Impact of mycobacterial porins on growth and intracellular persistence

(Einfluss mykobakterieller Porine auf Wachstum und intrazelluläre Persistenz)

Dissertation

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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 the 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).

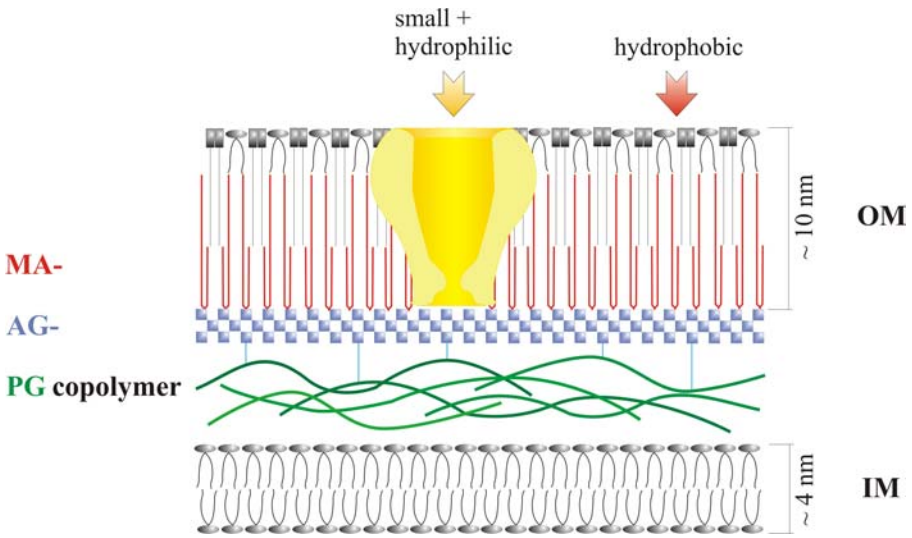

Figure 1: Structural model of the mycobacterial cell wall. The asymmetric OM is composed of lipids and mycolic acids, which are covalently bound to the arabinogalactan-peptidoglycan co-polymer. The OM is penetrated by porins mediating the uptake of hydrophilic substances across the membrane. (Abbreviations: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

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 number 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 persistence of *M. tuberculosis*, such as dormancy and persistence remain barely investigated.

1.4 Pathogenic rapidly growing mycobacteria

The species of rapidly growing mycobacteria (RGM) 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 affect 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.

2 Materials and Methods

2.1 Strains and growth conditions

All mycobacterial strains (Table 2) were grown in Middlebrook 7H9 medium (BD Biosciences, Heidelberg, Germany) supplemented with ADC (BD Biosciences) and 0.05% Tween 80 at 37°C (except of *M. peregrinum* strains, which were grown at 30°C) without shaking or on Middlebrook 7H10 agar (BD Biosciences) supplemented with OADC (BD Biosciences), respectively. Media were supplemented when required with 25 µg ml^{-1} kanamycin or 75 µg ml^{-1} hygromycin B for selection of recombinant mycobacteria. *E. coli* DH5α was grown in LB medium at 37°C (Sambrook et al., 1989). Media were supplemented with 100 µg ml^{-1} kanamycin or 200 µg ml^{-1} hygromycin B for selection of recombinant *E. coli*. 10 µg ml^{-1} tertracycline was added for selection of transposon mutants of *E. coli* DH5α.

Table 2: Mycobacterial strains used in this work.

Strains	Characteristics	Reference
M. bovis BCG (Copenhagen)	Vaccine strain	
M. bovis BCG (pMV306)	BCG derivative, harboring the plasmid pMV306, KM^R	This study
M. bovis BCG (pSSa100)	BCG derivative, harboring the plasmid pSSa100, KM^R	This study
M. smegmatis mc^2155	Type strain	
M. smegmatis SMR5	*M. smegmatis* mc^2155 derivative, SM^R	(Sander et al., 1995)
M. smegmatis MN01	SMR5 derivative, Δ*mspA*	(Stahl et al., 2001)
M. smegmatis MN01 (pMN013)	MN01 derivative, harboring the plasmid pMN013, HYG^R	This study
M. smegmatis MN01 (pSSa100)	MN01 derivative, harboring the plasmid pSSa100, KM^R	This study
M. smegmatis ML10	SMR5 derivative, Δ*mspA* and Δ*mspC*	(Stephan et al., 2004b)
M. smegmatis ML10 (pMN013)	ML10 derivative, harboring the plasmid pMN013, HYG^R	This study
M. smegmatis ML10 (pSSa100)	ML10 derivative, harboring the plasmid pSSa100, KM^R	This study
M. fortuitum DSM 46621	Type strain; HYG^R	
M. fortuitum 10851/03	Human patient isolate, KM^R	This study
M. fortuitum 10860/03	Human patient isolate, KM^R and HYG^R	This study
M. peregrinum 9912/03	Human patient isolate	This study
M. peregrinum 9926/03	Human patient isolate	This study

(HYG: hygromycin; KM: kanamycin; SM: streptomycin)

2.2 Cell line and culture conditions

The mouse macrophage cell line J774A.1 (DSMZ No. ACC170) and the human type II pneumocyte cell line A549 (ATCC No. CCL185) were maintained and passaged twice weekly in DMEM and RPMI 1640 (Gibco^{TM}, Grand Island, NB, USA) respectively. Both media were supplemented with 10% fetal bovine serum (Bio Whittaker, Walkersville, MD, USA). Cultivation of cells was performed in BD Falcon^{TM} 75 cm^2 flasks (BD Biosciences) at 37°C and 5% CO_2 (Lewin et al., 2003).

Murine bone marrow macrophages (BMMs), kindly provided by Stefan Kaulfuss, were derived in vitro from bone marrow progenitors of black female C57BL/6 mice as described previously (Dorner et al., 2002). Prior to infection BMMs were maintained in D-MEM supplemented with 10% foetal calf serum (Biochrom AG, Berlin, Germany), 5% horse serum (Biochrom AG), 1% 1mM Na-Pyruvate and 1% L-Glutamin.

Axenic *A. castellanii* (Walochnik et al., 2000) was grown to 90% confluence at 28°C in the dark in BD Falcon^{TM} 75 cm^2 flasks (BD Biosciences) containing PYG broth (Moffat & Tompkins, 1992).

2.3 Molecular biology techniques

Common molecular biology techniques were carried out according to standard protocols (Sambrook et al., 1989) or according to the recommendations of the manufacturers of kits and enzymes. Sequencing reactions were performed by using the Prism Big Dye^{TM} FS Terminator Cycle Sequencing Ready Reaction Kit from PE Applied Biosystems (Darmstadt, Germany). Transformation of *E. coli* was performed according to the method of Hanahan (Hanahan, 1983).

2.4 In silico analysis

Protein and nucletide sequence analysis was performed using the software packages MacVector^{TM} 7.2.3 (Accelrys, Cambridge, UK) and Lasergene (DNASTAR, Inc., Madison, WI, USA). Signal peptides were predicted using the SignalP 3.0 Server at http://www.cbs.dtu.dk/services/SignalP/ (Bendtsen et al., 2004).

Phylogenetic relationships among the RGM were analyzed using the program ClustalW in the MacVector^{TM} 7.2.3 package. Before analyzing the phylogenetic relationships,

sequences were trimmed in order to start and finish at the same nucleotide position for all employed strains. Phylograms were obtained from nucleotide sequences using the neighbor-joining method with Kimura 2-Parameter distance correction (Kimura, 1980).

2.5 Isolation of genomic DNA from mycobacteria

Mycobacteria were grown to an OD_{600} of 1-1.5. 5 ml of the culture was harvested by centrifugation at 6000 × g and 4°C. The pellet was resuspended in 400 µl TE-buffer (pH 8) and mycobacteria were inactivated at 80°C for 30 min. After cooling down the samples to room temprature, 5 µl of lysozyme (150mg/ml) was added to the suspension and was subsequently incubated at 37°C over night. 70 µl of 10% SDS and 5 µl Proteinase K (20 mg/ml) were added to the lysate. The sample was then incubated for 1 h at 60°C. After addition of 100 µl 5M NaCl and 100 µl CTAB the sample was incubated at 65°C for 10 min. After an initial chloroform extraction, DNA was purified by phenol/chloroform extraction followed by precipitation of DNA by ethanol. The genomic DNA was used for experiments as Southern Blots, cloning or was applied as template in PCRs, respectively.

2.6 Protein preparation and Western Blot

Selective extraction of MspA from *M. smegmatis* was carried out with the detergent n-octylpolyoxyethylene (nOPOE) from Bachem (Heidelberg, Germany) according to Heinz and Niederweis (2000). Isolation of recombinant MspA (rMspA) from *M. bovis* BCG and porins from the *M. fortuitum*-group was performed in PBS buffer supplemented with 0,5% (w/v) nOPOE and 0.2% EDTA (POP05) by slightly modifying the method of Heinz and Niederweis. *M. bovis* BCG was grown to an OD_{600} of up to 3 and members of the *M. fortuitum*-group were grown to an OD_{600} of up to 1. Subsequently about 350 mg of *M. bovis* BCG or 150 mg of *M. fortuitum*-group (wet weight) were washed twice in PBS buffer supplemented with 0.2% EDTA. Pellets were resuspended in POP05 using a ratio of 200 µl POP05 per 100 mg mycobacteria and were incubated at 100°C for 30 min. Afterwards the cell debris was pelleted by centrifugation at 27 000 × g and 4°C, the supernatant was transferred to a new tube and quick-freezed in liquid nitrogen. Quantification of protein samples was carried out using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA).

Protein samples were mixed with 4x loading buffer (Heinz et al., 2003b), incubated for 10 min at room temperature, separated by SDS-PAGE (10%) and transferred at 1.2 mA/cm^2 constant current for 50 – 90 min to a PVDF membrane (Bio-Rad Laboratories GmbH, München, Germany) in the semidry blotter Trans Blot SD (Bio-Rad Laboratories) using Towbin buffer (25 mM Tris, 192 mM glycine and 20% methanol, pH 8.3). The detection of porins in immunoblot experiments was carried out with the polyclonal rabbit antiserum pAK MspA#813; kindly provided by Dr. M. Niederweis; (Heinz & Niederweis, 2000). Western blotting and detection was performed with the BM Chemiluminescence Western Blotting Kit (Roche Diagnostics GmbH, Mannheim, Germany) using a 1:4000 dilution of the antiserum.

2.7 *M. bovis* BCG experiments

2.7.1 Cloning of *mspA* in *M. bovis* BCG

A 3429 bp fragment including *mspA* was obtained from genomic DNA of *M. smegmatis* by PCR using the primers mspA2-I 5'-CGA TAT CCC GAC CGT GAC TGG CTC AAG-3' and mspA2-II 5'-GAA GCT TGT CCA TGA CGG AGT TGG CGG-3' with the LA PCR Kit (TaKaRa BIO Europe S.A., Gennevilliers, France). Preliminary sequence data was obtained from The Institute for Genomic Research through the website at http://www.tigr.org. The 3429 bp fragment was digested with EcoRV and SmaI and cloned into the unique EcoRV site of the plasmid pMV306 (Stover et al., 1991), which is an integrative vector carrying the kanamycin resistance gene *aph* from transposon Tn903, the gene for the integrase and the attP site of phage L5. The recombinant plasmid (pSSa100, Figure 2) was introduced into *M. bovis* BCG by electroporation. For electroporation, competent *M. bovis* BCG were pulsed with 1000 Ω, 25 µF, 2.5 kV in 2 mm electroporation cuvettes.

2.7.2 RT-PCR with mRNA from *M. bovis* BCG derivatives

RNA was extracted from recombinant strains of *M. bovis* BCG according to the method of Bashyam (Bashyam & Tyagi, 1994). 250 ng of the RNA was treated prior to RT-PCR with RQ1 RNase-Free DNase (Promega GmbH, Mannheim, Germany). Half of the treated RNA was added to the RT-PCR reaction carried out with the Access RT-PCR System (Promega). The following specific primers were designed to prove the expression of *mspA* by

amplifying a fragment of 228 bp: AT/POR/FW (5'-TGG ACC GCA ACC GTC TTA CC-3') and AT/POR/BW (5'-GGG TGA TGA CCG AGT TCA GGC-3'). The expression of the selection marker *aph* conferring kanamycin resistance was demonstrated by amplifying a fragment of 556 bp using the primers Tn903/AS1 (5'-TGA GTG ACG ACT GAA TCC GGT GAG A-3') and Tn903/S1 (5'-CGA GGC CGC GAT TAA ATT CCA AC-3'). A non-reverse-transcribed PCR control was performed with the same samples to guarantee the absence of contaminating genomic DNA.

2.7.3 Growth experiments

Dilutions of cultures of recombinant strains of *M. bovis* BCG were grown on Middlebrook 7H11 (BD Biosciences) agar plates supplemented with 0.5% glycerol and OADC (BD Biosciences) and were incubated at 37°C. Colonies per plate were counted, washed from the plate with Middlebrook medium and transferred into a collection tube; afterwards the volume was adjusted to 3 ml. The colonies were resuspended by sonicating in sealed tubes for 10 seconds at 4°C and 450 W with the Branson Sonifier 450 (Branson Ultrasonics Corporation, Danbury, USA). ATP synthesis was chosen as a reference for growth of mycobacteria on plates. The ATP concentration of appropriate dilutions of the resuspended colonies was quantified by three measurements for each sample using the ATP Bioluminescence Assay Kit HS II (Roche) and the microplate luminometer LB96V (Microlumat Plus, EG & G Berthold, Bad Wildbad, Germany) according to the recommendations of the manufacturer. All ATP values were standardized to relative light units (RLU)/100 colonies.

Determination of growth of recombinant strains of *M. bovis* BCG in broth was carried out in Middlebrook 7H9 medium. Afterwards 120 ml of medium was inoculated with log phase *M. bovis* BCG to an OD_{600} of 0.02, evenly distributed into three flasks and incubated at 37°C without shaking. Growth was determined during six weeks by measuring the OD_{600} of cultures and determination of the cellular ATP content using the ATP Bioluminescence Assay Kit HS II (Roche).

2.7.4 In vitro mutagenesis of pSSa100

Mutants of the recombinant plasmid pSSa100 were obtained by in vitro random insertion of a transposon with the EZ::TN^{TM} <TET-1> Insertion Kit (Epicentre, Madison, WI, USA) with tetracycline as a marker for selection of mutants. *E. coli* DH5α was transformed with mutagenized DNA and was grown on plates containing tetracycline and kanamycin. Extracted plasmid DNA of several clones was sequenced to identify insertion loci and an insertion mutant of pSSa100 containing the transposon in *mspA* (Tn#39, Figure 2) was finally introduced into *M. bovis* BCG by electroporation.

2.7.5 Infection of cells and measurement of intracellular growth

Infections of the macrophage cell line J774A.1 and the pneumocyte cell line A549 were performed in 24 well plates (BD Biosciences). 5×10^4 cells of J774A.1 or 7×10^4 cells of A549 per well were seeded and allowed to adhere over night. Cells were then infected at a multiplicity of infection (MOI) of 10 CFU with *M. bovis* BCG. J774A.1 was infected for 6 hours and A549 for 24 hours. Afterwards the supernatants were removed and adherent cells were washed twice with PBS buffer. The cells were then treated with 200 µg ml^{-1} amikacin for two hours to kill the unphagocytosed *M. bovis* BCG. After washing twice with PBS buffer, 1 ml medium supplemented with 1 µg ml^{-1} amikacin was given to each well. Samples for quantification of intracellular bacteria were taken at the end of the infection time after removal and killing of extracellular bacteria and then daily for four days.

The intracellular growth was determined by measuring the DNA synthesis of *M. bovis* BCG by Real-time PCR with the ABIPrism 5700 Sequence detection System (PE Applied Biosystems). For the extraction of DNA 100 µl of the cell lysate was added to 200 µl Te9 buffer (Goelz et al., 1985). The mixture was first incubated at 58°C for 60 min and then at 97°C for 30 min. DNA was extracted with phenol/chloroform, precipitated with ethanol and the pellet was resuspended in 25 µl sterile dH_2O. The *M. bovis* BCG DNA was quantified by amplifying a fragment of 130 bp from the 85B α antigen using the primers MY85FW/BW (5'-TCA GGG GAT GGG GCC TAG-3' and 5'-GCT TGG GGA TCT GCT GCG TA-3', (Desjardin et al., 1996) and the dually labelled detector probe 5'-(FAM)-TCG AGT GAC CCG GCA TGG GAG CGT-3'-(TAMRA) (Hellyer et al., 1999). The reaction was performed with the MBI Fermentas PCR Kit (Fermentas GmbH, St. Leon-Roth, Germany) in 50 µl reaction mix containing 5 µl of the DNA sample as template, 0.2 mM of each dNTP, 3 mM

$MgCl_2$, 150 ng of each primer, 184 nM probe, 1 U Taq DNA polymerase, PCR buffer and 1µM ROX (6-Carboxy-X-rhodamin) as passive reference dye. Amplification was carried out by running a first step at 97°C for 5 min followed by 40 cycles with 30 s at 95°C and 1 min at 63°C. DNA amounts were determined by three measurements for each sample using a standard established with known amounts of genomic *M. bovis* BCG DNA.

2.8 RGM experiments

2.8.1 Construction of *M. smegmatis* deletion mutants and complementation of the mutations

In strain *M. smegmatis* MN01 (Δ*mspA*) the *mspA* gene was partially deleted by homologous recombination as described by Stahl et al. (2001). In strain *M. smegmatis* ML10 (Δ*mspA*Δ*mspC*) the *mspA* and the *mspC* gene were deleted (Stephan et al., 2004b). Both strains were kindly provided by Dr. M. Niederweis.

The plasmids pSSa100 (Sharbati-Tehrani et al., 2004) and pMN013 (Mailaender et al., 2004) were used for complementation of porin mutations in the strains MN01 and ML10. As mentioned above, pSSa100 is an integrative plasmid harboring *mspA* including its own promoter. pMN013 carries a transcriptional fusion of the promoter p_{imyc} with the *mspA* gene. Plasmids were introduced into *M. smegmatis* derivatives via electroporation as described above.

2.8.2 Growth experiments in broth

Determination of growth of *M. smegmatis* strains SMR5, MN01 and ML10 in vitro was carried out in Middlebrook 7H9 medium at the pH 5.0 and pH 6.7. Prior to inoculation, log phase *M. smegmatis* were washed twice with PBS supplemented with 0.05% Tween 80 (PBS-T) to minimize the formation of aggregates. Afterwards 120 ml of medium was inoculated with 3×10^7 CFU, evenly distributed into three flasks and incubated at 37°C without shaking. Growth was determined by measuring the OD_{600} of cultures in triplicate.

2.8.3 Infection of macrophages and measurement of intracellular growth

Infection of the macrophage cell line J774A.1 as well as BMMs was performed as described above for *M. bovis* BCG with the following modifications. 5×10^4 cells/well were seeded in 24 well plates (BD Biosciences). J774A.1 were allowed to adhere for two hours and BMMs over night. Cells were then infected with log phase *M. smegmatis* strains at an MOI of 1 in triplicate. After 4 hours the supernatants were removed and adherent cells were washed twice with medium. The cells were then treated with 200 µg ml^{-1} amikacin for one hour to kill the non-phagocytosed *M. smegmatis*. After washing twice with medium, 1 ml medium supplemented with 2 µg ml^{-1} amikacin was given to each well to prevent extracellular growth. Samples for quantification of intracellular bacteria were taken at the end of the infection time after removal and killing of extracellular bacteria and then twice per day until 54 hours post infection. After removal of supernatants lysis of cells was performed by addition of 1ml sterile dH_2O and incubation at 37°C until complete lysis. The intracellular persistence of *M. smegmatis* was determined by plating and colony counting.

