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The mycolyltransferase 85A, a putative drug target of *Mycobacterium tuberculosis*: Development of a novel assay and quantification of glycolipid-status of the mycobacterial cell wall

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Abstract:

The enzymes of the Antigen 85 complex (Ag85A, B, and C) possess mycolyltransferase activity and catalyze the synthesis of the most abundant glycolipid of the mycobacterial cell wall, the cord factor. The cord factor (trehalose 6,6'-dimycolate, TDM) is essential for the integrity of the mycobacterial cell wall and pathogenesis of the bacillus. Thus, TDM biosynthesis is regarded as a potential drug target for control of *Mycobacterium tuberculosis* infections. Trehalose 6,6'-dimycolate (TDM) is synthesized from two molecules of trehalose-6'-monomycolate (TMM) by antigen 85A. We report here a novel enzyme assay using the natural substrate TMM. The novel colorimetric assay is based on the quantification of glucose from the degradation of trehalose, which is the product from catalytic activity of antigen 85A. Using the new assay, K_m and K_{cat} were determined with values of 129.6 ± 8.1 µM and 65.4 ± 4.1 min^{-1}, respectively. This novel assay is also suitable for robust high-throughput screening (HTS) for compound library screening against mycolyltransferase (antigen 85A). The assay is significantly faster and more convenient to use than all assays currently in use. The assay has a very low coefficient of variance (0.04) in 96-well plates and shows a Z' factor of 0.67-0.73, indicating the robustness of the assay. In addition, this new assay is highly suitable for the quantification of total TMM of the mycobacterial cell envelope.

Key words: *Mycobacterium tuberculosis*; HTS-assay; Antigen 85; cord factor; TMM.

1. Introduction

Tuberculosis (TB) caused by the facultative intracellular bacterial pathogen *Mycobacterium tuberculosis* (MTB) remains a serious global problem, with approximately 8 million new cases per year (Harries and Dye, 2006). Due to the occurrence of Multi-Drug Resistant TB (MDR-TB) which is resistant to Rifampicin (Rif) and Isoniazid (INH), patients require a prolonged treatment with second-line drugs for up to 2 years, which increases the cost of treatment drastically. The epidemiological situation is deteriorating further due to the emergence of the so-called Extreme Drug Resistant (XDR) strains of MTB which are resistant not only to Rif and INH, but also to at least one of the second line TB drugs (White and Moore-Gillon, 2000).

The cell wall is critical for long-term persistence of MTB in the hostile environment of the host's macrophage and for progression of tuberculosis (Barry et al., 1998; Brennan and Nikaido, 1995). Mycobacteria are gram-positive bacilli, but the cell wall structures are different from those of other gram-positive bacteria (Brennan and Nikaido, 1995). Approximately one-half of the cell wall mass is comprised of mycolic acids. In the mycobacterium cell envelope, mycolic acids are esterified to the terminal pentaarabinofuranosyl unit of arabinogalactan, which is a peptidoglycan-linked polysaccharide (McNeil et al., 1991; Mikusova et al., 1995). The inner layer of the cell wall is composed of mycolic acids covalently linked to the distal portion of the arabinogalactan (AG) moiety (Brennan and Nikaido, 1995; Liu and Nikaido, 1999). The outer layer is composed of extractable glycolipids containing mycolic acids, such as trehalose 6'-monomycolate (TMM) and trehalose 6,6'-dimycolate (TDM), which accumulates in a cord-like fashion on the surface of the cells. Both mycolyl glycolipids TDM and TMM (the biosynthetic precursor of TDM), have been reported as powerful antigens (Fujita et al., 2006; Pan et al., 1999; Steingart et al., 2009; Wang et al., 1999).

