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Subfractionation and analysis of the cell envelope (lipo)polysaccharides of *Mycobacterium tuberculosis*

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Abstract

The cell envelope of *Mycobacterium tuberculosis*, the causative agent of tuberculosis in humans, is the source of carbohydrates of exceptional structure which play essential roles in the physiology of the bacterium and in its interactions with the host during infection. Much of what is known about their biosynthesis was derived from the phenotypic analysis of knock-out or conditional knock-out mutants of *Mycobacteria* generated by random or specific insertional mutagenesis. Here, we describe the current techniques used to subfractionate *M. tuberculosis* cells and investigate major quantitative and qualitative changes in their cell envelope (lipo)polysaccharides.

Keywords

Mycobacterium tuberculosis; arabinogalactan; lipoarabinomannan; lipomannan; glucan; capsule

1. Introduction

The structure and composition of the cell envelopes of *Mycobacterium tuberculosis* and other *Mycobacteria* differentiate these microorganisms from other prokaryotes (reviewed in ref 1). In particular, it contains a number of unique (lipo)polysaccharides which have been the focus of much attention both from the perspective of drug development and understanding the host-pathogen interactions underlying the characteristic immunopathology of tuberculosis (1,2-5). In spite of being Gram positive microorganisms, *Mycobacteria* are endowed with an inner (IM) and an outer (asymmetrical) membrane (OM) separated by a periplasmic space (6-7) (Fig. 1). Beyond the IM, a covalently-linked complex consisting of mycolic acids, the heteropolysaccharide arabinogalactan (AG) and peptidoglycan (PG), constitutes the ‘core’ of the cell wall. Interspersed somehow within this structure are the free (glyco)lipids of the OM with their fatty acyl chains intercalating into the mycolic acid layer. Lipomannan (LM) and lipoarabinomannan (LAM), two lipopolysaccharides (also referred to as ‘lipoglycans’) present in abundant quantities in the cell envelope of mycobacteria, possess a mannosylated phosphatidyl-myo-inositol moiety that mediates their non-covalent anchoring to the IM and OM (8) (Fig. 1). Finally, the outermost compartment of the cell envelope of *M. tuberculosis* consists of a loosely bound structure, referred to as ‘capsule’,

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which is essentially composed of polysaccharides and proteins (9-11) (Fig. 1). The three major capsular polysaccharides are: a high molecular weight α -D-glucan with a structure similar to that of glycogen (12) (see Note 1), a D - arabino- D -mannan (AM), and a D - mannan (1). The D-arabino-D-mannan and a D-mannan share structural resemblance with some segments of LM and LAM and are thought to be metabolically-related (Fig. 2). Likewise, the arabinan domains of AG and that of LAM are structurally very similar and their biosynthesis involves the participation of common enzymes (Fig. 2).

The remarkable progress that was made over the last 15 years toward the elucidation of the biosynthetic pathways of *M. tuberculosis*' unique carbohydrates has heavily relied on genetic approaches to create mutants deficient in various aspects of their synthesis (reviewed in refs. 1,5,13). The phenotypic characterization of these mutants has allowed much of the glycosyltransferases involved in the biosynthesis of virulence-associated glycolipids, LM, LAM and AG to be identified (1). How these macromolecules - which are synthesized in part in the cytosol - reach their final periplasmic or OM location is less well documented and remains an area of intensive investigation. This chapter describes the current subfractionation and analytical methods that are used to investigate quantitative and qualitative changes in the capsular, cell wall- and IM/OM-associated (lipo)polysaccharides of *M. tuberculosis* (see Note 2). The analysis of *M. tuberculosis* (glyco)lipids and the detailed structural characterization of (lipo)polysaccharides are out of the scope of this review.

2. Materials

2.1. Isolation of the capsular material

1. *M. tuberculosis* H37Rv, frozen stock.
2. Gas medium: add 0.3 g of Bacto casitone, 0.05 g of Ferric ammonium citrate, 4.0 g of K_2HPO_4 , 2.0 g of Citric acid, anhydrous, 1.0 g of L-alanine, 1.2 g of $MgCl_2 \cdot 6H_2O$, 0.6 g of K_2SO_4 and 2.0 g of NH_4Cl to 800 ml of Milli-Q water. Adjust pH to 6.8 with 10 M NaOH. Add 10 ml of glycerol and bring volume to 1 L with Milli-Q water. Sterilize by autoclaving on liquid cycle at 121°C for 15 min.
3. Glass beads (4 mm-diameter).
4. Vacuum filtration system (0.22 μ m).
5. 50-mL Falcon tube.

