The MspA porin promotes growth and increases antibiotic susceptibility of both Mycobacterium bovis BCG and Mycobacterium tuberculosis

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Porins mediate the diffusion of hydrophilic solutes across the outer membrane of mycobacteria, but the efficiency of this pathway is very low compared to Gram-negative bacteria. To examine the importance of porins in slow-growing mycobacteria, the major porin MspA of Mycobacterium smegmatis was expressed in Mycobacterium tuberculosis and Mycobacterium bovis. Approximately 20 and 35 MspA molecules per μm² cell wall were observed in M. tuberculosis and M. bovis BCG, respectively, by electron microscopy and quantitative immunoblot experiments. Surface accessibility of MspA in M. tuberculosis was demonstrated by flow cytometry. Glucose uptake was twofold faster, indicating that the outer membrane permeability of M. bovis BCG to small and hydrophilic solutes was increased by MspA. This significantly accelerated the growth of M. bovis BCG, identifying very slow nutrient uptake as one of the determinants of slow growth in mycobacteria. The susceptibility of both M. bovis BCG and M. tuberculosis to zwitterionic β-lactam antibiotics was substantially enhanced by MspA, decreasing the minimal inhibitory concentration up to 16-fold. Furthermore, M. tuberculosis became significantly more susceptible to isoniazid, ethambutol and streptomycin. Fluorescence with the nucleic acid binding dye SYTO 9 was 10-fold increased upon expression of mspA. These results indicated that MspA not only enhanced the efficiency of the porin pathway, but also that of pathways mediating access to large and/or hydrophobic agents. This study provides the first experimental evidence that porins are important for drug susceptibility of M. tuberculosis.

INTRODUCTION

Tuberculosis (TB) causes approximately two million deaths per year and is still a major global health problem, although it can be efficiently cured by a 6-month chemotherapy with four drugs. The unusual length of the chemotherapy, the increasing spread of multi-drug resistant strains, and the current failure to treat persistent infections with Mycobacterium tuberculosis, have intensified worldwide efforts to find new antitubercular drugs and to understand the mechanisms of action of the current drugs (Kremer & Besra, 2002a, b).

It has long been suggested that the extremely low permeability of the unusual mycobacterial cell wall renders mycobacteria intrinsically resistant to many antibiotics such as β-lactams, macrolides, tetracyclines, novobiocin and chloramphenicol (Nikaido & Jarlier, 1991). Based on X-ray diffraction experiments of purified mycobacterial cell walls, which showed that the mycolic acids are oriented perpendicular to the cell surface (Nikaido et al., 1993), it was proposed that the mycolic acids form the inner leaflet, and extractable lipids the outer leaflet, of an outer membrane (OM)-like structure (Brennan & Nikaido, 1995). It is assumed that at least two general diffusion pathways across the mycobacterial OM exist: the 'hydrophobic' (or lipid) pathway, which is characterized by the nature and the interactions of the membrane lipids; and the 'hydrophilic' (or porin) pathway, whose properties are determined by water-filled channel proteins, the porins, which span the
OM (Niederweis, 2003). Nikaido and co-workers showed that the fluidity of the OM of *Mycobacterium chelonae* is very low and strongly depends on the nature of the mycolic acids (Liu et al., 1995, 1996). They proposed that the unique structure and composition of the outer lipid bilayer make mycobacteria exceptionally impermeable to lipophilic solutes, but quantitative data are lacking so far. The uptake pathways for cephaloridine of *Mycobacterium smegmatis* and *M. tuberculosis* (Chambers et al., 1995; Trias & Benz, 1994) and of *C. chelonae* (Jarlier & Nikaido, 1990) were shown to be 100-fold and 1000-fold, respectively, less efficient than that of *Escherichia coli* (Nikaido, 1986). We provided evidence that permeation of cephaloridine across the OM of *M. smegmatis* is mainly mediated by the porin MspA (Stahl et al., 2001). Furthermore, the 45-fold lower number of pores and the 2.5-fold longer pore channels compared to *E. coli* were identified as two determinants of the low efficiency of the porin pathway in *M. smegmatis* (Engelhardt et al., 2002). Similar causes for low OM permeability for hydrophilic solutes are likely to exist for all mycobacteria (Niederweis, 2003). These results implied that the low porin permeability of *M. tuberculosis* may limit (i) the efficiency of hydrophilic drugs in TB chemotherapy as suggested by many authors (Brennan & Nikaido, 1995; Draper, 1998; Jarlier & Nikaido, 1994; Lambert, 2002) and (ii) the growth rate of mycobacteria due to restricted uptake of polar nutrients (Jarlier & Nikaido, 1990). Considering the importance of *M. tuberculosis* as a bacterial pathogen, and the need to understand how nutrients and drugs are transported inside the cell, it is surprising that the importance of porins for these processes has not been experimentally examined yet. For example, it is not known which proteins mediate the diffusion of hydrophilic solutes across the OM of *M. tuberculosis*. OmpATb displays a low channel activity in vitro (Senaratne et al., 1998) and has transport activity for serine at low pH in vivo, but is unlikely to be a major general porin of *M. tuberculosis*, because the uptake rates for serine and glycine at pH 7.2 were not greatly affected by deletion of the *ompATb* gene (Raynaud et al., 2002). The existence of other porins of *M. tuberculosis* has been demonstrated (Kartmann et al., 1999), but these proteins still await identification. It is also unknown which antitubercular drugs are capable of diffusion through mycobacterial porins.

Since porin-negative mutants of *M. tuberculosis* or *M. bovis* BCG are lacking, we used a different approach to examine the importance of porins for the OM permeability of slow-growing mycobacteria. To this end, the porin MspA of *M. smegmatis* was expressed in the OMs of *M. bovis* BCG and *M. tuberculosis* and the OM permeability for glucose, the sensitivity to antibiotics and the growth rate of the recombinant strains were analysed.

