Inhibition of mycolic acid transport across the *Mycobacterium tuberculosis* plasma membrane

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**Supplementary Information**

**I. Supplementary Methods**

**Macromolecular effects of AU1235 on whole *M. tb* cells** - The effects of AU1235 on the biosynthesis of DNA, RNA, proteins and cell envelope components (fatty acids, mycolic acids, (glyco)lipids and polysaccharides) of whole *M. tb* H37Ra cells were assessed by metabolic labeling. To this end, bacterial cultures were grown in 7H9-OADC-Tween 80 medium. The radiolabeled precursors and different concentrations of the inhibitors (0.5 to 10x MIC) were added simultaneously to the cultures, which were then allowed to grow for another 12-16 hr at 37ºC. *M. tb* cultures treated with different concentrations of control drugs with known mechanisms of action were analyzed alongside to assess the specificity of the inhibitor for any observed effects. Cultures aimed at analyzing cell envelope components were radiolabeled with [U-14C]glucose (1 µCi ml⁻¹; specific activity, 250-360 Ci mol⁻¹, NEN Radiochemicals) or [1,2-14C]acetate (0.5 µCi ml⁻¹; specific activity, 57 Ci mol⁻¹, NEN Radiochemicals). Protein synthesis was monitored by radiolabeling with (Expres35S35S, 20 µCi ml⁻¹; specific activity >1000 Ci mmol⁻¹; NEN Radiochemicals), RNA synthesis by labeling with [5,6-3H]uracil (0.5 µCi ml⁻¹; specific activity, 40 Ci mmol⁻¹, NEN Radiochemicals) and DNA synthesis by labeling with [8-3H]guanine (1 µCi ml⁻¹; specific activity, 20 Ci mmol⁻¹, American Radiolabeled Chemicals Inc.).

Untreated and inhibitor-treated bacteria were collected by centrifugation, washed and their lipids and mycolyl-arabinogalactan-peptidoglycan (mAGP) complex extracted essentially as described (Mikušová et al., 1995). [14C]-glucose- and [14C]-acetate-derived lipids and fatty acids (including mycolates) were analyzed by TLC in a variety of solvent systems (Rousseau et al., 2003) as indicated under the Method section. The amount of radioactivity incorporated into the individual sugars of the mAGP complex was determined by hydrolysis of the [14C]-glucose-derived material with 2M CF₃COOH for 3 hr at 120°C and separation of the individual monosaccharides on TLC plates developed twice in [pyridine: ethyl acetate: acetic acid: water] (5:5:1:3, by vol.). Autoradiograms were produced by exposure of the TLC plates to KODAK-Biomax MR films at -80°C.

In the case of DNA and RNA labeling, radiolabeled bacterial cultures were applied to glass fiber filters (Whatman GF/A 25mm) to get rid of the culture medium and bacterial pellets were then washed with cold PBS, quenched with 10% cold trifluoroacetic acid (TFA) for 15 min at 4°C, and further washed with 10% TFA and 95% ethanol. Radioactivity incorporation was determined by scintillation counting. 35S-protein-labeling mix incorporation was determined upon breaking washed cells in cold PBS using a bead beater, TCA precipitation of the lysates, and scintillation counting. All results were expressed as a percentage of incorporation in untreated controls.

**LC/MS analysis of total lipids** - Total lipids were analyzed by LC/MS as described by Sartain *et al.* (2011) in both positive and negative mode and the released fatty acids/mycolic acids in negative mode only, on a high resolution Agilent 6220 TOF mass spectrometer interfaced to a LC. Data files were analyzed with Agilent’s Mass hunter work station software (Version B.02.00, build 2.0.197.0) to identify compounds using ‘molecular feature extractor’. Agilent’s mass profiler program was used to compare the various lipid and mycolic acid forms present in analogous conditional mutant and wild-type, or untreated and inhibitor-treated, fractions. Most compounds were identified using a database of *M. tb* lipids.
developed in-house (Sartain et al., 2011). Quantification of the compounds of interest was achieved by comparing their relative ratios in the samples.

