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GENERAL INTRODUCTION

TUBERCULOSIS

Since ancient history, the infectious pulmonary disease tuberculosis (TB) has claimed the lives of numerous human beings. Today, its causative agent *Mycobacterium tuberculosis* is still responsible for 1.4 million deaths annually [1]. After the human immunodeficiency virus (HIV), TB ranks the second leading cause of death from an infectious disease, with the highest burden in Africa and South-East Asia. Since 1993, when the World Health Organization declared TB a global public health emergency, increased efforts to improve prevention, detection and treatment have effectively reduced the number of new cases and deaths. However, the variable efficacy of the current vaccine and increasing emergence of antibiotic-resistant strains are major drawbacks in the control of TB. Treatment of TB requires a minimum course of six months with a combination of drugs. Over the years, inefficient bacterial clearance due to inappropriate treatment or failure to complete treatment has given rise to antibiotic-resistant *M. tuberculosis*. Multi-, extensive- and even total-drug resistant TB strains, that display resistance to an increasing number of drugs, have now been described. The current vaccine that is used to prevent disease, was developed almost a century ago by continuous passaging of the cattle-tuberculosis causing species *Mycobacterium bovis*. The resulting attenuated strain *M. bovis BCG*, named after its founders Calmette and Guérin, shows highly variable protective effects against pulmonary tuberculosis, ranging from 0 to 80% [2]. As BCG offers a reasonably good protection against disseminated TB in children, it is still used. Altogether, the low efficacy of the vaccine, increase in antibiotic-resistant *M. tuberculosis* strains and lack of proper diagnostic tools in endemic areas urges the development of new preventive and curative treatment options.

EVOLUTION OF MYCOBACTERIA

Over the millennia, *M. tuberculosis* has successfully adapted to its human host. This has become clear from genetic studies on mycobacterial evolution and adaptation, which became possible with the availability of complete genome sequences in 1998 [3]. One major advantage for these studies is that *M. tuberculosis* does not show signs of recent horizontal gene transfer, which means that genomic trees can be produced with high confidence. *M. tuberculosis* belongs to the *M. tuberculosis* complex (MTBC), a collection of TB-causing bacterial agents that share identical 16S RNA and have greater than 99.9% nucleotide identity (Figure 1). Other members of the MTBC include *M. bovis*, *Mycobacterium canettii*, *Mycobacterium africanum* and *Mycobacterium microti*, each with their own host tropism [4]. Based on analyses of deleted genomic regions, two major MTBC lineages have been found to originate from a common mycobacterial ancestor around 40,000 years ago [5], coinciding with the proposed foundation of modern human populations in the horn of Africa [6]. In the following 20,000 years, further deletion events resulted in the individual members of the MTBC [4]. These deletions may be seen as evolutionary markers of host-specific adaptation. For *M. tuberculosis*, they have led to the restriction and specialization to the human host. The MTBC comprises just a few of the more than 120 species that belong to the genus Mycobacterium. Phylogenetic

analysis based on 16s rRNA gene sequences has revealed that species of this family can be divided into slow and fast growing mycobacteria, depending on whether visible colonies appear before or after 7 days of incubation (Figure 1) [7]. The cluster of slow growing mycobacteria, which includes most of the pathogenic species such as the MTBC, seems to have evolved from a fast growing ancestor [8].

MYCOBACTERIAL DISEASE

Genetically most closely related to the MTBC are *Mycobacterium ulcerans* and *Mycobacterium marinum*. Although these species are very closely related to each other and share identical 16s rRNA sequences, there are some major differences. *M. ulcerans* is subjected to ongoing degenerative evolution and has a large number of pseudogenes, whereas *M. marinum* has a large genome with hardly any pseudogenes and shows signs of recent horizontal gene transfer. *M. ulcerans* is a human pathogen that causes Buruli ulcer, a (sub)tropical disease characterized by ulcerated lesions of the skin and subcutaneous tissues. After TB and leprosy, Buruli ulcer is the third most common human disease caused by mycobacteria. *M. marinum* on the other hand, causes TB in a wide-range of ectothermic animals such as fish and frogs. In addition, it can also cause disease in humans. However, due to its restricted growth temperature with a maximum of 33°C, it only gives rise to local skin infections known as fish tank granuloma. Despite