2.8.4 *A. castellanii* Infection

Prior to infection *A. castellanii* monolayers were washed with *A. castellanii* buffer (Moffat & Tompkins, 1992), were harvested and resuspended in *A. castellanii* buffer. 10^5 *A. castellanii*/well were seeded in 24 well plates (BD Biosciences) and allowed to adhere for one hour. Afterwards amoebae were infected with log phase *M. smegmatis* strains at an MOI of 10 (Cirillo et al., 1997) in triplicate. After an initial infection time of 2 h further treatment was performed according to the infection procedure for J774A.1 cells by replacing the medium with *A. castellanii* buffer, except that no amikacin was added to *A. castellanii* buffer after the washing procedure. Intracellular mycobacteria were recovered by lysing the amoebae with PBS supplemented with 0.5% SDS (Cirillo et al., 1997). The intracellular persistence of *M. smegmatis* was determined by plating and colony counting.

At the time point 4 h post infection quantification of DNA of intracellular mycobacteria was performed to confirm consistent uptake of different strains by amoebae using the Mx3000P™ Real-time PCR System (Stratagene, La Jolla, CA, USA). Extraction of DNA was performed as described above. *M. smegmatis* DNA was quantified by amplifying a fragment of 91 bp from the 16S rRNA using the primers myco16STaq FW and BW (5'-AGG GTG ACC GGC CAC ACT G-3' and 5'-ATC AGG CTT GCG CCC ATT GT-3') and the

dually labeled detector probe 5'-FAM-TGA GAT ACG GCC CAG ACT CCT ACG GGA-TAMRA-3'. The reaction was performed with the MBI Fermentas PCR Kit (Fermentas GmbH) in 50 µl reaction mix containing 7 µl of the DNA sample as template, 0.2 mM of each dNTP, 3 mM $MgCl_2$, 100 ng of each primer, 60 nM probe, 1 U Taq DNA polymerase, PCR buffer and 30 nM ROX as passive reference dye. Amplification was carried out by running a first step at 97°C for 5 min followed by 40 cycles with 30 s at 95°C and 1 min at 63°C. DNA amounts were determined by three measurements for each sample using a standard established with known amounts of genomic *M. smegmatis* DNA.

2.8.5 Quantification of expression of porin genes by RT-Real-time PCR

Expression of porin genes in the different strains was determined by means of RT-Real-time PCR using the Mx3000P^{TM} Real-time PCR System (Stratagene). *M. smegmatis* derivatives were grown to an OD_{600} of 0.8 and RNA was extracted according to Bashyam and Tyagi (1994). 1 µg of the RNA was treated prior to RT-Real-time PCR with RQ1 RNase-Free DNase (Promega GmbH). The expression of porin genes from *M. smegmatis* and memebers of the *M. fortuitum*-group was quantified by amplifying a fragment of about 100 bp using the primers and probes as indicated in Table 3. The reaction was performed with the Access RT-PCR System (Promega) in 50 µl reaction mix containing 1 µl (100 ng) of the DNase treated RNA as template, 0.2 mM of each dNTP, 1 mM $MgSO_4$, 40 pmol of each primer, 50 nM probe, 5 U AMV Reverse Transcriptase, 5 U Tfl DNA Polymerase, AMV/Tfl Reaction Buffer and 30 nM ROX as passive reference dye. Amplification was carried out by running a first reverse transcription step at 48°C for 45 min followed by 2 min at 94°C and 40 cycles with 30 s at 94°C and 1 min at 58°C. RNA amounts were determined by three measurements for each sample using a calibration curve established with known amounts of linearized pSSa100. Non-reverse-transcribed PCR controls were performed with the same samples to guarantee the absence of contaminating genomic DNA.

Table 3: Primers and probes used for quantification of porin expression by RT-Real-time PCR.

Gene	Primers and probes	Sequence 5'-3'
mspA		
	mspATaqFW	5'-CGT GCA GCA GTG GGA CAC CTT-3'
	mspATaqBW	5'-CCA CGA TGT ACT TGG CGC GAC-3'
	mspATaqProbe	5'-FAM-TGG ACC GCA ACC GTC TTA CCC GTG AGT G-TAMRA-3'
porM1		
	mfpqPCRfw	5'-CGT TCA GCA GTG GGA CAC CTT-3'
	mfpqPCRrev	5'-CCA CGG TGT ACT TGG CCC GGC-3'
	mfpqPCRprobe	5'-FAM-TGG ACC GCA ACC GGC TGA CCC GTG AGT G-TAMRA-3'

2.8.6 Electron microscopy

For transmission electron microscopy (TEM) of uninfected and infected *A. castellanii*, the *A. castellanii* buffer was replaced by glutaraldehyde (2.5%, v/v) buffered with 0.05 M Hepes (pH 7.2) and fixed first for 1 h at room temperature, then stored at 4°C in the same solution. The cells were first agarose-block embedded by mixing equal volumes of cells and low melting point agarose (3% PBS), postfixed with OsO_4 for 1 h (1% in ddH_2O; Plano, Wetzlar, Germany) and block-stained with uranyl acetate for 1 h (2% in ddH_2O; Merck, Darmstadt, Germany). The samples were then dehydrated stepwise in graded alcohol and embedded in LR-White resin (Science Services, Munich, Germany), which was polymerized at 60°C over night. Ultra thin sections were prepared with an Ultramicrotome (Ultracut S, Leica, Germany) and placed on naked 400-mesh grids. The sections were stained with lead citrate and stabilized with approximately 1.5 nm carbon (carbon evaporation; BAE 250, Bal Tec, Liechtenstein). Transmission electron microscopy was performed with an EM 902 (Zeiss, Oberkochen, Germany) using a slow scan CCD-camera (pro scan, Scheuring, Germany). All electron microscopy experiments were performed in collaboration with Dr. M. Özel and G. Holland (Robert Koch-Institute, Berlin, Germany).

2.8.7 Detection of porin genes from the *M. fortuitum*-group by Southern Blotting

About 1 µg genomic DNA from *M. fortuitum* strains and *M. peregrinum* strains was isolated as described above and was then digested to completion with the restriction enzyme SacII (Cfr42I) and separated by agarose gel electrophoresis. The DNA was then transferred to the Hybond+ membrane (Amersham Biosciences, Freiburg, Germany) as described by

Sambrook et al. (1989). Porin genes were detected using a Flourescein labeled probe of 700 bp, which was established from *M. fortuitum* 10860/03 genomic DNA using the primers Mf-4IV-fw (5'-TCT CCA GGG GCT GCT TTT G-3') and Mf-4-bw (5'-CGG GAC GCC AAC CAC ATA AC-3') and the PCR Fluorescein Labeling Kit (Roche) according to the manufacturers instructions.

2.8.8 Cloning of the porin *porM1* from *M. fortuitum* 10860/03 and its detection in other members of the *M.fortuitum*-group

Genomic DNA from *M. fortuitum* 10860/03 was digested with the restriction enzyme SacII and separated by agarose gel electrophoresis. The region about 3000 bp was cut out of the gel and DNA was eluted using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Afterwards the eluted DNA was ligated into the unique SacII site of the plasmid pIV2 (Strauch et al., 2000) and the ligation was transformed into *E. coli* DH5α. Transformants were screened by Dot Blot analysis using the probe mentioned above. Inserts of the identified recombinant plasmids pSSp107 and pSSp108, which harbored porin sequences, were then sequenced. Identification of orthologus genes from other members of the *M. fortuitum*-group was performed by PCR using the primers KOMF 3F (5'-CTG AAG CTT CAC CGA GCT GAG CAT CCT CAC-3') and KOMF 4B (5'-GAC ACT AGT CGT TGG CTA CAG AAC AAC ATT CC-3') and the Advantage GC 2 PCR Kit (BD Biosciences). Both strands of the PCR products were then sequenced.

2.8.9 Detection of porins by 2-D Electrophoresis

About 75 µg of protein was precipitated by aceton and pellets were washed with 70% aceton to desalt the sample. Afterwards pellets were resuspended in 200 µl Rehydration solution (8M urea, 0.5% CHAPS, 0.2% DTT, 0.5% Pharmalyte, 0.002% bromphenol blue), incubated for 5 h at room temperature and loaded on IPG strips pH 3-5.6 NL, 11 cm (Amersham Biosciences). The strips were focused on an Ettan IPGphorII unit and the second dimension was run on vertical 10% SDS-PAGE gels using the Ettan Daltsix electrophoresis unit (Amersham Biosciences) according to the manufacturers instructions. The gels were stained by silver using Roti-Black P (Carl Roth GmbH, Karlsruhe, Germany). Porins were detected by Western Blotting as described above.

2.8.10 Quantification of porin by ELISA

In addition to the RT-Real-time PCR experiments the amount of porin in members of *M. fortuitum*-group and *M. smegmatis* was determined by Enzyme-Linked Immunosorbent Assay (ELISA). Protein was isolated from mycobacteria using the detergent nOPOE as described above. The protein isolation (15 µl corresponding approximately to 25 µg) was diluted in 50 mM $NaHCO_3$, pH 9.6 to yield a protein concentration of about 5 µg/100 µl. Aliquots (100 µl) of the sample and dilutions thereof were loaded to wells of a Nunc-Immuno Maxisorp Module (Nalge Nunc International, NY, USA). After incubating the samples at 4°C overnight, wells were washed twice with TBS-T (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM $MgCl_2$ and 0.05% Tween 80). The surface was blocked with 3% powdered skim milk in TBS for 1.5 h at room temperature followed by three steps of washing with TBS-T. Samples were then treated with the primary antibody for 1.5 h at room temperature, using a 1:1500 dilution of the antiserum pAK MspA#813 in TBS. The wells were washed five times with TBS-T and were incubated for 1 h at room temperature with a 1:7500 dilution of Peroxidase-conjugated AffiniPure F (ab') 2 Fragment Goat Anti-Rabbit IgG (H+L) (Jackson Immuno Research, Soham, UK) in TBS. After five steps of washing the reaction was performed using the SureBlue™ TMB Microwell Peroxidase Substrate (KPL, Geithersburg, MD, USA) according to the instructions of the manufacturer. Absorption at 450 nm was measured with the microplate reader SPECTRA Fluor (TECAN, Crailsheim, Germany).

2.8.11 Detection of PorM1 at the surface of mycobacteria by means of flow cytometry and quantitative microwell immunoassays

For flow cytometry experiments 40 ml of mycobacterial culture was harvested at O_{600} of 0.8, washed with PBS-T and the pellet was resuspended in 1 ml PBS-T. 50 µl aliquots were then incubated for 30 min on ice with a 1:100 dilution of antiserum (MspA#813) in PBS-T. Afterwards 1 ml PBS-T was given to each sample, mycobacteria were harvested by centrifugation and washed once with PBS-T. Pellets were resuspended in 30 µl of a 1:100 dilution of the secondary R-Phycoerythrin-conjugated AffiniPure F(ab') Fragment Donkey Anti-Rabbit IgG (H+L) (Jackson Immuno Research) and were incubated on ice for 30 min. After addition of 1 ml PBS-T, mycobacteria were harvested by centrifugation and were washed once with PBS-T. The pellet was then resuspended in 500 µl of PBS-T supplemented

with 2% formaldehyde. Fluorocytometric analysis was carried out using a FACScalibur cytometer (BD Biosciences).

40 ml of mycobacterial culture was harvested at OD_{600} of 0.8, washed with PBS-T and the pellet was resuspended in 1 ml PBS-T. 200 µl aliquots were then incubated for 30 min on ice with 1 µl of antiserum (pAK MspA#813); for detection of background no antiserum was given to the samples. Afterwards 1 ml PBS-T was given to each sample, mycobacteria were harvested by centrifugation and washed once with PBS-T. Pellets were resuspended in 100 µl of PBS-T and 1 µl of the secondary Peroxidase-conjugated AffiniPure F (ab') 2 Fragment Goat Anti-Rabbit IgG (H+L) (Jackson Immuno Research) was added to each sample and incubated on ice for 30 min. After addition of 1 ml PBS-T, mycobacteria were pelleted by centrifugation and were washed once with PBS-T. Pellets were then resuspended in 500 µl of PBS-T, and 100 µl of dilutions thereof were transferred to wells of a Nunc-Immuno Polysorp Module (Nalge Nunc International). After addition of 100 µl SureBlue™ TMB Microwell Peroxidase Substrate (KPL) and stopping the reaction by addition of 50 µl 1M HCl, the reaction was detected by the reader SPECTRAFluor (TECAN).

2.9 Construction of the suicide plasmid pSSs003

An all-purpose suicide plasmid harboring only one resistance gene was designed to construct allelic exchange mutants of mycobacteria, in particular *M. fortuitum*. For this purpose the plasmids pMN437 (Kaps et al., 2001) and pMN013 were used. The origin of replication for *E. coli* ColE1 was obtained by digestion of pMN437 with the restriction enzymes XbaI and Bsp68I. The hygromycin resistance cassette was amplified from pMN013 by PCR using primers with overhanging 5' ends, which introduced the restriction sites Bsp68I and BspT1 upstream and XbaI and Bsu15I downstream to the gene (VTlongfw2: 5'-CGG TCG CGA TAG GCT TAA GGG TAG CGG GTA GCG GTG GTT TTT TTG TTT GC-3'; VTlongbw: 5'-GCG CGT CTA GAG CAT CGA TCG ACT GTC CTC GTT GAT CCT TG-3') . The precursor plasmid pVTs001 was obtained after ligation of the mentioned fragments. A fragment of the multiple cloning site (MCS) of pMV306 was obtained after digestion with KspAI and EcoRV and was cloned into the unique Bsp68I site of pVTs001 to obtain the suicide plasmid pSSs003. Afterwards both strands of pSSs003 were sequenced.

For deletion of *porM1* in *M. fortuitum* 10851/03 parts of the *porM1* gene including its flanking regions were obtained by PCR using primers with overhanging 5'-ends, which

introduced additional restriction sites into the PCR products (Figure 20). PCR products were then cloned into the restriction sites HindIII/PstI and XbaI/BcuI to obtain the plasmid pSSs110 as indicated in Figure 20.

3 Results

3.1 Heterologous expression of the porin MspA from *M. smegmatis* in *M. bovis* BCG affects its in vitro growth and intracellular persistence.

3.1.1 Introduction of *mspA* into *M. bovis* BCG

The lack of efficient porins like MspA in the cell wall of slow-growing mycobacteria like *M. bovis* BCG motivated me to transfer a genomic region of *M. smegmatis* comprising *mspA* into *M. bovis* BCG. A 3429 bp DNA fragment containing *mspA* was obtained by PCR and was cloned in the integrative plasmid pMV306 (see section 2.7.1). The generated recombinant plasmid (pSSa100) contained besides *mspA* three other open reading frames (ORFs). Two ORFs were located upstream and one downstream of *mspA* (Figure 2). A blast search (http://www.ncbi.nlm.nih.gov/BLAST/) revealed ORF1 to be highly homologous to a TetR-family transcriptional regulator of *Streptomyces coelicolor*. ORF2 had a low homology to a putative oxidase of *S. coelicolor*. ORF3 located downstream of *mspA* showed a high homology to a putative protein of *M. tuberculosis* CDC1551. Sequencing of the insert of pSSa100 revealed rMspA to contain no amino acid replacements.

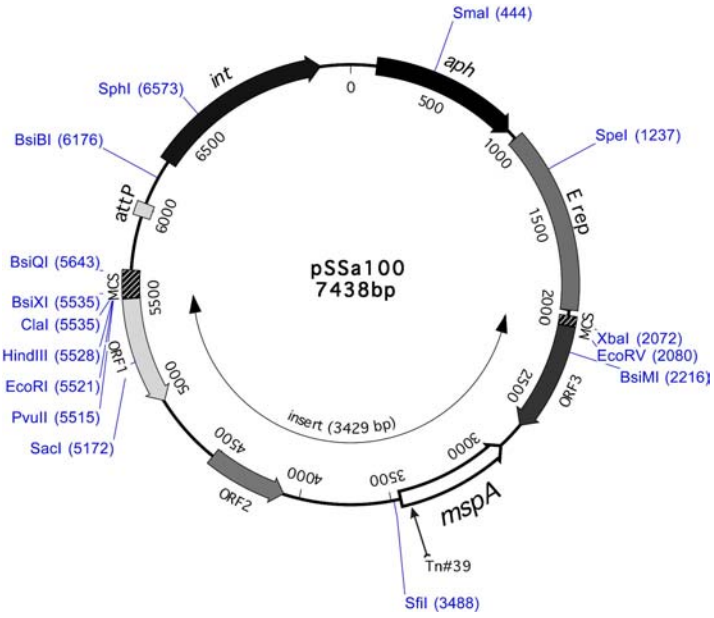

Figure 2: Map of the plasmid pSSa100. A 3429 bp fragment containing the porin gene *mspA* was obtained from genomic DNA of *M. smegmatis* by PCR and cloned into the unique *Eco*RV site of the plasmid pMV306. The arrow indicates the location of a transposon insertion in a mutant derivative (Tn#39) of pSSa100. *aph*: aminoglycoside phosphotransferase; *att*P: attachment site of phage L5; Erep: origin of replication of pUC plasmids; *int*: integrase gene; MCS: multiple cloning site.

After introduction of pSSa100 into *M. bovis* BCG via electroporation I could detect a growth advantage of *M. bovis* BCG (pSSa100) in comparison with the reference *M. bovis* BCG (pMV306). Colonies of *M. bovis* BCG containing the *mspA* gene appeared about one or two days earlier than the reference (data not shown). By performing PCR with primers specific for the *aph* gene of pMV306 (conferring kananmycin resistance) and for the *mspA* gene, I could show that *mspA* and *aph* were stably integrated into the genome of the *M. bovis* BCG transformants and were not lost in the course of the experiments (data not shown).

3.1.2 *MspA* is expressed in *M. bovis* BCG containing pSSa100

To investigate the expression of *mspA* in the *M. bovis* BCG derivative, I performed RT-PCRs. In addition, the expression of the selection marker *aph* of pSSa100 was chosen as a reference for expression of a gene introduced with the same vector system into *M. bovis* BCG. Total RNA was isolated from cultures of *M. bovis* BCG (pSSa100) and *M. bovis* BCG (pMV306) and RT-PCRs were performed using specific primers binding to *mspA* and *aph*. As shown in Figure 3 A, lane 2 and 3, an *mspA* specific RT-PCR product of 228 bp was only obtained by using RNA from *M. bovis* BCG (pSSa100). In addition a 556 bp fragment specific for *aph* was amplified using RNA preparations of both strains (Figure 3 A, lanes 5 and 6). These results demonstrate the successful transcription of the recombinant *mspA* in the *M. bovis* BCG derivative.

Next it had to be answered, if translation of *mspA* mRNA in *M. bovis* BCG occured. Therefore, Western Blots with protein isolations from both *M. bovis* BCG strains and *M. smegmatis* were performed. The selective extraction of MspA from *M. smegmatis* according to the method of Heinz and Niederweis (2000) showed on a 10% SDS-PAGE the oligomer of MspA (data not shown), which was recognized by the polyclonal rabbit antiserum (pAK MspA#813) in Western Blot experiments (Figure 3B, lane 1). However, the selective isolation of rMspA with nOPOE from *M. bovis* BCG was not as effective as the isolation of MspA from *M. smegmatis*. Therefore, the cell mass of *M. bovis* BCG used for the isolation of proteins was increased and about 350 mg of *M. bovis* BCG (wet weight) was employed. Although SDS-PAGE of these extracts did not show a defined band for rMspA even after silver staining (data not shown), a clear signal for the oligomeric form of rMspA was detected in extracts from the *M. bovis* BCG derivative with pSSa100 in Western Blot experiments (Figure 3 B, lane 2). Hereby I evidenced the synthesis of oligomeric rMspA in *M. bovis* BCG

(pSSa100). However the amount of rMspA in *M. bovis* BCG was clearly decreased if compared with *M. smegmatis*. As expected, the reference strain *M. bovis* BCG (pMV306) produced no signal with the MspA antiserum (Figure 3 B, lane 3).

