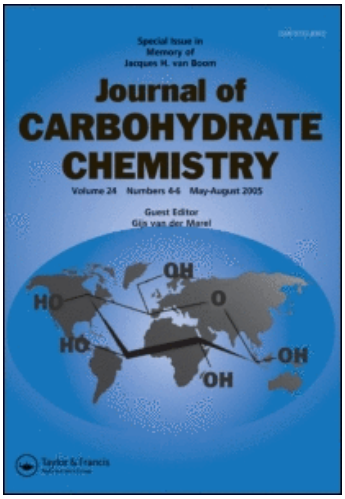

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Purification and LSIMS Analysis of Methyl Glucose Polysaccharides from *Mycobacterium xenopi*, a Slow Growing Mycobacterium[1]

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PURIFICATION AND LSIMS ANALYSIS OF METHYL GLUCOSE POLYSACCHARIDES FROM *Mycobacterium xenopi*, A SLOW GROWING MYCOBACTERIUM[1]

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ABSTRACT

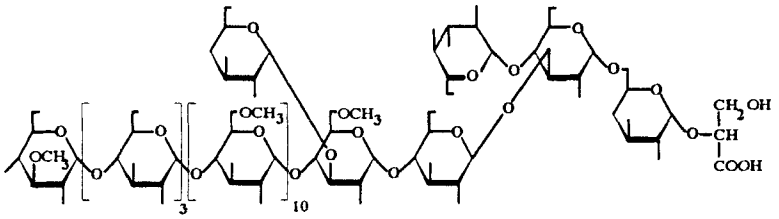
Methyl glucose lipopolysaccharides (MGLP) were first isolated in 1964 from a fast growing, nonpathogenic mycobacterial strain, *Mycobacterium smegmatis*. Their complete structure was achieved in 1982 by Forsberg *et al.*[2] It was established that the *M. smegmatis* MGLP heterogeneity arises from the acyl appendages borne by the polysaccharidic core. In the present study, we report the occurrence of MGLP from a slow growing strain *M. xenopi* and the structure of the deacylated derivatives (MGP). A new analytical strategy, based on the use of High pH Anion Exchange liquid Chromatography (HPAEC) and Liquid Secondary Ion Mass Spectrometry (LSIMS) was successfully developed. Thanks to HPAEC, the MGP mixture was fractionated and from LSIMS data, it was clearly established that the heterogeneity of the MGP polysaccharidic core arises from the number of glycosyl and methoxyl units.

INTRODUCTION

The key step in the mycobacterial infection is the ability of the pathogenic strains to survive in the potentially hostile macrophage cells. Indeed it is admitted that the

mycobacterial cell wall must play a crucial role in the resistance to intramacrophagic lysis. The main feature of the mycobacterial cell wall resides in its high lipidic content that constitutes a hydrophobic barrier. It is characterized by the presence of mycolic acids which are long chain fatty acids specific of the mycobacterial genus. These mycolic acids esterify the terminal arabinose units of the arabinogalactan.

Mycolic acids are synthesized from shorter fatty acids precursors (C16-C24)[3]. Twenty years ago, the fatty acid synthetase I (FAS I), a cytoplasmic multienzymatic complex, involved in the biosynthesis of fatty acids, was purified by Bloch *et al.* from *Mycobacterium smegmatis*.[4] The FAS I was stimulated by the PolyMethyl PolySaccharides (PMPS) described by Gray & Ballou.[5] It was postulated that PMPS give tight complexes with newly synthesized fatty acyls-CoA which would otherwise inhibit their own biosynthesis.[4] A helical conformation for the PMPS was advanced allowing the inclusion of fatty acids as proposed for complexes between amylose and fatty acids.[6] PMPS from the nonpathogenic fast growing strain *M. smegmatis* are composed of methyl mannose polysaccharides: **MMP** and methyl glucose lipopolysaccharides: **MGLP** (scheme 1). Here we report the purification and structural elucidation of MGP (the polysaccharidic core of MGLP) from a slow growing species: *M. xenopi*.