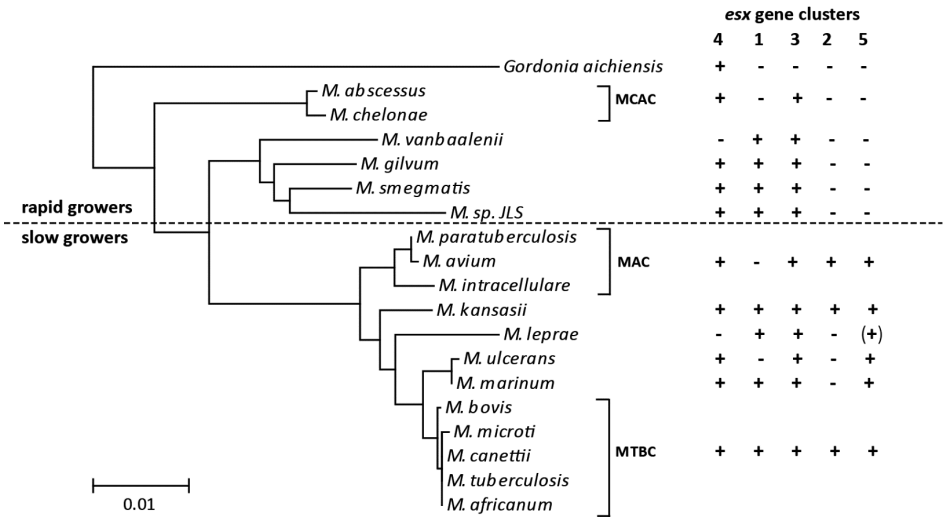

Figure 1. Phylogenetic tree and presence of esx gene clusters in several mycobacterial species. Based on 16s rRNA sequences, a phylogenetic tree showing evolutionary relationships between a selection of mycobacterial species was constructed. The presence of the five *esx*-gene clusters in the genomes of these species are indicated, based on the presence of the *eccBCDE* genes which encode the conserved structural components of the T7ss. MTBC; *M. tuberculosis* complex, MAC; *M. avium* complex, MCAC; *M. chelonae-abscessus* complex. Scale bar represents 0,01 changes per nucleotide. This figure was adapted with permission, courtesy of M. Daleke.

its wide host range, the course of an *M. marinum* infection in ectothermic animals is strikingly similar to that of *M. tuberculosis* in humans [9]. In both cases, mycobacteria are initially phagocytosed by macrophages, which are unable to completely clear the infection. As a result of cytokine and chemokine secretion, infected macrophages are rapidly surrounded by other immune cells, leading to the formation of granulomas. These large immune aggregates can contain the infection for a long period of time whilst offering bacteria a protective niche. When the immune system of the host is weakened, granulomas can rupture, enabling bacterial spread and transmission to a new host. During this phase, infection progresses from latent to active disease [10,11].

DORMANCY

Upon their encapsulation in granulomas, mycobacteria undergo a series of transcriptional changes that enable them to survive for a prolonged time period in this hostile and oxygen-poor environment. As a result of these changes, bacteria are thought to enter a non- or low-replicating state of dormancy, which is characterized by metabolic inactivity and accumulation of lipids [10]. In addition, mycobacteria develop phenotypic tolerance to antibiotic drugs. The replication state of mycobacteria during latent infection is still under debate. The view of a static equilibrium of dormant bacteria that remain viable but do not divide, which is based on the amount of CFU over time, is generally accepted. However, there is increasing evidence that there is substantial replication of *M. tuberculosis* during latency. For instance, the dynamic nature of the immune response during chronic infection and the loss of an experimentally introduced plasmid during prolonged infection *in vivo* point to continued cell division [12-14]. However, it is unclear whether all bacteria are dividing at a continuous but reduced rate or whether an actively dividing subpopulation of mycobacteria is present amid the dormant bacilli. The second option seems to be more likely and suggests that the balance between dormant and replicating *M. tuberculosis* could be a distinguishing factor between latent infection and active disease [10].

