Hyperglycosylation of glycopeptidolipid of *Mycobacterium smegmatis* under nutrient starvation: structural studies

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The presence of a polar species of glycopeptidolipid (GPL) in carbon-starved *Mycobacterium smegmatis* has been reported previously. In this study, the complete structure of this GPL is established with the help of MALDI-TOF (matrix assisted laser desorption/ionization time of flight) and ESI (electrospray ionization) -MS, ^{13}C-SEFT (spin echo Fourier transform) -NMR spectroscopy, and HPLC analysis. In the molecule, two units of a 3,4-di-*O*-methyl derivative of rhamnose are attached to L-alaninol via a 1→2 linkage. Various methyl derivatives of rhamnose and 6-deoxytalose were synthesized as standards to establish this structure. The accumulation of this polar GPL in *M. smegmatis* is *sigB* dependent, as a SigB-overproducing strain of *M. smegmatis* shows the presence of this spot in the exponential phase, and a *sigB*-knockout strain of *M. smegmatis* does not show the presence of any polar GPLs.

INTRODUCTION

The *in vivo* environment encountered by the persistent mycobacteria is thought to be nutritionally deprived. However, nutritionally starved bacteria bear significant similarity with these natural persistors (Nyka, 1974). Studies to understand the metabolic changes that facilitate dormancy in mycobacteria are difficult due to the slow growth rate of these persistors. Therefore, searches for suitable *in vitro* models have always been an important area of research. *Mycobacterium smegmatis* grown in minimal media has been established as a suitable model for studying persistence (Ojha *et al.*, 2000). We have previously reported that nutrient starvation, in *M. smegmatis* mc²155, induces a prolonged generation time with a change in the colony morphology from the regular rugose, large colony to a small and smooth colony. Moreover, a change in cell-surface glycopeptidolipid (GPL) composition was also noted (Ojha *et al.*, 2002). Though there are reports on species-specific variations of GPL composition in non-tuberculous mycobacteria, we have observed stationary-phase-induced biosynthesis of polar GPL in *M. smegmatis* (Ojha *et al.*, 2002). The mycobacterial cell wall has a multilamellar structure with the outer layer consisting of an asymmetric lipid bilayer (Billman-Jacobe, 2004). In this lipid bilayer, mycolic acids are found in the inner layer, while GPLs occupy the outermost layer. Alkali-stable C-type GPLs are often found among the lipids of the outer layer of some non-tuberculous fast-growing mycobacteria that cause opportunistic infections, such as *Mycobacterium avium*, *Mycobacterium peregrinum*, *Mycobacterium chelonae*, *Mycobacterium abscessus*, and also in the saprophyte *Mycobacterium smegmatis* (Ortalo-Magné *et al.*, 1996; Daffé *et al.*, 1983). However, alkali-labile serine-containing GPLs are also reported in *Mycobacterium xenopi* (Riviere & Puzo, 1991).

GPLs are among the major antigens that are non-covalently attached to the cell surface. They contain a tetrapeptide-amino alcohol core (D-Phe-D-alloThr-D-Ala-L-alaninol), linked to a 3-hydroxy or 3-methoxy, C_{26}–C_{34} fatty acyl chain at the N-terminal of D-Phe through an amide bond (Brennan & Goren, 1979). The best known serovar-nonspecific GPLs (nsGPLs), which are found in all species of the *M. avium* complex, consist of a 2,3,4-tri-*O*-methylrhamnose or a 3,4-di-*O*-methylrhamnose, linked to the terminal L-alaninol, along with a glycosylated 6-deoxytalose unit at the D-allothreonine (Brennan & Goren, 1979; Belisle & Brennan, 1989). However, these nsGPLs are further glycosylated at the 6-deoxytalose with an oligosaccharide appendage, to produce the serovar-specific polar GPLs (ssGPLs). The ssGPLs are sometimes associated with smooth colony morphologies in some *M. avium* strains

(Barrow *et al.*, 1980). *M. smegmatis* produce only a simple range of nsGPLs, which are of four major types. The species vary in the degree of methylation of the fatty acyl chain and of the rhamnose (Billman-Jacobe *et al.*, 1999). As *M. smegmatis* does not possess ssGPLs, it has been exploited as a natural mutant and used for the identification of genes encoding specific glycosyltransferases from *M. avium* required for the synthesis of haptenic disaccharides linked to 6-deoxytalose (Belisle *et al.*, 1991). It was also shown that the simpler GPLs found in *M. smegmatis* could serve as intermediates in the biosynthesis of recombinant ssGPLs in *M. smegmatis* (Eckstein *et al.*, 1998). This led to the characterization of the *ser2* gene cluster, encoding enzymes like rhamnosyltransferase (encoded by *ser2*A), required for biosynthesis of GPLs in *M. avium* (Belisle *et al.*, 1991; Eckstein *et al.*, 1998; Maslow *et al.*, 2003). Subsequently, the GPL-biosynthesis gene cluster of *M. smegmatis* was found to encode a peptidylsynthetase, four methyltransferases and three putative glycosyltransferases among other proteins (Billman-Jacobe *et al.*, 1999; Patterson *et al.*, 2000; Jeevarajah *et al.*, 2002, 2004).