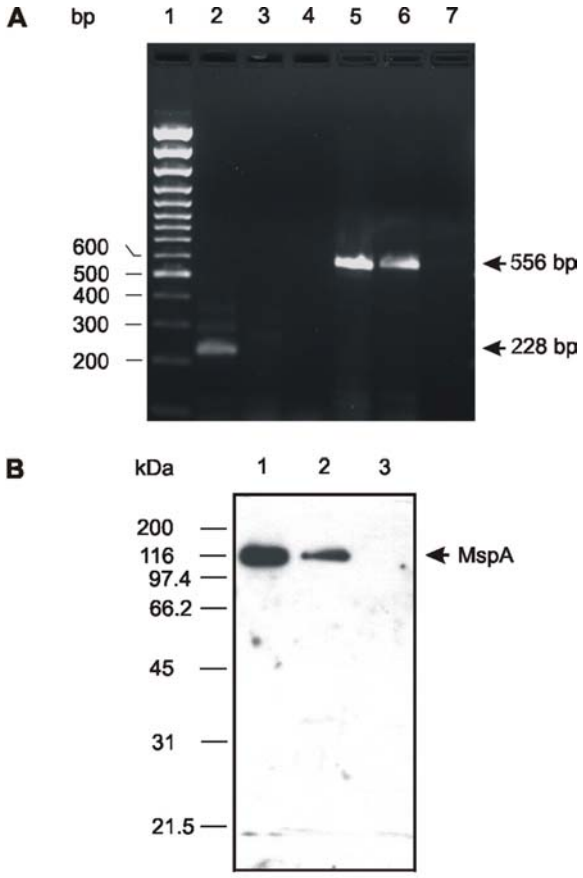

Figure 3: Expression analysis of *mspA* in *M. bovis* BCG derivatives by RT-PCR and Western Blotting. A. Transcription analysis of *mspA* in *M. bovis* BCG derivatives by RT-PCR. The successful transcription of the recombinant porin in *M. bovis* BCG (pSSa100) is demonstrated by the band of 228 bp (see lane 2) using specific primers for *mspA*. As a control the transcription of the selection marker *aph* is demonstrated by amplifying a fragment of 556 bp (see lanes 5 and 6). Lane 1, 100 bp DNA ladder; lanes 2-4, RT-PCR with specific primers for *mspA*; lane 2, *M. bovis* BCG (pSSa100); lane 3, *M. bovis* BCG (pMV306); lane 4, no template control; lanes 5-7, RT-PCR with specific primers for *aph*; lane 5, *M. bovis* BCG (pSSa100); lane 6, *M. bovis* BCG (pMV306); lane 7, no template control. B. Western Blot analysis of isolated proteins from *M. bovis* BCG derivatives and *M. smegmatis*. Proteins from *M. bovis* BCG derivatives and *M. smegmatis* were isolated using the detergent nOPOE. The samples from the *M. bovis* BCG derivatives and *M. smegmatis* were separated on a 10% SDS-PAGE and transferred to a PVDF membrane and MspA was detected with the polyclonal antiserum pAK MspA#813. The arrow indicates the location of the oligomeric form of MspA. Lane 1, 14 ng of protein extract from *M. smegmatis*; lane 2, 17 µg of protein extract from *M. bovis* BCG (pSSa100); lane 3, 17 µg of protein extract from *M. bovis* BCG (pMV306).

3.1.3 *M. bovis* BCG containing *mspA* shows an enhanced growth on plate

To investigate the influence of rMspA on growth of *M. bovis* BCG in vitro growth experiments with *M. bovis* BCG (pSSa100) and *M. bovis* BCG (pMV306) were performed in Middlebrook broth and on Middlebrook plates. Both strains showed a similar growth rate in broth measured over a period of 6 weeks (data not shown). However, if the strains were plated on agar, a clearly enhanced growth of the *M. bovis* BCG derivative with the porin was noticed, which became apparent as an earlier appearance of colonies and a faster increase in colony size (Figure 4 A and C).

In order to quantify the growth differences between the two strains, the ATP content of colonies washed away from agar plates was measured. The *M. bovis* BCG derivative with rMspA always showed higher ATP amounts per 100 colonies than the reference strain. The growth advantage of *M. bovis* BCG (pSSa100) was reflected as two to four-fold higher cellular ATP-contents in different experiments (Figure 4 B and D). Thus, it was shown that the 3429 bp fragment from *M. smegmatis* transferred into *M. bovis* BCG ameliorated its growth on agar plates.

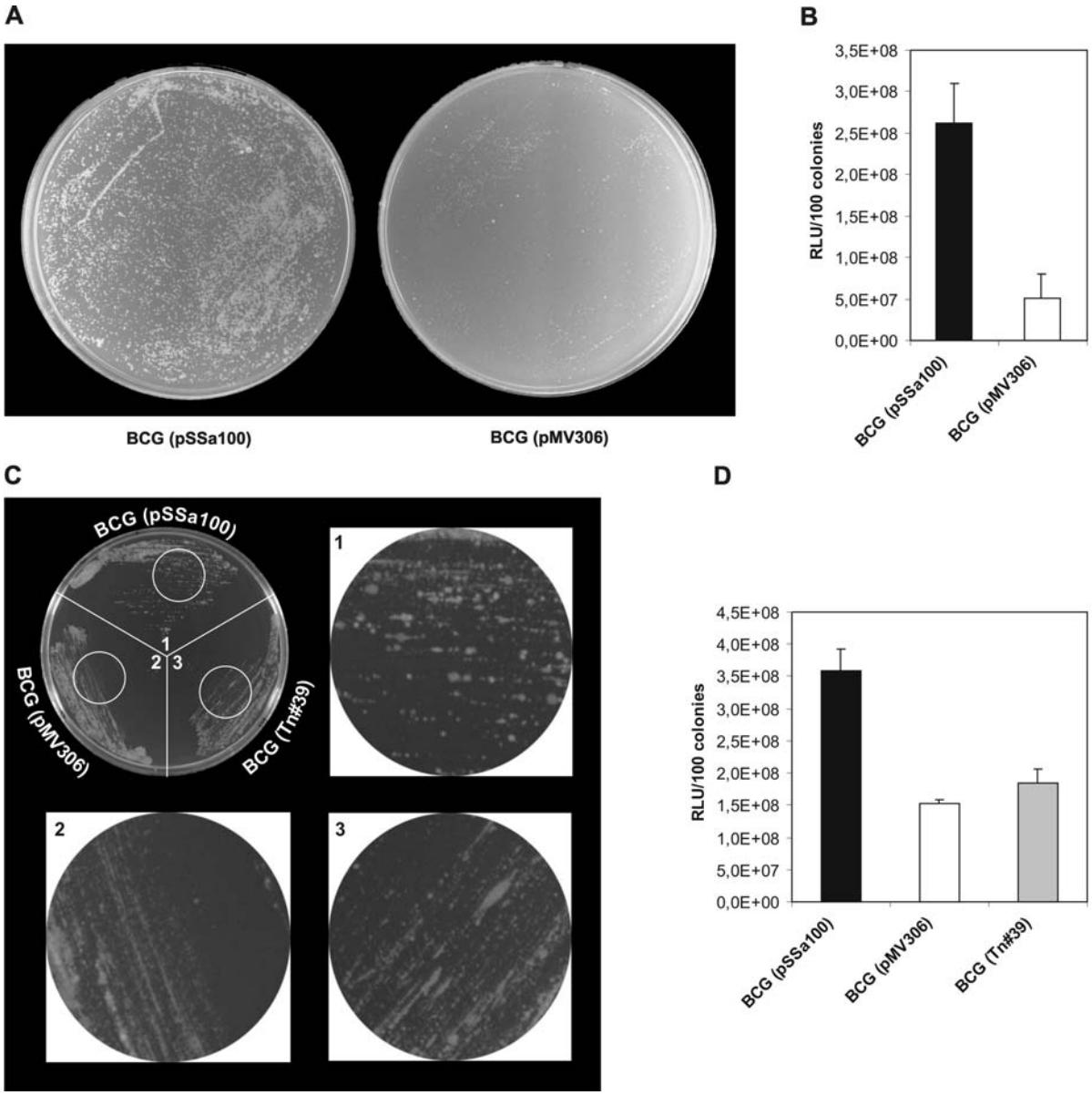

Figure 4: Enhanced growth of the *M. bovis* BCG derivative with rMspA on solid medium. A. Growth of the porin derivative *M. bovis* BCG (pSSa100) and the reference strain *M. bovis* BCG (pMV306) on Middlebrook agar plates 12 days after plating. B. Representative quantification of growth on plate by measuring the ATP content reflected as RLU in the luminescence assays. All values are standardized to 100 colonies. The *M. bovis* BCG derivative with rMspA showed up to four-fold higher cellular ATP concentrations if compared with the reference. C. Growth of *M. bovis* BCG (pSSa100), *M. bovis* BCG (pMV306) and *M. bovis* BCG (Tn#39, see section 4.1.4) streaked out on a Middlebrook agar plate. Sectors 1-3: Single colonies of *M. bovis* BCG (pSSa100) appeared after an incubation time of 16 days, whereas the reference and mutant strains showed either very small or no single colonies on plate. D. Effect of mutagenesis of *mspA* on growth on plates determined by representative quantification of the cellular ATP content. The *mspA* insertion mutant *M. bovis* BCG (Tn#39) showed comparable ATP concentrations as the reference, which were markedly lower than those of the *M. bovis* BCG derivative containing pSSa100.

3.1.4 Mutagenesis of *mspA* in pSSa100 reveals impairment of growth

Further proof that the *mspA* gene was responsible for the observed growth enhancement was adduced by transposon mutagenesis. The recombinant plasmid pSSa100 was mutagenized by an in vitro random insertion of a transposon with a tetracycline marker. A mutant with an insertion located in the coding sequence of *mspA* (Figure 2, Tn#39) was introduced into *M. bovis* BCG. The effect of the insertion on growth of *M. bovis* BCG on agar plates was measured by quantifying the ATP content of colonies containing the *mspA* gene with and without insertion. As shown in Figure 4 C and 4 D, the mutant strain *M. bovis* BCG (Tn#39) showed cellular ATP concentrations similar to the reference strain *M. bovis* BCG (pMV306), while *M. bovis* BCG (pSSa100) showed an enhanced growth. Hereby I demonstrated that the growth enhancement of *M. bovis* BCG (pSSa100) was caused by the introduced *mspA* gene.

3.1.5 Intracellular growth of *M. bovis* BCG containing the *mspA* gene

The intracellular growth of *M. bovis* BCG (pSSa100) and the reference strain was investigated by infection of non-activated J774A.1 macrophages and A549 pneumocytes by quantification of *M. bovis* BCG DNA via Real-time PCR. The *M. bovis* BCG strain with the recombinant porin showed in J774A.1 cells an initial growth phase until 56 hours post infection pronounced by a rapid increase of DNA (Figure 5 A). Until the end of the experiment the amount of intracellular *M. bovis* BCG (pSSa100) increased slightly. In contrast the reference strain showed a delayed growth until 80 hours post infection with markedly lower amounts of intracellular bacteria. After 80 hours post infection a decline in measurable *M. bovis* BCG (pMV306) occurred (Figure 5 A).

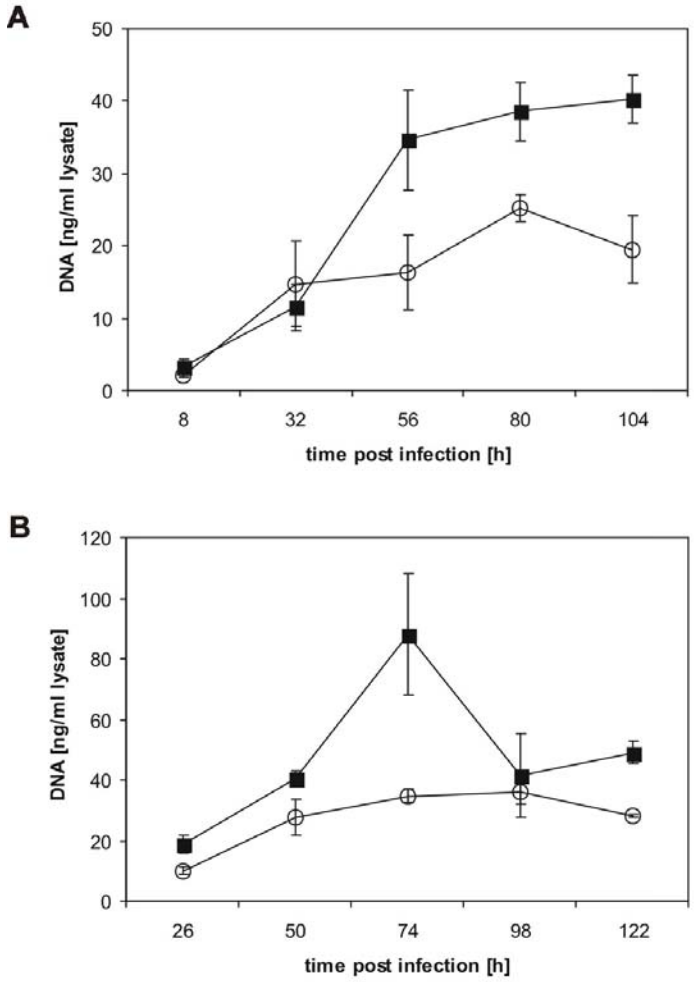

Figure 5: Representative measurement of intracellular growth of the *M. bovis* BCG derivative with rMspA and the reference strain. The DNA of intracellular bacteria was quantified by Real-time PCR. The closed squares demonstrate growth of *M. bovis* BCG (pSSa100) and the open circles growth of the reference *M. bovis* BCG (pMV306). Each value represents the mean (±SD) of DNA amounts from three independent experiments. A. Growth of *M. bovis* BCG derivatives in non-activated J774A.1. The values at 8 hours represent the amount of viable intracellular bacteria post infection. B. Growth of *M. bovis* BCG derivatives in the pneumocytic cells A549. The values at 26 hours represent the amount of viable intracellular bacteria post infection.

In the pneumocytic cells A549 an enhanced growth of intracellular *M. bovis* BCG (pSSa100) could be noticed if compared with the reference until 74 hours post infection (Figure 5 B). During the rest of the experiment a decline of intracellular *M. bovis* BCG (pSSa100) was detected. On the other hand the reference strain *M. bovis* BCG (pMV306) showed a very slow increase in bacterial amounts until 74 hours post infection followed by a declining phase (Figure 5 B).

These results indicate that *M. bovis* BCG containing the *mspA* gene shows an enhanced growth in monocytic and pneumocytic cells if compared with the reference.

3.2 Porins limit the intracellular persistence of *M. smegmatis*

To detect the potential impact of porins on survival in eukaryotic cells, I investigated the intracellular persistence of the wild type SMR5 and two porin deletion mutant strains of *M. smegmatis* MN01 (Δ*mspA*) and ML10 (Δ*mspA*Δ*mspC*) in different models. *MspA* and *mspC* were chosen for deletions, since they are the only genes of this type of porin, which are expressed in the parental strain. After deletion of *mspA*, however, the homologous genes are expressed in the mutant strains (Dr. M. Niederweis, personal communication). Macrophages and amoebae were chosen as model systems to analyze intracellular survival.

3.2.1 Intracellular persistence of *M. smegmatis* strains in macrophages

I began by investigating the degradation of *M. smegmatis* strains in J774A.1. As shown in Figure 6, a decrease in the number of intracellular mycobacteria was noticed during the course of the experiment. However, the double porin mutant ML10 showed significantly enhanced intracellular persistence when compared with the two other strains. After 54 h post infection SMR5 and MN01 showed bacterial loads of 2.7% and 3.4% when compared to bacterial loads after 6 h, whereas the bacterial load of J774A.1 infected with ML10 at 54 h amounted to 12.7%. The infection of BMMs produced more distinct differences between the three strains. ML10 exhibited throughout the entire experiment significantly higher CFU after plating when compared with the other two strains (Figure 6). After 30 h post infection also BMMs infected with MN01 showed significantly higher bacterial loads than BMMs infected with SMR5. In BMMs infected with ML10, 18.7% of bacteria remained viable after 54 h as compared to the 6 h value. With respect to the other two strains, 16.8% of MN01 and only 3.7% of SMR5 were viable after 54 h (Figure 6).

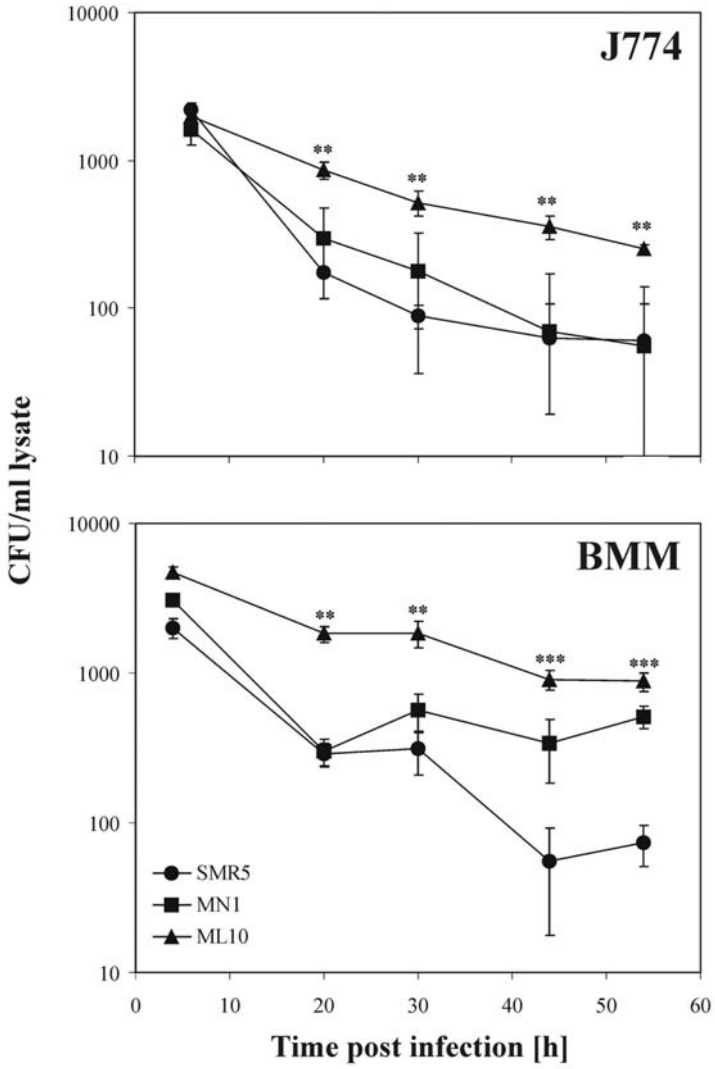

Figure 6: Intracellular persistence of *M. smegmatis* strains SMR5, MN01 and ML10 in J774A.1 and BMMs. Each value represents the mean (± SD) of three independent experiments. The values at 6 h represent the amount of viable intracellular *M. smegmatis* post infection. The double asterisks indicate values that varied significantly between ML10 and the other strains and the triple asterisks indicate significant differences among all three strains according to the paired Student's *t*-test ($P<0.001$).

3.2.2 Intracellular persistence of *M. smegmatis* strains in *A. castellanii*

A. castellanii was shown to serve as a host for mycobacteria such as *M. avium*. Additionally, *M. smegmatis* was reported to be killed by *A. castellanii* within a few days (Cirillo et al., 1997). Therefore, also this model was chosen to investigate intracellular persistence. As indicated in Figure 7 A, a pronounced initial killing phase occurred within the

first 20 h post infection. Afterwards the amount of intracellular ML10 did not decrease significantly while other strains showed a decline. While in *A. castellanii* infected with ML10 about 28% of bacteria remained intracellular and viable (relative to the 4 h values), MN01 and SMR5 showed levels between 5% and 0.66%, respectively (Table 4).