**Reagents and instrumentation** – All reagents were purchased from Sigma Aldrich (Milwaukee, WI) and were used without any further purification. Silica gel flash columns were purchased from Biotage Inc. (Lake Forest, VA). Biotage Isolera FLASH column chromatography system was used to purify AU1235. Melting points were obtained on the OptiMelt MPA100 Automated Melting Point System. Chemical shifts (δ) are reported in parts per million relative to solvent peak and coupling constants (J) are reported in Hz. High resolution mass spectra were recorded on a Waters Xevo G2 QTOF LCMS using ESI. Purity of >98% for AU1235 was determined using RP-HPLC on a Shimadzu HPLC system. Gradient Conditions: solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in MeOH): 0-1.00 min 95% A, 1.00-6.00 min 0-95% B (linear gradient), 6.00-9.50 min 100% B, 9.50-9.75 min 0-95% A, 9.75-10.0 min 95% A, detection by UV at 254 nm and by ELSD.

**Synthesis of 1-(2-adamantyl)-3-(2,3,4-trifluorophenyl)urea (AU1235)** – 2,3,4-Trifluorophenyl isocyanate(173 mg, 1.0 mmol), 2-adamantyl amine (181 mg, 1.2 mmol) and triethylamine (502 µL, 3.6 mmol) were dissolved in methylene chloride (10 ml) and stirred at room temperature overnight. The solvent was then removed under reduced pressure and the crude residue was purified by flash column chromatography using a hexane to ethyl acetate gradient. Pure compound (303 mg, 93%) was isolated as a white powder.

**Analysis of AU1235 accumulation in M. tb cells by LC/MS** – *M. tb* H37Ra (wild-type strain and spontaneous mutant isolates # 5 and 25) were grown up to mid to late log phase in 7H9-OADC without Tween 80. Pellets from 2-5 ml cultures were resuspended in 1 ml of media containing 5 µM AU1235 and incubated at 37°C for 4 hr with shaking. The cells were then extensively washed with PBS and broken in 1 ml ddH2O in a bead beater. Broken cells and beads were spun down at 12,000 x g for 10 min and kept as ‘cell wall’ fraction. The supernatant was removed and ~9 ml of ddH2O was added followed by ultracentrifugation for 1 hr at 4°C at 100,000 x g to separate membrane and cytosolic fractions. Each of the 3 fractions was transferred into glass tubes and AU1235 was extracted with 3 ml of ethyl acetate (containing 0.17 µg of a related urea compound used as an internal standard) and water. The ethyl acetate layer was removed and dried down using a nitrogen bath and reconstituted in 100 µl of methanol. Two µl were analyzed by LC/MS as described below and the M+H ions at m/z 325 (AU1235) and m/z 345 (internal standard) were integrated. Using a standard curve, the amount of AU1235 per cell pellet was calculated.

**LC/MS analysis of adamantyl ureas:** Samples were run on a Zorbax 2.1x50 mm SB-C18 1.8 µm (Agilent Technologies) column coupled to a high resolution Agilent 6220 TOF mass spectrometer. A 22 min solvent gradient, with a flow rate of 0.32 ml min⁻¹ started with 90% solvent A (10 mM formic acid in water), 10% solvent B (10 mM formic acid in acetonitrile). After 2 min, the gradient was started and the solvent ramped to 10% solvent A in 10 min. This was held for 8 min followed by a gradient back to 90% solvent A in 2 min.

**References**

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II. Supplementary Results

**Supplementary Table 1:** AU1235 susceptibilities for various Gram positive and Gram negative bacteria.

MIC determination was completed by the microbroth dilution method in Mueller-Hinton (MH) broth (Difco) according to the Clinical Laboratory Standards Institute (CLSI). *Corynebacterium glutamicum* was propagated at 30°C in Brain Heart Infusion agar (pH 7.4) (Becton Dickinson, Sparks, MD). For growth of *Streptococcus pyogenes* and *S. pneumonia*, MH was supplemented with 5% lysed horse blood. MRSA, methicillin-resistant *Staphylococcus aureus*. AU1235 was dissolved in DMSO at a concentration of 10 mg ml\(^{-1}\) and stored at -80°C until used. AU1235 serial dilutions with concentrations ranging from 400 µg ml\(^{-1}\) to 0.2 µg ml\(^{-1}\) were prepared in MH media in 96-well plates. 100 µl of bacterial culture (corresponding to 10\(^5\) CFU ml\(^{-1}\)) was added to each well. The 96-well plates were incubated overnight at 37°C and MIC determination was made by visual inspection where no bacterial growth could be seen.