The transcriptional adaptation to the conditions encountered in the granuloma is in part regulated by the mycobacterial dormancy survival regulon (dos). DosR, the key player of this regulon, induces transcription of 48 genes upon hypoxia and redox sensing by DosS and DosT [15,16]. In general, this transcriptional response leads to the down-regulation of central metabolism and the induction of stress response proteins and alternative metabolism pathways. Although glycerol is used as the standard carbon source to grow *M. tuberculosis in vitro*, during the onset and chronic phase of infection mycobacteria predominantly use host-derived fatty acids and cholesterol as a source of energy [17,18]. The glyoxylate pathway enzyme isocitrate lyase is essential for this process and has an important role in persistence in the host [19,20]. Thus far, little is known about the exact mechanisms of lipid transport across the mycobacterial cell wall. The *mce4* operon has been shown to encode a cholesterol import system that can provide energy to *M. tuberculosis* and plays an important role in bacterial growth during the chronic phase of infection [18]. The bacterial factors that are involved in the import of host-derived fatty acids however, remain to be determined. Lipids such as

cholesterol and triacylglycerol (TAG) are abundantly present in foamy macrophages of the *M. tuberculosis* granuloma [21]. Mycobacteria are able to store the host-derived fatty acids primarily as mycobacterial TAG, in a process mediated by triacylglycerol synthase 1 [22-24]. These TAG storages have been suggested to serve as an energy source for utilization during dormancy and reactivation of mycobacteria [22]. The TAG-hydrolyzing protein LipY may be involved in this process [25]. At a certain point during latent infection, the dormant bacteria will reactivate. This process probably occurs in response to lowered host immune defenses and has been shown to be mediated by resuscitation promoting factors encoded by the *rpf* genes [26]. The five members of this secreted protein family serve as bacterial growth factors that are dispensable for *in vitro* growth but required for the initiation of bacterial growth from dormancy [27]. Although the Rpf proteins are predicted to disrupt cell wall peptidoglycan structure, the exact mechanism behind the induction of resuscitation is thus far unknown [28].

VIRULENCE MECHANISMS WITHIN THE MACROPHAGE

In order to establish a successful infection, pathogenic mycobacteria need to survive the defense mechanisms of the host's innate immune system. The first event of infection is the phagocytosis of mycobacteria by macrophages, which in case of *M. tuberculosis* occurs in the human lungs after inhalation of bacteria-containing droplets. The phagosome is a very hostile environment for most bacteria. Upon phagocytosis of microbes, this compartment matures by a series of fusion events with intracellular organelles such as endosomes and lysosomes, resulting in a lowering of the pH, activation of hydrolytic enzymes and antimicrobial peptides and production of reactive oxygen and nitrogen species [29]. Although this is usually sufficient to kill microorganisms, pathogenic mycobacteria have developed a range of molecular mechanisms to counteract the macrophage's defense system. An important asset of *M. tuberculosis* is its capability to prevent phagosomal maturation. Several mycobacterial cell wall lipids and (de) phosphorylating proteins have been implicated in this process [30,31]. These factors act by reducing the drop in phagosomal pH and inhibiting the fusion with lysosomes, thereby preventing mycobacterial degradation. In addition to the arrest of phagosomal maturation, pathogenic mycobacteria have also developed a mechanism that allows them to survive phago-lysosomal fusion. For several pathogenic mycobacteria, such as *M. marinum*, *M. tuberculosis*, *M. leprae* and *M. bovis*, translocation from the phago-lysosomal compartment into the cytosol of the macrophage has been shown [32-35]. Although its exact molecular mechanism has not been elucidated, this process is dependent on the mycobacterial protein secretion system ESX-1 (Figure 2) [33-35].

ESX GENE CLUSTERS

The ESX-1 protein secretion system is encoded by the *esx-1* gene cluster, named after one of its substrates, EsxA (also known as ESAT-6). Genome sequence analysis of *M. tuberculosis* revealed the presence of five homologous copies of this gene cluster, which were named *esx-1* to *esx-5*. Phylogenetic analysis indicated that *esx-4* is the most

