

Multidrug Resistance of a Porin Deletion Mutant of *Mycobacterium smegmatis*

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Mycobacteria contain an outer membrane of unusually low permeability which contributes to their intrinsic resistance to many agents. It is assumed that small and hydrophilic antibiotics cross the outer membrane via porins, whereas hydrophobic antibiotics may diffuse through the membrane directly. A mutant of *Mycobacterium smegmatis* lacking the major porin MspA was used to examine the role of the porin pathway in antibiotic sensitivity. Deletion of the *mspA* gene caused high-level resistance of *M. smegmatis* to 256 µg of ampicillin/ml by increasing the MIC 16-fold. The permeation of cephaloridine in the *mspA* mutant was reduced ninefold, and the resistance increased eightfold. This established a clear relationship between the activity and the outer membrane permeation of cephaloridine. Surprisingly, the MICs of the large and/or hydrophobic antibiotics vancomycin, erythromycin, and rifampin for the *mspA* mutant were increased 2- to 10-fold. This is in contrast to those for *Escherichia coli*, whose sensitivity to these agents was not affected by deletion of porin genes. Uptake of the very hydrophobic steroid chenodeoxycholate by the *mspA* mutant was retarded threefold, which supports the hypothesis that loss of MspA indirectly reduces the permeability by the lipid pathway. The multidrug resistance of the *mspA* mutant highlights the prominent role of outer membrane permeability for the sensitivity of *M. smegmatis* to antibiotics. An understanding of the pathways across the outer membrane is essential to the successful design of chemotherapeutic agents with activities against mycobacteria.

The prevalence and spread of antibiotic resistance are increasingly serious problems that hamper the effective treatment of infectious diseases (26). The search for new antibiotics is mainly based on novel bacterial targets and high-throughput screening assays (10). However, many lead compounds discovered in vitro may fail because they do not reach their targets at sufficiently high concentrations in vivo (7). This is true in particular for gram-negative bacteria, which, in contrast to gram-positive bacteria, are protected from the toxic actions of certain antibiotics, dyes, and detergents and host defense factors such as lysozyme by an additional outer membrane (OM) (49). The OM can be crossed by at least two general pathways: 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 of gram-negative bacteria (49). It has been shown by the pioneering work of Nikaido and collaborators (21, 46) that *Escherichia coli* and *Salmonella* porins play a major role in the transport of β-lactam antibiotics. Subsequent studies showed that porin-deficient mutants of gram-negative bacteria were also more resistant to quinolones, tetracyclines, chloramphenicol, nalidixic acid, and trimethoprim (6, 15, 25, 52). These data suggest that porins are involved in the transport of these antibiotics, but the levels of resistance were rather low, as re-

flected by the 1.5- to 4-fold increased MICs for the porin mutants. However, combination of low-level OM permeability with drug efflux produces high-level resistance in gram-negative bacteria (44, 45) and is a powerful mechanism leading to multidrug resistance (51).

The permeability of the cell envelope of mycobacteria is several orders of magnitude lower than that of the cell envelope of gram-negative bacteria, which is believed to be the major reason for the intrinsic resistance of mycobacteria to most antibiotics and chemotherapeutic agents (31). This is likely due to a unique arrangement of the longest fatty acids known to date in nature, the mycolic acids, which can consist of up to 90 carbon atoms and which are covalently bound to a single polymeric head group, the arabinogalactan (4). Porins in the mycobacterial OM mediate the diffusion of hydrophilic solutes, although the efficiency of this pathway is 100- to 1,000-fold lower than that in *E. coli* (32). The porin MspA was identified in CHCl₃-methanol and in detergent extracts of *Mycobacterium smegmatis* (24, 41). Expression of the *mspA* gene encoding the mature MspA protein in *E. coli* yielded a folded but inactive 20-kDa monomer (23) and an oligomer with an apparent molecular mass of 100 kDa, which formed channels similar to those of the native protein (41). The genome of *M. smegmatis* encodes three additional very similar porins, designated MspB, MspC, and MspD, that differ from MspA only at 2, 4, and 18 positions of the mature protein, respectively (61). Detergent extracts of a Δ*mspA* *M. smegmatis* mutant exhibited significantly lower channel activities in lipid bilayer experiments and contained fewer porins than extracts of wild-type *M. smegmatis*. The lower porin content reduced the cell wall per-

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meability of the $\Delta mspA$ mutant for cephaloridine and glucose nine- and fourfold, respectively. However, the growth rate was not significantly altered upon deletion of the *mspA* gene (61). These results show that MspA is the main general diffusion pathway for hydrophilic molecules across the OM of *M. smegmatis* and is only partially replaced by fewer porins in the cell wall of the $\Delta mspA$ mutant. Other fast-growing mycobacteria, such as *M. chelonae* and *M. phlei*, appear to encode porins similar to MspA (41, 54), but no sequence homologs were identified in slowly growing mycobacteria, such as *M. tuberculosis*, or in any other organism outside the genus *Mycobacterium* (40). Determinants of the low efficiency of the porin pathway in *M. smegmatis* are the 45-fold lower number of pores and the 2.5-fold longer pore channels compared to the numbers and the sizes of the pores in *E. coli* (12). Similar causes for low permeability are likely to exist for all mycobacteria (40). However, the relative importance of the porin pathway and the lipid pathway across the OM of mycobacteria is unknown for any antibiotic, although the synergistic effects of cell wall-damaging agents such as ethambutol with many other drugs have been known for a long time (22, 67).

