Supplemental Figures S1-S5.

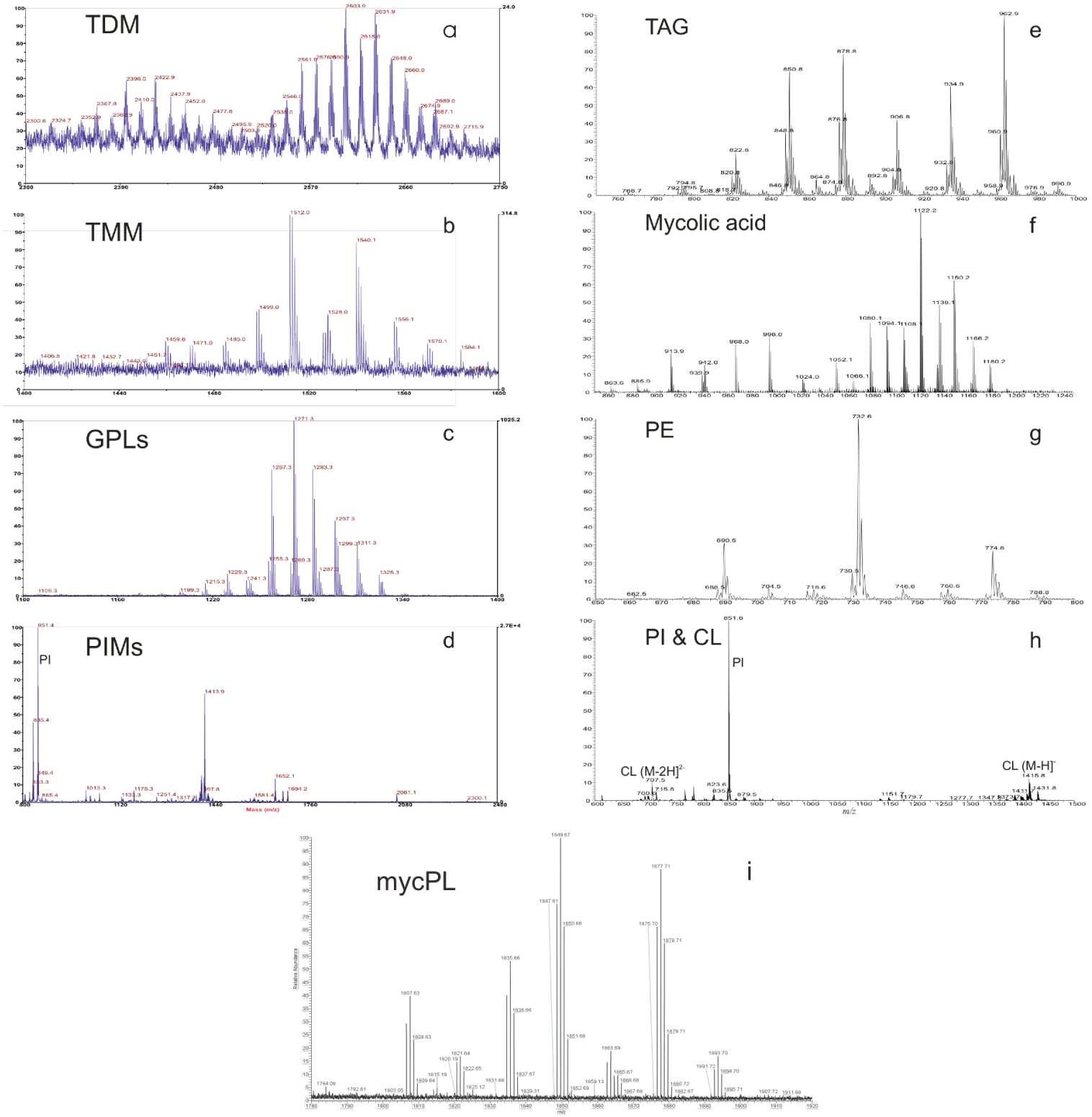

Fig. S1. The mass spectra of lipids extracted from wild-type *M. smegmatis*. (a) Trehalose 6,6'-dimycolate (TDM) ($[M + Na]^+$ ions), (b) Trehalose monomycolate (TMM) ($[M + Na]^+$ ions), (c) Glycopeptidolipids (GPLs) ($[M + Na]^+$ ions), (d) phosphatidylinositol mannosides (PIMs) ($[M - H]^-$ ions), (e) Triacylglyceride (TAG) ($[M + NH_4]^+$ ions), (f) mycolic acids ($[M - H]^-$ ions), (g) Phosphatidylethanolamine (PE) ($[M - H]^-$ ions), (h) Phosphatidylinositols and cardiolipin (PI & CL) ($[M - H]^-$ ions) and (i) mycolylphospholipid (mycPL) ($[M - H]^-$ ions). The total lipid extract was fractionated using a Macherey-Nagel amino Chromabond Sep-Pak column and mass spectra were obtained by ESI-MS or MALDI-TOF-MS.

