Biocides are chemical agents that inactivate microorganisms and include skin and mucosa antiseptics, disinfectants of surfaces and medical devices, and preservatives of food, feed, pharmaceuticals, and other industrial products (11, 20). They comprise a wide variety of chemical classes, such as phenols, aldehydes, biguanides, surface-active agents, halogens, and others (20). It is generally assumed that the majority of biocidal molecules and their formulations target multiple sites of the bacterial cell (19). This general mode of action depends on the physicochemical nature of the given molecule. Some biocides act as membrane destabilizers, and others are alkylating or oxidizing agents or intercalate with nucleic acids. Exceptions include triclosan and isothiazolinones, which inhibit specific enzymes (3, 20, 34).

Whereas the vast majority of Gram-positive and Gram-negative bacteria pose no potential challenge for most biocidal substances, mycobacteria are among the microorganisms least susceptible to biocides (17, 33). However, mycobacteria are important human pathogens, with Mycobacterium tuberculosis remaining a major cause of global mortality and with nontuberculosis mycobacteria increasingly causing opportunistic infections in hospitals and in other environments (9, 30). Therefore, mycobacteria are important organisms to study in infection control, and a better understanding of the mode of action of biocides against mycobacteria will help to fight mycobacterial infections more effectively. The exceptional resistance of mycobacteria to toxic solutes is due to an unusual outer membrane, which acts as an efficient permeability barrier (2), in synergy with other resistance mechanisms, such as efflux or enzymatic inactivation of the target molecule (25). Porins are water-filled channel proteins in the outer membrane of mycobacteria (21). MspA was discovered as the major porin (24) and was later found to be the most abundant protein of Mycobacterium smegmatis (29). Deletion of mspA reduced outer membrane permeability toward glucose (38), phosphate (44), and amino acids (40), indicating that MspA represents the major general diffusion pathway in M. smegmatis. The loss of MspA and MspC, a porin very similar to MspA, reduced the growth rate of M. smegmatis (40, 44), indicating that the influx of hydrophilic nutrients through Msp porins is required for normal growth. Importantly, it was shown that the Msp porins also provide an entry pathway for some antibiotics and drugs (6, 41). These results gave rise to the hypothesis that porins might also be involved in the susceptibility of mycobacteria to biocides. This assumption has been confirmed recently for glutardialdehyde (42). However, it is unknown how mycobacteria take up other biocides (34) and whether these biocidal agents enter mycobacteria by diffusing directly through the lipid bilayers of the cell envelope or by using porins for this purpose.

In this study we examined the role of porins in the susceptibility of M. smegmatis to biocides. Importance of Porins for Biocide Efficacy against Mycobacterium smegmatis

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MATERIALS AND METHODS

Bacterial strains and standard growth conditions. To assess the porin-dependent efficacy of biocides against *M. smegmatis*, a set of well-characterized isogenic porin mutants was chosen. Construction of the porin mutants *M. smegmatis* ML02 (the ΔmspA mutant), ML10 (the ΔmspA ΔmspC mutant), and ML16 (the ΔmspA ΔmspC ΔmspD mutant) was described previously (38, 40). Unless stated otherwise, all strains were cultured on Middlebrook 7H10 agar plates supplemented with 0.2% glycerol or under agitation in Middlebrook 7H9 liquid medium supplemented with 0.2% glycerol and 0.05% Tween 80 at 37°C. All media, chemicals, and the OADC enrichment were purchased from BD Biosciences, Difco Laboratories, and Merck.

