1 Introduction

Mycobacteria constitute a heterologous genus comprising highly pathogenic species like the members of the *Mycobacterium tuberculosis*-complex as well as less pathogenic or opportunistic species like *Mycobacterium fortuitum* or *Mycobacterium smegmatis*. Many species of the genus are of particular medical importance because they cause severe diseases. In general mycobacteria are distinguished by their growth rates between fast- and slow-growers.

1.1 Mycobacteria are distinguished by their growth characteristics

It is eye-catching that the highly pathogenic *Mycobacterium* species like members of the *M. tuberculosis*-complex, *Mycobacterium leprae* or *Mycobacterium avium* belong to the slow-growing mycobacteria with generation times of more than 5 hours, whereas many apathogenic or opportunistic *Mycobacterium* species like for example *M. peregrinum, M. smegmatis* or *M. fortuitum* belong to the rapidly growing mycobacteria (RGM) having generation times of less than 5 hours (Table 1). *M. tuberculosis*, for example, has a generation time of 14 – 15 hours under optimal conditions (1999). It is unknown, if and in which way the differences in their growth rates may contribute to the virulence and intracellular persistence of different *Mycobacterium* species.

Table 1: Classification of mycobacteria associated with their growth characteristics. *: Members of the *M. tuberculosis*-complex.

Species	Risk group	Growth	Intracellular persistence
M. tuberculosis *	3	slow-growing	+
M. africanum *	3	slow-growing	+
M. bovis *	3	slow-growing	+
M. leprae	3	non-cultivable	+
M. ulcerans	3	slow-growing	+
M. avium	2	slow-growing	+
M. intracellulare	2	slow-growing	+
M. chelonae	2	fast-growing	?
M. fortuitum	2	fast-growing	+
M. smegmatis	2	fast-growing	-
M. peregrinum	1	fast-growing	-
M. phlei	1	fast-growing	?

Several hypotheses are discussed as possible reasons for the different growth rates of slow- and fast-growing mycobacteria. Hiriyanna et al. (1986) found the DNA elongation rate in *M. tuberculosis* to be eleven times slower than in *M. smegmatis*. Similarly, the RNA elongation rate of *M. tuberculosis* was shown to be ten times slower compared with *Escherichia coli* (Harshey & Ramakrishnan, 1977). Another hypothesis assumed that the amount of rRNA molecules influences the growth rates. While slow-growing mycobacteria often only possess one rRNA operon, RGM usually have two rRNA operons and/or strong promoters in front of their rRNA operons (Bashyam et al., 1996; Bercovier et al., 1986; Gonzalez-y-Merchand et al., 1996; Gonzalez-y-Merchand et al., 1997; Gonzalez-y-Merchand et al., 1998; Ji et al., 1994b; Ji et al., 1994a; Verma et al., 1999). The DNA-binding protein MDP1 (mycobacterial DNA-binding protein 1) identified in *M. bovis* BCG (Matsumoto et al., 1999) was also assumed to slow down growth (Furugen et al., 2001; Matsumoto et al., 2000). An important characteristic biological property of mycobacteria probably influencing the growth characteristics is their thick hydrophobic cell wall (Jarlier & Nikaido, 1990). The present model of the mycobacterial cell wall (Figure 1) includes the presence of an outer membrane (OM). The OM is composed of long fatty acids, the mycolic acids (up to 90 carbon atoms), and non-covalently bound lipids, which complement the ordered arrangement of mycolic acids to an asymmetric bilayer (Niederweis, 2003).