94 The pathogenesis of tuberculosis is poorly understood, especially secondary and
95 cavitary disease. There is evidence that suggests that cord factor (trehalose 6,6'-dimycolate,
96 TDM) is a key driver of these processes. TDM is the most abundant lipid released by virulent
97 MTB. TDM exists as cell wall bound and secreted forms. TDM bound to the mycobacterial
98 cell wall is non-toxic and protects mycobacteria from killing by the host's macrophages
99 (Hunter et al., 2006; Pan et al., 1999). Secreted TDM accumulates with host lipids in alveoli, a
100 process that rapidly produces caseation necrosis that leads to cavities. Virulent MTB release
101 large amounts of TDM during growth as a pellicle within cavities. This process is thought to
102 be crucial to perpetuate the cavity (Hunter et al., 2006). The cord factor has been suggested to
103 be an important determinant for successful infection and survival of MTB within
104 macrophages (Indrigo et al., 2003). Furthermore, it has been shown that 85A and 85B proteins
105 are involved in the covalent attachment of mycolic acids to the mycobacterial cell wall (Puech
106 et al., 2002). Several studies strengthen the hypothesis that differential expression of the
107 antigen 85 genes may be a mechanism utilized by mycobacteria to confuse and evade the host
108 immune system (Armitige et al., 2000; Harth et al., 2002; Ronning et al., 2004).

109 The cord factor (TDM) is synthesized by Antigen 85 (Ag85) complex proteins
110 (Ag85A, Ag85B and Ag85C). The antigen 85 (Ag85) complex proteins are acyltransferases
111 and convert TMM to TDM (Belisle et al., 1997). Two molecules of TMM are bound to the
112 substrate-binding pockets of the enzyme, and the mycolyl acyl group of the TMM substrate in
113 the donor site is transferred to the TMM substrate bound in the acceptor site (Fig.1). The
114 reaction generates one molecule of TDM and one molecule of trehalose (Anderson et al.,
115 2001; Toida et al., 1989). Antigen 85 proteins are important for the survival of MTB in the
116 host macrophage. An antigen 85C knockout strain of MTB has a 40% decrease in the amount
117 of cell wall linked mycolic acid (Jackson et al., 1999; Sanki et al., 2008). Furthermore, an
118 fbpA^{-} mutant is not able to grow in macrophage like cell-lines (Armitige et al., 2000),
119 pinpointing the importance of 85A for virulence of this organism. Due to the importance for

120 survival of MTB in macrophages, antigen 85 (Ag85) complex proteins are promising targets
121 for novel antimycobacterial drugs.

122 Despite the knowledge concerning protein/substrate interactions and co-crystal
123 structure of Antigen 85 (Klegerman et al., 1994; Ronning et al., 2004) the prerequisite for
124 designing potent inhibitors to the mycolyltransferase activity is still the design of rapid and
125 easy assays, suitable for HTS. Several synthetic analogues of trehalose and TMM, putative
126 competitive inhibitors of their metabolism, have been shown to have dramatic effects on the
127 glycolipid metabolism of mycobacteria. 6-azido-6-deoxy-α,α`-trehalose (ADT), completely
128 suppresses the growth of *M. aurum* (Belisle et al., 1997; Hanessian and Lavallee, 1972), and
129 inhibits the in vitro mycolyltransferase activity of the purified recombinant antigen 85C about
130 60%. Finally, ADT reduces the synthesis of TMM (44 %) and TDM (87 %) (Belisle et al.,
131 1997).

132 Two mycolyltransferase assays have been reported, the first method is not suitable for
133 HTS, due to the complexity and use of radioactive substances (Kremer et al., 2002;
134 Sathyamoorthy and Takayama, 1987). Another test published (Boucau et al., 2009) uses
135 substrate analogues and the assay requires a synthetic carbohydrate based substrate that
136 functions as the acyl donor for the second TMM molecule. The use of synthetic substrates and
137 the use of D-glucose as acyl acceptor (Matsunaga et al., 2008) may not reflect the natural
138 activity of the enzyme and may not be suitable for the characterization of the enzyme's kinetic
139 properties.

140 The level of TMM of the cell wall might give an indirect indication of the fitness of
141 the cell within the macrophage. Until now there is no method for the quantification of
142 glycolipids that is suitable for HTS. We present for the first time a novel mycolyltransferase
143 activity assay that determines the TMM status of the cell wall and reflects the natural activity
144 of mycolyltransferase enzyme based on simple steps. Antigen 85A converts 2 molecules of
145 TMM to trehalose dimycolate (TDM) (cord factor) and one molecule trehalose. Trehalose is

146 converted by trehalase to produce two molecules of glucose. Glucose is assayed by the
147 glucose oxidase assay (Washko and Rice, 1961). The amount of glucose is proportional to the
148 trehalose concentration and so to the TMM concentration. A brief overview of the assay is
149 depicted in Fig. 2. We show that this simple spectrophotometric assay can be used for HTS
150 for inhibitors targeting mycobacterium antigen 85 and for TMM quantification.

