**Title:** Exquisite specificity of Pks13 defines the essentiality of trehalose in mycobacteria

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**Abstract**

Mycolic acids (MAs) are the major component of the mycobacterial outer membrane, a key contributor to the intrinsic resistance of mycobacteria to external insults, including multiple antibiotics. After being synthesized in the cytoplasm, MAs are transported across the cell envelope in a sugar-esterified form, generally believed to be trehalose monomycolates (TMMs). However, whether trehalose is the only mycolate carrier during transport is under debate, and why this highly abundant disaccharide is essential for mycobacterial growth is unclear. To address these questions, we leveraged a trehalose-auxotrophic *Mycobacterium smegmatis* strain to investigate the final biosynthetic steps affording TMMs. We show that in addition to TMMs, new MAs are also not produced in the absence of trehalose. During trehalose depletion, mycolate precursors accumulate on Pks13, the polyketide synthase catalyzing the condensation of MAs, and ligation to the sugar head group, possibly blocking the enzyme and preventing the synthesis of new mature MAs. We establish that the native mycolate precursors can only be released by trehalose in vitro, and subsequently processed in a final reduction step. Furthermore, we demonstrate that only trehalose can re-activate MA biosynthesis in trehalose-depleted cells. Our work reveals the exquisite specificity of Pks13 for trehalose, providing a compelling reason for the its essentiality in mycobacteria, as well as new ideas towards the future development of anti-mycobacterial strategies.

30 **Significance Statement**

31 Tuberculosis and other diseases caused by mycobacteria pose a persistent threat to human health and well-
32 being. One reason why mycobacterial infections are hard to treat is the presence of the outer membrane, which
33 serves as a barrier that confers resistance against multiple antibiotics. The mycobacterial outer membrane
34 comprises mycolic acids, whose biosynthesis at late stages remains to be clarified. In this study, we characterize
35 the penultimate step in mycolic acid biosynthesis, and discover extremely high specificity for the usage of the
36 disaccharide trehalose by the synthase enzyme. Our work answers key questions in outer membrane biogenesis,
37 and provides a molecular basis for why trehalose is essential for mycobacterial growth, a novel insight that is
38 exploitable for future anti-mycobacterial strategies.

## Introduction

Mycobacteria are the cause of a number of severe human diseases, such as tuberculosis (TB) and leprosy. There were more than 10 million new TB cases in a single year, and the disease alone claimed 1.1 million lives in 2023[1]. This situation is compounded by the spread of multi-drug resistant (MDR) *Mycobacterium tuberculosis* strains, and the higher risk of co-infection with TB in patients with human immunodeficiency virus. Non-tuberculous mycobacterial infections, caused by *Mycobacterium abscessus* and related organisms, have also been on the rise[2]. The intrinsic resistance of mycobacteria to multiple antimicrobials and their emerging resistance to current drugs mark an urgent need for the identification of new drug targets.

Mycobacteria possess a distinct cell envelope. Besides the cytoplasmic membrane (IM) and the peptidoglycan (PG) cell wall, cells also contain an outer membrane (OM) bilayer, with the inner leaflet covalently linked to arabinogalactan (AG) polysaccharides extending outward from PGs[3,4]. The essential mycobacterial OM serves as an effective permeability barrier[5], which is largely contributed by the abundance of tightly packed mycolic acids (MAs) in the bilayer. These MAs are either linked to AG-PG as the entire inner leaflet (termed mycolyl-AGP or mAGP), or found as free lipids at the outer leaflet in the forms of trehalose monomycolates (TMMs) and trehalose dimycolates (TDMs). MAs are 2-alkyl, 3-hydroxyl, very long chain, branched fatty acids ($C_{58}$-$C_{86}$) present in mycobacteria, as well as phylogenetically related genera[6,7]. They comprise the shorter α branch ($C_{22}$-$C_{26}$) and the longer meromycolate chain ($C_{36}$-$C_{60}$), which are synthesized by a single FAS-I protein and a multi-protein FAS-II system, respectively[8]. Different functional groups are introduced onto the meromycolate chain, giving rise to different MA classes - *M. tuberculosis* produces α-, methoxy-, and keto-MAs while *Mycobacterium smegmatis* contains α- and epoxy-MAs, in addition to α'-MAs with a much shorter overall chain length ($C_{60}$-$C_{66}$) [8]. Fully extended α-alkyl chains are carboxylated, and then condensed with meromycolate chains by polyketide synthase 13 (Pks13) [9], affording the MA precursors α-alkyl-β-ketoacids that are reduced by CmrA, [10] and esterified by trehalose to form TMMs[11]; the order of these