To examine the role of porins in the antibiotic sensitivity of *M. smegmatis*, we determined the MICs of many antibiotics for an *M. smegmatis mspA* mutant. Furthermore, we did transport experiments using radiolabeled compounds to confirm that reduced sensitivity correlated with slower uptake. This study demonstrates for the first time the importance of porins for the sensitivity of *M. smegmatis* to both hydrophilic and hydrophobic antibiotics.

MATERIALS AND METHODS

Chemicals, bacterial strains, media, and growth conditions. Chemicals were purchased from Merck, Roth, or Sigma at the highest purity available. [^{14}C]chenuodeoxycholate was obtained from NEN Life Science Products, and [^{14}C]isoniazid was obtained from Hartmann Analytic. *M. smegmatis* SMR5 is a derivative of *M. smegmatis* mc²155 and is streptomycin resistant due to a mutation in ribosomal protein S12 (55). The construction of $\Delta mspA$ mutant *M. smegmatis* MN01 was described previously (61). All mycobacterial strains were grown in Middlebrook 7H9 liquid medium (Difco) supplemented with 0.2% glycerol and 0.05% Tween 80 or on Middlebrook 7H10 plates (Difco) containing 0.2% glycerol at 37°C.

Drug sensitivity by agar dilution. The MICs for *M. smegmatis* SMR5 and *M. smegmatis* MN01 were determined in triplicate by serial dilutions in 7H10 agar. Each strain was first grown in a 4-ml culture for 2 days at 37°C to an optical density at 600 nm of 0.6 to 0.8. The cultures were diluted with 7H9 liquid medium to yield approximately 5,000 CFU per ml. Approximately 500 CFU was streaked on plates containing the appropriate antibiotic concentrations. As a reference, 500 CFU was also streaked on plates without antibiotic. The number of surviving cells was normalized to the number of cells counted on plates without antibiotic for each strain and was expressed as the relative CFU (percent CFU). For all drugs tested, the concentration that resulted in the complete inhibition of bacterial growth after 3 days of incubation at 37°C was determined as the MIC of the drug.

Transport experiments. Transport experiments were carried out as described previously (61). To reduce aggregation and clumping of the cells, *M. smegmatis* SMR5 and MN01 were each first grown in 4-ml cultures for 2 days at 37°C and then filtered through a 5- μm -pore-size filter (Sartorius). The filtrates were grown for 2 days at 37°C and then used to inoculate 100-ml cultures, which were grown to an optical density at 600 nm of 0.5. The cells were harvested by centrifugation ($1,250 \times g$ at 4°C for 10 min), washed once in 2 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (pH 6.5)–0.05 mM MgCl_2 , and resuspended in the same buffer. The ^{14}C -labeled compounds and their nonlabeled analogs were mixed and added to the cell suspensions to obtain final concentrations of 5 and 20 μM . The mixtures were incubated at 37°C, and 1-ml samples were removed at the indicated times. The cells were filtered through a 0.45- μm -pore-size filter (Sartorius), washed with 0.1 M LiCl, and counted in a liquid scintillation counter. The

mean dry weight of the cells in these samples was 1.4 ± 0.4 mg. The uptake rate was expressed as nanomoles per milligram of cells.

Analysis of cell wall lipids of *M. smegmatis*. *M. smegmatis* mc²155 and its isogenic porin-deficient mutant strain, MN01, were grown as surface pellicles on Sauton's medium for 6 days at 37°C. The strains were maintained by selection with streptomycin, which was added to a final concentration of 20 $\mu\text{g}/\text{ml}$. Lipids were isolated and characterized as described previously (13). Wet cells were first extracted with chloroform-methanol (1:2; vol/vol), then five times with chloroform-methanol (1:1; vol/vol), and finally, once with chloroform-methanol (2:1; vol/vol). These extracts were pooled, dried under vacuum, and partitioned between water and chloroform (1:1; vol/vol). The organic phase was extensively washed with distilled water, evaporated to dryness to yield the crude lipid extract, and weighed. Cell-bound fatty acids (mycolates) were obtained by saponification of delipidated cells with 5% KOH in methanol-benzene (8:2; vol/vol) for 16 h at 80°C. After acidification with sulfuric acid, fatty acids were extracted with diethyl ether and extensively washed with water. The washed lipid extract was dissolved in chloroform and analyzed by thin-layer chromatography (TLC) on silica gel plates (thickness, 0.25 mm; Macheray-Nagel) precoated with Durasil 25. Lipid spots were resolved by TLC run in the following solvent mixtures: petroleum ether-diethyl ether (9:1; vol/vol) for analysis of triacyl glycerols (TAGs), chloroform-methanol (9:1; vol/vol) for analysis of glycopeptidolipids (GPLs) and trehalose dimycolates (TDMs), and chloroform-methanol-water (60:30:8; vol/vol) for analysis of trehalose monomycolates (TMMs) and phospholipids. Sugar-containing compounds (GPLs, TDMs, TMMs and phosphatidylinositol mannosides) were visualized by spraying the plates with 0.2% anthrone in concentrated sulfuric acid, followed by heating at 110°C. The Dittmer-Lester reagent (9) was used to detect phosphorus-containing substances. The ninhydrin reagent was used to reveal the presence of free amino groups. A spray with 10% molybdophosphoric acid in ethanol solution, followed by heating at 110°C, was used to detect all of the lipids spots, including TAGs.