**METHODS**

**Chemicals, enzymes and DNA.** Hygromycin B was obtained from Calbiochem; all other chemicals were from Merck, Roth or Sigma at the highest purity available. Enzymes for DNA restriction and modification were from New England Biolabs, Boehringer, Stratagene or Pharmacia. Isolation and manipulation of DNA was done as described by Ausubel et al. (1987). Oligonucleotides were obtained from PerkinElmer Applied Biosystems.

**Bacterial strains and growth conditions.** *Mycobacterium bovis* BCG, strain Institut Pasteur, was obtained from the American Type Culture Collection (ATCC 27291). *M. tuberculosis* H37Rv was kindly provided by Dr Peter Sander (Institute of Medical Microbiology, University of Zurich, Switzerland). Unless otherwise noted, *M. bovis* BCG and *M. tuberculosis* H37Rv were grown in Middlebrook 7H9 broth (Difco) or on 7H10 agar plates supplemented with 0.05% Tween 80 (Sigma), 0.2% glycerol and ADS (0.5% bovine serum albumin fraction V, 0.2% glucose and 14 mM NaCl) enrichment at 37°C. All experiments with live *M. tuberculosis* were carried out under biosafety level 3 conditions. *E. coli* DH5α was used for all cloning experiments and was routinely grown in LB medium. Hygromycin B was used when required at the following concentrations: 200 μg ml⁻¹ for *E. coli* and 75 μg ml⁻¹ for mycobacteria.

**Construction of plasmids.** The hsp60 promoter was amplified from pSTM3 by PCR (kindly provided by Dr Sabine Ehrn, Cornell University, New York, USA) with the oligonucleotides hsp60_fwd (5'AAACGCGTACCAACGAGGCAGCGGACC-3'), which has a half-side of the Pmec site and hsp60_rev (5'GCTCTAGATTATTACTCTACCGGTTCGCGAGTGCCAACG-3'), which introduced a Pac site. The PCR fragment was digested with PacI, purified by preparative gel electrophoresis and ligated with PacI-digested pMS2 DNA to give pMS3. The mspA gene was isolated from pMN014 as a PacI–SwaI fragment and cloned via the same restriction sites into pMS3 to yield the mspa expression vector pMN066. All plasmid constructions were verified by restriction enzyme digestion and double-stranded DNA sequencing. The plasmids used in this study are summarized in Table 1.

**Preparation of detergent extracts and immunoblot analysis.** The whole-cell extracts were prepared as described previously (Heinz & Niederweis, 2000). Fifteen microlitres of the supernatant of each strain were used for protein gel electrophoresis. The immunoblot analysis was performed exactly as described by Stahl et al. (2001). For specific detection of MspA, the murine monoclonal antibody (mAb) A15 was used in a dilution of 1:500 and horseradish peroxidase coupled to a second anti-mouse antibody (dilution 1:10 000, Dianova) oxidized the luminol (ECL plus kit, Amersham) whose chemoluminescence was detected by X-AR-5 films (Kodak).

**Electron microscopy.** Cell suspensions of *M. bovis* BCG (0.5 ml) were sedimented in a table top centrifuge, at low speed, to collect intact cells only. The pellet was resuspended in distilled water. The suspension was cooled in ice-water and sonified in a Branson Sonifier for two or three pulses (50 W). This was sufficient to break part of the cells and to obtain cell wall fragments of reasonable size. Five microlitres of this suspension was put on carbon-coated copper grids. The liquid was blotted after 20 seconds of adsorption and the sample was negatively stained with 2% uranyl acetate. The specimens were inspected in the electron microscope (Philips EM420 or CM12) at a nominal magnification of x 36 000 and recorded using Agfa film material.

**Transport measurements.** Glucose uptake experiments were carried out as described previously (Bardou et al., 1998) with minor modifications. *M. bovis* BCG, with the control plasmid (pMN006) and with the mspa expression vector (pMN013), were grown as 10 ml precultures for 10–15 days in Middlebrook 7H9 medium containing 0-05% Tween 80 and ADS enrichment. The cells were passed through a filter with a pore size of 5 μm (Sartorius) to remove cell aggregates. After filtration more than 95% of all bacteria
were single, viable cells as demonstrated by staining with the LIVE/DEAD kit (Molecular Probes) and fluorescence microscopy (Axioskop2, Zeiss). The preculture was grown to an OD$_{600}$ of 0 to 32 min. The bacteria were separated from the liquid by filtration through a 0.45 µm pore size filter (Sartorius), washed with 0.1 M LiCl and the radioactivity was determined in a liquid scintillation counter. Five independent experiments were carried out in triplicate and uptake of glucose was expressed as pmol (mg dry weight cells)$^{-1}$. The condition of the cells after the transport measurements was checked by fluorescence microscopy using the LIVE/DEAD kit. In all experiments more than 97 % of all bacteria were single, live cells.

**Cephaloridine hydrolysis assay.** The hydrolysis of cephaloridine by β-lactamases of *M. bovis* BCG was measured spectrophotometrically using the method of Zimmermann and Rosselet as described previously (Stahl et al., 2001).

**Growth experiments.** *M. bovis* BCG/pMN006 (control strain) and the *mspA*-expressing strain BCG/pMN013 were grown as 10 ml precultures for 15 days in Middlebrook 7H9 broth supplemented with 0.05 % Tween 80 and ADS enrichment. The cells were passed through a filter with a pore size of 5 µm (Sartorius) to remove cell clumps. The new 10 ml cultures, containing more than 97 % live, single bacteria, were grown until an OD$_{600}$ of 1 was reached. The clumps. The new 10 ml cultures, containing more than 97 % live, single, viable cells as demonstrated by staining with the LIVE/DEAD kit. In all experiments more than 97 % of all bacteria were single, live cells.