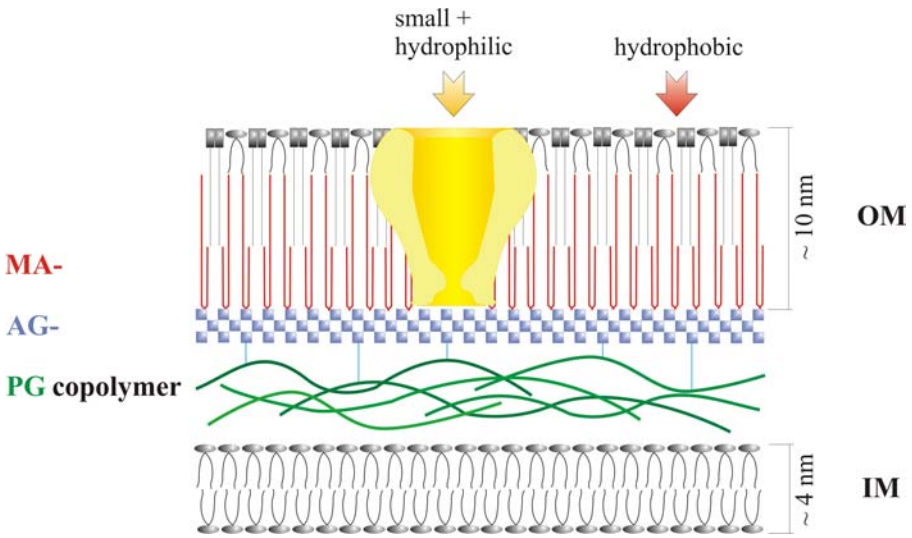

Figure 1: Structural model of the mycobacterial cell wall. The asymmetric OM is composed of lipids and mycolic acids, which are covalently bound to the arabinogalactan-peptidoglycan co-polymer. The OM is penetrated by porins mediating the uptake of hydrophilic substances across the membrane. (Abbreviations: AG: arabinogalactan; IM: inner membrane; MA: mycolic acid; OM: outer membrane; PG: peptidoglycan).

Because of the length of mycolic acids, the mycobacterial OM is the thickest known biological membrane with a very low fluidity. Brennan and Nikaido (1995) proposed the mycobacterial OM to be an efficient permeability barrier protecting the bacilli from toxic compounds. It is also thought to be the main determinant of mycobacterial resistance to most common antibiotics or chemotherapeutic agents. Diffusion of small hydrophilic nutrients across this extraordinary hydrophobic barrier is mediated by porins penetrating the OM.

1.2 The extremely hydrophobic mycobacterial OM is penetrated by porins

Niederweis et al. (1999) identified a new type of porin (MspA) in *M. smegmatis* and indicated that homologous genes seem to be present in RGM, but apparently absent in slow-growers (Niederweis et al., 1999). MspA is an extremely stable octameric protein composed of 20 kDa monomers (Faller et al., 2004; Heinz et al., 2003a). It has a selectivity for cations and the single channel conductance amounts to 4.6 nS (Niederweis et al., 1999). Besides the *mspA* gene, *M. smegmatis* possesses three homologous genes named *mspB*, *mspC*, and *mspD*. The main diffusion pathway of *M. smegmatis* is provided by MspA (Engelhardt et al., 2002; Stahl et al., 2001). A mutant strain with a deletion of *mspA* exhibited a 9 fold reduced permeability for cephaloridine and a 4 fold reduced permeability for glucose. However, the growth rate of the *mspA* deletion mutant in minimal medium with glucose as carbon source did not differ from the growth rate of the wild type (Stahl et al., 2001). Lichtinger et al. (1999) detected in detergent extracts of *M. bovis* BCG a porin that produced channels with a conductance of 0.8 nS with selectivity for anions and another channel of 4 nS. In accordance with these experiments, two other research groups demonstrated the existence of channels in the cell wall of the closely related species *M. tuberculosis*. Kartmann et al. (1999) described two porins in *M. tuberculosis*. One of them is composed of 15 kDa subunits and has a channel conductance of 0.7 nS. The other porin is a 60 kDa protein with a conductance of 3 nS. Based on nucleic acid sequence homology, Senaratne et al. (1998) identified the channel protein OmpATb from *M. tuberculosis*. OmpATb has a MW of 38 kDa, a pore diameter of 1.4 to 1.8 nm and a single channel conductance of only 0.7 nS. An *ompATb* deletion mutant was shown to be impaired in growth at low pH and in the ability to grow in macrophages (Raynaud et al., 2002). However, in general the amount of protein was too low to allow a characterization of proteins. Porins of members of the *M. tuberculosis*-complex are of particular interest because three of four first line tuberculosis drugs are small hydrophilic molecules and understanding

the porin pathway would promote the design of new drugs to fight tuberculosis (Niederweis, 2003).

Porins not only facilitate the diffusion of small hydrophilic molecules into the cell, but can also be involved in various stages of the infection process. For example, porins can function as binding sites of components of the complement cascade; and as adhesin they can fortify invasiveness. Porins can influence apoptosis, inhibit phagocyte function and induce cytokine expression (Achouak et al., 2001; Galdiero et al., 2003). Porins from *Neisseria*, for example, are involved in multiple functions during the infection process. They can activate B cells and other antigen-presenting cells thereby acting as adjuvants. Their effect on the immune response is mediated by upregulation of the costimulatory molecule B7-2 on the surface of antigen-presenting cells. Neisserial porins can also interact with components of the complement cascade and by co-localization with mitochondria modulate apoptosis (Massari et al., 2003).

