**Mycobacterium tuberculosis phoP mutant: lipoarabinomannan molecular structure**

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**INTRODUCTION**

The capacity of *Mycobacterium tuberculosis* to establish infection within an individual and efficiently disseminate within a human population is mediated by its ability to remain dormant over long periods of latent infection and resume growth upon disease reactivation (Parrish et al., 1998). Both environmental and physiological signals during periods of active disease, dormancy or disease reactivation could be involved in the adaptation of *M. tuberculosis* during various stages of infection.

Two-component systems, composed of a membrane-associated sensor kinase that uses energy released from ATP hydrolysis to modulate a cytoplasmic transcriptional regulator activity (Stock et al., 2000), exhibit multiple cellular implications. For instance, the two-component systems of some bacterial pathogens have been shown to regulate virulence factor biosynthesis pathways (Bernardini et al., 1990; Engleberg et al., 2001; Kinnear et al., 2001; Miller et al., 1989; Solan-Landa et al., 1998; Yarwood et al., 2001), modulating the response to immune defence of the infected host. This was particularly evidenced with the PhoP/PhoQ system in *Salmonella typhimurium* which regulates genes implicated in the biosynthesis of LPS (Guo et al., 1997), resulting in the addition of aminoarabinose and 2-hydroxy-myristate to lipid A. Consequently, this structurally modified lipid A altered expression of the adhesion molecule E-selectin by endothelial cells, and tumour necrosis factor-α expression by adherent monocytes, and thus represents a potential mechanism for bacteria to gain advantage within host tissues (Guo et al., 1997).

Recently, Pérez et al. (2001) have disrupted the phoP...
gene from *M. tuberculosis* MT103, which encodes a putative transcription regulator factor of the two-component system PhoP/PhoR. Interestingly, the phoP mutant strain exhibited impaired multiplication in *vitro*, brought into evidence when cultured in mouse bone marrow-derived macrophages, and also in *vivo* as the viability was attenuated in a mouse infection model (Perez et al., 2001). These results suggest that the phoP gene is required for intracellular growth of *M. tuberculosis* but open up the question of a hypothetical mutagenesis (Perez et al., 1991).

Mannosylated lipoarabinomannans (ManLAMs) are lipoglycans ubiquitously found in the mycobacterial cell wall (Brennan & Nikaido, 1995; Vercellone et al., 1998) and play an important role in tuberculosis immunopathogenesis (Chatterjee et al., 1992; Roach et al., 1993; Vercellone et al., 1998). Indeed, ManLAMs were found in *vitro* to inhibit IL-12 production by human dendritic cells (Nigou et al., 2001) or human mononuclear phagocytes (Knutson et al., 1998), to block the transcriptional activation of interferon-γ (Riedel & Kaufmann, 1997) and to neutralize the potentially cytotoxic oxygen free radicals (Chan et al., 1991). All these activities require the acylation of the mannosyl-phosphatidyl-inositol (MPI) anchor. Moreover, the degree of MPI acylation was found to control IL-12 inhibitory activity of ManLAMs (Nigou et al., 2001). In addition, the manno-oligosaccharide caps mediate the ManLAM binding to the mannose receptor (Schlesinger et al., 1994, 1996; Venisse et al., 1995) involved in ManLAM signalling; consequently, ManLAM devoid of the mannooligosaccharide caps failed to inhibit IL-12 production by human dendritic cells (Nigou et al., 2001).

In the present study, the structure of the two major functional domains of ManLAM from *phoP* mutant and wild-type *M. tuberculosis* MT103 were investigated as follows (i) the cap motifs by capillary electrophoresis and (ii) the MPI anchor by NMR spectroscopy. These structural findings will be discussed so as to evaluate their potential implication in the different growth rates observed between *phoP* mutant and wild-type *M. tuberculosis* MT103.

**METHODS**

**Bacteria cells.** The clinical isolate *M. tuberculosis* MT103 (Camacho et al., 1999) and *phoP* mutant (Perez et al., 2001) strains were both routinely grown using Middlebrook 7H9 supplemented with ADC and 0.05% Tween 80 (Jacobs et al., 1991).

