The cell envelope structure and properties of *Mycobacterium smegmatis* mc²155: is there a clue for the unique transformability of the strain?

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**INTRODUCTION**

*Mycobacterium smegmatis* is often used as a surrogate host for pathogenic mycobacteria, especially since the isolation of the transformable smooth morphotype strain mc²155 from the isogenic rough wild-type strain ATCC 607. Biochemical analysis of the cell envelope components revealed a lack of polar glycolipids, namely the lipooligosaccharides and the polar subfamilies of glycopeptidolipids, in the mc²155 strain. In addition, the latter strain differs from its parent by the distribution of various species of glycolipids and phospholipids between the outermost and deeper layers of the cell envelope. The presence of filamentous and rope-like structures at the cell surface of mc²155 cells grown in complex media further supported an ultrastructural change in the cell envelope of the mutant. Importantly, a significantly more rapid uptake of the hydrophobic chenodeoxycholate was observed for the mutant compared to wild-type cells. Taken together, these data indicate that the nature of the surface-exposed and envelope constituents is crucial for the surface properties, cell wall permeability and bacterial phenotype, and suggest that the transformable character of the mc²155 strain may be in part explained by these profound modifications of its cell envelope.

**Abbreviations:** ATCC, American Type Culture Collection; ept, efficient-plasmid-transformation; GPL, glycopeptidolipid; LOS, lipooligosaccharide; MALDI-TOF, matrix-assisted laser-desorption/ionization-time of flight; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositolmannoside; SXM, surface-exposed material; TAG, triacylglycerol; TDM, trehalose dimycolate; TMM, trehalose monomycolate.

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Chatterjee & Khoo, 2001) has been correlated with the rough morphotype of mutants of M. smegmatis (Billman-Jacobe et al., 1999; Recht et al., 2000) or Mycobacterium avium (Barrow & Brennan, 1982; Belisle et al., 1993a, b) and even in the distantly related Gordonia hydrophobica (Moormann et al., 1997).

In this study, we compared the cell-surface properties of the wild-type strain of M. smegmatis ATCC 607 with those of its transformable mutant mc²155. We showed that strain mc²155 displays profound modifications of its cell envelope composition and structure, and we suggest that its transformable character may be in part explained by these alterations of its cell envelope.

**METHODS**

**Bacterial strains and cultures.** M. smegmatis strain ATCC 607, obtained from the ATCC (http://www.lcpromochem.com/atcc/) at different times (see Results), and the transformable strain mc²155 (Snapper et al., 1990; Jacobs, 2000), were routinely grown at 37 °C with shaking at 250 r.p.m. (Incubator Shaker, New Brunswick Scientific) in Trypcase-Soy (TS broth, bioMérieux) medium plus 0-05 % Tween 80. Alternatively, the strains were grown in Luria–Bertani (LB broth, Euromedx) medium for microscopic studies and as surface pellicles on Sauton’s medium, at 37 °C without agitation, when information was needed on extracellular materials. Growth curves were determined as previously described (Raynaud et al., 1998). Single-cell suspensions were prepared with latex-exponential-phase cultures and their density adjusted to 1 OD₆₅₀ unit (Etienne et al., 1995).

**Mycobacterial cell-surface properties.** The determination of mycobacterial cell surface properties was as described elsewhere (Etienne et al., 2002); it is summarized here. (i) The motility assay was adapted from Martinez et al. (1999). Middlebrook 7H9 base medium (Difco) was solidified with 0-4 % high-grade agarose (Eurogentec). Plates were inoculated in their centre with 10 µl single-cell suspension (OD₆₅₀ 1) and motility was evaluated by measuring the diameter of the halo of growth formed by the mycobacteria. (ii) Cellular aggregation was quantified by cultivating mycobacteria in TS broth without Tween 80. The unicellular mycobacteria were separated from the aggregates by differential centrifugation (Couguoule et al., 2002) and the cellular aggregation was calculated as the percentage of aggregate-containing pellets versus total cell weight. (iii) A Congo red accumulation assay (Cangelosi et al., 1999) was adapted as follows. Mycobacteria were cultivated in TS broth plus 100 µg Congo red ml⁻¹ and 0-05 % Tween 80. The cells were then washed extensively with distilled water and the Congo red that remained associated with the cells was extracted with acetone. The Congo red binding index was defined as the percentage reduction in the OD₆₅₀ of the aqueous phase after complete separation of the two phases. (v) The bacterial cell surface charge (Bayer & Sloyer, 1990) was determined by measuring the zeta-potential (ζ) of a single-cell suspension (OD₆₅₀ 1) in a zetameter Zetasizer 3000 (Malvern Instruments).

**Fractionation and analysis of the extracellular, surface-exposed and cell-bound components.** Surface-exposed material (SXM) and extracellular compounds were extracted and analysed as previously described (Lemassu et al., 1996; Ortalo-Magné et al., 1996). Briefly, surface pellicles of mycobacteria grown on Sauton’s medium were treated with glass beads (Ortalo-Magné et al., 1995) and suspended in distilled water; bacilli were removed by filtration on a 0-2 µm Nalgene filter. Similarly, the culture broth, containing the extracellular materials, was filter-sterilized. Portions of the crude filtrates were separately concentrated under vacuum, extensively dialysed against distilled water and analysed for their carbohydrate and protein contents by colorimetric assays (Dische, 1962, and the Lowry method, respectively). Their glycosyl composition was also determined by acid hydrolysis followed by analysis of the trimethylsilylated sugar derivatives by gas chromatography using erythritol as internal standard (Lemassu et al., 1996). Alternatively, the various enzyme activities detectable in the SXMs or in the culture broth were assayed as previously described (Raynaud et al., 1998).

