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 are being isolated with increasing frequency from washer disinfector systems, with recent reports suggesting that RGM outbreaks are being associated with the development of resistance to GTA (4, 8, 10, 17, 30).

GTA is thought to be predominantly a surface-reactive biocide which forms bridges or cross-links with amino groups of proteins exposed at the surface of bacterial cells (17). Although the mechanisms of resistance of mycobacteria to the disinfectant are not known, it is thus reasonable to assume that changes in the cell surface resulting in decreased binding and/or penetration of GTA may be mechanisms through which RGM develop resistance. Because of the significant role played by the mycobacterial outer membrane in drug susceptibility (3, 12) and host-pathogen interactions (5), there is thus some concern that the widespread use of GTA in clinical settings selects for resistant populations of bacteria, with possible consequences on antibiotic resistance and pathogenicity.

Amino group-containing compounds susceptible to binding GTA at the surface of RGM include surface-exposed proteins—among which are porins—and glycopeptidolipids. In addition, 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 mycothiol, were also included in this study. The mutants fell into roughly three categories: (i) those whose susceptibility to GTA was not significantly altered (mc2155ΔmsmE250, mc2155ΔΔpimE, mc2155Δlsr2, Mcc55, A1) (data not shown), (ii) those showing a slight increase in susceptibility (mc2155ΔmsmE245, mc2155ΔembA, 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, respec-

FIG. 1. Chemical structures of monomeric GTA and OPA.

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tively (28). Complementation of MN01 and ML10 with plasmid pMN013 carrying a WT copy of the mspA gene restored the sensitivity of both mutants to GTA (Fig. 2B). Interestingly, ML10 and MN01 were also the only mutants to be significantly more resistant to ortho-phthalaldehyde (OPA) (Fig. 1 and 2C), implying 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 in field isolates of RGM, we next analyzed a clinical isolate of M. chelonae ATCC 35752 in 7H9 broth at 30°C (Fig. 4A) and was on average 5.7 ± 0.2-fold less proficient at taking up [U-14C]glucose (Fig. 4B). As expected, expression of the M. smegmatis mspA gene from the replicative plasmid pZS01 in M. chelonae 9917 partially restored growth (Fig. 4A). Importantly, expression of mspA in both M. chelonae 9917 and ATCC 35752 also resulted in increased susceptibilities to GTA (Fig. 3A and B) and OPA (Fig. 3C), although this effect was significantly more marked with the latter disinfectant in strain 9917. These different effects of expressing mspA on the susceptibility of M. chelonae 9917 and ATCC 35752 to OPA and GTA may be

**TABLE 1.** Isogenic insertional 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>mc&lt;sup&gt;2&lt;/sup&gt;155ΔpimE</td>
<td>pimE</td>
<td>KO mutant deficient in polar PIM synthesis</td>
<td>Our work</td>
</tr>
<tr>
<td>mc&lt;sup&gt;2&lt;/sup&gt;155ΔpimE</td>
<td>pimE</td>
<td>KO mutant deficient in polar PIM synthesis</td>
<td>14</td>
</tr>
<tr>
<td>mc&lt;sup&gt;2&lt;/sup&gt;155ΔMSMEG4245</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>13</td>
</tr>
<tr>
<td>mc&lt;sup&gt;2&lt;/sup&gt;155ΔMSMEG4250</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>9</td>
</tr>
<tr>
<td>mc&lt;sup&gt;2&lt;/sup&gt;155ΔembA</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>mc&lt;sup&gt;2&lt;/sup&gt;155ΔembB</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>mc&lt;sup&gt;2&lt;/sup&gt;155ΔembC</td>
<td>embC</td>
<td>KO mutant deficient in LAM synthesis</td>
<td>32</td>
</tr>
<tr>
<td>mc&lt;sup&gt;2&lt;/sup&gt;155Δlsr2 (DL2008)</td>
<td>lsr2</td>
<td>KO mutant deficient in the regulatory histone-like protein Lsr2</td>
<td>2</td>
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<tr>
<td>MN01</td>
<td>mspA</td>
<td>KO mutant deficient in the production of the major porin MspA</td>
<td>27</td>
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<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 mycobiosynthesis</td>
<td>19</td>
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</table>

<sup>a</sup> KO, knockout; PIM, phosphatidylinositol mannosides; LM, lipomannan; LAM, lipoarabinomannan.

