membrane includes an outer membrane (the mycomembrane). It was speculated that the mycomembrane might be functionally equivalent to the classical Gram-negative outer membrane despite a different chemical composition (Hoffmann et al. 2008).

In *M. tuberculosis*, ESAT-6 (EsxA) and its protein partner CFP-10 (10-kDa culture filtrate protein, EsxB) are representative members of the large Esx protein family that is constituted of 23 small (size of ~100 amino acids (aa)), highly immunogenic secreted proteins, sharing a conserved Trp-Xaa-Gly (WXG) motif and a characteristic hairpin structure with the WXG domain at the helix-turn-helix bend (Pallen 2002; Cole et al. 1998; Gey Van Pittius et al. 2001). Genes encoding for Esx family members usually lie in tandem pairs and, in five cases, are flanked by blocks of conserved gene clusters coding for components of the ESX secretory apparatus responsible for secretion of the corresponding ESAT-6-like proteins (Cole et al. 1998; Tekaija et al. 1999; Gey Van Pittius et al. 2001). Bioinformatic analyses identified genes encoding ESAT-6-like proteins both in the genomes of other *Actinobacteria* (*Nocardia*, *Corynebacteriae*, *Streptomyces*) (Gey Van Pittius et al. 2001) and in a number of bacterial species belonging to the phylum *Firmicutes* (Pallen 2002). Although the homology in the primary sequence is low relative to ESAT-6 from *M. tuberculosis*, certain features allow to classify these proteins into the WXG-100 superfamily (Pallen 2002). Bacteria with WXG proteins

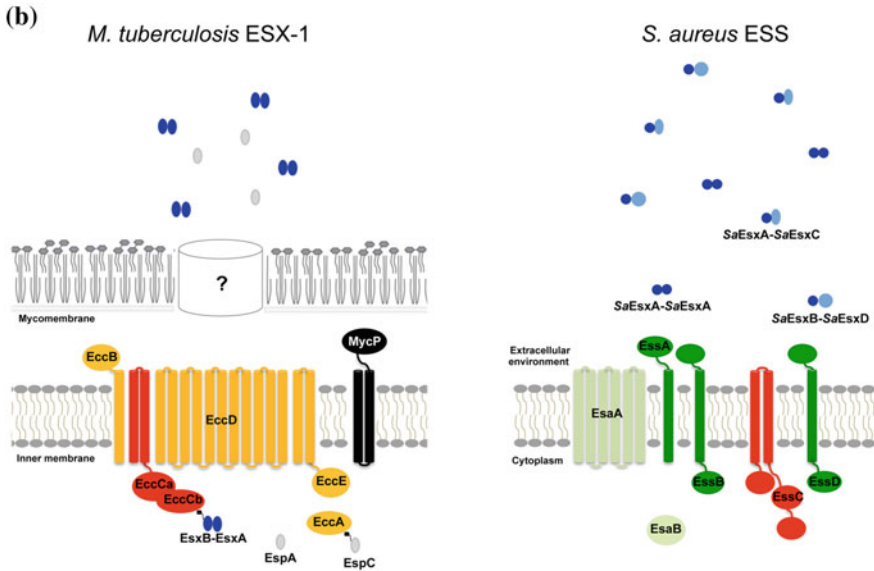
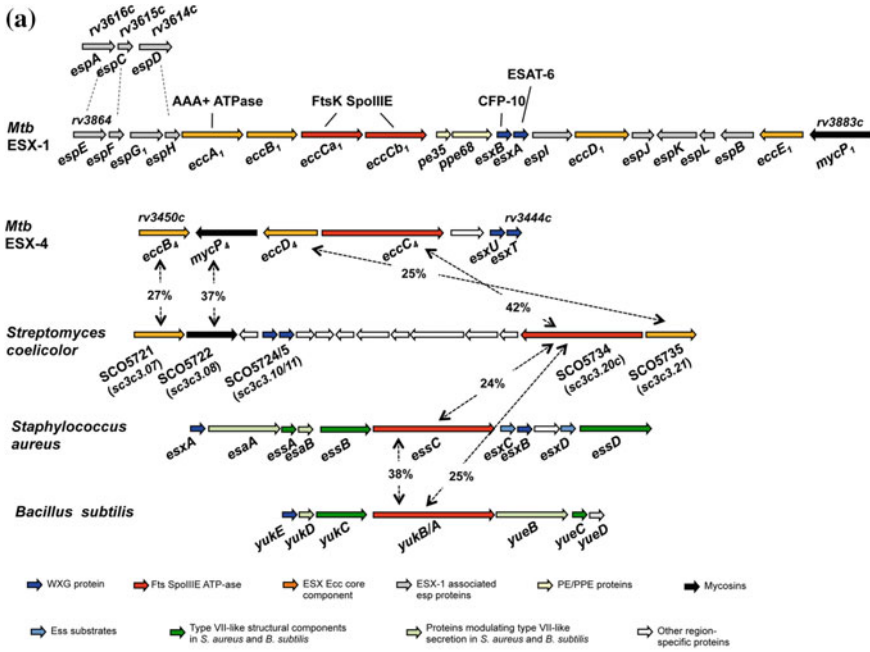


Fig. 1 **a** Genetic organization of type VII system genetic loci in *Mycobacterium tuberculosis* (Mtb) showing ESX-1 and ESX-4 clusters in comparison with type VII-like loci in *Streptomyces coelicolor*, *Staphylococcus aureus* and *Bacillus subtilis*. **b** Working model of the type VII- and type VII-like secretion machineries of *M. tuberculosis* (ESX-1) and *S. aureus* (ESS), respectively. Note that the colours of the represented proteins refer to the same colour code as used in panel A

also often harbour ATP-binding proteins belonging to the FtsK/SpoIIIE family, whose encoding genes are encoded next to those encoding WXG-100 proteins (Fig. 1) (Bitter et al. 2009a). FtsK/SpoIIIE ATPases are involved in translocation of macromolecules (proteins and DNA) across a membrane-bound channel in a wide range of cellular processes, including protein secretion, DNA conjugation, cell division and sporulation (Iyer et al. 2004). In the Gram-negative type IV secretion pathway, for example, FtsK/SpoIIIE family proteins play a key role in substrate recognition and ATP-mediated protein transport (Christie 2001). Consistently, they have been supposed to be involved in generating energy for translocation of WXG proteins/ESX substrates (Pallen 2002). Functionally active type VII-like secretion systems have been described in *Streptomyces* (Fyans et al. 2013; Akpe San Roman et al. 2010), *Staphylococcus aureus* (Burts et al. 2005), *Streptococcus agalactiae* (Shukla et al. 2010), *Listeria monocytogenes* (Way and Wilson 2005), *B. subtilis* (Baptista et al. 2013; Huppert et al. 2014) and *Bacillus anthracis* (Garufi et al. 2008).

3 Gene Clusters Encoding Type VII Secretion Systems

Typical features of the gene clusters encoding type VII secretion machineries both in *Actinobacteria* and *Firmicutes* are the presence of one or two genes encoding WXG-100 proteins, and a gene encoding an FtsK/SpoIIIE ATPase family member (Fig. 1) (Bitter et al. 2009a, b; Pallen 2002). Apart from these conserved genes, each locus includes a variable number of genus-specific genes encoding for structural or accessory system-specific components, essential for type VII- or type VII-like secretion in mycobacteria or other Gram-positive bacterial species, respectively. These differences in gene content may result in varying complexity and functionality of the corresponding secretion machineries and may account for their different roles in various biological processes and/or adaptation to specific host/environments.

3.1 The Mycobacterial ESX Loci

The *M. tuberculosis* genome harbours five highly conserved ESX gene clusters encoding type VII secretion systems, designated ESX-1 to ESX-5 (Tekaiia et al. 1999; Gey Van Pittius et al. 2001). Phylogenetically, the ESX-4 locus is considered the most ancestral ESX cluster in mycobacteria, showing a relatively small number of genes and a simple gene organization relative to the other ESX clusters (Fig. 1) (Gey Van Pittius et al. 2001; Gey van Pittius et al. 2006). ESX-4 also displays similarity to the ESX-related loci identified in the genome of a wide range of *Actinobacteria* (Gey Van Pittius et al. 2001; Gey van Pittius et al. 2006). It was hypothesized that other ESX clusters might have evolved from ESX-4 through gene

duplication/diversification events and insertions of additional genes. Conversely, ESX-5 is considered the most recent ESX locus, whose evolution correlates with the differentiation of slow-growing mycobacterial species. ESX-5 orthologous loci are exclusively conserved in the genomes of mycobacterial species belonging to the slow-growing group including several human pathogens (*M. tuberculosis*, *Mycobacterium leprae* and *Mycobacterium ulcerans*) or the fish pathogen *Mycobacterium marinum*. In contrast, ESX-5-like loci are not present in the genomes of phylogenetically more distant, fast-growing mycobacterial species, which are mainly constituted by saprophytic mycobacteria.

Although the gene content may vary in different ESX loci (in terms of genes encoding chaperones, ATPases, transcription factors and substrates), a set of genes is highly conserved in all ESX clusters. Each ESX locus consists of the following: (i) a pair of *esx* genes, coding for WXG-100 proteins (ESAT-6 and CFP-10 in the case of ESX-1 system/locus, the paradigm of ESX loci); (ii) *ecc* (*esx conserved components*) genes, which encode proteins with one or more transmembrane domain(s) and ATP-binding proteins, representing core components of the secretion machinery responsible for the ATP-dependent translocation of ESX substrates; (iii) a *mycP* gene, coding for mycobacteria-specific membrane-anchored proteins belonging to the subtilisin-like serine protease family; *iv*) *pe* and *ppe* genes, which encode for two large classes of mycobacteria-specific proteins, named after their conserved proline–glutamic acid (PE) or proline–proline–glutamic acid (PPE) motifs at their N-termini, respectively (Bitter et al. 2009a; Tekaiia et al. 1999; Cole et al. 1998). The ESX-associated *pe* and *ppe* genes are included in all ESX loci with the exception of ESX-4 and might represent the most ancestral *pe* and *ppe* genes of mycobacteria from which other members of these large gene families seem to have evolved by duplication and diversification (Gey van Pittius et al. 2006). Interestingly, selected *pe* and or *ppe* gene family members carrying characteristic GC-rich repetitive sequences (Cole et al. 1998) have also been acquired by horizontal gene transfer. This is the case of the *pe_pgrs33* (*rv1818*) gene that is present in all members of the *M. tuberculosis* complex but is missing from the supposed ancestral gene pool of tubercle bacilli, represented by the different *Mycobacterium canettii* strains (Supply et al. 2013; Boritsch et al. 2014). In addition to *ecc* genes, ESX loci may also contain genes encoding for ESX secretion-associated proteins (Esp) (Fortune et al. 2005; Bitter et al. 2009a). Several *esp* gene products are secreted via the type VII pathway and/or are involved in modulation of the ESX activity. In other cases, Esp proteins may act as chaperones in assisting the secretion of ESX substrates (as detailed below). Some Esp proteins, e.g. EspB or EspI, are part of the core ESX-1 cluster (Chen et al. 2013; Zhang et al. 2014) whereas others, such as EspA, EspC, EspD or EspR, exert fundamental roles in the regulation and secretion of ESX-1 proteins, but are not encoded in the core region (MacGurn et al. 2005; Frigui et al. 2008; Blasco et al. 2012; Pang et al. 2013; Majlessi et al. 2015). This is particularly relevant for the *espACD* locus which in contrast to the core ESX-1 region is exclusively present in the genomes of pathogenic mycobacteria, such as *M. tuberculosis*, *M. marinum* or *M. leprae*. It is noteworthy that in these different mycobacterial species, the *espACD* region is located in non-syntenic

genomic sections (Simeone et al. 2012), which are reminiscent of genomic islands and suggest an independent acquisition of the *espACD* region in the different mycobacterial species (Majlessi et al. 2015). Future work will have to elucidate how these *espACD* loci have been interconnected with the ESX-1 secretion activity in the concerned mycobacterial species.