151 **2. Materials and Methods**

152 *2.1. Bacterial culture*

153 *Mycobacterium smegmatis* (mc^2155) was grown in Middlebrook 7H9 medium supplemented
154 with ADC enrichment (BD Diagnostic Systems, Sparks, MD). The cultivation was performed
155 with shaking at 37 °C, and cells were harvested at their midlog phase of growth.

156 2.2. *Extraction, isolation and purification of total lipids*

157 Bacterial culture (0.5l) was centrifuged at 10.810g for 20 min. Lipids were extracted from the
158 cell pellet with 20 volumes of chloroform/methanol (2:1, v/v) with stirring for overnight. The
159 two phases were separated in a funnel. After the lower phase containing major glycolipid was
160 collected, it was added to 500 ml of acetone at –20°C for 12 h. The solvent was evaporated off
161 with a rotary evaporator. The total lipids were first separated by solvent fractionation and then
162 by thin-layer chromatography (TLC) on silica plates (Macherey-Nagel, Germany) with the
163 solvent system of chloroform/methanol/ammonium hydroxide (80:20:2, v/v) or with
164 chloroform/methanol (9:1, v/v). For analytical purposes, glycolipid spots were visualized with
165 a 9M H_2SO_4 spray followed by charring at 110°C for 2 minutes or with Coomassie blue
166 according to the method described previously with some modification (Nakamura and Handa,
167 1984). Each developed TLC plate was air dried and immersed in a staining solution consisting
168 of 0.04% Brilliant blue R (Sigma) in 25% methanol. After 30 min, the plate was removed
169 from the staining solution, immersed in 25% methanol (destaining solution), and allowed to
170 stand for 2 to 5 min with occasional agitation. Then the plate was removed from the
171 destaining solution and excess liquid on the surface of the plate was soaked up with filter

172 paper by gentle pressing. For preparative purposes the spots were visualized by exposure to
173 iodine vapour for a few minutes.

174 *2.3. TMM purification*

175 TMM was recovered from TLC plates after the iodine colour had disappeared by scratching
176 out the spot from the plate and subsequent dissolving in chloroform/methanol (2:1, v/v).
177 TMM was purified until a single spot was obtained by repeating TLC (Fujita et al., 2005). The
178 yield was determined by weighing the dried product.

179 *2.4. Plasmids and primers*

180 The full-length of the *M. tb H37Rv fbpA* gene encoding the secreted form of antigen 85A
181 lacking a stop codon was amplified by PCR. The sequence of PCR sense primer for *Rv3804c*
182 gene was 5`- ggc tgt cat atg gca ttt tcc cgg ccg ggc tt -3` and that of anti-sense primer was 5`-
183 ggt acc gga tcc cta ggc gcc ctg ggg cgc gg -3`. The PCR product was digested with FastDigest
184 *NdeI* and FastDigest *BamHI* (Fermentas*)* to generate a sticky end insert. The agarose-gel-
185 purified DNA was ligated with T4 DNA ligase (Roche) in FastDigest *NdeI* and FastDigest
186 *BamHI* digested pET-28a(+) (Novagen). The final plasmid pET28–Ag85A contained the
187 DNA-sequence coding for antigen 85A with a C-terminal 6x histidine tag. The expression
188 plasmid pAg85A was transformed into BL21 (DE3) cells (Novagen). The correct orientation
189 and integrity of inserts from plasmid constructions were confirmed by PCR amplification and
190 sequence analysis. Plasmids were purified with the QIAprep Spin Miniprep Kit (Qiagen).