RESULTS

The *mspA* mutant of *M. smegmatis* is more resistant to most β -lactam antibiotics. It has been well established that β -lactam antibiotics cross the OM of gram-negative bacteria by diffusing through porin channels (29, 48, 56). Therefore, we first examined whether deletion of the major porin MspA of *M. smegmatis* has any influence on the resistance of mycobacteria to β -lactam antibiotics. MICs were determined by agar dilution with Middlebrook 7H10 plates. Figure 1A shows that wild-type strain *M. smegmatis* SMR5 is drastically more susceptible to the zwitterionic agent ampicillin than the isogenic *mspA* mutant strain, *M. smegmatis* MN01. Thus, deletion of *mspA* increased the ampicillin MIC for *M. smegmatis* by 16-fold, to 256 $\mu\text{g}/\text{ml}$ (Table 1). Expression of the *mspA* gene on a plasmid (pMN014) in *M. smegmatis* MN01 established again the sensitivity of the wild type, demonstrating that the loss of *mspA* caused the resistance phenotype. *M. smegmatis* was also more sensitive to the zwitterionic agent cephaloridine than to ampicillin. Deletion of the *mspA* gene increased the cephaloridine MIC fourfold (Table 1). No significant difference in the susceptibilities of the two strains to the anionic agent cephalothin was observed (Table 1). It should be noted that the growth rates of both strains are similar in standard 7H9 and minimal liquid media (61) and on 7H10 agar plates.

The susceptibility of *M. smegmatis* to vancomycin but not to aminoglycosides is affected by deletion of the *mspA* gene. Hydrophilic solutes up to an exclusion limit of approximately 600 to 700 Da are thought to prefer the porin pathway across the OM of gram-negative bacteria (43). Since the exclusion limit of mycobacterial porins for hydrophilic solutes is unknown, we examined whether the susceptibility of *M. smegmatis* to hydrophilic antibiotics larger than β -lactam antibiotics was also affected by deletion of the *mspA* gene. The susceptibility of *M.*