A new type VII locus has recently been identified in the conjugative pRAW plasmid isolated from *M. marinum* (Ummels et al. 2014). This locus, which was referred to as ESX-P1, harbours genes highly homologous to the genome-associated ESX-5 cluster, although the gene order and the gene content closely resembles to the ESX-2 locus. The ESX-P1 locus differs from all known ESX loci for the presence at the 5' terminus of two genes coding for members of the NLP60 family of peptidoglycan-associated glycosides hydrolases, which might be predictive of a novel and different function for ESX-P1. In addition to ESX-P1, the pRAW plasmid also contains genes homologous to type IV secretion system components and proteins with predicted relaxase activity. The ESX-P1-harbours plasmid seems to be efficiently transferred among various slow-growing mycobacterial species and can also be transferred into *M. tuberculosis* under certain experimental conditions (Ummels et al. 2014). The conjugative process linked to the pRAW plasmid requires both, type VII and type IV secretion machinery components, with the ESXP-1-encoded EccC_{P1} and VirB4 homologs being involved in the gene transfer process. The implication of a secretion system in conjugal gene transfer is reminiscent of some biological properties of type IV secretion systems in Gram-negative bacteria. For mycobacteria, conjugative gene transfer has also been reported. Apart from the aforementioned implication of the pRAW plasmid in conjugation within the group of selected slow-growing mycobacteria, conjugative processes have been demonstrated for *M. smegmatis*. In this fast-growing mycobacterial model organism, an involvement of the ESX-1 type VII secretion machinery in distributive conjugal transfer has been described that results in genome-wide mosaicism (Flint et al. 2004; Gray et al. 2013). ESX-1 seems to play a dual role in the process: while ESX-1 components are negative regulators of DNA transfer in donor cells (Flint et al. 2004), the ESX-1 activity is required for DNA acquisition in recipient cells (Coros et al. 2008; Gray et al. 2013). Future studies will show whether distributive conjugal transfer may also play a role in the evolution of other (myco)bacterial species.

3.2 Type VII-Like Gene Clusters in Actinobacteria and Firmicutes

Apart from *M. tuberculosis* and other mycobacterial species, type VII-like secretion systems are found in the genomes of other mycolic acid-containing genera (mycolata), such as *Nocardia* or *Corynebacterium*, as well as in *Streptomyces* species (Gey Van Pittius et al. 2001). The genomic loci encoding type VII-like secretion

systems in mycolata show the highest degree of homology with the ESX-4 system of *M. tuberculosis*. In the more closely related *Corynebacterium diphtheriae*, an ESX-4-encoding gene cluster is located in a genomic region that shares a syntenic gene organization with *M. tuberculosis*, which suggests that ESX-4 like type VII systems might have played an important role in the evolution of selected branches of *Actinobacteria*. The genes in this region are predicted to be functional, as no deletions, frameshifts or stop codons were detected (Gey Van Pittius et al. 2001). The genome of the more distantly related *Streptomyces coelicolor* harbours an ESX locus including four out of six orthologous genes conserved in the *M. tuberculosis* ESX-4 cluster (*eccB₄*, *eccC₄*, *eccD₄* and *mycP₄*), and other species-specific genes (Fig. 1) (Gey Van Pittius et al. 2001).

However, the simplest gene organization compared to the mycobacterial ESX loci was found in gene clusters encoding type VII-like secretion systems in *Firmicutes*. The *Ess* locus of *S. aureus* consists of 11 type VII system-associated genes (Burts et al. 2005; Anderson et al. 2013), which include *esxA*, *esxB* and *essC* genes encoding WXG-100 proteins (the *S. aureus* EsxA and EsxB variants) and a membrane-anchored FtsK/SpoIIIE-like ATPase (*EssC*) (Fig. 1). The *Ess* locus also contains *essA*, *essB* and *essD* genes coding for membrane-embedded proteins required for secretion of *Ess* substrates, as well as *esaC/esxC* and *esaD/esxD* genes, which encode small proteins that were recently identified as specific *Ess* substrates of staphylococci (Burts et al. 2008; Anderson et al. 2011, 2013, Chen et al. 2012). Finally, among the genes at this locus *esaB* encodes a negative regulator of *Ess* secretion activity (Burts et al. 2008).

WXG-100-encoding genes have also been identified in different species of the genus *Bacillus*, both in the non-pathogenic species *B. subtilis* and in the virulent species *Bacillus cereus*, *Bacillus thuringiensis* and *B. anthracis* (Baptista et al. 2013; Huppert et al. 2014). The genome of *B. subtilis* harbours two genes encoding WXG-100 proteins, *yukE* and *yfiA*, situated in distant genomic regions. *YukE* is the first gene of a cluster consisting of five annotated genes (*yukEABCD*), which is conserved in the genomes of *B. cereus*, *B. thuringiensis*, but not in *B. anthracis* (Baptista et al. 2013; Huppert et al. 2014). For some *Bacillus* strains, the split *yukA/B* gene encodes for a predicted FtsK/SpoIIIE ATPase, homologous to *EccCa₁* and *EccCb₁* from *M. tuberculosis* or *EssC* from *S. aureus*. The *yukC* and *yukD* genes encode two additional structural proteins, thought to be required for *YukE* secretion (Baptista et al. 2013; Huppert et al. 2014). Also, the *yueB/C* cluster, lying adjacent to the *yuk* locus in the genome of *B. subtilis*, has recently been demonstrated to encode components of the secretion apparatus involved in *YukE* secretion (Huppert et al. 2014). Both *YueB* and *YueC* are homologous to *Ess* proteins of *S. aureus* and are conserved in putative type VII-like secretion systems of other species within the *Firmicutes*, such as *Streptococcus agalactiae* or *L. monocytogenes* (Huppert et al. 2014).

Inspection of the genome of *B. anthracis* identified six genes encoding for WXG proteins. One of them, the *Ba-esxB* (BAS2036), shows similarity with the mycobacterial *esxB* and staphylococcal *esxA* and lies in a gene cluster immediately downstream the *Ba-essC* gene (BAS2035), encoding an FtsK/SpoIIIE-like ATPase

(Garufi et al. 2008). Four other WXG encoding genes, namely *Ba-esxL*, *Ba-esxQ*, *Ba-esxV* and *Ba-esxW*, are located elsewhere on the *B. anthracis* chromosome, whereas the fifth gene, *Ba-esxP*, is encoded on the virulence plasmid pOX1. All these five genes encode for proteins harbouring a large C-terminal domain that is not found in mycobacterial and staphylococcal Esx proteins and appears to be a specific signature of the *B. cereus* group (Garufi et al. 2008).

4 Type VII Secretion Machineries

Because of their relatively recent discovery, almost no structural data on type VII secretion machineries are available (Rosenberg et al. 2015; Korotkova et al. 2014, 2015; van der Woude et al. 2013). However, in silico predictions and homology studies, in combination with comparative secretome analyses from different type VII mutant strains suggest that each type VII secretion apparatus secretes multiple substrates and constitutes a complex, multi-protein machinery, consisting of several components (structural and accessory factors). Mycobacterial ESX/type VII secretion machineries are the most extensively characterized, although some data on the composition and regulation of type VII-like secretion machineries in *Firmicutes* became recently available.

4.1 *Mycobacterial ESX Secretion Machineries: The Paradigm of Type VII Secretion Systems*

Most of the current knowledge on ESX secretion machineries comes from the characterization of ESX-1, ESX-3 and ESX-5 systems from *M. marinum*, *M. tuberculosis* or *M. smegmatis* (van der Woude et al. 2013).

The different ESX secretion machines are predicted to contain an inner-membrane-bound protein complex, thought to drive ATP-dependent translocation of ESX substrates across the cytoplasmic membrane in association with a yet un-identified outer-membrane-embedded channel, which seem to manage substrate export across the mycomembrane to the extracellular environment (see below). Structural components of the membrane-anchored apparatus are three transmembrane-domain-containing proteins (EccB, EccD, EccE), a membrane-anchored ATP-binding protein (EccC) and a cytosolic ATP-binding protein belonging to the AAA+ATPase family (EccA). Based on mass spectrometry analyses and the evaluation of the apparent molecular weight of the ESX-5 complex from *M. marinum*, each ESX membrane complex seems to be composed of 6 copies of EccB, EccC and EccD components and three copies of EccE (Houben et al. 2012b). EccB, EccC and EccD proteins have been demonstrated to be required for secretion of the corresponding ESX substrates in all ESX systems characterized so far (ESX-1, ESX-3

and ESX-5) (Siegrist et al. 2014; Di Luca et al. 2012; Brodin et al. 2006; MacGurn et al. 2005; Guinn et al. 2004; Hsu et al. 2003). Among these components, EccD is a highly hydrophobic protein, containing 11 predicted transmembrane domains. This protein is predicted to build the channel for transport of substrates across the cytoplasmic membrane (Bitter et al. 2009a). EccC proteins are thus key components of the ESX core complex and are encoded either by a single gene, or, as in the case for ESX-1, by two adjacent genes (*eccCa*₁ and *eccCb*₁), whose products are predicted to form a single functional unit (Cole et al. 1998; Bitter et al. 2009a). EccC proteins have functional ATP-binding domains, which are homologous to the FtsK/SpoIIIE family of ATPases. In contrast to the FtsK/SpoIIIE ATPases characterized so far, which contain only one ATPase domain, the ESX-encoded EccC component has a unique multi-domain structure consisting of three functionally distinct FtsK/SpoIIIE-like ATP-binding domains, supposed to play distinct roles in substrate translocation and complex formation (Ramsdell et al. 2015; van der Woude et al. 2013). While the most N-terminally situated ATPase domain seems to be involved in ATP hydrolysis, the second and third ATPase/nucleotide binding domains seem to be required for the assembly of a functional ESX secretion machinery (Ramsdell et al. 2015). The ESX-1-encoded EccC component interacts with the ESX-1 substrates EsxB, via the last seven C-terminal residues of EsxB (Stanley et al. 2003; Champion et al. 2006). This C-terminal short domain represents a signal sequence that is linked to secretion via ESX-1 (Champion et al. 2006). The involvement of an ATPase in the contact between ESX substrates and their corresponding secretion apparatus resembles type IV secretion systems, where a FtsK/SpoIIIE ATPase recognizes an unstructured C-terminal sequence and hence directs substrates across the cytoplasmic membrane. It has recently been observed that the binding of EsxB via its signal sequence at the C-terminal domain to EccC₁ induces the activation of EccC₁ by stimulating its multimerization, thus resulting in increased ATPase activity (Rosenberg et al. 2015). These findings suggest a model in which ESX substrates (e.g. EsxB) might modulate the coordinated release of substrates from the bacterium (Rosenberg et al. 2015). Apart from the FtsK/SpoIIIE ATPase, ESX secretion machineries also include a cytosolic ATP-binding protein (EccA) belonging to the AAA+ family (ATPase associated with various cellular activities). Proteins belonging to this family play a role in various cellular processes: assembly and disassembly of protein complexes, protein degradation and signal transduction. Although some EccA protein family members have been demonstrated to be involved in ESX activity (EccA₁ and EccA₅ are implicated ESX-1 and ESX-5 secretion, respectively, while the ESX-3-encoded EccA₃ is required for viability of *M. tuberculosis*) (Siegrist et al. 2014; Bottai et al. 2012; Brodin et al. 2006), the specific function of AAA+ ATPases in ESX-mediated secretion remains unknown. Structural data on the ESX-1-encoded EccA₁ revealed that the C-terminal part of EccA₁ contains an oligomerization domain, which might induce the formation of a hexamer. It was also proposed that EsxA₁ possesses ATPase activity, possibly regulated by the N-terminal part of the protein (Wagner et al. 2013). The finding that EccA₁ interacts with EspC and EspF₁ in two-hybrid studies in vitro (Champion et al. 2009) suggests that these proteins might be involved in the

assembly/disassembly of secreted substrate molecules, or alternatively, secreted substrates and their chaperones.

In addition to the ESX secretion components mentioned above, ESX activity also involves mycosins (MycP₁–MycP₅). These proteins correspond to specific subtilisin-like serine proteases (Brown et al. 2000; Dave et al. 2002) and are predicted to be anchored in the cytoplasmic membrane via the C-terminal transmembrane domain, with the active site exposed to the extracytoplasmic space (Sonnhammer et al. 1998; Gardy et al. 2005). This protein topology suggests a role for these proteins in proteolytic digestion of ESX substrates. To date, proteolytic activity has been demonstrated for MycP₁, the ESX-1 associated mycosin. MycP₁ was shown to digest EspB at its C-terminal domain, thereby modulating ESX-1 activity (Ohol et al. 2010). More recently, the ESX-3-encoded mycosin MycP₃ has been demonstrated to be involved in stabilization or/and optimal secretion of the EsxG–EsxH complex in *M. smegmatis* (see below) (Siegrist et al. 2014).

The actual way of how ESX substrates are transported across the mycobacterial outer membrane to the extracellular environment remains unknown. In Gram-negative bacteria, protein secretion may occur by a “one-step” mechanism, in which a protein channel spanning both the inner and the outer membrane mediates the substrate translocation across the diderm cell envelope (type I, type III and type VI secretion pathways). Alternatively, this transport can also occur by a “two-step” mechanism, in which the Sec or TAT machineries are responsible for substrate transport across the cytoplasmic membrane, and a different, more specialized secretion apparatus is involved in subsequent substrate translocation across the outer membrane (type II and type IV secretion pathways). In mycobacteria, the absence of a Sec or TAT signal sequence in ESX substrates and the presence of transmembrane domains in all ESX core components suggest that type VII protein transport across—at least the inner membrane—occurs by the ESX systems. It has been speculated that some of the Ecc-membrane-bound components, such as EccE or EccC, might span across the mycomembrane (Houben et al. 2012b), favouring a one-step secretion mechanisms of ESX substrates. However, as detailed structural data of the type VII apparatus are missing, it is also possible that proteins with yet undefined roles might mediate the ESX protein translocation to the extracellular environment.