Scheme 1 Structure of the MGP of *M. smegmatis* (Forsberg *et al*?)

RESULTS

Purification of MGLP from *M. xenopi*.

The lipidic fraction containing the MGLP was isolated from *M. xenopi* cells as previously described by Lee[7] and Hindsgaul & Ballou[8] for the MGLP of *M. smegmatis*. Then, the mixture was fractionated by FPLC (Fast Protein Liquid Chromatography) using a preparative C18 reverse phase column. The elution was run by steps of solvent containing increasing amounts of methanol in water. Fractions 1, 2 and 3 were eluted by water and mixtures of 20% and 40% of methanol in water (v/v), respectively. After methanolysis and *O*-trimethylsilyl derivatization, all these fractions were found to be composed of arabinose, galactose, mannose and inositol suggesting the presence of

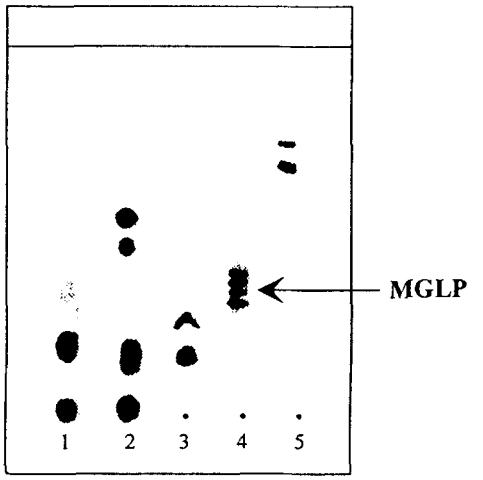


Figure 1 Silica gel TLC analysis of the fractions issued from the C18 preparative column. The plate was developed in $CHCl_3/CH_3OH/H_2O$ (56/38/10) and revealed with sulfuric anthron and heating. Lanes 1, 2, 3, 4 and 5 corresponds to fraction from the C18 column, eluted with 0, 20, 40, 70 and 100% of methanol in water, respectively.

arabinogalactan and phosphatidyl inositol mannosides. Fraction 4 was eluted by 70% of methanol in water. GC analysis, after methanolysis and TMS derivatization, showed the presence of four peaks, three of which were assigned, using authentic standards, to glyceric acid, 3-*O*-Me-Glc*p* and Glc*p*. The remaining one was identified as 6-*O*-Me-Glc*p* by GC/MS using a mixture of partially *O*-Me-D-Glc*p*s synthesized as previously described[9].

By analogy to the MGLP structures from *M. smegmatis* proposed by Ballou and co-workers, it can be proposed that the fraction 4 contains structurally related compounds. As shown by the TLC analysis (Figure 1, lane 4, mean Rf = 0.35), this fraction appears heterogeneous. This molecular heterogeneity could arise from the presumed acyl appendages described for the MGLP of *M. smegmatis*.[10,15] However, our attempt to fractionate MGLP using anion exchange chromatography (DEAE) or reverse phase HPLC (C18) were unsuccessful suggesting that heterogenity not only results from the net charge as previously proposed but also from variation of the oligosaccharidic core (MGP). Thus, our effort was focused on the purification and structural analysis of MGP, obtained after mild alkalinolysis of MGLP.

MGP HPAEC separation.

1μg of the MGP fraction was analyzed by HPAEC equipped with a Carbopac PA1 column, eluted by a gradient of sodium acetate (500 mM) in sodium hydroxide (100 mM) and monitored by pulsed electrochemical detection (PED).