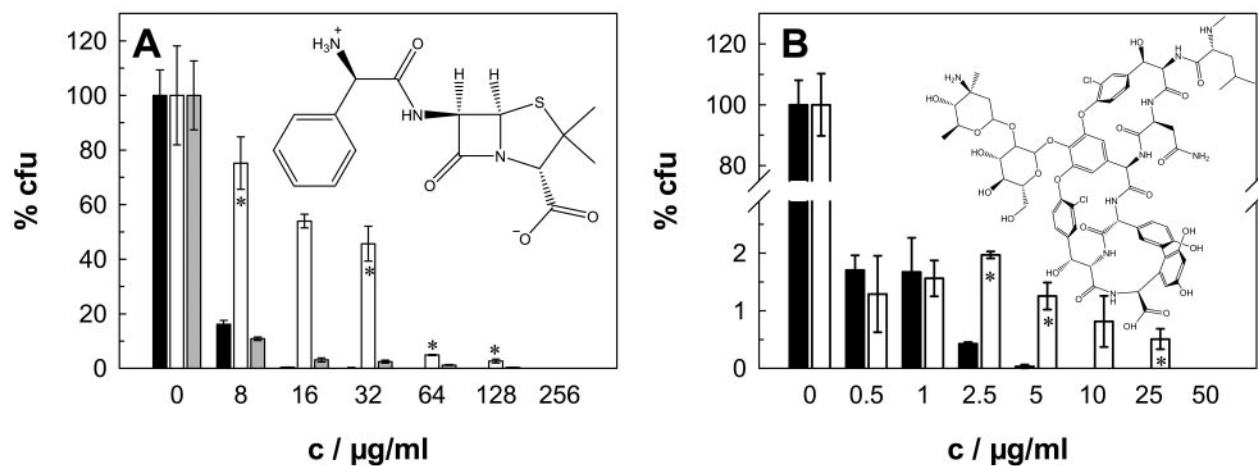


FIG. 1. MspA-dependent sensitivity of *M. smegmatis* to hydrophilic antibiotics. The sensitivities of wild-type *M. smegmatis* (black bars), the $\Delta mspA$ mutant (white bars), and the $\Delta mspA$ mutant transformed with the *mspA* expression vector pMN014 (gray bars) to ampicillin (A) and vancomycin (B) were determined by serial dilution in Middlebrook 7H10 agar. The number of surviving cells was normalized to the number cells counted on plates without antibiotic for each strain, and the results are expressed as relative CFU (percent CFU). The sensitivity experiments were done in triplicate, and the concentrations (c) are shown with their standard deviations. The structures of the antibiotics are shown in the corresponding graphs. The asterisks denote the datum points that differed significantly ($P < 0.05$) between the wild-type strain and the *mspA* mutant strain by the paired Student *t* test.

smegmatis to kanamycin was high, and that of the *mspA* mutant was increased only slightly (data not shown). This result is similar to that obtained for porin mutants of *E. coli*, which did not show altered susceptibilities to aminoglycoside antibiotics (16, 18). Surprisingly, the *mspA* mutant was clearly more resistant to the much larger hydrophilic peptide antibiotic vancomycin (Fig. 1B). This result was also reflected by a 10-fold increased MIC (Table 1). It should be noted that only a small population of vancomycin-resistant cells caused the increased MIC for the *mspA* mutant (Fig. 1B) and that *M. smegmatis* was 50-fold more sensitive to vancomycin than *E. coli* (64).

The *mspA* mutant of *M. smegmatis* is more resistant to hydrophobic antibiotics. The rates of penetration of compounds through water-filled protein channels such as the porins decrease with increasing hydrophobicity (46). This explains why *E. coli* mutants lacking one or more porins have nearly unaltered sensitivities to hydrophobic antibiotics (49). A similar behavior was expected for porin mutants of *M. smegmatis*. Surprisingly, deletion of the *mspA* gene increased the resistance of *M. smegmatis* to the hydrophobic antibiotic erythromycin, resulting in a more than twofold increased MIC (Fig. 2A; Table 1). The level of resistance mediated by the loss of

TABLE 1. Physicochemical properties of antibiotics and the efficiencies of their activities against *M. smegmatis*^a

Antibiotic	Molecular mass (g/mol)	Partition coefficient (P_{ow})	MIC ($\mu\text{g/ml}$) for <i>M. smegmatis</i>		R factor
			Wild type	$\Delta mspA$	
Ampicillin	349	0.002–0.2 (pH 7.2) ^b , <0.01 (pH 7.0) ^c	16	256	16
Cephaloridine	416	0.02–0.03 (pH 7.4) ^b	16	128	8
Cephalothin	395	0.006–3.6 (pH 7.4) ^b , <0.01 (pH 7.0) ^c	250	500	2
Kanamycin A	488	NF ^d	1	1	1
Vancomycin	1486	<0.01 (pH 7.0) ^c	5	50	10
D-Cycloserine	102	<0.01 (pH 7.0) ^c	125	150	1.2
Ethambutol	204	NF	0.5	0.5	1
Isoniazid	137	0.07 (pH 7.4) ^b	>40	>40	1
Tetracyclin	444	0.04–0.07 (pH 7.0) ^{b,c}	0.2	0.25	1.25
Chloramphenicol	323	12.3–12.4 (pH 7.0) ^{b,c}	16	32	2
Erythromycin A	734	4.6–18.2 (pH 7.4) ^b	128	>256	2
Novobiocin	613	5.2–38.0 (pH 7.4) ^b , >20 (pH 7.0) ^c	25	25	1
Rifampin	821	20.9 (pH 7.5) ^b	25	75	3

^a The molecular masses of the antibiotics, their hydrophobicities as apparent *n*-octanol–water partition coefficients (P_{ow}), and their MICs for *M. smegmatis* SMR5 (wild type) and mutant strain MN01 ($\Delta mspA$) are listed. Apparent *n*-octanol–water partition coefficients were determined in triplicate and are shown with their standard deviations. The resistance (R) factor for *M. smegmatis* is given by the ratio of the MIC for $\Delta mspA$ mutant/MIC for the wild type.

^b Data are from reference 20.

^c Data are from reference 42.

^d NF, not found.