**Antibiotic sensitivity experiments.** Minimal inhibitory concentrations (MICs) were determined for the control strain *M. tuberculosis* H37Rv/pMN006 and the *mspA*-expressing strain H37Rv/pMN013, as well as for *M. bovis* BCG/pMN006 and BCG/pMN013 by agar dilution experiments. Each strain was grown as 10 ml preculture for 15 days and then passed through a filter with a pore size of 5 µm to remove cell clumps. The filtrate, containing only single bacteria, was grown until an OD$_{600}$ of 0.6–0.8 was achieved. A reference curve was constructed with a correlation between the number of colony-forming units (c.f.u.) and OD$_{600}$. Using this curve, dilutions of each strain were made to obtain a final concentration of 5000 c.f.u. ml$^{-1}$. Five hundred colony-forming units were streaked out on plates with rising antibiotic concentrations. The MIC was defined as the lowest drug concentration inhibiting the visible growth of 99 % of all cells after 25 days of incubation at 37 °C.

**Flow cytometry.** Aliquots of *M. tuberculosis* suspensions were thawed and centrifuged for 10 min at 1000 g. Bacteria were resuspended in phosphate-buffered saline (PBS) and several times passed through a syringe with a 26 gauge needle to disrupt aggregates. Aliquots of 2 × 10$^{8}$ bacteria of the *MspA*-expressing *M. tuberculosis* strain, containing the plasmid pMN013, and of the control strain, containing the plasmid pMN006 with a promoterless *mspA* gene, were incubated with a murine monoclonal antibody directed against MspA (mAb A15) for 30 min at 4 °C. Cells were washed, and binding of the primary antibodies was detected by the use of Cy5-labelled goat anti-mouse antiserum (GaMCy5) (Dianova) for 30 min. In experiments using the nucleic acid staining SYTO dyes (Molecular Probes), cells were incubated with 25 µM SYTO 9 and SYTO 12 for the times indicated. After staining the cells were washed, resuspended and fixed in PBS containing 1.5 % paraformaldehyde until analysis in a FACScalibur flow cytometer (BD Bioscience) using CellQuest Pro software (BD Bioscience).

**Apparent n-octanol/water partition coefficients $P_{ow}$.** Apparent partition coefficients of SYTO 9 and SYTO 12 were determined by the ‘shake flask’ method (OECD, Paris, 1981, Test Guideline 107; http://www.oecd.org/dataoecd/17/35/1948169.pdf). Solutions of the dyes were diluted 50-fold in n-octanol, saturated with water, to a final concentration of 0.5 µM. Reference UV spectra of each compound were recorded (Novaspec II spectrophotometer, Pharmacia). Five hundred microlitres of the 10 µM solution in n-octanol was mixed with the same volume of fresh 7H9 broth. Growth rates were determined in three independent cultures by OD$_{600}$ measurements. Condition of precultures and cultures was followed by staining of cell aliquots with the LIVE/DEAD kit and fluorescence microscopy.

### Table 1. Plasmids used in this work

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<th>Plasmid</th>
<th>Characteristics</th>
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<tr>
<td>pSMT3</td>
<td>Hyg$^R$, shuttle vector <em>E. coli/mycobacteria</em>, $P_{pnap}$ from <em>M. bovis</em> BCG, 5700 bp</td>
<td>Gaora (1998)</td>
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<td>pMS2</td>
<td>Hyg$^R$, shuttle vector <em>E. coli/mycobacteria</em>, 5229 bp</td>
<td>Kaps et al. (2001)</td>
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<td>Scholz et al. (2000)</td>
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<td>pMN006</td>
<td>pMS2 derivative, Hyg$^R$, promoterless <em>mspA</em>, 5868 bp</td>
<td>Stahl et al. (2001)</td>
</tr>
<tr>
<td>pMN012</td>
<td>pMS2 derivative, Hyg$^R$, <em>Ppap–mspA</em>, 6152 bp</td>
<td>This study</td>
</tr>
<tr>
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<td>pMS2 derivative, Hyg$^R$, <em>Ppap–mspA</em>, 6000 bp</td>
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<td>pMS2 derivative, Hyg$^R$, <em>Ppap–mspA</em>, 6177 bp</td>
<td>This study</td>
</tr>
<tr>
<td>pPOR6</td>
<td>pOLYG derivative, Hyg$^R$, 8394 bp</td>
<td>Niederweis et al. (1999)</td>
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cross-linked polyethylene glycol capillary column (HP-INNOWAX, 1-20 m length) of a gas chromatograph coupled with a mass selective infrared detector (HP 5971A MSD, HP 5965B ID). Data analysis was performed using the HP 5965B/Chemstation software using a NIST/2001 database.

**Fluorescence analysis of SYTO 9 and SYTO 12.** The fluorescence properties and the binding specificity of the dyes SYTO 9 and 12 were examined using a fluorimeter (Fluorolog-3, Jasco) equipped with two monochromators at an excitation and emission bandpass of 2 nm. One millilitre samples of 10 μM of the SYTO dyes were measured in the presence of increasing amounts of DNA ranging from 1 to 2000 ng, or 5 and 10 μg purified MsPA. SYTO 9 was excited at 470 nm and the fluorescence emission was recorded between 500 and 700 nm. SYTO 12 was excited at 499 nm and the fluorescence emission was recorded between 510 and 700 nm. All experiments were done at a sample temperature of 25 °C.