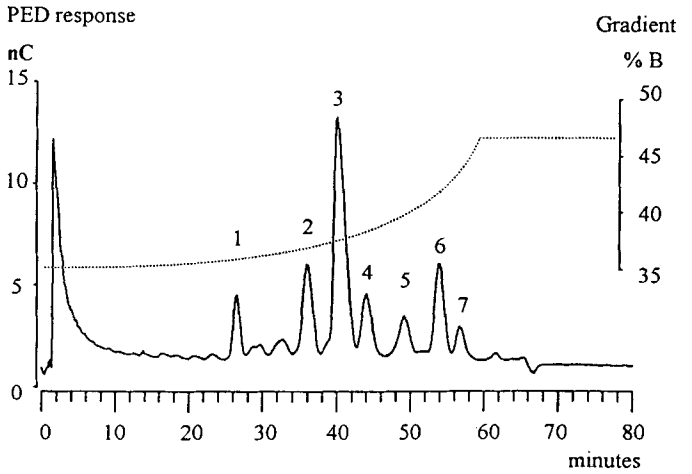


Figure 2. HPAEC analysis of the MGP mixture from *M. xenopi*. Fraction 5 was not analysed.

The chromatogram (Figure 2) showed one major peak (3) and at least six peaks of lower intensity (1, 2, 4, 5, 6 and 7). From the integration values, it was found that these compounds represent approximately 53 % of the MGP fraction, while the major peak (3) accounts for almost 40 %. GC analysis, after methanolysis and TMS derivatization, of each of these fractions revealed the presence of the MGP reporter monosaccharides. Thus, HPAEC appeared to be fully suited to separate the MGP mixture. Subsequently, 50 to 100 µg of each of these MGP were collected by this procedure using a semi-preparative Carbopac PA1 column. Each of these MGP were further analyzed by LSIMS.

LSIMS analysis of the major MGP 3

Prior to the mass analysis, the sodium salts arising from HPAEC separation were removed by reverse phase Sep Pak chromatography. Thanks to this step, MGP were successfully analyzed by LSIMS.

The high mass range of the spectrum (Figure 3), shows two intense peaks at *m/z* 3537 and 3559 assigned to $[M+Na]^+$ and $[M-H+2Na]^+$ respectively. This is supported by the presence, in the negative LSIMS spectrum, of an intense peak found at *m/z* 3513, assigned to the pseudomolecular ion $[M-H]^-$. From the molecular weight of 3514 Da, it was proposed that MGP 3 is composed of 8 Glc*p*s, 12 mono-*O*-Me-Glc*p*s (either 6-*O*-Me or 3-*O*-Me) and one glyceric acid unit. Thus, this MGP was called $MGP_{20,12}$, where the first number represents the total number of glucopyranose units and the second, the number of O-methylated ones. This analysis indicates that HPAEC fraction 3 is composed of one MGP molecular species revealing the performance of the chromatographic strategy applied.