62 final two reactions in cells remain to be clarified. TMMs are generally believed to be the final MA product in
63 the IM and are continuously transported across the cell envelope to build the OM. The transport process is
64 largely elusive, however, except for the identification of MmpL3 as the TMM flippase at the IM[12], and Ag85
65 enzymes as the mycolyltransferases for mAGP and TDM formation at the OM[13]. Targeting the MA biosynthetic
66 and transport pathways has proven to be an important anti-mycobacterial strategy, exemplified by the
67 prevailing application of isoniazid, a first-line anti-TB drug targeting FAS-II[14].

68 There are knowledge gaps in mycolate biosynthesis that would be important for our understanding of
69 mycobacterial physiology. One intriguing question is why trehalose is an essential metabolite for growth[15]. It
70 has been proposed that the role of trehalose as the final acceptor for MAs accounts for its essentiality. However,
71 in vitro evidence suggested that Pks13 is not strictly specific for trehalose; several other mono- and
72 disaccharides can release α-alkyl-β-ketoacids, albeit non-native short chain versions, from the enzyme to form
73 the corresponding ester[11]. Glycerol and glucose monomycolates have also previously been detected in
74 lipidomic analyses[16,17], although it is unclear whether they are derived directly from Pks13 or via mycolyl
75 transfer reactions catalyzed by Ag85s, leading to sugar headgroup exchange[18]. Therefore, is the predominance
76 of TMM as the mycolate carrier in cells due to the high abundance of trehalose, or the hitherto unknown in
77 vivo specificity of Pks13, which may then explain trehalose essentiality?

78 To elucidate acceptor specificity in cells, we need to devise a way to study the release of native mycolates
79 from Pks13 by trehalose (or other sugars). We reasoned that we might be able to accumulate mycolates or its
80 precursors on Pks13 by depleting the acceptor and stalling release. In this study, we exploited a trehalose
81 auxotrophic *M. smegmatis* strain (TKO) lacking all three trehalose biosynthetic pathways[19], namely the OtsAB,
82 TreYZ, and TreS pathways[20]. This strain can only grow in the presence of exogenous trehalose actively taken
83 up via the LpqY-SugABC ATP-binding-cassette (ABC) transporter[21]. We conducted extensive lipid profiling
84 of TKO cells depleted of trehalose, and reconstituted the final steps leading to TMM formation using native

85 substrates from endogenously purified Pks13. We show that upon trehalose depletion, cells stop synthesizing
86 mature MAs and TMMs, likely due to product accumulation on Pks13 that prevents further substrate turnover.
87 Interestingly, only adding back trehalose, but not other sugars, can reactivate MA biosynthesis. Furthermore,
88 we demonstrate that only trehalose can be used by purified Pks13 to release unreduced mycolates, for
89 subsequent reduction by CmrA. Our work highlights the exquisite specificity of trehalose as the mycolate
90 acceptor in cells, providing a molecular basis for its essentiality in mycobacteria.
91