FIG. 2. GTA and OPA susceptibility of defined isogenic mutants of M. smegmatis mc<sup>2</sup>155. Results are expressed as CFU counts upon exposure of the test organisms to the indicated concentrations of disinfectants for 0 to 15 min. (A) mc<sup>2</sup>155ΔembA (closed circles), mc<sup>2</sup>155ΔembB (closed triangles), and mc<sup>2</sup>155ΔMSMEG4245 (closed diamonds) mutants are slightly more sensitive to GTA than is their WT parent, mc<sup>2</sup>155 (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 the reference strain to erythromycin, rifampin, linezolid, and tetracycline and 2- to 20-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 on 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.
TABLE 2. MICs of various drugs against *M. chelonae* ATCC 35752, *M. chelonae* 9917, *M. chelonae* 9917 expressing *mepA* (9917/pZS01) and *M. chelonae* ATCC 35752 expressing *mepA* (ATCC/pZS01)

<table>
<thead>
<tr>
<th>Drug</th>
<th>ATCC 35752</th>
<th>ATCC/pZS01</th>
<th>9917</th>
<th>9917/pZS01</th>
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<tbody>
<tr>
<td>AMP</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
<tr>
<td>KAN</td>
<td>50</td>
<td>ND</td>
<td>10</td>
<td>ND</td>
</tr>
<tr>
<td>STR</td>
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<tr>
<td>VAN</td>
<td>5</td>
<td>5–10</td>
<td>&gt;25</td>
<td>25</td>
</tr>
<tr>
<td>CLA</td>
<td>25–50</td>
<td>50</td>
<td>&gt;25</td>
<td>25</td>
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<tr>
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<td>500</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>HYG</td>
<td>&gt;500</td>
<td>ND</td>
<td>&gt;500</td>
<td>ND</td>
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<tr>
<td>ERY</td>
<td>20–25</td>
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<td>RIF</td>
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<td>&gt;500</td>
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<td>&gt;500</td>
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<tr>
<td>TOB</td>
<td>25–50</td>
<td>ND</td>
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<td>ND</td>
</tr>
<tr>
<td>GEN</td>
<td>7.5</td>
<td>7.5</td>
<td>15</td>
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<tr>
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<td>PZA</td>
<td>&gt;500</td>
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<tr>
<td>TET</td>
<td>50</td>
<td>5–10</td>
<td>&gt;500</td>
<td>25</td>
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<tr>
<td>LIN</td>
<td>25</td>
<td>5</td>
<td>100</td>
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</tr>
<tr>
<td>CIP</td>
<td>1.5</td>
<td>2.5</td>
<td>5</td>
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<tr>
<td>NOR</td>
<td>5</td>
<td>ND</td>
<td>5</td>
<td>ND</td>
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</table>

* MICs were determined using the colorimetric resazurin microtiter assay in 7H9-oleic acid-albumin-dextrose-catalase broth at 30°C (16), and results were confirmed by visually scanning for growth. All assays were performed on well-dispersed bacteria and repeated at least three times on independent culture batches. AMP, ampicillin; AZI, azithromycin; CHL, chloramphenicol; CIP, ciprofloxacin; CLA, clarithromycin; ERY, erythromycin; ETH, ethambutol; GEN, genamycin; HYG, hygromycin; INH, isoniazid; KAN, kanamycin; LIN, linezolid; NOR, norfloxacain; PZA, pyrazinamide; RIF, rifampin; STR, streptomycin; TET, tetracycline; TOB, tobramycin; VAN, vancomycin; ND, not determined.

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).

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References.


