Synthesis of an unusual polar glycopeptidolipid in glucose-limited culture of Mycobacterium smegmatis

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INTRODUCTION

Studies on starvation physiology of mycobacteria is considered to provide an insight into the long-term persistence of the bacilli, which is an important challenge to the successful therapy of mycobacterial infections (for review see Parrish et al., 1998). Moreover, nutritionally starved bacteria have been demonstrated to bear significant similarity with the natural persistors (Nyka, 1974). In fact, an in vitro starvation model has led to the discovery of at least two genes, sigF (Chen et al., 2000) and relA/spoT (Primm et al., 2000; Ojha et al., 2000) which are involved in the long term persistence of Mycobacterium tuberculosis. With an objective of gaining further insight into the physiological changes that occur during nutritional starvation in mycobacteria, we have studied the change in the profile of cell surface composition among different species of mycobacteria, the findings reported here may suggest a general mechanism of environmental regulation of cell wall biosynthesis.

GPs is an important cell-surface antigen in Mycobacterium avium, Mycobacterium intracellulare and Mycobacterium scrofulaceum. As the nomenclature suggests, GPs contain a tripeptide-amino alcohol core, D-Phe-D-allo-Thr-p-Ala-l-alaminol, which is linked with hydroxylated fatty acyl chain at the N-terminal D-Phe through amide linkage (Brennan & Goren, 1979). The D-allo-Thr and the terminal l-alaminol are further linked with 6-deoxytalose (6-dTal) and O-methyl rhamnose, respectively, to form serovar non-specific GPL (nsGPL) that are found in all species of M. avium complex (Brennan & Goren, 1979; Belisle & Brennan, 1989; Aspinall et al., 1995). The nsGPL, also called core GPL, is further glycosylated at 6-dTal to produce serovar-specific polar GPL (Brennan & Nikaido, 1995) (Fig. 1a). The antigenic polar GPL (serovar specific) has also been associated with the change in colony morphology among natural isolates of M. avium. For example, smooth-colony-forming serovar 20 of M. avium has polar glycopeptidolipids (Barrow et al., 1980).

GPs are also an important cell-surface constituent in some fast-growing mycobacteria such as Mycobacterium chelonae, Mycobacterium fortuitum and M. smegmatis (Brennan & Nikaido, 1995). However, M. smegmatis, a fast-growing non-pathogenic species, has been reported to produce only the apolar nsGPL, structurally similar to those produced by M. avium (Billman-Jacobe et al., 1999; Daffe et al., 1983; Patterson...
Based on the fact that *M. smegmatis* does not produce multiglycosylated polar GPLs, it has been genetically exploited as a surrogate host for identification of *M. avium* genes encoding specific glycosyltransferases, which add sugars to 6-dTal (Belisle *et al.*, 1991, 1993; Eckstein *et al.*, 1998). Through this strategy a *ser2* locus was identified in *M. avium* that had genes encoding rhamnosyl transferase and fucosyl transferase (Belisle *et al.*, 1991). In this study we report a conditional synthesis of a polar GPL in glucose-limited culture of *M. smegmatis*. Mass spectrometric analysis suggests that the polar GPL is a hyperglycosylated derivative of the apolar species. Further chemical analysis, however, indicates that the newly found polar species is structurally different from the usual polar GPLs found in *M. avium* in a way that the additional sugar is not linked to 6-dTal.

### METHODS

**Bacterial strain, medium and growth.** *M. smegmatis mc²155* (Snapper *et al.*, 1990), was used for all experiments. Bacteria were grown in 7H9 medium supplemented with 2% (w/v) glucose and 0.05% (v/v) Tween-80. Where necessary, glucose was replaced by 2% (v/v) glycerol as an enriched carbon source. For carbon-limited cultures the glucose concentration was reduced to 0.02% without any change in the concentration of the detergent. For plate culture 1.5% agar was added to the broth without Tween-80. The concentration of glucose for normal and starved plate cultures were the same as for the respective liquid cultures. For GPL analysis, the bacteria from the preinoculum (fully grown enriched culture) were harvested, washed and then subcultured in the appropriate medium. The growth profile of liquid cultures was obtained by measuring the OD₆₅₀ at regular time intervals. Colony morphology was observed under the microscope (Zeiss) at 10 × magnification.