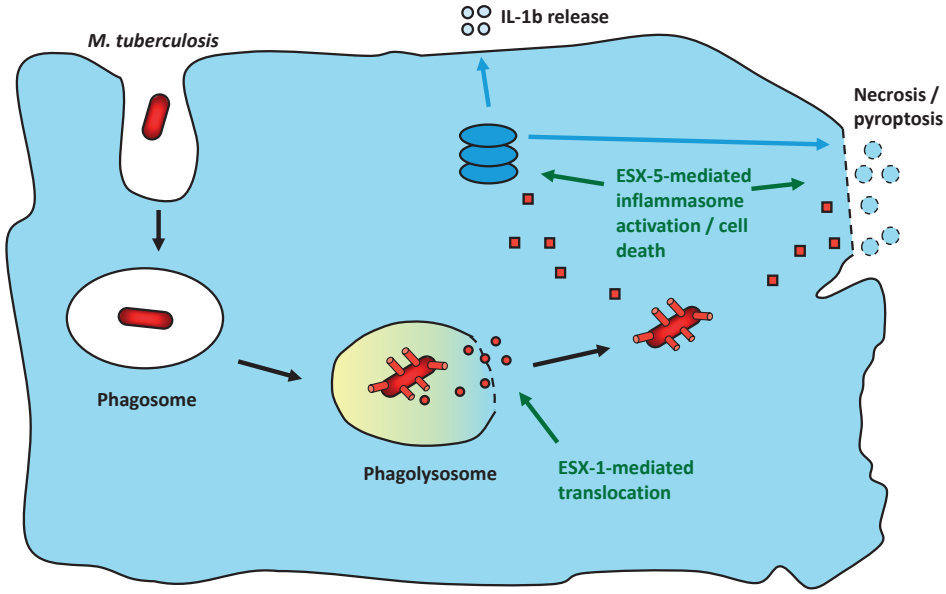

Figure 2. Proposed effects of Mycobacterium tuberculosis T7ss during infection of macrophages. As a first step of infection, *M. tuberculosis* is phagocytosed by a macrophage. The phagosomal compartment subsequently matures into the more hostile phago-lysosome by a series of events that can be inhibited by the bacterium. Probably as a result of ESX-1-dependent release of specific proteins, *M. tuberculosis* is able to lyse the membrane of the phago-lysosome and translocate into the cytosol of the cell [33,35]. Mycobacteria localized in the cytosol induce cell death of the macrophage by production of toxic factors. In addition, inflammasome activation leads to the release of IL-1b. In both of these processes, ESX-5 has been proposed to play a role [68].

ancestral of these regions. By subsequent duplication events, *esx-1*, *esx-3*, *esx-2* and, most recently, *esx-5* evolved [36]. The *esx* gene clusters are specific for Actinobacteria, which include the genus Mycobacterium and Cornyebacterium. The ancestral *esx-4* cluster was found to be present in the genome of *Cornyebacterium diphteriae*, suggesting that Mycobacteria and Cornyebacteria have a common ancestor [36]. As the fast growing mycobacterial species do not contain the most recently duplicated cluster *esx-5*, this cluster probably appeared after the divergence of the slow-growing species (Figure 1). In some mycobacterial species, specific *esx* clusters have been deleted from their genome. For example, *M. leprae*, a species that has undergone severe genome down-sizing, only contains an intact copy of the *esx-1* and *esx-3* clusters and the core genes from *esx-5*, suggesting that these clusters are essential for mycobacterial viability or pathogenicity [36]. However, different pathogens might have evolved different solutions to compensate for the loss of these gene clusters. For instance, the opportunistic avian pathogen *Mycobacterium avium* is still able to establish a successful infection in macrophages, despite the loss of *esx-1* from its genome [37].

