SUPPLEMENTAL MATERIAL: LIPIDS ANALYSES

MATERIALS AND METHODS

Lipids extraction. Lipids from bacterial cells grown as surface pellicle on Sauton's fluid medium were extracted with mixtures of $CHCl_3$ and CH_3OH according to standard protocols as described previously (1,2).

Mild alkaline deacylation of lipids. In order to eliminate alkali-labile lipids such as TAG, phospholipids and trehalose esters, lipid fractions were suspended in $CHCl_3$/CH_3OH (2:1 v/v) and incubated at 37°C for 60 min with an equal volume of 0.2 M NaOH in methanol for mild alkaline deacylation. The mixture was neutralized with glacial acetic acid, then concentrated and finally dissolved in $CHCl_3$. The organic phase was washed with water and dried.

Saponification of lipids. Strong saponification of lipids was realized with 5% KOH in methoxyethanol at 110°C for 3 hours. After acidification, the resulting fatty acids were extracted with diethyl ether and methylated by the addition of a diazomethane solution in ether. The resulting esters were analysed both by TLC with CH_2Cl_2 as running solvent and characterized by their Mc-Lafferty rearrangements (*m/z* 74 or m/z 88 for methyl branch esters) using a gas chromatography/mass spectrometry (GC/MS) apparatus.

Acid methanolysis of lipids for cleavage of osidic bonds. Acid methanolysis of lipids was realized in a screw-capped tube using 1M CH_3OH/HCl (prepared by the acetyl chloride reaction on methanol in anhydrous conditions) at 80°C overnight under nitrogen atmosphere. After evaporation by successive additions of methanol and drying and after the separation on preparative TLC, the aglycone lipid was purified and analysed by MALDI mass spectrometry.

Trimethylsilyl derivatisation of hydroxyl functions (*TMS).* TMS was performed as described previously (3). Lipid samples were dissolved in 200 µl of anhydrous pyridine

followed by the addition of 100 µl hexamethyldisilazane and 50 µl of trimethylchlorosilane. The mixture was incubated at room temperature for 30 min and then dried under nitrogen. The derivatives were solubilised in petroleum ether for GC and GC/MS analyses.

Lipid purification and visualisation. Intact and deacylated lipids from mild alkaline treatment were first comparatively analysed by thin-layer chromatography (TLC) Silica Gel 60 plates (Macherey-Nagel) in various solvent systems, mainly petroleum ether/diethyl ether 9:1 (v/v) for phthiocerol dimycocerosates (PDIM) and triacyl glycerol (TAG) and $CHCl_3/CH_3OH$ 9:1 (v/v) for phenol glycolipid (PGL). Visualisation was performed using 10% phosphomolybdic acid and charring for PDIM and 0.2% anthrone solution in concentrated H_2SO_4 followed by heating for PGL (4).

In order to allow further characterizations, purification of complex lipids as PDIM and PGL was realized by Florisil column chromatography followed by preparative TLC and infrared (IR) spectroscopy. For apolar lipids such as DIM, the samples were dissolved in petroleum ether and loaded onto a Florisil column prepared in petroleum ether and compounds were eluted with a gradient of diethyl ether in petroleum ether. For glycolipids, the samples were dissolved in $CHCl_3$ and lipids were eluted with increasing concentrations of methanol in $CHCl_3$, the major phenol glycolipid of BCG being eluted with $CHCl_3/CH_3OH$ 95:5 (v/v). The separation was followed on thin-layer chromatography (TLC).

We also performed lipids labelling using [^{14}C]acetate (Amersham). After 16 h incorporation in 30 ml Sauton's fluid medium containing 1.5% glycerol and 1·2 MBq sodium [^{14}C]acetate (Amersham) and mid-exponential-phase surface-pellicle grown bacteria, the reaction was stopped by centrifugation and washing. The lipids were isolated by extraction with glass beads as previously described. The ^{14}C-labelled lipids were separated on TLC and visualised with a Typhoon PhosphorImager (Amersham Biosciences).

Instrumentation for lipid characterization

GC/MS analyses were performed in the electron-impact mode on an HP5889X mass spectrometer (electron energy, 70 eV) coupled to an HP5890 series II gas chromatograph fitted with OV1 capillary column (0.30 mm x 25 m) using helium gas. The injector temperature was set at 260°C and the temperature separation program increased from 100°C to 300°C at the rate of 5°C min^{-1} followed by 10 min at 300°C

^{1}H-Nuclear magnetic resonance spectroscopy experiments were carried out at 296°K on a on a Bruker AVANCE spectrometer operating at 600,13 MHz with a 5mm triple resonance TCI ^{1}H ^{13}C ^{15}N pulsed field z-gradient cryoprobe. Samples were dissolved in 99.9% $CDCl_3$. Chemical shifts are expressed in ppm using the chloroform signal as an internal reference (7.23 ppm).

Infrared spectra of samples placed as films on NaCl discs were recorded using a Perkin-Elmer Life Sciences Fourier transform IR 1600 spectrometer.