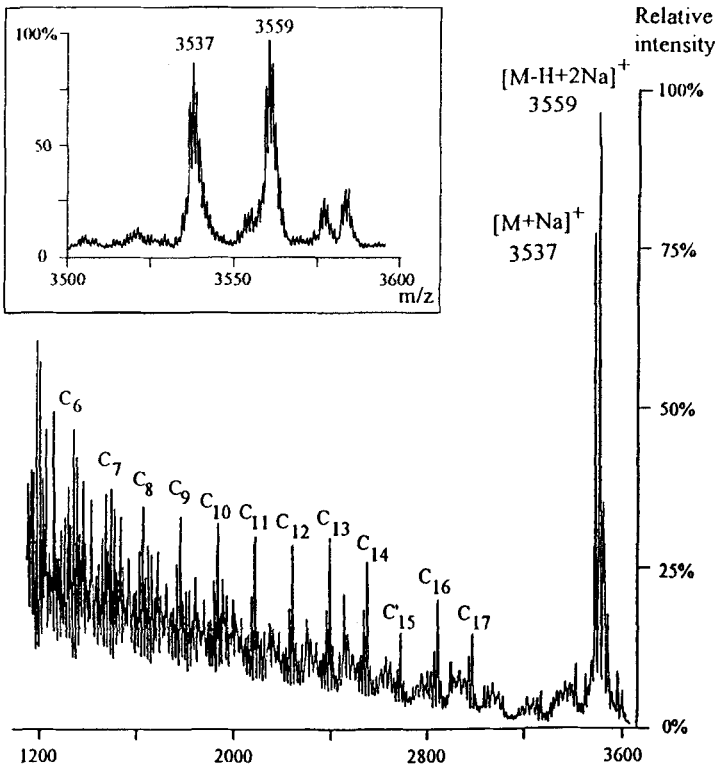
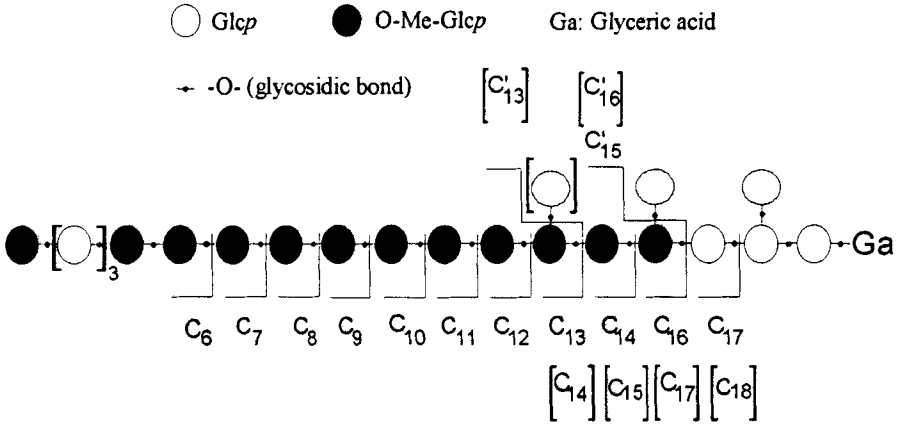


Figure 3 LSIMS spectrum of the MGP 3 ($MGP_{20,12}$). Pseudomolecular ion masses (top) corresponds to nominal mass (C:12,O:16, H:1, Na:23). Sequence ions (bottom) are named according to the nomenclature of Domon & Costello.[11]

The $MGP_{20,12}$ LSIMS spectrum shows, besides the cationized molecular ions, fragment ions arising from the glycosidic bound cleavages. Most of the fragment ions called C_n ions, according to the nomenclature of Domon and Costello,[11] retain the non-reducing end and the glycosidic oxygen (Scheme 2).

The first fragment ion C_{17} localized at *m/z* 2963 (Figure 3) arises from the $[M+Na]^+$ ion by the loss of 574 Da corresponding to three Glc*p* and one glyceric acid residues. The next one C_{16} at *m/z* 2801 distant of 162 amu agrees with the loss of one anhydro Glc*p*. The next fragment ion with the same intensity is the C_{14}, found at *m/z* 2463. The mass difference of 338 corresponds to the loss of the anhydro disaccharide Glc*p*-*O*-Me-Glc*p*. Between both C_{16} and C_{14} peaks, it can be observed the C'_{15} peak of half intensity suggesting the presence of a branched Glc*p* or *O*-Me-Glc*p*. From the mass difference between C'_{15} and C_{16} and C_{14} of 158 and 180 amu, respectively, it was hazardous to determine the nature of the branched monosaccharide. Thus, the $MGP_{20,12}$



Scheme 2 Origin of the sequence ions. Ions are called C_n according the nomenclature of Domon and Costello,[11] where n refers to the number of monosaccharides. Central part of the scheme represents the sequence of the 19 and 20 monosaccharides comprising MGP.
The corresponding sequence for the 21 monosaccharides containing MGP is drawn between brackets.

