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

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There has been a general understanding that *Mycobacterium smegmatis* produces only apolar glycopeptidolipid (GPL), similar in structure to serovar non-specific GPL of *Mycobacterium avium*. In this study, synthesis of polar GPL in carbon-starved *M. smegmatis* is reported. Mass spectrometric analysis suggests the polar GPL to be a hyperglycosylated species. The earlier structural studies of polar GPLs from *M. avium* have invariably shown the presence of an oligosaccharide appendage to D-allo-Thr. However, a further chemical analysis using β -elimination of the newly found polar GPL in *M. smegmatis* shows that the molecule still contains a monosaccharide at the D-allo-Thr, thus suggesting a new form of polar GPL.

Keywords: polar glycopeptidolipid, carbon starvation

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 biologically active lipid, glycopeptidolipid (GPL), of *Mycobacterium smegmatis* in response to carbon starvation. Although there is a variation in the cell surface composition among different species of mycobacteria, the findings reported here may suggest a general mechanism of environmental regulation of cell wall biosynthesis.

GPL is an important cell-surface antigen in *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum*. As the nomenclature suggests, GPLs contain a tripeptide-amino alcohol core, D-Phe-D-allo-Thr-D-Ala-L-alaninol, 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-alaninol 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).

GPLs 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

Abbreviations: 6-dTal, 6-deoxy talose; ESI-MS, electrospray ionization mass spectrometry; GPL, glycopeptidolipid; MALDI-TOF, matrix assisted laser desorption ionization-time of flight; nsGPL, non-specific GPL.

Fig. 1. (a) General structure of myco-bacterial GPLs. The arrow heads show further addition of sugars on 6-dTal in polar species of GPLs reported in *M. avium*. The rhamnose appendage is not found in GPLs from an enriched culture of *M. smegmatis*. (b) The permethylated derivative of GPL.

et al., 2000). 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 glycosyl-transferases, 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 OD₆₀₀ 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 deacylated 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% H_2SO_4 (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 NaBH_4 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 by 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/acetic 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

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

Carbon source (% glucose)	Generation time (h)*	Maximum cell density†
2	2.40	5.5
0.2	2.73	2.7
0.02	2.83	0.8
0.0	4.65	0.6

* Calculated as a function of OD_{600} during the fastest-growing phase of the culture.

† OD_{600} at stationary phase.

Fig. 2. Comparison of normal (2% glucose) (a) and glucose-limited (0.02% glucose) (b) colonies of *M. smegmatis*. Both plates were incubated for one week and photographed at the same magnification.

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. Deacylated GPL was isolated from different growth phases of *M. smegmatis*. Deacylation 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

Fig. 3. Profile of GPLs of enriched (2% glucose, lanes 1), glucose-limited (0.02% glucose, lanes 2) and glucose-starved (0.0% glucose, lanes 3) cultures of *M. smegmatis* identified by TLC on silica-coated plates. The cultures were harvested at two different growth phases, i.e. exponential (a) and stationary phase (b). The OD₆₀₀ reading of each culture at the time of harvest is written below its respective lane. Each spot was assigned the corresponding number based on its mass.

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 (0.02% 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

Fig. 4. The synthesis of polar GPL is activated by a lack of carbon source and not derepressed by the absence of glucose. GPL profile of a glycerol-enriched culture (2%) in exponential phase (lane 1), is similar to that of a glucose-enriched culture in exponential phase (lane 2). Both the cultures lack the polar species, spot 1, which is found in the GPL profile of carbon-starved (0% glucose) culture (lane 3). The identity of each spot annotated here was confirmed by mass spectrometry.

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

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

* 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).

Patterson *et al.*, 2000). Structures of the four apolar GPLs from *M. smegmatis* strain mc²155 have been fully characterized by Billman-Jacobe *et al.* (1999) and Patterson *et al.* (2000) using GC-MS as well as ESI-MS. The findings from their studies are as follows. 1) Spots 4 and 5 have 2,3,4-trimethyl rhamnose whereas spots 2 and 3 have 3,4-dimethyl rhamnose. 2) Spots 3 and 5 have methoxylated while spots 2 and 4 have hydroxylated fatty acyl chains. 3) All the four deacylated GPLs have unsubstituted 6-dTal. Thus, it implies that the combined total number of free OH groups on the fatty acyl chain and sugars in spots 5, 4, 3 and 2 would be 3, 4, 4 and 5, respectively. Furthermore, the mass of spots 4 and 3 would remain the same, although their structure and thus, polarity would be different. Consistent with their conclusions we find that spot 5, the most apolar species, has combined total number of three free OH groups on the fatty acyl chain and the sugars (see the last column of Table 2), all accounted for by 6-dTal. Similarly, the mass and the combined total of free OH groups on the fatty acyl chains and the sugars of spots 4, 3 and 2 exactly follow the structures elucidated in the reported studies (Billman-Jacobe *et al.*, 1999; Daffe *et al.*, 1983; Patterson *et al.*, 2000).

Spot 1, which has a mass of 1334 a.m.u., is heavier than spot 5 by 146 a.m.u. and has a combined total of five free OH groups on the fatty acyl chain and the sugars. The increment in mass and number of free OH groups, along with the increased polarity of spot 1, can be easily satisfied when a deoxyhexose (with an appropriate number of methyl substitutions depending on the precursor) is linked to one of the apolar species. However, the position of linkage can either be 6-dTal or the terminal O-methyl rhamnose.

Interestingly, there was at least one more species of polar GPL, spot 0, which had a relative increase in its quantity upon carbon starvation. The preliminary mass spectrometric analysis showed its mass to be 1320 a.m.u., 14 a.m.u. less than the mass of spot 1 (Fig. 5a). This clearly suggested that spot 0 differs 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.

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 Eickstein *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 D-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 D-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

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 β -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 β -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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