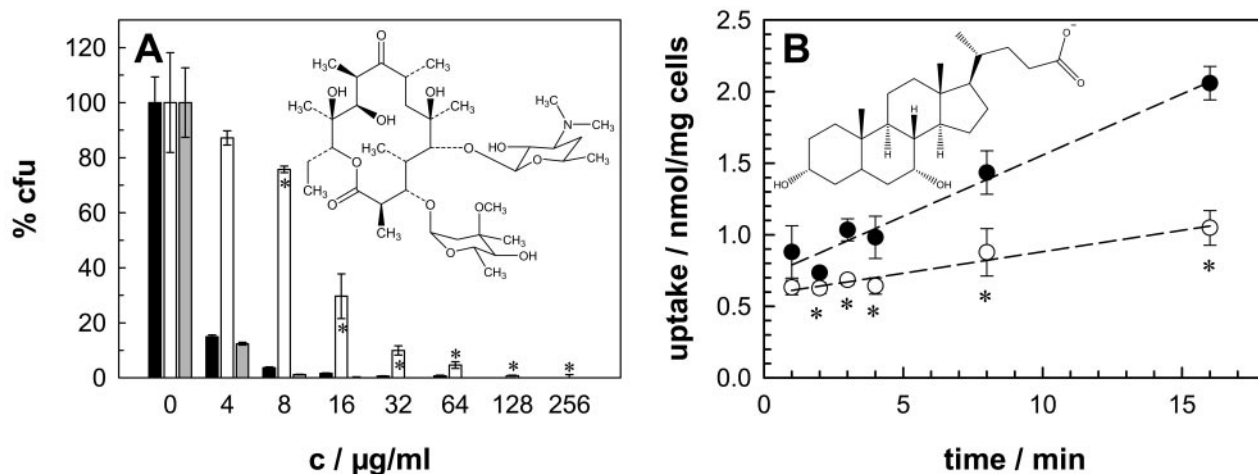


FIG. 2. MspA-dependent sensitivities and permeabilities of *M. smegmatis* to hydrophobic compounds. (A) The sensitivities of wild-type *M. smegmatis* (black bars), the $\Delta mspA$ mutant (white bars), and the $\Delta mspA$ mutant transformed with the *mspA* expression vector pMN014 (gray bars) to erythromycin were determined by serial dilution in Middlebrook 7H10 agar (c, concentration). The number of surviving cells was normalized to the number of cells counted on plates without antibiotic for each strain, and the results are expressed as relative CFU (percent CFU). (B) The accumulation of [^{14}C]chenodeoxycholate by *M. smegmatis* SMR5 (wild type; closed circles) and *M. smegmatis* MN01 ($\Delta mspA$; open circles) was measured. Regression analysis yielded uptake rates of 0.09 and 0.03 $\text{nmol mg}^{-1} \text{min}^{-1}$ for 20 μM chenodeoxycholate for the wild-type and mutant strains of *M. smegmatis*, respectively. The assay was performed at 37°C. The structures of the compounds are shown in the corresponding graphs. The sensitivity and uptake experiments were done in triplicate, and the results are shown with their standard deviations. The asterisks denote the datum points that differed significantly ($P < 0.05$) between the wild-type strain and the *mspA* mutant strain by the paired Student *t* test.

MspA was even more pronounced than that obtained for the hydrophilic antibiotic cephaloridine, although this is not reflected in the MICs of the two antibiotics (Table 1). The susceptibility of the mutant to erythromycin was restored by expression of the *mspA* gene (Fig. 2A), demonstrating that deletion of the *mspA* gene caused this resistance phenotype. The sensitivities of *M. smegmatis* MN01 to rifampin, novobiocin, and chloramphenicol were determined to examine

whether deletion of *mspA* also affected sensitivities to other hydrophobic compounds or was an erythromycin-specific effect. Rifampin was less effective against *M. smegmatis* MN01 than against the wild type (Table 1). Similarly, the hydrophobic antibiotics novobiocin and chloramphenicol were less effective against the *mspA* mutant, although the difference was smaller than that for erythromycin and was detectable at only a few concentrations (data not shown). These results indicate that