1.3 Many slow-growing mycobacteria cause severe diseases

Various slow-growing mycobacteria are capable to cause serious diseases. For example *M. leprae*, the causative agent of leprosy, is despite decreasing global prevalence still endemic in countries such as India, Vietnam or the Philippines. Leprosy is a transmissible infectious disease, which leads to skin lesions and peripheral nerve enlargement and impairment (Boggild et al., 2004). But the most common pathogen among the genus is *M. tuberculosis*, the causative agent of human tuberculosis, which causes latent and acute illness. Tuberculosis is regarded as re-emerging disease causing more than 1.5 million deaths per year. Every second someone in the world is newly infected with *M. tuberculosis* and one third of the world's population is latently infected and is at risk to develop active tuberculosis during the lifetime. *M. tuberculosis* world-wide kills more people than any other bacterial pathogen (www.who.int/topics/tuberculosis/en/). Owing to the persistence of *M. tuberculosis* in infected individuals and the increasing frequency of antibiotic resistant strains, treatment of tuberculosis requires medication with a combination of different antibiotics (first line tuberculosis drugs: rifampin, isoniazid, pyrazinamide and ethambutol) during at least six months. In many countries, such a long and expensive therapy cannot reliably be administered. The only vaccine available, the attenuated *M. bovis* derivative BCG (Bacillus Calmette Guérin) is non-satisfying because of its poor protective effect (Dietrich et al., 2003).

The most frequent disease pattern caused by *M. tuberculosis* is a disease of lungs. Inhaled droplets containing a few numbers of bacilli are engulfed by alveolar macrophages and through interaction of mycobacterial components with Toll-Like receptors (TLR) the macrophages produce cytokines and chemokines that serve as signals of infection. The signals result in migration of monocyte-derived macrophages and dendritic cells to the site of infection in the lungs. Dendritic cells containing mycobacteria migrate to the local lymph nodes and recruit CD4+ and CD8+ T cells, which are primed against mycobacterial antigens. These T cells expand and migrate back to the lungs (the origin of infection). The migration of macrophages, T cells and B cells to the site of infection results in formation of a granuloma, a characteristic element of tuberculosis. Also dendritic cells, endothelial cells and fibroblasts participate in formation of a granuloma. Mycobacteria remain in this restricted environment but are not eradicated and the host is latently infected. (Tufariello et al., 2003). Although *M. tuberculosis* persists intracellularly in the early phagosomal compartment by inhibiting the phagolysosome fusion, activated macrophages, which produce reactive oxygen and nitrogen intermediates, are able to kill a part of the bacteria or prevent them from replication. Due to the infection with *M. tuberculosis*, different T cell populations (T helper 1) produce interferon γ (IFN-γ), which is the major mediator of macrophage activation beside tumor necrosis factor α (TNFα) (Kaufmann, 2002). While the molecular components and pathways of the host immune response are well studied, the mechanisms of virulence of *M. tuberculosis*, such as dormancy and persistence remain barely investigated.

1.4 Pathogenic rapidly growing mycobacteria

The species of rapidly growing mycobacteria able to cause human disease belong basically to the *M. fortuitum*-group, the *Mycobacterium chelonae/abscessus*-group and the *M. smegmatis*-group. Members of these groups are commonly seen in municipal tap water and health care associated outbreaks are often associated with contact to tap water or water sources such as ice (Brown-Elliott & Wallace, 2002).

The *M. fortuitum*-group includes three taxa: *M. fortuitum*, *M. peregrinum* and a third biovariant complex. The *M. fortuitum*-group is involved in 60% of localized cutaneous infections in immunocompetent persons caused by RGM but is a rare cause of pulmonary disease. Most or all of the cases of community-acquired or health care-associated diseases caused by the *M. fortuitum*-group are due to *M. fortuitum*. This species basically causes skin

lesions, wound infections, postinjection abscesses, postsurgical wound infections or pulmonary disease in previously healthy hosts. Little is known about the virulence mechanisms and persistence of this human pathogen. However, Cirillo et al. (1997) showed *M. fortuitum* to be capable to replicate in amoebae. Unlike *M. fortuitum*, there is no published review evaluating the clinical significance of *M. peregrinum* (Brown-Elliott & Wallace, 2002).