4.2 ESX Substrates

In addition to the above-described Esx/WXG-100 proteins representing the first known substrates of mycobacterial ESX/type VII secretion systems, PE/PPE protein family members and a number of Esp proteins are also transported by ESX machineries.

Although Esx, PE/PPE and Esp proteins display very low similarity in their primary amino acid sequence, all these classes of ESX substrates share several typical features: (a) the ability to be secreted as heterodimers (for Esx and PE/PPE

proteins); (b) the codependent secretion where deletion/absence of one substrate affects the secretion of the cognate ESX substrate; (c) the presence of a C-terminal signal domain responsible for protein targeting to the type VII secretion pathway. The resolved structure of the ESAT-6-CFP-10 heterodimer, representative of the Esx protein complexes, revealed the presence of two helices on each Esx protein connected by a turn formed by the WXG motif. In this complex, the two proteins are in antiparallel orientation and have flexible N- and C-terminal tails (Renshaw et al. 2005). A similar structure has been reported for the PE25-PPE41 heterodimer, representative of the PE/PPE protein complexes: the domains of the proteins that are responsible for interaction form a four-helix bundle in an antiparallel fashion, and a WXG motif is located in the turn between the two helices of the PPE protein (Strong et al. 2006). Both, EsxB and PE25 proteins in the ESAT-6-CFP-10 and PE25-PPE41 heterodimers, respectively, have an identical C-terminal short motif, which is crucial for secretion (Champion et al. 2006; Renshaw et al. 2005; Strong et al. 2006). Although ESX substrates lack a classical N-terminal signal sequence, they share a conserved C-terminal secretion domain which interacts with a conserved ESX structural component (e.g. the short C-terminal domain of CFP-10 is recognized by the FtsK/SpoIIIE ATPase EccC), thus targeting the substrate and its protein partner to the corresponding secretion machinery. More recently, a conserved C-terminal YxxxD/E motif was identified as the general sequence required for targeting proteins to type VII secretion pathway (Daleke et al. 2012). This YxxxD/E domain seems also to be located adjacent to the helix-turn-helix motif of several PE proteins and EspB (Daleke et al. 2012). Moreover, an additional hydrophobic residue located seven positions downstream the YxxxD/E motif (the Y subdomain) was proposed to be involved in ESX substrate recognition (Poulsen et al. 2014). However, the signal sequences that specifically target each ESX substrate to the corresponding ESX secretion apparatus are still unknown. In this respect, it has been hypothesized that selected EspG proteins (e.g. the *M. marinum* ESX-1- and ESX-5-encoded EspG) might act as chaperons in directing type VII substrates to the corresponding ESX machineries (see below).

Apart from Esx and PE/PPE proteins, several ESX-1-related Esp proteins such as EspA, EspC, EspE, EspF, EspJ, EspK, EspB have been proposed as ESX-1 substrates (Champion et al. 2009; McLaughlin et al. 2007; Sani et al. 2010; Carlsson et al. 2009). Several of these Esp proteins carry the C-terminal YxxxD/E motif, conserved in type VII substrates or substrate complexes (Daleke et al. 2012). Secretion of some Esp proteins (e.g. EspA and EspC) or Esp protein activity (e.g. EspB, EspD) impact on ESAT-6 secretion and in turn, on mycobacterial virulence. The mutually codependent secretion of EspA and EspC with ESAT-6 and CFP-10 represents a key element of the regulation of the ESX-1 activity (see below). Differently, EspB secretion via ESX-1 requires neither EspA/EspC/EspD expression nor ESAT-6/CFP-10 secretion (Chen et al. 2013). However, the proteolytic digestion of EspB by MycP₁ modulates the amount of proteins secreted by ESX-1. EspB is able to bind bioactive host phospholipids such as phosphatidic acid and phosphatidylserine, thus interfering with eukaryotic cell signalling (Chen et al. 2013). This feature reveals an additional, ESAT-6-independent impact of EspB on

mycobacterial virulence. Recent data on the resolved crystal structure of EspB demonstrated that the protein can adopt a PE/PPE-like fold and oligomerize to form a barrel-shaped structure with heptameric symmetry, thus suggesting the possibility that EspB might be part of a structural subunit of a cell-wall-associated complex (Solomonson et al. 2015; Korotkova et al. 2015).

Other ESX-1-associated Esp proteins (e.g. EspD) do not seem to be ESX-1 substrates, but are involved in modulation of the ESX-1 activity as they seem to influence EspA and EspC stabilization and secretion (Chen et al. 2013). Moreover, a species-specific impact on ESX-1 secretion has been reported for some Esp proteins (EspF and EspG₁) (Bottai et al. 2011; Gao et al. 2004; Converse and Cox 2005), suggesting that the function of similar proteins in related bacterial species might have changed during evolution due to adaptation processes. EspF and EspG₁ have been reported to be implicated in ESAT-6 and CFP-10 secretion in *M. smegmatis* and *M. marinum*, whereas they are dispensable for secretion, post-transcriptional modification, and immunogenicity of ESAT-6 in *M. tuberculosis*. However, both, EspF and EspG₁, are required for full virulence of *M. tuberculosis* (Bottai et al. 2011). Although the function of EspG₁ is still unknown, the impact of the protein on virulence of *M. tuberculosis* might be related to specific interactions with other ESX components, such as PE/PPE proteins (Teutschbein et al. 2009). Deletion of *espG₁* from *M. tuberculosis* results indeed in lower amounts of PPE68 in cell lysates, suggesting a possible role for EspG₁ in folding or stability of PPE68 (Bottai et al. 2011), in accordance with the proposed chaperone activity of EspG-like proteins encoded by other ESX systems (Ekiert and Cox 2014; Korotkova et al. 2014).

EspI is an ESX-1-associated protein for which a putative function as negative regulator of ESX-1 activity has been postulated. Although dispensable for ESX-1-mediated secretion and full virulence of *M. tuberculosis*, EspI seems to be involved in the shutdown of ESX-1 secretion activity in case of ATP depletion (Zhang et al. 2014).

4.3 Regulation of ESX Secretion in Mycobacteria

Because of the relatively recent discovery of type VII systems, the regulation of ESX activity is a rather unexplored field of research on mycobacterial type VII secretion. As opposed to type III and type IV secretion in Gram-negative bacteria, where substrate secretion occurs after host–cell contact, ESX/type VII secretion occurs also in in vitro growing mycobacteria. This feature suggests that mycobacterial ESX secretion systems might play more general roles in the physiology of the bacteria, whereby their implication in virulence could have evolved as an adaptation to the hostile intracellular environment. Recent experimental data provide evidence that ESX-mediated secretion and/or ESX gene cluster expression is controlled by different global transcriptional regulators. These include the putative transcriptional repressors Lrs2 and CRP (Rickman et al. 2005; Gordon et al.

2010) and the activators PhoP and EspR (Frigui et al. 2008; Blasco et al. 2012), which regulate ESX-1 secretion; the iron-dependent regulator IdeR (Rodriguez et al. 2002) and the zinc uptake regulator Zur (Maciag et al. 2007), which control the expression of the ESX-3 gene cluster; the global transcriptional regulator WhiB5 and the alternative sigma factor SigM which regulate genes at ESX-2 and ESX-4 loci (Casonato et al. 2012; Raman et al. 2006; Agarwal et al. 2007).

The regulation of ESX-1-mediated secretion of ESAT-6 and CFP-10 mainly involves the *espACD* locus due to the codependent secretion of these proteins with EspA and EspC. According to the currently proposed model, PhoP/R, a major two-component regulatory system of *M. tuberculosis* (Walters et al. 2006), influences the expression of the nucleoid-associated protein/regulator EspR (Blasco et al. 2012; Raghavan et al. 2008), which controls the *espACD* operon (Blasco et al. 2012; Hunt et al. 2012). The link between a functional PhoP protein and ESAT-6 secretion was first demonstrated in *M. tuberculosis* H37Ra (Frigui et al. 2008), the paradigm attenuated *M. tuberculosis* strain, which carries a point mutation in its *phoP* gene that interferes with the DNA binding capacities of PhoP (Wang et al. 2007). Complementation of H37Ra with a wild-type copy of the *phoP* locus restored ESAT-6 secretion and partially increased the virulence of the recombinant H37Ra::*phoP* strain (Frigui et al. 2008). Moreover, a second two-component signal transduction system named MprA/B (for mycobacterial persistence regulator) was also found to modulate ESX-1 functions via regulation of the *espACD* locus (Pang et al. 2013). Interestingly, regulation of the *espACD* locus by two-component regulators may also be bypassed, as observed in a lineage of tubercle bacilli that have the region of difference 8 (RD8) deleted, leading to ESAT-6 secretion despite PhoP/R mutations (Gonzalo-Asensio et al. 2014). The RD8 deletion is located just upstream of the *espACD* operon in *Mycobacterium africanum* lineage 6 strains, as well as in animal-adapted members of the *M. tuberculosis* complex, such as *M. microti* or *M. bovis* (Boritsch et al. 2014).

However, apart from regulation of ESAT-6 secretion via EspA/C expression, regulation of ESX-1 genes may also occur directly at the ESX-1 locus by WhiB6, a member of large WhiB protein regulatory family (Solans et al. 2014). A point mutation in the putative *whiB6* promoter region present in the reference strain *M. tuberculosis* H37Rv determines the formation of a stem-loop structure in the binding region of the regulator PhoP, resulting in lower expression of ESAT-6 in *M. tuberculosis* H37Rv relative to *M. tuberculosis* clinical isolates and other reference strains (e.g. CDC1551) (Solans et al. 2014).

4.4 Type VII-like Secretion Machineries in Non-mycobacterial Species

The Ess secretion system of *S. aureus* is one of the best-characterized type VII secretion systems in *Firmicutes*. It consists of four structural components (EssA, EssB, EssC and EssD), all required for transport of Ess substrates across the

staphylococcal cell envelope and two accessory proteins (EsaA and EsaB) (Burts et al. 2005, 2008, Anderson et al. 2013, 2011, Chen et al. 2012, Kneuper et al. 2014). Among the Ess core components, EssC is a membrane-bound FtsK/SpoIIIE ATPase, which represents a hallmark for type VII secretion machineries. Moreover, the locus also encodes phylum-specific EssA, EssB and EssD, which are membrane-anchored proteins that are not conserved in the mycobacterial ESX systems (Fig. 1) (Burts et al. 2005, 2008, Anderson et al. 2011, 2013). The Ess secretion machineries also include EsaB, a small cytosolic protein, for which a role in regulation of the expression and production of a specific subset of Ess substrates (e.g. EsxC/EsaC, see below) has been demonstrated (Burts et al. 2008, Kneuper et al. 2014). Secretome analysis of a panel of *S. aureus* Ess mutant strains in two different genetic backgrounds (*S. aureus* USA300 and *S. aureus* Newman clinical isolates) identified four Ess substrates: the canonical *SaEsxA* and *SaEsxB* (Burts et al. 2005), as well as the non-canonical type VII substrates *SaEsxC* and *SaEsxD* (Anderson et al. 2011, 2013). *S. aureus* EsxA and EsxB share some features with the *M. tuberculosis* ESAT-6-CFP-10, including the presence of a WXG motif (located in the middle of a 100-aa-long protein) and the codependent secretion (genetic deletion of *SaEsxA* or *SaEsxB* impairs the secretion of the related WXG protein). However, unlike ESAT-6 and CFP-10 from *M. tuberculosis*, *SaEsxA* and *SaEsxB* from *S. aureus* do not seem to interact (Anderson et al. 2013). Instead, *SaEsxA* dimerizes with itself or associates with *SaEsxC*, while *SaEsxB* interacts with *SaEsxD* (Anderson et al. 2013). The recently resolved crystal structure of the EsxA-EsxA homodimer reveals that each EsxA subunit folds into an elongated cylinder of two helices bent by a hairpin, carrying the WXG motif (Sundaramoorthy et al. 2008), in a structure that is reminiscent of the *M. tuberculosis* ESAT-6-CFP-10 or EsxG-EsxH heterodimers secreted by ESX-1 and ESX-3 systems, respectively.

