Role of Porins in the Susceptibility of *Mycobacterium smegmatis* and *Mycobacterium chelonae* to Aldehyde-Based Disinfectants and Drugs

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Nosocomial outbreaks attributable to glutaraldehyde-resistant, rapidly growing mycobacteria are increasing. Here, evidence is provided that defects in porin expression dramatically increase the resistance of *Mycobacterium smegmatis* and *Mycobacterium chelonae* to glutaraldehyde and another aldehyde disinfectant, ortho-phthalaldehyde. Since defects in porin activity also dramatically increased the resistance of *M. chelonae* to drugs, there is thus some concern that the widespread use of glutaraldehyde and ortho-phthalaldehyde in clinical settings may select for drug-resistant bacteria.

Rapidly growing mycobacteria (RGM) are ubiquitous in hospitals’ water sources and cause outbreaks in health care settings throughout the world (7, 22, 23, 31). Among the effective options for the disinfection of semicritical, temperature-sensitive medical devices, glutaraldehyde (GTA) remains the most widely used chemical disinfectant in hospitals worldwide due to its effective mycobactericidal activity and relatively low cost (Fig. 1). Recent reports suggest, however, that RGM may be mechanisms through which RGM develop resistance. Because of the significant role played by the mycobacterial outer surface resulting in decreased binding and/or penetration of GTA—known, it is thus reasonable to assume that changes in the cell wall (lipo)polysaccharides have been proposed to affect the susceptibility of *M. chelonae* to GTA (15). To assess the impact of these cell envelope compounds on the resistance of *Mycobacterium smegmatis* to GTA, mc2155 isogenic mutants deficient in different aspects of their biosynthesis (Table 1) were compared to their respective wild-type (WT) parent for GTA resistance, using the suspension test described by Griffiths et al. (10). Mutants deficient in other factors known to significantly affect the susceptibility of *M. smegmatis* to biocides, such as phosphatidylinositol mannosides, the Lsr2 protein, and mycothiols, were also included in this study. The mutants fell into roughly three categories: (i) those whose susceptibility to GTA was not significantly altered (mc2155ΔMSMEG4250, mc2155ΔpimE, mc2155Δlsr2, Myc55, A1) (data not shown), (ii) those showing a slight increase in susceptibility (mc2155ΔMSMEG4245, mc2155ΔembB, mc2155ΔembB) (Fig. 2A), and (iii) those displaying a significantly increased resistance to the disinfectant. The last category clearly included the *mspA* and *mspA-mspC* porin mutants, MN01 and ML10 (Fig. 2B). MspA is the main porin, constituting more than 70% of all pores of *M. smegmatis* (27). The ML10 mutant has at least 15- and 5-fold less porins than WT *M. smegmatis* and MN01, respectively.

GTA at the surface of RGM include surface-exposed proteins—among which are porins—and glycopeptidolipids. In addition, cell wall polysaccharides have been proposed to affect the susceptibility of *M. chelonae* to GTA (15). To assess the impact of these cell envelope compounds on the resistance of *Mycobacterium smegmatis* to GTA, mc2155 isogenic mutants deficient in different aspects of their biosynthesis (Table 1) were compared to their respective wild-type (WT) parent for GTA resistance, using the suspension test described by Griffiths et al. (10). Mutants deficient in other factors known to significantly affect the susceptibility of *M. smegmatis* to biocides, such as phosphatidylinositol mannosides, the Lsr2 protein, and mycothiols, were also included in this study. The mutants fell into roughly three categories: (i) those whose susceptibility to GTA was not significantly altered (mc2155ΔMSMEG4250, mc2155ΔpimE, mc2155Δlsr2, Myc55, A1) (data not shown), (ii) those showing a slight increase in susceptibility (mc2155ΔMSMEG4245, mc2155ΔembB, mc2155ΔembB) (Fig. 2A), and (iii) those displaying a significantly increased resistance to the disinfectant. The last category clearly included the *mspA* and *mspA-mspC* porin mutants, MN01 and ML10 (Fig. 2B). MspA is the main porin, constituting more than 70% of all pores of *M. smegmatis* (27). The ML10 mutant has at least 15- and 5-fold less porins than WT *M. smegmatis* and MN01, respect-