**ManLAM extraction and purification.** ManLAMs from *phoP* mutant and wild-type *M. tuberculosis* MT103 cells were purified as described (Nigou et al., 1997). Briefly, bacterial cells were pelleted by five extractions using CHCl3/CH3OH (1:1, v/v), the resulting cells were washed, weakened in ice by sonication, disrupted using a cell disrupter (one-step lysis at a pressure maintained at 2.6 kPa) and extracted (refluxing three times at 65 °C) for 8 h with ethanol/water (1:1, v/v). Most of the cellular proteins contained in this extract were removed by a hot 80% (w/w) aqueous phenol biphasic wash. Then each aqueous extract was treated to remove DNA, RNA and glucose leading to glycemic- and lipoglycan-rich extracts.

**Hydrophobic interaction chromatography of lipoglycans.** Hydrophobic interaction chromatography was used to separate lipoglycans, mainly arabinomannan from lipoglycans [ManLAMs and lipoarabinomannans (LMs)]. *M. tuberculosis* (9 mg) was reconstituted in 0.05 M ammonium acetate solution containing 5% (v/v) propan-2-ol and loaded on an Octyl-Sepharose CL-4B (Pharmacia) column (20 x 1.5 cm) pre-equilibrated in the same buffer. The column was then eluted with 3 column vols 5% then 50% (v/v) propan-2-ol containing 0.05 M ammonium acetate at a flow rate of 3 ml h⁻¹. Lipoarabinomannans and LMs were only recovered in the 50% (v/v) propan-2-ol/0.05 M ammonium acetate fractions, as observed by SDS-PAGE (Venisse et al., 1993), and were dialysed and separated by gel filtration as previously described (Nigou et al., 1997).

**Capillary electrophoresis.** Analyses were performed on a P/ACE 5000 Capillary zone electrophoresis system (Beckman Instruments) operating in reverse mode and monitored on-column with a Beckman laser-induced fluorescence detection system. The electropherograms were acquired and stored on a Dell XPS 60 computer using the System Gold software package (Beckman Instruments).

Dried (30–60 pmol) mild hydrolysed (0.1 M HCl for 20 min at 110 °C) ManLAMs were derivatized with 1-aminopyrene-3,6,8-trisulfonate (APTS; Interchim) (Guttman et al., 1995) in the presence of mannoheptose (100 pmol) as the internal standard. The reaction was performed for 90 min at 55 °C and was quenched by adding 20 µl water. Separations were performed as previously described (Nigou et al., 2000).

**MALDI-TOF mass spectrometry experiments.** MALDI-TOF/MS (matrix-assisted laser desorption ionization/time of flight mass spectrometry) analyses were carried out on a Voyager DE-STR (Applied Biosystems) using the linear mode. Lipoglycan mass spectra were recorded in the negative mode using a 300 ns time delay with a grid voltage of 94% of full acceleration. All samples were prepared for MALDI-TOF mass spectrometry experiments.

**GC and GC/MS analysis.** GC was performed on a Girdel series 30 chromatograph equipped with an OVI capillary column (0.22 mm x 25 m) using helium gas with a flow rate of 2.5 ml min⁻¹ and a flame-ionization detector at 310 °C. The injector temperature was 260 °C and the temperature separation program was from 100 to 290 °C, rising at 3 °C min⁻¹. GC/MS analysis were performed on a Hewlett Packard 5890X mass spectrometer (electron energy, 70 eV) working in electron impact coupled to a Hewlett Packard 5890 gas chromatography series II fitted with a similar OVI column (0.30 mm x 12 m).
NMR analysis. NMR spectra were recorded with an Avance DMX500 spectrometer (Bruker) equipped with an Origin 200 SGI using Xwinnmr 2.6. Samples were exchanged in $^2$H$_2$O with intermediate freeze-drying, then dissolved in Me$_2$SO-d$_6$ (deuterated dimethylsulfoxide) and analysed in $200 \times 5$ mm 535-PP NMR tubes at 343 K. Proton chemical shifts are expressed in p.p.m. downfield from the signal of the methyl of Me$_2$SO-d$_6$ ($\delta_H$/TMS 2.52 and $\delta_d$/TMS 40.98). The one-dimensional (1-D) phosphorus ($^{31}$P) spectra were measured at 202 MHz with phosphoric acid (85 %) as the external standard ($\delta_H$/00). All the details concerning NMR sequences used and experimental procedures were detailed in previous studies (Gilleron et al., 1999, 2000; Nigou et al., 1999).