TYPE VII SECRETION SYSTEMS

The discovery of the ESX protein secretion systems did not come as a surprise, since mycobacteria have a distinctively thick and waxy cell wall that forms a highly impermeable barrier. The main constituents of the cell wall are long-chain mycolic acids that make it difficult to stain these bacteria with the water-based Gram stain. For diagnostic purposes, an acidic stain is therefore often used to identify mycobacteria. Over the last years, several structural studies have shed light on the composition of the mycobacterial cell wall. These studies revealed that the cell wall contains a second lipid bilayer, which is present in addition to the cytoplasmic (or inner) membrane [38-40]. This lipid bilayer, which is also referred to as the mycobacterial outer membrane, mainly consists of mycolic acids, together with a range of other unusual lipids. The mycolic acids in the inner layer of the mycobacterial outer membrane are covalently linked to a peptidoglycan-arabinogalactan polymer that is located between the inner and outer membrane [38]. In order to ensure import of nutrients and export of bacterial virulence factors across this complex cell wall, mycobacteria contain the ESX or type VII secretion systems (T7ss), which have unique properties that distinguish them from all (six) other known bacterial secretion systems [41]. Each ESX system consists of a set of core components, the Ecc proteins (ESX-conserved component) [42]. These components include proteins that provide the energy for substrate secretion (ATP-dependent chaperones of the AAA family and membrane-bound ATP-ases), several transmembrane proteins and a subtilisin-like serine protease, MycP. Four of these proteins have been shown to form a large complex embedded in the membrane, which might form the actual transport machinery (EccBCDE, Figure 3) [43]. The substrates of the ESX-systems can be encoded by the *esx* regions, but genes coding for additional substrates are also found elsewhere in the genome. Recently, a conserved secretion signal was detected in all known mycobacterial T7s substrates or secreted protein pairs. This signal consists of two α-helices, separated by a turn and followed by a conserved secretion motif, YxxxD/E [44]. Although this signal can be used to identify new type VII substrates, its role is still under investigation. The exact mechanism of secretion and structure of the T7ss remain to be determined as well. Of the five mycobacterial T7ss, only ESX-1, ESX-3 and ESX-5 have been proven to be functional [41]. In contrast to ESX-1 and ESX-5, ESX-3 is conserved among all sequenced mycobacterial species, suggesting a crucial function. Indeed, in *M. tuberculosis*, ESX-3 is essential for bacterial survival [45,46]. The ESX-3 secretion system has been shown to be involved in metal acquisition, in particular of iron and zinc [45,47]. However, its exact working mechanism and role in virulence is thus far unknown. In contrast, many studies have established an important role for ESX-1 in virulence. In fact, the attenuation of the vaccine strain BCG has been shown to be attributable to a deletion of part of *esx-1* from the *M. bovis* genome, as reintroduction of this region (RD1; region of difference 1) in BCG could greatly increase virulence [48]. Moreover, deletion of RD1 from the genomes of *M. tuberculosis* and *M. marinum* severely decreased virulence [49,50]. Abrogation of ESX-1-mediated protein secretion has been shown to greatly attenuate mycobacterial infection in several animal models