We have previously reported on the conditional synthesis of a novel polar GPL in carbon-starved cultures of *M. smegmatis*, and have speculated that the normal apolar GPL species are hyperglycosylated (Ojha *et al.*, 2002). In this paper, we describe the complete structure of a novel class of GPLs. We also propose that one of the glycosyltransferases in the GPL locus is regulated in response to environmental signals.

This work describes an approach using MALDI-TOF-MS and an HPLC ESI-MS technique, along with ^{13}C-NMR, to obtain the primary sequence and glycosidic composition of this polar GPL.

METHODS

Bacterial strains and growth conditions. *M. smegmatis* mc^{2}155 (Snapper *et al.*, 1990), recombinant clones of this strain and mutants derived from it were grown in 7H9 (Difco) medium supplemented with 2 or 0·02 % (w/v) glucose and 0·05 % (v/v) Tween 80. The recombinant clones and mutants were grown in the presence of kanamycin (20 μg ml^{-1}). For plate cultures 1·5 % (w/v) agar was added to the broth without Tween 80; kanamycin was added (50 μg ml^{-1}) as and when required. The growth profiles of liquid cultures were obtained by measuring the OD$_{600}$ at regular intervals.

Chemical reagents. All chemicals, including L-rhamnose monohydrate and D-talose, were obtained at the highest grade from Aldrich unless otherwise specified. Milli-Q (Millipore) water was used for all chemical reactions.

Purification and analysis of GPLs. The GPLs from *M. smegmatis* mc^{2}155, its recombinant clones and mutants were purified as mentioned previously (Khoo *et al.*, 1999). Briefly, cells were harvested at exponential and stationary phases of growth, and separated from the culture medium by centrifuging at 4000 r.p.m. for 15 min. Lipids were extracted with CHCl$_3$/CH$_3$OH (2:1, v/v) at room temperature for 24 h. The organic supernatant was dried and dissolved in CHCl$_3$/CH$_3$OH (2:1, v/v), and deacylated by treating with an equal volume of 0·2 M NaOH in CH$_3$OH at 37 °C for 30 min, then

neutralized with a few drops of glacial acetic acid. After drying to remove the solvents, lipids were dissolved in CHCl$_3$/CH$_3$OH/H$_2$O (4:2:1, by vol.) and centrifuged. The aqueous layer was discarded, and the organic layer containing the lipids was washed with supersaturated brine and concentrated. The deacylated lipids were spotted onto silica-coated TLC plates (Merck) and developed with CHCl$_3$/CH$_3$OH (9:1, v/v). The sugar-containing lipids were visualized by spraying the plates with 10 % H$_2$SO$_4$ in ethanol, followed by charring the separated spots at 120 °C for 10 min.
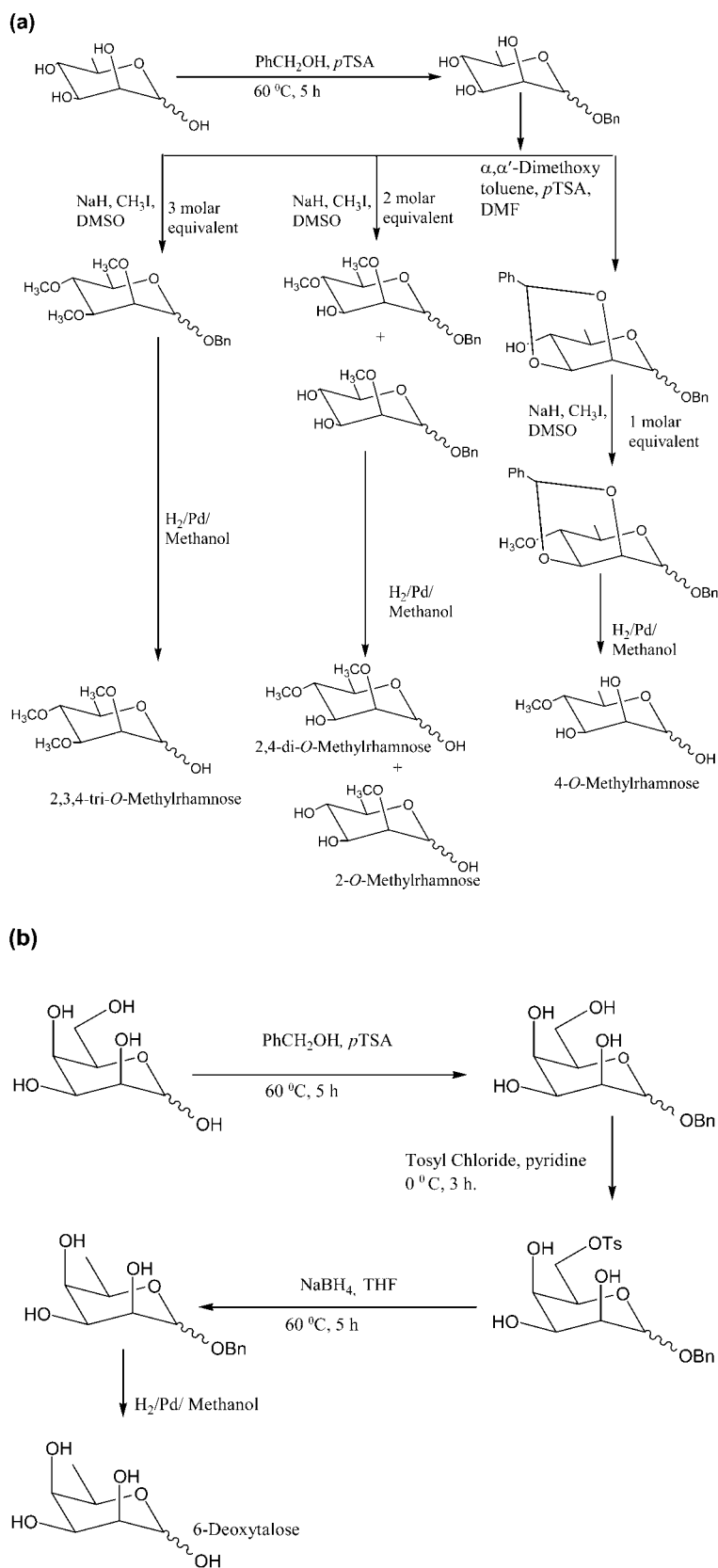
Spectroscopy. ^{13}C-NMR spectra were obtained in CD$_3$OD at 300 K on a Bruker AMX-400 instrument using the SEFT (spin echo Fourier transform) pulse programme (Brown *et al.*, 1981). Tetramethylsilane ($\delta_{ppm}=0$) was used as an internal calibrant.