92 **Results**

93

94 **Trehalose depletion causes cessation of de novo MA biosynthesis**

95 To study MA biosynthetic steps involving Pks13 and trehalose in mycobacteria, we first profiled newly
96 synthesized lipids upon trehalose depletion in the *M. smegmatis* Δ*otsA*Δ*treYZ*Δ*treS* (TKO) trehalose auxotroph.
97 When trehalose was depleted, cells stopped growing (Fig. S1). At that point, cells were labeled with [$^{14}$C]-
98 acetate, and newly synthesized lipids in the forms of total extractable lipids (containing TMMs) or total
99 mycolates (esterified as MA methyl esters, MAMEs) were isolated and analyzed by thin layered
100 chromatography (TLC). As expected, mutant cells depleted of trehalose (**tre**$^{-}$ cells) were deficient in TMM
101 formation (Figure 1a). Surprisingly, trehalose depletion also resulted in essentially no newly synthesized
102 mycolates (Figure 1b), indicating that MA biosynthesis was possibly disrupted at steps before TMM formation.
103 When the same cells were exogenously re-supplemented with trehalose during [$^{14}$C]-labeling, both MA
104 biosynthesis and TMM formation were restored (Figure 1). Importantly, restoration of MA biosynthesis was
105 not inhibited by several antibiotics that stopped cellular growth (Fig. S2), suggesting that the cessation of MA
106 synthesis was not due to growth arrest caused by lack of trehalose. The newly synthesized MAs and TMMs,
107 immediately upon trehalose re-supplementation, exhibited a higher α' to α ratio than those in wild-type cells

108 (Figure 1), a phenomenon also observed in cells when FAS-II was perturbed[22-24]. Transcriptomic analyses
109 revealed that genes in the *fabD-acpM-kasA-kasB-accD6* operon but not those encoding other FAS-II
110 components were up-regulated in trehalose-depleted cells (Figure 2a), consistent with FAS-II perturbation.
111 Furthermore, we observed up-regulation of the *acpS-fas*, *accD4-pks13-fadD32*, and *iniBAC* operons (Figure
112 2a and S3), perhaps a result of stalled MA biosynthesis, reminiscent of cellular responses of *M. tuberculosis* to
113 FAS-I/II inhibitors (cerulenin, thiolactomycin and isoniazid) [25,26]. It is likely that TKO cells exhibit different
114 metabolic states with or without trehalose. Following prolonged trehalose re-supplementation, the cellular
115 transcriptomic profile of TKO cells became more similar to wild type cells (Figure 2a). In addition, the ratio
116 of α'- to α-mycolates was fully restored in a manner dependent on active transcription and translation (Figure
117 2b). Taken together, our results establish that trehalose is required for de novo MA biosynthesis.
118

119 **Trehalose depletion causes accumulation of keto-mycolates on Pks13 that must be released and reduced**
120 **sequentially to form TMMs**

121 Both mycolate biosynthesis and TMM formation were abolished in trehalose-depleted cells. We reasoned
122 that these observations may indicate accumulation of lipids on Pks13, which prevents further turnover of
123 mycolate substrates. To test this idea, we labeled *M. smegmatis* **tre**$^{-}$ cells with [$^{14}$C]-acetate and analyzed the
124 whole cell protein profile via SDS-PAGE. Notably, a radioactive band showed up at ~200 kDa (Fig. S4), which
125 is consistent with the size of Pks13 (197.1 kDa). The radioactivity on the band could be reduced by either
126 trehalose or isoniazid treatment (Fig. S4), suggesting that the labeled band contains mycolate-related lipids.
127 We next expressed C-terminally His-tagged Pks13 from an integrative plasmid, and isolated it from [$^{14}$C]-
128 acetate-labeled **tre**$^{-}$ cells via affinity purification. Remarkably, we detected significant radioactivity on Pks13-
129 His from these cells (Figure 3a); the signal was much weaker when the same cells had been treated with
130 trehalose or isoniazid (Figure 3b). These results indicate that mycolate precursors accumulate on Pks13 upon

131 trehalose depletion, likely accounting for why de novo biosynthesis of mycolates is abolished in the absence
132 of trehalose.

