GENERAL INTRODUCTION

Tuberculosis

Mycobacteria form a group of environmental and pathogenic bacteria that distinguish themselves by unique capacities of their cell envelope. Several species of mycobacteria are able to cause diseases in humans and other animals. The most well-known of these pathogenic mycobacteria is *Mycobacterium tuberculosis*, the causative agent of tuberculosis. In western countries tuberculosis is often seen as a solved problem of the past. However, *M. tuberculosis* is still the most deadly bacterial pathogen worldwide. Each year nine million new cases of tuberculosis occur, and one-and-a-half million people die of this infectious disease (World Health Organization 2013). Tuberculosis is spread through inhalation of contaminated aerosols and only a low dose of *M. tuberculosis* bacilli is required to establish infection. Although *M. tuberculosis* spreads efficiently, the bacteria themselves grow very slowly. In comparison: *Escherichia coli* is able to divide every 20 minutes, while *M. tuberculosis* divides every 24 hours. This sounds as a disadvantage for the bacterium, but is actually an important part of the successful infection strategy of *M. tuberculosis*. The slow growth of mycobacteria is combined with the ability to slow down the bacterial metabolism into a state of dormancy, where by active infection turns to latent infection. This allows *M. tuberculosis* to stay undetected in the body of the host for years, until the infection is reactivated, usually because of an imbalance in the host's immune system. Malnourishment and HIV infection are important risk factors for reactivation to severe forms of tuberculosis that often result in death (World Health Organization 2013). Tuberculosis also used to be referred to as “the consumption”, which gives an indication of the state of a patient with progressive tuberculosis. *M. tuberculosis* primarily causes infection of the lungs, which in most cases remains occurs without symptoms. However, reactivation of the disease can occur in a situation of reduced immunity of the host, leading to dissemination of the bacteria within and outside of the lungs. This may lead to chronic coughing associated with bloody sputum and weight loss as important symptoms. In advanced tuberculosis, other organs can also be infected with very serious symptoms and death as a consequence. Treatment of tuberculosis consists of a cocktail of at least 4 different antibiotics that need to be taken for several months (World Health Organization 2013). This long and intense treatment leads to compliance problems in the treatment of tuberculosis, which in turn have led to antibiotic resistance of *M. tuberculosis* against first- and second-line antibiotics. Rising drug resistance has resulted in multi-drug-resistant (MDR) or extremely-drug-resistant (XDR) strains of *M. tuberculosis*. The prevalence of these resistant strains is increasing (World Health Organization 2013). Treatment of XDR-tuberculosis is extremely challenging, requiring cocktails of over ten different second-line antibiotics, associated with severe side-effects, that need to be taken for a period of at least 18 months years (World Health Organization 2013). In some cases, treatment of XDR-tuberculosis needs to be performed in quarantine of the patient in the first
months of treatment. This leads to stigmatization of patients, fear of healthcare facilities and rips apart families and social structures. Even if proper healthcare is available, treatment success rates of XDR-tuberculosis remain below 50%, especially in patients co-infected with HIV. Treatment of a single XDR-tuberculosis patient in a western country is estimated to cost approximately €450.000,- (World Health Organization 2013). Even though XDR-tuberculosis is still rare, the rise of these antibiotic resistant strains make it evident that improvements of current tuberculosis treatment and prevention strategies urgently need to be developed. The current vaccine against tuberculosis is a live attenuated bacterium called BCG. BCG was developed in the 1920’s by Calmette and Guérin in France. Their vaccine was based on Mycobacterium bovis, which causes a tuberculosis-like disease in cattle and humans. This strain was cultured in the laboratory for 10 years; over this period it accumulated genetic mutations. These mutations resulted in attenuation of the strain, i.e. it lost its ability to cause disease in humans, although vaccination with BCG still causes immune responses that help protect the vaccinated individuals. These immune-responses elicited by BCG confer partial protection against the most severe forms of tuberculosis in children, but unfortunately are not able to prevent reactivation of disease and development of open lung tuberculosis (hence transmission of M. tuberculosis) in adults and adolescents.

The worldwide increase in incidence of tuberculosis cases that was partially caused by the HIV epidemic has been halted in the last decade. However, only a marginal reduction in the number of tuberculosis cases and deaths has been achieved, indicating that the elimination of tuberculosis still requires intensified efforts (World Health Organization 2013). In order to achieve the millennium goal of the World Health Organization to eradicate tuberculosis by 2050, both new vaccines and new antibiotics are needed.