Mass spectrometry. Each GPL species was eluted from preparative TLC silica plates (20 × 20 cm) by dissolving the GPLs in CHCl$_3$/CH$_3$OH (2:1, v/v). Samples were mixed with an equal volume of matrix solution (dihydroxybenzoic acid in ethanol), and then allowed to crystallize at room temperature. MALDI-TOF-MS and MS-MS spectra were acquired on a Bruker Daltonics ULTRAFLEX TOF-TOF instrument equipped with a pulsed N$_2$ laser, and analysed in the reflectron mode using a 90 ns time delay, and a 25 kV accelerating voltage in the positive ion mode. To improve the signal-to-noise ratio a mean of 500 shots were taken for each spectrum. External calibration was done to a spectrum acquired for a mixture of peptides with masses ranging from 1046–2465 Da. MS-MS spectra were acquired by selecting the precursor mass with a 10 Da window, and fragments were generated by collision-induced dissociation using He. In this case 1000 laser shots were acquired and averaged to generate the MS-MS spectra (Mechref *et al.*, 2003).

Analytical procedures. The *N*-acyl-phenylalanyl methyl ester was produced from the pure native GPL by strong-acid methanolysis with anhydrous 1·5 M CH$_3$OH/HCl for 16 h at 80 °C, as described previously (Villeneuve *et al.*, 2003). Then this *N*-acyl-phenylalanyl methyl ester was permethylated (Ojha *et al.*, 2002). Briefly, 0·5 ml anhydrous DMSO was mixed with one pellet of NaOH and the slurry was added to a vial containing the *N*-acyl-phenylalanyl methyl ester. To this mixture 0·5 ml CH$_3$I was added and stirred at room temperature for 10 min. This reaction was quenched by the slow addition of 1 ml water. The permethylated product was extracted by adding 2 ml CHCl$_3$ and washing the CHCl$_3$ layer three times with water. The organic phase was dried and concentrated. After every step, the mass of the compounds was checked by MALDI-TOF-MS.

Carbohydrate composition analysis. For monosaccharide composition analysis, pure GPL species were hydrolysed in 2 M trifluoroacetic acid for 2 h at 100 °C as described previously (Billman-Jacobe *et al.*, 1999), and the acid was removed at 40 °C under a high vacuum. 2-Propanol (0·5 ml) was added to the dried tube and evaporated. For HPLC-MS analysis of the released monosaccharides, the trifluoroacetic acid hydrolysate as well as the standard monosaccharides were passed through a Phenomenex LUNA/NH$_2$ column, with 2 % water in acetonitrile as a mobile phase at a flow rate of 0·3 ml min^{-1} at 20 °C, in a Waters HPLC instrument equipped with a refractive index detector. The eluted fractions were analysed by ESI-MS and ESI MS-MS, at a 270 °C nebulizer temperature with N$_2$ as the nebulizer and dry gas in a Bruker Daltonics ESQUIRE 3000 mass spectrometer equipped with an ion trap mass detector.