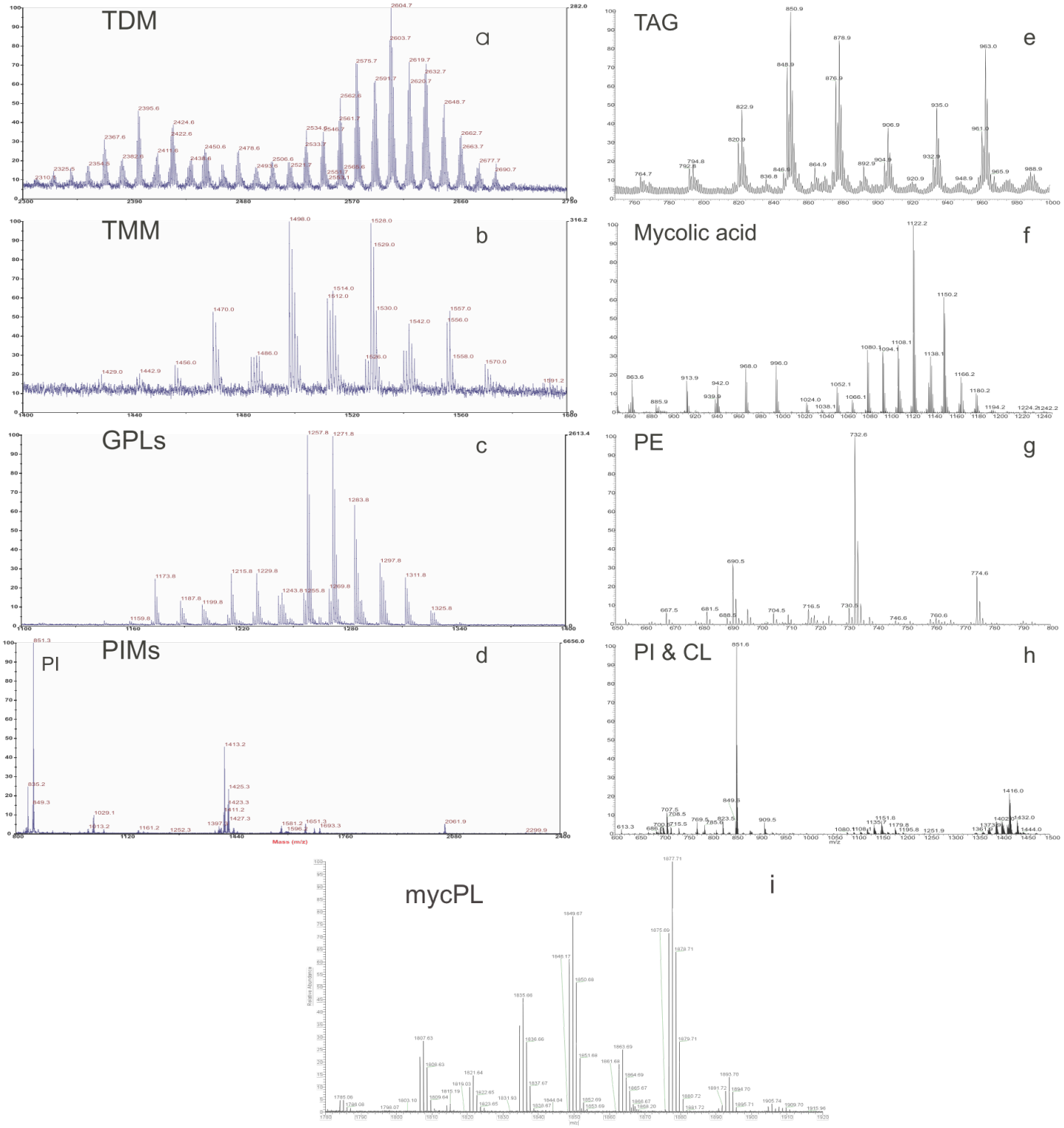

Fig. S2. The mass spectra of lipids extracted from the *M. smegmatis mmpL11* mutant GP02. (a) Trehalose 6,6'-dimycolate (TDM) ([M + Na]$^+$ ions), (b) Trehalose monomycolate (TMM) ([M + Na]$^+$ ions), (c) Glycopeptidolipids (GPLs) ([M + Na]$^+$ ions), (d) phosphatidylinositol mannosides (PIMs) ([M – H]$^-$ ions), (e) Triacylglyceride (TAG) ([M + NH_4]$^+$ ions), (f) mycolic acids ([M - H]$^-$ ions), (g) Phosphatidylethanolamine (PE) ([M – H]$^-$ ions), (h) Phosphatidylinositols and cardiolipin (PI & CL) ([M – H]$^-$ ions) and (i) mycolylphospholipid (mycPL) ([M – H]$^-$ ions). The total lipid extract was fractionated using a Macherey-Nagel amino Chromabond Sep-Pak column and mass spectra were obtained by ESI-MS or MALDI-TOF-MS.

Fig S3-S4. Structural Characterization of MMDAGs

The elemental compositions $C_{73}H_{138}(CH_2)_nO_6$ (n=1,2, …, 25) of this lipid family deduced from high resolution mass measurements on the $[M + NH_4]^+$ (not shown) and the corresponding $[M + Li]^+$ adduct ions (Figure 4) point to the structures of this lipid family consisting of three fatty acyl groups similar to TAG. The later HPLC elution time of this apolar lipid (25-26 min) than that of TAG (18-23.5 min) in a C8-reversed phase column is consistent with the notion that MMDAG contains a very long meromycolyl fatty acyl chain at sn-3 (or sn-1), which permits its distinction from the TAG lipids by mass spectrometry. The structural characterization was achieved by MS^n on the $[M + Li]^+$ adduct ions at m/z 1326 (Fig. S3), which is one of the major species consisting of several isomeric structures, as described below.