| Bacterial species                                   | AU1235 MIC (µg ml\(^{-1}\)) | Tetracycline MIC (µg ml\(^{-1}\)) |
|-----------------------------------------------------|-----------------------------|-----------------------------------|
| *Staphylococcus aureus* 8325                        | > 200                       | < 0.1                             |
| MRSA strain ATCC 33591                               | > 200                       | 3.12                              |
| *Enterococcus faecalis*                              | > 200                       | 0.2                               |
| *Streptococcus pyogenes* MGAS1                       | > 200                       | < 0.1                             |
| *Streptococcus pneumoniae* DAW30                     | > 200                       | 0.4                               |
| *Bacillus anthracis* Sterne                         | > 200                       | < 0.1                             |
| *Bacillus subtilis*                                  | > 200                       | < 0.1                             |
| *Pseudomonas aeruginosa*                             | > 200                       | 1.6                               |
| *Escherichia coli* K12                               | > 200                       | 1.6                               |
| *Corynebacterium glutamicum*                         | > 40                        | nd                                |

**Supplementary Table 2:** Drug susceptibilities of wild-type *M. tb* H37Rv and H37Ra and corresponding spontaneous AU1235-resistant mutants.

The MIC of the drugs that inhibited 100% of the growth of the different strains was determined in 7H9-S-OADC-Tween 80 broth (for H37Ra) or 7H9-S-OADC broth without Tween 80 (for H37Rv) at 37°C as described under the Methods section. MIC values are expressed in µg ml\(^{-1}\). INH, isoniazid; RIF, rifampicin; EMB, ethambutol; STR, streptomycin; CIP, ciprofloxacin; AMI, amikacin. nd, not determined.

| *M. tb* strain          | AU1235 | INH   | RIF  | EMB | STR | CIP  | AMI |
|-------------------------|--------|-------|------|-----|-----|------|-----|
| *M. tb* H37Rv TMC102    | 0.125  | 0.0625| 0.03 | 1   | 1   | 0.25 | 1   |
| AU1235 isolate # 2      | 1      | 0.0625| 0.03 | 1   | 1   | 0.25 | 1   |
| AU1235 isolate # 3      | 1      | 0.0625| 0.03 | 1   | 1   | 0.125| 1   |
| AU1235 isolate # 4      | 1      | 0.0250| nd   | 0.7 | nd  | nd   | nd  |
| AU1235 isolate # 5      | 1      | 0.0625| 0.03 | 1   | 1   | 0.125| 1   |
| AU1235 isolate # 6      | 1      | 0.0625| 0.03 | 1   | 1   | 0.25 | 1   |
| *M. tb* H37Ra           | 0.25   | 0.03  | 0.001| 2   | >4  | 0.25 | 0.25|
| H37Ra AU1235 # 1        | 4      | 0.03  | 0.001| 2   | >4  | 0.25 | 0.25|
| H37Ra AU1235 # 2        | 2      | 0.03  | 0.002| 2   | >4  | 0.25 | 0.25|
| H37Ra AU1235 # 5        | 4      | 0.02  | 0.002| 4   | >4  | 0.25 | 0.25|
| H37Ra AU1235 # 25       | 4      | 0.03  | 0.001| 2   | >4  | 0.5  | 0.25|
**Supplementary Table 3:** Effect of expressing the wild-type and point-mutated (G253E) *mmpL3tb* genes on the susceptibility of *M. smegmatis* mc²155Δ*mmpL3* to various antibiotics.

The MIC of the various drugs that inhibited 100% of bacterial growth was determined in 7H9-S-OADC-Tween 80 at 37°C as described under the Methods section.

INH, isoniazid; EMB, ethambutol; STR, streptomycin; RIF, rifampicin; CIP, ciprofloxacin.

MIC values are given in µg ml⁻¹.

| Mycobacterial strain | AU1235   | INH      | EMB      | STR      | RIF      | CIP      |
|----------------------|----------|----------|----------|----------|----------|----------|
| mc²155Δ*mmpL3*/pMVG1-*mmpL3tb* | 0.4 - 0.8 | 10 – 20  | 0.4 – 0.8 | 0.1 – 0.3 | 1.25     | 0.4 – 0.8 |
| mc²155Δ*mmpL3*/pMVG1-*mmpL3tb-G253E* | 3.2 – 6.4 | 10 – 20  | 0.4 – 0.8 | 0.1 – 0.3 | 1.25     | 0.4 – 0.8 |
**Supplementary Figure 1:** AU1235 has no detectable inhibitory effect on DNA, RNA, protein and polysaccharide synthesis in *M. tb*.