Table 1 Per-*O*-ethylation analysis of the $MGP_{20,12}$

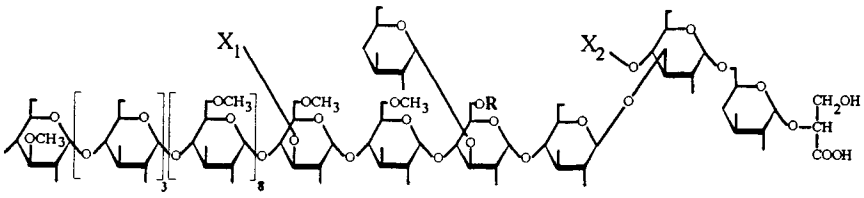
O-TMS methyl glycopyranosides	EI/GC/MS reporter fragments	CI/GC/MS molecular ions $[M+NH_4\text{-MeOH}]^+$	Ratio[b] calcd	Ratio[b] found
3-*O*-Me-2,4,6-tri-*O*-Et	75, 102, 129	278	1	0.65
2,3,4,6-tetra-*O*-Et	89, 116, 129	292	2	1.3
2,3-di-*O*-Et-6-*O*-Me	89, 116, 160, 173	322	10	10
2,3,6-tri-*O*-Et	89, 116, 160, 173	336	4	4.3[a]
2,3,4-tri-*O*-Et	89, 116, 117, 129	336	1	
2-*O*-Et-6-*O*-Me	133, 160, 173, 204	366	1	1.3
2,6-di-*O*-Et	133, 160, 173, 204	380	1	1.2

a. Those two monosaccharides were not resolved on the GC capillary column used.

b. Ratio normalized on the intensity of the 2,3-di-*O*-Et-6-*O*-Me-4-*O*-TMS-Glc*p*.

was per-*O*-ethylated and analyzed by GC/MS after methanolysis and trimethylsilylation of the resulting monosaccharides.

Data summarized in the Table I clearly established the presence of 2-*O*-Et-3,4 di-*O*-TMS-6-*O*-Me-Glc*p* and 2,3,4-6-tetra-*O*-Et-Glc*p* units indicating that the Glc*p* unit is



Scheme 3

branched on the 6-*O*-Me-Glc*p*. From the C_{14} ion, a series of sequence ions (C_{13} to C_6) distant of 176 amu arises from the successive loss of anhydro *O*-Me-Glc*p* indicating a linear structure. Moreover, the occurrence of 2,6-di-*O*-Et-3,4-di-*O*-TMS-Glc*p* in equal amount with 2-*O*-Et-3,4-di-*O*-TMS-6-*O*-Me-Glc*p* is in agreement with the presence of a tri-linked Glc*p* characteristic of another branch point. Thus, data from the $MGP_{20,12}$ mass spectrum and from linkage studies are consistent with the structure proposed for the MGP synthesized by the fast growing *M. smegmatis* (Scheme 3).

MGP 2 and 4 mass spectra

Compared to the $MGP_{20,12}$, the pseudomolecular ions of MGP 2 and 4 were found 14 amu up and down, respectively. Thus, HPAEC fractions 2 and 4 were identified as $MGP_{20,13}$ and $MGP_{20,11}$.

It was found that the $MGP_{20,13}$ C_n ions (C_6 to C_{17}) have the same mass values as those from the $MGP_{20,12}$ (Table 2). Thus the additional methoxyl group, found in $MGP_{20,13}$, was localized on one of the three Glc*p*s which compose the reducing end. GC/MS analysis after per-*O*-ethylation revealed that the methoxyl group is not localized on the tri-linked Glc*p* since the 3,4-di-*O*-TMS-2,6-di-*O*-Et derivative is still present in the GC chromatogram. However a slight increase in the intensity of the 3-*O*-Me-2,4,6-tri-*O*-Et-Glc*p* suggests that the branching monosaccharide X_2 (scheme 3) is also 3-*O*-methylated.

Likewise, in the mass spectrum of the $MGP_{20,11}$, the C_{17}, C_{16} and C'_{15} fragment ions are 14 amu down shifted while the C_{14} to C_6 ions are present at the same mass value as those from the $MGP_{20,12}$ (Table 2). Thus, it can be advanced that the tri-linked 6-*O*-Me-Glc*p* in the $MGP_{20,12}$ is no longer methoxylated in the $MGP_{20,11}$. This was supported by the absence of the 2-*O*-Et-3,4-di-*O*-TMS-6-*O*-Me-Glc*p* peak in the GC analysis of the methanolysis products of the per-*O*-ethylated $MGP_{20,11}$.