2.2 Preparation of lipoglycans (LAM and LM)

1. *M. tuberculosis* H37Rv, frozen stock.
2. Gas medium (see Subheading 2.1, item 2).

¹Although mycobacterial glycogen is very similar in structure to capsular α -D-glucan, glycogen is localized intracellularly and the capsular α -D-glucan will thus readily be separated from glycogen using the subfractionation procedure with glass beads described under Subheading 3.1. A description of how intracellular glycogen may be prepared can be found in ref. 12.

²Although this chapter refers of *M. tuberculosis*, the same procedures can be used to analyze the cell envelope (lipo)polysaccharide content of other *Mycobacterium* species.

3. Chloroform:methanol:water (10:10:3, v/v/v).
4. Endotoxin free water (commercially available)
5. Endotoxin-free phosphate buffer saline (PBS) (commercially available)
6. 32% Triton X-114 solution in sterile endotoxin free PBS.
7. Breaking buffer: add 70 μ l of 1 mg/ml Pepstatin A, 50 μ l of 1mg/ml Leupeptin, 200 μ l of 1 mg/ml PMSF, 150 μ g of DNase I, 165 μ g of RNase I to 100 ml of 8% Triton X-114.
8. Acid fast staining reagent.
9. Cold 95% ethanol.
10. Proteinase K solution: 2 mg/ml in 10 mM Tris-HCl (pH 7.5), 20 mM CaCl₂, 50% (v/v) glycerol.
11. Slide-A-Lyzer Casette 3.5 kDa MWCO (molecular weight cut off).
12. Vacuum concentrator, speed - vac.
13. Air bath.
14. Adjustable tilt rocking platform shaker.
15. French Press.
16. Oakridge Teflon FEP tubes
17. 7H10-OADC agar, (commercially available).
18. 13×100 mm glass tubes.
19. 200-ml Falcon tubes.
20. 4-L conical flasks.
21. High- and low-speed centrifuges and rotors.

2.3 Separation of lipoglycans (LAM and LM)

1. Lipoglycans extracted from *M. tuberculosis* H37Rv (~15 mg dry weight).
2. Column running buffer: 10 mM TRIS-HCl, 0.2M NaCl, 0.02% (w/v) sodium azide, 0.25%(w/v) deoxycholic salt and 1 mM EDTA, pH 8.0.
3. Dialysis buffer: 10 mM TRIS-HCl, 0.2M NaCl, 0.02% (w/v) sodium azide and 1mM EDTA, pH 8.0.
4. 1 M NaCl
5. Endotoxin free water.
6. 0.22 μ m syringe filters.
7. 10 ml syringe.
8. High performance liquid chromatography system (HPLC).

9. Sephacryl S-100HiPrep 16/60 column.
10. Sephacryl S-200HiPrep 26/60 column.
11. Fraction collector.
12. Spectra/Por Dialysis Membranes (6-8 kDa MWCO and 12-14kDa MWCO).

2.4 Tricine SDS/Polyacrylamide Gel Electrophoresis of lipoglycans followed by Periodic Acid Silver Staining

1. 5 to 10 µg of lipoglycans.
2. PageRuler Prestained Protein Ladder, 10-170 kDa.
3. 10-20 % gradient Tricine SDS/polyacrylamide gels, commercially obtained.
4. 10X Tricine SDS running buffer, commercially obtained.
5. 2X Tricine SDS sample buffer and 10X Reducing agent, commercially obtained.
6. First fixative: 40 % (v/v) methanol, 10 % (v/v) acetic acid in distilled water.
7. Second fixative: 5 % (v/v) methanol, 7 % (v/v) acetic acid in distilled water.
8. 0.7 % (w/v) periodic acid solution in the first fixative.
9. 2.5 % (v/v) glutaraldehyde solution in distilled water.
10. 0.0025 % (w/v) α-dithiothreitol solution (DTT) in distilled water.
11. 0.1 % (w/v) silver nitrate solution in distilled water.
12. 3% (w/v) sodium carbonate solution in distilled water.
13. 37 % (v/v) formaldehyde solution in distilled water
14. Developer solution: to 200 ml of 3% sodium carbonate add 100 µl of 37% formaldehyde.
15. 50 % (w/v) citric acid solution in distilled water
16. Adjustable tilt rocking platform shaker.

