## 4 Discussion

The majority of the members of the genus *Mycobacterium* are opportunistic or even non-pathogenic environmental species. Some members of the genus, however, are highly successful pathogens like *M. tuberculosis*, *M. leprae* and *M. ulcerans*, which cause severe human diseases and are of immense medical importance. *M. tuberculosis* is the causative agent of human tuberculosis and due to the infection with this bacterium two million persons are killed annually (WHO, Tuberculosis Fact Sheet N°104, 2004). Three characteristics of *M. tuberculosis* result in its successful expansion as a hardy pathogen and are commonly used to describe this bacterium: latency, dormancy and persistence. *M. tuberculosis* is able to persist in various ways. The most important way to persist is on the cellular level. It resides intracellularly within macrophages, cells that are intended to prevent infections by eradication of pathogens. Moreover, *M. tuberculosis* is able to prevent elimination from the host despite of development of cell-mediated immunity. Another nontrivial way of persistence is the slow elimination of *M. tuberculosis* by anti-tuberculosis drugs (Gomez & McKinney, 2004). It is of particular interest to understand the mechanisms of persistence of mycobacteria and to understand why slow-growing and highly pathogenic mycobacteria are such successful persisters, whereas fast-growing and non-pathogenic species are eradicated by phagocytes within few days (Barker et al., 1996; Cirillo et al., 1997).

Being concerned with mycobacteria the following questions arise. Why do the highly pathogenic species belong to the slow-growers, whereas the non-pathogenic or opportunistic mycobacteria are fast-growers? And is slow growth in principle a factor of virulence? As already mentioned in the introduction, several hypotheses have been discussed during the past years, which itemize probable reasons for the slow growth. Jarlier & Nikaido (1990) discussed the extremely hydrophobic cell wall of mycobacteria to limit the permeability for small hydrophilic molecules and to restrict their growth. The principal structure of the mycobacterial cell wall is known to be similar among the members of the genus (Paul & Beveridge, 1992). However, the equipment of the OM of fast- and slow-growing mycobacteria with porins differs (Niederweis, 2003). The major porin MspA from *M. smegmatis* is apparently absent in slow-growing pathogenic mycobacteria (Niederweis et al., 1999). Porins forming water-filled channels in the cell wall permit diffusion of small and hydrophilic molecules through the mycobacterial OM and are consequently of importance for nutrient supply (Stahl et al., 2001). On the other hand, mycobacterial porins have also been

associated with secretion of proteins from the periplasmic space into the environment (Wiker, 2001). The intention of this study was to find out if and to which degree the presence of porins in the OM of mycobacteria affects their growth characteristics and intracellular persistence.

Heterologous porin expression is an adequate approach to study the importance of the cell wall permeability for slow-growing mycobacteria like *M. bovis* BCG. For this purpose the coding sequence of *mspA* was cloned behind the *hsp60* promoter in a shuttle vector. However, this construct revealed to be unstable after transformation into *M. bovis* BCG (data not shown). This is in accordance with observations of other authors (Al-Zarouni & Dale, 2002; Haeseleer, 1994; Kumar et al., 1998), who demonstrated structural instability of transcriptional fusions with the *hsp60* promoter in *M. bovis* BCG. Al-Zarouni & Dale (2002) have reported frequent deletions in the *hsp60* promoter and the tagged gene.

Therefore, a 3429 bp DNA fragment from *M. smegmatis* carrying the porin gene *mspA* with its own promoter as well as three additional ORFs was inserted into the integrative vector pMV306 and the recombinant plasmid (pSSa100) was transformed into *M. bovis* BCG. Since pMV306 integrates with one copy at the *att* integration site for phage L5 and does not carry a mycobacterial promoter in front of the cloning site, the *mspA* promoter drives the expression of *mspA* in the transformed *M. bovis* BCG. The plasmid pSSa100 pointed out to be stably maintained in *M. bovis* BCG.