RESULTS AND DISCUSSION

Extraction and purification of ManLAM

Wild-type and $phoP$ mutant $M$. $tuberculosis$ MT103 ManLAMs were purified in accordance with the protocol previously described (Nigou et al., 1997). However, no ethanol/water extracts were performed on the delipidated mycobacteria and consequently both the so-called cellular and parietal ManLAMs were obtained in one pool. Furthermore, amphipathic mannoconjugates [ManLAMs and their hypothetical biosynthetic precursor lipomannans (LMs)] were separated from the hydrophilic polysaccharides arabinomannans using hydrophobic interaction chromatography (Leopold & Fischer, 1993) instead of the Triton X-114 phase separation system. Indeed, this latter technique appeared more suitable for large amounts of material. Finally, ManLAMs and LMs were fractionated using a Bio-Gel P-100 gel filtration column and the elution profile

controlled by SDS-PAGE. This protocol enabled the recovery of 6 and 9 mg $phoP$ mutant and wild-type $M$. $tuberculosis$ MT103 ManLAMs, respectively.

General structural features

ManLAMs arising from both $phoP$ mutant and wild-type $M$. $tuberculosis$ MT103 strains were analysed by linear MALDI-TOF/MS in negative mode (Fig. 1). The $phoP$ mutant ManLAM mass spectrum (Fig. 1a) shows one intense broad signal, approximately centred around $m/z$ 19000 assigned to pseudomolecular ions revealing a molecular mass of 19 kDa with an evaluated molecular heterogeneity of 8–9 kDa. Likewise, the wild-type ManLAM mass spectrum shows a similar signal centred at the same value (Fig. 1b). These experiments reveal that the $phoP$ mutation appears to affect neither
ManLAM molecular mass nor the observed molecular heterogeneity for these lipoglycans.

Subsequently, to gain insight into these macromolecules, 1-D $^1$H NMR spectra of ManLAMs from both strains were recorded in Me$_2$SO-$d_6$ at 343 K and compared (Fig. 2). Anomeric proton resonances were assigned from two-dimensional (2-D) homonuclear ($^1$H-$^1$H) and heteronuclear ($^1$H-$^13$C) experiments as previously described on ManLAM ( Gilleron et al., 2000; Nigou et al., 1999) and LM ( Gilleron et al., 1999). Fig. 2a shows the 1-D $^1$H NMR spectra obtained for the ManLAM arising from the $phoP$ mutant. Anomeric resonances (Fig. 2c) were tentatively assigned to the following O-linked monosaccharides: 3,5-$\alpha$-Araf (I), 5-$\alpha$-Araf (II), 2-$\alpha$-Araf (III), t-$\alpha$-Manp (IV), t-$\beta$-Araf (V), 6-$\alpha$-Manp (VI), 2-$\alpha$-Manp (VII) and 2,6-$\alpha$-Manp (VIII). A similar anomeric region was observed for the wild-type ManLAM (Fig. 2d), suggesting that the $phoP$ mutation does not affect genes implicated in the biosynthesis of ManLAM arabinomannan backbone structures.