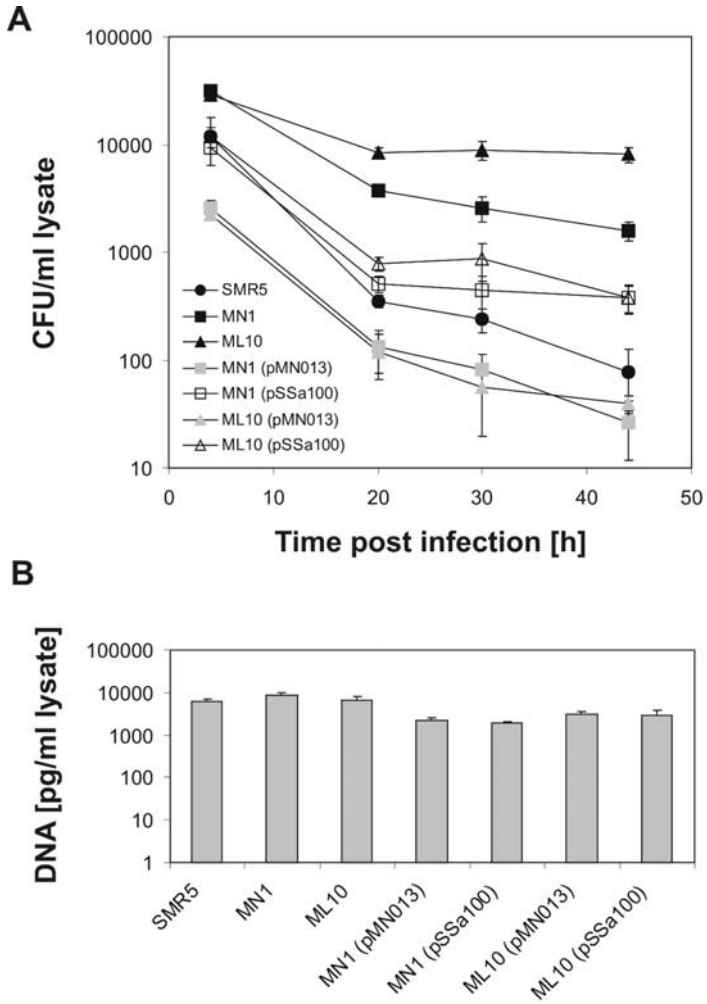

Figure 7: A. Intracellular persistence of *M. smegmatis* strains in *A. castellanii*. Each value represents the mean (± SD) of three independent experiments. The values at 4 h represent the amount of viable intracellular *M. smegmatis* post infection. B. Determination of consistent uptake of mycobacteria after 4 h post infection. DNA from intracellular mycobacteria was quantified by Real-time PCR. Columns show the mean (± SD) of three independent experiments.

To prove that the enhanced persistence of the mutants was in fact caused by the deletion of the porin genes, I performed complementation experiments by transforming the mutants with plasmids carrying the *mspA* gene. Plasmid pSSa100 (Figure 2) carries a 3.4 kb

DNA fragment from *M. smegmatis* containing the *mspA* gene with its own promoter in an integrative vector, while plasmid pMN013 (see appendix) carries the *mspA* gene fused to the *M. smegmatis* promoter p_{imyc} on a shuttle vector. The intracellular persistence of the mutants carrying either pSSa100 or pMN013 was tested in *A. castellanii,* because the differences between the mutants were most pronounced in this test system. As shown in Figure 7 A and Table 5, complementation of the porin deletion in mutant strains was achieved by introducing pMN013 as well as pSSa100. While complementation of porin deletion by pMN013 resulted in a higher *mspA* expression in both mutant strains than in the parental strain, complementation by pSSa100 was only partial (Table 5). Transfer of pSSa100 only provides one copy of *mspA* to the recipients and *mspA* is under control of its own promoter. The shuttle plasmid pMN013 is present in several copies in mycobacteria and the promoter p_{imyc} is a relatively strong promoter (Kaps et al., 2001).

Table 4: Bacterial load in *A. castellanii* infected with *M. smegmatis* strains. Values demonstrate the percentage of viable intracellular mycobacteria at 44 h post infection when compared to the 4 h values.

Strains	Intracellular persistence after 44h
SMR5	0.66%
MN01	5.0%
ML10	28.1%
MN01 (pMN013)	1.0%
MN01 (pSSa100)	4.0%
ML10 (pMN013)	1.8%
ML10 (pSSa100)	3.1%

RT-Real-time PCR experiments confirmed a much stronger expression of *mspA* from pMN013 compared to pSSa100 (Table 5). Transcription of *mspA* in the mutants carrying pMN013 was about 7 to 10 times stronger than in wild type. Nevertheless, both plasmids affected an adaptation towards the phenotype of the wild type SMR5 (Figure 7A). The complemented strains with pSSa100 were degraded similar to the wild type up to 20 h post infection. However, after this time point, their number remained relatively constant until the end of the experiment. MN01 (pMN013) and ML10 (pMN013) were degraded more efficiently by *A. castellanii* than all the other tested strains including SMR5. The decrease of

the intracellular persistence of the mutants harboring either pSSa100 or pMN013 confirmed that the deletion of the porin genes was responsible for the enhanced persistence of the mutants in the amoeba (Figure 7 A).

Table 5: Porin expression in the wild type, deletion mutants and complemented strains. Values represent the quantification of porin mRNA measured via RT-Real-time PCR. Data are the means (± SD) of three measurements.

Strains	Porin expression [pg cDNA / ng RNA]	% of porin expression referring to SMR5
SMR5	15.76 ± 4.89	100
MN01	0.36 ± 0.02	2.3
ML10	0.82 ± 0.17	5.2
MN01 (pMN013)	144.43 ± 10.89	916.3
MN01 (pSSa100)	2.90 ± 0.12	18.4
ML10 (pMN013)	111.38 ± 20.80	706.6
ML10 (pSSa100)	1.73 ± 0.56	11.0

Although all strains were applied at an MOI of 10, the CFU values of the first samples taken 4 h after infection already diverged, which is consistent with a kill off of mycobacteria by the amoebae starting directly after phagocytosis. Cirillo et al. (1997), for example, reported that 30 min after entry of *M. smegmatis* into *A. castellanii* the majority of bacteria were partially degraded. To ensure that equal numbers of bacteria of all strains had been taken up by the amoebae, the DNA of intracellular *M. smegmatis* after the initial infection time was quantified. There was no significant difference in the amount of intracellular DNA of all tested strains at 4 h post infection (Figure 7 B).

So far few studies have been performed, which illustrate the intracellular persistence of mycobacteria in *A. castellanii* particularly with regard to *M. smegmatis*. Therefore, I also examined the infection of *A. castellanii* with the wild type SMR5, MN01 and ML10 by means of transmission electron microscopy of samples taken after 18 h (Figure 8), 30 h (Figure 9) and 42 h (data not shown) post infection. Phagosomes were detected containing intact bacteria as well as degradation products up to 42 h post infection. Most of the phagosomes contained more than one *M. smegmatis* (typically up to 15), as indicated by the arrows in Figure 8 and 9.

At 18 h post infection the wild type SMR5 showed incipient degradation (see arrowhead in Figure 8 B) whereas less detritus was observed in phagosomes containing the porin deletion mutants (Figure 8 C and D). However, increased degradation was observed over the course of infection (Figure 9 B-D).

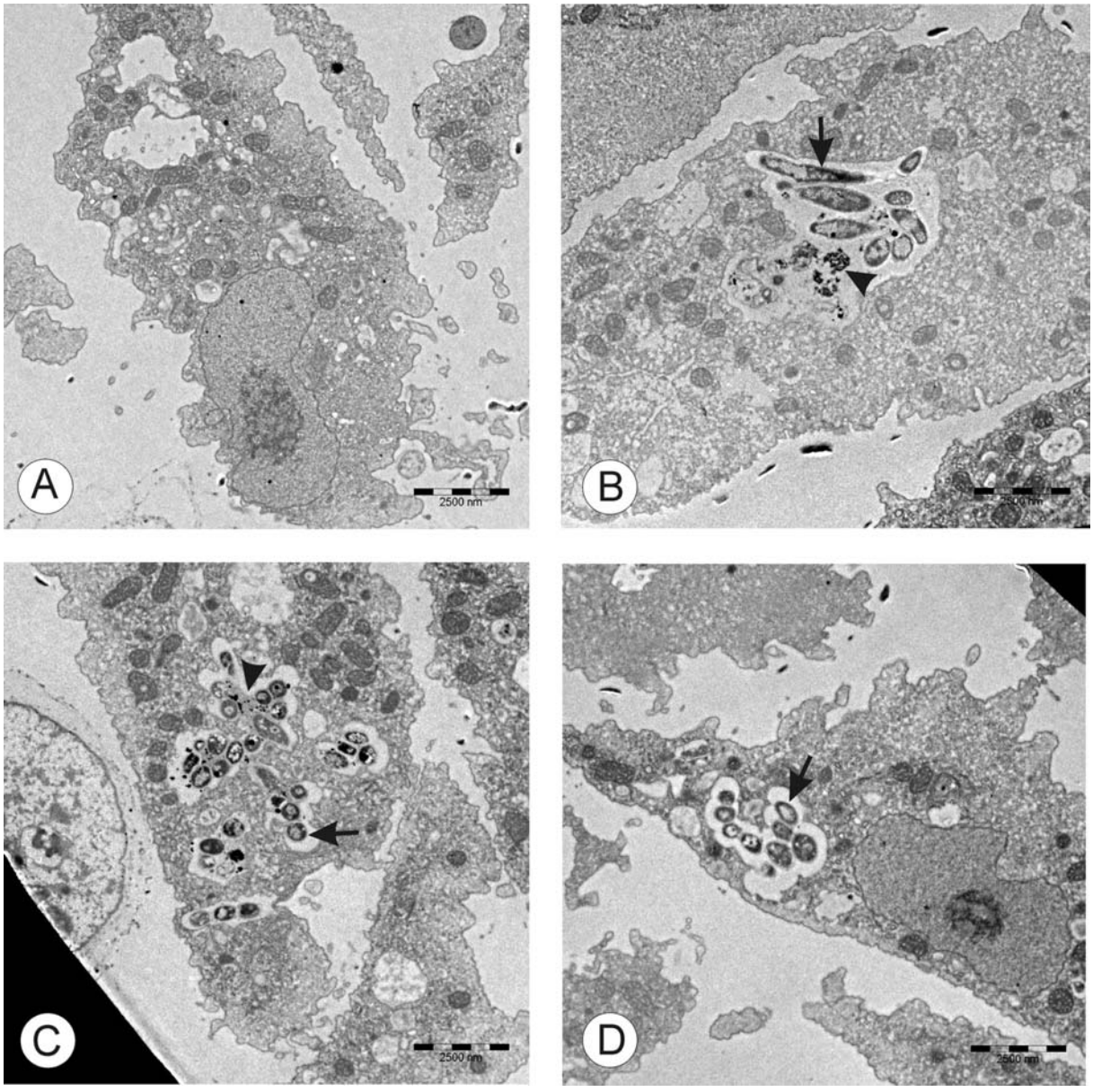

Figure 8: TEM of cultured *A. castellanii* 18 h post infection. A. Non-infected control. B. Infected with *M. smegmatis* SMR5 (wild type). C. Infected with *M. smegmatis* MN01 (Δ*mspA*). D. Infected with *M. smegmatis* ML10 (Δ*mspA*Δ*mspC*). Arrows indicate *M. smegmatis*, whereas detritus is indicated by arrowheads.

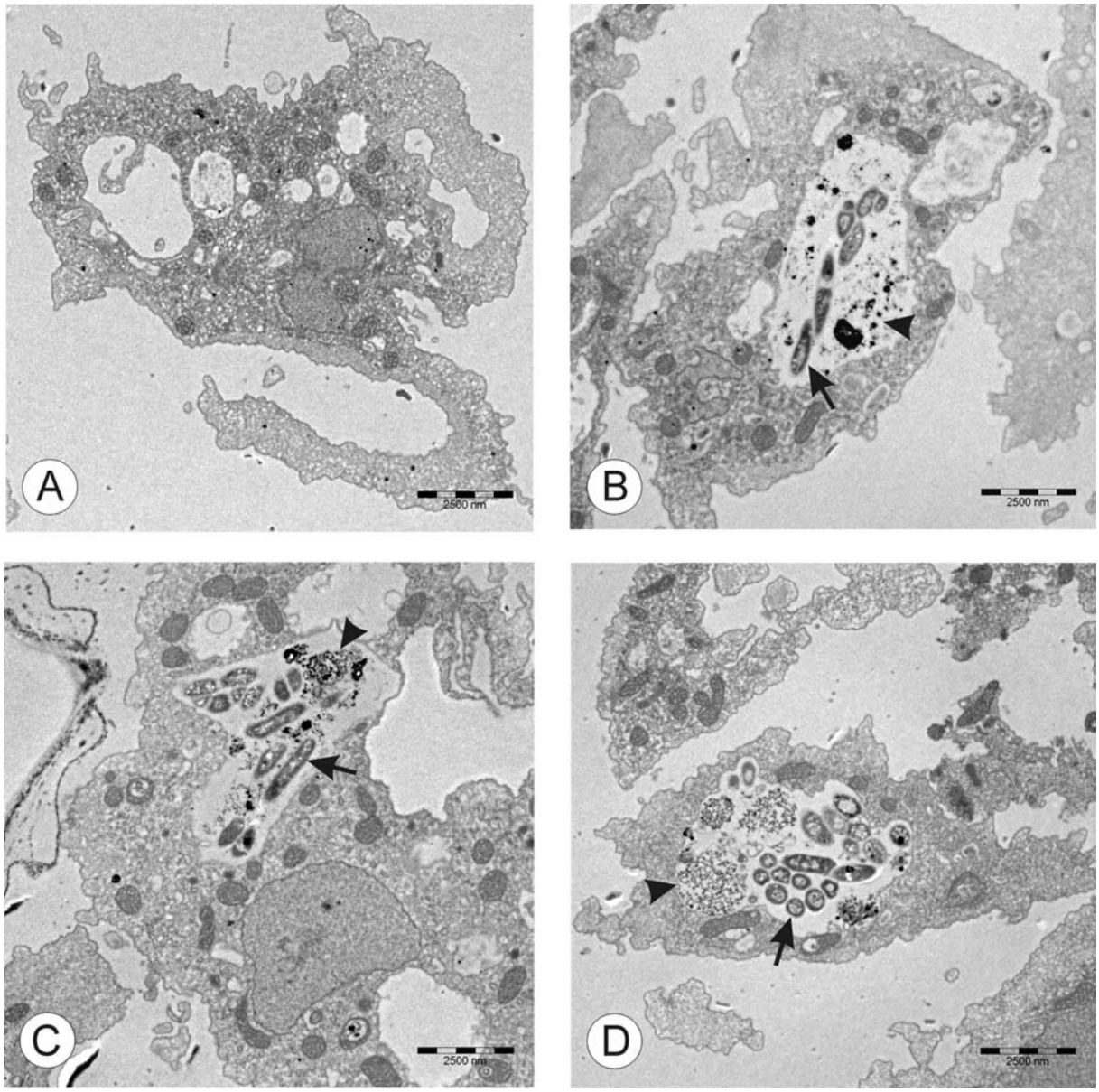

Figure 9: TEM of cultured *A. castellanii* 30 h post infection. A. Non-infected control. B. Infected with *M. smegmatis* SMR5 (wild type). C. Infected with *M. smegmatis* MN01 (Δ*mspA*). D. Infected with *M. smegmatis* ML10 (Δ*mspA*Δ*mspC*). Arrows indicate *M. smegmatis*, whereas detritus is indicated by arrowheads.

It should be noted that after 30 h post infection some trophozoites rounded up and encystment of *A. castellanii* was observed. Electron micrographs revealed double-walled cysts. *M. smegmatis* was not only present in phagosomes but also was found to be embedded in walls of cysts of *A. castellanii* (Figure 10).

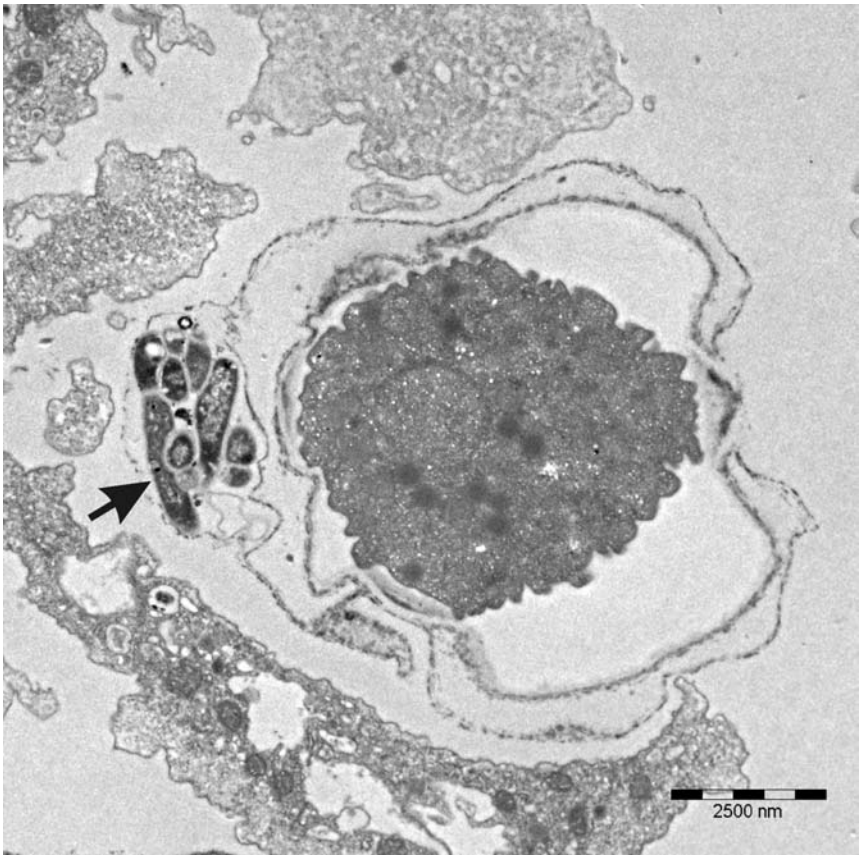

Figure 10: TEM of amoeba cyst including mycobacteria (*M. smegmatis* MN01) in the cyst-wall as indicated by the arrow.

3.2.3 Growth of *M. smegmatis* strains in broth culture at different pH

As the double mutant lacking *mspA* and *mspC* exhibited an enhanced survival in phagocytes, the question arose, if this was caused by an improved resistance to acidic pH conditions present in the phagosomes. To answer this question, the growth of SMR5, MN01 and ML10 were compared in Middlebrook 7H9 medium at pH 6.7 and pH 5.0 over 8 days. The double mutant strain ML10 exhibited a decreased growth at pH 6.7 and pH 5.0 across the course of the experiment when compared with SMR5 and MN01 (Figure 11). No significant differences were noticed between the growth of SMR5 and MN01. The differences in the growth rates in broth culture between the three strains were independent of the pH of the medium.