In contrast to *SaEsxA* and *SaEsxB*, staphylococcal EsxC and EsxD do not share obvious sequence features with WXG proteins, nor with other reported ESX substrates, such as PE/PPE or Esp protein family members (Anderson et al. 2013). However, *SaEsxD* contains a C-terminal YxxD/E motif (Anderson et al. 2013) identical to that involved in targeting substrates to the type VII secretion pathways in mycobacteria. It was found that *SaEsxD* can play a role in the interplay among Ess substrates and Ess core components. Deletion of the entire *SaEsxD*, as well as deletion of its C-terminal domain, abolished the production of *SaEsxB* and affects the secretion of *SaEsxA* and *SaEsxB*. These effects on Ess secretion seem to be related to a direct effect of the absence of *SaEsxD* on EssD stability (Anderson et al. 2013). This suggests a model in which *SaEsxD* might contribute to the stability of EssD via the last 6 amino acids at the C-terminal domain, thus influencing the secretion of other Ess substrates. Recently, some data on regulation of Ess activity became available: similarly to the mycobacterial ESX loci, whose expression is under the control of different global transcriptional regulators, the Ess locus is negatively regulated by the response regulator SaeR (Anderson et al. 2013). SaeR is the transcriptional regulator of the two-component regulation system SaeR/SaeS, which modulates the secretion of a plethora of virulence factors in *S. aureus*

(Giraud et al. 1997; Cheung et al. 2004; Novick 2003). Transposon insertion in SaeR or SaeS results in an increased production of both Ess core components (EssD) and Ess-dependent substrates (*SaEsxA* and *SaEsxB*) (Anderson et al. 2013). The SaeR-/SaeS-dependent Ess expression might account for the increased Ess secretion activity observed in the *S. aureus* USA300 strain relative to that observed for the Newman strain (Anderson et al. 2013), where *SaEsxA* and *SaEsxB* are produced and secreted at low levels, and *SaEsxC* is produced only after *SaEsxB* deletion (Burts et al. 2005, 2008). A point mutation in the SaeS encoding gene in the Newman strain results indeed in a constitutively active variant of the signalling kinase SaeS (Adhikari and Novick 2008).

The simplest protein composition was predicted for the Yuk/Yue type VII-like system in *B. subtilis*. In addition to the FtsK/SpoIIIE-like ATPase Yuka/B, other components of the secretion machinery are the ubiquitin-like YukC and YukD proteins, and the membrane-bound YueB/YueC proteins, homologous to the Ess components in *S. aureus* and conserved in type VII-like secretion systems of other *Firmicutes* (*Streptococcus agalactiae* and *L. monocytogenes*) (Baptista et al. 2013; Huppert et al. 2014). Interestingly, YueB is a membrane receptor essential for phage infection (Huppert et al. 2014). The WXG-100 protein member Yuke is the only type VII-like substrate identified so far (Huppert et al. 2014). Yuke accumulates in stationary growth phase culture supernatants as homodimer (Sysoeva et al. 2014), whose predicted structure consists in a putative helix-loop-helix fold, with the WXG domain lying in the loop and the two helices in an antiparallel configuration (Huppert et al. 2014). Mutagenesis studies combined with cross-linking experiments revealed that the C-terminal residues of Yuke are important for secretion, thus providing further evidence for a general mode of recognition of type VII-like and ESX substrates in *Firmicutes* and *Actinobacteria*, respectively (Sysoeva et al. 2014). However, the tryptophan and glycine residues of the WXG motif of Yuke seem also to be required for an efficient translocation of the protein homodimer towards the outside of the cell (Sysoeva et al. 2014). Based on structural data, Yuke homodimers contain two sites composed by the C terminus and the WXG turn, but only one intact bipartite site seems to be required for protein export. These features suggest that substrate secretion via the type VII-like pathway in *B. subtilis* requires a composite, bipartite signal formed by two folded Yuke polypeptides (Sysoeva et al. 2014).

Yuk/Yue expression in *B. subtilis* is regulated by the two-component system DegU–DegS (Ogura et al. 2001; Mader et al. 2002; Kobayashi 2007), which controls several post-exponential processes (genetic competence, biofilm formation and cell motility) (Murray et al. 2009; Hsueh et al. 2011; Lopez and Kolter 2010). Stable production of Yuke requires high levels of phosphorylated transcriptional regulator DegU (Baptista et al. 2013), whose levels increase in cells upon transition to the stationary growth phase. This could explain the reason why no Ess activity could be demonstrated in the *B. subtilis* strain 168, which is impaired in DegU-P-dependent processes (Baptista et al. 2013).

5 Type VII Secretion Systems in Bacterial Virulence and Pathogenicity

The biological function of several type VII-secreted substrates and effector molecules is still unknown. Likewise, it remains unclear whether type VII secretion machineries might share conserved functions. It is well established that mycobacterial ESX secretion systems (ESX-1, ESX-3 and ESX-5) are virulence determinants in *M. tuberculosis* and other pathogenic mycobacteria (ESX-1 and ESX-5), or play crucial roles in essential metabolic pathways (ESX-3) (Bottai et al. 2015; Bottai et al. 2014; Majlessi et al. 2015). Similarly, type VII-like secretion systems have a strong impact on bacterial virulence and pathogenicity in *S. aureus* (Korea et al. 2014; Burts et al. 2005) and *B. anthracis* (Garufi et al. 2008). However, some other bacterial pathogens are known, whose type VII-like secretion systems do not seem to impact on virulence, as this is, for example, the case for *L. monocytogenes* (Way and Wilson 2005). Likewise, type VII-like secretion is also not required for the virulence of the plant pathogen *Streptomyces scabies* (Fyans et al. 2013), or of *S. coelicolor*, where the type VII system plays a role in modulation of sporulation and development (Akpe San Roman et al. 2010).

5.1 The Mycobacterial ESX-1, ESX-3 and ESX-5 Secretion Systems

The ESX-1 system impacts on the ability of *M. tuberculosis* and other pathogenic mycobacteria to establish infection due to its role as modulator of the mycobacterial trafficking in phagocytic host cells (macrophages and dendritic cells). Several cellular events have been reported to be related to the activity of a functional ESX-1 system: inhibition of phagosomal maturation and acidification, cytosolic access, autophagy, host cell-death, and modulation of the inflammatory response. ESX-1-proficient mycobacterial species *M. tuberculosis*, *M. leprae* and *M. marinum*, as well as recombinant BCG::ESX-1 variants were shown to access the host cytosol of infected phagocytic cells, while strains with an interrupted ESX-1 system remained enclosed in the phagovacuole (Stamm et al. 2003; van der Wel et al. 2007; Simeone et al. 2012; Houben et al. 2012a; Simeone et al. 2015). Translocation into the cytosol plays a key role not only in cultured phagocytic cells, but was also demonstrated for a murine infection model (Simeone et al. 2015). Such ESX-1-mediated access to the cytosolic compartment seems to be related to the ability of ESAT-6 and potential, other ESX-1-secreted effector molecules to interact with biomembranes under specific conditions and induce membrane lysis (de Jonge et al. 2007; De Leon et al. 2012). The finding that recombinant ESX-1 strains expressing mutated variants of ESAT-6 are unable to induce phagosomal rupture despite secretion of the protein further supports a role for ESAT-6 in this process (Simeone et al. 2012; Houben et al. 2012a). The ability to lyse vacuolar

membranes, thereby allowing pathogenic mycobacteria and/or bacterial components to gain access to the cytosolic compartment of the host cell, might account for several features which characterize the pathogenic potential of *M. tuberculosis* and other ESX-1-proficient mycobacterial species: cell-to-cell spread (Guinn et al. 2004; Hsu et al. 2003), autophagy induction and/or impairment (Watson et al. 2012; Romagnoli et al. 2012), and access of mycobacterial proteins to the class I-processing machinery contained in the proteasome, with impact on NLRP3 inflammasome activation (Mishra et al. 2010; Wong and Jacobs 2011; Dorhoi et al. 2012), type I interferon (type I-IFN) responses (Stanley et al. 2007) and induction of increased CD8+ T-cell responses (Ryan et al. 2009). Recent studies demonstrated a role of cytosolic *M. tuberculosis* DNA in the induction of the synthesis of type I-IFN, via the induction of the cGAMP signalling cascade, both in human and murine macrophages (Wassermann et al. 2015; Watson et al. 2015; Collins et al. 2015). This DNA-dependent induction was suggested to be linked to ESX-1-mediated access of the pathogen to the host cytosol (Majlessi and Brosch 2015).

In addition to ESX-1, ESX-5 is a key virulence determinant of pathogenic mycobacteria, due to its role as specialized secretion system devoted to the export of PE and PPE proteins. Apart from EsxM (the ESAT-6 homolog encoded at the ESX-5 locus), a number of PE/PPE proteins have been identified as ESX-5 substrates, both in *M. tuberculosis* and in *M. marinum*. At first, the representative PE/PPE proteins PE25-PPE41 (Abdallah et al. 2006, 2009; Bottai et al. 2012) were found to be ESX-5 substrates, similar to LipY (Daleke et al. 2011), which represents a mycobacterial lipase that is involved in degradation of long chain triacylglycerols during late phases of infection (Deb et al. 2006). Other putative ESX-5 substrates are the members of PE-PGRS and PPE-MPTR subfamilies (Abdallah et al. 2009; Houben et al. 2012b). These phylogenetically most recent subclasses of the PE and PPE proteins seem to have emerged from ancestral PE and PPE proteins encoded at the ESX-5 locus (Gey van Pittius et al. 2006).

Although the biological function of most of the PE/PPE proteins remains unknown, some of them have been suggested to play a role in mycobacterial virulence. They are involved in mycobacterial growth in macrophages, efficiency of phagocytosis, inhibition of phagosome maturation, and virulence in the mouse infection model (Ramakrishnan et al. 2000; Brennan et al. 2001; Sampson et al. 2001; Li et al. 2005; Goldstone et al. 2009; Brodin et al. 2010; Dong et al. 2012; Iantomasi et al. 2012; Bottai et al. 2012). PE/PPE proteins have high immunogenic potential and represent a rich source of B- and T-cell epitopes (Copin et al. 2014). Their polymorphic nature, in combination with their predicted localization at the mycobacterial surface (Sampson et al. 2001; Banu et al. 2002; Cascioferro et al. 2007; Song et al. 2008; Chaturvedi et al. 2010) and the large variability in expression at different stages of the infection (Voskuil et al. 2004) suggested a role for PE/PPE proteins as a source of antigenic variation (Delogu and Brennan 2001; Bottai and Brosch 2009; Cole et al. 1998). However, it was recently reported that the T-cell epitopes were located within the conserved N-terminal parts of the PE/PPE proteins and not in the variable PGRS and MPTR sections of the proteins,

which questions the previous hypothesis (Copin et al. 2014) and suggests that the variable parts might have different, possibly structural, roles for the mycobacteria. Indeed, when ESX-5-associated PE and PPE proteins were investigated, which harbour mainly the conserved N-terminal part of PE/PPE proteins, strong, specific T-cell responses were recorded (Sayes et al. 2012). The responses were directed against the ESX-5-associated PE/PPE proteins (PPE25, PE18, PPE26, PPE27, PE19), but also against a number of non-ESX-5-encoded homologs (Sayes et al. 2012). These responses were completely abolished in mutant strains where the ESX-5 secretion system was non-functional (Sayes et al. 2012). These findings further confirm a role for ESX-5 in the release of PE and PPE proteins from the bacterial cell during infection and emphasize the impact of ESX-5 as key modulator of the adaptive antimycobacterial host immune response against PE/PPE proteins.

A functionally active ESX-5 system is essential for full virulence of pathogenic mycobacteria. In *M. tuberculosis*, ESX-5 inactivation via disruption of single components of the ESX-5 secretion machineries (e.g. the predicted transmembrane channel EccD₅) caused strong attenuation of the corresponding mutant, which was neither able to replicate in murine macrophages nor in severely combined immunodeficient (SCID) mice (Bottai et al. 2012). Furthermore, an intact ESX-5 system is required for in vitro growth of several mycobacterial species (*M. tuberculosis*, BCG and *M. marinum*) (Di Luca et al. 2012; Ates et al. 2015). Deletion of large portions of the *M. tuberculosis* ESX-5 locus (e.g. the *eccB₅-eccC₅* operon that encodes key building blocks of the ESX-5-membrane-bound protein complex) results in the loss of viability of tubercle bacilli (Di Luca et al. 2012). Moreover, a putative role for certain duplicated regions of the ESX-5 locus was recently suggested, whereby knockout mutants of the so-called ESX-5a region, comprising *esxI*, *esxJ*, *ppe15* and *pe8*, of *M. marinum* and/or *M. tuberculosis* showed differences in secretion of selected PE/PPE proteins and some other proteins that were not members of these protein families (Shah et al. 2015). The impact of ESX-5 on *M. tuberculosis* viability and virulence seems to be related to its role in transport of PE/PPE proteins as well as its involvement in maintaining the cell envelope stability and functionality. ESX-5 disruption results in extensive damage of the mycobacterial cell envelope, as revealed by increased sensitivity of ESX-5 mutants to detergents and hydrophilic antibiotics to which mycobacteria are naturally resistant (Bottai et al. 2012). The detrimental effect of ESX-5 disruption on cell envelope functionality and mycobacterial viability can be restored by increasing the permeability of the mycomembrane, by altering its lipid composition or by introducing the heterologous outer-membrane-associated mycobacterial porin MspA (Ates et al. 2015). The existence of a functional link between ESX-mediated secretion and cell-wall biogenesis is supported by results of ChIP-on-chip analyses, which demonstrated that genes at the ESX-2 and ESX-5 loci, as well as a number of genes encoding enzymes involved in cell-wall biosynthesis were regulated by EspR (Blasco et al. 2012).