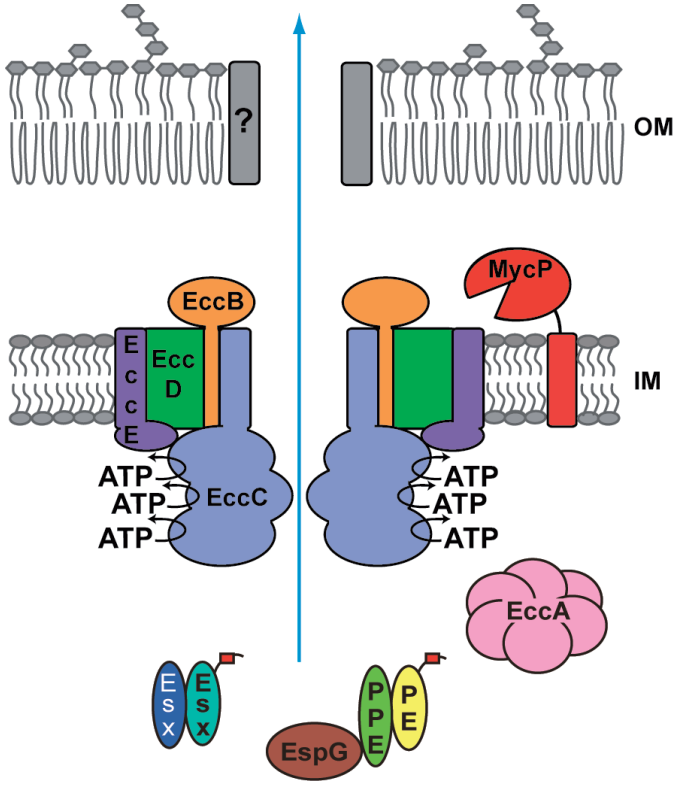

Figure 3. Proposed model of the T7S membrane channel. Substrate transport by T7ss may occur according the model depicted. The conserved structural components EccB, EccC, EccD and EccE of ESX-5 have been shown to form a major complex that is localized in the cell envelope [43]. This complex may be responsible for substrate translocation across the inner membrane (IM) after which a second structure in the outer membrane (OM) needs to be crossed for secretion. Alternatively, the membrane complex forms a channel across both membranes. Although the membrane protein MycP and the cytosolic components EspG and EccA have not been shown to be part of the complex, they are involved in the substrate translocation process [50,69,87-90]. This figure was published in [43], and reprinted by permission of John Wiley & Sons, Inc.

[51]. Highly decreased bacterial burdens, loss of granuloma formation and an enhanced inflammatory response in these animals demonstrate the importance of ESX-1 for pathogenic mycobacteria. Remarkably, in the non-pathogenic environmental species *M. smegmatis*, ESX-1 is involved in conjugative DNA transfer [52,53].

ESX-1 SUBSTRATES AND FUNCTION

So far, ESX-1 has been shown to be responsible for secretion of EsxA, EsxB (also known as CFP-10), EspB, EspE, EspK, EspF and PPE68, which are all encoded within the *esx-1* gene cluster [40,54-56]. In addition, ESX-1 transports proteins that are encoded by the *espACD* operon located ~300 kb upstream, and possibly one of their regulators, EspR

[54,57,58]. The proteins encoded by the *espACD* operon are all secreted, although EspD is not transported exclusively via ESX-1 [59]. A complicating factor in the analysis of the ESX-1 secretion system is that some of the substrates have been shown to be mutually dependent, meaning that secretion of each substrate is dependent on secretion of the others [54]. Although all ESX-1 substrates therefore appear to be important for virulence, the exact function of each individual substrate is unknown. Of all substrates, EsxA and EsxB have gained most attention. This protein pair, which is encoded within *esx-1* and conserved across the other *esx* clusters, forms a stable 1:1 dimer [60]. According to one study, the EsxA/B protein pair dissociates at low pH [61], exposing host membranes to the lysing capacities of EsxA [50,62]. Although this strongly suggests that EsxA is responsible for ESX-1 mediated phago-lysosomal escape, the homology between EsxA of *M. tuberculosis* and the translocating species *M. leprae* is very low, whereas the homology with EsxA of the non-translocating species *Myobacterium kansasii* and *Mycobacterium smegmatis* is quite high [33]. Furthermore, other structural and biochemical studies have not been able to confirm the EsxA/B dissociation data [63,64]. Disruption of ESX-1-mediated protein secretion leads to a growth defect in macrophages [51], which may be attributable to the inability to escape the hostile phago-lysosomal compartment and/or inhibiting phagosomal maturation [35,65]. Other proposed intracellular functions of ESX-1 substrates include inflammasome activation and cell to cell spread of bacteria [50,66,67]. However, as ESX-1 is required for phagosome escape, it is not clear whether these observed effects are a direct result of ESX-1 substrate activity or occur via other mechanisms that follow upon translocation (Figure 2) [68].

ESX-5 AND ITS SUBSTRATES

The most recently evolved T7ss is ESX-5. This system is exclusively present in the slow-growing, more pathogenic mycobacterial species, suggesting that ESX-5 is involved in virulence [36]. In addition to secretion of the EsxA/B homologues EsxN/M that are encoded within *esx-5*, the primary substrates of this system appear to be proteins of the PE and PPE families [69,70]. Members of these protein families are characterized by a Pro-Glu (PE) or Pro-Pro-Glu (PPE) motif near their conserved 110 and 180 amino acid N-termini, respectively (Figure 4). Although a large number of *pe* and *ppe* genes are spread across the whole genome of *M. tuberculosis* and *M. marinum*, the most ancient of these genes seem to have been inserted in *esx-1* and duplicated together with the *esx* clusters. With the duplication of each *esx* cluster, a limited number of secondary sub-duplications of the *pe/ppe* genes occurred. After the introduction of *esx-5* however, multiple copies of *pe/ppe* genes were inserted in the genome [8]. This large expansion of *pe/ppe* family genes occurred not only within the *esx-5* region, but especially outside this cluster. During the expansion process, several different subfamilies arose, of which the *pe_prgs* and *ppe_mptr* gene families are the largest (Figure 4) [3]. Both subfamilies are characterized by multiple tandem repeats in their C-terminal domain, which in case of PE_PGRS primarily encompass Gly and Ala residues. Around 10% of the genomic coding potential of *M. tuberculosis* is