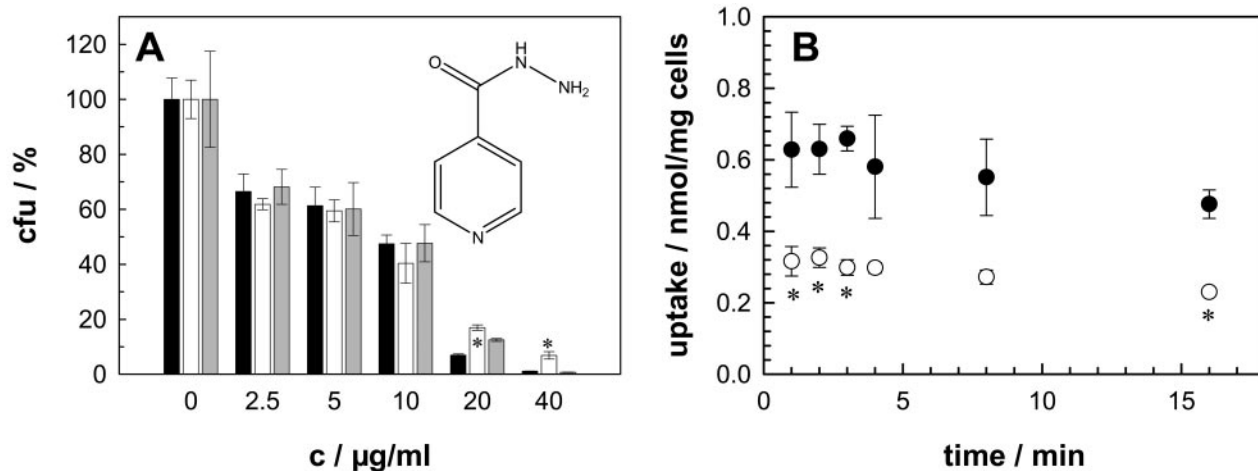


FIG. 3. MspA-dependent sensitivity and permeability of *M. smegmatis* to isoniazid. (A) The sensitivities of wild-type *M. smegmatis* (black bars), the $\Delta mspA$ mutant (white bars), and the $\Delta mspA$ mutant transformed with the *mspA* expression vector pMN014 (gray bars) to isoniazid were determined by serial dilution in Middlebrook 7H10 agar (c, concentration). The number of surviving cells was normalized to the number of cells counted on plates without antibiotic for each strain, and the results are expressed as relative CFU (percent CFU). The structure of isoniazid is shown. (B) The accumulation of 20 μM [^{14}C]isoniazid by *M. smegmatis* SMR5 (wild type; closed circles) and *M. smegmatis* MN01 ($\Delta mspA$; open circles) was measured. The assay was performed at 37°C. The sensitivity and uptake experiments were done in triplicate, and the results are shown with their standard deviations. The asterisks denote the datum points that differed significantly ($P < 0.05$) between the wild-type strain and the *mspA* mutant strain by the paired Student *t* test.

the reduced porin expression increased the level of resistance of the *mspA* mutant not only to hydrophilic antibiotics but also to hydrophobic antibiotics.

Considering the reduced susceptibility of the *M. smegmatis mspA* mutant to hydrophobic antibiotics, we suspected that a less efficient permeation of hydrophobic solutes might be responsible for this phenotype. To this end, we measured the rates of uptake of the very hydrophobic steroid chenodeoxycholate, which is considered an indicator of the permeability of the lipid pathway in mycobacteria, by *M. smegmatis* and the *mspA* mutant (28, 36). The uptake rate was $0.09 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ for the wild type, but the rate was reduced threefold for the *mspA* mutant (Fig. 2B). This result indicates that, indeed, a slower rate of transport of hydrophobic compounds across the OM increased the level of resistance of the *mspA* mutant to hydrophobic antibiotics.

Quantitative analysis of the cell wall lipids of the *M. smegmatis* wild type and the $\Delta mspA$ strain. Alteration of the lipid composition of the cell wall of *M. smegmatis* might explain the decreased permeability of the $\Delta mspA$ mutant to hydrophobic compounds. To test this possibility, noncovalently bound lipids of the cell wall of the *M. smegmatis* wild type and the $\Delta mspA$ mutant were extracted with organic solvents and analyzed by TLC. The cell-bound fatty acids (mycolates) were obtained by saponification of the delipidated cells and extraction with ether oxide. The following lipids were detected in similar amounts in both strains of *M. smegmatis*: TAGs, GPLs, TDM, TMM, phosphatidylinositol mannosides 2 and 6, phosphatidylinositol, phosphatidylglycerol, and phosphatidylethanolamine. These results show that deletion of the porin gene *mspA* does not significantly alter the lipid composition of the *M. smegmatis* cell wall.

Deletion of the *mspA* gene does not affect the susceptibility of *M. smegmatis* to small and hydrophilic chemotherapeutic agents for tuberculosis. Mycolic acids and arabinogalactan are essential components of the mycobacterial cell wall. Isoniazid and ethambutol interfere with the synthesis of mycolic acids (65) and with the assembly and synthesis of the arabinogalactan (62), respectively. Both drugs are essential in present tuberculosis chemotherapy regimens (34). Since isoniazid and ethambutol are small and hydrophilic molecules, it was assumed that they use the porin pathway for entry into mycobacteria (35). Similar considerations apply to cycloserine, which inhibits peptidoglycan synthesis and which is used as a second-line drug in tuberculosis chemotherapy. Despite these assumptions, the effect of deletion of the *mspA* gene on the sensitivity of *M. smegmatis* to these drugs was negligible. The *mspA* mutant showed marginally increased levels of resistance to all three drugs only at high concentrations. This is shown for isoniazid in Fig. 3A. Interpretation of the results of drug sensitivity experiments in terms of cell wall permeability is always difficult and might be additionally hampered for isoniazid, because *M. smegmatis* is naturally about 100-fold more resistant to isoniazid than *M. tuberculosis* (66). Therefore, we examined the transport of $20 \mu\text{M}$ [^{14}C]isoniazid directly. The amount of isoniazid associated with wild-type *M. smegmatis* was significantly higher than that associated with the *mspA* mutant, but it did not increase between 1 and 15 min for both strains in several independent experiments at 37°C (Fig. 3B), indicating that an equilibrium was already reached in these experiments.