191 *2.5. Purification of recombinant antigen 85A*

192 BL21(DE3) cells containing pAg85A were cultured at 37°C in LB Broth until an OD_{600} of
193 0.8. Then the cells were plated on APS agar (BD Diagnostic Systems, Sparks, MD) plates for
194 3 days at 18°C. The bacterial cells were suspended in 20 mM Tris buffer pH 8 and harvested
195 by centrifugation at 7,945g for 25 min, the pelleted cells were resuspended in 5 volumes of
196 buffer (containing 50 mM $NaH_2PO_4.H_2O$, 300 mM NaCl and 10 mM imidazole). The cells
197 were sonicated and the disruption conditions were: sonic treatment 3 times (time for each

198 treatment was 3 min, with a 30 s interval) (Labsonic U, Braun Biotech). The suspension was
199 clarified by centrifugation (10,866g) for 20 min. The antigen 85A was purified by affinity
200 chromatography using a Ni-NTA column on a ÄKTA purifier system (GE-Healthcare).
201 Antigen 85A was purified by applying a linear imidazole gradient from 10 – 500 mM. The
202 fractions containing antigen 85A were pooled, the enzyme was detected by using SDS/PAGE
203 and western blotting. The final purification step used size exclusion chromatography on a
204 Superdex 200 prep-grade column (XK 26/60, Amersham Biosciences). The fractions
205 containing antigen 85A were pooled and stored at 4°C.

206 *2.6. Protein analysis*

207 The purity of purified proteins was assessed by SDS/PAGE on 12% (w/v) polyacrylamide
208 gels stained with Coomassie Brilliant Blue R-250. Molecular mass marker proteins were from
209 Fermentas. The immune blotting was conducted by using a semi-dry blotting device (Sigma).
210 The fractions were electrophoresed through preparative 12% (w/v) polyacrylamide gels and
211 were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore), then the
212 membranes were placed in a blocking solution consisting of 1X PBS plus 0.5% Tween 20
213 (PBST) and 2% (w/v) bovine serum albumin (Sigma). After blocking for 2 hour, the
214 membrane was incubated with mouse anti 85A mAb IgG (Lionex, Germany) for overnight
215 with gentle rocking. Then the membrane was washed three times with PBST and was
216 incubated with horseradish peroxidase-conjugated anti-mouse IgG (goat origin; Pierce
217 Chemical Co.) diluted 1:10,000 in PBST for 30 minutes. Blots were again washed three times
218 with PBST and finally developed in membrane peroxidase substrate (Seramun Diagnostica).

219 *2.7. Determination of enzyme concentration*

220 The enzyme concentration was determined by absorbance spectroscopy with 280 nm
221 wavelength light. Additionally, absorbance at 260 nm was measured to calculate the A_{280nm}
222 $/A_{260nm}$ ratio. The readings were performed on a geneQuant spectrophotometer (Pharmacia).
223 The extinction coefficient was obtained from computational calculations on the ExPASy

224 website (Wilkins et al., 1999). The predicted extinction coefficient for the recombinant
225 antigen 85A protein was 73.005 M^{-1} cm^{-1}.
226 *2.8. Mycolyltransferase assay*
227 The mycolyltransferase assay was carried out with a POLARstar OPTIMA (BMG Labtech)
228 device. The assay was accomplished in F-bottom 96-well plates (Greiner Bio-One) in total
229 volumes of 300 µl. First, 5 µl of TMM (150 µM sonicated in 20 mM Tris buffer pH 7.5 and
230 10 mmol DTT, the solution was sonicated because it is an essential to improve the substrate
231 solubility) were incubated with 5 µl antigen 85A at final concentration of 175 nM for 30 min
232 at 37°C. Then 20 µl of 135 mM citric acid buffer (pH 5.7) and 6.5 µl trehalase enzyme
233 solution (0.3 unit/ml in 135 mM citric acid buffer pH 5.7) were added. After 15 min, 33.5 µl
234 of 500 mM Tris buffer (pH 7.5) were added to stop the reaction. The glucose content was
235 determined with a glucose (GO) Assay Kit (Sigma). In short, 140.5 µl of a solution containing
236 1.75 units glucose oxidase from *Aspergillus niger*, 0.35 units horseradish peroxidase and
237 0.014 mg *o*-dianisidine. The reaction was stopped after 30 min by the addition of 100 µl of 6
238 M H_2SO_4. The absorption at 540 nm was measured and the glucose concentration was
239 calculated using a glucose equilibration curve (111, 222, 333 and 444 µM/ml), prepared with
240 a D-(+)-glucose standard solution and blank with water. An experiment without antigen 85A
241 served as negative control.
242 In order to determine the range of linearity, the substrate concentration was varied
243 from 25-225 µM with 175 nM antigen 85A under the same reaction conditions as mentioned
244 above. Various reactions were carried out by adding different amounts of enzyme (125, 150,
245 175, 200, 225, and 250 nM) under the standard reaction conditions. The influence of a number
246 of organic solvents on the reaction rate was studied by adding 50 µM of TMM dissolved in
247 methanol and mixture of methanol and chloroform (1:1 v/v) to 175 nM of enzyme. Fifty µM
248 of TDM dissolved in the same solvents was used as the negative control and for the positive
249 control 50 µM TMM dissolved in the standard buffer (20 mM Tris buffer pH 7.5 and 10