As shown in Figure S4a, the MS^2 spectrum of the $[M + Li]^+$ ion at m/z 1326 contained prominent ions at m/z 1070, 1044 and 583 arising from losses of 16:0-, 18:1- and 51:2-FA substituents, respectively, and the ions at m/z 1064, 1038 and 577, arising from losses of the corresponding fatty acid substituents as lithium salt, respectively. The presence of 51:2-FA is consistent with the observation of the ion at m/z 749, representing a lithiated mycolyl 51:2-FA (m51:2-FA) ion. Further dissociation of the ion of m/z 1044 (1326 → 1044; Fig. S4b) also gave rise to the ion at m/z 749, corresponding to the lithiated m51:2-FA ion; together with the abundant ions at m/z 789 arising from loss of 16:0-FA moiety as an α,β-unsaturated fatty acid (loss as 16:1-FA), indicating that the 16:0-fatty acid substituent is located at sn-2 (1, 2). The results indicate the presence of the structure of 18:1/16:0/m51:2-TAG. The confirmation of 18:1- and 16:0-FA at sn-1 and sn-2, respectively, is further supported by the MS^3 spectrum of the ion of m/z 583 (1326 → 583; data not shown), which is identical to the MS^3 spectrum arising from 18:1/16:0/18:0-TAG (951 → 867) (2). These results also readily located the double bond of the 18:1-FA substituent at C-9. The combined structural information indicates the presence of the major $\Delta^9$18:1/16:0/m51:2-TAG structure.

In Figure S4b, minor ions at m/z 761 arising from loss of 18:0-FA as α,β-unsaturated fatty acid (loss as 18:1-FA) together with the ion at m/z 721 representing a lithiated m49:2-FA cation were also observed, indicating the presence of $\Delta^9$18:1/18:0/m49:2-TAG isomer. This structural assignment is further supported by the observation of the minor ions at m/z 1042 and 1036 arising from losses of 18:0-FA as acid and as lithium salt respectively, and of the ion of m/z 611 arising from loss of m49:2-FA moiety seen in Figure S4a.