### RESULTS

**Expression of the porin gene mspa in slow-growing mycobacteria**

MsPA is the main porin of *M. smegmatis* and constitutes a wide, water-filled central pore of 10 nm length (Engelhardt et al., 2002). Since all mycobacterial species are thought to have a similar general cell wall architecture (Niederweis, 2003), expression of the *mspa* gene might result in a functional insertion of the porin into the OM of *M. tuberculosis* and *M. bovis* BCG, and increase its permeability for small and hydrophilic compounds. To evaluate this hypothesis, transcriptional fusions of the *mspa* gene with six different promoters were constructed. Four of the *mspa* expression vectors complemented the low porin level of the OPOE extracts was quantified by image analysis using control vector, demonstrating that the antibody mAb A15 observed in extracts of *M. bovis* BCG transformed with a *mspA* mutant (not shown). Fusions of the *hcpr60* or the

<table>
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<th>kDa</th>
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<td>30</td>
<td>16</td>
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**Fig. 1.** Expression of the *mspa* gene in *M. bovis* BCG and *M. tuberculosis* immuno blot analysis of whole-cell extracts of different strains of *M. bovis* BCG and *M. tuberculosis*. The samples were separated on a denaturing 10% polyacrylamide gel and blotted on a nitrocellulose membrane. Proteins were visualized using the murine mAb A15 and a chemiluminescence reaction (ECLplus kit, Amersham). The letters denote the functional MsPA oligomer (O) and the monomer (M). Lanes: 1, *M. tuberculosis* control strain (pMN006); 2, *M. tuberculosis* p*pimyc−mspa* (pMN013); 3, 1 ng purified MsPA from *M. smegmatis*; 4, *M. bovis* BCG control strain (pMN006); 5, *M. bovis* BCG p*pimyc−mspa* (pMN012); 6, *M. bovis* BCG p*pimyc−mspa* (pMN013); 7, *M. bovis* BCG p*hcpr60−mspa* (pMN066).

**Visualization of MsPA in isolated cell walls of *M. bovis* BCG by electron microscopy**

Electron microscopy analysis of negatively stained cell wall fragments of *M. smegmatis* revealed clearly visible pores with a central stain-filled cavity. In contrast to porins in the cell walls of Gram-negative bacteria, the MsPA pores sharply contrasted with the surrounding lipid matrix, probably due to the large amount of stain in these wide and exceptionally long channels (Engelhardt et al., 2002). We therefore wanted to demonstrate, by electron microscopy, the localization of MsPA and the number of MsPA pores in the cell wall of *M. bovis* BCG. Furthermore, we envisaged that endogenous porins of *M. bovis* BCG might be visible in a similar manner as in *M. smegmatis*. Electron microscopy of negatively stained cell wall fragments of *M. bovis* BCG showed many small black dots in an area of about 0.1 μm² (Fig. 2A). Larger black dots with the diameter of typical MsPA pores were only observed in cell wall fragments of *M. bovis* BCG expressing the *mspa* gene (Fig. 2B). Analysis of nine cell wall fragments revealed 302 pores with a diameter similar to that of MsPA, yielding a mean of 34 ± 11 large pores per μm² cell wall. Thus, the
density of larger pores in *M. bovis* BCG is approximately 30-fold less than that in *M. smegmatis*, which has approximately 1000 MspA-like pores per μm² cell wall (Engelhardt et al., 2002). This is consistent with the 40-fold lower amount of MspA extracted from *M. bovis* BCG cells compared to *M. smegmatis* cells as determined by quantitative immunoblot analysis using the monoclonal anti-MspA mAb A15 (Fig. 1). It should be noted that the MspA-like pores lack the halo in cell walls of *M. bovis* BCG which is clearly visible in *M. smegmatis*. It is concluded that the low amount of MspA in OPOE extracts was due to a low number of pores in the cell wall and not to inefficient extraction from *M. bovis* BCG in comparison to *M. smegmatis*. These results showed that the *mspA* gene is expressed in *M. bovis* BCG and that the MspA porin is integrated into the cell wall of *M. bovis* BCG.

**MspA is accessible on the cell surface of *M. tuberculosis***

The yield of MspA in OPOE extracts of *M. tuberculosis* H37Rv containing the *mspA* expression vector pMN013 was 1.7-fold less than in extracts of *M. bovis* BCG as shown by quantitative image analysis of an immunoblot using the MspA-specific monoclonal antibody A15 (Fig. 1, lanes 2 and 6). This results in a very low number of approximately 20 MspA pores per μm² cell wall of *M. tuberculosis*, assuming that MspA is as efficiently extracted from *M. tuberculosis* cells as from cells of *M. bovis* BCG, and taking the density of MspA pores in *M. bovis* BCG as a reference (Fig. 2). Cells of *M. tuberculosis* H37Rv/pMN013 were incubated with the anti-MspA antiserum #813 (Niederweis et al., 1999) to demonstrate the OM localization of MspA. However, no MspA-specific signal was obtained in flow cytometry experiments compared with cells of a control strain which did not express *mspA* (data not shown), although MspA was easily detected on the surface of *M. smegmatis* in enzyme-linked immunofluorescence assays using this antiserum (Stahl et al., 2001). By contrast, the antibody mAb A15 bound specifically to *M. tuberculosis* cells, containing the *mspA* expression vector, but not to cells containing the control vector pMN006 (Fig. 3). The small but significant fluorescence increase of the *mspA*-expressing **Fig. 2. Visualization of MspA in isolated cell walls of *M. bovis* BCG by electron microscopy.** Isolated cell wall fragments of *M. bovis* BCG (A) and *M. bovis* BCG expressing the *M. smegmatis* porin MspA from the plasmid pMN013 (B) negatively stained with uranyl acetate. Cell wall pores are stain-filled and appear as small black dots. Scale bar, 100 nm. For size comparison, an isolated cell wall fragment of *M. smegmatis* negatively stained with uranyl acetate is shown with the same magnification as the cell fragments of *M. bovis* BCG in the inset. The inset represents an area of 136 nm × 136 nm and shows mainly MspA pores (Engelhardt et al., 2002). Larger pores with a diameter similar to that of the MspA pores of *M. smegmatis* were only observed in cell wall fragments of *M. bovis* BCG expressing the *mspA* gene and are indicated by arrows. The visible membrane areas in the large square and in the inset represent 0.061 μm² and 0.18 μm², respectively (B). Thus, four and 28 pores with a diameter similar to that of MspA pores were found in the two areas, corresponding to a density of 65 and 1514 pores μm⁻² in these cell wall fragments of *M. bovis* BCG and *M. smegmatis*, respectively.