2.5 Preparation of the mycolate-arabinogalactan peptidoglycan (mAGP) complex

1. Cell walls from *M. tuberculosis* H37Rv (i.e., remaining pellet after lipoglycans extraction, see Subheading 3.2 step 18).
2. Oakridge Teflon FEP tube.
3. 2% SDS in PBS.
4. Proteinase K solution: add 10mg in 1ml of 10 mM Tris-HCl (pH 7.5), 20 mM CaCl₂, 50% (v/v) glycerol.
5. Acetone.
6. Magnetic stir/heat plate.

7. High-speed centrifuges and rotors.

2.6 Preparation of arabinogalactan (AG)

1. Purified mAGP (50 to 150 mg dry weight).
2. Oakridge Teflon FEP tube.
3. 0.5% KOH in methanol.
4. Methanol.
5. Chloroform.
6. 0.05 M sulfuric acid.
7. Barium carbonate.
8. Vacuum concentrator, speed - vac.
9. Air bath.
10. Magnetic stir/heat plate.
11. 16x100 mm glass tubes.
12. High-speed centrifuges and rotors.

2.7 Analysis of monosaccharide composition by Gas Chromatography/Mass spectrometry

1. 100 µg/ml of monosaccharide standards stock solutions (arabinose, fucose, galactose, glucose, mannose, rhamnose, ribose, xylose and *myo* – inositol) in Milli-Q water
2. 100 µg/ml of 3-*O*-methyl-glucose (internal standard) in MilliQ water.
3. 13 × 100 mm glass tubes with Teflon lined lids.
4. 2 M Trifluoroacetic acid (TFA) in Milli-Q water.
5. Reducing agent: 10 mg/ml sodium borodeuteride in 1 M aqueous ammonium hydroxide/95% ethanol (1:1, v/v).
6. Acetic acid, glacial.
7. Acetic anhydride.
8. Chloroform.
9. Methanol.
10. Vacuum concentrator, speed - vac.
11. Heat block.
12. Air bath.
13. CP 3800 gas chromatograph equipped with an MS320 mass spectrometer and fitted with a DB 5 column (30 m × 0.20 mm i.d.).

14. Low speed centrifuge and rotor.

3. Methods

3.1. Isolation of the capsular material

To isolate the capsule, i.e., the outermost compartment of the mycobacterial cell envelope, a gentle extraction with glass beads is applied. The mechanical treatment of mycobacterial cells with glass beads disperses the cells by extracting the amorphous material without causing cell disruption (10). The monosaccharide composition of the capsule (9-10) is determined by gas chromatography/mass spectrometry analysis of alditol acetate derivatives.

1. *M. tuberculosis* H37Rv is grown in 200 ml of Gas medium at 37°C with shaking to late-log phase (see Note 3).
2. Harvest the cells by centrifugation at $3,000 \times g$ for 15 min at 4°C and combine all pellets in a 50 ml falcon tube.
3. To isolate the capsule, add 10 g of glass beads per 2 g of the wet bacterial pellet and gently shake for 2 min.
4. Suspend the bacterial pellet and beads in 50 ml of Milli-Q water and spin down at $3,000 \times g$ for 15 min at 4°C. Collect the supernatant and filter immediately through a 0.22 μ M pore-size-filter.
5. Freeze-dry the filtered supernatant (capsule material) by lyophilization.
6. Weigh the lyophilized material and dissolve in MilliQ-water.
7. Use a 50 μ g aliquot of this preparation for alditol acetate preparation for monosaccharide composition determination (see Subheading 3.7).

3.2 Preparation of lipoglycans (LAM and LM)

To prepare mycobacterial lipoglycans suitable for structural analyses, the cells are first delipidated. The delipidation step removes all peripheral, noncovalently bound lipids. The delipidated cells are disrupted mechanically by French Press and lipoglycans are extracted with detergent and precipitated with ethanol. Proteinase K treatment is applied to digest and remove proteins. This procedure yields a mixture of LAM and LM (see Note 4).