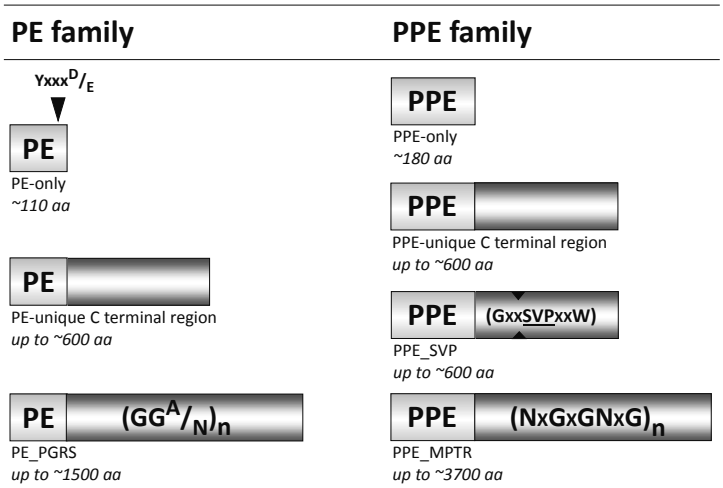

Figure 4. Schematic representation of members of the PE and PPE family. PE and PPE family proteins can be divided into several subgroups based on their C-terminal sequences. The most ancestral of these proteins consist of a PE or PPE domain only. The conserved YxxxD/E secretion signal is often found at the end of the N-terminal PE domain [44]. The most numerous PE and PPE subfamilies are shown here, which comprise 24 (PPE_SVP), 23 (PPE_MPTR), or 65 (PE_PGRS) members in *M. tuberculosis* H37Rv [76]. This figure was adapted from [75].

devoted to the PE/PPE proteins [3], indicating that they must play an important role in virulence. Their exclusive expansion in pathogenic species such as those of the MTBC, *M. marinum* and *M. ulcerans*, supports this theory [8].

ROLE OF PE/PPE PROTEINS IN VIRULENCE

The ancient *pe* and *ppe* genes often form an operon, in which the *pe* gene is located upstream of the *ppe* gene. Some of the resulting protein pairs have been shown to form a complex that can be secreted via a T7ss [44,71,72]. Although several PE and PPE proteins can be actively secreted via ESX-5 [69,70], numerous of these proteins are also associated with the bacterial cell wall. Especially the PE_PGRS proteins are abundantly present on the cell surface [40,73,74], suggesting that these proteins are ideally placed for interaction with the host's immune system. Several studies have indeed established immuno-modulatory capacities for PE_PGRS and other PE/PPE proteins. The proposed mechanisms by which individual PE/PPE proteins interact with the immune system include TLR2 signaling, induction of cell death, promotion of uptake by macrophages, modulation of vacuole acidification and inhibition of antigen presentation [75]. This diverse range of proposed functions suggests that individual PE/PPE proteins may play distinct roles in the infection process. It has also been speculated that the PE/PPE and in particular PE_PGRS family proteins provide pathogenic mycobacteria with a diverse antigenic profile [3]. As the C-terminal domains of these cell surface- exposed proteins can vary substantially, the large number of PE/PPE proteins could be a source of antigen