The ions at m/z 1098 arising from loss of 14:0-FA substituent, and at m/z 555 arising from loss of 53:2-FA substituent were also observed in Figure S4a, indicating that a minor 18:1/14:0/m53:2-TAG isomer is also present. This structural assignment is also in agreement with the observation of the ions at m/z 777, representing a lithiated m53:2-FA; and at m/z 817, arising from loss of 14:0-FA as α,β-unsaturated fatty acid (loss as 14:1-FA), and supporting the presence of 14:0-FA substituent at sn-2 (Fig. S4b). Further dissociation of the ion at m/z 1070 (1326 → 1070; Fig. S4c) gave rise to ions at m/z 789 and 329 arising from losses of 18:1- (loss as 18:2-FA) and 51:2-Fatty acids (loss as 51:3-FA) as α,β-unsaturated fatty acids respectively, together with ions at m/z 805 and 345 arising from losses of the 18:1- and m51:2-FA as ketenes, respectively. These results located the 18:1- and m51:2-fatty acid at sn-1 and sn-3 (or vice versa), respectively. The structural information combined with the prominent ion seen at m/z 749, representing a lithiated m51:2-FA, gave the assignment of the major 18:1/16:0/m51:2-TAG isomer, consistent with the structure assignment as described earlier. The spectrum (Fig. S4c) also contained the minor ions at m/z 817 (loss of 16:1-FA as α,β-unsaturated fatty acid), and at m/z 777 (lithiated 53:2-FA), suggesting the presence of 16:1/16:0/m53:2-TAG isomer. The assignment is also consistent with the observation of the ion of 1072 (loss of 16:1-FA) in Figure S4a. The above structural assignments of the fragment ions were confirmed by high resolution mass measurements (data not shown). The combined results revealed that the $[M + Li]^+$ ion of m/z 1326 consists of the major $\Delta^9$18:1/16:0/m51:2-TAG structure together with minor isomers of $\Delta^9$18:1/14:0/m53:2-TAG, 16:1/16:0/m53:2-TAG , and $\Delta^9$18:1/18:0/m49:2-TAG.

1. Hsu F-F, Turk J. 1999. Structural characterization of triacylglycerols as lithiated adducts by electrospray ionization mass spectrometry using low-energy collisionally activated dissociation on a triple stage quadrupole instrument. *Journal of The American Society for Mass Spectrometry* 10: 587-99
2. Hsu F-F, Turk J. 2010. Electrospray ionization multiple-stage linear ion-trap mass spectrometry for structural elucidation of triacylglycerols: Assignment of fatty acyl groups on the glycerol backbone and location of double bonds. *Journal of The American Society for Mass Spectrometry* 21: 657-69