MGP 1 mass spectrum.

LSIMS spectra of the MGP 1 are dominated in the molecular mass range by two intense peaks at *m/z* 3375 and 3397 assigned to $[M+Na]^+$ and $[M-H+2Na]^+$, respectively. These two signals are down shifted of 162 amu compared to the $MGP_{20,12}$. Thus, the

Table 2 Nominal mass (C:12, O:16, H:1, Na:23) of molecular and sequence ions from LSIMS spectra of MGP resolved by HPAEC.

MGP		1	2	3	4	6	7
		$MGP_{19,12}$	$MGP_{20,13}$	$MGP_{20,12}$	$MGP_{20,11}$	$MGP_{21,12}$	$MGP_{21,11}$
M.W.		3352	3528	3514	3500	3676	3662
Molecular ions	$[RCOOH,Na]^+$	3375	3551	3537	3523	3699	3685
	$[RCOONa,Na]^+$	3397	3573	3559	3545	3721	3707
Sequence ions	C_{18}					3125	3111
	C_{17}	2963	2963	2963	2949	2963	2949
	C_{16}	2801	2801	2801	2787		
	C'_{16}					2783	2769
	C_{15}					2625	2625
	C'_{15}	2621	2621	2621	2607		
	C_{14}	2463	2463	2463	2463	2449	2449
	C_{13}	2287	2287	2287	2287		
	C'_{13}					2269	2269
	$\leq C_{12}$	C_{12}:2111, C_{11}:1935, C_{10}:1759, C_9:1583, C_8:1407, C_7:1231, C_6:1055					

MGP 1 can be assigned to the $MGP_{19,12}$ according to our nomenclature. Interestingly, the analysis of the fragment ions indicates that the sequence ions have the same mass as those from the $MGP_{20,12}$. Particularly, the presence of the C_{17} ions at *m/z* 2963 supports the absence of one of the three Glc*p*s near the reducing end (Scheme 3). Since GC/MS analysis after per-*O*-ethylation indicated the absence of the 2,6-di-*O*-Et-3,4-di-*O*-TMS-Glc*p* derivative, we propose that the branching Glc*p* near the reducing end is lost in the $MGP_{19,12}$.

MGP 6 and 7 mass spectra.

The high mass range of the LSIMS spectrum of fraction 6 shows two intense peaks at *m/z* 3699 and 3721 assigned to $[M+Na]^+$ and $[M-H+2Na]^+$, leading to a molecular weight of 3676 Da. Compared to the $MGP_{20,12}$, it was proposed that the MGP 6 bears one additional Glc*p* residue and corresponds to the $MGP_{21,12}$ according to our

Table 3 Structural features of MGP (cf. scheme 3)

	$MGP_{19,12}$	$MGP_{20,13}$	$MGP_{20,12}$	$MGP_{20,11}$	$MGP_{21,12}$	$MGP_{21,11}$
X_1	H	H	H	H	Glc*p*	Glc*p*
X_2	H	3-*O*-Me-Glc*p*	Glc*p*	Glc*p*	Glc*p*	Glc*p*
R	CH_3	CH_3	CH_3	H	CH_3	H

nomenclature. The first fragment ion localized at *m/z* = 3125 (Table 2) arises from the $[M+Na]^+$ ion by the loss of 574 Da assigned to three Glc*p* and one glyceric acid residues. However, this ion shows a mass increment of 162 amu from the similar one of the $MGP_{20,12}$ indicating the presence of an additional Glc*p* residue and was thus noted C_{18}. From this ion, we can observe the successive losses of 162, 178, 160 and 176 giving rise respectively to the ions C_{17} (*m/z* 2963), C'_{16} (*m/z* 2783), C_{15} (*m/z* 2625) and C_{14} (*m/z* 2449). All these C_n ions are up shifted of 162 amu compared to the $MGP_{20,12}$ ions, suggesting a similar sequence. Moreover, the C_{12} to C_6 ions have the same mass values indicating that the additional Glc*p* is branched on the third 6-*O*-Me-Glc*p* starting from the reducing end. Indeed, from the C_{14} it can observed losses of 180 (C'_{13} *m/z* 2269) and 158 amu (C_{12} *m/z* 2111) characteristic of the presence of an additional branch point. So, this compound differs from the major $MGP_{20,12}$ by the presence of a third ramified Glc*p* (Scheme 3).