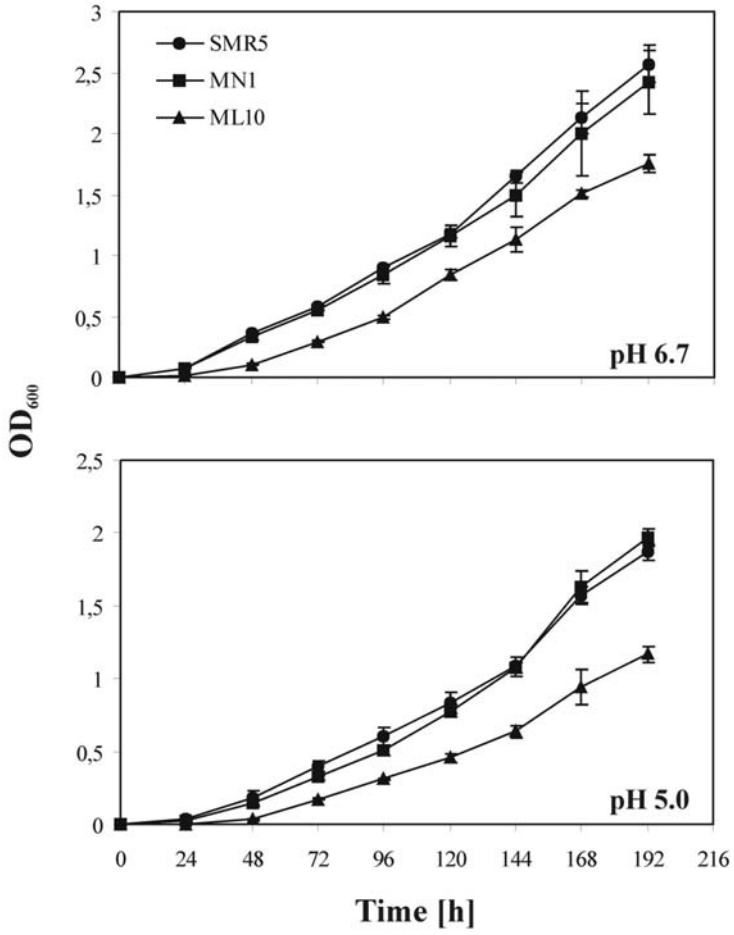

Figure 11: Impact of porin deletions on growth of *M. smegmatis* under different pH-conditions. The strains were grown in Middlebrook 7H9 at pH 6.7 or pH 5.0. Measuring points represent the mean (± SD) of three independent cultures.

3.3 Characterization of porins from members of the *M. fortuitum*-group

Since the saprophytic bacterium *M. smegmatis* causes disease only in rare cases (Brown-Elliott & Wallace, 2002), it is important to investigate the role of porins on virulence in more pathogenic members of RGM. Members of the *M. fortuitum*-group were therefore chosen to detect and analyze homologous porins of the *mspA* class. For this purpose, two different *M. fortuitum* strains (10851/03 & 10860/03) and two *M. peregrinum* strains (9912/03 & 9926/03) were employed, which originally were isolated from human patients and were provided by the National Reference Center for Mycobacteria at the Research Center Borstel, Germany. Comparative analysis was performed using also the type strain *M. fortuitum* DSM 46621. In order to verify the taxonomic classification and to define the phylogenetic relationship

between the named strains or species, complete sequences of the 16S rRNA genes were determined using the primers, which were approved by Adekambi & Drancourt (2004). As shown in Figure 14, phylogenetic analysis of the 16S rRNA sequences confirmed the taxonomic classification of the employed members of the *M. fortuitum*-group.

3.3.1 *PorM1* is present in different members of the *M. fortuitum*-group

TO DETECT PORINS HOMOLOGOUS TO *MSPA* IN MEMBERS OF THE *M. FORTUITUM*-GROUP, HYBRIDIZATION EXPERIMENTS WERE PERFORMED WITH A PROBE DERIVED FROM PRELIMINARY PORIN GENE SEQUENCES OF *M. FORTUITUM*. PRILIMINARY PORIN GENE SEQUENCES FROM *M. FORTUITUM* WERE OBTAINED BY PERFORMING PCRS UNDER LOW STRINGENCY CONDITIONS USING PRIMERS DERIVED FROM THE *MSPA* SEQUENCE. THE PROBE HYBRIDIZED TO THE GENOMIC DNA FROM THE *M. FORTUITUM* STRAINS AS WELL AS TO THE GENOMIC DNA FROM *M. PEREGRINUM* STRAINS. THUS, HOMOLOGOUS PORINS SEEM TO EXIST IN ALL STRAINS. THE PROBE HYBRIDIZED TO TWO FRAGMENTS OF THE SACII-DIGESTED GENOMIC DNA OF *M. FORTUITUM* AND *M. PEREGRINUM*. HOWEVER THE FRAGMENT SIZES DIFFERED AMONG THE MEMBERS OF THE *M. FORTUITUM*-GROUP (FIGURE 12). HENCE, THE *M. FORTUITUM* AND *M. PEREGRINUM* GENOMES CONTAIN AT LEAST TWO COPIES OF THE PORIN GENE.

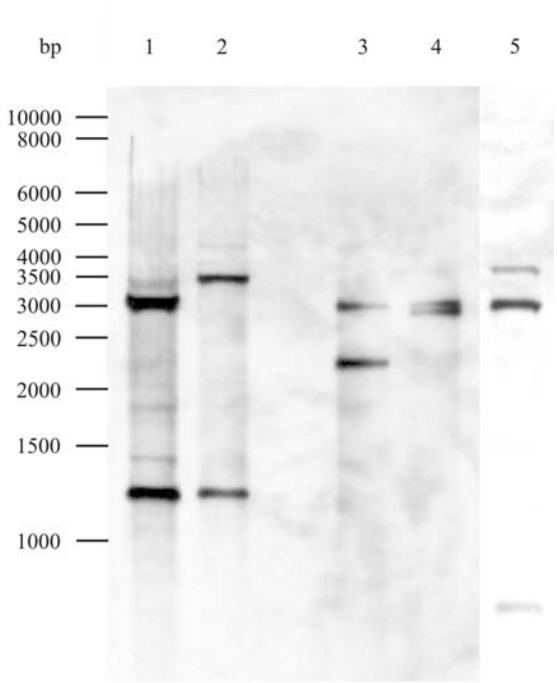

Figure 12: Occurrence of porin genes among members of the *M. fortuitum*-group. Chromosomal DNA of different strains was digested with SacII and analyzed by Southern blotting. Lanes: 1, *M. peregrinum* 9912/03; 2, *M. peregrinum* 9926/03; 3, *M. fortuitum* 10851/03; 4, *M. fortuitum* 10860/03; 5, *M. fortuitum* DSM 46621.

3.3.2 *PorM1* genes from members of the *M. fortuitum*-group are highly homologous to *mspA*

To clone porins from *M. fortuitum*, genomic DNA of *M. fortuitum* 10860/03 was digested with SacII and a 3000 bp fragment was cut out of the preparative gel. The DNA was ligated into the unique SacII site of pIV2 and transformed into *E. coli*. After screening the transformants by Dot Blot analysis, two clones were identified to contain porin sequences, from which the plasmids pSSp107 and pSSp108 were isolated. Both plasmids turned out to contain the same genomic region of 2895 bp including one porin gene and were chosen for further characterization. The inserts were sequenced by primer walking, whereas both strands of the porin genes and their surrounding regions were sequenced twice at least. As shown in Figure 13, the insert of the plasmids contained several ORFs, one of which (*porM1*) was homologous to *mspA*. It contained 636 bp, encoding a protein of 211 amino acids with an N-terminal signal sequence of 27 amino acids, which was predicted using the SignalP 3.0 Server at http://www.cbs.dtu.dk/services/SignalP/ (Bendtsen et al., 2004).

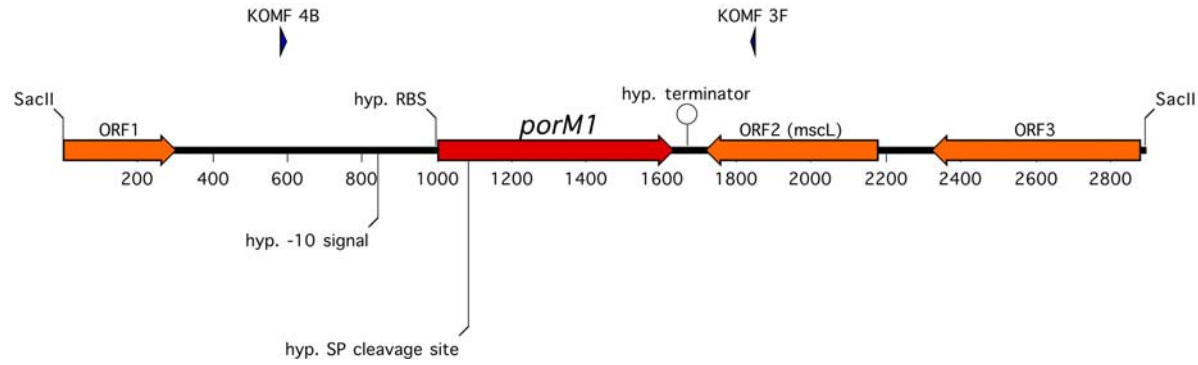

Figure 13: Map of the insert of plasmid pSSp107. The insert includes the *porM1* gene and three other ORFs. Up- and downstream to *porM1* various nucleotide signal sequences were detected: -10 signal of a promoter (TATGTT), a ribosome binding site (RBS: GGAGA), a signal peptide (SP) of 81 bp and a hairpin structure, which could represent a terminator. The binding sites of the primers used for detection of the genomic regions of *porM1* in other employed strains are indicated as KOMF 4B and KOMF 3F.

A hypothetical –10 region of a promoter and a ribosome binding site (RBS) were identified upstream of the coding sequence. Downstream of the ORF a hairpin sequence was detected, which could function as a terminator (Figure 13). It has to be noted that the sequence homology between *M. fortuitum* and *M. smegmatis* was only restricted to the coding

sequence. According to the designation of other mycobacterial porin genes and to the instructions of EMBL nucleotide sequence database, the gene was named *porM1*.

Besides the porin gene two other complete ORFs and a part of another ORF were detected. ORF1 was interrupted by one of the SacII sites and showed a high homology to a molybdopterin biosynthesis protein of *M. tuberculosis* CDC 1551 (accession-nr.: AAK 45260). ORF2 turned out to be a mechanosensitive channel homologous to the gene *mscL* from *M. avium subsp. paratuberculosis* str. 10 (accession-nr.: NP 959854). ORF3 was homologous to the hypothetical protein Rv0990c from *M. tuberculosis* H37Rv (accession-nr.: NP 215505).

Next it had to be answered, whether *porM1* was present in other employed strains. Thus, the primers KOMF3F and KOMF4B were chosen to amplify a fragment of approximately 1250 bp including the porin gene and flanking regions. PCRs, using a polymerase-mix with proofreading activity, revealed the specific fragment to be present in all strains. Several PCRs were performed and both strands of the different fragments were sequenced. *PorM1* was detected in all members of the *M. fortuitum*-group and the nucleotide sequences were submitted to the EMBL nucleotide sequence database (Table 6). The nucleotide sequences are given in the section Appendix. The nucleic acid subsequences such as the -10 signal of a promoter, the RBS, the signal peptide of 81 bp and the hairpin structure were also present and were conserved among all tested strains (data not shown).

Table 6: Nucleotide sequence homology between *porM1* from members of the *M. fortuitum*-group and *mspA*.

Species	Nucleotide similarity index	Accession-nr. to the EMBL nucleotide sequence database
M. fortuitum DSM 46621	88.2%	AJ880097
M. fortuitum 10851/03	88.4%	AJ880098
M. fortuitum 10860/03	87.4%	AJ874299
M. peregrinum 9912/03	86%	AJ880099
M. peregrinum 9926/03	86.9%	AJ880100

As already indicated in Table 6, the nucleotide sequences of the gene *porM1* differed among members of the *M. fortuitum*-group. The phylogenetic comparison of the employed strains based on their porin sequences showed the closest relationship between the type strain *M. fortuitum* DSM 46621 and *M. fortuitum* 10851/03, it could, however, not reflect the phylogenetic relationships among the *M. fortuitum*-group based on 16S rRNA sequences (Figure 14).

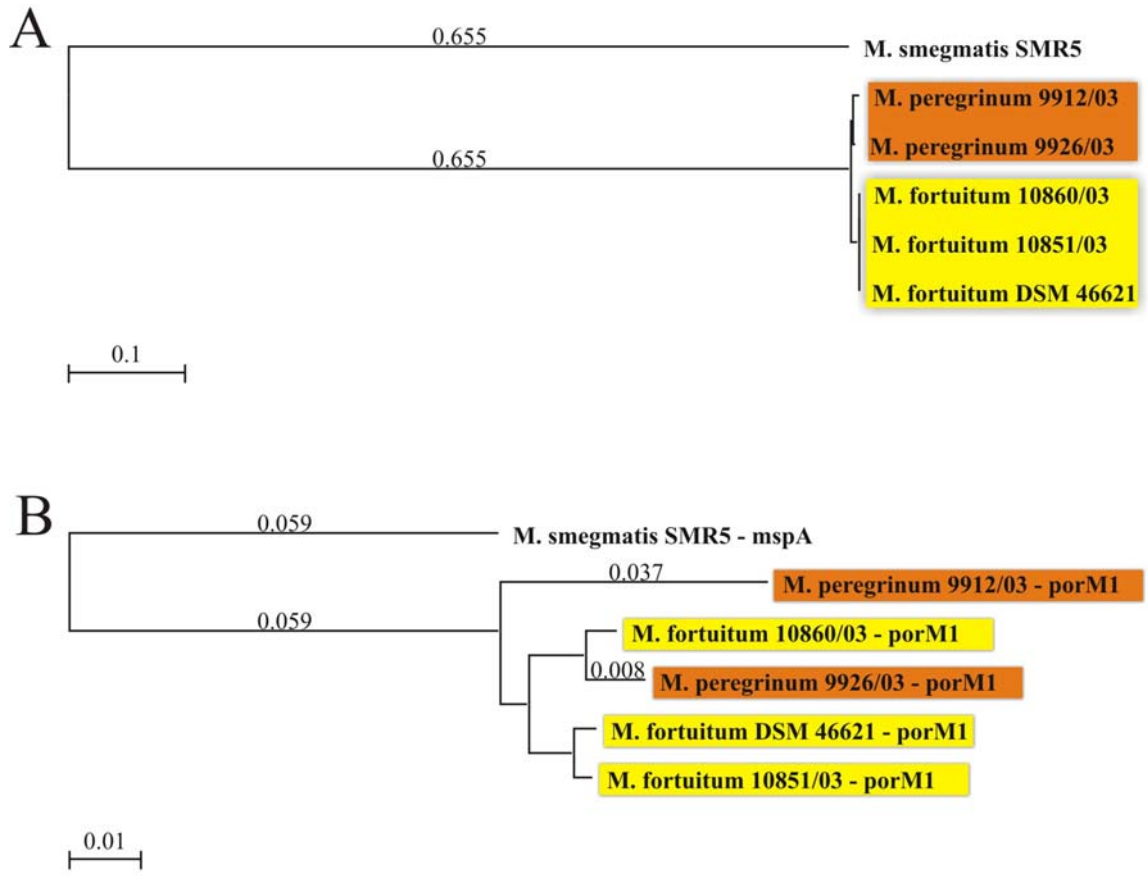

Figure 14: Phylograms of the *M. fortuitum*-group based on 16S rRNA and *porM1* sequences using the neighbor-joining method with Kimura 2-Parameter distance correction. The branch lengths indicate the evolutionary distance relative to the scale. *M. smegmatis* was used as the outgroup. All *M. fortuitum* strains are shaded yellow and the *M. peregrinum* strains are shaded orange. A. Phylogenetic tree of 16S rRNA gene sequences of employed mycobacteria. B. Phylogenetic tree of porin gene sequences of employed mycobacteria.

The amino acid sequences of PorM1 among the *M. fortuitum*-group were however highly conserved (Figure 15). All *M. fortuitum* strains possessed 100% identical PorM1 amino acid sequences, whereas *M. peregrinum* 9926/03 exhibited only one and *M. peregrinum* 9912/26 only two amino replacements compared to the sequence from *M. fortuitum*. According to studies about typical amino acid replacements by Betts & Russel (2003) all amino acid substitutions were exchanges between amino acids preferred for membrane proteins. The aliphatic and hydrophobic amino acid Isoleucine was substituted with the similar aliphatic and hydrophobic amino acid Leucine. The negatively charged and

polar amino acid Glutamate was replaced by the negatively charged (and very similar) amino acid Aspartate.

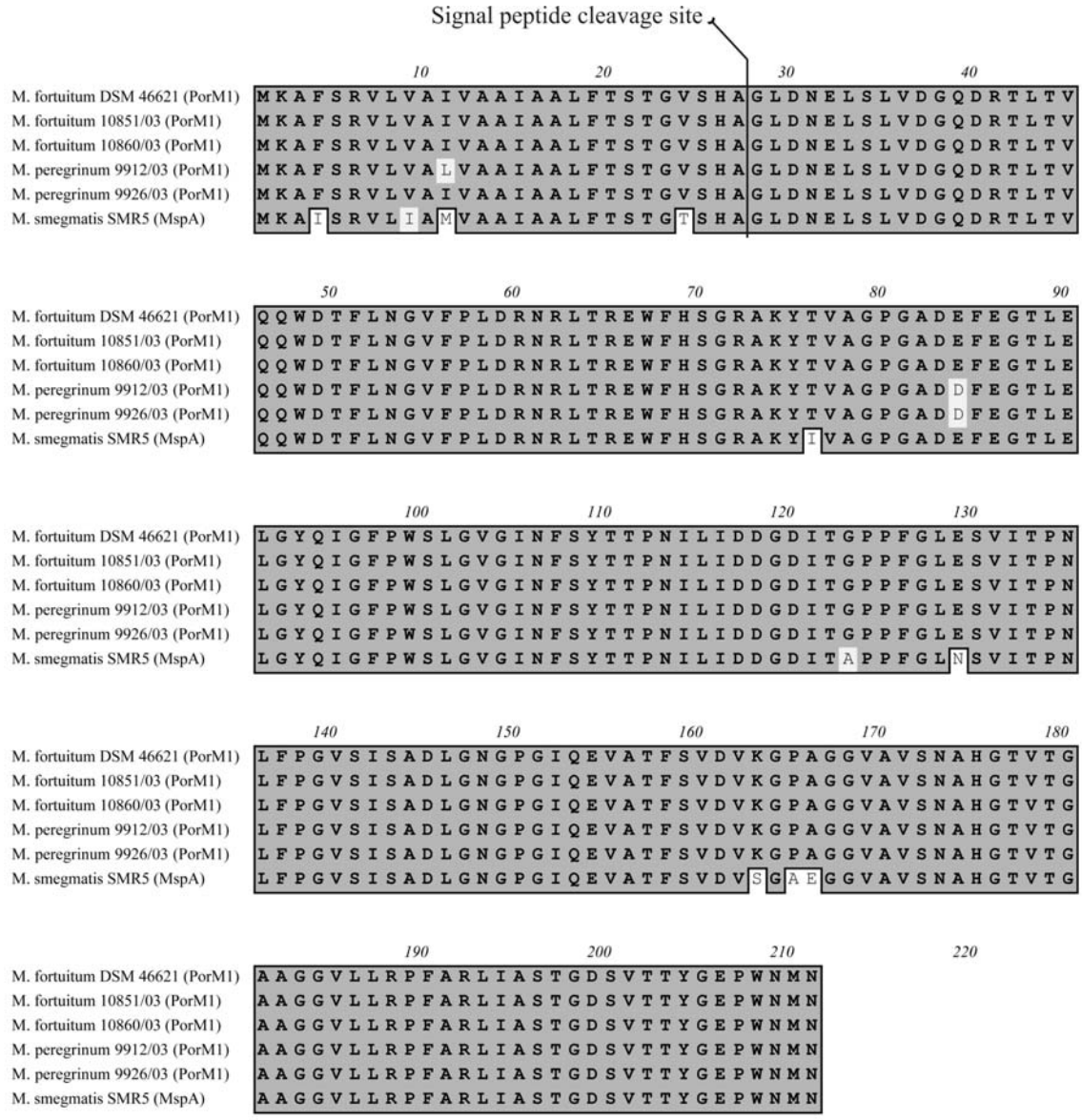

Figure 15: Alignment of PorM1 from members of the *M. fortuitum*-group and MspA. The start codon ATG and the stop codon TGA were chosen according to the sequence of *mspA*. The cleavage recognition site of the signal peptidase was predicted for PorM1 using the SignalP 3.0 Server at http://www.cbs.dtu.dk/services/SignalP/ (Bendtsen et al., 2004). The predicted signal peptide cleavage sites corresponded to those from MspA. Identical amino acids are shaded in dark gray, similar amino acids are shaded in light gray and different amino acids are non-shaded.