The expression of the *mspA* gene in *M. bovis* BCG was demonstrated by RT-PCR and by Western blot analysis. Using the polyclonal rabbit antiserum to purified MspA (pAK MspA#813) I could illustrate the presence of the oligomeric form of rMspA in protein extracts from *M. bovis* BCG containing pSSa100. However, the amount of rMspA in the *M. bovis* BCG derivative was much lower than in the protein extracts from *M. smegmatis*. Heinz and Niederweis (2000) had enriched the oligomeric form of MspA from *M. smegmatis* by preparing detergent extracts using Genapol X-80 or nOPOE and had shown the intact oligomeric form of MspA to be detectable by means of Western blotting using the antiserum pAK MspA#813. The selective isolation of rMspA from the cell wall of *M. bovis* BCG using detergents like nOPOE yielded lower amounts as the extraction of MspA from *M. smegmatis*. Similar to these results Lichtinger et al. (1999) observed channel forming activity in detergent extracts of *M. bovis* BCG and were also not able to show a defined band of the protein on SDS-PAGE. This suggests that the expression of *mspA* in *M. bovis* BCG was less efficient compared with *M. smegmatis*. Nevertheless the clear signal on the Western blots with a

protein of an apparent molecular mass of 116 kDa proves the successful expression and assembly of rMspA in the *M. bovis* BCG derivative. Heinz and Niederweis (2000) showed the molecular mass of the oligomeric form of MspA to be dependant on the electrophoretic conditions and to vary between 100 and 115 kDa, which is in good agreement with the results in this study (Figure 3 B, lanes 1 and 2).

Growth of the *M. bovis* BCG transformant containing pSSa100 on agar plates was clearly enhanced as shown by measuring the cellular ATP concentrations. The cellular ATP content of the *mspA*-expressing *M. bovis* BCG was two to four-fold higher than that of *M. bovis* BCG containing the vector pMV306. This in turn reflects the faster growth of the porin expressing *M. bovis* BCG on plate. Accuracy of growth measurement by this method requires homogenous lysis of the bacteria. This was guaranteed by the use of a lysis buffer, which ensured complete lysis. The quantification of the cellular ATP concentration of colonies turned out to be a reproducible and convenient method to determine the growth of *M. bovis* BCG on plates and was more precise than the observation of the time of colony appearance or measuring of colony size. Mycobacterial colonies in general show variations in colony sizes caused by aggregate formation (Fenner, 1951) in cultures. This variation in colony size is also visible in Figure 4 C and supports the need for other methods, like measurements of cellular ATP contents, for the determination of growth on plates.

Mutagenesis analysis confirmed the *mspA* gene to be responsible for the growth improvement. This result strongly supports the hypothesis that differences concerning the equipment with porins and consequently the permeability of the cell wall for nutrients influence the generation time of mycobacteria. These results are also supported by the observations of Mailaender et al. (2004), who also introduced *mspA* into *M. bovis* BCG and *M. tuberculosis*. By using a transcriptional fusion of the coding sequence of *mspA* with the relatively strong promoter $p_{imyc}$ (Kaps et al., 2001), they could show increased cell wall permeability of the *mspA* expressing *M. bovis* BCG for glucose. They also showed a very small but significant growth advantage of the *mspA* expressing *M. bovis* BCG in broth.

At first sight these observations seem to be in contrast to observations of Stahl et al. (2001), who found only a very slight growth retardation of a Δ*mspA* mutant of *M. smegmatis* compared with the wild type in minimal medium with either 1mM glucose or 1 mM glucosamine. It must, however, be taken into consideration that *M. smegmatis* possesses additionally to *mspA* the three highly homologous genes *mspB*, *mspC* and *mspD*, which may partially compensate the *mspA* mutation.

Although the presence of rMspA enhanced the growth of *M. bovis* BCG on plates, the *mspA* expressing *M. bovis* BCG derivatives were still "slow-growing" and by far not as fast as any fast-growing *Mycobacterium*. The generation time of mycobacteria is determined by several concomitant factors (see introduction), one of which according to the results of this study is the equipment of the OM with porins and hence, the permeability of the mycobacterial cell wall.

After it was shown that heterologous expression of *mspA* in *M. bovis* BCG affects its growth in vitro, it was of great interest to find out if and how MspA influenced intracellular persistence of the *M. bovis* BCG derivative. The murine macrophage cell line J774A.1 as well as the human alveolar pneumocyte epithelial cell line A549 were chosen for persistence experiments. Both cell lines were shown to be efficiently infected by *M. tuberculosis* (Mehta et al., 1996). The prerequisite for measurement of intracellular multiplication is a method producing results, which are not biased by extracellular bacteria, and therefore much effort was put in establishing and verifying the infection experiments. Preliminary tests were performed by infecting both cell lines with a *gfp*-marked *M. bovis* BCG strain and observed the kinetics of phagocytosis by scanning electron microscopy and confocal laser scanning fluorescence microscopy. This permitted identification of the optimal infection time for the two cell lines and proof of almost complete absence of extracellular bacteria at this point of time. Real-time PCR was employed to quantify intracellular *M. bovis* BCG, because this method is more precise and even faster than colony counting. Colony counting is known to be inexact due to clumping of mycobacteria, particularly upon growth in macrophages. Furthermore, it has been reported that CFU plating underestimates the number of intracellular viable mycobacteria (Biketov et al., 2000). As has been discussed in the context of the measurement of cellular ATP content for growth measurements, the accuracy of the use of Real-time PCR for this purpose also requires complete lysis of the mycobacteria. This was achieved by boiling the samples after incubation in the presence of proteinase K and SDS followed by phenol/chloroform extraction (Lewin et al., 2003). Quantification of intracellular mycobacteria by Real-time PCR bears the risk of including dead bacteria. Studies of Barrera et al. (1993) have, however, shown that measurement of growth of intracellular *M. bovis* BCG within macrophages during four days by a PCR method yielded results equivalent to those obtained by CFU counting or measurement of [$^3$H]uracil incorporation.