Preparation of partially *O*-methylated monosaccharides. For the synthesis of partially methylated rhamnose, L-rhamnose monohydrate was taken as a starting material; mono-*O*-benzylated rhamnose was produced by the reaction scheme as depicted in Fig. 1(a). This mono-*O*-benzylated rhamnose was then methylated with one, two and three equivalents of CH$_3$I to produce mono-*O*-methylated,

(a)

(b)

Fig. 1. (a) Scheme showing synthesis of variably-*O*-methylated rhamnose from L-rhamnose. (b) Scheme showing synthesis of 6-deoxytalose from D-talose.

di-*O*-methylated and tri-*O*-methylated rhamnose. In addition, methylation was carried out at the C-4 position after protecting the C-2 and the C-3 positions as benzylidine acetal. After the desired synthetic manipulations, the protected, methylated monosaccharides were deprotected using H$_2$/Pd under pressure, and purified to homogeneity in a silica column by adsorption chromatography. A

mixture of mono- and di-O-methylrhamnose obtained upon methylation with CH_3I (Fig. 1a) was also separated by silica column. 6-Deoxytalose was prepared using D-talose as the starting material, following the reaction scheme shown in Fig. 1(b). O-Benzylated talose was tosylated at the C-6 position, and then reduced with $NaBH_4$ to generate 6-deoxytalose. This was deprotected under H_2/Pd to obtain 6-deoxytalose. In each case the purity of the product was checked by TLC and by elemental analysis (Thermo Finnigan; FlashEA 1112 CHNS).

Creation of a SigB-overexpressing construct in *M. smegmatis*. *Mycobacterium tuberculosis sigB* cloned in pARC8193 (Amp$^+$) (a kind gift from AstraZeneca) was digested with *PstI*/*Hin*dIII, and the insert was subcloned into a mycobacterium–*Escherichia coli* shuttle vector pMV261 (Kan$^+$) (Stover *et al.*, 1991). After subcloning, this modified vector pMV-SigB was used to transform competent *M. smegmatis* mc^{2}155 cells by electroporation with a BTX electroporator system at $2 \cdot 5$ kV min^{-1}. Recombinant clones of PMVSigB were selected on 7H9 plates containing 20 µg kanamycin ml^{-1}.
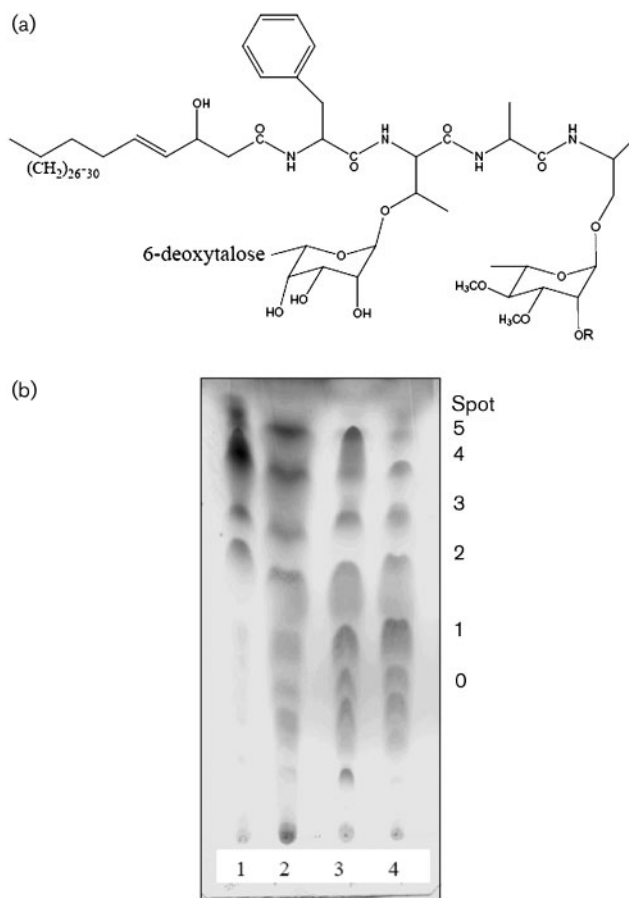
Creation of an *M. smegmatis sigB* mutant. A $2 \cdot 2$ kb fragment of the *M. smegmatis* chromosome containing *sigB* was cloned into plasmid pAlter, resulting in plasmid pSM140. Then a kanamycin-resistance cassette was cloned into the *Bam*HI site of *sigB*, in pSM140, inactivating this gene, to create plasmid pSM187. This plasmid was linearized and was used to transform *M. smegmatis* mc^{2}155, with selection for kanamycin resistance. Disruption of *sigB* in one of the transformants was verified by Southern hybridization (data not shown), and this strain, SM140, was used in the studies described here.