**Fig. 3. Detection of MspA on the cell surface of *M. tuberculosis* by flow cytometry.** *M. tuberculosis* carrying the control plasmid with the promoterless *mspA* gene (fine line) and the *mspA*-expressing strain containing the episomal copy of the P𝑟𝑝𝑟𝑛–*mspA* fusion (bold line) were incubated with a murine monoclonal antibody against MspA (mAb A15). The binding of mAb A15 was detected by a Cy5-labelled goat anti-mouse antiserum using flow cytometry. As controls, the non-specific binding of the secondary antibody to both strains is shown as broken lines.
M. tuberculosis cells upon binding of the antibody mAb A15 is consistent with a low amount of MspA in the OM of M. tuberculosis (Fig. 3). This result showed that the epitope recognized by mAb A15 is accessible on the surface of M. tuberculosis, and indicated that MspA was integrated into the OM of M. tuberculosis upon expression of the mspa gene, consistent with the results obtained for M. bovis BCG.

MspA increases the OM permeability of M. bovis BCG

Glucose was chosen as a hydrophilic solute to probe the porin-mediated cell wall permeability of M. bovis BCG, because, in contrast to glycerol (Jackson et al., 1999), it does not diffuse through lipid membranes and it has an intermediate size. Furthermore, it has been demonstrated previously that MspA is the main porin of M. smegmatis for glucose diffusion (Stahl et al., 2001). In five experiments (each done in triplicate), glucose uptake was taken by wild-type M. bovis BCG at a mean rate of $0.7 \pm 0.3$ pmol min$^{-1}$ (mg dry weight cells)$^{-1}$, at a concentration of 6-4 mM and a temperature of 37°C. Glucose uptake was increased 2.3-fold upon expression of mspa as shown in Fig. 4. Thus, it is concluded that MspA has maintained its porin function in the OM of M. bovis BCG.

MspA accelerates the growth of M. bovis BCG

To examine whether the slow flux of nutrients across the OM limits the growth rate of slow-growing mycobacteria, we compared the growth of M. bovis BCG transformed with the mspa expression vector (pMN013) and with the control vector (pMN006). To minimize clumping of the bacteria, the medium was inoculated with a suspension of single cells of both strains. Cells from cultures of both strains were stained with propidium iodide and SYTO 9 (LIVE/DEAD Kit), which has been shown to differentiate between live and dead M. tuberculosis (Förtsch et al., 2000). Fluorescence microscopy showed that more than 97% of all cells were viable and did not aggregate under those conditions (see supplementary data with the online version of this paper at http://mic.sgmjournals.org). This allowed us to follow bacterial growth by measuring the optical density. At low inocula of approximately $2.5 \times 10^5$ c.f.u., the mspa-expressing M. bovis BCG strain grew significantly faster than the control strain. The generation times were 27 h for the wild-type and 25 h for the mspa-expressing strain in three independent cultures containing the standard Middlebrook 7H9 medium plus ADS enrichment (38 mM glycerol, 11 mM glucose) (Fig. 5). When the inoculum was increased two- and fourfold, the lag phase was reduced by 2 and 3 days, respectively. However, the growth rate was again significantly faster for the mspa-expressing strain, although the growth difference was less pronounced with larger inocula (not shown). Thus, three experiments (each done in triplicate) showed that expression of mspa increased the growth rate of M. bovis BCG.

MspA increases the antibiotic susceptibility of M. bovis BCG and M. tuberculosis

An inherent disadvantage of transport experiments with radiolabelled compounds is the difficulty of distinguishing between transport across the membrane(s), and other

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**Fig. 4.** Cell wall permeability of M. bovis BCG and the mspa-expressing strain for glucose. Wild-type M. bovis BCG contained a promoterless mspa gene on the plasmid pMN006 (●); the mspa-expressing strain carried the p$_{mpc}$-mspA fusion on the plasmid pMN013 (○). The standard deviations of three experiments are shown as error bars. Regression analysis of the first three data points yielded glucose uptake rates of 0.6 and 1.4 pmol min$^{-1}$ (mg dry weight cells)$^{-1}$ with correlation coefficients of 0.96 and 0.98 for wild-type M. bovis BCG and the mspa-expressing strain, respectively (dotted lines). The glucose concentration was 6-4 mM. This assay was done at 37°C.

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**Fig. 5.** Influence of mspa expression on growth of M. bovis BCG. Wild-type M. bovis BCG contained a promoterless mspa gene on the plasmid pMN006 (●) and the mspa-expressing strain carried the p$_{mpc}$-mspA fusion on the plasmid pMN013 (○). Both strains were grown in Middlebrook 7H9 medium containing 0.05% Tween 80 and ADS enrichment. The optical densities of three independent cultures for each strain were averaged. Standard deviations are shown as error bars. The double asterisks denote data points that differed significantly between the wild-type and the mspa-expressing strain according to the paired Student’s $t$-test ($P<0.01$). The generation times were 27 h for the wild-type and 25 h for the mspa-expressing strain.
processes such as adsorption, which also lead to a time-dependent increase of radioactivity pelleted with the cells. Since the targets of most antibiotics are within bacterial cells, we also determined the sensitivity of the mspA-expressing strain of M. bovis BCG to different antibiotics. Surprisingly, the small amount of MspA in the OM drastically increased the sensitivity of M. bovis BCG to the zwitterionic cephaloridine on plates (Fig. 6A). Approximately 70% of the wild-type cells survived on plates containing rising concentrations of: A, cephaloridine; B, ampicillin; C, amoxycillin; D, ethambutol. The plates were incubated at 37°C for 25 days. The number of c.f.u. on plates with antibiotic was normalized to the total c.f.u. as obtained from control plates without antibiotic and was expressed as percentage c.f.u. The experiments were done in triplicate. Standard deviations are shown as error bars. The double asterisks denote data points that differed significantly between the wild-type and the mspA-expressing strain according to the paired Student’s t-test (P < 0.05).