Some data are now available on the functional role of ESX-3. The finding that the expression of the ESX-3 locus is regulated by the iron-dependent transcriptional repressor IdeR (Rodriguez et al. 2002) and the zinc uptake repressor Zur (Maciag

et al. 2007) and is up-regulated in iron/zinc limiting conditions provides evidence for the implication of this secretion system in homeostasis of ferric and zinc metal ions (Siegrist et al. 2009, 2014, Serafini et al. 2009). Consistent with its involvement in fundamental metabolic pathways, ESX-3 is highly conserved in the genome of all mycobacterial species. In *M. tuberculosis*, where the uptake of ferric ions exclusively occurs via the mycobactin-mediated siderophore pathway, ESX-3 is essential for viability (Serafini et al. 2009). In contrast, in *M. smegmatis*, where the iron uptake also occurs by the alternate exochelin pathway, ESX-3 is not essential for in vitro growth (Siegrist et al. 2014). Selective inactivation of different *M. smegmatis* ESX-3 genes (*eccC₃*, *eccD₃* and *espG₃*) confirmed the implication of the ESX-3 secretion system in mycobactin-mediated iron acquisition. EsxG and EsxH form a characteristic heterodimer resembling the ESAT-6-CFP-10 complex (Ilghari et al. 2011), whose secretion is enhanced in iron-limiting conditions (Siegrist et al. 2014). NMR spectroscopy of the *M. tuberculosis* EsxG–EsxH complex revealed the presence of a specific Zn²⁺ binding site in EsxH (Ilghari et al. 2011). The Zn²⁺ binding site is specific for EsxH proteins of mycobacterial species belonging to the *M. tuberculosis* complex, and it is not conserved in EsxH paralogs from other mycobacterial species (*M. smegmatis*, *M. marinum* and *M. ulcerans*), nor in other Esx proteins. Although the functional role of this site is still unknown, the Zn²⁺ binding site might regulate the stability of the EsxG–EsxH complex, its interactions with other protein partners, or might be implicated in zinc uptake.

Apart from the functional role in iron uptake, a role of ESX-3 in virulence of mycobacteria has been recently reported. The EsxG–EsxH complex was found as being involved in inhibition of phagosome maturation. This process involves the disruption of the host endosomal sorting complex required for transport (ESCRT) responsible to the delivery of *M. tuberculosis*-loaded phagosomes to the lysosomes (Mehra et al. 2013).

5.2 *Ess* Secretion System in Virulence of *S. Aureus*

Several observations indicate that *Ess* plays an important role in virulence of *S. aureus*. *SaEsxA* and *SaEsxB* are required for *S. aureus* replication in organs and tissues of infected mice (Burts et al. 2005). Moreover, *Ess* secretion activity is also required for the establishment of staphylococcal abscesses wherein the pathogen can persist and evade the host immune response (Burts et al. 2005, 2008; Anderson et al. 2011). Inactivation of the *Ess* pathway by the disruption of key components of the *Ess* secretion machinery (*EssC*) as well as the deletion of *esxA* and *esxB* causes a significant reduction in the ability of *S. aureus* to establish kidney or liver abscesses in a murine model of staphylococcal blood-borne dissemination and abscess formation (Burts et al. 2005, 2008; Anderson et al. 2011). Other *Ess* substrates, such as the staphylococcal-specific *EsaC* protein, although being dispensable for the establishment of acute infections, were required for the formation of persistent infection in animal models (Burts et al. 2008). Moreover, the *Ess*

secretion system has recently been demonstrated to be required for nasal colonization and virulence in a murine lung pneumonia model, using two different *S. aureus* strains (RN6390 and COL) for infection (Kneuper et al. 2014). More insights on the cellular mechanisms responsible for the impact of EsxA in virulence of *S. aureus* have recently been provided (Korea et al. 2014). Although *S. aureus* is primarily an extracellular pathogen, whose presence in intracellular environment during in vivo infections remains unclear, *S. aureus* was recently reported to be able to invade and replicate in several non-phagocytic cells (Garzoni and Kelley 2009; Clement et al. 2005; Sachse et al. 2010). It has also been proposed that a transient intracellular lifestyle might potentially provide protection against exposure to antibiotics and the host immune response and might represent a favourable environment for the formation of resistant variants (Tuchscherr et al. 2011; Fraunholz and Sinha 2012). SaEsxA is able to interfere with the host cell apoptotic pathways in human epithelial cells, thus affecting bacterial survival and mediating the release of *S. aureus* from the host cells (Korea et al. 2014).

5.3 The ESAT-like Secretion System in Virulence of *Bacillus Anthracis*

B. anthracis encodes six WXG proteins, referred to as EsxB, EsxL, EsxP, EsxQ, EsxV and EsxW. EsxB and EsxW are secreted into the extracellular environment during growth in liquid medium, although the secretion process seems to not require the BaEssC ATPase (Garufi et al. 2008). In contrast, no expression was found for other Esx-like proteins under laboratory growth conditions (Garufi et al. 2008). To date, the impact of ESX-like secreted substrates in virulence of *B. anthracis* is still unknown. However, the finding that specific humoral immune responses against EsxB-, EsxP- and EsxW-like proteins are detectable in *B. anthracis*-infected guinea pigs suggests that the type VII-like secretion pathway is activated during *B. anthracis* in vivo infections.

6 Concluding Comments

Taken together, the different aspects of type VII- and type VII-like secretion in Gram-positive bacteria, reviewed in this book chapter, emphasize the actuality of this topic for bacterial physiology and host-pathogen interaction. Despite considerable advances in understanding the mechanisms and functions of the different type VII- and type VII-like systems in high- and low-C + G-content Gram-positive bacteria, namely *Actinobacteria* and *Firmicutes*, many questions remain to be answered (Schneewind and Missiakas 2012). These challenges for future work have relevance for pathogenicity research (Le Chevalier et al. 2014; Majlessi and Brosch 2015),

novel treatment strategies (Rybniker et al. 2014; Christophe et al. 2009), or vaccine design (Bottai et al. 2015; Pym et al. 2003) and shall thus receive the continued attention of a broad spectrum of fundamental and applied research.

Acknowledgements Support by the European Community (Grant H2020-PHC-643381) and the Fondation pour la Recherche Médicale FRM (DEQ 20130326471) is gratefully acknowledged. M.I.G. is supported by an MD–PhD grant of the University of Groningen, The Netherlands.

References

- Abdallah AM, Verboom T, Hannes F, Safi M, Strong M, Eisenberg D, Musters RJ, Vandenbroucke-Grauls CM, Appelmelk BJ, Luirink J, Bitter W (2006) A specific secretion system mediates PPE41 transport in pathogenic mycobacteria. *Mol Microbiol* 62:667–679
- Abdallah AM, Gey van Pittius NC, Champion PA, Cox J, Luirink J, Vandenbroucke-Grauls CM, Appelmelk BJ, Bitter W (2007) Type VII secretion system of mycobacteria show the way. *Nat Rev Microbiol* 5:883–891
- Abdallah AM, Verboom T, Weerdenburg EM, Gey van Pittius NC, Mahasha PW, Jimenez C, Parra M, Cadieux N, Brennan MJ, Appelmelk BJ, Bitter W (2009) PPE and PE_PGRS proteins of *Mycobacterium marinum* are transported via the type VII secretion system ESX-5. *Mol Microbiol* 73:329–340
- Adhikari RP, Novick RP (2008) Regulatory organization of the staphylococcal *sae* locus. *Microbiology* 154:949–959
- Agarwal N, Woolwine SC, Tyagi S, Bishai WR (2007) Characterization of the *Mycobacterium tuberculosis* sigma factor SigM by assessment of virulence and identification of SigM-dependent genes. *Infect Immun* 75:452–461
- Akpe San Roman S, Facey PD, Fernandez-Martinez L, Rodriguez C, Vallin C, Del Sol R, Dyson P (2010) A heterodimer of EsxA and EsxB is involved in sporulation and is secreted by a type VII secretion system in *Streptomyces coelicolor*. *Microbiology* 156:1719–1729
- Anderson M, Chen YH, Butler EK, Missiakas DM (2011) EsaD, a secretion factor for the Ess pathway in *Staphylococcus aureus*. *J Bacteriol* 193:1583–1589
- Anderson M, Aly KA, Chen YH, Missiakas D (2013) Secretion of atypical protein substrates by the ESAT-6 secretion system of *Staphylococcus aureus*. *Mol Microbiol* 90:734–743
- Ates LS, Ummels R, Commandeur S, van der Weerd R, Sparrius M, Weerdenburg E, Alber M, Kalscheuer R, Piersma SR, Abdallah AM, Abd El Ghany M, Abdel-Haleem AM, Pain A, Jimenez CR, Bitter W, Houben EN (2015) Essential role of the ESX-5 secretion system in outer membrane permeability of pathogenic mycobacteria. *PLoS Genet* 11:e1005190
- Banu S, Honore N, Saint-Joanis B, Philpott D, Prevost MC, Cole ST (2002) Are the PE-PGRS proteins of *Mycobacterium tuberculosis* variable surface antigens? *Mol Microbiol* 44:9–19
- Baptista C, Barreto HC, Sao-Jose C (2013) High levels of DegU-P activate an Esat-6-like secretion system in *Bacillus subtilis*. *PLoS ONE* 8:e67840
- Bitter W, Houben EN, Bottai D, Brodin P, Brown EJ, Cox JS, Derbyshire K, Fortune SM, Gao LY, Liu J, Gey van Pittius NC, Pym AS, Rubin EJ, Sherman DR, Cole ST, Brosch R (2009a) Systematic genetic nomenclature for type VII secretion systems. *PLoS Pathog* 5: e1000507
- Bitter W, Houben EN, Luirink J, Appelmelk BJ (2009b) Type VII secretion in mycobacteria: classification in line with cell envelope structure. *Trends Microbiol* 17:337–338
- Blasco B, Chen JM, Hartkoorn R, Sala C, Uplekar S, Rougemont J, Pojer F, Cole ST (2012) Virulence regulator EspR of *Mycobacterium tuberculosis* is a nucleoid-associated protein. *PLoS Pathog* 8:e1002621

- Boritsch EC, Supply P, Honore N, Seemann T, Stinear TP, Brosch R (2014) A glimpse into the past and predictions for the future: the molecular evolution of the tuberculosis agent. *Mol Microbiol* 93:835–852
- Bottai D, Brosch R (2009) Mycobacterial PE, PPE and ESX clusters: novel insights into the secretion of these most unusual protein families. *Mol Microbiol* 73:325–328
- Bottai D, Majlessi L, Simeone R, Frigui W, Laurent C, Lenormand P, Chen J, Rosenkrands I, Huerre M, Leclerc C, Cole ST, Brosch R (2011) ESAT-6 secretion-independent impact of ESX-1 genes *espF* and *espG1* on virulence of *Mycobacterium tuberculosis*. *J Infect Dis* 203:1155–1164
- Bottai D, Di Luca M, Majlessi L, Frigui W, Simeone R, Sayes F, Bitter W, Brennan MJ, Leclerc C, Batoni G, Campa M, Brosch R, Esin S (2012) Disruption of the ESX-5 system of *Mycobacterium tuberculosis* causes loss of PPE protein secretion, reduction of cell wall integrity and strong attenuation. *Mol Microbiol* 83:1195–1209
- Bottai D, Stinear TP, Supply P, Brosch R (2014) Mycobacterial Pathogenomics and Evolution. *Microbiol Spectr* 2:MGM2-0025-2013
- Bottai D, Frigui W, Clark S, Rayner E, Zelmer A, Andreu N, de Jonge MI, Bancroft GJ, Williams A, Brodin P, Brosch R (2015) Increased protective efficacy of recombinant BCG strains expressing virulence-neutral proteins of the ESX-1 secretion system. *Vaccine* 33:2710–2718
- Brennan MJ, Delogu G, Chen Y, Bardarov S, Kriakov J, Alavi M, Jacobs WR Jr (2001) Evidence that mycobacterial PE_PGRS proteins are cell surface constituents that influence interactions with other cells. *Infect Immun* 69:7326–7333
- Brodin P, Eiglmeier K, Marmiesse M, Billault A, Garnier T, Niemann S, Cole ST, Brosch R (2002) Bacterial artificial chromosome-based comparative genomic analysis identifies *Mycobacterium microti* as a natural ESAT-6 deletion mutant. *Infect Immun* 70:5568–5578
- Brodin P, Majlessi L, Brosch R, Smith D, Bancroft G, Clark S, Williams A, Leclerc C, Cole ST (2004a) Enhanced protection against tuberculosis by vaccination with recombinant *Mycobacterium microti* vaccine that induces T cell immunity against region of difference 1 antigens. *J Infect Dis* 190:115–122
- Brodin P, Rosenkrands I, Andersen P, Cole ST, Brosch R (2004b) ESAT-6 proteins: protective antigens and virulence factors? *Trends Microbiol* 12:500–508
- Brodin P, Majlessi L, Marsollier L, de Jonge MI, Bottai D, Demangel C, Hinds J, Neyrolles O, Butcher PD, Leclerc C, Cole ST, Brosch R (2006) Dissection of ESAT-6 system 1 of *Mycobacterium tuberculosis* and impact on immunogenicity and virulence. *Infect Immun* 74:88–98
- Brodin P, Poquet Y, Levillain F, Peguillet I, Larrouy-Maumus G, Gilleron M, Ewann F, Christophe T, Fenistein D, Jang J, Jang MS, Park SJ, Rauzier J, Carralot JP, Shrimpton R, Genovesio A, Gonzalo-Asensio JA, Puzo G, Martin C, Brosch R, Stewart GR, Gicquel B, Neyrolles O (2010) High content phenotypic cell-based visual screen identifies *Mycobacterium tuberculosis* acyltrehalose-containing glycolipids involved in phagosome remodeling. *PLoS Pathog* 6:e1001100
- Brown GD, Dave JA, Gey van Pittius NC, Stevens L, Ehlers MR, Beyers AD (2000) The mycosins of *Mycobacterium tuberculosis* H37Rv: a family of subtilisin-like serine proteases. *Gene* 254:147–155
- Burts ML, Williams WA, Debord K, Missiakas DM (2005) 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 U S A* 102:1169–1174
- Burts ML, DeDent AC, Missiakas DM (2008) EsaC substrate for the ESAT-6 secretion pathway and its role in persistent infections of *Staphylococcus aureus*. *Mol Microbiol* 69:736–746
- Carlsson F, Joshi SA, Rangell L, Brown EJ (2009) Polar localization of virulence-related Esx-1 secretion in mycobacteria. *PLoS Pathog* 5:e1000285
- Cascioferro A, Delogu G, Colone M, Sali M, Stringaro A, Arancia G, Fadda G, Palu G, Manganelli R (2007) PE is a functional domain responsible for protein translocation and localization on mycobacterial cell wall. *Mol Microbiol* 66:1536–1547