RESULTS AND DISCUSSION
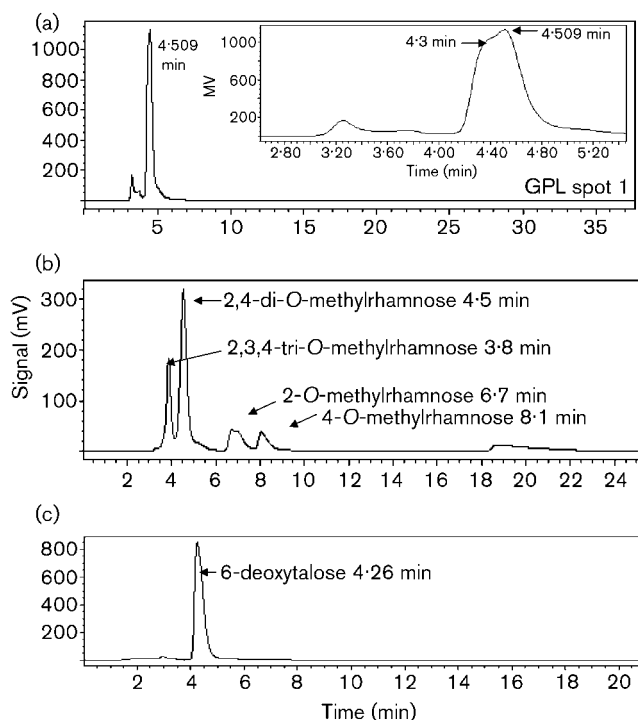
The new GPL is hyperglycosylated

Fig. 2(a) shows the core structure of the GPLs present in *M. smegmatis*, which was established previously by several groups (Daffé *et al.*, 1983; Brennan & Goren, 1979). Variations in the GPLs occur by way of introducing functional groups or sugar units to the core structure. Fig. 2(b) shows the presence of the apolar GPLs (spots 2, 3, 4 and 5) and a new polar GPL (spot 1), which we reported previously (Ojha *et al.*, 2002), appears during exponential phase in a carbon-starved culture of *M. smegmatis*. We have shown (Ojha *et al.*, 2002) that the same species also appeared during the stationary phase of a carbon-fed culture and has a *m/z* value of 1334 a.m.u. $[M+Na]^+$ (spot 1). As we noticed that this polar GPL still contains only a monosaccharide of 6-deoxytalose attached to the D-allo Thr, the next obvious position for the presence of an extra partially methylated deoxysugar, is the terminal alaninol. There is a possibility for a monosaccharide linked to rhamnose, unlike other non-tuberculous mycobacteria, where the oligosaccharide appendage is attached to the 6-deoxytalose (Belisle & Brennan, 1989).

In order to characterize the structure of this spot 1 satisfactorily, we undertook the following experiments. This new polar GPL firstly was subjected to strong-acid hydrolysis, whereupon monosaccharides were released. These released products were passed onto an amino HPLC column (Fig. 3a). Upon carefully examining the elution pattern one may notice that between $4 \cdot 3$ and $4 \cdot 5$ min a broad peak

Fig. 2. (a) Structure of the core nsGPLs from *M. smegmatis*. R = H or CH_3 for 3,4-di-O-methylrhamnose and 2,3,4-tri-O-methylrhamnose, respectively. (b) Profiles of GPLs of enriched (2 % glucose at exponential phase, lane 1; and at stationary phase, lane 2) and carbon-starved (0·02 % glucose at exponential phase, lane 3; and at stationary phase, lane 4) cultures of *M. smegmatis* identified by TLC on silica-coated plates.