Expression of the mspA gene in the OM of M. tuberculosis offers the opportunity to examine whether porins really limit the uptake and efficiency of TB drugs as is often

**Table 2.** MIC values for M. bovis BCG and M. tuberculosis containing either the control plasmid (wt) or an mspA expression vector (mspA)

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<th>Drug</th>
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<td></td>
<td>wt</td>
<td>mspA</td>
<td>wt</td>
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<td>Ampicillin</td>
<td>128</td>
<td>64</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Amoxycillin</td>
<td>32</td>
<td>8</td>
<td>ND</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>32</td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>Cephapirin</td>
<td>ND</td>
<td>ND</td>
<td>&gt;64</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0·25</td>
<td>0·1</td>
<td>2</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>&gt;0·05</td>
<td>&gt;0·05</td>
<td>0·1</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>&lt;0·025</td>
<td>&lt;0·025</td>
<td>0·5</td>
</tr>
</tbody>
</table>

ND, Not determined.

*Lowest concentration inhibiting growth of 99% of all cells after 25 days incubation at 37°C.
assumed (Brennan & Nikaido, 1995; Lambert, 2002). In plate assays, the $mspA$-expressing strain of $M. tuberculosis$ H37Rv was clearly more sensitive to the hydrophilic and negatively charged $\beta$-lactam antibiotic ampicillin than the wild-type strain, harbouring the control plasmid (Fig. 7A). Approximately 50% of the wild-type cells survived on plates containing 64 $\mu$g ml$^{-1}$ ampicillin, compared to less than 3% of the cells of the recombinant strain. The increased sensitivity of the $mspA$-expressing strain was also evident for the zwitterionic cephapirin: approximately 85% of the wild-type cells survived on plates containing 4 $\mu$g ml$^{-1}$ cephapirin compared to less than 25% of the cells of the recombinant strain. Thus, $M. tuberculosis$ displayed a substantially increased sensitivity to $\beta$-lactam antibiotics, as did $M. bovis$ BCG, upon expression of $mspA$.

Ethambutol and isoniazid interfere with the assembly and synthesis of the arabinogalactan (Takayama & Kilburn, 1989) and with the synthesis of mycolic acids (Winder & Collins, 1970), respectively, which are essential components of the mycobacterial cell wall. Both drugs are essential in current TB therapy regimens (Kremer & Besra, 2002b). Since ethambutol and isoniazid are small, hydrophilic molecules it was assumed that they use the porin pathway for entry into mycobacteria (Lambert, 2002). This assumption was now experimentally tested with the $mspA$-expressing strains of $M. bovis$ BCG and $M. tuberculosis$. The presence of MspA in the OM significantly increased the sensitivity of $M. bovis$ BCG and $M. tuberculosis$ to ethambutol and isoniazid (Figs 6D, 7B and 7C). However, the difference was small compared to $\beta$-lactam antibiotics and did not result in an altered MIC (Table 2).

Streptomycin is an aminocyclitol glycoside antibiotic and is highly efficient against $M. tuberculosis$ (Kremer & Besra, 2002b). Streptomycin is a large, polar molecule and was thought to be too large to diffuse via mycobacterial porins (Senaratne et al., 1998). Surprisingly, expression of MspA in the OM significantly increased the sensitivity of $M. tuberculosis$ to streptomycin (Fig. 7D).

**MspA specifically increases the uptake of a fluorescent dye by $M. tuberculosis$**

Fluorescent stains are widely used in microscopy and flow cytometry to visualize bacteria and to report on their viability and other cellular parameters (Joux & Lebaron, 2000; Novo et al., 2000). A comprehensive study revealed...
that mycobacteria are stained equally well by the membrane-permeant SYTO stains, whose fluorescence is approximately 40-fold enhanced upon binding to nucleic acids (Molecular Probes, www.probes.com). Quantitative staining experiments with different SYTO dyes were performed to analyse whether the presence of MspA had an influence on uptake of these dyes. The mspA-expressing strain of *M. tuberculosis* showed a 10-fold increased fluorescence compared to the control when stained with 25 μM SYTO 9 for 30 min at 25 °C and analysed by flow cytometry (Fig. 8A). By contrast, staining of *M. tuberculosis* with SYTO 12 was not dependent on MspA (Fig. 8B). The kinetics of the staining of *M. tuberculosis* with SYTO 9 and 12 was determined to examine whether staining was saturated for both dyes. These experiments revealed that *M. tuberculosis* is stained at the same rate by both dyes at concentrations of 25 μM and that staining was already saturated after 5 min (Fig. 8C). Strikingly, staining of *M. tuberculosis* by SYTO 9 was clearly faster in the presence of MspA, whereas staining with SYTO 12 did not depend on MspA. Similar results, at reduced staining rates, were obtained with lower dye concentrations of 2·5 and 0·25 μM (data not shown). Although nucleic acids are the only molecules known to enhance the fluorescence of the SYTO stains, we wanted to exclude the possibility that the fluorescence enhancement of *M. tuberculosis* was caused by a direct binding of SYTO 9 to MspA. The fluorescence intensity of SYTO 9 at 500–700 nm, when excited at the absorption maximum at 470 nm, did not increase upon addition of MspA, indicating that MspA increased the permeability of SYTO 9 across the OM of *M. tuberculosis* and thereby the access of SYTO 9 to nucleic acids inside *M. tuberculosis* (data not shown). A possible explanation for the apparently increased permeability for SYTO 9 compared to SYTO 12 is that the former might be significantly smaller and more hydrophilic than the latter. Therefore, we measured both the hydrophobicities of the dye molecules and their masses. The octanol/water partition coefficients *P*<sub>ow</sub> were 8·5 ± 0·9 and 13·2 ± 1·5 for SYTO 9 and SYTO 12, respectively. Mass spectroscopy revealed one mass of 355 (m/z) for SYTO 9 and several masses between 330–430 (m/z) for SYTO 12. Thus, the gross physico-chemical properties of both dyes are similar and do not explain the drastic permeability differences in the mspA-expressing strain of *M. tuberculosis*.