**Purification and analysis of GPLs.** The GPLs from *M. smegmatis* were purified as mentioned earlier (Khoo *et al.*, 1999). Briefly, lipids were extracted by treating 5 g (wet wt) cells at various growth phases (see legend for Fig. 3) with chloroform/methanol (2:1) for 24 h at room temperature. The organic supernatant was dried and redissolved in chloroform/methanol (2:1) and treated with an equal volume of 0.2 M NaOH in methanol at 37 °C for 30 min then neutralized with a few drops of glacial acetic acid. After drying the solvent lipids were redissolved in chloroform/methanol/water.
(4:2:1) and centrifuged. The aqueous layer was discarded and the organic layer containing the lipids was concentrated. The decylated lipids were spotted on to a silica-coated TLC plate (Merck) and developed with chloroform/methanol (9:1). The sugar-containing lipids were visualized by spraying the plate with 10% H2SO4 (in ethanol) followed by heating at 120 °C for 10 min. The relative intensity of each spot was determined by densitometric scanning (Bio-Rad) against the plate background.

**MS of GPLs.** Each species of GPL (marked as spots 1–5) was eluted from a preparative TLC silica plate (20 × 20 cm) and dissolved in chloroform/methanol (2:1). The purity of each species was confirmed by TLC. MALDI-TOF (matrix assisted laser desorption ionization-time of flight; Kratos) was used to obtain the mass of each GPL species of M. smegmatis in positive ion mode using gentisic acid as the matrix. For further analysis of structure, GPLs were permethylated as described earlier (Khoo et al., 1996). Briefly, 0.5 ml DMSO was mixed with one pellet NaOH and the slurry was added to a vial containing 500 µg GPL. To the mixture, 0.5 ml methyl iodide was added and stirred at room temperature for 10 min. The reaction was quenched with slow addition of 1 ml water. The methylated GPL was extracted by adding 2 ml chloroform and washing the chloroform layer three times with water. The organic phase was dried, concentrated and used for MALDI analysis.

**β-Elimination of GPLs.** The β-elimination of sugars from GPLs was carried out as described earlier (McNeil et al., 1989; Eckstein et al., 1998). The purified ~50 mg of each spot (1 and 3) were resuspended in 1 ml absolute ethanol and then 1 ml 1 M NaOH was added to it. To each reaction mix, 10 mg NaBH4 was added and the resulting mixture was incubated at 60 °C for 24 h. Then the reaction mix was cooled to room temperature and neutralized with Dowex 50 (H+) resins. Then the solution was filtered and concentrated, and the residue was dissolved in 7 ml chloroform/methanol/water (4:2:1, by vol.). The organic phase containing the lipid fraction was concentrated and analysed by MALDI-TOF. The aqueous layer containing sugar was separated and concentrated. The excess of borate salt was removed by several cycles of co-evaporation with methanol. The sugar was peracetylated with 1 ml pyridine/acetone anhydride (1:1) at 80 °C for 2 h. The solution was evaporated and the residues were redissolved in acetone and analysed by ESI-MS (electrospray ionization mass spectroscopy; Hewlett Packard) in positive ion mode.

**RESULTS**

**Glucose-starved M. smegmatis forms a smooth colony**

The basis of studying the GPL in glucose-limited culture stems from the observation that there is a marked difference in the colony morphology of M. smegmatis under glucose limitation (Fig. 2). As we can see from Fig. 2 that the M. smegmatis colony grown on 0.02% glucose assumes a uniform margin, smooth surface and very small size (proportionate to the growth in liquid culture, Table 1) whereas the normal colony of M. smegmatis has non-uniform margin, rugose surface and comparatively very large size. Moreover, the colony on glucose-limited medium was translucent as against the normal opaque colony.

**A polar species of GPL in glucose-limited culture of M. smegmatis**

After observing the changes in colony morphology upon glucose limitation we asked whether it could be translated into variation in the molecular composition of the cell surface. As GPL forms the outermost layer of the cell surface of M. smegmatis, we analysed this important cell-surface lipid. Decylated GPL was isolated from different growth phases of M. smegmatis. Decylation was carried out using methanolic NaOH (Brennan & Goren, 1979). The treatment with methanolic NaOH esterifies contaminating fatty acyl chains and enriches the alkali-stable GPLs. Fig. 3 shows four major species of apolar GPLs (spots 2–5), observed in normal (2% glucose), glucose-limited (0.02%) and glucose-starved (0%) cultures. The growth of bacteria in the complete absence of glucose is facilitated by the trace amount of carbon source present in the 7H9 medium and also perhaps from the Tween-80. The GPL profile of the enriched culture is consistent with an earlier one shown by Patterson et al. (2000). Upon comparing the quantity of each spot by densitometric scanning and normalizing