Biocides for susceptibility testing. Formaldehyde-releasing biocides hexahydropyrimidine (1,3,5-tris (2-hydroxyethyl)-hexahydropyrimidine; HHT) (Grotan BK) and methylenbisoxazolidine (N,N'-methylene-bis-5-methyl-oxazolidine; MBO) (Grotan OX) and a mycobactericidal disinfectant formulation consisting of the active ingredients phenoxy-propanol (35%, wt/wt), cocospropylene-diamine-guaidinidine diacetate (14%, wt/wt), and benzalkonium chloride (2.5%, wt/wt) (Gigasept Instru AF) were provided by Schülke & Mayr (Germany). The isothiazolinones Kathon 886 and Kathon 893 were purchased from Rohm & Haas (Germany) and Thor (Germany). Polyhexamethylene biguanide hydrochloride (PHMB) solution (also termed Vantocil IB) was from Avecia (Germany), and octenidine dihydrochloride (octenidine) was from Schülke & Mayr GmbH (Germany). See Fig. 1 for the chemical structures.

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Susceptibility determination by MABA. A microplate-based alamarBlue assay (MABA) was performed to determine the MIC as described in reference 5, with slight modifications (6). All solutions of antimicrobial compounds were made freshly and were sterilized by filtration (0.2-μm pore size) prior to each assay. Stock solutions of solid substances were prepared with ultrapure water. Biocide and antibiotic stock solutions were diluted with 7H9-T medium (without Tween 80) to the required test concentrations. In brief, well-dispersed cell inocula (optical density of 600 nm [OD600] of 0.01 to 0.02) were exposed to serially diluted biocidal agents. The microplates were incubated overnight at 37°C under shaking (300 rpm). After addition of alamarBlue (Biosource, Belgium), incubation was continued for 3 to 4 h until the control wells containing no biocides turned from blue to pink due to the metabolic reduction of the redox-active dyes. Fluorescence was measured with a Synergy HT reader (Bio-Tek) at 590 nm after excitation at 530 nm in the top-reading mode. Biocide concentrations that reduced the fluorescence by more than 90% were considered MICs as defined previously (5).

Quantitative suspension test EN 14348. Quantitative suspension tests were carried out according to the EN 14348 testing method for the evaluation of mycobactericidal activity of chemical disinfectants in the medical area, including instrument disinfectants (phase 2/step 1) (8). Cell suspensions were prepared from three agar plates according to EN specifications. Removal of cell aggregates was achieved by grinding the suspension with a cooled, sterile agitate mortar at 4°C. The suspension was left to settle for 30 min, and the supernatant was adjusted to 10^9 to 5 x 10^9 CFU/ml with sterile, purified water. Complete homogenization and integrity of the cells were subsequently monitored by fluorescence staining (LIVE/DEAD BacLight bacterial viability kit; Molecular Probes, OR) according to the instructions of the manufacturer. For fluorescence microscopy, an Olympus BX61 microscope equipped with a U-WIBA filter cube (excitation filter, 460- to 490-nm band pass; barrier filter, 510- to 550-nm narrow band pass) was used.

The disinfectant formulation (Gigasept Instru AF) was diluted with standardized hard water (CEN-HW; 300 ppm CaCO3) to 1.25-fold of the desired test concentrations to achieve final concentrations of 0.0625% and 0.125% after addition of the cell inoculum. Experiments were carried out in the presence of 0.3% bovine serum albumin (BSA) (“clean conditions”) with contact times of 5, 15, and 30 min. The antimicrobial efficacy of the disinfectant formulation was quenched in a neutralizer solution consisting of 3.0% (wt/vol) saponin, 1.0%...
This increased resistance to ampicillin has been attributed to the drastic reduction in the number of porins from 1,000 to less than 60 porins/µm² cell wall (6, 40).