Chloroform and methanol were added to the remaining portions of the crude filtrates to obtain partition mixtures composed of chloroform/methanol/water (3 : 4 : 3, by vol.); the organic phases were washed with water and evaporated to dryness to yield crude lipid extracts. The mycolate-containing lipids were separated from the other types of lipids by precipitation with methanol (Villeneuve et al., 2003). Both kinds of lipids were further fractionated on a Florisil column irrigated with chloroform and then with a stepwise gradient of increasing concentrations of methanol and water in chloroform (Ortalo-Magné et al., 1996). Finally, the various classes of lipids were identified by TLC analysis as previously described (Etienne et al., 2002) and by matrix-assisted laser-desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (see below). The N-acylphosphatidyl moiety of GPLs was produced as methyl ester from native GPLs by methanalysis with anhydrous 1 M CH₃OH/HCl for 16 h at 80 °C; a portion of the products was further perdeutero-acetylated in anhydrous 1 : 1 (CD₃CO)₂O/pyridine (100 °C, 1 h). The resulting products were analysed by MALDI-TOF mass spectrometry.

The cell-bound fatty acids were released by saponification of the delipidated cells for 16 h at 80 °C in 5 % (w/v) KOH in methanol/benzene (8 : 2, v/v), extracted with diethyl ether and methylated. They were further analysed by TLC (Jackson et al., 1999) or MALDI-TOF mass spectrometry as previously described. Quantification of the mycolic acids resulted from the saponification of at least 300 mg (dry weight) of delipidated cells in triplicate.

**Spectrometric methods.** MALDI-TOF mass spectrometry analysis of lipids was performed as previously described (Laval et al., 2001). Sample solutions (1 mM) were directly applied onto the sample plate as a 1 µl droplet, followed by the addition of 0-5 µl of the matrix solution [10 mg 2,5-dihydroxybenzoic acid ml⁻¹ in chloroform/methanol (1 : 1, v/v)]. After crystallization of the samples, MALDI-TOF spectra were acquired on a Voyager-DE STR mass spectrometer (PerSeptive Biosystems) equipped with a pulsed nitrogen laser emitting at 337 nm and were analysed in the reflectron mode using an extraction delay time set at 100 ns and an accelerating voltage operating in positive-ion mode of 20 kV. An external mass spectrum calibration was performed using calibration mixture 1 of the Sequazyme Peptide Mass Standards Kit (PerSeptive Biosystems), including known peptide standards in a mass range from 900 to 1600 Da.

**Labelling of lipids.** The various classes of extractable and cell surface lipids were quantified by labelling: 1-14C acetate (Amersham) was added to 100 ml (Sauton broth) of 2-day-old cultures containing mid-exponential-phase bacteria. After 16 h incubation, the reaction was stopped by centrifugation and the SXMs were isolated by extraction with glass beads (Ortalo-Magné et al., 1995). Lipids from these latter materials and those from bead-treated cells were extracted with chloroform/methanol (1 : 2, v/v). Both types of lipid extracts were analysed by TLC using the solvent chloroform/methanol/water (3 : 4 : 3, by vol.).
mixtures described above; the radioactivity was located and measured on plates using an automatic TLC linear analyser (Berthold LB 2832). Then, the lipid spots were visualized by spraying with the appropriate reagents, with charring when necessary.

**Permeability assays.** The permeability of the strains of *M. smegmatis* to chenodeoxycholate was assessed as previously described (Bardou et al., 1998; Jackson et al., 1999). Exponentially growing cells were first labelled for 16 h with [5,6-3H]ursolic (2 x 10^{-5} M, 1-85 TBq mol^{-1}; DuPont NEN) to quantify the biomass and aliquots of labelled cells were used to measure radioactivity, then dried and weighed to correlate 3H-labelling with cell dry weight. Accumulation assays were performed under continuous agitation. [14C]Chenodeoxycholate (2 x 10^{-5} M, 1-8 GBq mmol^{-1}; DuPont NEN) was added to 1 ml 10 mM HEPES pH 7-2 buffer containing about 40 mg 3H-labelled cells. Aliquots (0-1 ml) were removed at different time intervals and added at the top of an Eppendorf centrifuge tube containing 0-25 ml silicone oil/paraffin oil (1:0-2, v/v). Cells were separated from the accumulation medium by centrifugation (13 000 g, 1 min). Centrifuged tubes were frozen on dry ice and the pellets were dropped into counting flasks by cutting the cone top. Scintillation solution (Aqualuma) was added and the vials were counted on a liquid scintillation counter.

**Transmission electron microscopy.** The method for preparing samples for transmission electron microscopy (Etienne et al., 2002) was based on procedures of Daffe et al. (1989) and Paul & Beveridge (1992). Briefly, early-exponential-phase bacteria (TS medium) were fixed in 2-5% (v/v) glutaraldehyde, 0-05% (w/v) ruthenium red in cacodylate buffer for 2 h in the dark at room temperature. Cells were washed three times, postfixed for 2 h in the dark in 1% (w/v) osmium tetroxide, 0-05% (w/v) ruthenium red and then washed twice each in cacodylate buffer and then water. Cells were dehydrated by exposure to increasing ethanol concentrations for 5 min each, washed twice in 100% ethanol and then twice in propylene oxide. Cells were suspended in 1:1 propylene oxide/Spurr resin for 2 h. After infiltration overnight, samples were transferred to 100% Spurr resin and left overnight. Resin was replenished the next morning and samples were left to cure at 60 °C overnight. Blocks were thin-sectioned on a Reichert–Jung microtome and mounted on copper grids. Sections were post-stained with uranyl acetate and Reynolds’s lead citrate. Microscopy was performed on a JEOL 120 EX electron microscope.