Structure of the manno-oligosaccharide caps

The structure of manno-oligosaccharide cap motifs in both $phoP$ mutant and wild-type ManLAMs was then investigated by capillary electrophoresis (CE) analysis. Both $phoP$ mutant and wild-type ManLAMs were submitted to (i) mild acid hydrolysis (0.1 M HCl, 30 min, 110 °C), (ii) APTS tagging by reductive amination (Gutman et al., 1996) and (iii) analysis by CE monitored by laser-induced fluorescence. The electropherogram obtained for the $phoP$ mutant ManLAM is presented in Fig. 3. The peaks were assigned from APTS standards and previous CE/electrospray ionization-MS (Monsarrat et al., 1999) and CE/MALDI-TOF MS (Ludwiczak et al., 2001) studies concerning the structure elucidation of the manno-oligosaccharide cap motifs from Mycobacterium bovis BCG and M. tuberculosis H37Rv ManLAMs respectively. The peaks of interest were peaks V, VI and VII assigned to $\alpha$-d-Manp-(1$\rightarrow$5)-Ara-APTS, $\alpha$-d-Manp-(1$\rightarrow$2)-$\alpha$-d-Manp-(1$\rightarrow$5)-Ara-APTS and $\alpha$-d-Manp-(1$\rightarrow$2)-$\alpha$-d-Manp-(1$\rightarrow$2)-$\alpha$-d-Manp-(1$\rightarrow$5)-Ara-APTS respectively (Fig. 3). The relative abundance of the different cap motifs was directly determined by integration of these peaks, revealing that the major structural motif was the dimannosyl unit (75%), while the mannosyl and the trimannosyl ones only represented 12% and 13% respectively. Moreover, an accurate cap motif quantification was determined as precise ManLAM-solution molarity was corrected from the knowledge of ManLAM molecular mass determined through MALDI-TOF MS as previously described (Nigou et al., 2000). The mono-, di- and trimannoside motifs were found at 0.8, 4.9 and 0.9, respectively, per ManLAM molecule for the $phoP$ mutant strain. Likewise, the electropherogram obtained after mild acid hydrolysis and APTS-tagging of the wild-type ManLAM (not shown) revealed identical cap structures. Moreover, the three manno-oligosaccharide cap motifs were found with the same relative abundance as those observed for the $phoP$ mutant strain (12%, 75% and 13% for the mono-, di- and the trimannoside motif respectively) as well as the number of each cap per ManLAM molecule (0.8, 4.6 and 0.8 for the mono-, di- and the trimannoside motifs). Thus, even if it is now well established that the ManLAM manno-oligosaccharide caps are significantly involved in the biological activities of the ManLAM molecules, the $phoP$ gene seems not to be implicated in the regulation of the genes involved in the biosynthesis of the ManLAM capping motifs.

MPI anchor characterization

To determine the nature of the acyl groups present on the MPI anchor, ManLAM from the two strains were submitted to alkalineolysis and the fatty acids liberated were then analysed as their methyl ester derivatives by GC and GC/MS analysis. Palmitic and tuberculostearic acids were the major fatty acids liberated, but heptadecanoic and stearic acids were also recovered in small amounts in both strains, thus indicating no significant difference between ManLAM from the $phoP$ mutant and the wild-type strains. In addition, the presence of hydroxylated fatty acids was investigated but not found in the $phoP$ mutant ManLAM. It is interesting to note that similar investigations with an S. typhimurium PhoplPhoQ mutant led to the identification of the addition of 2-hydroxy-myristate to the lipid A portion of the LPS. Thus, in M. tuberculosis MT103 the $phoP$ gene does not appear to be involved in the control of genes implicated in ManLAM fatty acid modification.