**Role of porins in the efficacy of isothiazolinones against M. smegmatis.** Isothiazolinones are broad-spectrum biocidal agents used to control the growth of bacteria, fungi, and algae in cooling water systems, storage tanks, emulsions, or paints. Methylchloroisothiazolinone (MCI) is used as a preservative in many water-based cosmetics because of its effectiveness against Gram-positive and Gram-negative bacteria, yeast, and fungi (32). Kathon 886 is a mixture (3:1) of MCI and methylisothiazolinone (MI) and killed *M. smegmatis* efficiently with a MIC of 0.2 µg/ml (Table 1), in line with previously reported MICs ranging from 0.2 to 15 µg/ml against different bacteria (3, 7). The porin mutants ML02, ML10, and ML16 were 2- to 4-fold less susceptible to MCI/MI (Table 1). It is likely that methylchloroisothiazolinone and methylisothiazolinone diffuse through the pores of Msp porins of *M. smegmatis* due to their small sizes and their hydrophilicity (Fig. 1). This hypothesis is supported by the observation that *Pseudomonas aeruginosa* isolates not expressing the outer membrane porin OmpD lacked susceptibility to the more effective methylchloroisothiazolinone (4).

Octylisothiazolinone (2-n-octyl-4-isothiazolin-3-one [OIT]) is a biocide that is used in technical applications, such as paints or metalworking fluids for suppressing growth of bacteria and fungi (16). We tested the role of porins in the susceptibility of *M. smegmatis* to OIT (Kathon 893). Deletion of mspA reduced the susceptibility of *M. smegmatis* to OIT 2-fold (Fig. 2A). Deletion of the two porins MspA and MspC, which are the only Msp porins expressed under laboratory growth conditions (40), enhanced the resistance of *M. smegmatis* to OIT 16-fold (Table 1 and Fig. 2A). However, octylisothiazolinone is more hydrophobic and larger than methylisothiazolinone and methylchloroisothiazolinone (Fig. 1). Increased hydrophobicity and larger solute size are molecular properties known to slow down diffusion through water-filled channel proteins in general (27, 28) and in particular through Msp pores, in which 16 aspartate residues form the constriction zone (10). Hence, the reduced susceptibility of porin mutants to octylisothiazolinone might be increased by an indirect effect. It was shown that the loss of porins decreases the permeability of the outer membrane of *M.

**RESULTS AND DISCUSSION**

Susceptibility of *M. smegmatis* and isogenic porin mutants to biocides by alamarBlue assay. We showed previously that antibiotics cross the mycobacterial cell envelope either by porin-facilitated diffusion or by direct diffusion through the lipid membranes, with the entry route being dependent on the size and the hydrophobicity of the solute (6, 41). The aim of this study was to examine which pathways biocides use to penetrate the cell walls of mycobacteria. To examine the role of porins in the susceptibility of mycobacteria to biocides, a well-characterized set of isogenic porin mutants of *M. smegmatis* was employed. These mutants lack the major porin gene *mspA* (ML02), *mspA* and *mspC* (ML10), and *mspA*, *mspC*, and *mspD* (ML16) (40). The efficacies of biocides with various physicochemical properties and modes of action (Table 1 and Fig. 1) and of a more complex mycobacterial disinfectant formulation against *M. smegmatis* and the isogenic porin mutants were determined by the microplate-based alamarBlue assay (MABA). MICs were defined as the concentration of the antimicrobial agent which caused a reduction of cell viability by ≥90% (Table 1). Ampicillin is a small and hydrophilic antibiotic which diffuses through porins in *M. smegmatis* (6, 41). In our study, ampicillin served as a standard for solutes whose uptake was largely porin mediated. Deletion of the major porin MspA (∆mspA; strain ML02) reduced the susceptibility of *M. smegmatis* against ampicillin 2-fold (Table 1). The lack of additional porins in the mutant strains ML10 (∆mspA ∆mspC mutant) and ML16 (∆mspA ∆mspC ∆mspD mutant) enhanced the β-lactam resistance of *M. smegmatis* 16-fold (Table 1). This increased resistance to ampicillin has been attributed to the drastic reduction in the number of porins from 1,000 to less than 60 porins/µm² cell wall (6, 40).