**RESULTS**

**Cell surface properties of the *M. smegmatis* strains**

*M. smegmatis* mc^2^155 is a transformable strain that was isolated from the non-transformable ATCC 607 in a three-step procedure (Snapper et al., 1990; Jacobs, 2000). The two strains also differ in the aspect of their colonies, i.e. rough for ATCC 607 (Jacobs, 2000) and smooth for mc^2^155 (data not shown). Distinguishable macroscopic features could also be observed in liquid cultures whatever the broth used, provided that it did not contain Tween 80: under agitation, ATCC 607 cells were found to aggregate fivefold more than did mc^2^155 (Table 1). These phenotypic changes could not be attributed to a difference in the growth rate of the strains, since they exhibited similar generation times (3-5-4 h in TS medium). The diazo dye Congo red, previously known to provide information on cell-surface hydrophobicity in mycobacteria (Cangelosi et al., 1999; Etienne et al., 2002), was used in the comparison of the two strains. Consistent with the more aggregative character of strain ATCC 607, cells from this strain bound significantly more dye than those of mc^2^155 (Table 1). When the surface motility of these strains on agar plates was compared, cells from the ATCC 607 strain were found to display a spreading halo consistently smaller than those from the mc^2^155 strain (Table 1). Finally, the cell surface of the ATCC 607 strain appeared to display a significantly more negative net charge than that of mc^2^155 (Table 1). In contrast, no significant difference was found between the strains when the cell-surface hydrophobicity was assessed by the hexadecane partition procedure (Rosenberg et al., 1980).

**Ultrastuctural features of the *M. smegmatis* strains**

Cells from the ATCC 607 and mc^2^155 strains of *M. smegmatis* grown in Sauton’s medium were examined by electron microscopy (Daffe et al., 1989; Etienne et al., 2002). Ultrathin sections were stained with ruthenium red, a stain that strongly reacts with the surface of mycobacteria (Etienne et al., 2002; Rastogi et al., 1984). Examination of thin sections revealed for both strains a cell envelope structure (Fig. 1a, b), composed of (i) a plasma membrane, (ii) a hypothetical ‘periplasmic’ space (Daffe & Draper, 1998), (iii) a thick internal electron-dense layer, (iv) a thin electron-transparent layer, difficult to distinguish in these sections from the electron-dense layer, and (v) an electron-dense outer layer. This ultrastructural appearance is similar to that previously found in other mycobacterial species (Daffe & Draper, 1998; Draper, 1982; Paul & Beveridge, 1992; Rastogi et al., 1986) and particularly in *M. smegmatis* mc^2^155, where the staining of the electron-dense outer layer with ruthenium red has been attributed to GPLs (Etienne et al., 1986).

**Table 1. Surface properties of the wild-type (ATCC 607) and the transformable (mc^2^155) strains of *M. smegmatis***

<table>
<thead>
<tr>
<th>Surface property</th>
<th>ATCC 607</th>
<th>mc^2^155</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility*</td>
<td>11·7 ± 3·1</td>
<td>29·1 ± 18·7</td>
</tr>
<tr>
<td>Cellular aggregation†</td>
<td>96·4 ± 0·7</td>
<td>20·1 ± 3·1</td>
</tr>
<tr>
<td>Congo red binding§</td>
<td>310 ± 12</td>
<td>211 ± 81</td>
</tr>
<tr>
<td>Hydrophobicity index§</td>
<td>38·0 ± 16·5</td>
<td>34·9 ± 19·7</td>
</tr>
<tr>
<td>Zeta potential†Il</td>
<td>-34·4 ± 4·6</td>
<td>-27·6 ± 1·2</td>
</tr>
</tbody>
</table>

* Diameter (mm) of the halo formed.
† Percentage of aggregated cells.
§ Arbitrary units expressed as the percentage of cells recovered in the organic phase.
||ln mV.
¶ P<0·05 (a) or P<0·01 (b) compared with the control, calculated with paired Student’s t test.
et al., 2002). The fact that the two strains displayed no marked difference in the electron density of their outer layer (Fig. 1a, b) was in accordance with the similar amounts of GPLs found in both strains (see below). We have previously reported that the mc²155 strain grown in complex media, such as LB, exposed large amounts of GPLs at the cell surface, leading to the formation of filamentous and rope-like structures stained by the dye (Fig. 4D in Etienne et al., 2002). This was also the case with TS broth, another complex medium suitable for cultivating fast-growing mycobacteria (Fig 1e). The ATCC 607 strain never exposed these structures, whatever the medium used for growth (Fig. 1a, c, d).

Quantitative analysis of the composition of the envelope and the SXM

The mycobacterial cell envelope (Minnikin, 1982; Brennan & Nikaido, 1995; Liu et al., 1995) is particularly rich in genus-specific lipids. Many of these lipids (glycolipids and phospholipids) have been detected in the surface-exposed material (SXM) of various strains of mycobacteria (Ortalo-Magné et al., 1996). In a first approach to tentatively explain the marked differences in the cell surface properties of strains ATCC 607 and mc²155, the nature and relative amounts of the components of their SXM and of their envelope were analysed. Gentle shaking of the bacterial cells with glass beads, a technique that disperses aggregates by removing the amorphous material covering the cells (Ortalo-Magné et al., 1995, 1996), was used to extract the SXM. For strains ATCC 607 and mc²155, the amounts of SXM (3·8 ± 0·8 and 3·3 ± 0·4 % of the cell dry weight, respectively; means ± SD), and the lipid (2·6 ± 0·4 and 2·2 ± 1·5 % of the SXM, respectively), carbohydrate (13·1 ± 3·4 and 15·3 ± 3·2 %, respectively) and protein (84·3 ± 32·9 and 82·5 ± 32·4 %, respectively) contents of the SXMs, were found to be comparable. The sugar composition of the SXMs from both origins was similar and consisted of arabinose, mannose, xylose and glucose (Lemassu et al., 1996). The amounts of lipids extracted from the whole cells (15·27 ± 1·10 and 18·13 ± 5·57 % of the cell dry weight for ATCC 607 and mc²155, respectively), as well as those of cell-bound fatty acids, extracted after saponification of the whole delipidated cells (ATCC 607, 6·23 ± 0·64; mc²155, 5·57 ± 0·50 % of the cell dry weight), were similar.