with a shoulder appears, indicating the presence of a mixture of sugar units. Thus, we determined the flow time of standard O-methyl derivatives of rhamnose and 6-deoxytalose units (Fig. 1a, b) as shown in Fig. 3(b) and (c), respectively. Interestingly, the 6-deoxytalose unit elutes at more or less the same position as that of 2,4-di-O-methylrhamnose, which apparently gives a broad peak in the HPLC elution pattern as shown in the inset of Fig. 3(a). In order to further confirm our assignment, we subjected these eluates to ESI-MS. However, as we could collect only a single fraction from the HPLC column, this fraction was subjected to MS analysis and further fragmented. Fig. 4a shows two major masses corresponding to 202 and 210 a.m.u. The former could be due to the presence of a potassiated species of 6-deoxytalose, whereas the latter could be due to the presence of a water adduct of di-O-methylrhamnose. This was further confirmed by fragmenting these species, where the loss of a potassium ion and the
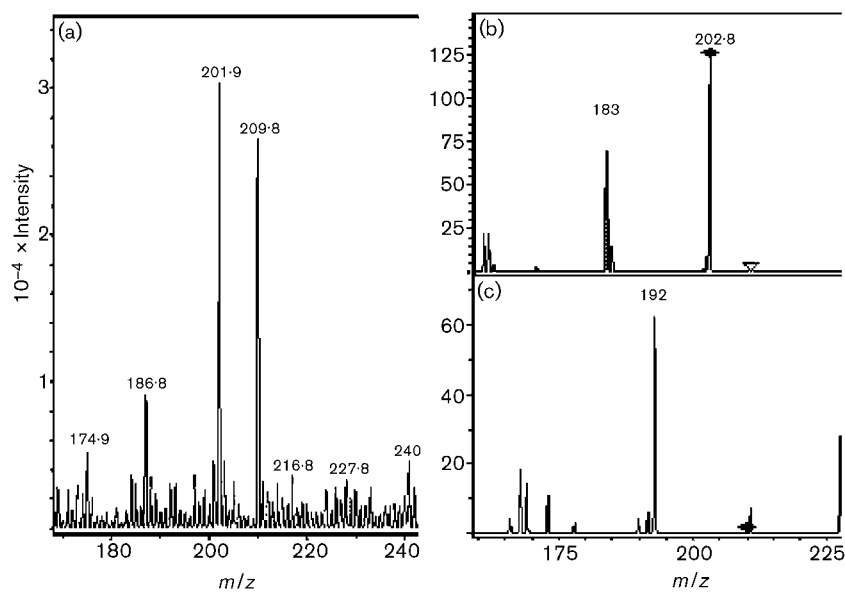
Fig. 3. Glycosidic composition analysis of TFA hydrolysate. HPLC elution profiles of TFA hydrolysate of the polar GPL (spot 1) (a), variably *O*-methylated rhamnose (b) and 6-deoxytalose (c). The inset in (a) shows a blow-up of the elution profile.

formation of a water adduct upon fragmentation gives rise to a mass of 183 a.m.u. (Fig. 4b), while the loss of water generates a species of mass 192 a.m.u. (Fig. 4c). Thus, the probable structure of this GPL species appears to have a diglycosyl unit with two molecules of di-*O*-methylrhamnose linked to L-alaninol, similar to what was described earlier
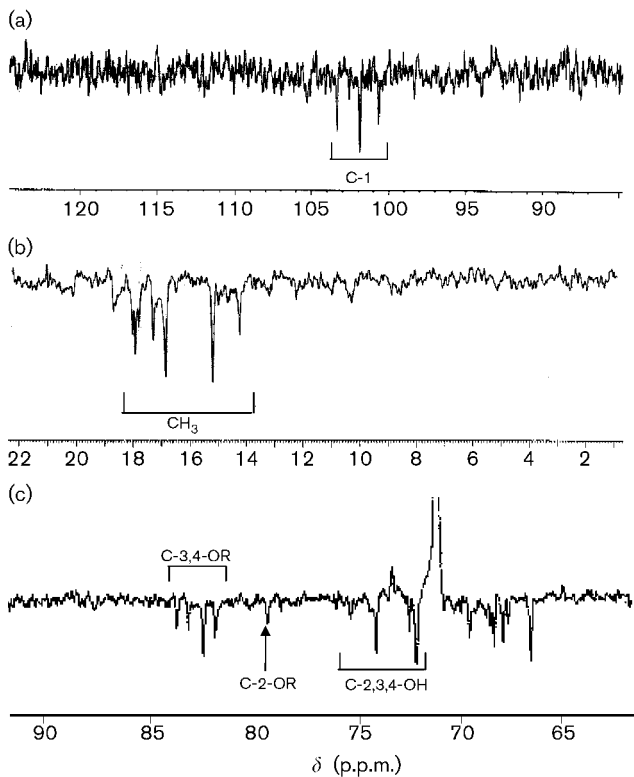
(Villeneuve *et al.*, 2003). The presence of three sugar units in the new polar GPL species was further confirmed by ^{13}C-SEFT-NMR spectroscopy (Brown *et al.*, 1981) as shown in Fig. 5(a). This clearly indicates the presence of three C-1 anomeric carbons (out of phase) within δ_{ppm} 95 and 105. In addition, Fig. 5(b) shows the presence of seven methyl groups between δ_{ppm} 14 and 18, out of phase. Upon per-methylation, as presented in our previous report (Ojha *et al.*, 2002), this polar GPL was found to have five free hydroxyl groups. On accounting for three free hydroxyl groups on deacylated 6-deoxytalose and one free hydroxyl group on the terminal 3,4-di-*O*-methylrhamnose, the last hydroxyl group may originate from the C-3 of the fatty acyl chain. To reconfirm this, the mass of native GPL cleaved by methanolysis was compared to the products obtained after cleaving by methanolysis and further permethylation. The MALDI-TOF-MS spectra showed an increment of 28 Da from native to permethylated products, accounting for two methyl groups being added, one on the amide nitrogen of D-phenylalanine, with the other at the C-3 of the fatty acyl chain (data not shown). The presence of masses $m/z =$ 213, 678, 749, 806, 980 and 1171 in the MALDI-TOF MS-MS fragment spectra (Fig. 6) brings out the same apolar backbone with a dirhamnosyl hyperglycosylation at alaninol and a C_{26}–C_{30} β-hydroxy fatty acyl chain with one unsaturation.