- Casonato S, Cervantes Sanchez A, Haruki H, Rengifo Gonzalez M, Provvedi R, Dainese E, Jaouen T, Gola S, Bini E, Vicente M, Johnsson K, Ghisotti D, Palu G, Hernandez-Pando R, Manganeli R (2012) WhiB5, a transcriptional regulator that contributes to *Mycobacterium tuberculosis* virulence and reactivation. *Infect Immun* 80:3132–3144
- Champion PA, Stanley SA, Champion MM, Brown EJ, Cox JS (2006) C-terminal signal sequence promotes virulence factor secretion in *Mycobacterium tuberculosis*. *Science* 313:1632–1636
- Champion PA, Champion MM, Manzanillo P, Cox JS (2009) ESX-1 secreted virulence factors are recognized by multiple cytosolic AAA ATPases in pathogenic mycobacteria. *Mol Microbiol* 73:950–962
- Chaturvedi R, Bansal K, Narayana Y, Kapoor N, Sukumar N, Togarsimalemath SK, Chandra N, Mishra S, Ajitkumar P, Joshi B, Katoch VM, Patil SA, Balaji KN (2010) The multifunctional PE_PGRS11 protein from *Mycobacterium tuberculosis* plays a role in regulating resistance to oxidative stress. *J Biol Chem* 285:30389–30403
- Chen YH, Anderson M, Hendrickx AP, Missiakas D (2012) Characterization of EssB, a protein required for secretion of ESAT-6 like proteins in *Staphylococcus aureus*. *BMC Microbiol* 12:219
- Chen JM, Zhang M, Rybniker J, Boy-Rottger S, Dhar N, Pojer F, Cole ST (2013) *Mycobacterium tuberculosis* EspB binds phospholipids and mediates EsxA-independent virulence. *Mol Microbiol* 89:1154–1166
- Cheung AL, Bayer AS, Zhang G, Gresham H, Xiong YQ (2004) Regulation of virulence determinants in vitro and in vivo in *Staphylococcus aureus*. *FEMS Immunol Med Microbiol* 40:1–9
- Christie PJ (2001) Type IV secretion: intercellular transfer of macromolecules by systems ancestrally related to conjugation machines. *Mol Microbiol* 40:294–305
- Christophe T, Jackson M, Jeon HK, Fenistein D, Contreras-Dominguez M, Kim J, Genovesio A, Carralot JP, Ewann F, Kim EH, Lee SY, Kang S, Seo MJ, Park EJ, Skovierova H, Pham H, Riccardi G, Nam JY, Marsollier L, Kempf M, Joly-Guillou ML, Oh T, Shin WK, No Z, Nehrbass U, Brosch R, Cole ST, Brodin P (2009) High content screening identifies decaprenyl-phosphoribose 2' epimerase as a target for intracellular antimycobacterial inhibitors. *PLoS Pathog* 5:e1000645
- Clement S, Vaudaux P, Francois P, Schrenzel J, Huggler E, Kampf S, Chaponnier C, Lew D, Lacroix JS (2005) Evidence of an intracellular reservoir in the nasal mucosa of patients with recurrent *Staphylococcus aureus* rhinosinusitis. *J Infect Dis* 192:1023–1028
- Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry CE 3rd, Tekaia F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Krogh A, McLean J, Moule S, Murphy L, Oliver K, Osborne J, Quail MA, Rajandream MA, Rogers J, Rutter S, Seeger K, Skelton J, Squares R, Squares S, Sulston JE, Taylor K, Whitehead S, Barrell BG (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 393:537–544
- Collins AC, Cai H, Li T, Franco LH, Li X-D, Nair VR, Scharn CR, Stamm CE, Levine B, Chen ZJ, Shiloh MU (2015) Cyclic GMP-AMP Synthase (cGAS) is an innate immune DNA sensor for *Mycobacterium tuberculosis*. *Cell Host Microbe* 17:820–828
- Converse SE, Cox JS (2005) A protein secretion pathway critical for *Mycobacterium tuberculosis* virulence is conserved and functional in *Mycobacterium smegmatis*. *J Bacteriol* 187:1238–1245
- Copin R, Coscolla M, Seiffert SN, Bothamley G, Sutherland J, Mbayo G, Gagneux S, Ernst JD (2014) Sequence diversity in the pe_pgrs genes of *Mycobacterium tuberculosis* is independent of human T cell recognition. *MBio* 5:e00960–13
- Coros A, Callahan B, Battaglioli E, Derbyshire KM (2008) The specialized secretory apparatus ESX-1 is essential for DNA transfer in *Mycobacterium smegmatis*. *Mol Microbiol* 69:794–808
- Costa TR, Felisberto-Rodrigues C, Meir A, Prevost MS, Redzej A, Trokter M, Waksman G (2015) Secretion systems in Gram-negative bacteria: structural and mechanistic insights. *Nat Rev Microbiol* 13:343–359

- Daleke MH, Cascioferro A, de Punder K, Ummels R, Abdallah AM, van der Wel N, Peters PJ, Luirink J, Manganelli R, Bitter W (2011) Conserved Pro-Glu (PE) and Pro-Pro-Glu (PPE) protein domains target LipY lipases of pathogenic mycobacteria to the cell surface via the ESX-5 pathway. *J Biol Chem* 286:19024–19034
- Daleke MH, Ummels R, Bawono P, Heringa J, Vandenbroucke-Grauls CM, Luirink J, Bitter W (2012) General secretion signal for the mycobacterial type VII secretion pathway. *Proc Natl Acad Sci U S A* 109:11342–11347
- Dave JA, Gey van Pittius NC, Beyers AD, Ehlers MR, Brown GD (2002) Mycosin-I, a subtilisin-like serine protease of *Mycobacterium tuberculosis*, is cell wall-associated and expressed during infection of macrophages. *BMC Microbiol* 2:30
- de Jonge MI, Pehau-Arnaudet G, Fretz MM, Romain F, Bottai D, Brodin P, Honore N, Marchal G, Jiskoot W, England P, Cole ST, Brosch R (2007) 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
- De Leon J, Jiang G, Ma Y, Rubin E, Fortune S, Sun J (2012) *Mycobacterium tuberculosis* ESAT-6 exhibits a unique membrane-interacting activity that is not found in its ortholog from non-pathogenic *Mycobacterium smegmatis*. *J Biol Chem* 287:44184–44191
- Deb C, Daniel J, Sirakova TD, Abomoelak B, Dubey VS, Kolattukudy PE (2006) A novel lipase belonging to the hormone-sensitive lipase family induced under starvation to utilize stored triacylglycerol in *Mycobacterium tuberculosis*. *J Biol Chem* 281:3866–3875
- Delogu G, Brennan MJ (2001) Comparative immune response to PE and PE_PGRS antigens of *Mycobacterium tuberculosis*. *Infect Immun* 69:5606–5611
- Di Luca M, Bottai D, Batoni G, Orgeur M, Aulicino A, Counoupas C, Campa M, Brosch R, Esin S (2012) The ESX-5 associated *eccB-eccC* locus is essential for *Mycobacterium tuberculosis* viability. *PLoS ONE* 7:e52059
- Dong D, Wang D, Li M, Wang H, Yu J, Wang C, Liu J, Gao Q (2012) PPE38 modulates the innate immune response and is required for *Mycobacterium marinum* virulence. *Infect Immun* 80:43–54
- Dorhoi A, Nouailles G, Jorg S, Hagens K, Heinemann E, Pradl L, Oberbeck-Muller D, Duque-Correa MA, Reece ST, Ruland J, Brosch R, Tschopp J, Gross O, Kaufmann SHE (2012) Activation of the NLRP3 inflammasome by *Mycobacterium tuberculosis* is uncoupled from susceptibility to active tuberculosis. *Eur J Immunol* 42:374–384
- Ekiert DC, Cox JS (2014) Structure of a PE-PPE-EspG complex from *Mycobacterium tuberculosis* reveals molecular specificity of ESX protein secretion. *Proc Natl Acad Sci U S A* 111:14758–14763
- Flint JL, Kowalski JC, Karnati PK, Derbyshire KM (2004) The RD1 virulence locus of *Mycobacterium tuberculosis* regulates DNA transfer in *Mycobacterium smegmatis*. *Proc Natl Acad Sci U S A* 101:12598–12603
- Fortune SM, Jaeger A, Sarracino DA, Chase MR, Sasseti CM, Sherman DR, Bloom BR, Rubin EJ (2005) Mutually dependent secretion of proteins required for mycobacterial virulence. *Proc Natl Acad Sci U S A* 102:10676–10681
- Fraunholz M, Sinha B (2012) Intracellular *Staphylococcus aureus*: live-in and let die. *Front Cell Infect Microbiol* 2:43
- Frigui W, Bottai D, Majlessi L, Monot M, Josselin E, Brodin P, Garnier T, Gicquel B, Martin C, Leclerc C, Cole S, Brosch R (2008) Control of *M. tuberculosis* ESAT-6 secretion and specific T cell recognition by PhoP. *PLoS Pathog* 4:e33
- Fyans JK, Bignell D, Loria R, Toth I, Palmer T (2013) The ESX/type VII secretion system modulates development, but not virulence, of the plant pathogen *Streptomyces scabies*. *Mol Plant Pathol* 14:119–130
- Gao LY, Guo S, McLaughlin B, Morisaki H, Engel JN, Brown EJ (2004) A mycobacterial virulence gene cluster extending RD1 is required for cytolysis, bacterial spreading and ESAT-6 secretion. *Mol Microbiol* 53:1677–1693