The MGP 7 LSIMS spectrum shows a peak at *m/z* 3685 assigned to the pseudomolecular ion $[M+Na]^+$. Thus, from the deduced molecular weight of 3662 Da, this MGP was identified as the $MGP_{21,11}$. Compared to the $MGP_{21,12}$, the C_{18}, C_{17} and C'_{16} fragment ions are 14 amu down shifted while the C_{15} to C_6 ions are still present at the same mass value (Table 2), suggesting that the penultimate tri-linked 6-*O*-Me Glc*p* is no longer methoxylated in the $MGP_{21,11}$ (Scheme 3).

Linkage analysis after per-*O*-ethylation was consistent with the presence of one additional branch point in $MGP_{21,12}$ and $MGP_{21,11}$. Indeed, the ratio of 2-*O*-Et-6-*O*-Me-3,4-di-*O*-TMS-Glc*p*/2,6-di-*O*-Et-3,4-di-*O*-TMS-Glc*p* derivatives represented 1/1.85 (calcd 1/2) and 1.82/1 (calcd 2/1) for the $MGP_{21,11}$ and $MGP_{21,12}$, respectively.

CONCLUSION

MGLP were first discovered in a fast growing nonpathogenic mycobacteria *M. smegmatis* ATCC 356. They were also suspected in slow growing strains like *M.*

tuberculosis (Lee & Ballou,[13] Lornitzo & Goldman[14]) but for those species, the structural data were limited to GC analysis of constitutive monosaccharides. In the present report, we have shown that MGLPs are also present in a slow growing pathogenic mycobacterium, *M. xenopi*.

Moreover, this work clearly established that MGLPs of this strain were in a mixture and that the heterogeneity observed does not only result from the lipidic appendages as previously proposed but also from the polysaccharidic core. By HPAEC, we could resolve the mixture of MGP (deacylated MGLP) some of which differing only by one methoxyl group. Subsequent LSIMS analysis has allowed their individual molecular mass determination and the monosaccharide sequence has been deduced from the fragment ions observed in the LSIMS spectra and from linkage studies after per-*O*-ethylation. It results from this study that the heterogeneity of MGP from *M. xenopi* arises both from the number of glycosyl and methoxyl units. From a biological point of view, quantitative analysis of the mixture shows that the major compound, $MGP_{20,12}$ reported in *M. smegmatis*, accounts for less than 50% of the mixture in the slow growing *M. xenopi*. Since it is assumed that this compound is involved in mycolic acid biosynthesis, which rate has been shown to be related to the generation time of mycobacterial cells,[18] one can speculate on the presence of altered form of MGP in a slow growing strain. These products could lower the specific activity of the mixture due to their different affinities for fatty-acyl CoAs which could in turn affect mycolic acid biosynthesis.

EXPERIMENTAL

Preparation of MGP. *M. xenopi* (CIPT 140 35004 Institut Pasteur reference strain) was grown as surface pellicules on 7 litres Sauton's medium at 42 °C.[12]

Preparation of MGP was adapted from the procedure proposed by Hindsgaul & Ballou using affinity chromatography.[8] Briefly, the cells were extracted thoroughly with a mixture of $CHCl_3/CH_3OH$ (2/1). The crude lipidic extract was dried, diluted in chloroform and partitioned against water. MGLP were recovered from the aqueous phase using reverse phase-FPLC. The system was composed of a Gilson apparatus (two 302 pumps and an 806 manometric module) equipped with a 60 mL column (Merck) containing HPLC reverse phase (C18, 25-60µm, Merck). Elution was run by steps of 200 mL of solvent constituted by increasing amounts of methanol in water at 2.5 mL/min. The first solvent contained water and then mixtures containing 20%, 40%, 70% of methanol in water (v/v) were used, respectively. Finally the column was eluted by pure methanol.