The amino acid sequences of porins from members of the *M. fortuitum*-group and *M. smegmatis* were also highly conserved. The mature proteins (without signal peptide) possessed 97.3% similar and at least 96.2% identical amino acids (Betts & Russel, 2003).

3.3.3 PorM1 is expressed in the members of the *M. fortuitum*-group

THE EXPRESSION OF THE PORIN *PORM1* AND DETECTION OF THE MATURE OLIGOMERIC PORIN WAS EXAMINED BY SDS-PAGE, 2D-ELECTROPHORESIS AND WESTERN BLOTTING. THEREFORE, PROTEIN WAS ISOLATED FROM *M. FORTUITUM* 10860/03 USING THE DETERGENT NOPOE AND SEPARATED BY 2D-ELECTROPHORESIS. AS SHOWN IN FIGURE 16 A, ABOUT 50 PROTEINSPOTS WERE DETECTED ON THE 2D-GEL. WESTERN BLOT EXPERIMENTS WITH IDENTICAL GELS SHOWED ONLY ONE DEFINED SPOT DETECTED BY THE ANTISERUM PAK MSPA#813 (FIGURE 16 A I). THE PROTEIN HAD AN APPARENT MOLECULAR MASS OF APPROXIMATELY 120 KDA AND AN APPARENT ISOELECTRIC POINT (IP) OF ABOUT 4, WHICH CORRESPONDED WELL TO THE PREDICTED IP OF THE MATURE PROTEIN OF 4.1.

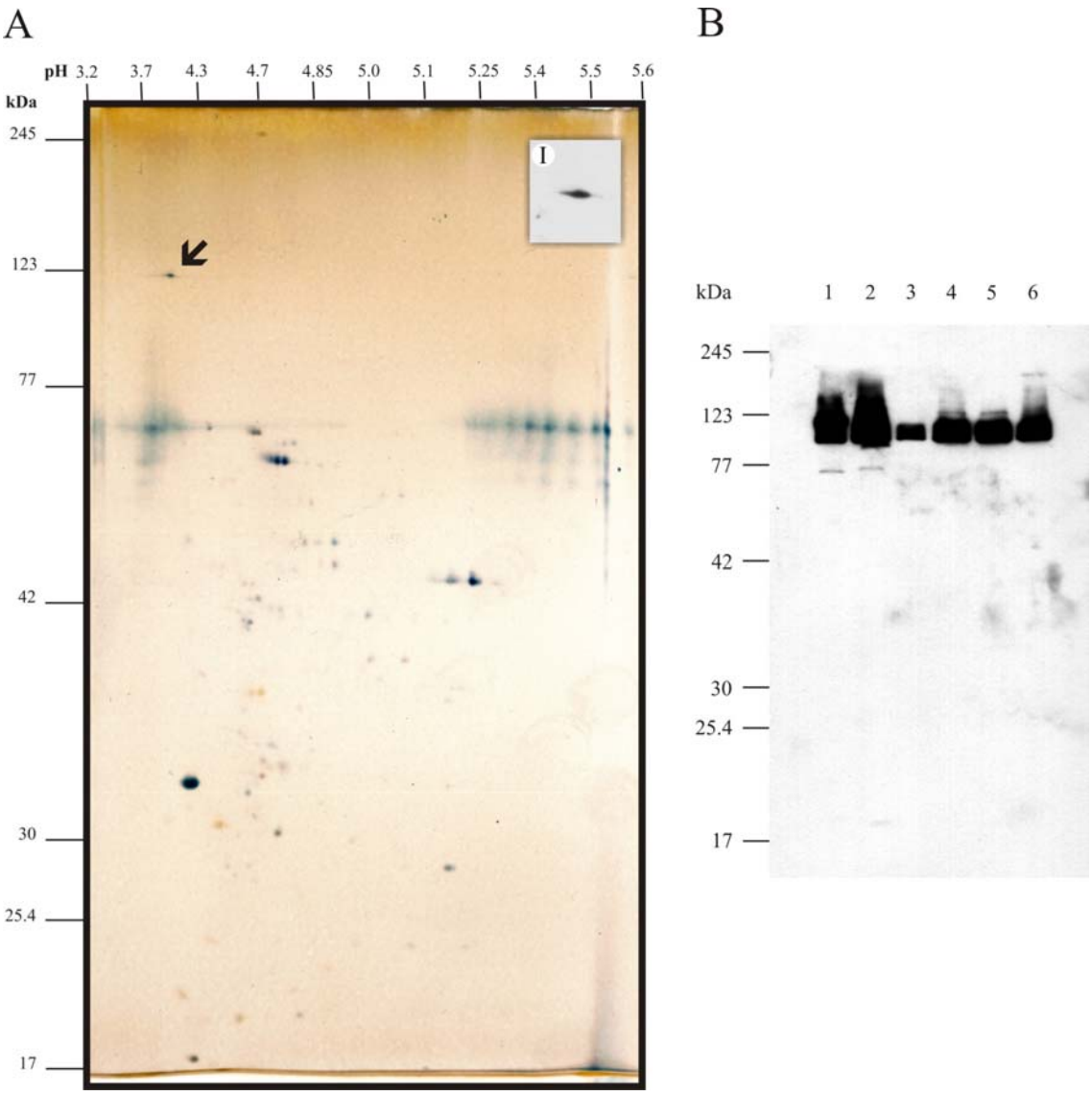

Figure 16: Detection of PorM1 in members of the *M. fortuitum*-group. 2D-Electrophoresis and Western Blot experiments proved PorM1 to be expressed in the analyzed strains. A. 2D-Electrophoresis of protein isolation from the strain *M. fortuitum* 10860/03 using the detergent nOPOE. The arrow indicates the porin spot as proved by Western Blot analysis (see section I). B. Detection of the porin in members of the *M. fortuitum*-group by Western Blotting. 10-30 µg of protein was detected by the antiserum pAK MspA#813. Lanes 1-6: 1, *M. smegmatis* SMR5 (10 µg); 2, *M. fortuitum* DSM 466211 (30 µg); 3, *M. fortuitum* 10851/03 (30 µg); 4, *M. fortuitum* 10860/03 (30 µg); 5, *M. peregrinum* 9912/03 (30 µg); 6, *M. peregrinum* 9926/03 (30 µg).

Since the oligomeric porin was detected in cell extracts of *M. fortuitum* 10860/03 (Figure 16 A), other members were tested for expression of PorM1. The mature oligomeric form of the porin was shown in cell extracts of the members of the *M. fortuitum*-group as well as in extracts from *M. smegmatis*, which served as a positive control (Figure 16 B). After

extended exposition times also the monomeric form of the porin was detected on Western Blots (data not shown). Although equal amounts (30 µg) of the cell extracts from members of the *M. fortuitum*-group were blotted on the PVDF membrane, the signals on the Western Blots and thus the amount of PorM1 seemed not to be equal among the tested strains (Figure 16 B).

3.3.4 Members of the *M. fortuitum*-group express less porin than *M. smegmatis*

The disparate signal intensities on Western Blot experiments among the *M. fortuitum*-group gave rise to analyze the expression profile of *porM1*. For this purpose, cell extracts with the detergent nOPOE were employed in ELISA experiments to quantify the amount of porin in different strains. Furthermore, the expression of *porM1* in the members of *M. fortuitum*-group was determined by means of RT-Real-time PCR using sequence specific primers and probe and was compared to porin expression in *M. smegmatis*.

Different dilutions of the cell extracts from the various strains were loaded to the wells of a microtitre plate and porins were detected by an antiserum raised against MspA (pAK MspA#813). Higher amounts than 5 µg per well turned out to be inapplicable due to saturation effects and the detection of porin in cell extracts failed at concentrations of about 0.04 µg per well (Figure 17 A). Therefore, the most eligible working range turned out to be 1 µg cell extract per well. Indeed, the amount of porin differed in various strains. The highest amount of porin was detected in the internal control *M. smegmatis* SMR5. The type strain *M. fortuitum* DSM 46621 exhibited porin amounts close to *M. smegmatis*, whereas all of the strains, which originally were isolated from human patients showed clearly decreased porin amounts (Figure 17 A). I observed with particular interest the amount of porin in *M. fortuitum* 10851/03, which was the lowest amount among the analyzed RGM. Since *M. bovis* BCG does not possess homologous porins (Niederweis et al., 1999), extracts of *M. bovis* BCG were employed to detect the background. Figure 17 A demonstrates, that the antibody specifically recognized MspA and the homologous porin PorM1.

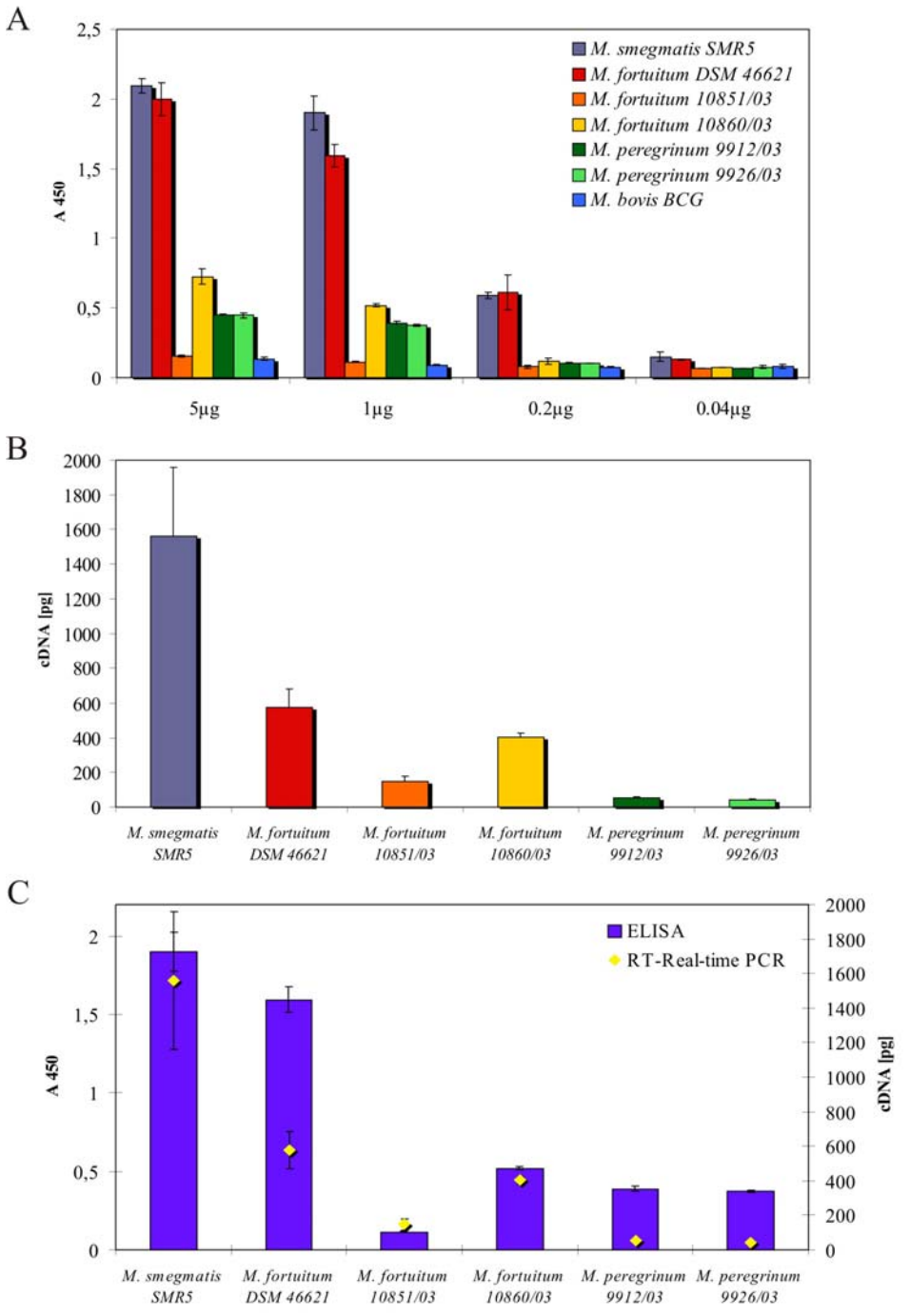

Figure 17: Comparative analysis of porin expression among RGM. Expression of porin was detected by means of ELISA and RT-Real-time PCR. Each value represents the mean (±SD) of at least three independent experiments. A. Quantification of porin in cell extracts of different mycobacteria using the polyclonal antibody pAk MspA#813. B. Quantification of porin transcription in various RGM using specific primers and probes for *mspA* or *porM1*, respectively. C. Combined illustration of the results from ELISA- and RT-Real-time PCR-experiments. The left ordinate and the blue columns demonstrate the results from ELISA-experiments using 1 µg protein per well, whereas the right ordinate and the yellow rhombs show the results from RT-Real-time PCR-experiments.

Comparative expression analysis was also performed by means of RT-Real-time PCR using specific primers and probes for *porM1* or *mspA*, respectively. 100 ng of RNA preparations from RGM were employed to compare the transcription of the porin genes among the strains. As it was already proven by the ELISA-results, the highest porin transcription was measured in *M. smegmatis*. It showed about twice as high transcription rates as the highest transcription rate among the *M. fortuitum*-group, which belonged to the type strain *M. fortuitum* DSM 46621. *M. fortuitum* 10851/03 exhibited the lowest transcription rate among all *M. fortuitum* strains, whereas the two *M. peregrinum* strains showed even lower amounts of porin mRNA than any other tested strain (Figure 17 B). The combined illustration (Figure 17 C) of the amounts of *porM1* protein and mRNA isolated from various strains demonstrates the clear concordance of transcription rates with translation rates of porin genes among the RGM.

These results show that although the amino acid sequences of porin genes among the RGM are highly conserved, the expression profiles of porins differ even among the strains of one species.

3.3.5 PorM1 is exposed to the surface of *M. fortuitum*

MspA was shown to be accessible on the cell surface of *M. smegmatis* by using the antiserum pAK MspA#813 (Stahl et al., 2001). Since the expression analysis showed a decreased amount of porin in members of the *M. fortuitum*-group, *M. fortuitum* DSM 46621 and *M. fortuitum* 10860/03 (the two strains with the highest porin expression rates) were exemplary employed for detection of porins at the surface of intact mycobacteria. I first started with detection of porins at the surface of *M. fortuitum* by means of flow cytometry experiments, however no MspA-specific signal was measured compared to the autoflourescence of untreated mycobacteria (data not shown). Thus, the detection of the primary MspA-specific antibody was performed by using a secondary horseradish peroxidase conjugated anti-rabbit IgG in quantitative microwell immunoassays. Every molecule of peroxidase catalyzes the oxidation of many molecules of substrate, which results in amplification of the signal and in turn in a higher sensitivity of the assay compared to flow cytometry experiments. As shown in Figure 18, porins were accessible at the surface of intact cells of *M. fortuitum* and were detected by the porin specific antibody. Significantly higher absorption at 450 nm was measured for *M. fortuitum* DSM 46621 as well as *M. fortuitum*

10860/03 compared to the relative backgrounds, which were measured by applying only the secondary antibody and measuring its non-specific binding.

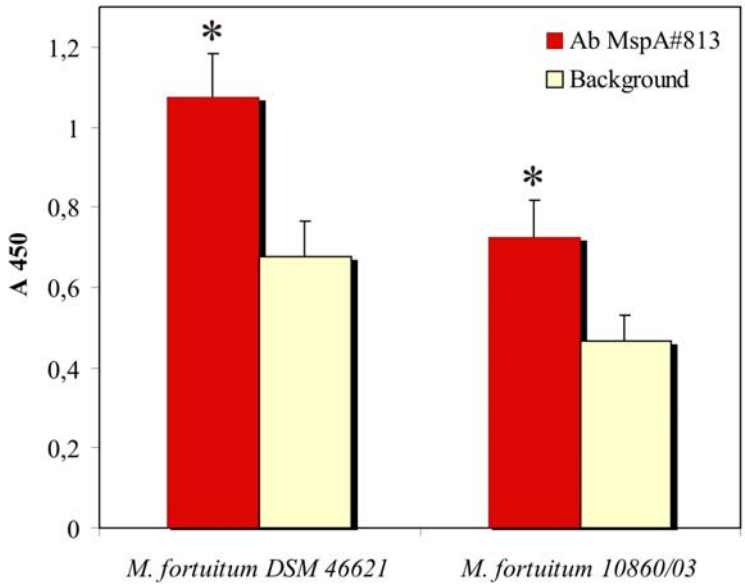

Figure 18: Detection of PorM1 at the surface of *M. fortuitum* using the porin-specific antiserum pAK MspA#813 in quantitative microwell immunoassays. Each column represents the mean (±SD) of 8 measurements. Asterisks indicate significant differences between the samples, which were treated with pAK MspA#813 and backgrounds according to the paired Student's *t*-test ($P<0.001$).

The ratio of detected porin at the surface between the two strains was consistent with ratios, which were obtained by analyzing the expression profiles (see above). Because of this finding and because *M. smegmatis* expresses higher levels of porin (Figure 17 C), the density of porins in the OM of members of the *M. fortuitum*-group is probably lower than in the OM of *M. smegmatis*.

3.4 Construction of the suicide plasmid pSSs003

As a consequence of at least eight *Mycobacterium* genome-sequencing projects, which are at or near completion, plenty of sequence data is provided, which remains to be functionally characterized. Identification and characterization of new genes, however, requires in many cases the mutagenesis of genes and study of phenotypes of the mutant strains. I therefore constructed a suicide plasmid suitable for mutagenesis of any mycobacterial gene with known nucleotide sequences. There are few appropriate and functional resistance genes, which are qualified for mycobacteria. One of those is the *hyg*

resistance gene, which was applied to construct pSSs003. This plasmid provides two multiple cloning sites for cloning of flanking regions from target genes to enable allelic exchange between the target sequence and the *hyg* gene by homologous recombination. The inserted fragments can be checked by the two sequencing primers hygseq_1 (5'-TCG CCT TCA CCT TCC TGC-3') and hygseq_2 (5'-GTA ACA GGG ATT CTT GTG TCA C-3'), which allow sequencing reactions at an annealing temperature of 54°C (Figure 19 and 20).