250 mmol DTT). The pH of the reaction mixture was not adjusted in the experiments of TMM
251 determination from organic solvents. The Z′ factor was determined for 200, 300 and 350 µl
252 reaction mix volume according to the method of Zhang et al. (1999).

253 *2.9. Analysis of kinetic parameters*

254 To determine K_m and K_{cat}, the following setup was used: TMM concentrations ranging from
255 25 to 275 µM and enzyme at 175 nM. A time-course experiment at fixed enzyme
256 concentration and different substrate concentrations was performed to determine the initial
257 velocity values. The initial velocities were plotted versus substrate concentrations and kinetic
258 constants were obtained by fitting the experimental data to the appropriate rate equations by
259 nonlinear regression ($Y = (V_{max} . X)/ (K_m + X)$, where X is substrate concentration; Y, the
260 enzyme velocity; V_{max}, the maximum enzyme velocity; K_m, the Michaelis-Menten constant).
261 The enzyme obeyed the Michaelis-Menten equation. The kinetic data were calculated and
262 plotted by using GraphPad Prism 5.02 (GraphPad Software, Inc.).

263 **3. Results and Discussion**

264 *3.1. Isolation and purification of TMM*

265 *Mycobacterium smegmatis* cells were harvested at the midlog phase of growth. The total
266 lipids were extracted and applied to a silica gel TLC plate, followed by development in
267 chloroform/methanol/ammonium hydroxide (80:20:2, by vol.) or chloroform/methanol (9:1,
268 by vol.). Both mixtures achieved a fairly good resolution of the mycolate-containing
269 glycolipids TMM and TDM. The chloroform/methanol (9:1 by vol.) allowed a wider
270 inclination (Fig. S1B). Using chloroform/methanol/ammonium hydroxide as mobile phase
271 TMM and TDM had an R_f of 0.16 and 0.36. The R_f ratio obtained were 0.26 and 0.68,
272 respectively with chloroform/methanol.

273 *3.2. Purification of antigen 85A*

274 After the two-step purification (Ni-NTA and size exclusion chromatography), antigen 85A
275 was purified near to homogeneity. The protein showed a single band in a Coomassie stained

276 SDS-PAGE with an MW of 32 kDa (Fig. S2). An additional absorbance reading at 260 nm
277 allowed the calculation of the A_{280nm}/A_{260nm} ratio to confirm that the A280 nm value represents
278 the true protein concentration and not the concentration of a protein/nucleic acid mixture. The
279 A_{280nm}/A_{260nm} ratio for purified antigen 85A used in the assay was 1.9, indicating that little or
280 no nucleic acid was present in the sample because nucleic acid contamination decrease the
281 ratio from approximately 2.0 to 1.57-1.28.

282 *3.3. Assay design and substrate specificity*

283 The basic idea for this assay was the need for a fast and stable assay for the analysis of kinetic
284 parameters of antigen 85A and secondly, a method for the quantification of glycolipids in cell
285 extracts. Known assays use synthetic substrates or radioactive labelled substrates. Boucau et
286 al.(2009) describe for example an assay using the artificial substrate p-nitrophenyl-6-O-
287 octanoyl-β-D-glucopyranoside. Since this substrate, is different form the natural substrate we
288 invented an assay using the natural substrate TMM, which can be easily isolated from several
289 organisms (Fujita, et al., 2005) and reflects best the kinetic parameters of the enzyme. Antigen
290 85A produces one molecule of trehalose per reaction cycle. We used trehalase to convert
291 trehalose to glucose, which can be easily measured. Trehalase hydrolyzes the O-glycosyl bond
292 of trehalose to yield two molecules of glucose. Quantification of glucose is finally achieved
293 by a commercial glucose quantification assay. A prerequisite for the assay was the
294 observation that trehalase from porcine kidney neither uses TMM nor TDM as substrate
295 because if these compounds will react in the assay may give a false-positive result (Data not
296 shown).