Metabolic labeling of *M. tb* H37Ra with various radiolabeled precursors was performed as described in the Supplementary Methods. The percentages of radioactivity incorporation are expressed relative to the untreated control. For RNA and DNA, the mean values and standard deviations of two experimental points are shown. ISO, isoxyl; KAN, kanamycin; RIF, rifampicin; INH, isoniazid; CIP, ciprofloxacin.

**Effect of AU1235 on protein synthesis**

**Effect of AU1235 on RNA synthesis**

**Effect of AU1235 on DNA synthesis**

**Effect of AU1235 on arabinogalactan synthesis**

Ara  
Glc  
Gal  

[AU1235]  
µg ml⁻¹  
0 0.15 0.5
Supplementary Figure 2: Effect of AU1235 on the export of TMM in *M. tb*.

(a) *M. tb* H37Ra cultured in 7H9-OADC broth was treated for 5 hr at 37°C with either no inhibitor (0) or with AU1235 (AU) at a concentration of 0.5 µg ml⁻¹ (5 x MIC). Surface-exposed lipids were extracted from whole cells in water-saturated 1-butanol as described (Morita *et al.*, 2005), and the butanol-treated cells were then re-extracted overnight with chloroform/methanol (2:1, by vol.) to recover all remaining extractable lipids. The lipids from the butanol and chloroform/methanol fractions were resuspended in 400 µl of [chloroform: methanol] (2:1) and 5 µl of lipid sample were loaded per TLC lane. As a reference and control for inhibition, total lipids were also extracted from whole untreated and AU1235-treated cells. The TLC was developed in the solvent system [chloroform:methanol:water] (20:4:0.5, by vol.) and revealed and semi-quantified using a PhosphorImager. TMM, trehalose monomycolates; TDM, trehalose dimycolates.

The relative distribution of TMM and TDM between the butanol (black bars) and chloroform/methanol fractions (red bars) is indicated on the adjacent graphs. A 3-fold decrease in the proportion of TMM found in the butanol fraction was observed in the treated cells (3.2% of total TMM) compared to the untreated ones (9.7% of total TMM). In comparison, the decrease in the proportion of TDM found in the butanol fraction of the treated cells was only 1.4-fold (1.1% in untreated cells compared to 0.8% in the treated ones). A cpm to cpm comparison is not possible here due to the fact that AU1235-treated whole bacilli (not pre-extracted with butanol) contained 3.7 times more TMM and 22 times less TDM than the untreated *M. tb* H37Ra bacilli.

(b-c) *M. tb* H37Ra cultured in 7H9-OADC broth was treated for 4.5 hr at 37°C with either no inhibitor (0) or with AU1235 (AU) at a concentration of 1 µg ml⁻¹ (10 x MIC). [¹⁴C]-acetate was added to the cultures at the same time as the inhibitor. Bacterial cultures were collected and subfractionated into membranes, cytosol and ‘20,000 x g fraction’. To this end, cells were broken in a bead beater. Broken cells and beads were spun down at 3,000 rpm for 10 min to remove unbroken and beads and the supernatant was further centrifuged at 20,000 x g for 40 min to pellet the ‘20,000 x g cell wall fraction’ containing both cell wall and membrane components. Indeed, owing to the apparent tight association of membranes with cell wall components in mycobacteria (Morita *et al.*, 2005), the 20,000 x g ‘cell wall’
fraction still contains significant amounts of membranes. The supernatant resulting from the 20,000 x g centrifugation was further centrifuged at 100,000 x g for 30 min to separate membranes from cytosol.

(b) The total lipids from each fraction were extracted and resuspended in 50 µl of [chloroform: methanol] (2:1) and 10 µl of lipid sample were loaded per lane. As a reference and control for inhibition, total lipids were also extracted from whole untreated and AU1235-treated cells (non-subfractionated). The TLC was developed and revealed as described above.

(c) The amount of radioactivity incorporated in TMM in each subfraction was semi-quantified using a PhosphorImager and the results are expressed as fold increases over the values measured in the untreated subfractions (all arbitrarily set to 1).

Reference

Morita, Y. S. et al. Compartmentalization of lipid biosynthesis in mycobacteria. J. Biol. Chem 280, 21645-21652 (2005).
Supplementary Figure 3: Effect of AU1235 on mycolic acid biosynthesis and transfer in *M. smegmatis*.

