Supporting Information

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Fig. S1. TLC profiles of extracted lipids revealed with phosphospray. TLC was developed with chloroform-methanol-water (CMW) (30:8:1). "CMW" and "RMS" indicate CMW and reverse micellar solution (RMS) extracts of intact cells, whereas "IM" indicates CMW extract of residues of RMS-extracted cells. (A) TLC of *Mycobacterium smegmatis* lipids shows the absence of phospholipids (PLs) in the RMS extract [outer membrane (OM) lipids], all of them being present in the inner plasma membrane (IM) extract. (B) TLC of the RMS extract of *Escherichia coli* sprayed with phosphospray shows the presence of PLs in its OM.



Fig. S2. Identification of the predominant lipid in the IM extract of *M. smegmatis*. (*A*) TLC profile of the IM extract developed using chloroform–methanol–13 M ammonia–1 M ammonia actate–water (180:140:9:9:23, vol/vol). The suspected Ac_2PIM_2 and other phosphatidylinositol mannosides (PIMs) were visualized by specific labeling of cells with [¹⁴C]mannose (¹⁴C man) and also by spraying with anthrone for glycolipids (GL). (*B*) electrospray ionization–mass spectrometry (ESI-MS) profile of the suspected Ac_2PIM_2 isolated from the TLC plate of the IM extract. The predicted structure of the major species (*m*/*z* 1,654) is shown. (*C*) The predicted structure of the second most abundant species (*m*/*z* 1,694) in *B*. (*D*) GC-MS analysis of the fatty acid methyl esters obtained by alkaline hydrolysis of Ac_2PIM_2 .



Fig. S3. Detection of PIMs larger than $PIM_{6.}$ (*A*) Separation by SDS/PAGE stained with basic fuchsin. (*B*) Separation of IM extract by SDS/PAGE visualized by modified silver staining. (*C*) SDS/PAGE of various extracts of cells pulse-labeled with [¹⁴C]mannose, followed by detection of radioactivity. (*D*) TLC of various extracts of cells specifically labeled with [¹⁴C]mannose developed with chloroform–methanol–13 M ammonia–1 M ammonium acetate–water (180:140:9:9:23, vol/vol). CMW, CMW extract of intact cells; IM, CMW extract of residues of RMS-extracted cells; IM_{aq}, aqueous wash following CMW extraction of RMS-treated cells; Lyso, the extract obtained after treating intact cells with aqueous lysozyme solution; RM_{aq}, aqueous washes following RMS extraction; and RMS, RMS extract of intact cells.



Fig. 54. Identification of trehalose monomycolate (TMM). (A) TLC of the CMW extract of intact cells, developed with chloroform-methanol-water (60:16:2, vol/vol) and visualized by spraying with anthrone reagent. Note that trehalose-containing lipids appear bluish violet with anthrone and other glycolipids such as glycopeptidolipids (GPLs) appear bright blue. (*B*) TLC profile of the alkaline hydrolysis product of the suspected ¹⁴C-labeled TMM band (isolated using preparative TLC), developed using petroleum ether:diethylether (85:15). FAMEs, fatty acid methyl esters.



Fig. S5. Two-dimensional TLC to separate GPLs from trehalose dimycolates (TDMs) and other GLs, using CMW (100:14:0.8) in the first direction and C-acetone-MW (50:60:2.5:3) in the second direction. All lipids were quantified and visualized by phosphorimaging of ¹⁴C-labeled lipids whereas the GLs were visualized by spraying with anthrone. In all of the figures, CMW indicates CMW extract of intact cells, RMS indicates RMS extract of intact cells, and IM indicates CMW extract of residues of RMS extracted cells. Std. TDM, standard *Mycobacterium tuberculosis* TDM from Sigma–Aldrich.







Fig. 57. Two-dimensional TLC profiles of ¹⁴C-labeled lipids using CMW (30:8:1) in the first direction and hexane–diethyl ether–acetic acid (70:30:1) in the second direction, followed by phosphorimaging. CMW indicates CMW extract of intact cells, RMS indicates RMS extract of intact cells, and IM indicates CMW extract of residues of RMS extracted cells. Ac₂PIM₂, diacyl phosphatidylinositol dimannosides; DAGs, diacyl gycerols; MAs, mycolic acids; PE, phosphatidyl-ethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; and TAGs, triacylgycerols.



Fig. S8. Two-dimensional TLC profiles of lipids using CMW (30:8:1) in the first direction and chloroform–methanol (9:1) in the second direction. All lipids were quantified and visualized by phosphorimaging of ¹⁴C-labeled lipids whereas the GLs were visualized by spraying with anthrone. CMW indicates CMW extract of intact cells, RMS indicates RMS extract of intact cells, and IM indicates CMW extract of residues of RMS extracted cells.



Fig. 59. Fatty acid composition of the lipids in the CMW extract of intact cells determined using GC-MS. Lipids were subjected to alkaline hydrolysis followed by transmethylation of the released fatty acids as described in *Experimental Procedures* and then separated and quantitated by using GC-MS. Note that glycopeptidolipids are alkali-stable whereas mycolic esters are not eluted in the GC system used, and hence they are not represented in this figure. TS, tuberculostearic acid.