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 lines 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 DMEM 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 & 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 4 × 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 s 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 *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* genes 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 1 ml 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 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 members 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 in 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 cell extract (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 OD_{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 (pAK 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, 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 bacilli were 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.