Surface lipids represent a very minor fraction (usually less than 5 % of the dry weight) of mycobacteria grown on Sauton’s medium (Lemassu et al., 1996; Ortalo-Magné et al., 1996). Therefore the lipids of exponential-phase cultures from the two strains were metabolically labelled with sodium [¹⁴C]acetate before extracting the SXMs and the bead-treated cells to make it easier to quantify and compare the surface-exposed and the cell lipids. Triacylglycerols (TAGs) were the predominant class of lipids, representing 25·6 % and 57·2 % of the total [¹⁴C]acetate incorporated in the bead-treated bacterial and the surface-exposed lipids, respectively, of the wild-type strain of M. smegmatis grown on the glycerol-rich Sauton’s medium (Fig. 1a, b). The other major lipids identified in the ATCC 607 strain were glycopeptidolipids (GPLs), trehalose monomycolates (TMMs) and phospholipids; the trehalose dimycolates (TDMs) were not detected in the surface-exposed lipids, as previously observed (Ortalo-Magné et al., 1996; Etienne et al., 2002). The major classes of lipids found in the wild-type strain were also predominant in the mc²155 mutant strain, but their relative distribution between the surface and the deeper layers of the envelope was dramatically different from that found in the parent strain (Fig. 2). While the bacterial surface of the wild-type strain was 2·2 times enriched in TAGs, compared to the deeper layers of the envelope, TMMs

Fig. 1. Transmission electron micrographs of M. smegmatis strains ATCC 607 (a, c, d) and mc²155 (b, e). Strains were grown either in Sauton’s medium (a, b) or in LB (c) or TS (d, e) broths.
and phospholipids were respectively 14·6 and 1·6 times more abundant in the bead-treated cells compared to the outermost layer. In contrast, the mc²155 cellular envelope seemed to have lost this differentiation: the TAGs and phospholipids displayed comparable amounts in both compartments (Fig. 2). The difference in the TMMs between the outermost and the deeper layers of the envelope was also reduced to 1·8 times. These observations may account for the difference in negative net charge observed between the surfaces of the two strains (Table 1), although this can not be precisely correlated to a distinctive lipid species.

**Isolation and identification of the bacterial lipids**

Subtle modifications of the structure of the cell-surface components could result in a dramatic phenotypic change for the mutant strains. For instance, the absence of GPL acetylation in *M. smegmatis* resulted in the loss of sliding motility (Recht & Kolter, 2001). To carry out a more precise comparison at the molecular level, lipids of both strains were extracted from whole cells and fractionated by adsorption chromatography on a Florisil column. Lipids were eluted with chloroform, increasing concentrations of methanol in chloroform and finally with a mixture of chloroform, methanol and water. The mycolate-containing lipids were further separated from the other lipids by chloroform precipitation. The ATCC 607 and mc²155 strains were extracted from whole cells and fractionated by methanol precipitation. The ATCC 607 and mc²155 strains exhibited the same values in both strains (Table 2), suggesting identical structures. TDMs and TMMs were not detected by MALDI-TOF mass spectrometry analysis. GPLs and LOSs are discussed below.

Cell-bound mycolates were prepared by saponification of the delipidated cells, extraction with diethyl ether and methylation. TLC analysis showed identical mycolate profiles between the materials derived from the ATCC 607 and mc²155 strains (data not shown). *M. smegmatis* contains χ-, χ'– and epoxymycolic acids (Daffe et al., 1983a; Minnikin et al., 1984), which were separated by preparative TLC. Purified methyl mycolates were analysed by MALDI-TOF mass spectrometry; in both strains the major mycolates displayed pseudomolecular masses identical to that previously published for *M. smegmatis* (Laval et al., 2001). The prominent sodium adduct of methyl mycolates corresponded to C77–80 χ–, C60–64 χ’– and C77–80 epoxymycolic acids (data not shown). As a consequence of the fact that the ATCC 607 and mc²155 strains exhibited the same species of cell-bound mycolates it could be concluded that the TDM and TMM mycoloyl-containing glycolipids were identical in both species.

**Table 2.** Pseudomolecular ion [M+Na]+ mass values determined by MALDI-TOF mass spectrometry and proposed acyl substituents of the major lipids of the *M. smegmatis* strains

<table>
<thead>
<tr>
<th>Lipid</th>
<th>ATCC 607 Mass (a.m.u.)</th>
<th>ATCC 607 Fatty acid</th>
<th>mc²155 Mass (a.m.u.)</th>
<th>mc²155 Fatty acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAGs</td>
<td>869</td>
<td>C₁₆/C₁₆ : 1/TBS</td>
<td>869</td>
<td>C₁₆/C₁₆ : 1/TBS</td>
</tr>
<tr>
<td>GPLs</td>
<td>1283</td>
<td>C₃₀,₁₁(OH)</td>
<td>1257</td>
<td>C₂₉(OH)</td>
</tr>
<tr>
<td>Iia</td>
<td>1199</td>
<td>C₃₀,₁₁(OH)</td>
<td>1159</td>
<td>C₂₉(OH)</td>
</tr>
<tr>
<td>Iib</td>
<td>1443</td>
<td>C₃₀,₁₁(OH)</td>
<td>1319</td>
<td>C₂₉(OH)</td>
</tr>
<tr>
<td>IIa</td>
<td>1383</td>
<td>C₃₀,₁₁(OH)</td>
<td>1343</td>
<td>C₂₉(OH)</td>
</tr>
<tr>
<td>Iib</td>
<td>1543</td>
<td>C₃₀,₁₁(OH)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>LOS-A</td>
<td>1689</td>
<td>C₆/C₁₄/C₂₂:1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>LOS-B1</td>
<td>1429</td>
<td>C₆/C₁₄/C₂₂:1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>PE</td>
<td>756</td>
<td>C₁₆/TBS</td>
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<td>PG</td>
<td>809</td>
<td>C₁₆/TBS</td>
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<td>C₁₆/TBS</td>
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<tr>
<td>PI</td>
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<tr>
<td>PIMs</td>
<td>1459</td>
<td>C₁₆/C₁₆/TBS</td>
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<td>C₁₆/C₁₆/TBS</td>
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<tr>
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<tr>
<td>PIMs</td>
<td>2107</td>
<td>C₁₆/C₁₆/TBS</td>
<td>2107</td>
<td>C₁₆/C₁₆/TBS</td>
</tr>
</tbody>
</table>

ND, Not detected.