variation aiming at misleading the host's immune system. However, the available data for this is limited [3,76-78]. Both options, i.e. individual (unrelated) functions and antigenic variation, are in accordance with the observed transcriptional regulation of *pe/ppe* genes, which does not occur as one group but rather independently [77]. Individual *pe/ppe* genes are controlled by regulatory circuits that also control expression of other genes and several transcriptional regulators of *pe/ppe* genes have been identified [79]. Furthermore, different clinical isolates of *M. tuberculosis* show quite variable patterns of *pe/ppe* gene expression [80,81], supporting the theory that the highly immunogenic PE/PPE proteins act as a decoy for the immune system and may undergo immune selection *in vivo*, resulting in altered expression patterns. Also on the genetic level, a relatively high variation in *pe/ppe* genes between clinical isolates has been documented [76]. Comparison of *pe/ppe* expression levels between the MTBC members *M. tuberculosis* and *M. bovis* revealed that some of these genes are highly over-expressed in *M. tuberculosis*, suggesting a role for their coding products in host-specific processes [82,83]. Furthermore, expression of specific *M. tuberculosis pe* and *ppe* genes was found to be up-regulated in lung granulomas of the human host [84]. More recently, a proteomic study in guinea pigs showed consistent expression of a significant number of PE/PPE proteins during the early and chronic phase *of M. tuberculosis* infection [85]. Together, these data point to an important but not well-understood role for PE and PPE proteins in virulence.

SCOPE OF THE THESIS

The objective of the research described in this thesis was to identify and characterize the specific virulence mechanisms used by pathogenic mycobacteria. It is important to obtain knowledge on these processes as this may lead to new drug targets and vaccine strategies to combat TB. In this thesis, special emphasis was placed on ESX-5. As this T7ss is exclusively present in the slow growing and mostly pathogenic mycobacterial species, it is likely to play a role in virulence. In all studies described in this thesis, *M. marinum* was used as a model for pathogenic mycobacteria. In **Chapter 2**, the secretome of ESX-5 was determined by a proteomic approach using a secretion defective mutant. The data revealed ESX-5 as a major secretion pathway for mycobacteria, responsible for the secretion of a large number of PE_PGRS, PPE and some ESAT-6-like proteins. In **Chapter 3**, the ESX-5-defective secretion mutant was used to study the effect of ESX-5-dependent proteins on virulence. Surprisingly, inactivation of ESX-5 was found to result in mycobacterial hypervirulence in adult zebrafish. As the ESX-5 mutant displayed normal to slightly attenuated virulence in macrophages and zebrafish embryos, the observed effects of increased bacterial growth and granuloma formation were specific for conditions encountered in adult fish. A possible role for the adaptive immune system was ruled out in this study. In **Chapter 4,** the granulomas that were formed in adult zebrafish upon *M. marinum* infection were characterized in more detail. The presence of a surrounding ring of CD4+ T cells and hypoxic core indicate that the zebrafish *M. marinum* granuloma is highly similar to the human *M. tuberculosis*

granuloma. Additionally, the mechanism behind hypervirulence of the ESX-5 mutant was studied in this chapter. The results of these experiments showed that ESX-5 is involved in the uptake of extracellular fatty acids. The ESX-5 dependent lipase LipY was found to play a role in this process, after rerouting it to the ESX-1 secretion system. However, LipY-mediated fatty acid uptake could not be linked to the hypervirulent phenotype of ESX-5-deficient mycobacteria in adult zebrafish. In **Chapter 5**, ESX-1–defective *M. marinum* and *M. tuberculosis* strains were used to study the effect of ESX-1 on gene transcription. In contrast to the systemic components of the secretion system, expression of the known substrates of ESX-1 was down-regulated in the mutant strains of both species. Based on these gene expression data, EspJ could be identified as a novel ESX-1 substrate. In addition, transcription profiles during early macrophage infection pointed towards an important role for the *espACD* operon in ESX-1-mediated virulence. **Chapter 6** describes a more general search for virulence factors that are differentially required by *M. marinum* in different hosts. In order to perform this study, the genome sequence of the *M. marinum* strain E11, used in all studies described in this thesis, was first determined. In a large scale cell infection study, Transposon Directed Insertion site Sequencing (TraDIS) of a large pool of mycobacterial transposon mutants subsequently shed light on the factors required for a successful infection of macrophages [86]. In this study, the bacterial requirements in phagocytic cells derived from mammals and fish were compared with two protozoan species. In addition to common mycobacterial virulence pathways, host-specific virulence mechanisms could be identified. The obtained data provides important insight in mycobacterial adaptation to the host and also shows that amoeba cannot merely be seen as a playground for pathogenic mycobacteria. In the summarizing final part, **Chapter 7**, the results of the work described above are discussed in a broader perspective and their implications for further research and solutions towards mycobacterial diseases are evaluated.

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