297 *3.4. Optimal enzyme concentration, general setup and linearity*

298 The assay was designed first for the kinetic characterization of antigen 85A. As substrate,
299 freshly isolated TMM was used. TMM was dissolved in Tris buffer with DTT using
300 ultrasonication. These assays were done with ultrasonicated TMM. TMM was weighted from
301 dried material obtained by TLC as described in the Materials and Methods section.

302 In this assay, glucose concentration increases with respect to time with a fixed amount
303 of substrate used. As shown in Fig. 3 and Fig. 4B, TMM is completely converted to glucose
304 within 30 minutes. In order to ensure that the assay was functioning as expected and that each
305 step of the assay was dependent on the presence of all components, control reactions were
306 carried out in the presence and absence of both enzymes antigen 85A and trehalase (Fig. 3).
307 Without any enzyme present there is no increase in absorbance observed over time, showing
308 that the chromophore is not released by non-enzymatic hydrolysis under our conditions. The
309 addition of trehalase caused a slight increase in absorbance over time. Addition of antigen
310 85A to the reaction mixture produced a significant increase of absorbance and glucose
311 concentrations over time. To further validate the method, the dependence of the reaction rate
312 on the antigen 85A and TMM concentrations was studied. Fig. 4B shows the plot
313 corresponding to different antigen 85A concentrations (125, 150, 175, 200, 225, and 250 nM).
314 The data illustrate the increasing rate of glucose production with increasing concentration of
315 antigen 85A. Fig. 4A shows the linearity observed between glucose and TMM concentration.

316 *3.5. TMM determination from organic solvents*

317 For the purpose of TMM quantification from total lipids extraction steps, the assay was
318 carried out in presence of different organic solvents. To investigate the influence of a number
319 of organic solvents on mycolyltransferase enzymatic reaction, 50 μM of TMM and TDM
320 (determined by weight) were dissolved in methanol and a mixture of methanol and
321 chloroform (1:1 v/v) followed by addition of 175 nM of enzyme. For the positive control, the
322 same concentration was used under standard buffer conditions. The results from these
323 experiments show that the mycolyltransferase activity was reduced more in the presence of
324 organic solvents than in standard buffer reaction (20 mM Tris buffer pH.7.5 and 10 mM
325 DTT). As indicated in Fig. S3, the enzyme retains 100, 82.9 and 49.1% of its activity in the
326 presence of standard buffer, methanol and methanol/ chloroform (1:1 v/v) mixture

327 respectively. In spite of it, the antigen 85A can be assayed in processes where methanol and
328 mixture of chloroform/methanol are used.

329 *3.6. Enzymatic characterization of antigen 85A*

330 With TMM as substrate, the enzyme had an apparent K_m of 129.6 ± 8.1 µM, a V_{max} of 11.4 ±
331 0.7 µM min^{-1} and K_{cat} of 65.4 ± 4.1 min^{-1}. These values are quite similar to those reported for
332 the (Ag85 fold family) α/β hydrolase superfamily of enzymes like acetylcholinesterase
333 (AChEs), polyketide synthase thioesterase (PKS–TE) from *Bacillus brevis* and
334 *Mycobacterium tuberculosis Rv3802c* (Parker et al., 2009; Rosenberry and Bernhard, 1971;
335 Trauger et al., 2000). Kinetic studies of antigen 85C reveal the K_m and K_{cat} values to be 47 ± 8
336 µM and 0.062 s^{-1} respectfully (Boucau et al., 2009). The lower K_{cat} value of 0.062 s^{-1} of the
337 antigen 85C may result from the artificial substrate used.