<table>
<thead>
<tr>
<th>Antimicrobial substance</th>
<th>Molecular mass (g/mol)</th>
<th>Partition coefficient (log <em>P</em>&lt;sub&gt;ow&lt;/sub&gt;)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MIC for <em>M. smegmatis</em> (µg/ml)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Resistance factor&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>349</td>
<td>0.43</td>
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</tr>
<tr>
<td>OIT (Kathon 893)</td>
<td>213</td>
<td>3.89</td>
<td>0.94</td>
<td>15</td>
</tr>
<tr>
<td>MCTM&lt;sup&gt;d&lt;/sup&gt; (Kathon 886)</td>
<td>150.115</td>
<td>0.72048</td>
<td>0.18</td>
<td>4</td>
</tr>
<tr>
<td>MBO (Grotan OX)</td>
<td>186</td>
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<td>0.01</td>
<td>2</td>
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<tr>
<td>HFTT (Grotan BK)</td>
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<tr>
<td>Octenidine dihydrochloride</td>
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<td>2</td>
</tr>
<tr>
<td>Gisagentes Intru AF</td>
<td>Formulation</td>
<td>ND</td>
<td>6.25</td>
<td>8</td>
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<sup>a</sup> Partition coefficients were calculated using ALOGPS 2.1 (available from the Virtual Computational Chemistry Laboratory at http://www.vcclab.org).

<sup>b</sup> MICs were determined by MABA (see Materials and Methods) and represent biocide concentrations that reduced viability of the cells by ≥90%.

<sup>c</sup> The resistance factor *R* is the quotient of the MIC of the triple porin knockout strain *M. smegmatis* ML16 and the MIC of the *M. smegmatis* wild type.

<sup>d</sup> Kathon 886 contains a mixture of the active ingredients methylchloroisothiazolinone (MCI) and methylisothiazolinone (MI) in a ratio of 3:1.

<sup>e</sup> PHMB is a mixture of hexamethylene biguanide hydrochloride polymers (n = 2 to 40) with an average chain length of 12 units. The partition coefficient cannot be calculated due to the polycationic nature of the molecules.

<sup>f</sup> ND, the partition coefficient cannot be determined for mixtures of compounds.

(wt/vol) Tween 80, 0.5% (wt/vol) sodium thiosulfate, and 0.1% (wt/vol) histidine in 0.08 M phosphate buffer, pH 7.0.

Briefly, 1 ml of the cell inoculum preincubated with 1 ml organic load was added to 8 ml Gigasentis Intru AF. After specified contact times, 1-ml aliquots were removed and quenched in 9 ml neutralizer solution. Further decimal dilutions were prepared in neutralizer and spread doubly on 7H10 plates to perform conventional plate counting. The level of mycobactericidal activity was calculated according to the guidelines (logarithmic cell count difference of *R*<sub>0</sub> = log *N*<sub>0</sub>− log *N*<sub>f</sub>, where *R*<sub>0</sub> is the reduction factor and *N*<sub>0</sub> and *N*<sub>f</sub> are the numbers of CFU per milliliter before and after exposure to disinfent, respectively). Additionally, standardized internal controls were carried out to verify the nontoxicity of hard water, BSA, and neutralizer exposure to each strain.

### TABLE 1. Physicochemical properties of biocides and their MICs for *M. smegmatis* SMR5 and isogenic porin mutants

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Efficacy of biocides against mycobacteria