1. *M. tuberculosis* is grown in 8 liters (2×4 -L conical flasks) of Gas medium to late-log phase (see Note 3).
2. Harvest the cells by centrifugation at $3,000 \times g$ for 15 min at 4°C. Wash the cell pellet 3 times with PBS and transfer to an Oakridge Teflon FEP tube.
3. Delipidate the cells with 10 ml of chloroform:methanol:water (10:10:3,v/v/v), for every 1 g of wet cells. After rocking the tubes for 2 hours, centrifuge at $27,000 \times g$ for 20 min at 15°C.

³All steps involving live *M. tuberculosis* H37Rv must be performed under Biosafety Level III conditions.

⁴Remaining phosphatidylinositol mannosides (PIMs), particularly the more polar (i.e., more mannosylated) forms of them, may contaminate this preparation.

4. The delipidation step is repeated two more times (see Note 5).
5. To remove residual organic solvents, the cells are dried on the air bath at room temperature overnight (see Note 6).
6. Grind the dried cell pellet to achieve a fine powder.
7. Delipidated cells are suspended in a minimal amount of breaking buffer and freeze-thawed three times to achieve complete suspension before passing over French Press 8 times at 1500 psi (see Note 7).
8. Transfer the suspension of broken cells to Oakridge Teflon FEP tubes and remove any remaining unbroken cells by centrifugation at $3,000 \times g$ for 5 min.
9. Add breaking buffer to the supernatant (broken cells) at a ratio of 1:1 (v:v) and rock overnight at 4°C.
10. Centrifuge the broken cells (from step 9) at $27,000 \times g$ for 1 hour at 4°C (see Note 8).
11. Transfer the clear supernatant to Oakridge Teflon FEP tubes and keep the pellet at 4°C.
12. Incubate the supernatant at 37°C to separate the aqueous layer from the detergent layer (see Note 9).
13. After the supernatant is partitioned into two layers, centrifuge at $27,000 \times g$ for 15 min at 25°C.
14. Carefully collect the aqueous layer (upper layer) into the tubes containing the pellet from step 11. Pool the detergent layer and store at 4°C.
15. Add Triton X-114 to the aqueous layer containing the pellet from step 11 to achieve the final concentration of 8% (v/v).
16. Rock the tubes gently at 4°C for 2 h.
17. Repeat detergent extraction two more times (steps 10-16).
18. Combine all detergent extracts and keep the pellet (later referred to as 'cell wall pellet'; see Subheading 3.5) for the preparation of mAGP.
19. To precipitate the extracted lipoglycans, add cold 95% ethanol to the combined detergent layers at a ratio 10:1 (v:v) and leave at -20 °C overnight.
20. Centrifuge at $27,000 \times g$ for 20 min at 4°C.

⁵Delipidated bacterial cell pellet may be brought outside the BSL-3 containment laboratory at this point since, if applied under the conditions described here, *M. tuberculosis* should be killed by the treatment with chloroform/methanol. To ensure that no viable bacteria remain, plate some of the non-diluted suspension on 7H10-OADC agar and incubate the plates for 3 to 4 weeks at 37°C.

⁶Cover tubes with foil and dry the sample under a gentle stream of air.

⁷You may perform an acid fast staining (14) on the suspension to ensure that at least 90% of the cells have been broken.

⁸If the supernatant is not clear, repeat centrifugation until a clear supernatant is obtained. It is very important to obtain a clear supernatant devoid of any residual contamination with mAGP.

⁹Triton X-114 is miscible with water at 4°C, however it separates from water at 37°C and a biphasic suspension forms. The detergent layer is the lower layer.

21. Collect the precipitate in one tube and dry using a vacuum concentrator.
22. Add proteinase K at a concentration 2 mg/ml and digest at 37°C overnight.
23. Dialyze the digest for 24 h against the endotoxin-free water using a 3.5 kDa MWCO Slide-A-Lyzer Cassette.
24. Transfer the sample to 13 × 100 mm glass tubes and dry using a vacuum concentrator.
25. To determine if the sample contains protein-free soluble lipoglycans, run a Tricine SDS/polyacrylamide gel followed by PAS (Periodic Acid Silver staining) (see Subheading 3.4 and Fig. 3). If proteins are still present in the sample, repeat the proteinase K digestion step.