Mycobacteria are responsible for other diseases than just tuberculosis. Leprosy for instance, which is caused by Mycobacterium leprae, is still not eradicated and remains a health problem in developing countries. But also the less commonly known disease Buruli ulcer, which is caused by Mycobacterium ulcerans, is a debilitating disease that requires complex treatments. Several other species of mycobacteria can cause disease in animals or can cause opportunistic infections in humans.

Mycobacterium marinum is the causative agent of a tuberculosis-like disease in poikilothermic animals such as fish and frogs. It is very closely related to M. tuberculosis and M. ulcerans, however, and can also cause an infection, known as fish tank granuloma, in humans (van der Sar et al. 2004). This has led to the use of M. marinum as a model organism for tuberculosis research.

A defining characteristic, which sets mycobacteria apart from Gram-negative and Gram-positive bacteria, is their unique cell envelope. Mycobacteria possess an outer membrane (Zuber et al. 2008; Hoffmann et al. 2008), which consists of mycolic acids. These long-chain (C_{60}-C_{90}) fatty acids are covalently linked to a peptidoglycan/arabinogalactan periplasmic layer (P J Brennan and Nikaido 1995). This hydrophobic
permeability barrier makes mycobacteria extremely resistant to the harmful effects of antibiotics and host defence mechanisms such as oxygen radicals or antibiotic peptides (Purdy, Niederweis, and Russell 2009; Gao et al. 2003). This feature also allows mycobacteria to withstand the extremely harsh environment in the macrophage compartment, known as the phagosome, for limited amounts of time. A more loosely associated capsular layer is present outside the outer membrane of the mycobacteria (Sani et al. 2010; Daffé and Etienne 1999; Ortalo-Magné et al. 1996). This capsule consists of proteins, polysaccharides and glycolipids, components that have been implicated in immune modulation and pathogenesis (Geurtsen et al. 2009; Gagliardi et al. 2007; Geijtenbeek et al. 2003), but the function of the capsule in pathogenesis remains largely unknown.

Pathogenesis and Protein secretion

When *M. tuberculosis* enters the body, usually by inhalation of infected aerosols, bacteria are recognized by lung macrophages. Macrophages take up the mycobacteria and contain them in their phagosomes. The phagosomal compartment is meant to fuse with the lysosome. For most other bacteria this process would mean quick death and eradication from the body as the lysosome is filled with anti-bacterial molecules such as antibiotic peptides, nitrogen and oxygen radicals, and proteases. However, *M. tuberculosis* is protected from these attacks by its impermeable cell envelope and additionally secretes proteinaceous virulence factors that allow the bacteria survive. These proteins allow *M. tuberculosis* to delay the fusion of phagosomes with lysosomes (Stewart et al. 2005), but also allow the mycobacteria to even rupture the phagosome and enter the macrophage cytoplasm (van der Wel et al. 2007; Simeone et al. 2012; Simeone et al. 2015). This environment is considered less hostile for mycobacteria than the phagolysosome, and it contains more nutrients. To secrete proteins through their unique cell envelope, mycobacteria have developed specialized secretion systems known as type VII secretion (T7S) systems (Bitter et al. 2009). *M. tuberculosis* contains five different of these T7S systems called ESX-1 to ESX-5 (Bitter et al. 2009). These secretion systems have only been discovered over the last two decades; they have been shown to be absolutely essential for the pathogenesis of tuberculosis (Reviewed in Chapter 2) (Pym et al. 2002). The ESX-1 system is the best-studied of the T7S systems and its substrates are responsible for the escape of pathogenic mycobacteria from the phagosome (van der Wel et al. 2007; Simeone et al. 2012). The ESX-3 system is involved in the uptake of iron and zinc, although it is not precisely understood by what mechanism this occurs (Serafini et al. 2013; Siegrist et al. 2009; Siegrist et al. 2014; Serafini et al. 2009). The ESX-5 system and its substrates are the subject of this thesis; they have many enigmatic characteristics. ESX-5, the most recently evolved T7S system, is only found in the slow-growing mycobacteria, most of which are pathogenic (Gey van Pittius et al. 2006). ESX-5 is responsible for the secretion of the majority of the so-called PE and PPE proteins (Abdallah et al. 2009; Ates et al. 2015). An important subgroup of the PE proteins, the PE_PGRS
proteins, so named because of their polymorphic GC-rich sequences (Cole et al. 1998). The biological role of ESX-5 and the PE and PPE proteins, is still largely unknown, although PE_PGRS proteins have been implicated to be in immune modulation (reviewed in chapter 2).