338 *3.7. Validation*

339 The Z′ factor is a measurement of the quality and power of a HTS assay and was developed
340 for the evaluation of the inherent signal- to-noise ratio and to assess the assay reproducibility
341 (Zhang et al., 1999). In order to calculate this factor and to assess the robustness of the assay
342 protocol in microplate format, a series of negative and positive controls in different volumes
343 were used. For the negative control assay, reactions were performed in the presence of
344 trehalase without antigen 85A. Using the same assay conditions mentioned previously, the
345 positive control assay was performed. Based on the data, the Z′ value was calculated to be
346 0.67-0.73 (Table S1), indicating an excellent signal/noise (S/N) ratio for the assay and its high
347 potential for HTS applications. Assay reproducibility was determined by repeating the assay
348 20 times and the assay has a very low coefficient of variance (0.04) in 96-well plates.

349 Any colorimetric assay for HTS is useful if the color developed remains stable for a
350 substantial period of time. A simple assay in a 96-well format for HTS of MTB antigen 85A
351 was developed to test the enzyme activity by using purified TMM as substrate. By this
352 method we can quantify the TMM, TDM and trehalose. Since antigen 85A converts TMM to

one molecule of TDM and one molecule of trehalose, which is converted to two molecules of glucose, we can determine the concentration of glucose and then calculate the original concentration of TMM as follows:

concentration of [TMM] = concentration of [glucose], concentration of [TDM] = concentration of [glucose]/2 and concentration of [trehalose] = concentration of [glucose]/2

The method described here is useful also for quantitating the TMM from the total lipid of mycobacterium cells referring to the dry weight of the cells. Since the total lipids extraction can also contain free trehalose and glucose, the final TMM concentration could be obtained from the same equation as above with the subtraction of the glucose concentration of the negative control without antigen 85A from the glucose concentration obtained by the reaction. This was confirmed by performing a number of assays (data not shown).

In conclusion, this article has described a new and low-cost colorimetric method based on the reaction with TMM as natural substrate, which brings flexibility and convenience in screening of antigen 85A inhibitors. This assay is significantly faster and more convenient to use than those described to date (Boucau et al., 2009; Kremer et al., 2002; Sathyamoorthy and Takayama, 1987), and enables the measurement of amounts of TMM, one of the most important lipids in MTB cell wall. We are currently using this method for TDM and TMM quantification in different strains and mutants to better understand the reactions that control the biosynthesis of the glycolipids and for the screening of drug libraries.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in supplementary material sheet.

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486 **Legends**

487 **Fig. 1.** Schematic representation of TDM synthesis catalysed by antigen 85
488 mycolyltransferase. In the mycolyltransferase reaction both the donor site and the acceptor
489 site of the enzyme interact with TMM, resulting in TDM formation and one molecule
490 trehalose. MA, mycolic acid.

491 **Fig. 2.** Reaction scheme for novel mycolyltransferase activity assay. Using trehalose that is
492 produced as one of final products of mycolyltransferase reaction by trehalase to produce
493 glucose, which is oxidized to gluconic acid and hydrogen peroxidase by glucose oxidase.
494 Hydrogen peroxide reacts with *o*-dianisidine in the presence of peroxidase to produce a
495 colored product (oxidized *o*-dianisidine), which will be converted to a stable colored product
496 by sulfuric acid. The colored product is measured at 540 nm.

497 **Fig. 3.** Time course and control of the assay. Without trehalase and antigen 85A (■), with
498 trehalase (○) and with antigen 85A and trehalase (Δ). Reactions were performed with 150 mM
499 TMM and 175 nM antigen 85A. The experiment was carried out in a final volume of 300 µl in
500 a 96-well microplate at 37°C. The results are the average of 3 identical experiments.

501 **Fig. 4.** Determination of linear range of the reaction and optimal antigen 85A concentration.
502 (A) Linearity between TMM and glucose concentration. (B) Glucose production with
503 different antigen 85A concentrations: 125 nM (▲), 150 nM (●), 175 nM (□), 200 nM (△),
504 225 nM (■) and 250 nM (○).

505 **Fig. 5.** Michaelis–Menten kinetics of antigen 85A using TMM as substrate. Different
506 substrate concentrations were fit using nonlinear regression to the Michaelis-Menten equation.
507 The calculated K_m is 129.6 ± 8.1 µM and V_{max} is 11.4 ± 0.7 µM min.