FIG. 1. Chemical structures of monomeric GTA and OPA.
level with the mature MspA protein from M. chelonae, sharing about 73% identity at the amino acid sequence level. M. smegmatis clustered open reading frames, MCH_4689c, MCH_4690c, MCH_4691c, the genome of M. chelonae in field isolates of RGM, we next analyzed a clinical isolate of M. chelonae ATCC 35752 displaying high levels of resistance to OPA and GTA may be surface exposure might contribute to GTA and OPA resistance to both aldehyde disinfectants. Implied the existence of at least one common mechanism of resistance to both aldehyde disinfectants.

To investigate whether defects in porin production and/or surface exposure might contribute to GTA and OPA resistance in field isolates of RGM, we next analyzed a clinical isolate of M. chelonae ATCC 35752 displaying high levels of resistance to both disinfectants (Fig. 3). A search for msp-like porin genes in the genome of M. chelonae ATCC 35752 identified three clustered open reading frames, MCH_4689c, MCH_4690c, and MCH_4691c, sharing about 73% identity at the amino acid level with the mature MspA protein from M. smegmatis mc²155. Sequencing of these genes in M. chelonae 9917 revealed a frame-shift mutation at codon 137 of MCH_4689c, resulting in a 40-amino-acid truncation of its protein product. Unfortunately, attempts to determine porin production in strains 9917 and ATCC 35752 by immunoblot analysis using polyclonal antibodies directed against the MspA protein of M. smegmatis (20) were unsuccessful. No proteins of the expected size were detected, suggesting that the anti-MspA antibodies do not cross-react with the porins of M. chelonae or that porin expression was too low under the experimental conditions used to detect by immunoblot analysis.

The porin-mediated influx of nutrients was shown to be a major determinant of the growth rates of M. smegmatis and Mycobacterium fortuitum (24, 28). Consistent with a defect in porin activity, strain 9917 grew significantly slower than did M. smegmatis mc²155. The porin-mediated influx of nutrients was shown to be a major determinant of the growth rates of M. smegmatis and Mycobacterium fortuitum (24, 28). Consistent with a defect in porin activity, strain 9917 grew significantly slower than did M. smegmatis mc²155.

Table 1. Isogenic insertion mutants of M. smegmatis with known defects in cell envelope composition and/or biocide susceptibility

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation</th>
<th>Description/phenotype*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mc²155ΔpimE</td>
<td>pimE</td>
<td>KO mutant deficient in polar PIM synthesis</td>
<td>Our work</td>
</tr>
<tr>
<td>mc²155ΔMSMEG4245</td>
<td>MSMEG_4241</td>
<td>KO mutant deficient in the production of WT LM and LAM; produces a truncated form of LM</td>
<td>14</td>
</tr>
<tr>
<td>mc²155ΔMSMEG4250</td>
<td>MSMEG_4247</td>
<td>KO mutant deficient in the production of LM; produces a truncated form of LAM lacking α-1,2 Manp branches on the mannan core</td>
<td>13</td>
</tr>
<tr>
<td>mc²155ΔembA</td>
<td>embA</td>
<td>KO mutant deficient in synthesis of the terminal hexa-arabinofuranoside motif of arabinogalactan; decreased cell wall-bound mycolic acid content</td>
<td>9</td>
</tr>
<tr>
<td>mc²155ΔembB</td>
<td>embB</td>
<td>KO mutant deficient in synthesis of the terminal hexa-arabinofuranoside motif of arabinogalactan; decreased cell wall-bound mycolic acid content</td>
<td>9</td>
</tr>
<tr>
<td>mc²155ΔembC</td>
<td>embC</td>
<td>KO mutant deficient in LAM synthesis</td>
<td>32</td>
</tr>
<tr>
<td>mc²155Δlsr2 (DL2008)</td>
<td>lsr2</td>
<td>KO mutant deficient in the regulatory histone-like protein Lsr2</td>
<td>2</td>
</tr>
<tr>
<td>MN01</td>
<td>mspA</td>
<td>KO mutant deficient in the production of the major porin MspA</td>
<td>27</td>
</tr>
<tr>
<td>ML10</td>
<td>mspA-mspC</td>
<td>KO mutant deficient in the production of the MspA and MspC porins</td>
<td>28</td>
</tr>
<tr>
<td>Myc55</td>
<td>MSMEG_0408</td>
<td>Transposon mutant deficient in glycopeptidolipid biosynthesis</td>
<td>26</td>
</tr>
<tr>
<td>A1</td>
<td>mshA</td>
<td>Transposon mutant deficient in mycothiol biosynthesis</td>
<td>19</td>
</tr>
</tbody>
</table>