*M. smegmatis* mc²155 cultured in 7H9-OADC-Tween 80 broth was treated for 16 hr at 37°C with either no inhibitor or with AU1235 at a concentration of 1.6, 6.4, or 12.8 µg ml⁻¹ (0.5x, 2x and 4x MIC to *M. smegmatis* mc²155). [1,2-¹⁴C]-acetate was added to the cultures at the same time as the inhibitor. (a) Bacterial cells were collected and their total lipid content extracted as described under the Methods section. The same volume of samples was loaded per lane. The TLC was developed in the solvent system [chloroform:methanol:water] (20:4:0.5, by vol.) and revealed by autoradiography. (b) The mycolic acids esterified to arabinogalactan were prepared from delipidated cells as described (48). α, α’ and epoxy refer to the three types of mycolic acids produced by *M. smegmatis*. The same volume of samples was loaded per lane. The TLC was developed thrice in the solvent system [n-hexanes:ethyl acetate] (95:5, by vol.) and revealed by autoradiography.

The amount of radioactivity incorporated in the products of interest was semi-quantified using a Phospholmager and the results (expressed as a % of the value measured in the untreated control) are presented as histograms under their corresponding autoradiograms.

TMM, trehalose monomycolates; TDM, trehalose dimycolates; PE, phosphatidylethanolamine; CL, cardiolipin; MAMEs, mycolic acid methyl esters.
Supplementary Figure 4: Comparative analysis of the effects of AU1235 and Isoxyl on TMM and TDM synthesis in *M. tb*.

*M. tb* H37Ra cultured in 7H9-OADC-Tween 80 broth was treated for a total of 44 hr at 37°C with either no inhibitor (CTL), AU1235 at a concentration of 12.8 µg ml\(^{-1}\) or 32 µg ml\(^{-1}\) or Isoxyl (ISO) at a concentration of 10 µg ml\(^{-1}\) or 25 µg ml\(^{-1}\) (4x and 10x MIC). [\(^{14}\)C]-acetate was added to the cultures after 20 hr of treatment and the cells further incubated for 24 hr. Bacterial pellets were collected and delipidated as described under the Methods section. The same volume of samples was loaded per lane. The TLC was developed in the solvent system [chloroform:methanol:water] (20:4:0.5, by vol.) and revealed by autoradiography. TMM, trehalose monomycolates; TDM, trehalose dimycolates; PE, phosphatidylethanolamine; CL, cardiolipin.
Supplementary Figure 5: AU1235 does not inhibit the mycolyltransferases FbpA, FbpB and FbpC in vitro.

The TMM transesterification assay described in reference 24 was used to measure the mycolyltransferase activity of the purified FbpA FbpB and FbpC proteins (30 µg) in the presence of cold TMM purified by preparative TLC from *M. tb* (15 µg), [U-14C]trehalose (1.25 µCi ml⁻¹; specific activity, 600 mCi mol⁻¹; American Radiolabeled Chemicals Inc.) and 0 (CTL) or 0.5 µg ml⁻¹ AU1235. Assays were conducted in a total volume of 200 µl and allowed to proceed for 60 min at 37°C. The glycolipid products of the reaction were analyzed by TLC run in the same solvent system as described in Supplementary Fig. 3a.
**Supplementary Figure 6: Construction and mycolic acid-containing acyltrehalose content of epoxide hydrolase (EH) mutants of M. tb.**

(a) Construction of EH knock-out mutants of *M. tb* - A two-step procedure employing the counterselectable marker *sacB* (45) was used to achieve allelic replacement at the *ephC* (*MRA_1133*), *ephE* (*MRA_3705*) and *ephF* (*MRA_0141*) loci of *M. tb* H37Ra. Allelic replacement was confirmed by PCR as detailed in the schemes below (red and black arrows indicate primer sets). Details about the construction of these mutants and primers sequences are available upon request.

The wild-type (WT) amplification signals are replaced by fragments 1.2 kb bigger in size in the knock-out H37RaΔ*ephC* mutant (Δ*ephC*) due to the insertion of a 1.2 kb-kanamycin resistance cassette at the Ascl site of *ephC*. The wild-type (WT) amplification signals are replaced by fragments approximately 1.1 kb bigger in size in the knock-out H37RaΔ*ephE* mutant (Δ*ephE*) due to a 140-bp NcoI deletion in *ephE* and insertion of a 1.2 kb-kanamycin resistance cassette. The wild-type (WT) amplification signals are replaced by fragments approximately 1.0 kb bigger in size in the knock-out H37RaΔ*ephF* mutant (Δ*ephF*) due to a 168-bp SphI deletion in *ephF* and insertion of a 1.2 kb-kanamycin resistance cassette.