To slow the uptake rate, the concentration of isoniazid was reduced to $5 \mu\text{M}$ and the experiments were performed at 20°C . Again, more isoniazid was associated with wild-type *M. smegmatis* than with the *mspA* mutant, but no significant uptake was observed at between 15 and 90 s after addition of isoniazid to the cell suspensions (data not shown).

DISCUSSION

The intrinsic resistance of mycobacteria to most antibiotics and chemotherapeutic agents is believed to be a result of their impermeable OM in synergy with other resistance mechanisms, such as enzymatic inactivation or active efflux of the drugs (4, 31). The role of the porin pathway across the OM in the sensitivity of *M. smegmatis* to antibiotics was examined by using a mutant of *M. smegmatis* lacking the main porin MspA. The MIC of cephaloridine for this mutant increased eightfold, and the OM of the mutant had a ninefold reduced permeability to cephaloridine, while the β -lactamase activity of *M. smegmatis* was not altered (61). Thus, slower uptake of cephaloridine is directly correlated with increased resistance. These results indicate further that cephaloridine mainly diffuses through the MspA channel across the OM of *M. smegmatis*. Similar conclusions were drawn for *E. coli*, in which the outstanding permeation of cephaloridine through the porin OmpF was paralleled by its strong activity (46). However, it should be noted that there are two major differences between *M. smegmatis* and *E. coli*. First, *M. smegmatis* is about eight-fold less sensitive to cephaloridine than *E. coli* (MIC = $2 \mu\text{g/ml}$) (21), consistent with a less permeable OM. Second, deletion of the porin gene *mspA* clearly increased the level of resistance of *M. smegmatis* to cephaloridine in contrast to that of *E. coli*, for which inactivation of the OmpF porin did not detectably alter the cephaloridine MIC (21, 29). The cephaloridine MIC for the *mspA* mutant was even higher than that for an *E. coli* mutant lacking both major porins, OmpC and OmpF (29). Deletion of *mspA* produced high-level resistance of *M. smegmatis* to ampicillin by increasing the MIC 16-fold (MIC = $256 \mu\text{g/ml}$). This is the largest increase in the MIC of an antibiotic ever observed for a single-porin-knockout strain, in which no or only small effects of gene knockouts on drug sensitivity are usually shown (21). Such a high level of resistance to ampicillin was not observed even when the two main porins of *E. coli*, OmpF and OmpC, were inactivated (29), underlining the importance of MspA for sensitivity to ampicillin. *M. smegmatis* was resistant to a 15-fold higher concentration of the monoanionic agent cephalothin (Table 1), consistent with the 10-fold lower OM permeability of cephalothin compared to that of the zwitterionic agent cephaloridine (63). The cephalothin MIC for the *mspA* mutant was increased only slightly, indicating that negative charges severely hamper diffusion through MspA. This is explained by the crystal structure of MspA, which shows two rings, each of which has eight aspartate residues, lining the channel at the constriction zone (14). Thus, penetration of cephalothin through a nonporin pathway may be significant, consistent with observations for other β -lactam antibiotics, which have low rates of diffusion through *E. coli* porins (47). Taken together, these results indicate that hydrophilic and cationic or zwitterionic β -lactam antibiotics cross the OM of *M. smegmatis* via the

porin pathway, similar to the situation in *E. coli*, in which most β -lactams were found to diffuse through porins (46, 47).

Isoniazid and ethambutol are small and hydrophilic drugs indispensable in present tuberculosis chemotherapy regimens (34) and are thought to penetrate the mycobacterial cell via porins (35). The same holds true for the second-line antituberculosis drug cycloserine. We observed an increased level of resistance of the *mspA* mutant to these drugs only at concentrations close to the MIC (Fig. 3A). Since small and hydrophilic molecules diffuse through porins very fast, permeation through the remaining porins of the *mspA* mutant, MspB, MspC, and MspD, might still be sufficient for these drugs to inactivate their target enzymes at similar rates in both strains. However, we cannot exclude the possibility that MspA does not contribute to the uptake of these drugs. We also could not measure any isoniazid uptake for *M. smegmatis* between 15 s and 15 min, in contrast to the uptake results in experiments with *M. tuberculosis* (3). Whether this is due to a faster drug efflux in *M. smegmatis* or to other mechanisms is not known.

The kanamycin MIC for the *mspA* mutant of *M. smegmatis* was not reduced. This is similar to the findings for *E. coli* porin mutants (18). These results could be explained by a very efficient diffusion of aminoglycosides through the remaining porins in the mutants, as suggested earlier for *E. coli* on the basis of liposome swelling experiments (39). An alternative route of cell entry for aminoglycosides was proposed for *Pseudomonas aeruginosa*, which has an exceptionally low permeability by the porin pathway (17). Aminoglycosides were shown to permeabilize the OM of *P. aeruginosa* (19) and *E. coli* (18) and thereby promote their own uptake. Self-promoted uptake may be significant for *M. smegmatis* when the even lower porin-mediated OM permeability in *M. smegmatis* compared to that in *P. aeruginosa* is considered (30) and might explain the unchanged sensitivity of the *mspA* mutant of *M. smegmatis* to kanamycin.