Each fraction was analysed by silicic TLC, GC and GC/MS after methanolysis and TMS derivatization. MGLP were found in the fraction eluted with 70% of methanol in water (v/v).

MGLP were eluted on a G50 column by 30 mM of ammonium acetate in water in order to remove dies and contaminating phosphatidyl inositol mannosides. Elution was monitored by refractive index and by dosing sugars with anthrone. Fractions containing MGLP were pooled and lyophilised rising to the stock of MGLP.

MGLP were incubated overnight. with 0.1M NaOH at room temperature leading to MGP.[15] The mixture was neutralised using HCl and MGP were desalted after elution on a biogel P4.

HPAEC analysis of MGP. HPAEC analysis was conducted with 1 μg of MGP using a Dionex DX 300 system equipped with a solvent degas module, a gradient pump and a Carbopac PA1 column (4x250 mm). Solvents were made using deionized water (Millipore, Waters), 50% dilute sodium hydroxide (Baker) and ACS grade sodium acetate (Merck). Solvent A was NaOH 0.1M and solvent B was 0.5M CH_3COONa in NaOH 0.1M. Elution was performed at 1mL/min and started with a step of 38% of solvent B for 2 minutes. Then, a concave gradient (n° 7) from 38 to 48% of solvent B was used over 60 minutes. Detection was monitored by a Dionex PED detector with a standard wave program for carbohydrate (Gold electrode without pH reference, E1=0.1 V (0.5s), E2= -0.6V (0.1s), E3= 0.6V (0.05s)).[16] Purification of MGP for LSIMS analysis was performed with the same system but using a semi-preparative column (9x250 mm), eluted at 5 mL/min. Approximately 400 micrograms of the mixture of MGP could be eluted in a run.

Desalting of MGP after HPAEC purification. HPAEC fractions were neutralized using HCl (~1N) and applied to a C18 Sep-Pak cartridge (Waters Associates) which was eluted with water and mixtures of 20 and 70% (v/v) of methanol in water. Salts were eluted in the water fraction and in the fraction containing 20% of methanol in water. MGP were eluted in the fraction containing 70% of methanol. Completion of the desalting was controlled by GPC on biogel P4.

LSIMS analysis of MGPs: MS spectra were recorded on a two sector instrument ZAB-2E (VG Analytical) in the positive and negative modes. The Cesium beam energy was of 35kV and the accelerating voltage was of 8kV. Full spectra were recorded using an 800 to 4000 mass/range with a resolution M/ΔM of 800, while molecular ion regions were recorded using a 2600 to 4000 mass/range and a resolution M/ΔM of 4000. Data were treated on a Vax 2000 station (Digital). For each sample, 1μL of MGP at ~5 μg /μL was applied to a matrix of thioglycerol containing 10% of acetic acid on a probe tip.

Linkage analysis. HPAEC purified MGP were per-*O*-ethylated in DMSO using the procedure of Ciucanu and Kerek.[17] Per-*O*-ethylated MGP were solvolyzed using methanolic HCl (1N) and analyzed by GC and GC/MS after TMS derivatization.

Analytical Methods: Routine gas chromatography was performed on a Girdel series 30 chromatograph equipped with an OV1 wall-coated open tubular capillary column (0.32mm x 25m, Spiral France) using nitrogen gas at a flow of 2.5mL/min with a flame

ionisation detector set at 310 °C. A temperature program from 100 °C to 250 °C at a speed of 3 °C/min was used for the trimethylsilyl methyl glycoside analysis.

GC/MS experiments were done on a Hewlett Packard 5889X mass spectrometer working in both EI and CI (NH_4^+) ionization modes.

TLC analysis were performed on silicic acid plates (DC Alurolle, Kieselgel 60 pF_{254} MERCK) eluted by a chloroform/methanol/water (56/38/10) mixture and revealed by anthron staining.

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