Role of porins in the efficacy of lipophilic biocides against *M. smegmatis*. Polyhexamethylene biguanide (PHMB) and octenidine are lipophilic biocides. PHMB is a fast-acting, broad-spectrum biocide effective at low concentrations against bacteria and viruses and is used as a preservative in cosmetics, personal care products, fabric softeners, contact lens solutions, and hand washes and as a sanitizer in swimming pools and hot tubs due to its stability in sunlight and its resistance against heat and pH fluctuations. Octenidine dihydrochloride is a cationic lipophilic biocide of high molecular weight and is an efficient antiseptic for skin, mucous membranes, and wounds. The efficacy of the bispyridine derivative octenidine was reduced 2-fold by deletion of the main porin MspA, but not by further deletions of *mspC* and *mspD* (Table 1; Fig. 2B). Likewise, loss of porins caused only a 2-fold increase in MIC values from 5 μg/ml to 10 μg/ml by exposure to PHMB (Table 1). These lipophilic biocides might cross the mycobacterial outer membrane directly, because it is known that the rate of permeation of lipophilic substances through lipid membranes increases with an increasing n-octanol–water partition coefficient. This may explain the minor role of porins in the efficacy of these biocides. However, it was proposed that relative hydrophobic polymeric biguanides, which constitute PHMB, may enter Gram-negative bacteria by destabilizing the outer membrane in a synergistic way, termed self-promoted uptake. However, it is unknown whether a similar process exists in mycobacteria.

Role of porins in the efficacy of a complex disinfectant formulation against *M. smegmatis*. In addition to pure biocide solutions, we examined the influence of Msp porins on the mycobacterial susceptibility against a complex mycobactericidal disinfectant formulation consisting of the biocides phe-noxypropanol, cocospropylene-diamine-guanidine diacetate, and benzalkonium chloride. We observed a 2-fold decreased susceptibility of the ∆mspA porin mutant ML02 and an 8-fold decreased susceptibility of the double and triple porin mutant strains to Benzalkonium chloride, which belongs to the group of surface-active quaternary ammonium compounds, is relatively hydrophobic and acts primarily on lipids of bacterial origin.

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**FIG. 2.** Porin-dependent susceptibility of *M. smegmatis* to Kathon 893 and octenidine dihydrochloride. After exposure to various concentrations (c) of octylisothiazolinone (Kathon 893) (A) and the cationic surfactant octenidine (B), the viability of *M. smegmatis* wild type (black bars) and the isogenic porin mutants ML02 (the ∆mspA mutant, dark gray bars), ML10 (the ∆mspA ∆mspC mutant, medium gray bars), and ML16 (the ∆mspA ∆mspC ∆mspD mutant, light gray bars) was determined by the alamarBlue assay. Significant differences in the efficacy of the biocides against the wild type in comparison to at least one of the porin deletion strains are denoted with black asterisks. Gray asterisks indicate significantly different susceptibilities between the single porin deletion ML02 and the double or triple porin mutants. Significance was calculated using the paired Student t test (P ≤ 0.05).

The experiments were done in triplicate. Error bars represent standard deviations.

**TABLE 1.** Initial and final minimum inhibitory concentration (MIC) values of octylisothiazolinone (Kathon 893) and octenidine dihydrochloride. After exposure to various concentrations of octylisothiazolinone and octenidine dihydrochloride, the viability of wild type and deletion strains was determined by the alamarBlue assay. Significant differences in the efficacy of the biocides against the wild type in comparison to at least one of the porin deletion strains are denoted with black asterisks. Gray asterisks indicate significantly different susceptibilities between the single porin deletion ML02 and the double or triple porin mutants. Significance was calculated using the paired Student t test (P ≤ 0.05).

<table>
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<tr>
<th>Biocide</th>
<th>Concentration (μg/ml)</th>
<th>Wild Type</th>
<th>ΔmspA</th>
<th>ΔmspC</th>
<th>ΔmspA ΔmspC</th>
<th>ΔmspA ΔmspC ΔmspD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octylisothiazolinone (Kathon 893)</td>
<td>0.12</td>
<td>98 ± 2</td>
<td>100 ± 1</td>
<td>100 ± 1</td>
<td>100 ± 1</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>Octenidine dihydrochloride</td>
<td>0.06</td>
<td>98 ± 2</td>
<td>100 ± 1</td>
<td>100 ± 1</td>
<td>100 ± 1</td>
<td>100 ± 1</td>
</tr>
</tbody>
</table>
membranes (12). Cocospropylene-diamine-guanidine diacetate is a relatively hydrophilic, surface-active polymeric guanidinium salt of high molecular weight. Hence, both of these compounds are unlikely to cross the outer membrane of M. smegmatis via porins. Thus, we assume that uptake of the hydrophilic and small bactericidal biocide phenoxyparanol (152 g/mol; \( P_{\text{cow}} = 1.66 \)) is impaired by the lack of porins, resulting in the observed decrease in susceptibility of M. smegmatis porin mutants to the disinfectant formulation.