3.3 Separation of lipoglycans (LAM and LM)

A size-exclusion chromatography system is used to separate LM from LAM (and contaminating PIMs; see Note 4). It is performed on a High Performance Liquid Chromatography (HPLC) system fitted with Sephacryl S-200HiPrep 26/60 column in tandem with a Sephacryl S-100HiPrep 16/60 column. Purified lipoglycans are analyzed by Tricine SDS/Polyacrylamide gel electrophoresis and alditol acetate derivatives are prepared to determine monosaccharides composition and ratios.

1. Dissolve 15 mg lipoglycans extracted from *M. tuberculosis* H37Rv in 5 ml of column running buffer.
2. Filter the sample through 0.22-μm syringe filter units (see Note 10).
3. Set HPLC flow rate at 1 ml/min. After collecting the column void volume collect about 100 fractions of 2 ml.
4. Determine the elution profile by running every other fraction on Tricine SDS/polyacrylamide gel and develop by PAS staining (see Subheading 3.4).
5. Based on the results of PAS staining make pools of pure LAM and LM.
6. Dialyze against the dialysis buffer at 37°C for 24 h. Use the following membranes for each pool: 12-14kDa MWCO for LAM and 6-8 kDa MWCO for LM.
7. Change the dialysis buffer to 1 M NaCl and continue dialysis at room temperature for another 24 h.
8. Dialyze against endotoxin-free water at room temperature for 24 h.
9. Transfer the dialyzed fractions to 100 ml round-bottom flask and freeze-dry by lyophilization.

3.4 Tricine SDS/Polyacrylamide Gel Electrophoresis of lipoglycans followed by Periodic Acid Silver Staining

Lipoglycans are run on a Tricine SDS/polyacrylamide gel and visualized by PAS staining.

¹⁰If the sample is too concentrated and doesn't easily go through the 0.2-μm filter, it can first be filtered through a 0.8 μm filter.

1. Dissolve 5 µg of purified lipoglycans in 8 µl of Milli-Q water.
2. Add 10 µl of 2X Tricine SDS Sample buffer, 2 µl of 10X reducing agent to the sample and incubate at 100°C for 5 min.
3. Load 10 µl of PageRuler Prestained Protein Ladder and the sample onto a gel.
4. Run the gel at constant voltage of 125 V. The expected current should be 80 mA/gel at the beginning and 40 mA/gel at the end of the run.
5. Transfer the gel to a dish containing 100 ml of the first fixative and rock for 45 min (see Note 11).
6. Discard the first fixative and add 100 ml of 0.7% periodic acid solution. Rock for 7 min (see Note 12).
7. Discard the periodic acid and add 100 ml of the second fixative and rock for 5 min.
8. Discard the second fixative, add enough 2.5% glutaraldehyde to cover the gel and rock for 5 min.
9. Remove the glutaraldehyde and wash the gel with 100 ml of distilled water for 10 min. Repeat washing step as needed (see Note 13).
10. Add the 0.0025% DTT solution for 6 min.
11. Discard the DTT solution and after adding 100 ml of 0.1% silver nitrate rock for 5 min.
12. Decant off the silver nitrate solution and rinse the gels briefly with Milli-Q water three times.
13. Develop the gel in 200 ml of developer solution. Start by incubating the gel in about 50 ml of the developer. After the solution becomes brown/yellow, discard the developer and replace with the fresh 50 ml of developer. Incubate for 10-20 sec. Replace again with 100 ml developer and incubate until the bands are as dark as desired.
14. Stop development by adding 10 ml of 50% citric acid for 10 min and wash the stained gels with water.

3.5 Preparation of the mycolate-arabinogalactan peptidoglycan (mAGP) complex

To prepare the mAGP complex, the cell wall pellet is extracted with 2% SDS, which disrupts membranes and hydrophobic interactions. Proteinase K digestion is applied to digest and remove any protein contaminants. Finally, the mAGP pellet is washed with acetone, dried, and alditol acetate derivatives are prepared to determine the monosaccharide composition of mAGP.