Nature of the linkage in the dirhamnoside

At this stage, we had established the presence of two di-*O*-methylrhamnose units attached to L-alaninol, but we were not yet certain about the nature of linkage between the two sugars. The nature of the dimethylrhamnose could be 2,3-di-*O*-methylrhamnose, 2,4-di-*O*-methylrhamnose or 3,4-di-*O*-methylrhamnose. Unfortunately, they cannot be separated by the HPLC technique employed here. In addition our successive attempts to separate them by
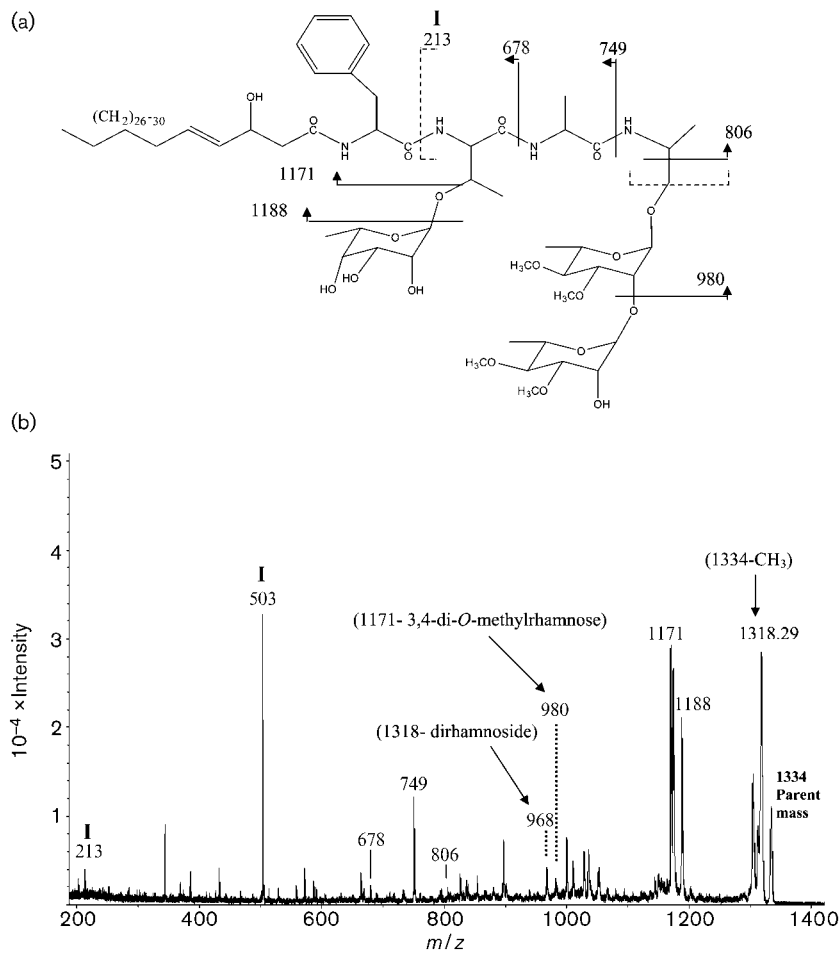

Fig. 4. ESI-MS spectra of the HPLC eluate (a), and the fragmentation pattern of the potassiated species of 6-deoxytalose of mass 203 a.m.u. (b) and the water adduct of di-*O*-methylrhamnose of mass 210 a.m.u. (c).
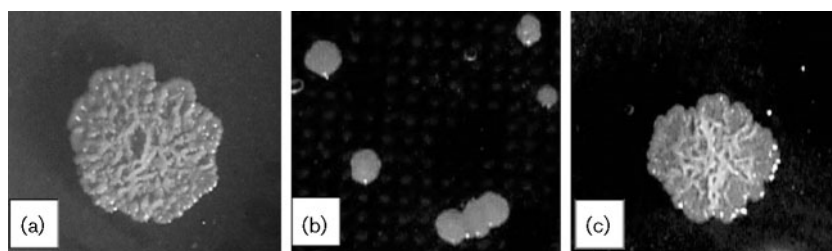
Fig. 5. (a, b) ^{13}C-SEFT-NMR spectra of spot 1 showing the three anomeric C-1 peaks of the three sugar units (a), and seven CH$_3$ groups, including the three C-6 of the three sugar units (b). (c) The presence of four C-3,4-OCH$_3$ peaks in the dirhamnoside structure is assigned along with four C-2,3,4-OH peaks.