Susceptibility of M. smegmatis and isogenic porin mutants to biocides in the quantitative suspension test EN 14348. To examine whether the reduced expression of porins would also affect the susceptibility of M. smegmatis to biocides under more complex conditions, quantitative suspension tests according to the EN 14348 testing method (8), used for the evaluation of the mycobactericidal efficacy of disinfectants, were carried out (8) with a low organic load (0.3% BSA, clean conditions). The aggregation of mycobacterial cells is a known problem in disinfectant testing (43). The M. smegmatis cultures were monitored prior to each experiment by fluorescence microscopy to ensure that they contained dispersed, viable cells with frequent cell clumps smaller than 10 cells (not shown). The initial cell count of the cultures ranged from \( 2 \times 10^9 \) to \( 5 \times 10^9 \) CFU/ml for both M. smegmatis SMR5 and ML16. Control experiments verified the nontoxicity and efficacy of the neutralizer, standard hard water, and BSA (data not shown).

Exposure to 0.0625% of the disinfectant Gigasept Instru AF reduced the viability of wild-type M. smegmatis 10,000-fold within a contact time of 5 min, whereas viability of the porin mutant ML16 was reduced only 100-fold (Fig. 3). In these quantitative suspension experiments, wild-type M. smegmatis was on average 100- to 1,000-fold more sensitive to disinfectant exposure than the porin triple mutant. Treatment with 0.125% of the disinfectant formulation led to an almost complete inactivation of the M. smegmatis SMR5 cell inoculum (Fig. 3). Only 0.0001% M. smegmatis SMR5 cells survived. As observed for the lower concentration, the ML16 strain was less susceptible to the disinfectant formulation at all contact times. Taken together, these findings make evident a strong correlation between the susceptibility of M. smegmatis to the disinfectant formulation and porin density. Thus, consistent with the data obtained in alamarBlue assays, outer membrane porins are important for the susceptibility of M. smegmatis also under more complex conditions, as used in the quantitative suspension test EN 14348.

Efficacy of the disinfectant Gigasept Instru AF against the standard test organisms Mycobacterium terrae and Mycobacterium avium was demonstrated in the quantitative suspension test EN 14348 (phase 2/step 1) and in the quantitative carrier test EN 14563 (phase 2/step 2) at a concentration of 1.5% of the disinfectant formulation for 60 min of contact time or at a concentration of 3% with 15 min of contact time (35, 36). Thus, a 24-fold higher concentration than that of M. smegmatis is necessary to kill M. avium and M. terrae, the latter being the surrogate organism for M. tuberculosis in European disinfectant testing procedures. According to the results of this study, the lower susceptibility of M. avium and M. terrae might be caused by a lower number of porins in the outer membrane of slow-growing mycobacteria, as pointed out earlier (22, 23).

Conclusions. In this study we showed that a lack of Msp porins, channel-forming proteins in the outer membrane, reduces the susceptibility of M. smegmatis against different classes of widely used biocides. This is of particular interest since Msp porins are widespread (21). For example, MspA-like porins have been identified in Mycobacterium chelonae (42), Mycobacterium ulcerans, and M. avium (21). Infections with these mycobacteria are increasingly common but difficult to treat (9, 30). The findings of this study underline the importance of porins for the susceptibility of M. smegmatis to chemically very different biocides. Better knowledge of the mode of how biocides enter mycobacterial cells and of their mode of action might help to prevent and treat mycobacterial infections more efficiently.

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