* Diacetylated form.

†TBS, tuberculostearic acid.
The glycopeptidolipids (GPLs)

GPLs have been previously largely implicated in biological and cell-surface properties of \textit{M. smegmatis}, e.g. receptors for mycobacteriophages (Furuchi & Tokunaga, 1972; Goren et al., 1972), colony morphology (Etienne et al., 2002; Billman-Jacobe et al., 1999) or binding to human monocyte-derived macrophages (Villeneuve et al., 2003). They are also involved in the sliding motility of mc\textsuperscript{2}155, as shown by the non-motility of GPL-deficient mutants (Martinez et al., 1999; Etienne et al., 2002). Therefore, we addressed the question of a possible difference in the GPL content of strains ATCC 607 and mc\textsuperscript{2}155. GPLs are among the characteristic lipids of mycobacteria (Daffe\`e & Lemassu 2000) and share a common lipopeptidyl core consisting of a mixture of 3-hydroxy and 3-methoxy C\textsubscript{26–34} fatty acids amidated by the tripeptide D-Phe-D-\textit{allo}-Thr-D-Ala and terminated by L-alaninol (Daffe\`e et al., 1983b). The diversity of GPLs has been recently extended in \textit{M. smegmatis} (Ojha et al., 2002; Villeneuve et al., 2003). The major and first-described GPL of \textit{M. smegmatis} (GPL I) is composed of, in addition to the core, a di-O-acetyl-6-deoxytalosyl residue linked to \textit{allo}-threoninyl and a tri-O-methylrhamnosyl unit attached to the alaninol residue (Brennan, 1988). Other components of this family include the deacetylated GPLs I (or GPLs IIa) and the hyperglycosylated GPLs I (or GPLs IIb), which possess a second di-O-methylrhamnosyl unit linked to alaninol (Ojha et al., 2002; Villeneuve et al., 2003). The succinylated GPLs (or GPLs IIIa and IIIb, with one and two rhamnosyl units, respectively) carry a succinyl substituent on the terminal di-O-methylrhamnosyl unit linked to the alaninol end (Villeneuve et al., 2003). Crude cellular GPLs were prepared by methanol precipitation of the total cellular lipids and analysed by TLC (Fig. 3a). Polar GPLs were more abundant in strain ATCC 607 than in strain mc\textsuperscript{2}155; it was not possible, however, to determine with certainty by this method whether GPLs II and GPLs III coexist in the mixtures, as the two types of GPLs co-migrated in TLC (Villeneuve et al., 2003). The MALDI-TOF mass spectra of the crude GPLs of strains ATCC 607 and mc\textsuperscript{2}155 are depicted Fig. 3(b). GPLs I were the prominent compounds, with the major pseudomolecular masses \((M+Na)\) at \(m/z\) 1283\textsuperscript{1} and 1257\textsuperscript{1} for strains ATCC 607 and mc\textsuperscript{2}155, respectively. GPLs IIb \((m/z\) at 1429\textsuperscript{1}, 1443\textsuperscript{1}), IIIa \((m/z\) at 1369\textsuperscript{0}, 1383\textsuperscript{0}) and IIIb \((m/z\) at 1543\textsuperscript{0}) were readily detectable in the mass spectrum of strain ATCC 607. In contrast, pseudomolecular masses corresponding to GPLs IIb and GPLs IIIb were not detected in the mass spectrum of strain mc\textsuperscript{2}155; in addition, the relative intensity of the peak corresponding to GPLs IIIa \((m/z\) at 1343\textsuperscript{3}) appeared to be considerably diminished in the mass spectrum of this strain. When the ratio of the peaks corresponding to GPLs II/I or III/I were estimated from the MALDI-TOF spectra (Fig. 3b) and compared, it appeared that while GPLs

![Fig. 3. TLC analysis (a) and MALDI-TOF mass spectra (b) of the crude GPL fractions of \textit{M. smegmatis} strains ATCC 607 and mc\textsuperscript{2}155. TLC was performed using chloroform/methanol 90:10 (v/v) as the solvent system and anthrone as a spray, followed by heating. Lane 1, ATCC 607; lane 2, mc\textsuperscript{2}155.](image-url)
Ill represented 51% of the GPLs I in strain ATCC 607, they accounted for only 13% in strain mc²155. The percentages of GPLs IIa, IIb and IIIb in strain ATCC 607 were, respectively, 3, 5, 14 and 10%, whereas they were below 1% in strain mc²155. Thus, the MALDI-TOF mass spectrometry data correlated well with the results of TLC and showed that both GPLs II and III were present in very small amounts in strain mc²155.