While in the macrophage cell line J774A.1 an increase in the number of *M. bovis* BCG expressing rMspA occurred during the entire course of the infection, the number of

intracellular bacilli in A549 transiently declined 74 h post infection. There is no evident explanation for this difference. Nevertheless, the outcome of the infection experiments clearly showed that the transfer of the *mspA* gene into *M. bovis* BCG enhanced its survival and multiplication in macrophages as well as in pneumocytes. It has been shown that nutrients available to the bacteria are limiting during infection (Sassetti & Rubin, 2003). Therefore, a feasible explanation for enhanced growth of the *M. bovis* BCG expressing rMspA may be improved nutrient uptake.

Mediation of enhanced survival in macrophages by a mycobacterial channel forming protein has also been described by Raynaud et al. (2002), who observed that an *ompATb* deletion mutant of *M. tuberculosis* was impaired in its growth at low pH and in macrophages. The *ompATb* deletion mutant furthermore was impaired in its ability to grow in normal mice. In mice lacking T cells, the mutant grew comparable to the wild type. This correlates with my observed positive effect of rMspA on intracellular growth of the *M. bovis* BCG derivative. Although OmpATb and MspA do not show any homology and belong to different classes of cell wall pores of mycobacteria, the effect of rMspA and OmpATb in intracellular *M. bovis* BCG and *M. tuberculosis* correlates. The results of this study and the results from Raynaud et al. (2002) indicate that channel forming proteins of mycobacteria may have functions important for host-pathogen interactions. For instance, porins have been shown to function as binding sites for components of the complement cascade (Achouak et al., 2001), to promote adhesion to macrophages (Negm & Pistole, 1999) and invasion of endothelial cells (Prasadarao et al., 1996), to influence maturation of dendritic cells (Jeannin et al., 2000) and phagosomes (Mosleh et al., 1998), to affect cytokine release (Iovane et al., 1998) and to modulate apoptosis (Buommino et al., 1999; Massari et al., 2003).

After it was shown that the heterologous expression of *mspA* affects the intracellular persistence of *M. bovis* BCG, the question arose whether or to which degree *mspA* and other homologous porins influence the intracellular persistence of the non-pathogenic and saprophytic *M. smegmatis*. It was reported that an *mspA* deletion mutant of *M. smegmatis* was impaired in nutrient uptake and also in the diffusion of harmful substances like antibiotics into the cell, which resulted in a multidrug resistant phenotype (Stahl et al., 2001; Stephan et al., 2004a). To investigate the significance of porins from *M. smegmatis* for intracellular survival two porin deletion mutant strains were provided by Dr. M. Niederweis. In *M. smegmatis* MN01 the *mspA* gene was partially deleted (Δ*mspA*) (Stahl et al., 2001) and in *M. smegmatis* ML10 *mspA* and *mspC* were deleted (Δ*mspA*Δ*mspC*) (Stephan et al., 2004b).

The viability of the mutants was compared to the parental strain in phagocytic cells. Compared with survival of the wild type strain, the mutants showed enhanced survival in murine macrophages as well as in *A. castellanii*. Complementation of the porin deletions using two different plasmids, which introduced *mspA* into the mutants, proved the mutation to be responsible for the improved persistence. The improvement of intracellular persistence by deletion of porin genes of *M. smegmatis* was most pronounced when *A. castellanii* was used as test system. Amoebae represent a natural test system to analyze the role of mycobacterial porins for persistence under natural conditions, and these experiments therefore reflect the impact of mycobacterial porins on intracellular survival under real-life conditions. I observed with particular interest the presence of *M. smegmatis* in the walls of cysts of *A. castellanii*. So far, this is the first proof showing that cysts of amoebae might serve as a niche for survival of *M. smegmatis.* The ability of *M. avium* to survive within cysts of *Acanthamoeba polyphaga* was previously demonstrated (Steinert et al., 1998). Cysts of amoebae are extremely resistant against adverse conditions like heat, dryness, lack of nutrients or presence of biocides (Miltner & Bermudez, 2000). The ability of *M. smegmatis* to survive in cysts might therefore be of major importance for its survival in unfavorable environments.