¹¹The gel can be kept in the first fixative and water indefinitely.

¹²Periodic acid enhances the staining of carbohydrates and shouldn't be used when the goal is to verify the contamination of lipoglycans with proteins.

¹³Background is reduced with increased washing with water. An overnight washing works well.

1. Transfer the cell wall pellet (see Subheading 3.2, step 18) to an Oakridge Teflon FEP tube.
2. Dissolve the cell wall pellet in 30 ml of 2% SDS solution and stir with a magnetic stir bar at room temperature for 30 min (See Note 14).
3. Centrifuge at $27,000 \times g$ for 10 min at 25°C . Decant the supernatant.
4. Repeat steps 2 to 3 two more times.
5. Add 26 ml of 2% SDS and 4 ml of Proteinase K solution to the pellet. Stir with a magnetic stir bar at room temperature for 30 min.
6. Centrifuge at $27,000 \times g$ for 10 min at 25° .
7. Discard the supernatant and add 30 ml of 2% SDS solution to the pellet.
8. After stirring with a magnetic stir bar at 90°C for 1 h, centrifuge at $27,000 \times g$ for 10 min at 25°C .
9. Repeat steps 7-8 two more times.
10. Discard the supernatant, add 30 ml of water to the pellet and stir at room temperature for 30 min.
11. Centrifuge at $27,000 \times g$ for 10 min at 25°C .
12. Discard the supernatant and after adding 5 ml of acetone to the pellet stir at room temperature for 10 min.
13. Centrifuge at $27,000 \times g$ for 10 min at 25°C , decant acetone and dry the pellet in a chemical fume hood.
14. Weight 1 mg of purified mAGP and perform alditol acetate derivatization for monosaccharide composition determination (see Subheading 3.7).

3.6 Preparation of AG

To isolate AG, the mAGP complex is treated with 0.5% KOH. The basic hydrolysis removes the cell wall bound mycolic acids from the mAGP complex. The released mycolic acids are extracted with organic solvents. At this step, the pellet consists mainly of AGP. AG is released from AGP by mild acid hydrolysis. The supernatant, which contains the solubilized AG is treated with barium carbonate to neutralize the acid. Alditol acetates are prepared to analyze the monosaccharide composition and purity of AG.

1. Transfer mAGP to an Oakridge Teflon FEP tube and resuspend in 30 ml of 0.5% KOH in methanol and stir with a magnetic stir bar at 37°C for 4 days.
2. Centrifuge at $27,000 \times g$ for 20 min.
3. Discard the supernatant and resuspend the pellet in 30 ml of methanol.
4. Centrifuge at $27,000 \times g$ for 20 min.

¹⁴Before each centrifugation step, remove the magnetic stir bar from the tube.

5. Resuspend the pellet in 20 ml of chloroform and mix well using a stir bar for 5-10 min at room temperature. Then add 10 ml of methanol and centrifuge at $27,000 \times g$ for 20 min (see Note 15).
6. Resuspend the pellet in 30 ml of methanol and centrifuge at $27,000 \times g$ for 20 min.
7. Discard the supernatant and dry the pellet on the air bath. The pellet now consists of rather pure AGP.
8. Transfer the AGP pellet to a 16×100 mm tube and add 5 ml of 0.05 M sulfuric acid.
9. Stir with a magnetic stir bar at 37°C for four days.
10. Centrifuge at $3,000 \times g$ for 15 min at 25°C .
11. Transfer the supernatant to a new 16×100 mm tube (see Note 16).
12. To neutralize the acid, add a few grains of barium carbonate and incubate overnight at room temperature.
13. Centrifuge at $3,000 \times g$ for 15 min at 25°C .
14. Transfer the supernatant to a new 16×100 mm tube and dry using a vacuum concentrator.
15. Dissolve in 5 ml of Milli-Q water and use 5 μl for alditol acetate preparation for monosaccharide composition determination (see Subheading 3.7).

3.7 Analysis of monosaccharide composition by Gas Chromatography/Mass spectrometry

To analyze the monosaccharide composition of the sample, it is necessary to derivatize the glycosyl residue to alditol acetates. This procedure involves three major steps: hydrolysis, reduction and acetylation.