In addition to the variations in the relative amounts of the subfamilies of GPLs displayed by strains ATCC 607 and mc²155, the MALDI-TOF mass spectrometry analysis also revealed some differences in terms of pseudomolecular masses of the prominent GPLs of the two strains (Fig. 3). Accordingly, GPL subfamilies were separated by adsorption chromatography using a Florisil column (Villeneuve et al., 2003) and analysed by mass spectrometry. The pseudomolecular masses of the major GPLs from strain ATCC 607 (Table 2) were consistent with previously described structures (Daffe et al., 1983b; Villeneuve et al., 2003). The m/z value of the major [M+Na]⁺ peaks of GPLs from mc²155 were 26–40 atomic mass units (a.m.u.) lower for GPLs I, IIa and IIIa than those of the corresponding GPLs from strain ATCC 607 (Table 2). For GPLs IIb, the m/z value was 124 a.m.u. lower in mc²155. Again, GPLs IIb were not detectable in strain mc²155. Saponification of the GPLs IIb from strain mc²155 had no impact on the mass values of the compounds, indicating the absence of the O-acyl substitution of the deoxytalosyl unit of GPLs IIb. Thus, 84 of the 124 a.m.u. which differentiate the GPLs IIb from the two strains could be attributable to the absence of the two acyl groups of the 6-deoxytalosyl moiety in mc²155. This observation implied again a 40 a.m.u. downshift for the lipopeptidic core of GPLs IIb from strain mc²155 compared to its ATCC 607 counterpart. The [M+Na]⁺ of the alkali-treated GPLs IIb of strain ATCC 607 decreased from 1429/1443 to 1359/1373 a.m.u., that is 26 to 40 a.m.u. higher than their mc²155 deacetylated counterparts (results not shown). Thus, the 26 to 40 a.m.u. downshift of the GPLs from strain mc²155 may reflect a difference in the structure of the lipopeptidyl core. To test this hypothesis GPLs I were perdeuterated with anhydrous 1:5 M CH₂OH/HCl to obtain the N-acylphenylalanyl methyl ester derivative of the fatty acid (Villeneuve et al., 2003). MALDI-TOF mass spectrometry analysis revealed a major [M+Na]⁺ peak at m/z 650 for strain ATCC 607; a minor [M+Na]⁺ peak was also observed at 822. These corresponded to the lipotripeptidyl moiety consisting of C₃₀:₁(OH) fatty acyl linked to D-Phe-D-alloThr-D-Ala of the GPL core, in agreement with previous reports (Daffe et al., 1983b; Villeneuve et al., 2003). The [M+Na]⁺ peaks for the methanolysis products of GPLs from strain mc²155 were observed at m/z 624 and 638, in a 10:1 ratio. Consequently, the observed downshift of 26 a.m.u., compared to the GPLs from strain ATCC 607, corresponded to a structural variation in the fatty acid chain. Perdeuterocacetylation of the methanalysis products of GPLs from strain mc²155 resulted in the upshift of peaks that were seen at 669 and 638 (10:1 ratio), indicating an increase of 45 Da (from 624 to 669 a.m.u.) for the [M+Na]⁺ peak of the major N-acylphenylalanyl moiety whereas the minor peak remained at 638 Da. Since the only possibility of acetylation in this molecule is the hydroxyl group of the fatty acid chain, the observed upshift of the m/z value upon acetylation indicated that this hydroxyl was free in the major mc²155 GPL I and methoxylated in the minor species (at m/z 638). It was thus concluded that strain mc²155 mainly synthesizes GPLs I possessing a fatty acyl chain of C₂₈(OH) with minor amounts of C₂₈(OCH₃)ᵣ containing GPLs whereas strain ATCC 607 mainly produces GPLs with a fatty acyl chain of C₃₀:₁(OH).

The lipooligosaccharides (LOSs)

LOSs are alkali-labile trehalose-containing lipids (Daffe & Lemassu, 2000) which have been characterized in some mycobacterial species, including M. smegmatis (Saada & Ballou, 1983; Kamisango et al., 1985). They were hardly detectable in the total lipid extracts by either TLC or MALDI-TOF mass spectrometry analyses. They were eluted from the Florisil column in the same fractions as the GPLs II subfamily, i.e. chloroform/methanol 80:20 (v/v), but co-migrated on TLC with these latter compounds (Fig. 4, lane 1). MALDI-TOF mass spectrometry analysis of these fractions revealed, besides the presence of GPLs II in both strains, the characteristic pseudomolecular ions

Fig. 4. TLC analysis of GPL- and LOS-enriched fractions of M. smegmatis strains ATCC 607 (lanes 1 and 2) and mc²155 (lanes 3 and 4) before (lanes 1 and 3) or after (lanes 2 and 4) an alkali treatment. The analysis was performed using chloroform/methanol 90:10 (v/v) as the solvent system and anthrone as a spray, followed by heating. The arrow indicates the position of LOS.
The ATCC 607 strain analysed above has been maintained in our laboratory for 3 decades. To check for a possible variation in its properties, we compared its characteristics with those of a culture of M. smegmatis ATCC 607 inoculated directly from cells newly received from the ATCC. All the distinctive features described above (Table I), cultural properties, particularly the orange rough colonial morphology that is the morphotype of ATCC 607 (Jacobs, 2000), as well as the occurrence of LOSs and the GPL pattern, were identical in the two ATCC 607 strains.

**Analysis of the extracellular materials**

GPLs are major components of the outermost layer of M. smegmatis (Etienne et al., 2002). One possible consequence of the absence of GPLs II and III from the mutant cells may be the release of surface-exposed substances into the culture medium. Moreover, the apparent disorganization of the cell envelope in strain mc²155 (Etienne et al., 2002) may also lead to the release of some of its components. To test this hypothesis, the composition of the extracellular material of the mutant was compared with that of the parent strain. The amounts of materials released by the two strains in the culture broth were similar: 3.1 ± 0.4 and 2.9 ± 0.4 mg per 100 mg dry cells for strains ATCC 607 and mc²155, respectively (means ±SD). The composition of the culture-broth substances was also comparable in the two strains. Extracellular material from strain ATCC 607 consisted of 6.7 ± 2.8% lipid, 53.5 ± 15.5% protein and 39.8 ± 29.7% carbohydrate, whereas these values were 7.9 ± 4.0%, 52.0 ± 4.1% and 40.1 ± 31.4%, respectively, in strain mc²155. In addition, both strains exhibited the same sugar composition (data not shown), which was similar to that previously found for M. smegmatis (Lemassu et al., 1996). Another approach used to detect the possible release of surface-exposed substances was to examine extracellular enzyme activities in the culture broth of strain mc²155. We have previously detected 14 extracellular enzyme activities in M. smegmatis ATCC 607; all of them were also surface-exposed, along with 11 additional activities which were more or less deeply located in the cell envelope of the bacteria (Raynaud et al., 1998). Examination of the enzyme activities present in the culture broth or surface-exposed in strain mc²155 revealed no difference between mc²155 and the ATCC 607 strains (data not shown). Thus, the absence of GPLs II and III from the mutant strain and the apparent disorganization of its cell envelope does not induce the release of surface components into the culture fluid.