The phosphatidyl-myo-Ins (phosphatidyl-myo-inositol) anchor structure of wild-type and $phoP$ mutant ManLAM were consequently investigated from 1-D and 2-D $^{31}$P NMR experiments. We have recently shown that the multiacylated structures of M. bovis BCG
ManLAMs and LMs can be efficiently displayed through 1-D $^{31}\text{P}$ NMR experiments by the use of Me$_2$SO-d$_6$ as a solvent system (Gilleron et al., 1999; Nigou et al., 1999). 1-D $^{31}\text{P}$ spectra of both phoP mutant and wild-type ManLAMs dissolved in Me$_2$SO-d$_6$ were thus recorded. The 1-D $^{31}\text{P}$ spectrum of the phoP mutant ManLAM (Fig. 4a) showed three signals, at 1.65 p.p.m., 1.83 p.p.m. and 3.54 p.p.m., attributed from their chemical shifts to P1, P3 and P5 and respectively assigned to ManLAM containing at least a tri-, di- and mono-acylated MPI anchor in agreement with our previous results (Gilleron et al., 2000; Nigou et al., 1999). Similarly, the 1-D $^{31}\text{P}$ spectrum obtained for the wild-type ManLAM (Fig. 4b) exhibited the same three resonance signals P1, P3 and P5 with almost identical chemical shifts ($\delta$ 1.67, $\delta$ 1.83 and $\delta$ 3.52 p.p.m., respectively). From these 1-D $^{31}\text{P}$ experiments it can be assumed that both phoP mutant and wild-type ManLAMs share the same acyl-form structures assigned to at least tri-, di- and mono-acylated MPI anchor. Nevertheless, the relative abundance of the different acyl forms, determined by their $^{31}\text{P}$ signal integration, was found to be different between the ManLAMs of the two strains. Indeed, whereas the abundance P1, 36%, P3, 55%, P5, 9% was observed for the wild-type ManLAM acyl forms (Fig. 4b), the determined ratio for the phoP mutant ManLAM acyl forms was P1, 25%, P3, 52%, P5, 23% (Fig. 4a). These data highlighted a decrease of the triacylated form and an increase in the monocacylated form, respectively typified by the P1 and the P5 resonances, for the phoP mutant compared to those observed for the wild-type strain.

From previous studies, four acylation sites were identified in ManLAMs, i.e. positions 1 and 2 of glycerol, position 6 of the Manp linked to C-2 of myo-Ins (Khoo et al., 1995; Nigou et al., 1997) and finally position 3 of myo-Ins (Nigou et al., 1999). To confirm these assignments, the acylation sites of the ManLAM from both strains were investigated with a strategy based on 2-D $^1\text{H}$-$^{31}\text{P}$ HMQC (heteronuclear multiple quantum correlation spectroscopy) and HMQC-HOHAHA (HMQC/heteronuclear Hartmann–Hahn spectroscopy) NMR experiments developed for structural elucidation of M. bovis BCG (Nigou et al., 1997) and M. tuberculosis H37Rv (Gilleron et al., 2000) ManLAM acyl forms. However, as previously noticed, this NMR approach failed to locate fatty acyl appendages on the Manp unit of the MPI anchor.

The P1 and P3 resonances of wild-type and phoP mutant ManLAM were assigned to at least tri- and diacylated ManLAM. The glycerol spin systems of P1 and P3 acyl forms were attributed by $^1\text{H}$-$^1\text{H}$ HOHAHA and $^1\text{H}$-$^{31}\text{P}$ HMQC experiments. The $^1\text{H}$-$^{31}\text{P}$ HMQC-HOHAHA spectra (Fig. 5) show the downfield resonances of glycerol methine protons H-2, at $\delta$ 5.10 and 5.12 p.p.m. for P1 and P3 of wild-type (Fig. 5b) and 5.11 and 5.12 p.p.m. for P1 and P3 of phoP mutant (Fig. 5a), revealing that P1 and P3 ManLAM acyl forms share 1,2-diacyl-3-phospho-sn-glycerol units. Likewise, the myo-Ins spin systems belonging to the MPI anchor characterized by P1 and P3 were then identified. The P1 and P3 myo-Ins H-1 resonances at 4.12 and 4.02 p.p.m. of both phoP mutant and wild-type ManLAMs were attributed from $^1\text{H}$-$^{31}\text{P}$ HMQC experiments. The remaining protons were identified from $^1\text{H}$-$^{31}\text{P}$ HMQC-HOHAHA and $^1\text{H}$-$^1\text{H}$ HOHAHA experiments and assigned from their multiplicity and $^3\text{J}_{^1\text{H}_-^1\text{H}}$ coupling constant values. The P3 myo-Ins spin system of the phoP mutant ManLAM was
Fig. 5. Expanded region (δ 1H, 300–530 and 31P, 1–40 p.p.m.) of the 31P-decoupled, 1H-detected HMQC-HOHAHA spectrum of ManLAM of the phoP mutant (a) and the wild-type (b) M. tuberculosis MT103 dissolved in Me2SO-d6 at 343 K. Numerals correspond to the proton number of the myo-Ins unit and numerals with letter G, to the proton number of the glycerol unit.