**SCOPE OF THIS THESIS**

The focus of this thesis is to acquire more insight into the functioning of the ESX-5 system, and the role of its substrates. A common first approach to investigate the role of a protein is to try to make a mutation in the gene that encodes this protein. However, work by my colleagues already indicated that it was not possible to make mutations in the conserved components of the ESX-5 system; i.e. the genes of interest were essential for growth. In chapter 3 we set out to prove that the ESX-5 system was indeed essential for growth. We discovered that when genes involved in the biogenesis of specific outer membrane lipids were mutated, the mutations in esx-5 were no longer lethal. Since the lack of these membrane lipids led to a more leaky outer membrane, we hypothesized that we could achieve the same effect by introducing an outer membrane porin from *M. smegmatis*, a non-pathogenic species of mycobacterium, which does not have ESX-5. After introduction of this porin in *M. marinum* we could indeed create several different ESX-5 mutants; this allowed us to study the ESX-5 system in much more detail then before. One of the results was that substrates of the ESX-5 system are involved in the uptake of fatty acids and probably also other nutrients.

In chapters 4 and 5 we focused on the role of two different substrates secreted by the ESX-5 system. Both studies were instigated by earlier screens in which we tried to identify ESX-5 negative secretion mutants (Abdallah et al. 2006; Ates et al. 2015; van der Woude et al. 2012). In chapter 4 we describe the identification of a mutant in the gene ppe10 which seemed to secrete increased amounts of PE_PGRS proteins. These proteins were, however, not produced or secreted in higher amounts, but were more loosely attached to the cell surface, because of reduced capsular integrity. We visualized the morphology of the capsule of this esx-5 mutant strain with electron microscopy and showed that the capsule of this mutant was malformed. Strains with reduced capsular integrity had fewer ESX-1 substrates on their cell surface and therefore were impaired in phagosomal rupture in the early stages of infection in both macrophage- and zebrafish infection experiments. That ESX-5 plays a role in capsule integrity is an important new finding; we were able to pinpoint this phenotype to a single ESX-5 substrate: PPE10.

In chapter 5 we identified a mutant in the gene encoding another substrate of ESX-5: PPE38. A *M. marinum* strain with a mutation in ppe38 appeared unable to secrete PE_PGRS proteins, although the ESX-5 system itself was not affected. When we made similar mutations in the orthologue of ppe38 in *M. tuberculosis* we saw that these mutants were also unable to secrete PE_PGRS proteins, while other T7S substrates were secreted normally. In earlier literature we found that certain clinical
M. tuberculosis strains, belonging to the ‘Beijing’ lineage, have naturally occurring mutations in ppe38, and surrounding genes, due to genetic recombination events (McEvoy, Warren, et al. 2009; McEvoy, van Helden, et al. 2009). We obtained these clinical strains from the group of Rob Warren at Stellenbosch University in South Africa. We were able to show that these clinical strains are also deficient in the secretion of specific subsets of the PE and PPE proteins (PE_PGRS and PPE_MPTR). Furthermore, when we reintroduced ppe38 and the surrounding genes in these isolates, these strains secreted PE_PGRS proteins like wild type M. tuberculosis. In collaboration with Professor Rogelio Hernandez-Pando in Mexico we performed in vivo experiments in mice to test the virulence of these strains. The naturally occurring ppe38 mutants were hypervirulent in this murine model. Mice infected with clinical isolates with the ppe38 mutation were more ill and died earlier than mice infected with wildtype strains. The hypervirulent phenotype could be partially reversed by reintroducing the ppe38 locus in the ppe38 mutants. This study showed that PPE38 is required for the secretion of specific groups of ESX-5 substrates and that loss of PPE38 and concomitant secretion defects lead to hypervirulence in mice of clinical M. tuberculosis isolates.

In chapter 6 we attempted to translate our findings on ESX-5 secretion to achieve secretion of non-mycobacterial proteins (heterologous secretion) through ESX-5; Heterologous secretion can be useful for vaccine design, or for fundamental research purposes. For this purpose, we investigated the possibility to use LipY, a substrate of the ESX-5 system which has been intensively studied by our group (Daleke et al. 2011; Daleke, Ummels, et al. 2012). We first defined the minimal domains that are required for the secretion of LipY. We fused these domains to an artificial protein derived from chicken egg ovalbumin (OVA), and investigated if this non-mycobacterial protein was secreted through the ESX-5 system. The secretion of the LipY-OVA fusion protein was indeed successful, but very inefficient. Therefore we set up a method to find mutations that would increase the secretion of this construct. By introducing random mutations in the construct with an error-prone DNA-polymerase and checking for colonies with more efficient secretion we were able to significantly optimize the secretion of LipY-OVA, thereby showing that this is a good method to optimize heterologous secretion.

The results obtained in this thesis are summarized and discussed in chapter 7, the summarizing discussion.