Surprisingly, the vancomycin MIC for the *mspA* mutant was increased 10-fold. It has been shown that OM proteins with large channels, such as TolC, which has a channel diameter of 3.5 nm and which translocates folded polypeptides across the OM of *E. coli* (33), or the phage export channel protein pIV (38), are needed to promote vancomycin uptake in *E. coli*. However, the crystal structure of MspA revealed a channel constriction with a diameter of 1 nm (14), which is too small to allow passage of vancomycin, which has molecular dimensions of 2.9 by 2.4 nm (57). Thus, it appears very unlikely that vancomycin can diffuse through the MspA channel. It should be noted that only a few percent of wild-type and *mspA* mutant cells were resistant to vancomycin at concentrations of 0.5 μ g/ml or higher, indicating that the majority of the cells of both strains were killed at concentrations far below the MIC (Fig. 1B). This is reminiscent to the observation of "heteroresistant staphylococci," in which the majority of a *Staphylococcus aureus* population is vancomycin susceptible and a minority population is vancomycin resistant (60). Deletion of *mspA* apparently increased the MIC for the resistant subpopulation of *M. smegmatis*. A thickened cell wall has been shown to be associated with vancomycin resistance in staphylococci (8). In the genus *Enterococcus*, resistance to vancomycin relies on the exchange of the terminal dipeptide in pentapeptide cell wall

precursors (2). However, the molecular mechanisms of vancomycin resistance in mycobacteria are unknown.

The hyperresistance of the *mspA* mutant of *M. smegmatis* to the large hydrophobic antibiotics rifampin and erythromycin was unexpected for two reasons. First, the rates of diffusion of β -lactam antibiotics through the porins of *E. coli* inversely depend on their hydrophobicities and are correlated with their efficacies (48). This was consistent with the observations that the sensitivities of *E. coli* and other gram-negative bacteria to hydrophobic antibiotics were not affected by the deletion of porins (49, 56) but were increased by mutation of the lipopolysaccharide or damage to the OM (42, 49). The highly negatively charged constriction site of the MspA channel (14) likely creates shells of strongly bound water molecules which should exclude hydrophobic solutes from passage. A similar mechanism, albeit based on a very different structure, was postulated and confirmed for the porin OmpF of *E. coli* (27, 58). Second, in addition to being hydrophobic, the large sizes of erythromycin and rifampin make it rather unlikely that these antibiotics diffuse through the MspA pore at a significant rate. An indirect effect of MspA on the OM permeability of *M. smegmatis* provides an alternative explanation for the increased resistance of the *mspA* mutant *M. smegmatis* to hydrophobic antibiotics. Alteration of the permeability of the lipid pathway by deletion of MspA is supported by the finding that the rate of uptake of the very hydrophobic agent chenodeoxycholate by the *mspA* mutant is reduced threefold compared to that by the wild type. Decreased permeability to chenodeoxycholate was also observed in a mutant of *M. tuberculosis* lacking oxygenated mycolic acids (11), which shows that the lipid arrangement is important for the permeability of the mycobacterial OM. Heterologous expression of a very small number of MspA porins increased the permeability of *M. tuberculosis* for the nucleic acid-binding, membrane-permeant stain SYTO 9 by 10-fold, indicating that MspA has a significant influence on OM properties (37). Thus, the lipid arrangement in the mycobacterial OM appears to depend on both the mycolic acid and the protein compositions. Although this model would explain the unexpected cross-resistance of the *mspA* mutant of *M. smegmatis* to hydrophobic antibiotics, it is in contrast to observations for *Salmonella*, in which reduced levels of OM proteins increased the rates of penetration of hydrophobic compounds (1). Experiments probing the fluidity of the OM of porin mutants are needed to solve this apparent contradiction. Taken together, this study highlights the importance of porins for the antibiotic resistance of *M. smegmatis*. This is of direct medical relevance for the treatment of nosocomial infections caused by the opportunistic pathogens *M. chelonae* and *M. fortuitum*, which have Msp-like porins (41), which are prevalent in up to 90% of biofilms (59), and which are associated with wound and catheter infections (5). Since the principal structure of the cell wall appears to be similar for many mycobacteria (50), it is anticipated that porins also play a role in determining the drug sensitivity of *M. tuberculosis*. However, the majority of clinically relevant multidrug-resistant *M. tuberculosis* strains arise as a result of the sequential accumulation of resistance mutations in known genes, which either encode the targets or are involved in activation of the individual drugs. Nevertheless, *M. tuberculosis* strains with unknown resistance mechanisms exist for each of the primary antituberculosis drugs (53), and such

strains should be analyzed for mutations involving porin genes and/or drug efflux.

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