Table 1. Proton chemical shifts of myo-Ins and glycerol units from ManLAMs of the phoP mutant and the wild-type M. tuberculosis MT103 MPI anchors characterized by P1, P3 and P5 phosphates

<table>
<thead>
<tr>
<th>Phosphatase Site</th>
<th>myo-Ins (δ p.p.m.)</th>
<th>Glycerol (δ p.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H-1</td>
<td>H-2</td>
</tr>
<tr>
<td>phoP mutant ManLAM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>4.12</td>
<td>4.17</td>
</tr>
<tr>
<td>P3</td>
<td>4.02</td>
<td>4.15</td>
</tr>
<tr>
<td>P5</td>
<td>4.01</td>
<td>4.17</td>
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<tr>
<td>Wild-type ManLAM</td>
<td></td>
<td></td>
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<tr>
<td>P1</td>
<td>4.12</td>
<td>4.18</td>
</tr>
<tr>
<td>P3</td>
<td>4.02</td>
<td>4.16</td>
</tr>
<tr>
<td>P5</td>
<td>4.01</td>
<td>4.18</td>
</tr>
</tbody>
</table>

Chemical shifts were recorded in Me2SO-d6 at 343 K, expressed in p.p.m. and referenced relative to the internal standard signal at 2.52 p.p.m. of the Me2SO. ND, Not determined.

found in agreement with a non-acylated phospho-myol-Ins due to the myo-Ins proton chemical shifts (see Table 1). Likewise, the P1 myo-Ins spin system was identified (see Table 1). Compared to the chemical shift of the P3 myo-Ins H-3 (δ 3.25 p.p.m.), the deshielding of the P1 myo-Ins H-3 (δ 4.59 p.p.m.; δ +1.34 p.p.m.) unambiguously indicated that the myo-Ins unit characterized by the P1 resonance was acylated on C-3. These data were also in agreement with our own previous work done on the tetra-acylated forms of PIM3 and ManLAM from M. bovis BCG and M. tuberculosis H37Rv ManLAMs (Gilleron et al., 1999, 2000; Nigou et al., 1999). Quite similar chemical shifts were also observed for the P1 and P3 myo-Ins spin systems of the wild-type ManLAM (Table 1). These data confirmed that P1 typified an at least triacylated ManLAM anchor comprising a diacylated glycerol and a myo-Ins acylated on C-3 while P3 characterized an at least diacylated ManLAM anchor.
Table 2. Proton chemical shifts of 5-methylthiopentose (5-MTP) (spin system IX, Fig. 2c, d) and 5-methylsulfoxypentose (5-MSP) (spin systems X and XI, Fig. 2c, d) in phoP mutant and wild-type ManLAMs.

<table>
<thead>
<tr>
<th>Chemical shift (p.p.m.)</th>
<th>H-1</th>
<th>H-2</th>
<th>H-3</th>
<th>H-4</th>
<th>H-5a</th>
<th>H-5b</th>
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</thead>
<tbody>
<tr>
<td><strong>phoP mutant ManLAM</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>MTP IX</td>
<td>5.24</td>
<td>3.92</td>
<td>3.99</td>
<td>4.19</td>
<td>2.70</td>
<td>2.53</td>
</tr>
<tr>
<td>MSP-1 XI</td>
<td>5.29</td>
<td>3.92</td>
<td>4.05</td>
<td>4.52</td>
<td>3.10</td>
<td>2.84</td>
</tr>
<tr>
<td>MSP-2 X</td>
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<td>3.93</td>
<td>4.05</td>
<td>4.44</td>
<td>2.91</td>
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<tr>
<td><strong>Wild-type ManLAM</strong></td>
<td></td>
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<tr>
<td>MTP IX</td>
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</table>

harbouring a diacylated glycerol in both strains. It is noteworthy that the presence of a fatty acyl appendage on the Manp unit cannot be excluded.