Purified molecules were analysed by matrix-assisted laser desorption-ionisation time-of-flight (MALDI TOF) mass spectrometry using the positive mode, as previously described (1, 5). Spectra were acquired in reflectron mode with an Applied Biosystems 4700 Analyzer mass spectrometer (Applied Biosystems, Voyager DE-STR, Framingham, MA) equipped with an Nd:YAG laser (wavelength 355 nm; pulse <500ps; repetition rate 200Hz). A total of 2500 shots were accumulated and mass spectrometry data were acquired with the default calibration for the instrument. The matrix was 2,5-dihydroxybenzoic acid (10 mg mL^{-1} in $CHCl_3$/CH3OH 1:1, v/v).

RESULTS

Data from TLC analyses and radio-TLC of ^{14}C-acetate labelled lipids indicated a similar composition in ubiquitous lipids in the *Δcpn60.1* mutant and wild type (WT) strain except for the absence of phthiocerol derivative waxes, the so-called PDIM A and B for phthiocerol and phthiodiolone dimycocerosates, in the *Δcpn60.1* mutant (data not shown). A complete

analysis of the extractable lipid of wild type, Δ*cpn*60.1 and complemented strains was performed in order to understand the cause of the alterations of the cell wall integrity.

Identification of waxes was realized by MALDI-TOF MS on the lipid extract after mild alkaline methanolysis in order to eliminate ubiquitous alkali-labile lipids such as glycerides and phospholipids. The remaining alkali-stable lipids (liberated mycolic acids, PDIM, and phenolic glycolipid PGL) were easily characterized by MALDI-TOF MS.

As shown in Fig. S1A, two main clusters of molecular ions with sodium adducts $[M+Na]^+$ were observed in the mass spectrum of the WT strain, one from *m/z* 1174 to 1390 and another one from *m/z* 1446 to 1572. The first cluster contained mycolic acid methyl esters (α-mycolates and keto-mycolates), diacyl phthiocerol (DIM A), and diacyl phthiodiolone (DIM B). The values at *m/z* 1146, 1174 were identified as α-mycolic acid methyl esters with C76 and C78 chain length and the values at *m/z* 1246 to 1330 were identified as free keto-mycolate with C82 and C88 chain length, a mycolic acid composition expected for *M. bovis* BCG. As determined by comparison with M. *canetti* lipids (data not shown), the same region contained peaks corresponding to the PDIM, diacyl phthiocerol (PDIM A) at *m/z* 1306, 1320, 1334, 1348, 1362, 1390, and diacyl phthiodiolone (PDIM B) at *m/z* 1290 to 1388 (spaced by 14 mass units)(6). The second series of peaks corresponded to the mass values of *M. bovis* phenolglycolipids (PGL).

The mass spectrum of the Δ*cpn*60.1 mutant strain was simpler (Fig. S1B). A series of peaks corresponding to mycolic acids methyl esters were observed, at *m/z* 1174 and 1202 for C78-C80 free acids α-mycolates and at *m/z* 1274 and 1302 for the C84 and C86 keto-mycolates major homologues. The longer chain lengths in α-mycolates (2 extra carbons) were in agreement with our previous data (7). A series of minor peaks at higher masses values (m/z 1642-1698) were also detected. The mass spectrum of the lipid extract of the Δ*cpn*60.1 mutant strain clearly confirmed the absence of intact *M. bovis* PDIM and PGL.

Interestingly, the phenotype was completely restored in the complemented strain *i.e*. the presence of PDIM and PGL (Fig S1C), indicative that Cpn60.1 is involved in the synthesis of these waxes.

To elucidate the nature of the minor peaks at higher masses values observed in the mutant strain (Fig. S1B), a more precise analysis of the glycolipid composition of the mutant strain was undertaken and the anthone-positive (sugar-containing) compound was isolated by TLC and analyzed by ^{1}NMR (data not shown) and MALDI-TOF mass spectrometry (Fig. S1D and E). The one-dimensional ^{1}H-NMR spectrum of the glycolipid was similar to that of the *M. bovis* BCG PGL, structurally related to mycoside B. MALDI-TOF mass spectrometry analyses of the purified PGL from the WT and mutant strains exhibited different profiles. A series of peaks at *m/z* 1390 to 1600 was observed for WT and the complemented strains while peaks at higher mass values, around *m/z* 1600 to 1726, were observed for the mutant strain. The mass spectra of mycoside B from *M. bovis* BCG showed a series of molecular ion [M+Na]+ at 1446, 1460, 1474, 1488, 1502, 1516, 1530, 1544, 1588, 1572. For the glycolipid of the mutant, peaks were seen at *m/z* 1614, 1628, 1642, 1656, 1670, 1684 and 1698, in agreement with the additional mass values observed in the total mass spectrum of the lipid from the mutant. Comparative analyses of MALDI-TOF mass spectra performed after acid methanolysis of WT and mutant lipids showed the loss of 160 mass units corresponding to the departure of the 2-*O*-methyl rhamnose as characterized by GC/MS analysis and confirmed by a shift of 72 amu after silylation. This showed that the mass difference in the mutant concerned the lipid moieties of the glycolipid. By comparison with the PGL aglycone from *M. canettii* (6), the glycolipid from the mutant was found to correspond to the higher mass values of the aglycone PGL. From all these data, it was concluded that the mycoside B-like glycolipid isolated from the mutant strain consists of a phenolphthiocerol core with chain