The most common member of the *M. smegmatis*-group is *M. smegmatis*, a saprophytic species, which occasionally is capable to cause skin and soft tissue lesions. It has been reported to be involved in cellulitis, localized abscesses and osteomyelitis of wound sites following traumatic events. Furthermore, health care-associated diseases, for example, catheter sepsis, infected pacemaker sites and sternal wound infections can be caused by *M. smegmatis* (Brown-Elliott & Wallace, 2002). Lung infections caused by *M. smegmatis* occur rarely (Daley & Griffith, 2002; Howard & Byrd, 2000; Kumar et al., 1995; Schreiber et al., 2001; Vonmoos et al., 1986). However, *M. smegmatis* has been identified as causative agent of fatal disseminated disease in patients with IFN-γ receptor deficiencies (Andrews & Sullivan, 2003; Howard & Byrd, 2000; Jouanguy et al., 1999; Pierre-Audigier et al., 1997). These patients are often heavily affected by otherwise poorly pathogenic mycobacteria, since the ability to respond to IFN-γ is of crucial importance for the destruction of intracellular pathogens. The ability of *M. smegmatis* to cause severe disease in patients not responding properly to IFN-γ gave rise to investigate the factors influencing its intracellular persistence. *M. smegmatis* is generally considered to be an environmental saprophytic bacterium. Unlike the typical intracellularly growing bacteria of the *M. tuberculosis*-complex, *M. smegmatis* is not able to inhibit the acidification of the phagosome (Kuehnel et al., 2001). Nevertheless, *M. smegmatis* has some capacity to persist intracellularly in mononuclear phagocytes and has been reported to grow during the first day after infection and to be partly eliminated during the second day (Lagier et al., 1998).

1.5 Environmental persistence of mycobacteria

Another interesting feature of some mycobacterial species is their ability to survive inside amoebae, classifying mycobacteria as “amoeba-resistant microorganisms” (Greub & Raoult, 2004). The mechanisms used by macrophages and amoebae for phagocytosis, phagolysosome formation and digestion of intracellular bacteria are very similar (Allen &

Dawidowicz, 1990a; Allen & Dawidowicz, 1990b; Brown & Barker, 1999; Greub & Raoult, 2004; Winiecka-Krusnell & Linder, 2001). Reciprocally, the strategies employed by bacteria to escape destruction by macrophages or amoebae are also similar. *M. avium*, for example, survives in macrophages by inhibiting lysosomal fusion and the same survival strategy is used in amoebae (Brown & Barker, 1999; Cirillo et al., 1997; Steinert et al., 1998). The parallels between the interaction of bacteria with macrophages and with amoebae are best studied for *Legionella pneumophila*. The *pmi* genes (protozoan and macrophage infection) of *L. pneumophila* are required for survival both in macrophages and in amoebae. Additionally, *L. pneumophila* possesses the *mil* genes (macrophage-specific infectivity loci), which are essential only for survival in macrophages (Kwaik Y.A et al., 1998). This supports the theory that an evolutionary selection for survival in environmental protozoa has enabled intracellular pathogenic bacteria to develop the capacities necessary for survival in macrophages (Brown & Barker, 1999; Steinert et al., 1998; Winiecka-Krusnell & Linder, 2001). In this context, it is interesting that passage through amoebae can enhance the virulence of pathogenic intracellular bacteria. As shown by Cirillo et al. (1997), growth of *M. avium* in amoebae enhances entry into epithelial cells and intracellular replication. Amoeba-grown *M. avium* are also more virulent in the beige mouse model of infection. Cirillo et al. observed a correlation between the virulence of mycobacterial species and survival in amoebae. This correlation was also reported by other authors (Neumeister, 2004; Pozos & Ramakrishan, 2004; Strahl et al., 2001) and supports the proposal to use amoebae, in addition to cell lines and animals, as model systems to study persistence.

1.6 Goals of this study

Mycobacteria differ not only by their various growth rates but also the ability to persist intracellularly. Whereas members of the *M. tuberculosis*-complex are known to persist intracellularly within macrophages (Taylor et al., 2003) and protozoa (Deretic & Fratti, 1999), *M. smegmatis* was shown to be killed by human monocytes and *A. castellanii* (Barker et al., 1996; Cirillo et al., 1997). As mentioned above, it is evident that a major difference between fast- and slow-growing mycobacteria lies in their equipment with porins (Niederweis, 2003). This work will address the question, how the diverse equipment of mycobacteria with porins affects their growth and intracellular survival.

The aim of the first part of this study was to express the *mspA* gene from *M. smegmatis* in *M. bovis* BCG and to analyze the effects of its expression on growth characteristics and intracellular persistence of *M. bovis* BCG.

To address the question if porins from RGM like *M. smegmatis* have the potential to influence the infection process, two porin mutants from *M. smegmatis* were used and their extracellular and intracellular growth was analyzed. In the first mutant *mspA* was deleted (Stahl et al., 2001), while in the second mutant *mspA* and *mspC* were deleted (Stephan et al., 2004). Intracellular persistence of the mutants compared to the parental strain was analyzed in different phagocytic cells including amoebae. For a better understanding of the impact of mycobacterial porins on virulence, porins from members of the *M. fortuitum*-group, which were isolated from human patients, were analyzed.