The ManLAM anchor typified by the P5 resonance was assigned to a 1-acyl-2-lyso-sn-3-phosphoglycerol unit. Indeed, in both strains, the absence of an acyl residue on the C-2 of the glycerol unit was unambiguously determined as no correlation peak was observed with methine protons around 5-10 p.p.m. (Fig. 5). Moreover, the chemical shifts of the glycerol protons of P5 (Table 1) were in total agreement with those obtained in a previous study on a standard, 1-α-lysophosphatidylinositol in Me2SO-d6 [1-acyl-2-lyso-sn-glycero-3-phospho-(1-d-mylo-Ins), δH/H 1/1' 3.99/3.97 p.p.m., δH 1/2 3.75 p.p.m., δH 2/3/4 3.78/3.76 p.p.m.] indicating that the glycerol was monoacylated on C-1 (Nigou et al., 1999). From the chemical shifts of the myo-Ins protons (Fig. 5a, Table 1), the absence of the fatty acyl appendage on the myo-Ins unit was confirmed.

In summary, these results demonstrate that both phoP mutant and wild-type M. tuberculosis MT103 ManLAM share the same anchor structure typified by three major resonances in 31P NMR, i.e. P1, P3 and P5 and assigned to at least tri-, di- and monoacylated ManLAM. Nevertheless their relative abundance was found to be different with an increase of the monoacylated form in the phoP mutant compared to the wild-type strain.

5-Methylthiopentose presence

The presence of 5-methylthiopentose was recently shown in M. tuberculosis H37Rv ManLAM (Treumann et al., 2002) and it has been postulated that these
particular substituents could be implicated in some of the biological properties of the ManLAM molecules. The presence of such residues was then investigated in both phoP mutant and wild-type ManLAM. In the anomic region of 1-D $^1$H NMR spectra of both strains, H-1 signals corresponding to 5-methylthiopentose at δ 5.24 p.p.m. and the oxidation products 5-methylsulfoxypentose at δ 5.28 and δ 5.29 p.p.m. were observed. The corresponding complete spin systems were then identified in the HOHAHA spectrum of the phoP mutant (Fig. 6, Table 2) and wild-type ManLAMs (Table 2), in agreement with literature data (Treumann et al., 2002). So, both phoP mutant and wild-type ManLAM share 5-methylthiopentose residues. So it seems that these particular monosaccharides are not implicated in the different growth rates observed between the two strains and that their biosynthesis is not under the control of the phoP system disrupted here.

Conclusion

In the present study, we investigated the structure of ManLAM, the major non-peptidic antigen of mycobacterial cell walls, of M. tuberculosis MT103 phoP mutant. This mutant strain was demonstrated to have reduced viability in macrophages and mice compared to the wild-type strain. We found that the phoP mutation altered neither the molecular mass and arabinomannan backbone structures of the ManLAM molecules nor the type and relative abundance of the fatty acids harboured on the MPI anchor. Moreover, no difference was found in the mannooligosaccharides caps between the mutant and the wild-type strains. Both strains were also demonstrated to harbour 5-methylthiopentose residues to identical extents on ManLAM molecules. Nevertheless, based on $^{31}$P NMR experiments, the relative abundance of the different ManLAM acyl forms was shown to be different, with an increase in the mono-acylated form in the mutant strain. This change is noteworthy as the monoacylated form was found to be the minor one in the ManLAM of M. tuberculosis MT103 and H37Rv (Gilleron et al., 2000) wild-type strains. These results showed that, in M. tuberculosis MT103, the phoP gene is not implicated in a drastic structural modification of ManLAM but could nonetheless be implicated in a modulation of the acyl forms resulting in an adaptation to the environment faced by the mycobacteria. Indeed, it was recently established that monoacylated ManLAM, contrary to multiacetylated ManLAM, failed to inhibit IL-12 production by human dendritic cells (Nigou et al., 2001). It can thus be postulated that the disruption of the phoP gene could decrease the ManLAM capacity to downregulate the inhibitory activity of IL-12. Consequently, this could participate in the impaired growth of the mutant strain observed when cultured in bone marrow-derived macrophages or in a mouse infection model (Perez et al., 2001).

This work represents a first step in the search for a better understanding of the biological processes which allow the persistence of mycobacteria in the infected host cells. Other components of the mycobacterial envelope will be next investigated to evaluate the impact of the disruption of such an environment-sensing system.

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