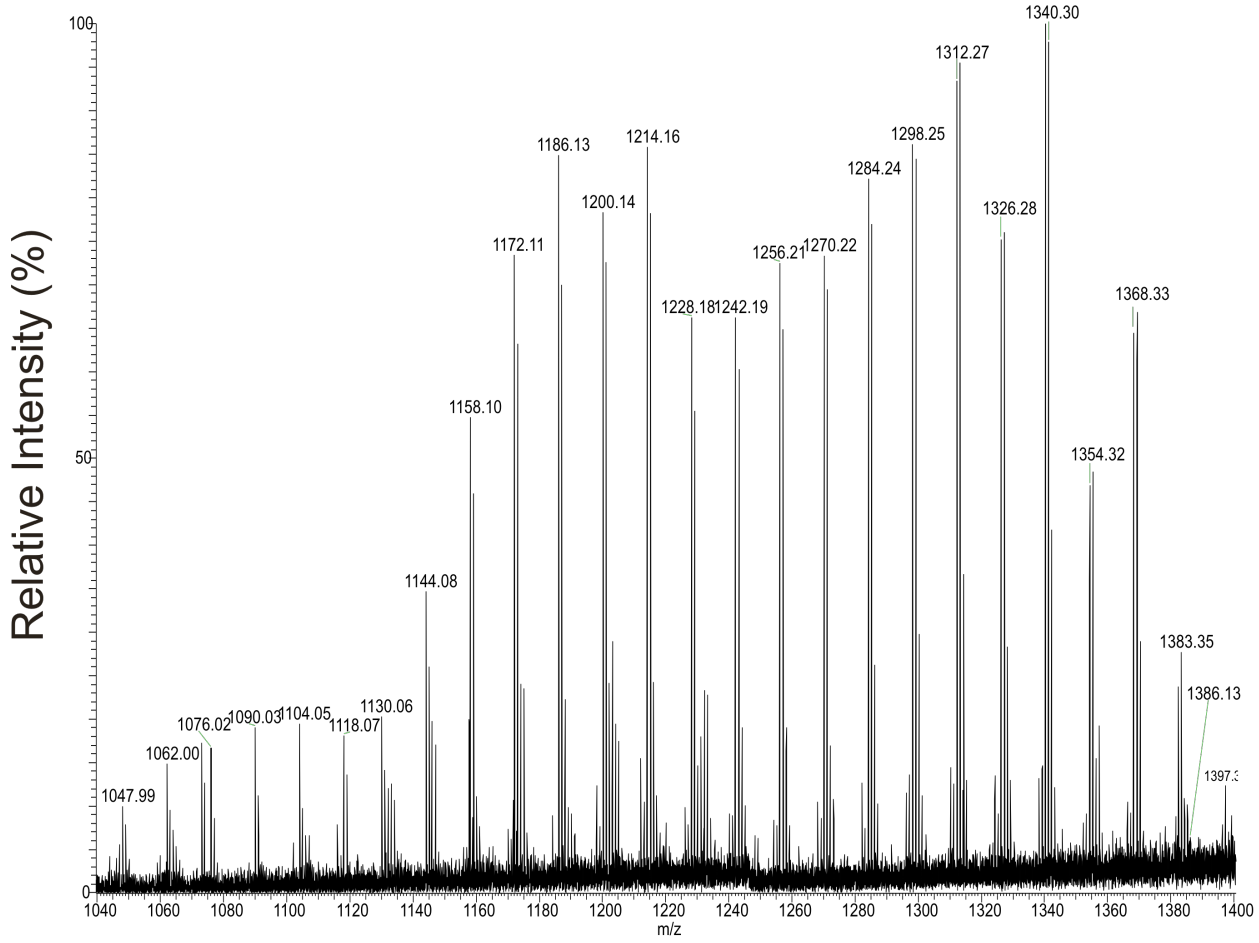

Fig. S3. High resolution mass spectra of the $[M + Li]^+$ ions of the apolar MMDAG lipid from *M. smegmatis*