* KO, knockout; PIM, phosphatidylinositol mannosides; LM, lipomannan; LAM, lipoarabinomannan.

FIG. 2. GTA and OPA susceptibility of defined isogenic mutants of M. smegmatis mc²155. Results are expressed as CFU counts upon exposure of the test organisms to the indicated concentrations of disinfectants for 0 to 15 min. (A) mc²155ΔembA (closed circles), mc²155ΔembB (closed triangles), and mc²155ΔMSMEG4245 (closed diamonds) mutants are slightly more sensitive to GTA than is their WT parent, mc²155 (open triangle). GTA (B) and OPA (C) sensitivity of the porin mutants MN01 (closed rectangles, solid line) and ML10 (closed triangles, solid line); the complemented porin mutants MN01/pMN013 (open rectangles, dashed line) and ML10/pMN013 (open triangles, dotted line); and their WT parent, SMR5 (closed circles, solid line).
accounted for by differences in composition and structure of the outer membranes of these two strains and the different modes of action of the two disinfectants (17).

Compared to the reference strain, *M. chelonae* ATCC 35752, strain 9917 displayed dramatically increased (4- to 100-fold) resistance to rifampin (rifampicin), vancomycin, ciprofloxacin, clarithromycin, erythromycin, linezolid, and tetracycline (Table 2). Strongly supporting the involvement of porins in the resistance phenotypes of 9917, expression of the *mspA* gene from *M. smegmatis* in this strain increased 5- to 500-fold its susceptibility to these drugs (Table 2). Interestingly, expression of *mspA* from pZS01 also increased 5- to 25-fold the susceptibility of both *M. chelonae* ATCC 35752 and 9917 to ethambutol, ethionamide, and chloramphenicol (Table 2). These results, which show a much more pronounced effect of porin expression on the drug susceptibility of *M. chelonae* than that of *M. smegmatis* (6, 29), could reflect important differences in the outer membrane organization and drug efflux mechanisms of these two rapidly growing *Mycobacterium* spp. (21).

Altogether, our data thus suggest that the Msp-like porin content of *M. smegmatis* and *M. chelonae* is a major determinant of the susceptibility of both species to GTA and OPA. Given the known functional similarities shared by the outer membranes of mycobacteria and gram-negative bacteria (3, 11–12, 18, 33), it is tempting to speculate that *Pseudomonas aeruginosa* and other gram-negative opportunistic pathogens may have or could adopt a similar strategy to resist aldehyde disinfectants.

Importantly, the results of this study support the hypothesis that GTA-resistant isolates are likely to develop cross-resistances to multiple antibiotics, including some used in the clinical treatment of RGM infections. Moreover, because porins have been shown to play important roles in the pathogenicity of a number of intracellular and extracellular pathogens (1), including *M. smegmatis* (25), our results also raise concerns that the selection of GTA-resistant organisms may impact their pathogenicity.
Accession numbers. The accession numbers corresponding to the porin genes of *M. chelonae* ATCC 35752 are FJ981588 (MCH_4689c), FJ981589 (MCH_4690c), and FJ981590 (MCH_4691c).


REFERENCES