1. Combine 10 μl of monosaccharides stock solutions and 10 μl of the internal standard in 13×100 mm glass tube (see Note 17).
2. Add your sample and 10 μl of the internal standard to another 13×100 mm glass tube.
3. Completely dry the samples using a vacuum concentrator.
4. Add 250 μl of TFA and heat at 120°C for 2 h.
5. Completely dry the content of each tube on the air bath.
6. Add 100 μl of methanol to each tube and dry on air bath.
7. Repeat step 6 one more time
8. Add 200 μl of the reducing agent and let it stand at room temperature for 2 h (see Note 18 and ¹⁹).

¹⁵The organic extract/supernatant contains mycolic acids.

¹⁶The pellet consists of peptidoglycan.

¹⁷³-O-methyl-glucose is used as an internal standard to determine quantitatively the amount of monosaccharides in the sample.

9. Stop the reaction by adding 2 drops (~50 µl) of glacial acetic acid and dry the samples on air bath.
10. Add 100 µl of 10 % glacial acetic acid in methanol and dry the samples on air bath.
11. Repeat step 10 three more times.
12. Add 100 µl of methanol and dry on air bath.
13. To acetylate, add 100 µl of acetic anhydride and heat at 120°C for 2 h in a heating block.
14. Cool the tubes at room temperature and dry the samples on the air bath under a gentle stream of air.
15. Add 1 ml of Milli-Q water and 2 ml of chloroform and mix thoroughly.
16. Centrifuge at $2,500 \times g$ for 5 min at 4°C.
17. Discard the upper aqueous layer.
18. Wash the organic layer with 1 ml of Milli-Q water.
19. Centrifuge at 2,500 g for 5 min at 4°C.
20. Transfer the lower organic layer to 13 × 100 mm glass tube and dry on air bath under a gentle stream of air (see Note 20).
21. Dissolve each sample in 50 µl of chloroform.
22. Inject 1 µl of the sample into the gas chromatograph-mass spectrometer. Use an initial temperature of 50°C for 1 min, increasing to 150°C at 30°C/min, followed by a 5°C/min increase to 275°C.

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¹⁸Reducing agent must be freshly made prior to use.

¹⁹The reduction reaction gives better results when performed overnight.

²⁰It is important not to contaminate the organic layer with the water layer.

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Figure 1.
Schematic representation of the *Mycobacterium tuberculosis* cell envelope.

Figure 2. Structures of the major cell envelope (lipo)polysaccharides from *Mycobacterium tuberculosis*.

(A) Mycolyl arabinogalactan attached to peptidoglycan (mAGP); AG is made of a galactofuran of 30 Galf residues and three arabinofurans (each made of 31 Araf residues) of which only one is shown here. Some of the interior α -3,5-Araf residues may be further substituted at position 2 with either galactosamine (GalNH₂) as shown herein, or with a succinyl residue (15). AG is connected to PG via a linker unit (made of a rhamnosyl residue attached to a *N*-acetylglucosaminosyl-1-phosphate residue) at the reducing end and to mycolic acids (MA) at the nonreducing ends, all together forming the mycobacterial cell wall complex mAGP.

(B) Lipoarabinomannan (LAM); the mannan moiety of LAM consists of approximately 20–30 Manp residues and the arabinan polymers of approximately 60 Araf units; the precise number of arabinan chain(s) attached to mannan is still uncertain. Lipomannan (LM) is devoid of the arabinan chains of LAM. The capsular D-mannan and D-arabino-D-mannan are similar in structure to LAM from which they are thought to be derived. Note that both capsular polysaccharides are devoid of the phosphatidylinositol anchor of LAM. R₁-tuberculostearic acid, R₂-palmitic acid.

(C) The repeating units of capsular α -D-glucan; the full-size molecule is >100,000 Da and represents about 80% of the capsular polysaccharides of *M. tuberculosis*.

Figure 3.

Tricine SDS polyacrylamide gel analysis of lipoglycans.

Purified lipoglycans were run on a Tricine SDS polyacrylamide gel and visualized by PAS staining. Lane 1, molecular weight marker; Lane 2, Purified LM; Lane 3, LAM and LM.