As the enhanced survival of the porin mutants occurred in all three phagocytic systems tested, the question came up, which bactericidal mechanism common to macrophages and amoebae was less effective in eliminating the porin mutants compared with the wild type. Also the diverging effects of the amount of porins on intracellular persistence of *M. bovis* BCG and *M. smegmatis* were interesting. While the transfer of the porin MspA into *M. bovis* BCG enhanced its intracellular survival, *M. smegmatis* showed better intracellular survival after deletion of one or two porin genes.

The cell wall permeability has been shown to influence the susceptibility of mycobacteria to host antimicrobial molecules like defensins and lysozyme (Gao et al., 2003). Differences in the equipment with porins of highly pathogenic slow-growing mycobacteria and the less pathogenic RGM may therefore be one of the factors accounting for their divergent intracellular persistence. The acidification of the phagosome similarly takes place in both macrophages as well as amoebae (McNeil et al., 1983). In contrast to the members of the *M. tuberculosis*-complex, *M. smegmatis* cannot prevent the acidification of the phagosome. After 5 h, the *M. smegmatis* containing phagosomes exhibit a pH of 5.2 (Cotter & Hill, 2003). It is known that *M. tuberculosis* produces ammonia and that ammonia inhibits phagosome-lysosome fusion (Clemens, 1996; Clemens et al., 1995; Gordon et al., 1980), which is one of

the strategies of pathogenic mycobacteria to survive inside macrophages. Export of ammonia by diffusion through rMspA may neutralize the acidification of the phagosome and thus enhance the intracellular growth of *M. bovis* BCG.

The enhanced intracellular survival of the *M. smegmatis* mutants is not caused by a higher resistance towards low phagosomal pH, because the differences in the growth rates between the three strains in broth cultures were not dependent on the pH of the medium. However, *M. smegmatis* is not able to prevent the phagosome maturation and consequently it is exposed to the entire mechanisms of host defense. Such bactericidal mechanisms of phagocytic cells consist in the production of reactive oxygen intermediates, reactive nitrogen intermediates and the activity of antimicrobial peptides and lysosomal enzymes. Reactive oxygen intermediates, lysosomal enzymes and antimicrobial peptides are employed by both macrophages and amoebae for the degradation of intracellular bacteria (Brooks & Schneider, 1985; Bruhn et al., 2003). A reduced permeability of the cell wall of the porin mutants for small hydrophilic molecules may implicate lower accessibility of anti-microbial substances and, as a consequence, better survival of the mutants compared with the parental strain.

In addition to the diffusion of molecules directly through the porin channels, the possibility of altered diffusion rates of molecules through the mycolic acid layer must also be considered. Stephan et al. (2004a) observed an increase in resistance of the Δ*mspA* mutant to hydrophobic antibiotics. They proposed that the integration of porins in the OM might reduce the strong interactions of the mycolic acids and thereby facilitate the diffusion of hydrophobic molecules. A reduced diffusion of harmful hydrophobic substances present in the phagolysosome of phagocytic cells through the mycolic acid layer of the Δ*mspA* and the Δ*mspA*Δ*mspC* mutants may therefore also contribute to their improved intracellular persistence.

Taken together, these data show that the permeability of the mycobacterial cell wall affects the intracellular persistence. The findings also suggest that intracellular persistence of mycobacteria depends, inter alia, on the balance between “walling-off” towards the hostile environment and the uptake of required compounds in the nutrient-depleted phagosomal environment.