*M. tb* CDC1551 knock-out mutants carrying a transposon insertion in the *ephA*, *ephB* or *ephD* genes of were obtained from TARGET ([http://webhost.nts.jhu.edu/target/](http://webhost.nts.jhu.edu/target/)) (Prof. W. Bishai). Note: The *ephA* and *ephD* transposon mutants were constructed in a Δ*sigF* background. Therefore, CDC1551Δ*sigF* was included as a control in this experiment.

(b) TMM and TDM content of *M. tb* EH mutant strains. Equal amounts of total cellular lipids from wild-type *M. tb* and the knock-out mutant strains were run in the solvent system [chloroform:methanol:water] (20:4:0.5, by vol.) and the plates were revealed with either cupric sulfate to reveal all organic compounds (CDC1551 WT and Δ*ephD*) or α-naphthol to reveal glycolipids (all other mutants and control strains).
Supplementary Figure 7: Protective effect of expressing mmpL3tb-G253E on mycolic acid transfer onto cell envelope acceptors in M. smegmatis.

*M. smegmatis* mc²155ΔmmpL3/pMVGH1-mmpL3tb (wt) and mc²155ΔmmpL3/pMVGH1-mmpL3tb-G253E (mut), cultured in 7H9-OADC-Tween 80 broth were treated for 16 hr at 37°C with either no inhibitor (CTL) or with AU1235 at a concentration of 0.32 µg ml⁻¹, 1.6 µg ml⁻¹, or 3.2 µg ml⁻¹ (4x to 40x MIC to mc²155ΔmmpL3/pMVGH1-mmpL3tb; 0.1x to 1x MIC to mc²155ΔmmpL3/pMVGH1-mmpL3tb-G253E). [1,2⁻¹⁴C]-acetate was added to the cultures at the same time as the inhibitor. (a) Bacterial cells were collected and their total lipid content extracted as described under the Methods section. The same volume of samples was loaded per lane. The TLC was developed in the solvent system [chloroform:methanol:water] (20:4:0.5, by vol.) and revealed by autoradiography. (b) The mycolic acids esterified to arabinogalactan were prepared from delipidated cells as described (48). The same volume of samples was loaded per lane. The TLC was developed thrice in the solvent system [n-hexanes:ethyl acetate] (95:5, by vol.) and revealed by autoradiography. α, α’ and epoxy refer to the three types of mycolic acids produced by *M. smegmatis*. TMM, trehalose monomycolates; TDM, trehalose dimycolates; PE, phosphatidylethanolamine; CL, cardiolipin; MAMEs, mycolic acid methyl esters.
**Supplementary Figure 8:** Accumulation of AU1235 in wild-type *M. tb* H37Ra cells and two spontaneous resistant mutants (isolates # 5 and 25).

AU1235 accumulation in *M. tb* cells was analyzed by LC/MS and quantified as detailed in the Supplementary Methods. Identical cell pellet weights were processed for all strains. The mean and standard deviations of two independent experiments in which each sample was analyzed in duplicate are shown. Wild-type *M. tb* H37Ra (black bars); Spontaneous resistant mutant (isolate # 5) (gray bars); Spontaneous resistant mutant (isolate # 25) (green bars). CYT, cytosol; MB, membranes; CW, ‘cell wall’ fraction (see Supplementary Fig. 2 b-c legend).
Supplementary Figure 9: \textit{mmpL3} is an essential gene of \textit{M. smegmatis}.

Full uncut gel from Figure 3. The portion of the gel presented in Fig. 3 only shows the profile of the two first conditional mutant clones (4 independent conditional mutant clones are shown here – lanes 3-6).

(a) Evidence for allelic replacement at the \textit{mmpL3} locus of \textit{M. smegmatis} mc\textsuperscript{2}155 in the presence of a rescue copy of \textit{mmpL3tb} expressed from an episomal plasmid. Allelic exchange mutants were rescued with the \textit{mmpL3tb} gene from \textit{M. tb} expressed from either pMVGH1-\textit{mmpL3tb}, pMVGH1-\textit{mmpL3tb-G253E} or pSETetR-\textit{mmpL3tb}. Allelic replacement was confirmed by PCR as described under the Methods section. The wild-type (WT) 3,752-bp amplification signal is replaced by a 3,358-bp fragment in the rescued mutants (r-mut) due to the 1,594-bp NotI deletion in the \textit{mmpL3} gene and insertion of a 1.2 kb-kanamycin resistance cassette.