- Gardy JL, Laird MR, Chen F, Rey S, Walsh CJ, Ester M, Brinkman FS (2005) PSORTb v. 2.0: expanded prediction of bacterial protein subcellular localization and insights gained from comparative proteome analysis. *Bioinformatics* 21:617–623
- Garufi G, Butler E, Missiakas D (2008) ESAT-6-like protein secretion in *Bacillus anthracis*. *J Bacteriol* 190:7004–7011
- Garzoni C, Kelley WL (2009) *Staphylococcus aureus*: new evidence for intracellular persistence. *Trends Microbiol* 17:59–65
- Gey Van Pittius NC, Gamiieldien J, Hide W, Brown GD, Siezen RJ, Beyers AD (2001) The ESAT-6 gene cluster of *Mycobacterium tuberculosis* and other high G + C Gram-positive bacteria. *Genome Biol* 2:RESEARCH0044
- Gey van Pittius NC, Sampson SL, Lee H, Kim Y, van Helden PD, Warren RM (2006) 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
- Giraudou AT, Cheung AL, Nagel R (1997) The *sae* locus of *Staphylococcus aureus* controls exoprotein synthesis at the transcriptional level. *Arch Microbiol* 168:53–58
- Goldstone RM, Goonesekera SD, Bloom BR, Sampson SL (2009) The transcriptional regulator Rv0485 modulates the expression of a *pe* and *ppe* gene pair and is required for *Mycobacterium tuberculosis* virulence. *Infect Immun* 77:4654–4667
- Gonzalo-Asensio J, Malaga W, Pawlik A, Astarie-Dequeker C, Passemar C, Moreau F, Laval F, Daffe M, Martin C, Brosch R, Guilhot C (2014) Evolutionary history of tuberculosis shaped by conserved mutations in the PhoPR virulence regulator. *Proc Natl Acad Sci U S A* 111:11491–11496
- Gordon BR, Li Y, Wang L, Sintsova A, van Bakel H, Tian S, Navarre WW, Xia B, Liu J (2010) Lsr2 is a nucleoid-associated protein that targets AT-rich sequences and virulence genes in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A* 107:5154–5159
- Gray TA, Krywy JA, Harold J, Palumbo MJ, Derbyshire KM (2013) Distributive conjugal transfer in mycobacteria generates progeny with meiotic-like genome-wide mosaicism, allowing mapping of a mating identity locus. *PLoS Biol* 11:e1001602
- Guinn KM, Hickey MJ, Mathur SK, Zakei KL, Grotzke JE, Lewinsohn DM, Smith S, Sherman DR (2004) Individual RD1-region genes are required for export of ESAT-6/CFP-10 and for virulence of *Mycobacterium tuberculosis*. *Mol Microbiol* 51:359–370
- Hoffmann C, Leis A, Niederweis M, Plietzko JM, Engelhardt H (2008) Disclosure of the mycobacterial outer membrane: cryo-electron tomography and vitreous sections reveal the lipid bilayer structure. *Proc Natl Acad Sci U S A* 105:3963–3967
- Houben D, Demangel C, van Ingen J, Perez J, Baldeon L, Abdallah AM, Caleechurn L, Bottai D, van Zon M, de Punder K, van der Laan T, Kant A, Bossers-de Vries R, Willemsen P, Bitter W, van Soolingen D, Brosch R, van der Wel N, Peters PJ (2012a) ESX-1-mediated translocation to the cytosol controls virulence of mycobacteria. *Cell Microbiol* 14:1287–1298
- Houben EN, Bestebroer J, Ummels R, Wilson L, Piersma SR, Jimenez CR, Ottenhoff TH, Luirink J, Bitter W (2012b) Composition of the type VII secretion system membrane complex. *Mol Microbiol* 86:472–484
- Houben EN, Korotkov KV, Bitter W (2014) Take five - Type VII secretion systems of Mycobacteria. *Biochim Biophys Acta* 1844:1707–1716
- Hsu T, Hingley-Wilson SM, Chen B, Chen M, Dai AZ, Morin PM, Marks CB, Padiyar J, Goulding C, Gingery M, Eisenberg D, Russell RG, Derrick SC, Collins FM, Morris SL, King CH, Jacobs WR Jr (2003) 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 U S A* 100:12420–12425
- Hsueh YH, Cozy LM, Sham LT, Calvo RA, Gutu AD, Winkler ME, Kearns DB (2011) DegU-phosphate activates expression of the anti-sigma factor FlgM in *Bacillus subtilis*. *Mol Microbiol* 81:1092–1108
- Hunt DM, Sweeney NP, Mori L, Whalan RH, Comas I, Norman L, Cortes T, Arnvig KB, Davis EO, Stapleton MR, Green J, Buxton RS (2012) Long-range transcriptional control of an

- operon necessary for virulence-critical ESX-1 secretion in *Mycobacterium tuberculosis*. J Bacteriol 194:2307–2320
- Huppert LA, Ramsdell TL, Chase MR, Sarracino DA, Fortune SM, Burton BM (2014) The ESX system in *Bacillus subtilis* mediates protein secretion. PLoS ONE 9:e96267
- Iantomasi R, Sali M, Cascioferro A, Palucci I, Zumbo A, Soldini S, Rocca S, Greco E, Maulucci G, De Spirito M, Fraziano M, Fadda G, Manganelli R, Delogu G (2012) PE_PGRS30 is required for the full virulence of *Mycobacterium tuberculosis*. Cell Microbiol 14:356–367
- Ilghari D, Lightbody KL, Veverka V, Waters LC, Muskett FW, Renshaw PS, Carr MD (2011) Solution structure of the *Mycobacterium tuberculosis* EsxG.EsxH complex: functional implications and comparisons with other *M. tuberculosis* Esx family complexes. J Biol Chem 286:29993–30002
- Iyer LM, Makarova KS, Koonin EV, Aravind L (2004) Comparative genomics of the FtsK-HerA superfamily of pumping ATPases: implications for the origins of chromosome segregation, cell division and viral capsid packaging. Nucleic Acids Res 32:5260–5279
- Kneuper H, Cao ZP, Twomey KB, Zoltner M, Jager F, Cargill JS, Chalmers J, van der Kooi-Pol MM, van Dijl JM, Ryan RP, Hunter WN, Palmer T (2014) Heterogeneity in *ess* transcriptional organization and variable contribution of the *Ess*/Type VII protein secretion system to virulence across closely related *Staphylococcus aureus* strains. Mol Microbiol 93:928–943
- Kobayashi K (2007) Gradual activation of the response regulator DegU controls serial expression of genes for flagellum formation and biofilm formation in *Bacillus subtilis*. Mol Microbiol 66:395–409
- Korea CG, Balsamo G, Pezzicoli A, Merakou C, Tavarini S, Bagnoli F, Serruto D, Unnikrishnan M (2014) Staphylococcal Esx proteins modulate apoptosis and release of intracellular *Staphylococcus aureus* during infection in epithelial cells. Infect Immun 82:4144–4153
- Korotkova N, Freire D, Phan TH, Ummels R, Creekmore CC, Evans TJ, Wilmanns M, Bitter W, Parret AH, Houben EN, Korotkov KV (2014) Structure of the *Mycobacterium tuberculosis* type VII secretion system chaperone EspG5 in complex with PE25-PPE41 dimer. Mol Microbiol 94:367–382
- Korotkova N, Piton J, Wagner JM, Boy-Rottger S, Japaridze A, Evans TJ, Cole ST, Pojer F, Korotkov KV (2015) Structure of EspB, a secreted substrate of the ESX-1 secretion system of *Mycobacterium tuberculosis*. J Struct Biol 191:236–244
- Le Chevalier F, Cascioferro A, Majlessi L, Herrmann JL, Brosch R (2014) *Mycobacterium tuberculosis* evolutionary pathogenesis and its putative impact on drug development. Future Microbiol 9:969–985
- Lewis KN, Liao R, Guinn KM, Hickey MJ, Smith S, Behr MA, Sherman DR (2003) Deletion of RD1 from *Mycobacterium tuberculosis* mimics bacille Calmette-Guerin attenuation. J Infect Dis 187:117–123
- Li Y, Miltner E, Wu M, Petrofsky M, Bermudez LE (2005) 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
- Lopez D, Kolter R (2010) Extracellular signals that define distinct and coexisting cell fates in *Bacillus subtilis*. FEMS Microbiol Rev 34:134–149
- MacGurn JA, Raghavan S, Stanley SA, Cox JS (2005) A non-RD1 gene cluster is required for Snm secretion in *Mycobacterium tuberculosis*. Mol Microbiol 57:1653–1663
- Maciag A, Dainese E, Rodriguez GM, Milano A, Provvedi R, Pasca MR, Smith I, Palu G, Riccardi G, Manganelli R (2007) Global analysis of the *Mycobacterium tuberculosis* Zur (FurB) regulon. J Bacteriol 189:730–740
- Mader U, Antelmann H, Buder T, Dahl MK, Hecker M, Homuth G (2002) *Bacillus subtilis* functional genomics: genome-wide analysis of the DegS-DegU regulon by transcriptomics and proteomics. Mol Genet Genomics 268:455–467

- Mahairas GG, Sabo PJ, Hickey MJ, Singh DC, Stover CK (1996) Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. *J Bacteriol* 178:1274–1282
- Majlessi L, Brosch R (2015) *Mycobacterium tuberculosis* meets the cytosol: The role of cGAS in anti-mycobacterial immunity. *Cell Host Microbe* 17:733–735
- Majlessi L, Prados-Rosales R, Casadevall A, Brosch R (2015) Release of mycobacterial antigens. *Immunol Rev* 264:25–45
- McLaughlin B, Chon JS, MacGurn JA, Carlsson F, Cheng TL, Cox JS, Brown EJ (2007) A mycobacterium ESX-1-secreted virulence factor with unique requirements for export. *PLoS Pathog* 3:e105
- Mehra A, Zahra A, Thompson V, Sirisaengtaksin N, Wells A, Porto M, Koster S, Penberthy K, Kubota Y, Dricot A, Rogan D, Vidal M, Hill DE, Bean AJ, Philips JA (2013) *Mycobacterium tuberculosis* type VII secreted effector EsxH targets host ESCRT to impair trafficking. *PLoS Pathog* 9:e1003734
- Mishra BB, Moura-Alves P, Sonawane A, Hacoen N, Griffiths G, Moita LF, Anes E (2010) *Mycobacterium tuberculosis* protein ESAT-6 is a potent activator of the NLRP3/ASC inflammasome. *Cell Microbiol* 12:1046–1063
- Murray EJ, Kiley TB, Stanley-Wall NR (2009) A pivotal role for the response regulator DegU in controlling multicellular behaviour. *Microbiology* 155:1–8
- Novick RP (2003) Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol Microbiol* 48:1429–1449
- Ogura M, Yamaguchi H, Yoshida K, Fujita Y, Tanaka T (2001) DNA microarray analysis of *Bacillus subtilis* DegU, ComA and PhoP regulons: an approach to comprehensive analysis of *B. subtilis* two-component regulatory systems. *Nucleic Acids Res* 29:3804–3813
- Ohol YM, Goetz DH, Chan K, Shiloh MU, Craik CS, Cox JS (2010) *Mycobacterium tuberculosis* MycP1 protease plays a dual role in regulation of ESX-1 secretion and virulence. *Cell Host Microbe* 7:210–220
- Pallen MJ (2002) The ESAT-6/WXG100 superfamily—and a new Gram-positive secretion system? *Trends Microbiol* 10:209–212
- Pang X, Samten B, Cao G, Wang X, Tvinnereim AR, Chen XL, Howard ST (2013) MprAB regulates the espA operon in *Mycobacterium tuberculosis* and modulates ESX-1 function and host cytokine response. *J Bacteriol* 195:66–75
- Poulsen C, Panjikar S, Holton SJ, Wilmanns M, Song YH (2014) WXG100 protein superfamily consists of three subfamilies and exhibits an alpha-helical C-terminal conserved residue pattern. *PLoS ONE* 9:e89313
- Pym AS, Brodin P, Brosch R, Huerre M, Cole ST (2002) Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines *Mycobacterium bovis* BCG and *Mycobacterium microti*. *Mol Microbiol* 46:709–717
- Pym AS, Brodin P, Majlessi L, Brosch R, Demangel C, Williams A, Griffiths KE, Marchal G, Leclerc C, Cole ST (2003) Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. *Nat Med* 9:533–539
- Raghavan S, Manzanillo P, Chan K, Dovey C, Cox J (2008) Secreted transcription factor controls *Mycobacterium tuberculosis* virulence. *Nature* 454:717–721
- Ramakrishnan L, Federspiel NA, Falkow S (2000) Granuloma-specific expression of *Mycobacterium* virulence proteins from the glycine-rich PE-PGRS family. *Science* 288:1436–1439
- Raman S, Puyang X, Cheng TY, Young DC, Moody DB, Husson RN (2006) *Mycobacterium tuberculosis* SigM positively regulates Esx secreted protein and nonribosomal peptide synthetase genes and down regulates virulence-associated surface lipid synthesis. *J Bacteriol* 188:8460–8468
- Ramsdell TL, Huppert LA, Sysoeva TA, Fortune SM, Burton BM (2015) Linked domain architectures allow for specialization of function in the FtsK/SpoIIIE ATPases of ESX secretion systems. *J Mol Biol* 427:1119–1132