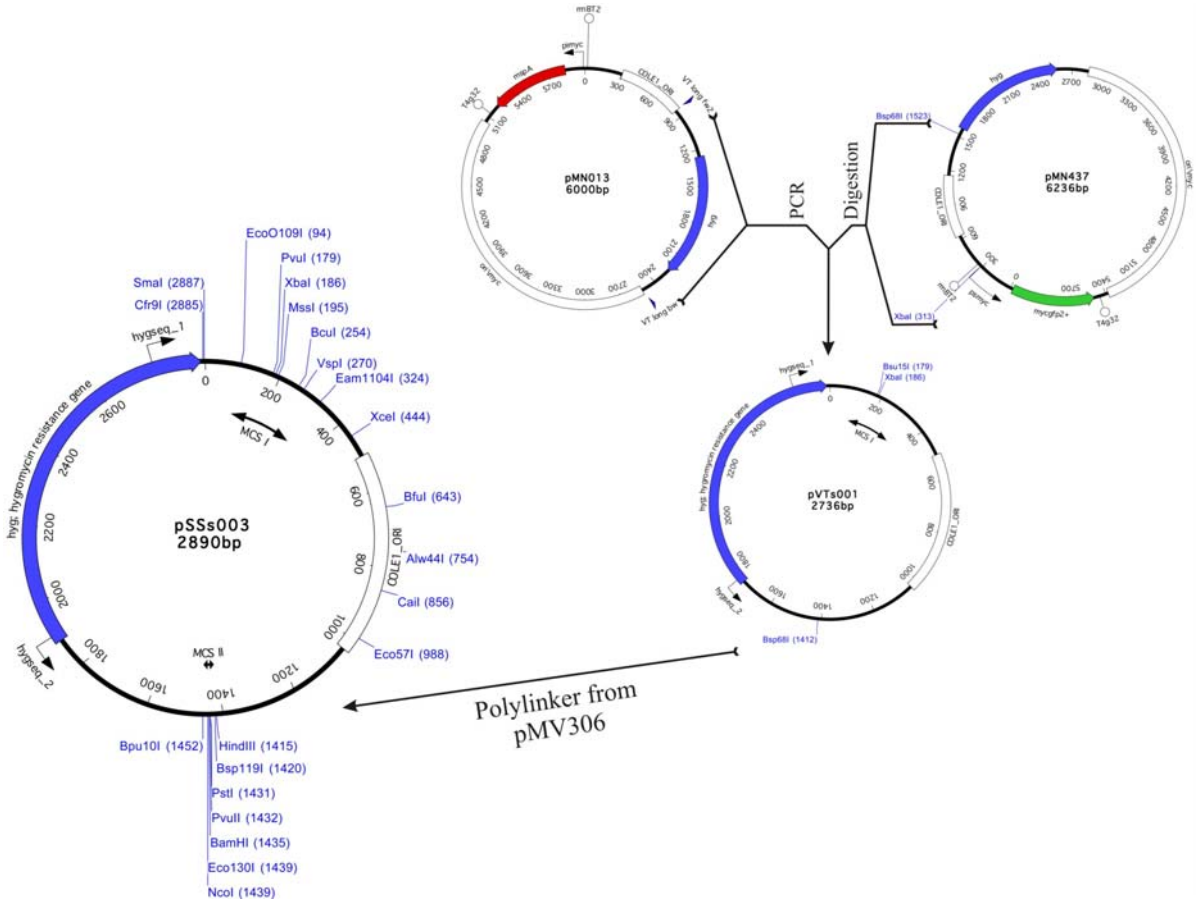

Figure 19: Construction and map of the suicide plasmid pSSs003. It possesses two multiple cloning sites (MCS), which flank the hygromycin resistance gene *hyg*. The binding sites for sequencing primers to prove the inserted sequences are indicated as hygseq_1 and 2.

Currently the flanking regions of *porM1* from *M. fortuitum* 10851/03 are transferred into both multiple cloning sites (Figure 20) and mutagenesis experiments have started. Deletion of *porM1* in *M. fortuitum* will provide an option to analyze the role of porins on pathogenicity of the species. This plasmid is also currently employed to delete the genes for the DNA binding proteins *mdp1* in *M. bovis* BCG and *hlp1* in *M. smegmatis* (data not shown).

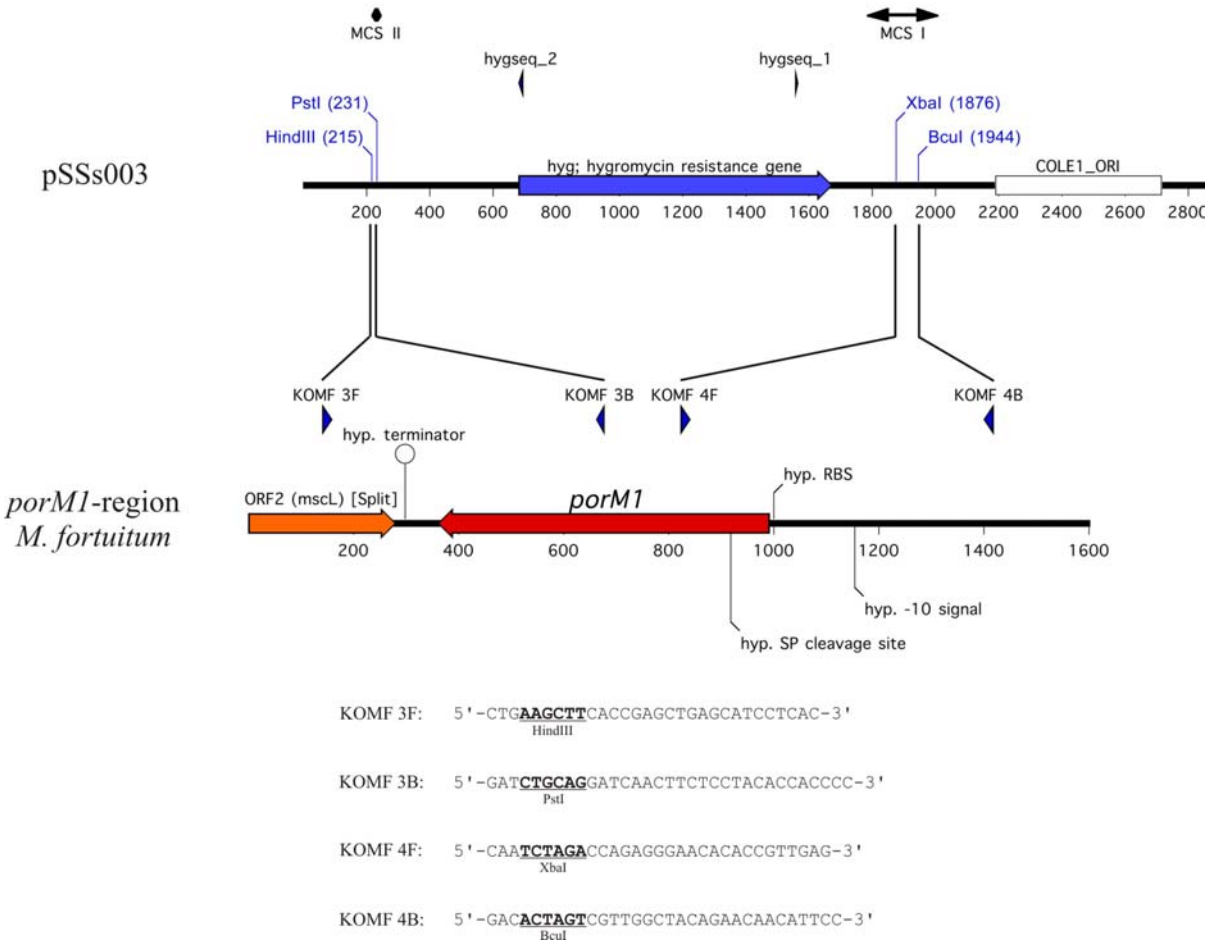

Figure 20: Cloning of flanking regions of *porM1* in pSSs003. Parts of porM1 and its flanking regions were obtained by means of PCR using the primer pairs KOMF 3F/3B and KOMF 4F/4B. These primers introduce the restriction sites as indicated by the underlined and bold letters, which enable the cloning of the PCR fragments in the relative restriction sites of pSSs003.

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 three million persons are killed annually (Dye et al., 1999). 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 one question arises, why the highly pathogenic species belong to the slow-growers, whereas the non-pathogenic or opportunistic mycobacteria are fast-growers and whether slow growth in principle could represent 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 and 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 excretion 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 different types of porins in the OM of mycobacteria affects their growth characetristics 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 transformed the recombinant plasmid (pSSa100) 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 those 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 observation of the time point 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* into 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 time point. 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.1an 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 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.

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 do not allow direct comparisons (Barry, 2001). Mutagenesis of porin genes in *M. smegmatis* allows investigating the impact of cell wall permeability on persistence. However, more appropriate species for these studies must naturally be able to persist on the cellular level and additionally possess a known class of porins, conditions that 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) as well as possesses *mspA* homologues.

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 and closely related *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. 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 protein (without signal peptide) exhibit only six or seven amino acid replacement, respectively if compared to MspA. These features imply very similar functions and characteristics of the two porins. Few studies are performed to enlighten the virulence mechanisms of pathogenic RGM like *M. fortuitum*. The investigation on the role of porins on persistence of *M. fortuitum* would not only contribute to the understanding of mycobacterial persistence but also elucidate the virulence mechanisms of this poorly investigated pathogen.

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. At the same time the porin expression in the members of the *M. fortuitum*-group was significantly lower than those in *M. smegmatis*. The differences in porin expression can be associated with different abilities of the strains to persist, which remain to be investigated. It was shown that *M. smegmatis* possesses 1000 MspA-like pores per μm^2 cell wall (Engelhardt et al., 2002). Since the analyzed strains 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 μm^2 cell wall.

An interesting result from the various genome-sequencing projects is the different genome size between the RGM and the pathogenic slow-growing mycobacteria. 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 that different data such as 16S rRNA sequences or genome sizes suggest 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 of the MspA class or a decreased density of porins in the cell wall play an important role to wall-off towards the hostile phagosomal environment and thus of particular importance to become an successful intracellular pathogen.

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.

5 Summary

The genus *Mycobacterium* comprises highly pathogenic as well as opportunistic or apathogenic species exhibiting a great variability with respect to their growth rates but also their ability to persist or multiply on the cellular level. Intracellular persistence is a key feature of virulence of *M. tuberculosis*, the causative agent of human tuberculosis. The intention of this work was to find out whether or to which degree the permeability of the mycobacterial outer membrane affects the intracellular persistence. For this purpose the major porin of *M. smegmatis* (*mspA*), which is lacking in slow-growing mycobacteria, was expressed in *M. bovis* BCG. Quantification of bacterial growth on agar plates demonstrated clearly increased growth of the *M. bovis* BCG derivative expressing MspA. Transposon mutagenesis proved the *mspA* gene to be responsible for the growth enhancement. Intracellular multiplication of the *M. bovis* BCG derivative in the mouse macrophage cell line J774A.1 and the human pneumocyte cell line A549 was also clearly enhanced.

In contrast to this finding, deletion of *mspA* in *M. smegmatis* increased its intracellular persistence in *A. castellanii* and murine bone marrow macrophages. Deletion of *mspA* together with another homologous porin *mspC* in another mutant strain of *M. smegmatis* resulted in decreased growth in broth culture while it significantly enhanced intracellular persistence in murine bone marrow macrophages, the mouse macrophage cell line J774A.1 and *A. castellanii*, respectively. Complementation of deletions by expression of *mspA* in the porin mutant strains resulted in restoration of the wild type phenotype with respect to intracellular persistence.

These data show that the permeability of the mycobacterial cell wall affects the intracellular persistence. These 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.

Furthermore, the gene *porM1* encoding a porin homologous to MspA was characterized in *M. fortuitum*. PorM1 was present in different strains of *M. fortuitum* and in the closely related non-pathogenic *M. peregrinum*. Analysis of expression patterns of *porM1* showed divergent expression profiles among the members of the *M. fortuitum*-group. Due to the ability of *M. fortuitum* to persist and due to the existence of porins like PorM1, this

species represent an appropriate model to study the impact of mycobacterial porins on persistence.

6 Zusammenfassung

Die Gattung *Mycobacterium* beherbergt sowohl hoch pathogene als auch opportunistische oder apathogene Arten, die unterschiedliche Wachstumsraten aufweisen und außerdem sich in der Fähigkeit intrazellulär zu persistieren unterscheiden. Ein Hauptmerkmal der Virulenz von *M. tuberculosis*, dem Erreger der Tuberkulose im Menschen, ist die intrazelluläre Persistenz. Das Ziel dieser Arbeit war herauszufinden, ob und inwiefern die Permeabilität der äußeren Membran von Mykobakterien die intrazelluläre Persistenz beeinflusst. Hierfür wurde das Porin MspA von *M. smegmatis*, welches in langsam wachsenden Mykobakterien nicht vorkommt, in *M. bovis* BCG expremiert. Die Quantifizierung des bakteriellen Wachstums auf Agarplatten zeigte einen deutlichen Wachstumsvorteil des *M. bovis* BCG Derivats, das *mspA* enthielt. Die Mutagenese von *mspA* mittels einer Transposon-Insertion zeigte, dass MspA den veränderten Phänotyp bedingte. Des Weiteren zeigte das *M. bovis* BCG Derivat mit MspA eine verbesserte intrazelluläre Persistenz in der murinen Makrophagen-Zelllinie J774A.1 und der humanen Lungenepithel-Zelllinie A549.

Im Gegensatz zu diesen Ergebnissen führte die Deletion von *mspA* in *M. smegmatis* zu einer verbesserten intrazellulären Persistenz in *A. castellanii* und in murinen Knochenmarksmakrophagen. Die Deletion von *mspA* und dem homologen Poringen *mspC* in einem anderen Mutantenstamm von *M. smegmatis* führte zu langsamerem Wachstum in Flüssigkultur und verbesserter intrazellulärer Persistenz in der murinen Makrophagen-Zelllinie J774A.1, in *A. castellanii* und in murinen Knochenmarksmakrophagen. Die Komplementation der Mutation durch die Expression von *mspA* in den Mutantenstämmen führte zur Wiederherstellung des Wildtyp-Phänotyps.

Diese Ergebnisse zeigen, dass die Permeabilität der mykobakteriellen Zellwand die intrazelluläre Persistenz und die Wachstumsrate beeinflusst. Außerdem deuten diese Daten daraufhin, dass die Persistenz von Mykobakterien abhängig ist von der Balance zwischen der Abschottung gegenüber des feindlichen phagosomalen Milieus und der Aufnahme von benötigten Nährstoffen in dieser nährstoffarmen Umgebung.

Weiterhin wurde das Gen *porM1*von *M. fortuitum* charakterisiert, das ein homologes Porin zu MspA kodiert. PorM1 konnte in allen untersuchten Stämmen von *M. fortuitum* und in zwei Stämmen der nahe verwandten und apathogenen Art *M. peregrinum* nachgewiesen werden. Die Expressionsanalyse von *porM1* zeigte unterschiedliche Expressionsmuster

innerhalb der untersuchten Stämme der *M. fortuitum*-Gruppe. Aufgrund der Fähigkeit von *M. fortuitum* intrazellulär persistieren zu können und aufgrund des Vorhandenseins von Porinen wie PorM1, stellt diese Art ein geeignetes Modell dar, um den Einfluss mykobakterieller Porine auf die Persistenz zu studieren.

7 References

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8 Appendix

8.1 Abbreviations

Table 7: List of abbreviations.

AG	Arabinogalactan
aph	Gene for aminotransferase
ATP	Adenosintriphosphate
att	Attachment site
BCG	Bacille Calmette-Guerin
bp	Basepare
CFU	Colony forming unit
CTAB	Hexa-decyltrimethylammonium bromide
dH_2O	Distilled H_2O
DNA	Desoxiribonucleic acid
dNTP	Desoxiribonucleoside triphosphate
ELISA	Enzyme linked immunosorbant assay
EMBL	European Molecular Biology Laboratory
FAM	6-Carboxyl-Flourecein
g	Acceleration of gravity
h	Hour
hsp60	Heat shock protein 60
HYG	Hygromycin
Ig	Immunoglobulin
IM	Inner membrane
int	Gene for integrase
Ip	Isoelectric point
kDa	Kilodalton
KM	Kanamycin
kV	Kilovolt
LAM	Lipoarabinomannan
LB	Luria Bertani medium
MA	Mycolic acid
Mb	Mega basepare
min	Minute
MOI	Multiplicity of infection
mspA	*Mycobacterium smegmatis* porin A
MW	Molecular weight
NCBI	National Center for Biotechnology Information
nOPOE	n-octylpolyoxyethylene
nS	Nanosiemens
OD	Optical density
OM	Outer embrane
ompATb	Outer membrane protein A *Tuberculosis*
ORF	Open reading frame
PBS	Phsphate buffered saline
PCR	Polymerase chain reaction
PG	Peptidoglycan
porM1	Porin *Mycobacterium* 1
RGM	Rapidly growing mycobacteria
rMspA	Recombinant MspA
RNA	Ribonucleic acid
ROX	6-Carboxy-X-rhodamine
rpm	Rounds per minute
rRNA	Ribosomal RNA
RT	Reverse transcriptase
s	Second
SD	Standard deviation
SDS	Sodium dodecyl sulfate
TAMRA	Tetramethylrhodamine
TEM	Transmission electron microscopy
U	Unit
WHO	World Health Organization

8.2 Maps of plasmids

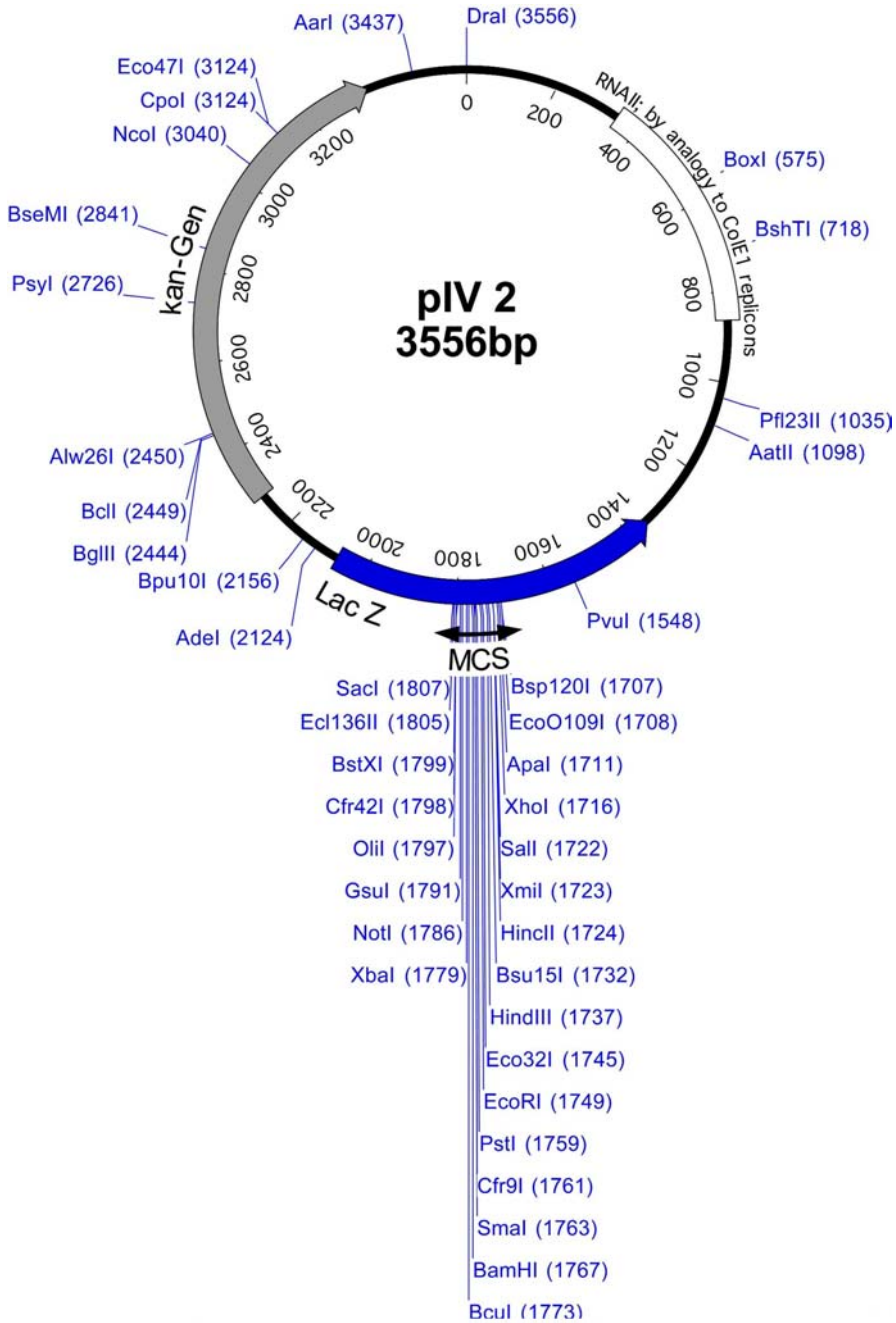

Figure 21: Cloning plasmid pIV2 (Strauch et al., 2000).