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**Table 1. The generation time and stationary phase cell density of M. smegmatis as determined from the liquid culture**

<table>
<thead>
<tr>
<th>Carbon source (% glucose)</th>
<th>Generation time (h)*</th>
<th>Maximum cell density†</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2.40</td>
<td>5.5</td>
</tr>
<tr>
<td>0.2</td>
<td>2.73</td>
<td>2.7</td>
</tr>
<tr>
<td>0.02</td>
<td>2.83</td>
<td>0.8</td>
</tr>
<tr>
<td>0.0</td>
<td>4.65</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*Calculated as a function of OD660 during the fastest-growing phase of the culture.
†OD660 at stationary phase.
with spot 2, equally present in each sample (data not shown), it was found that glucose-limited bacteria in stationary phase had an additional polar species (spot 1), which was almost absent in actively growing enriched culture. It can be seen from Fig. 3 that the exponential phase of both normal (2% glucose) and glucose-limited (0–0.2% glucose) cultures contain a negligible quantity of the polar species (lanes 1 and 2 of Fig. 3a). The presence of this species is more distinct in stationary phase of the glucose-limited culture (lane 2 of Fig. 3b). The result suggests that the presence of the polar GPL in M. smegmatis is dependent on the concentration of glucose in the medium. To test the conditional synthesis of polar GPLs, we analysed the GPLs of an exponentially growing culture that was completely devoid of glucose (0% glucose). As can be seen from lane 3 of Fig. 3a, actively dividing exponential-phase bacteria synthesize polar GPL in the absence of glucose. This observation also rules out the possibility of the growth-phase dependent synthesis of polar GPL, which is also supported by the fact that the stationary phase bacteria from the enriched culture have a very low level of polar GPL. This observation can, once again, be explained by the fact that the presence of polar species is strictly dependent on the concentration of glucose in the medium. Smeulders et al. (1999) have shown that bacteria in an enriched medium enter into the stationary phase at a higher level of carbon source than those grown in carbon-limiting medium. This could perhaps be due to accumulation of large amounts of growth inhibitory secondary metabolites at very high cell density.

The synthesis of polar species of GPL in glucose-starved medium means that the genetic apparatus for the synthesis of such species is either activated by the lack of a carbon source or derepressed by the lack of glucose (a catabolite repressor). To find out which of the two mechanisms is operating, the GPL profile of a glycerol-enriched culture was analysed. Fig. 4 shows that a glycerol-enriched culture doesn’t produce the polar species, thus indicating that the synthesis of polar GPL is activated by the lack of carbon source in the medium.

**Polar GPL is most likely to be a hyperglycosylated derivative of apolar GPL**

For preliminary identification of the most polar GPL, spot 1, MS of each spot was carried out in native and permethylated (Fig. 1b) state. The mass increment due to permethylation would unambiguously indicate the numbers of free OH groups that have undergone methyl substitution. Fig. 5a and b shows the m/z values of GPLs in native and permethylated forms, respectively. The analysis of mass spectra of apolar GPLs (spots 2–5) was carried out on the basis of the structural studies reported earlier (Billman-Jacobe et al., 1999; Daffe et al., 1983;
Table 2. m/z values of the major molecular ion peak of each GPL spot in native and permethylated states

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>m/z of native GPL*</th>
<th>m/z of permethylated GPL*</th>
<th>No. methyl groups transferred</th>
<th>No. free OH groups†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1334</td>
<td>1462</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>1160</td>
<td>1286</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>1174</td>
<td>1286</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>1174</td>
<td>1286</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>1188</td>
<td>1286</td>
<td>7</td>
<td>3</td>
</tr>
</tbody>
</table>

* The m/z values obtained in Fig. 5 are approximated within the error range of the instrument (0.1%).
† Free OH groups on the sugars and fatty acyl chain. The free OH groups are the equivalent O-methyl substitution to the sugars and fatty acyl chain after permethylation. The values are obtained by subtracting 4 methyl groups substituted to tetrapeptide core (see Fig. 1b).