- Renshaw PS, Lightbody KL, Veverka V, Muskett FW, Kelly G, Frenkiel TA, Gordon SV, Hewinson RG, Burke B, Norman J, Williamson RA, Carr MD (2005) Structure and function of the complex formed by the tuberculosis virulence factors CFP-10 and ESAT-6. *EMBO J* 24:2491–2498
- Rickman L, Scott C, Hunt DM, Hutchinson T, Menendez MC, Whalan R, Hinds J, Colston MJ, Green J, Buxton RS (2005) A member of the cAMP receptor protein family of transcription regulators in *Mycobacterium tuberculosis* is required for virulence in mice and controls transcription of the *rpfa* gene coding for a resuscitation promoting factor. *Mol Microbiol* 56:1274–1286
- Rodriguez GM, Voskuil MI, Gold B, Schoolnik GK, Smith I (2002) *ideR*, an essential gene in *Mycobacterium tuberculosis*: role of IdeR in iron-dependent gene expression, iron metabolism, and oxidative stress response. *Infect Immun* 70:3371–3381
- Romagnoli A, Etna MP, Giacomini E, Pardini M, Remoli ME, Corazzari M, Falasca L, Goletti D, Gafa V, Simeone R, Delogu G, Piacentini M, Brosch R, Fimia GM, Coccia EM (2012) ESX-1 dependent impairment of autophagic flux by *Mycobacterium tuberculosis* in human dendritic cells. *Autophagy* 8:1357–1370
- Rosenberg OS, Dovala D, Li X, Connolly L, Bendebury A, Finer-Moore J, Holton J, Cheng Y, Stroud RM, Cox JS (2015) Substrates control multimerization and activation of the multi-domain ATPase motor of type VII secretion. *Cell* 161:501–512
- Ryan AA, Nambiar JK, Wozniak TM, Roediger B, Shklovskaya E, Britton WJ, Fazekas de St Groth B, Triccas JA (2009) Antigen load governs the differential priming of CD8 T cells in response to the bacille Calmette Guerin vaccine or *Mycobacterium tuberculosis* infection. *J Immunol* 182:7172–7177
- Rybniiker J, Chen JM, Sala C, Hartkoorn RC, Vocat A, Benjak A, Boy-Rottger S, Zhang M, Szekely R, Greff Z, Orfi L, Szabadkai I, Pato J, Keri G, Cole ST (2014) Anticytolytic screen identifies inhibitors of mycobacterial virulence protein secretion. *Cell Host Microbe* 16:538–548
- Sachse F, Becker K, von Eiff C, Metzke D, Rudack C (2010) *Staphylococcus aureus* invades the epithelium in nasal polyposis and induces IL-6 in nasal epithelial cells in vitro. *Allergy* 65:1430–1437
- Sampson SL, Lukey P, Warren RM, van Helden PD, Richardson M, Everett MJ (2001) Expression, characterization and subcellular localization of the *Mycobacterium tuberculosis* PPE gene Rv1917c. *Tuberculosis (Edinb)* 81:305–317
- Sani M, Houben EN, Geurtsen J, Pierson J, de Punder K, van Zon M, Wever B, Piersma SR, Jimenez CR, Daffe M, Appelmelk BJ, Bitter W, van der Wel N, Peters PJ (2010) Direct visualization by cryo-EM of the mycobacterial capsular layer: a labile structure containing ESX-1-secreted proteins. *PLoS Pathog* 6:e1000794
- Sayes F, Sun L, Di Luca M, Simeone R, Degaiffier N, Fiette L, Esin S, Brosch R, Bottai D, Leclerc C, Majlessi L (2012) Strong immunogenicity and cross-reactivity of *Mycobacterium tuberculosis* ESX-5 Type VII secretion- encoded PE-PPE proteins predicts vaccine potential. *Cell Host Microbe* 11:352–363
- Schneewind O, Missiakas DM (2012) Protein secretion and surface display in Gram-positive bacteria. *Philos Trans R Soc Lond B Biol Sci* 367:1123–1139
- Serafini A, Boldrin F, Palu G, Manganelli R (2009) Characterization of a *Mycobacterium tuberculosis* ESX-3 conditional mutant: essentiality and rescue by iron and zinc. *J Bacteriol* 191:6340–6344
- Shah S, Cannon JR, Fenselau C, Briken V (2015) A duplicated ESAT-6 region of ESX-5 is involved in protein export and virulence of mycobacteria. *Infect Immun* 83:4349–4361
- Shukla A, Pallen M, Anthony M, White SA (2010) The homodimeric GBS1074 from *Streptococcus agalactiae*. *Acta Crystallogr, Sect F: Struct Biol Cryst Commun* 66:1421–1425
- Siegrist MS, Unnikrishnan M, McConnell MJ, Borowsky M, Cheng TY, Siddiqi N, Fortune SM, Moody DB, Rubin EJ (2009) Mycobacterial Esx-3 is required for mycobactin-mediated iron acquisition. *Proc Natl Acad Sci U S A* 106:18792–18797

- Siegrist MS, Steigedal M, Ahmad R, Mehra A, Dragset MS, Schuster BM, Philips JA, Carr SA, Rubin EJ (2014) Mycobacterial Esx-3 requires multiple components for iron acquisition. *MBio* 5:e01073–14
- Simeone R, Bobard A, Lippmann J, Bitter W, Majlessi L, Brosch R, Enninga J (2012) Phagosomal rupture by *Mycobacterium tuberculosis* results in toxicity and host cell death. *PLoS Pathog* 8: e1002507
- Simeone R, Sayes F, Song O, Groschel MI, Brodin P, Brosch R, Majlessi L (2015) Cytosolic access of *Mycobacterium tuberculosis*: Critical impact of phagosomal acidification control and demonstration of occurrence in vivo. *PLoS Pathog* 11:e1004650
- Solans L, Aguilo N, Samper S, Pawlik A, Frigui W, Martin C, Brosch R, Gonzalo-Asensio J (2014) A specific polymorphism in *Mycobacterium tuberculosis* H37Rv causes differential ESAT-6 expression and identifies WhiB6 as a novel ESX-1 component. *Infect Immun* 82:3446–3456
- Solomonson M, Setiawati D, Makepeace KA, Lameignere E, Petrotchenko EV, Conrady DG, Bergeron JR, Vuckovic M, DiMaio F, Borchers CH, Yip CK, Strynadka NC (2015) Structure of EspB from the ESX-1 type VII secretion system and insights into its export mechanism. *Structure* 23:571–583
- Song H, Sandie R, Wang Y, Andrade-Navarro MA, Niederweis M (2008) Identification of outer membrane proteins of *Mycobacterium tuberculosis*. *Tuberculosis (Edinb)* 88:526–544
- Sonnhammer EL, von Heijne G, Krogh A (1998) A hidden Markov model for predicting transmembrane helices in protein sequences. *Proc Int Conf Intell Syst Mol Biol* 6:175–182
- Sorensen AL, Nagai S, Houen G, Andersen P, Andersen AB (1995) Purification and characterization of a low-molecular-mass T-cell antigen secreted by *Mycobacterium tuberculosis*. *Infect Immun* 63:1710–1717
- Stamm LM, Morisaki JH, Gao LY, Jeng RL, McDonald KL, Roth R, Takeshita S, Heuser J, Welch MD, Brown EJ (2003) *Mycobacterium marinum* escapes from phagosomes and is propelled by actin-based motility. *J Exp Med* 198:1361–1368
- Stanley SA, Cox JS (2013) Host-pathogen interactions during *Mycobacterium tuberculosis* infections. *Curr Top Microbiol Immunol* 374:211–241
- Stanley SA, Raghavan S, Hwang WW, Cox JS (2003) Acute infection and macrophage subversion by *Mycobacterium tuberculosis* require a specialized secretion system. *Proc Natl Acad Sci U S A* 100:13001–13006
- Stanley SA, Johndrow JE, Manzanillo P, Cox JS (2007) The Type I IFN Response to Infection with *Mycobacterium tuberculosis* Requires ESX-1-Mediated Secretion and Contributes to Pathogenesis. *J Immunol* 178:3143–3152
- Strong M, Sawaya MR, Wang S, Phillips M, Cascio D, Eisenberg D (2006) Toward the structural genomics of complexes: crystal structure of a PE/PPE protein complex from *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A* 103:8060–8065
- Sundaramoorthy R, Fyfe PK, Hunter WN (2008) Structure of *Staphylococcus aureus* EsxA suggests a contribution to virulence by action as a transport chaperone and/or adaptor protein. *J Mol Biol* 383:603–614
- Supply P, Marceau M, Mangenot S, Roche D, Rouanet C, Khanna V, Majlessi L, Criscuolo A, Tap J, Pawlik A, Fiette L, Orgeur M, Fabre M, Parmentier C, Frigui W, Simeone R, Boritsch EC, Debie AS, Willery E, Walker D, Quail MA, Ma L, Bouchier C, Salvignol G, Sayes F, Cascioferro A, Seemann T, Barbe V, Loch C, Gutierrez MC, Leclerc C, Bentley SD, Stinear TP, Brisse S, Médigue C, Parkhill J, Cruveiller S, Brosch R (2013) Genomic analysis of smooth tubercle bacilli provides insights into ancestry and pathoadaptation of *Mycobacterium tuberculosis*. *Nat Genet* 45:172–179
- Sysoeva TA, Zepeda-Rivera MA, Huppert LA, Burton BM (2014) Dimer recognition and secretion by the ESX secretion system in *Bacillus subtilis*. *Proc Natl Acad Sci U S A* 111:7653–7658
- Tekaia F, Gordon SV, Garnier T, Brosch R, Barrell BG, Cole ST (1999) Analysis of the proteome of *Mycobacterium tuberculosis* in silico. *Tuber Lung Dis* 79:329–342

- Teutschbein J, Schumann G, Mollmann U, Grabley S, Cole ST, Munder T (2009) A protein linkage map of the ESAT-6 secretion system 1 (ESX-1) of *Mycobacterium tuberculosis*. *Microbiol Res* 164:253–259
- Tuchscher L, Medina E, Hussain M, Volker W, Heitmann V, Niemann S, Holzinger D, Roth J, Proctor RA, Becker K, Peters G, Löffler B (2011) *Staphylococcus aureus* phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. *EMBO Mol Med* 3:129–141
- Ummels R, Abdallah AM, Kuiper V, Aajoud A, Sparrius M, Naeem R, Spaink HP, van Soolingen D, Pain A, Bitter W (2014) Identification of a novel conjugative plasmid in mycobacteria that requires both type IV and type VII secretion. *MBio* 5:e01744–14
- van der Wel N, Hava D, Houben D, Fluittsma D, van Zon M, Pierson J, Brenner M, Peters PJ (2007) *M. tuberculosis* and *M. leprae* translocate from the phagolysosome to the cytosol in myeloid cells. *Cell* 129:1287–1298
- van der Woude AD, Luirink J, Bitter W (2013) Getting across the cell envelope: mycobacterial protein secretion. *Curr Top Microbiol Immunol* 374:109–134
- Voskuil MI, Schnappinger D, Rutherford R, Liu Y, Schoolnik GK (2004) Regulation of the *Mycobacterium tuberculosis* PE/PPE genes. *Tuberculosis (Edinb)* 84:256–262
- Wagner JM, Evans TJ, Chen J, Zhu H, Houben EN, Bitter W, Korotkov KV (2013) Understanding specificity of the mycosin proteases in ESX/type VII secretion by structural and functional analysis. *J Struct Biol* 184:115–128
- Walters SB, Dubnau E, Kolesnikova I, Laval F, Daffe M, Smith I (2006) The *Mycobacterium tuberculosis* PhoPR two-component system regulates genes essential for virulence and complex lipid biosynthesis. *Mol Microbiol* 60:312–330
- Wang S, Engohang-Ndong J, Smith I (2007) Structure of the DNA-binding domain of the response regulator PhoP from *Mycobacterium tuberculosis*. *Biochemistry* 46:14751–14761
- Wassermann R, Gulen MF, Sala C, Perin SG, Lou Y, Rybniker J, Schmid-Burgk JL, Schmidt T, Hornung V, Cole ST, Ablasser A (2015) *Mycobacterium tuberculosis* differentially activates cGAS- and inflammasome-dependent intracellular immune responses through ESX-1. *Cell Host Microbe* 17:799–810
- Watson RO, Manzanillo PS, Cox JS (2012) Extracellular *M. tuberculosis* DNA targets bacteria for autophagy by activating the host DNA-sensing pathway. *Cell* 150:803–815
- Watson RO, Bell SL, MacDuff DA, Kimmey JM, Diner EJ, Olivias J, Vance RE, Stallings CL, Virgin HW, Cox JS (2015) The cytosolic sensor cGAS detects *Mycobacterium tuberculosis* DNA to induce type I Interferons and activate autophagy. *Cell Host Microbe* 17:811–819
- Way SS, Wilson CB (2005) The *Mycobacterium tuberculosis* ESAT-6 homologue in *Listeria monocytogenes* is dispensable for growth in vitro and in vivo. *Infect Immun* 73:6151–6153
- Wong KW, Jacobs WR Jr (2011) Critical role for NLRP3 in necrotic death triggered by *Mycobacterium tuberculosis*. *Cell Microbiol* 13:1371–1384
- Zhang M, Chen JM, Sala C, Rybniker J, Dhar N, Cole ST (2014) EspI regulates the ESX-1 secretion system in response to ATP levels in *Mycobacterium tuberculosis*. *Mol Microbiol* 93:1057–1065
- Zuber B, Chami M, Houssin C, Dubochet J, Griffiths G, Daffe M (2008) Direct visualization of the outer membrane of mycobacteria and corynebacteria in their native state. *J Bacteriol* 190:5672–5680