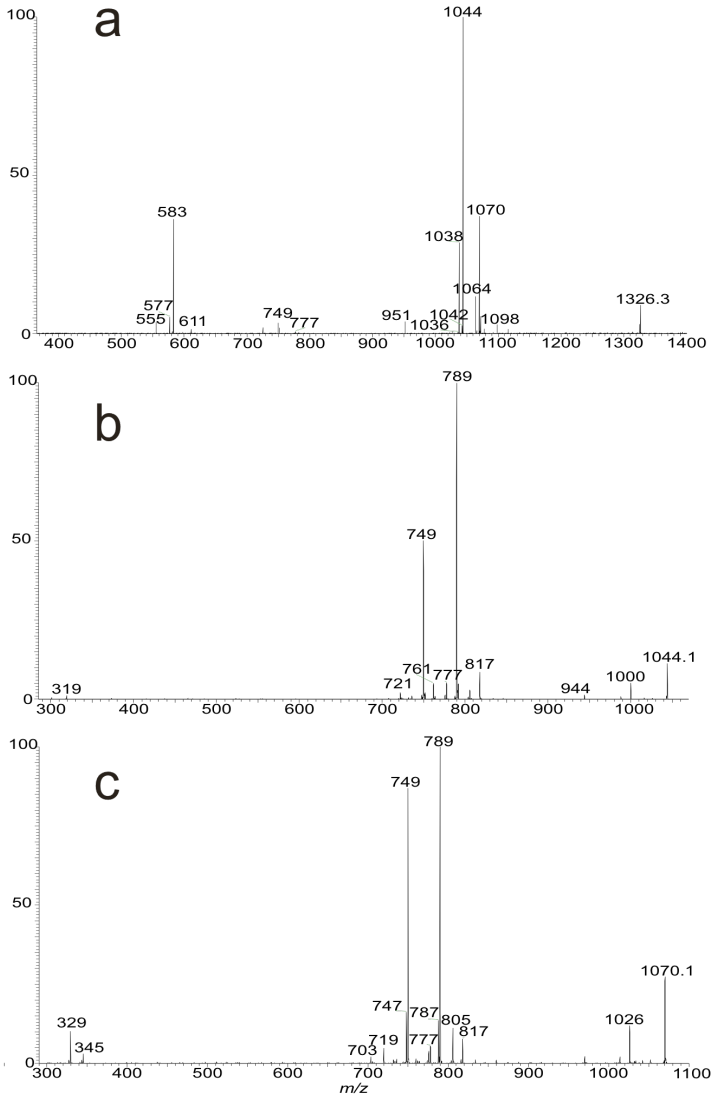

Fig. S4. MS^2 spectrum of the [M + Li]$^+$ ions of MMDAG at m/z 1326 (a), m/z 1044 (b) and m/z 1070 (c).

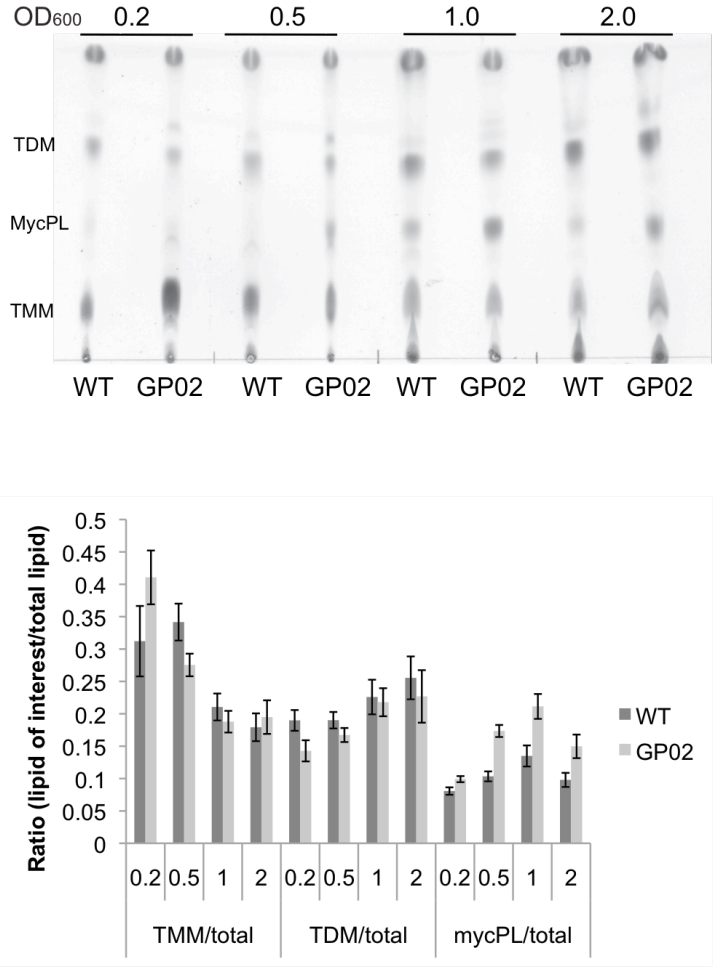

Fig S5. The *M. smegmatis mmpL11* mutant consistently accumulates MycPL relative to wild-type when grown planktonically. At the indicated OD_{600}, total lipids were extracted from wild-type (mc^2155/pVV16) and *mmpL11* mutant (GP02/pVV16) then resolved by TLC in chloroform: methanol: ammonium hydroxide (80:20:2, v:v:v). TLC plates were sprayed with ethanolic sulfuric acid and charred to visualize lipids. Densitometry was performed using the Image J software. Values of each lipid species were normalized to total lipid for each strain and timepoint and results presented graphically below as the average and standard deviation of three experiments. A representative TLC is shown.