**Fig. 8.** Influence of *mspA* expression on uptake of fluorescent dyes by *M. tuberculosis*. MapA-expressing *M. tuberculosis* (pMN013; bold lines) and control strain (pMN006; fine lines) were stained with 25 μM of the DNA-binding dyes SYTO 9 (A) and SYTO 12 (B) for 30 min and analysed by flow cytometry. (C) Time kinetics of dye uptake (median fluorescence).

**DISCUSSION**

To examine the function of the porin-mediated OM permeability in slow-growing mycobacteria, the major porin gene *mspA* of *M. smegmatis* was expressed in *M. tuberculosis* and *M. bovis* BCG. Quantitative immunoblot analysis of detergent extracts and counting of MspA-like pores in cell wall fragments by electron microscopy consistently yielded only about 35 pores μm<sup>−2</sup> in *M. bovis* BCG from the same expression vector pMN013, which gave rise to about 1000 MspA pores μm<sup>−2</sup> in *M. smegmatis*. However, expression of gfp genes from the same promoter (pimyc) yielded similar fluorescence intensities in both *M. smegmatis* and *M. bovis* BCG (I. Kaps & M. Niederweis, unpublished). Furthermore, the use of stronger promoters such as the hsp60 promoter did not increase the amount of MspA, in contrast to high-level expression of other genes from this promoter in *M. bovis* BCG (Stover et al., 1991). This indicated that the maximal amount of MspA in the OM of *M. bovis* BCG is limited to a very low level compared to *M. smegmatis*. Many sequence-specific mechanisms regulate expression of porin genes in *E. coli* (Delcour, 2003; Ferenci, 1999; Pratt et al., 1996), but such mechanisms are unlikely to limit *mspA* expression in the heterologous hosts *M. bovis* BCG and *M. tuberculosis*, which have no genes homologous to *mspA*.
Glucose was taken up by M. bovis BCG at a rate of approximately 0.7 pmol min\(^{-1}\) (mg dry weight cells\(^{-1}\)) at a concentration of 6-4 \(\mu\)M. This is 1430-fold slower than the rate of 1 nmol min\(^{-1}\) (mg dry weight cells\(^{-1}\)) measured for M. smegmatis under identical conditions (J. Stephan & M. Niederweis, unpublished) and 140-fold slower than that calculated for M. chelonae using the published \(V_{\text{max}}\) and \(K_m\) values (Jarlier & Nikaido, 1990). Glucose uptake kinetics for M. bovis BCG have been published previously (Yuan et al., 1998). An uptake rate of 0.04 pmol min\(^{-1}\) (mg dry weight cells\(^{-1}\)) was calculated for this experiment assuming the same specific activity as in our experiments. Uptake of 6.5 \(\mu\)M glycerol by M. tuberculosis was also very slow with a rate of 0.1 pmol min\(^{-1}\) (mg dry weight cells\(^{-1}\)) (Jackson et al., 1999). In conclusion, these results consistently indicated that the OM permeability of both M. bovis BCG and M. tuberculosis for small nutrient molecules is orders of magnitude lower than that of fast-growing mycobacteria. These data contrast with the observation that the OM permeability of M. tuberculosis for cephaloridine was similar to that of M. smegmatis (Chambers et al., 1995) and one order of magnitude higher than that of M. chelonae (Jarlier & Nikaido, 1990). This discrepancy might be caused by the different solutes, but usually both methods yielded consistent results: e.g. the permeability to cephaloridine decreased in the order E. coli, Pseudomonas aeruginosa and M. chelonae by almost four orders of magnitude as did the permeability to glucose (Jarlier & Nikaido, 1990). The observation that uptake of glucose was about twofold faster upon expression of \(m\sp{3}A\)-like pores, consistent with the apparent lack of MspA homologues in M. bovis BCG and M. tuberculosis (Niederweis, 2003), and corroborating the assumption that the endogenous porins of M. bovis BCG are substantially different from the M. smegmatis porins. Less efficient porins and/or a lower number of porins would explain the lower permeability to hydrophilic solutes of slow- compared to fast-growing mycobacteria and might have resulted from adaptations to the very different habitats of these bacteria as discussed recently (Niederweis, 2003). The antibiotic sensitivity experiments and the growth-promoting effect of MspA provided further support for the extremely low OM permeability of M. tuberculosis and M. bovis BCG compared to M. smegmatis. It is very unlikely that the same small increase of 20-35 porins per \(\mu\)m\(^2\) cell wall as was observed for the \(m\sp{3}A\)-expressing strains of M. tuberculosis and M. bovis BCG, respectively, would have led to any observable change of phenotype in M. smegmatis, which has 1000 MspA-like porins per \(\mu\)m\(^2\) cell wall (Engelhardt et al., 2002). Thus, the permeability through the endogenous porins of M. tuberculosis and M. bovis BCG must be considerably lower compared to M. smegmatis. Certainly, a systematic and quantitative analysis of the OM permeability and identification of the major porins of slow-growing mycobacteria are needed to solve this puzzle.