To prove this hypothesis more appropriate models are required. Different views have been expressed among scientists, whether *M. smegmatis* could serve as an appropriate model to study aspects related to virulence of highly pathogenic mycobacteria. A notable number of

*M. tuberculosis* genes, which are related to virulence but also play a housekeeping role share closely related homologues in *M. smegmatis*. In the case of common mycobacterial genes *M. smegmatis* was suggested as an appropriate model organism (Reyrat & Kahn, 2001; Tyagi & Sharma, 2002). On the other hand, the physiological differences between *M. smegmatis* and *M. tuberculosis* were mentioned to constrict the significance of direct comparisons (Barry, 2001). Mutagenesis of porin genes in *M. smegmatis* allows the investigation of the impact of cell wall permeability on persistence. However, more appropriate models for such studies must naturally be able to persist intracellularly and probably belong to the RGM. Additionally they must possess a known class of porins. All conditions are fulfilled by *M. fortuitum*. This species is able to infect and grow in phagocytic cells (Sharbati-Tehrani, S., Tykiel, V., Appel, B., and Lewin, A., unpublished data) and also possesses porins homologous to MspA.

Only few studies have been performed to enlighten the virulence mechanisms of pathogenic RGM like *M. fortuitum*. The investigation on the role of porins on persistence of *M. fortuitum* not only contributes to the understanding of mycobacterial persistence but also elucidates the virulence mechanisms of this poorly investigated pathogen. Therefore, I decided to characterize porin genes from *M. fortuitum* as well as from the closely related and non-pathogenic species *M. peregrinum*. The results of this study show that different strains of *M. fortuitum* – including the type strain – as well as two strains of the non-pathogenic *M. peregrinum* feature at least two copies of homologous porins of the MspA class. After cloning one of the two genes, *porM1* was shown to be present in all of the tested members of the *M. fortuitum*-group. The amino acid sequences of PorM1 among the species and strains are highly conserved, whereas the nucleotide sequences vary. Nucleotide sequences of bacterial porins can show large variations but mature proteins have still the same structure (Niederweis, 2003). PorM1 has the same apparent molecular mass as MspA, the antiserum raised against MspA binds well to PorM1, it is accessible at the surface of *M. fortuitum* and the mature PorM1 (without signal peptide) from *M. fortuitum* and *M. peregrinum* exhibits only six or seven amino acid substitutions, respectively if compared to MspA. These features imply very similar functions and characteristics of the two porins.

An interesting finding of this study was that the expression of *porM1* both at the transcriptional level and at the translational level differed among the tested species and strains. As shown by RT-Real-time PCR, ELISA and Western blotting, the porin expression in the members of the *M. fortuitum*-group was significantly lower than in *M. smegmatis*. It was shown that *M. smegmatis* possesses 1000 MspA-like pores per $\mu m^2$ cell wall (Engelhardt

et al., 2002). Since the analyzed strains of *M. fortuitum* and *M. peregrinum* showed clearly decreased *porM1* expression both at the transcriptional level and at the translational level, the amount of pores in the cell wall of *M. fortuitum* and *M. peregrinum* has to be distinctly lower than 1000 pores per $\mu m^2$ cell wall. The differences in porin expression may be associated with different abilities of the strains to persist, which remain to be investigated.

An interesting result from the various genome-sequencing projects is that genome sizes of RGM and the pathogenic slow-growing mycobacteria largely differ. Highly pathogenic species like *M. tuberculosis* or *M. leprae* have genome sizes of about 4.4 Mb and 3.27 Mb, respectively. On the other hand, *M. smegmatis* has a genome size of about 7 Mb, which is similar to that of the related actinomycete *Streptomyces coelicolor*. Brosch et al. (2001) reviewed different data such as 16S rRNA sequences or genome sizes suggesting that the branch of slow-growing mycobacteria represents the most recently evolved part of the genus. They proposed that the loss of genes, rather than gain of genetic material by horizontal transfer contributed to the pathogenicity of slow-growing mycobacteria or to the fine-tuning of their virulence, respectively. Loss of efficient porins like MspA or a decreased density of porins in the cell wall play an important role to "wall-off" towards the hostile phagosomal environment. These aspects are of particular importance for the evolution of a successful intracellular pathogen. In contrast, the presence of several copies of porin genes and in turn a high density of efficient porins in the OM of *M. smegmatis* would provide a selective advantage for saprophytes.

These data suggest that decreased amount of porins in the cell wall of *M. fortuitum* may represent an evolutionary intermediate stage between saprophytic mycobacteria like *M. smegmatis* and the highly pathogenic slow-growing mycobacteria.

Ongoing deletion experiments of *porM1* in *M. fortuitum* 10851/03, which natively shows the lowest porin expression levels among the analyzed strains of *M. fortuitum*, will provide an appropriate model to elucidate the role of mycobacterial porins on virulence. Future studies on mycobacterial porins will contribute to our understanding of pathogenicity and will give rise to design new drugs to fight these hardy pathogens.