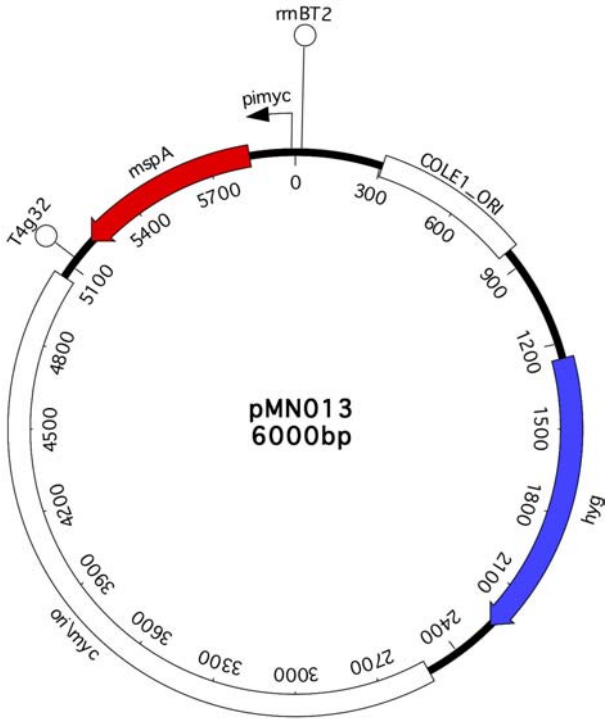

Figure 22: Plasmid pMN013 (Mailaender et al., 2004).

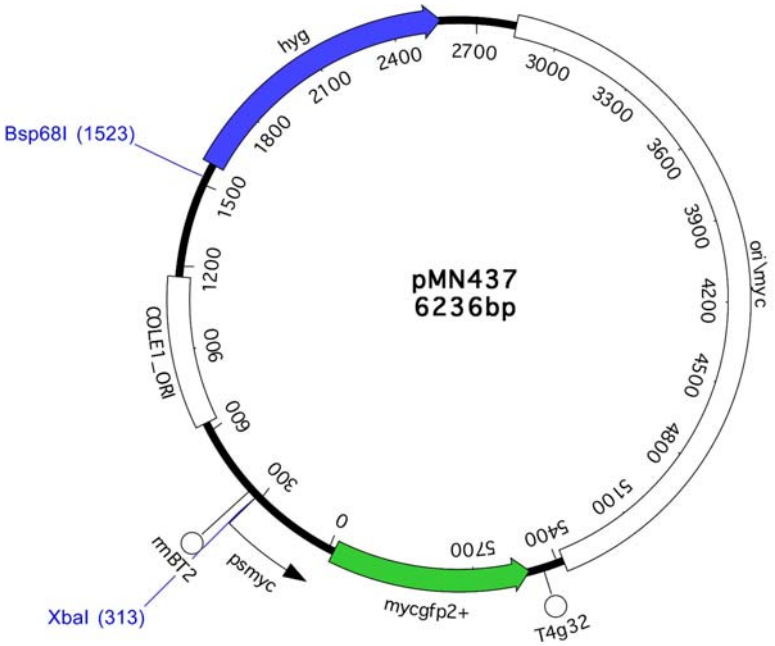

Figure 23: Plasmid pMN437 (Kaps et al., 2001).

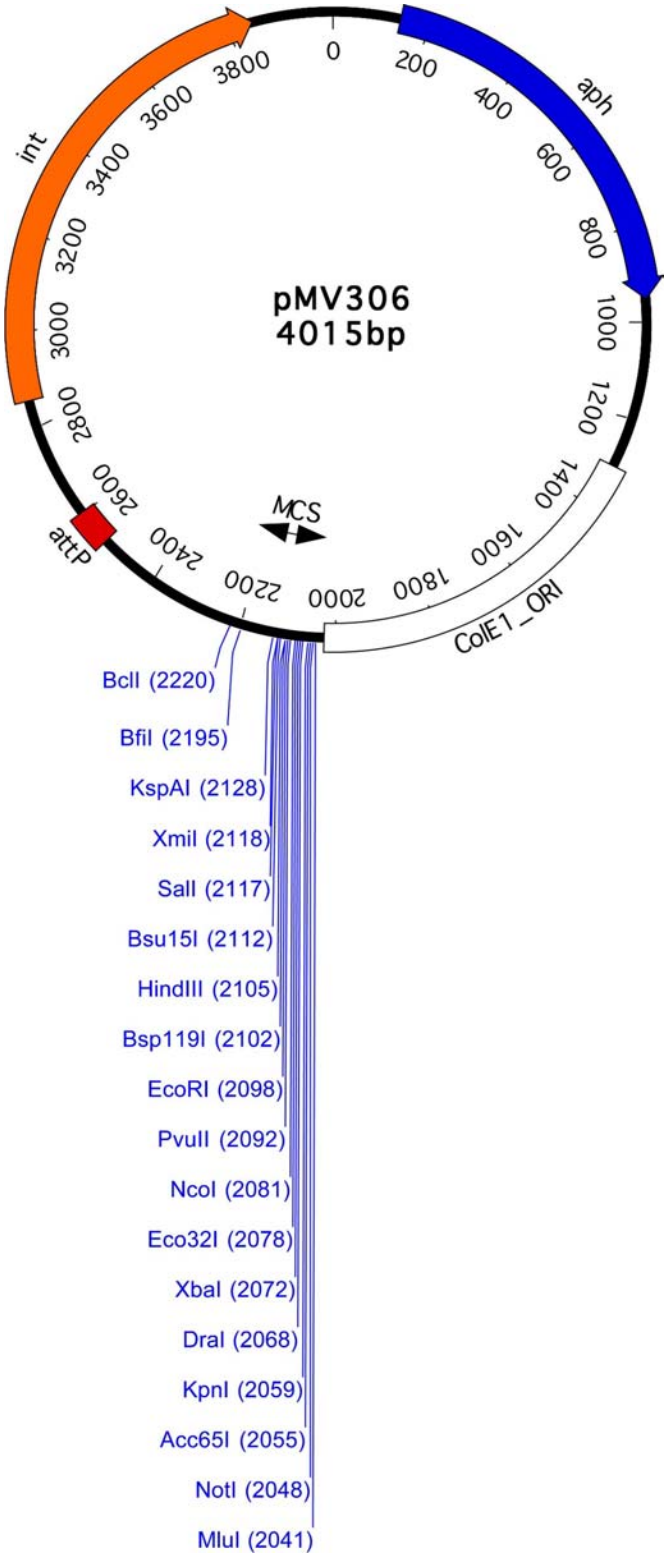

Figure 24: Cloning plasmid pMV306 (Stover et al., 1991).

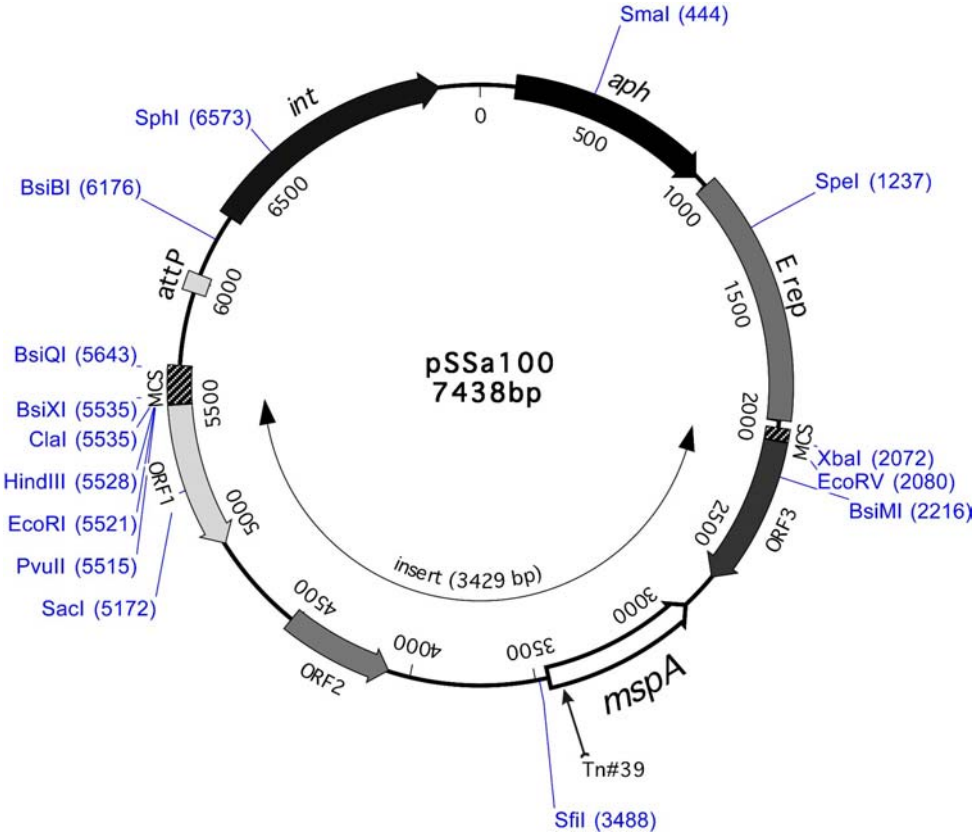

Figure 25: Plasmid pSSa100, prepared in this work (Sharbati-Tehrani et al., 2004).

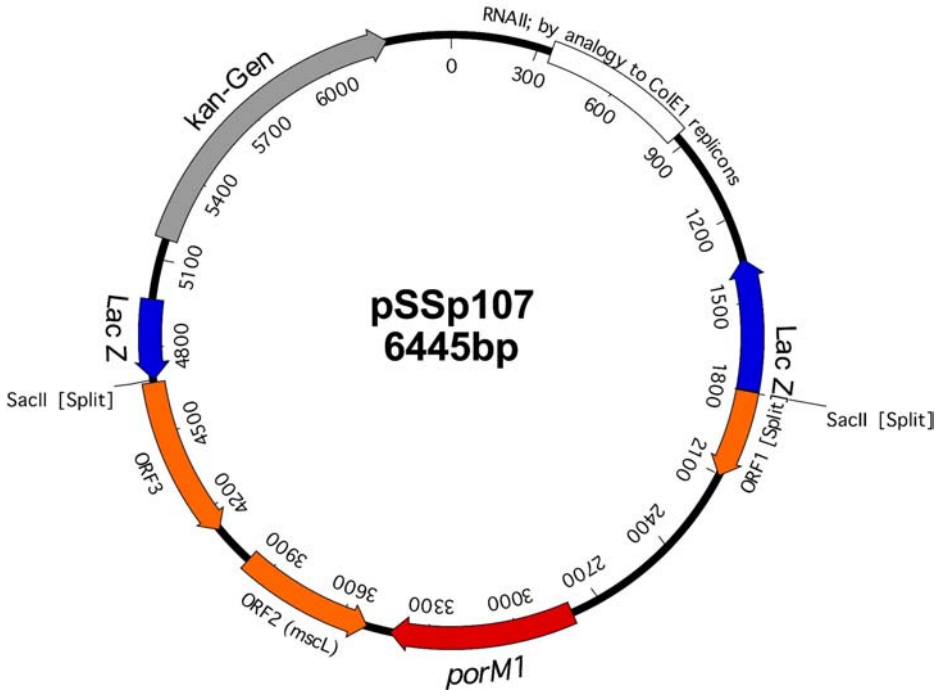

Figure 26: Plasmid pSSp107 (identical to pSSp108), prepared in this work.

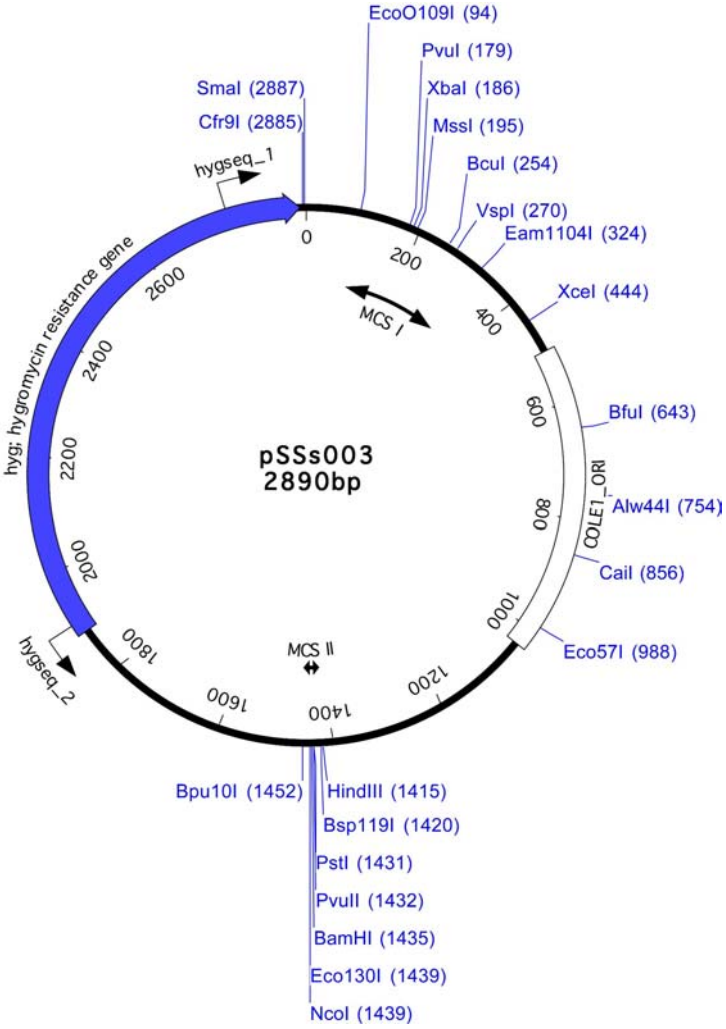

Figure 27: Mycobacterial suicide plasmid pSSs003, constructed in this work.

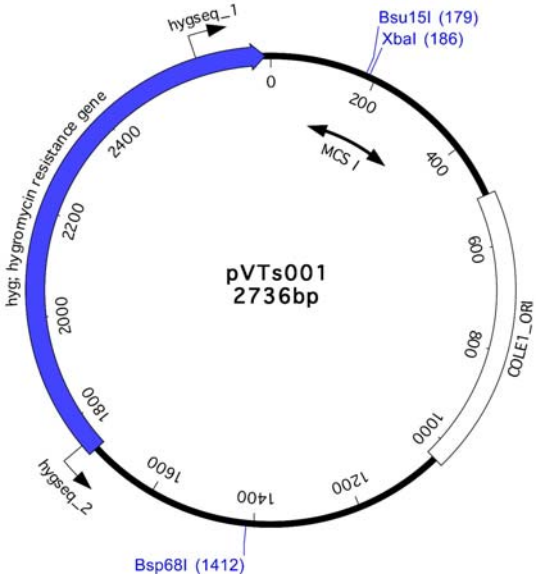

Figure 28: Precursor plasmid pVTs001, constructed in this work.

8.3 Nucleotide sequences

PorM1 sequence from *M. fortuitum* DSM 46621:

ATGAAGGCATTCAGTCGGGTGCTGGTCGCGATAGTTGCAGCTATCGCGGCGCTGTTTACGAG
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CTGACCCGTGAGTGGTTCCATTCCGGCCGGGCCAAGTACACCGTGGCCGGCCCGGGCGCCGA
TGAGTTCGAGGGCACCTTGGAGCTGGGCTACCAGATCGGCTTCCCCTGGTCGCTGGGTGTCG
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CCGGCGGTGTGGCGGTGTCCAACGCGCACGGCACCGTGACCGGTGCGGCCGGTGGTGTGCTG
CTGCGCCCGTTCGCCCGCCTGATCGCCTCCACCGGTGACAGCGTCACCACCTACGGCGAACC
CTGGAACATGAACTGA

PorM1 sequence from *M. fortuitum* 10851/03:

ATGAAGGCATTCAGTCGGGTGCTGGTCGCGATAGTTGCAGCTATCGCGGCGCTGTTTACGAG
CACGGGCGTATCTCATGCGGGTCTGGACAATGAACTGAGCCTGGTTGATGGGCAGGATCGGA
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CTGACCCGTGAGTGGTTCCATTCCGGCCGGGCCAAGTACACCGTGGCCGGCCCGGGCGCCGA
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PorM1 sequence from *M. fortuitum* 10860/03:

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PorM1 sequence from *M. peregrinum* 9912/03:

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PorM1 sequence from *M. peregrinum* 9926/03:

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16S rRNA sequence from *M. fortuitum* DSM 46621:

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16S rRNA sequence from *M. fortuitum* 10851/03:

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16S rRNA sequence from *M. fortuitum* 10860/03:

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16S rRNA sequence from *M. peregrinum* 9912/03:

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16S rRNA sequence from *M. peregrinum* 9926/03:

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Nucleotide sequence of pSSs003:

CCCGCGCCGAAGCCCGCTGCTGCGAGCCCGGAGCGGGCCGGCCGACGGCGGTGCGGGCCCGG
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GGAGACCCCGCTGGATCTCTCCGGCTTCACCGATCCGGAGGAACTGGCGCAGTTCCTCTGGG
GGCCGCCGGACACCGCCCCCGGCGCCTGACGCCCCGGG

8.4 Publications resulted from this study

Lewin, A., Freytag, B., Meister, B., Sharbati-Tehrani, S., Schafer, H., & Appel, B. (2003). Use of a quantitative TaqMan-PCR for the fast quantification of mycobacteria in broth culture, eukaryotic cell culture and tissue. *J. Vet. Med. B. Infect. Dis. Vet. Public Health* **50**, 505-509.

Sharbati-Tehrani, S., Freytag, B., Meister, B., Martinez-Moya, M., Appel, B., and Lewin, A. (2003). Introduction of a gene from *Mycobacterium smegmatis* encoding a porin into *Mycobacterium bovis* BCG and investigation of its growth physiology. *Infection* 31 (Suppl1): 104. 7. Kongress für Infektionskrankheiten und Tropenmedizin. 27.02.-01.03.2003, Berlin.

Sharbati-Tehrani, S., Meister, B., Appel, B., & Lewin, A. (2004). The porin MspA from *Mycobacterium smegmatis* improves growth of *Mycobacterium bovis* BCG. *Int. J. Med. Microbiol.* **294**, 235-245.

Sharbati-Tehrani, S., Stephan, J., Appel, B., Niederweis, M., and Lewin, A. (2005). Porins limit the intracellular persistence of *Mycobacterium smegmatis*. (submitted to *Microbiology*).

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8.6 Erklärung

Die dieser Dissertation zugrunde liegenden Arbeiten wurden am Robert Koch-Institut, Berlin, im Zeitraum Mai 2002 bis März 2005 durchgeführt.

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig verfasst und keine anderen als die angegebenen Hilfsmittel verwendet habe.

Berlin, _____

Soroush Sharbati-Tehrani