The polar species, spot 1, is structurally different from the usual polar species in other mycobacteria

The available literature on the structural studies of GPLs indicates that the polar species of the GPLs in *M. avium* invariably have oligosaccharide appendage linked to 6-dTal (Brennan & Nikaido, 1995; McNeil et al., 1989). Hence, we asked whether the polar species, spot 1, was also a result of similar linkage, although it was contrary to the idea suggested by Belisle et al. (1991) and Eckstein et al. (1998) that *M. smegmatis* is a suitable surrogate host for identification of *M. avium* genes which further glycosylate core GPLs at 6-dTal. For that, β-elimination of the sugar linked to δ-allo-Thr in spot 1 was carried out according to the protocol described earlier (McNeil et al., 1989). One of the apolar species, spot 3, known to have a single 6-dTal at δ-allo-Thr was used as a control. The eliminated sugar was reduced, peracetylated and subsequently analysed by ESI-MS. Fig. 6 shows that both spots 1 and 3 released 6-dTal which corresponded to [M + Na]+ of 399 and there was no major molecular ion peak corresponding to a disaccharide ([M + Na]+ of 629 or higher by a unit of 14) in the spectra of sugar released from spot 1. This was further corroborated by mass spectral analysis of the organic fraction, which contained the O-rhamnosylated lipotetrapeptide. The MALDI-TOF spectrum of spot 1 (Fig. 7a) expectedly showed a molecular ion peak of 1170 a.m.u., which resulted in the loss of 164 a.m.u. due to removal of 6-dTal. Similarly, spot 3 showed a major peak at 1010 (1174 – 164) a.m.u., Fig. 7b. The observation that the new sugar is not linked to 6-dTal is also consistent with the genetic data that provides the evidence for the lack of genes responsible for glycosyl transfer to 6-dTal in *M. smegmatis* and thus, making it a suitable host for the identification of such genes from spot 1 by a methyl substitution and thus raises the possibility that more than one species of apolar GPLs are hyperglycosylated. This spot was not analysed further.
Fig. 5. MS of GPLs from carbon-starved culture of *M. smegmatis*. (a) Mass spectrum of each species in native state using MALDI-TOF. The spot number is mentioned at the top left of each spectrum. *m/z* value of spot 4 (not shown here) is same as that of spot 3 (see Table 2). (b) Mass spectrum of permethylated derivative of each species using MALDI-TOF. The spot number is mentioned at the top left of each respective spectrum. The *m/z* value of spot 4 (not shown here) is same as that of spot 3 (see Table 2).
Fig. 6. ESI-MS spectra of the peracetylated derivative of reduced sugar released from spot 1 (a) and spot 3 (b) after $\beta$-elimination. The molecular ion peak of 399 a.m.u. corresponds to the 6-dTal derivative.

Fig. 7. MALDI-TOF spectra of the organic fractions, which contained the released lipidic portion of the GPL after $\beta$-elimination of sugars from spot 1 (a) and spot 3 (b). The release of 6-dTal from the native GPL will decrease the mass of the molecule by 164 a.m.u.
other species of mycobacteria. Hence, it is very likely that the deoxyhexose may be linked to the terminal rhamnose in the polar species.

**DISCUSSION**

The observations reported in this study suggest a possible correlation between the formation of smooth colonies and the synthesis of a polar GPL in *M. smegmatis* during carbon starvation. Although this study shows that *M. smegmatis* can synthesize polar GPL, the lack of additional sugar at 6-dTal in polar GPL still supports the strategy of using this species as a surrogate host for identifying glycosyltransferases from other species of mycobacteria that transfer sugars to 6-dTal. A comparative analysis of the *M. smegmatis* genome sequence shows that it contains at least two ORFs (contigs 3314 and 3310 in the TIGR database) that are significantly homologous to the glycosyltransferase gene of *M. avium*. This suggests that there may be two kinds of glycosylation in *M. smegmatis* GPL biosynthesis, i.e. a constitutive one for the synthesis of apolar GPLs and a regulated one for the synthesis of polar species. Although the structural finding reveals an unusual polar GPL, evidence for such polar species provides a new insight into the biosynthetic regulation of GPL in *M. smegmatis* and perhaps also in other mycobacterial species. The detailed structural studies of the polar species and the subsequent identification of genetic apparatus for the synthesis of such species would lead to a better understanding of the molecular basis of the observed phenotype. The involvement of GPL in sliding motility and biofilm formation by *M. smegmatis*, elegantly studied by Kolter and colleagues (Martinez et al., 1999; Recht et al., 2000; Recht & Kolter, 2001), demonstrate a wider role of the cell surface lipid in the physiology of the bacteria. In the light of their studies, the regulation of GPL biosynthesis in response to carbon starvation suggests that this cell surface change would be an important adaptive mechanism and may provide a functional advantage to bacilli during such stress. Thus the functional significance of the polar GPL in *M. smegmatis* would be an obvious follow-up to this study.

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**REFERENCES**


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