**Cell wall permeability of the M. smegmatis strains**

GPLs are major lipids of M. smegmatis and have been shown to significantly contribute to the permeability barrier of the cell envelope of the bacteria (Etienne et al., 2002). Considering the low level of GPLs II and III in strain mc²155, it was interesting to address the question of the importance of the GPL types in the permeability of the mycobacterial outer barrier. To determine whether it was altered in strain mc²155, the uptake of the hydrophobic chenoxycholate by cells of the wild-type strain ATCC 607 and the mutant mc²155 was compared. Chenoxycholate is a negatively charged hydrophobic molecule that has been previously used to evaluate the fluidity of mycobacterial cell wall lipids (Dubnau et al., 2000; Etienne et al., 2002;
Strain mc2155 showed an initial rate of uptake and a final accumulation of chenodeoxycholate significantly higher than those of the parent strain (Fig. 6).

DISCUSSION

We have addressed the question of possible structural and molecular variations between the cell envelopes of the M. smegmatis transformable mutant strain mc<sup>2</sup>155 and its parent ATCC 607, which could explain at least in part the distinctive phenotype of the mutant. Strain mc<sup>2</sup>155 was selected on the basis of an efficient-plasmid-transformation (ept) phenotype (Snapper et al., 1990) in order to overcome the low transformation efficiencies of the M. smegmatis wild-type strains. The major macroscopic feature that differentiates the two strains is the characteristic rough aspect of the ATCC 607 cultures, both as colonies on agar plates and as pellicles formed on static liquid media, compared to the smooth phenotype of strain mc<sup>2</sup>155 (Jacobs, 2000). The occurrence of rough and smooth morphotypic variations is rather common in mycobacterial species. Particularly well described in the M. avium complex (Barrow & Brennan, 1982), the appearance of natural rough variants in these species has been largely correlated with the absence of the C-mycoside GPLs (Belisle et al., 1993a, b). Isolation of M. smegmatis strains impaired in the biosynthesis of GPLs has confirmed these observations. The absence of C-type GPLs in the non-ribosomal protein synthetase mps (Billman-Jacobe et al., 1999; Recht et al., 2000) or the putative membrane protein tmptC (Recht et al., 2000) mutants has resulted in rough morphotypes. In this regard, the results reported in this paper appeared quite puzzling since the ATCC 607 strain displays a rough colony morphology while harbouring GPLs at its surface in quantities similar to those of the mc<sup>2</sup>155 strain. However, compared to ATCC 607, the mc<sup>2</sup>155 strain lacks polar glycolipids, either completely in the case of the LOSs or partially for the types II and III of GPLs, amounts of which were drastically reduced in the mutant. Such a case is not without precendents. Although many rough variants of M. avium are completely devoid of C-mycosides, others have been described which still produce the lipopeptidic core of GPLs (Belisle et al., 1993a). In a way more closely related to our data, Recht & Kolter (2001) described an atf1 strain of M. smegmatis which was deficient in the enzyme involved in the transfer of acetyl groups to the glycopeptidic core. This strain displayed a rough colony morphology and produced only deacetylated GPLs (Recht & Kolter, 2001), which correspond to the polar GPLs IIa. This pattern is reminiscent of the ATCC 607 strain, where polar GPLs II and III represent together more than 70 % of the apolar GPLs I. The combined data of Recht & Kolter (2001) and those presented here suggest that the nature of the surface-exposed GPLs could be as important as their absence for the development of a rough morphotype. Our data also showed that the nature of the substituents carried by the GPL molecules plays a fundamental role in other cell-surface properties, i.e. a reduced sliding motility, an enhanced Congo red binding and marked cellular aggregation. These distinctive properties are shared by the rough strains of M. smegmatis strains ATCC 607 (the present study), TM99 (Etienne et al., 2002) and atf1 (Recht & Kolter, 2001) but not by the smooth mc<sup>2</sup>155. A strong correlation has been previously found between the lack of GPLs and the inability to spread on a surface (Martinez et al., 1999). The major apolar GPLs of strain mc<sup>2</sup>155, GPLs I, presumably because they have a more pronounced amphipathic character than the deacetylated (type IIa), hyperglycosylated (type IIb) or succinylated (type III) GPLs harboured by strain ATCC 607, would minimize interactions at the cell surface and, consequently, allow the bacteria to spread on surfaces. Lipids commonly represent only a minor fraction of the outermost materials of the mycobacterial cell (Lemassu et al., 1996), of which GPLs represent 15–20 % in Sauton medium (this paper). In complex media they composed up to 85 % of the surface-exposed lipids (Etienne et al., 2002). In addition, it has been demonstrated that they are the substances that react with ruthenium red to give the electron-dense appearance of the outermost cell-envelope layer (Etienne et al., 2002). Thus, they appeared to cover all the mycobacterial envelope, in agreement with their putative role in dictating some of the surface properties of the mycobacteria. Other molecules that are not surface-exposed in strain mc<sup>2</sup>155 were the LOSs, a class of glycolipids which was found typifying the smooth Canetti strain but was not detected in typical rough strains of M. tuberculosis H37Rv and H37Rv (Daffe et al., 1991). A further and more comprehensive study of the Canetti strain, its spontaneous rough variant and 16 additional strains of the M. tuberculosis complex has shown that the presence of LOSs was not related to the colony morphotypes (Lemassu et al., 1992). Our observations clearly reinforce this latter conclusion, as the smooth
mc²155 strain lacks LOSs, in contrast to the rough ATCC 607.