GC-MS failed. However, we carried out ^{13}C-SEFT-NMR analysis, as described, to ascertain the linkage between the two sugar units. It was noticeable from Fig. 5(c) that the out of phase C-2 sugar position is predominant, with a δ_{ppm} value of 79·5 as reported earlier (Pozsgay *et al.*, 1981). We would like to mention at this point that the chemical shift values were assigned with respect to the tetramethylsilane internal standard (not shown). In addition, the other chemical shifts of the sugar units were assigned as shown in Fig. 5(c). Thus, from all these data we feel confident to conclude that the structure of the polar GPL of *M. smegmatis* induced upon carbon starvation is as shown in Fig. 6, and it is a dirhamnoside of 3,4-di-*O*-methylrhamnose with a 1→2 linkage, thus ruling out the presence of 2,4-di-*O*-methylrhamnose along with a 1→3 glycosidic linkage. It should be mentioned here that a 2,3-di-*O*-methylrhamnose together with a 1→4 glycosidic linkage has not so far been

Fig. 6. (a) Cleavage pattern of the new polar GPL spot 1 of mass 1334 a.m.u. (b) MALDI-TOF MS-MS fragment spectra of spot 1. Internal fragment ions are indicated by arrows.

Fig. 7. Comparison of colonies of wild-type mc²155 (a), PMVSigB (b) and *sigB*-knockout (c) cells of *M. smegmatis* grown for 5 days and photographed at the same magnification.

reported to occur between the sugars at the terminal alaninol of glycopeptidolipids in mycobacteria.

Cell surface composition and colony morphology in the *sigB* mutant and SigB-overexpressing *M. smegmatis*

The presence of an extra rhamnose unit, as discussed in the previous section, strongly suggests that the gene responsible for rhamnose transfer is upregulated during exponential growth in limiting carbon, or during stationary phase in high-carbon-containing cultures of *M. smegmatis* as these conditions show the accumulation of this polar GPL (Fig. 2b). We were interested to find out whether such regulation is dependent on SigB, thought to be a sigma factor with an important role in the stationary phase (Gomez & Smith, unpublished data). For this purpose, an *M. smegmatis sigB* mutant was constructed as described in Methods, and a strain that overexpressed SigB was also constructed. For these experiments we used the *M. tuberculosis* SigB, which has 93 % identity to *M. smegmatis* SigB. Interestingly, overexpression of SigB in mc²155 cells, i.e.

strain PMVSigB, caused a prolonged generation time concomitant with a marked change in the colony morphology (Fig. 7). We observed small, round and smooth colonies on 7H9 plates, in contrast to the normal wild-type colonies, which are large and have non-uniform margins with rugose morphology. However, the *sigB*-deleted mc²155 cells showed the usual wild-type phenotype. The GPL composition of strain PMVSigB was found to be altered. The PMVSigB clones produced the hyperglycosylated polar species of GPLs of masses 1334 and 1320 for spot 1 and spot 0 (which is 14 Da smaller than spot 1) respectively (Fig. 8), at the exponential phase of growth. This is the same polar GPL whose structure has been established here. The *sigB*-deleted *M. smegmatis* showed the absence of any hyperglycosylated or undermethylated species at its exponential phase of growth. It was difficult to grow this strain in 0·02 % glucose medium, and at very late stationary phase in 2 % glucose only a faint band at the spot 1 position appeared. We believe this observation will lead to further understanding of the network of regulation of gene expression in mycobacteria during nutrient starvation.
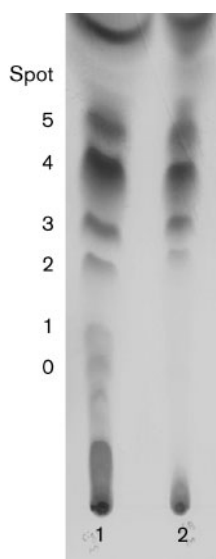
Concluding remarks

We have established here the structure of a polar GPL that is synthesized in *M. smegmatis* during growth in carbon-depleted medium or in stationary phase. It appears that the accumulation of the polar GPL may be *sigB* dependent. Identification of the genes responsible for the synthesis of this polar GPL is currently under way.

Fig. 8. Profiles of GPLs extracted from PMVSigB (lane 1) and *sigB*-knockout (lane 2) cells of *M. smegmatis* grown to exponential phase in a glucose-enriched medium, as identified on silica-coated TLC plates.

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