126 lengths that are 6-9 carbon atoms longer than those of the WT and complemented strains and
127 bearing a 2-*O*-methyl rhamnosyl moiety and polymethyl-branched fatty acyl residues.
128

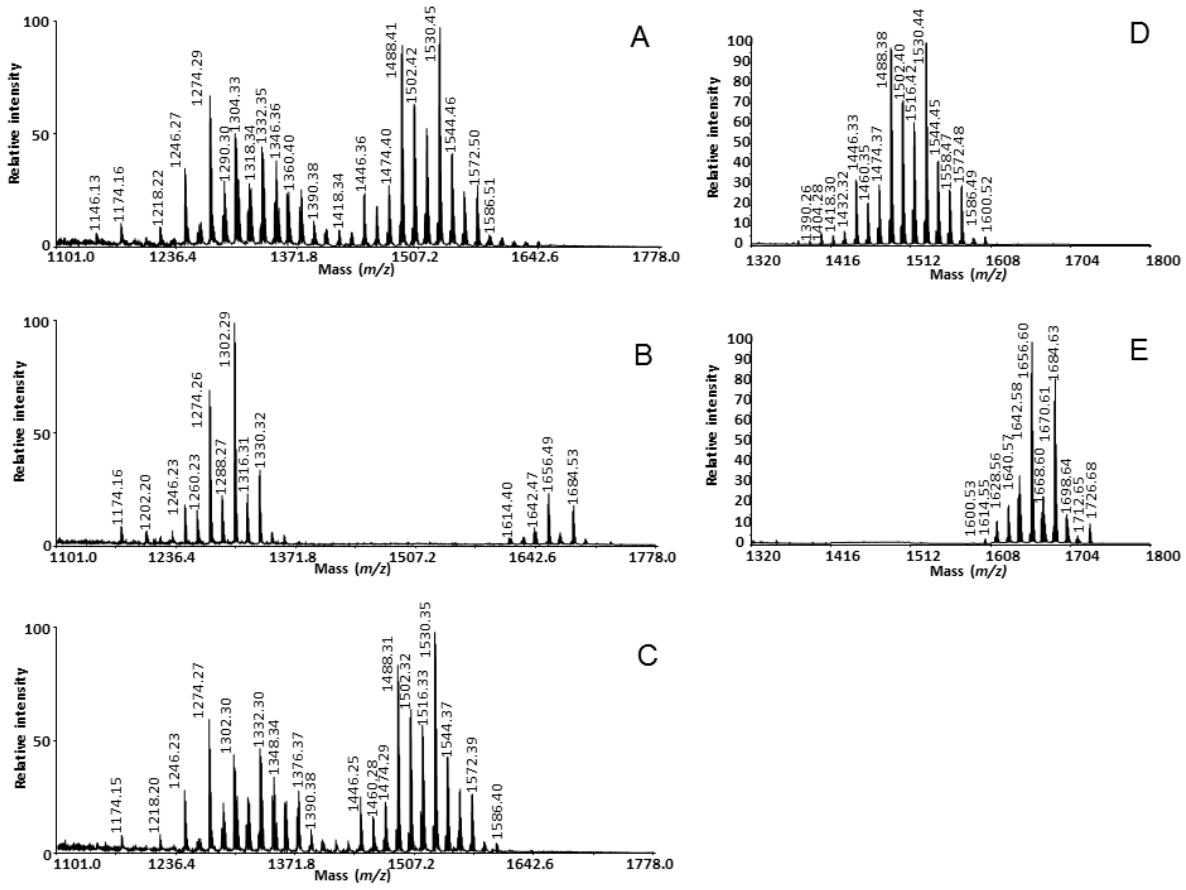

FIG S1 The lack of Cpn60.1 in a *M. bovis* BCG strain inhibits PDIM and PGL synthesis. Comparative analyses of MALDI-TOF mass spectra of alkali-stable lipids in WT (A, D), Δ*cpn*60-1 (B, E) and complemented (C) *M. bovis* BCG strains.

(A and C): m/z 1174, 1202 for C76 and C78 α-mycolates; m/z 1274, 1302, 1330 for C82 and C88 keto-mycolates,; diacyl phthiocerol (DIM A) at m/z 1306, 1320, 1334, 1348, 1362, 1390) and diacyl phthiodiolone (DIM B)at m/z 1290 to 1388.

(B): m/z 1146 to 1230 for C78 and C80 α-mycolates and m/z 1246 to 1330 for C84 and C86 keto-mycolates.

(A, C, D) mycoside B at m/z 1446, 1460, 1474, 1488, 1502, 1516, 1530, 1544, 1588, 1572.

(B, E) mycoside B at *m/z* 1614, 1628, 1642, 1656, 1670, 1684 and 1698.

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