The questions why the strictly pathogenic members of the genus Mycobacterium such as M. tuberculosis and M. leprae grow much more slowly than the non-pathogenic saprophytes such as M. smegmatis and M. phlei (generation times: >15 h vs <5 h; Rastogi et al., 2001) and whether slow growth is beneficial or even necessary for pathogenicity have puzzled generations of scientists. Thus, it is not surprising that many factors have been invoked to explain the slow growth of M. tuberculosis: (i) slow RNA synthesis (Harshley & Ramakrishnan, 1977), (ii) slow DNA elongation (Hiriyanna & Ramakrishnan, 1986), (iii) slow protein synthesis due to the lack of multiple copies of rRNA operons (Bercovier et al., 1986), (iv) slow porin-mediated uptake of nutrients (Jarlier & Nikaido, 1990) and (v) presence of the DNA-binding protein MDBP1 (Matsumoto et al., 2000). So far, experimental evidence demonstrating that any of these factors really limits the growth rate of slow-growing mycobacteria is lacking. In this study, we showed that even the presence of the very low number of about 35 MspA porins per \(\mu\)m\(^{-2}\) cell wall significantly accelerated the growth rate of M. bovis BCG. However, it is not clear whether increased nutrient influx directly affected the growth rate or whether this effect reflected regulatory events in the cell. In both cases, our results indicate that low porin permeability is probably one of multiple factors contributing to the slow growth of M. bovis BCG.

The intrinsic resistance of mycobacteria to most hydrophilic antibiotics and chemotherapeutic agents is believed to result from a low-efficiency porin pathway in synergy with other resistance mechanisms such as enzymic inactivation or active efflux of the drugs (Brennan & Nikaido, 1995; Jarlier & Nikaido, 1994). It is also assumed that porins are necessary for the uptake of the first line TB drugs isoniazid and ethambutol, since they are small and hydrophilic molecules (Jarlier & Nikaido, 1994; Lambert, 2002). MspA increased the susceptibility of both M. bovis BCG and M. tuberculosis to \(\beta\)-lactam antibiotics. The MspA-mediated sensitivity was still significant but smaller for the TB drugs isoniazid and ethambutol, consistent with the finding that, in general, differences in porin permeabilities have less pronounced effects for smaller solutes (Nikaido & Rosenberg, 1983). These results also suggested that \(\beta\)-lactam antibiotics and both isoniazid and ethambutol use the MspA porin pathway to enter the recombinant M. tuberculosis and M. bovis BCG strains. This assumption is consistent with the observation that the \(m\sp{3}A\) deletion mutant of M. smegmatis, which has a threefold reduced porin density, showed the reverse phenotype: It was 8- and 16-fold more resistant to cephaloridine and ampicillin, respectively, and slightly more resistant to ethambutol (J. Stephan, C. Mailaender, G. Etienne, M. Daffé & M. Niederweis, unpublished). The clear correlation of porin-mediated OM permeability and sensitivity indicated that cephaloridine, and most likely other zwitterionic \(\beta\)-lactam
antibiotics, use the porin pathway for cell entry in *M. smegmatis* (J. Stephan, C. Maiaender, G. Etienne, M. Daffé & M. Niederweis, unpublished). The interpretation is less clear for isoniazid, which is also able to diffuse directly through lipid membranes, as shown by liposome-swelling experiments (Jackson *et al*., 1999; Raynaud *et al*., 1999). However, it is likely that the diffusion rate of isoniazid through the mycolic acid layer of mycobacteria is much lower compared to bilayers from conventional C<sub>16</sub>-phosphatidylcholine lipids (Jackson *et al*., 1999). Therefore, further experiments are necessary to evaluate the relative importance of the porin and the lipid pathway for uptake of isoniazid by mycobacteria. The effect of MspA for all drugs tested was smaller in *M. tuberculosis*, consistent with the almost twofold lower amount of MspA compared to *M. bovis* BCG. These results provide the proof of principle that porins are essential for susceptibility of *M. tuberculosis* to drugs currently used in TB chemotherapy. However, it should be noted that these data do not allow us to clearly assign a particular OM pathway for the drugs examined in this study.

MspA also increased the sensitivity of *M. tuberculosis* to streptomycin, although aminoglycosides were believed to be too large to diffuse through porin channels (Senaratne *et al*., 1998). This assumption is supported by the observation that even disaccharides such as sucrose and maltose did not produce a significant swelling of multilamellar proteoliposomes containing MspA (C. Heinz & M. Niederweis, unpublished results). This suggested an indirect effect of MspA on the OM permeability of *M. tuberculosis* for streptomycin. Indeed, such an effect was observed for the *mspA* deletion mutant of *M. smegmatis*, which was hyper-resistant not only to β-lactam antibiotics, but also to very large antibiotics such as kanamycin and vancomycin, and to hydrophobic antibiotics such as rifampicin (J. Stephan, C. Maiaender, G. Etienne, M. Daffé & M. Niederweis, unpublished). This may be explained by rearrangement and stronger interactions of OM lipids due to the loss of MspA in *M. smegmatis*. Local interruptions of lipid–lipid interactions, which are not fully replaced by lipid–protein interactions, would explain the higher permeability of the OM of *M. tuberculosis* to large and hydrophobic compounds upon expression of *mspA*. Evidence that this model might be correct was provided by the observation that MspA drastically increased the fluorescence of *M. tuberculosis* upon staining with SYO 9. Binding of SYO 9 to DNA was demonstrated in fluorescence experiments, but binding to MspA and to other compounds of the cell wall of *M. tuberculosis* was excluded *in vitro* and *in vivo*, respectively. The hydrophobicity of SYO 9 makes it rather unlikely that it can diffuse directly through water-filled protein channels at significant rates (Nikaido *et al*., 1983). These results suggested that the presence of MspA indirectly increased the permeability of the OM of *M. tuberculosis* to streptomycin.

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REFERENCES


tion of the antigen 85C gene profoundly affects the mycolate content and alters the permeability of the Mycobacterium tuberculosis cell envelope. Mol Microbiol 31, 1573–1587.