One of the main differences revealed in the cell envelope composition of the two strains of *M. smegmatis* examined here is a marked dissimilarity in strain ATCC 607 of the lipid distribution between the outermost layer and the deeper compartments. One additional difference between the two strains is the formation of filamentous and rope-like structures at the surface of the mc²155 cells in complex media, when large amounts of GPLs are present on the cell surface. These structures were absent from a GPL-deficient mutant cultivated in the same conditions (Etienne *et al.*, 2002), and therefore can be attributed to GPLs budding from the surface. It seems that, in strain mc²155, a particular stratified organization of the envelope has been lost; the reason for such a loss deserves consideration, as the precise nature of this stratification remains elusive. It is worth noting, however, that the disturbance of the outermost layer was not the reflection of a marked disorganization of the envelope. Indeed, neither specific release of surface constituents into the culture broth nor the exposure at the cell surface of a compound located in deeper compartments of the mycobacterial cell envelope such as TDM (Ortalo-Magné *et al.*, 1996) were observed in the mutant.

We investigated the consequence of these differences in envelope composition on cell wall properties, especially their impact on the outer permeability barrier. In all currently proposed models, the outer permeability barrier of mycobacteria consists of a monolayer of mycoloyl residues covalently linked to the cell wall arabinogalactan and includes other lipids which are probably arranged to form a bilayer with the mycoloyl residues (Brennan & Nikaido, 1995; Daffe & Draper, 1998; Minnikin, 1982; Rastogi, 1991). Although cell-wall-linked mycolates certainly contribute to this barrier (Jackson *et al.*, 1999), the involvement of non-covalently bound lipids in the wall bilayer has been demonstrated to date for only phthiocerol dimycocerosates of *M. tuberculosis* MT103 (Camacho *et al.*, 2001) and GPLs of *M. smegmatis* mc²155 (Etienne *et al.*, 2002). The present work demonstrates that the events that have led to *M. smegmatis* mc²155 have a profound effect on the uptake of chenodeoxycholate, a hydrophobic molecule that diffuses through lipid domains of the mycobacterial cell wall (Dubnau *et al.*, 2000; Etienne *et al.*, 2002; Jackson *et al.*, 1999; Liu *et al.*, 1996; Yuan *et al.*, 1997). Considering the implication of lipids in the establishment of the outer permeability barrier of mycobacteria and knowing that the two strains examined elaborated comparable amounts of GPLs, the increased permeability of the cell envelope of strain mc²155 may originate either from the absence of LOSs or from a change in the chemical structure of the GPLs expressed by this strain. Although LOSs are hardly quantifiable by mass spectrometry or TLC analysis, they appeared not to be as major a component as GPLs or phospholipids in the cell envelope of strain ATCC 607. It follows that variations in the proportions of GPL subfamilies are likely to be the origin of the alterations of the envelope properties of strain mc²155. The fact that subtle modifications of some cell envelope constituents may lead to a dramatic phenotypic change is not without precedent. Indeed, it has been previously reported that the chemical structures of mycolic acids play a role in determining the fluidity and permeability of the mycobacterial cell wall (George *et al.*, 1995; Liu *et al.*, 1996; Dubnau *et al.*, 2000). Moreover, the O-succinylation of GPLs is critical in their ability to inhibit mycobacterial phagocytosis by human macrophages (Villeneuve *et al.*, 2003). Finally, should the fact that strain mc²155 displays an enhanced cellular permeability be correlated with its transformability? Snapper *et al.*, (1990), who isolated the strain, looked for a possible alteration of the cell wall as an alternative hypothesis for the ept phenotype. Electroporation, in contrast to plasmid transformation, of mc²155 and its parental strain mc²6 derived from strain ATCC 607 by DNA from mycobacteriophage D29 resulted in no significant difference between the two strains. In addition, transformation by an integrative plasmid yielded similar numbers of transformants for both strains mc²155 and mc²6, at frequencies that were four orders of magnitude lower than for transformation with replicative plasmids (Snapper *et al.*, 1990). Thus, it appears that the alteration of the cell envelope of mc²155, although enough to affect the uptake of small hydrophobic molecules, may not be sufficient to completely explain its transformable character.

An alternative explanation would be that genomic and phenotypic rearrangements have occurred as the result of the subcloning of strain ATCC 607 which has led to mc²6, the parental strain of mc²155 (Jacobs, 2000). In this connection, PFGE analysis has revealed that a large duplication exists in the mc²155 genome (Galamba *et al.*, 2001) but which is differently located in the ATCC 607 genome (A. Galamba & J. Content, unpublished results). In addition, it appears that another rearrangement of the genetic material, possibly a deletion, has occurred in the ATCC 607 chromosome to generate mc²155 (A. Galamba & J. Content, unpublished results). To our best knowledge, this would be the first report where the isolation of a mutant strain in *M. smegmatis* could be correlated with a major chromosomal rearrangement. But such events are not uncommon in mycobacteria. Although members of the *M. tuberculosis* complex display an unusually high degree of conservation in their housekeeping genes (Sreevatsan *et al.*, 1997), whole-genome comparison of strains has revealed a large polymorphism due to numerous insertion–deletion events (Behr *et al.*, 1999; Gordon *et al.*, 1999; Brosch *et al.*, 2002; Tsolaki *et al.*, 2004). These events have often been correlated with a smooth-to-rough variation in species of the *M. avium* complex (Belisle *et al.*, 1993b; Eckstein *et al.*, 2000; O’Shea *et al.*, 2004) or in the fast-growing *Mycobacterium abscessus* (Howard *et al.*, 2002). Thus, *M. smegmatis* strains appear to be, like other mycobacterial species, subject to chromosomal rearrangement. Taken together, these data indicate that the nature of the surface-exposed compounds and of
the envelope constituents is crucial for the mycobacterial phenotype, and suggest that the transformable character of the mc²155 strain may be in part explained by a profound modification of its cell envelope.

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