133 To elucidate what lipid species may have accumulated on Pks13, we attempted to use trehalose to release
134 lipids from affinity-purified Pks13-His in vitro. Interestingly, trehalose indeed facilitated the formation of a
135 new extractable lipid species in the reaction mixture. The lipid migrated slightly faster than TMM on normal-
136 phase TLC, suggesting that it is a less polar derivative of the latter (Figure 4a). By comparing the mobility of
137 this lipid to TMM species synthesized in a strain lacking the reductase CmrA[10], we assigned it to be trehalose
138 α-alkyl-β-ketomeromycolate, the unreduced keto form of TMM (TMM*k*). The release of the α-alkyl-β-
139 ketomeromycolate precursor to form TMM*k* is believed to be catalyzed by the thioesterase domain of Pks13.
140 While this reaction can happen in the absence of the ketoreductase CmrA both in vitro[11] and in cells[27], it is not
141 clear whether the reduction step actually takes place before or after the release. Notably, the observed
142 accumulation of α-alkyl-β-ketomeromycolates in the absence of trehalose demonstrates the inability of
143 endogenous CmrA to reduce this precursor directly on Pks13. Consistent with this idea, only when we also
144 added purified CmrA and NADPH during the reaction, we observed the conversion of TMM*k* to mature TMM.
145 A mutation at the putative CmrA active site ($CmrA^{Y157F}$) reduced the conversion significantly (Figure 3c and
146 d). Therefore, we have effectively recapitulated in vitro the final release and reduction steps in the formation
147 of TMM. We conclude that trehalose release of mycolates from Pks13 allows subsequent CmrA reduction to
148 form TMMs.

149

150 **Release of native ketomycolates from Pks13 is highly specific to trehalose**

151 Having established that unreduced MA precursors can be released from Pks13 and esterified by trehalose
152 in vitro, we further explored if other sugars could act as MA acceptors. In an earlier study, unreduced MA
153 analogs ($C_{30}$) could be synthesized in vitro and released from recombinant Pks13 by a few mono- and

154 disaccharides other than trehalose[11]. Surprisingly, none of these sugars, including glucose, facilitated formation
155 of ketomycolate sugar esters when added to Pks13 endogenously purified from **tre**$^{-}$ cells (Figure 4a); we
156 reasoned that the longer chain length of native ketomycolates can in fact contribute to the sugar specificity of
157 the MA acceptor. We also tested other trehalose analogs in the release assay. Notably, 6-azido-trehalose, but
158 not 6,6'-diazido-trehalose, could drive the formation of putative 6-azido-TMM*k* in the reaction mixture,
159 consistent with the requirement of a free 6'-OH group. To corroborate this finding on acceptor specificity, we
160 next examined the ability of the various sugars in activating de novo mycolate biosynthesis when re-
161 supplemented to **tre**$^{-}$ cells. Remarkably, only 6-azido-trehalose (in addition to trehalose) enabled the formation
162 of newly synthesized MAs (Figure 4b), consistent with its ability to release accumulated precursors from Pks13.
163 We conclude that Pks13 exclusively uses trehalose as the MA acceptor in cells, which can explain the
164 essentiality of trehalose in mycobacteria.
165
166 **TDM is dispensable for mycobacterial growth in vitro**
167 It is intriguing that 6-azido-trehalose is able to release mycolate precursors from Pks13 (Figure 4a) and
168 facilitate continuous MA biosynthesis (Figure 4b). In fact, we found 6-azido-trehalose alone could support the
169 growth of TKO cells, albeit at a slower rate than that supported by the same amount of trehalose (Figure 5a).
170 This observation implies that *M. smegmatis* cells can survive without TDM; in the trehalose auxotrophic strain
171 grown in 6-azido-trehalose, mAGP but not TDM could be formed, since the mycolyl chain from 6-azido-TMM
172 can still be transferred to AGs but not another 6-azido-TMM molecule lacking the 6-OH group. Using [$^{14}$C]-
173 acetate lipid profiling, we confirmed that 6-azido-trehalose-fed TKO cells made mAGP but not TDM (Figure
174 5b). Notably, we did not detect any major difference in drug sensitivity between TKO cells with and without
175 TDM (Table S1). Therefore, we assert that the TDM, despite its key role in pathogenicity as the cording factor[28],
176 is not essential for mycobacterial growth and OM permeability function *in vitro*.

177

178 **Discussion**

179 By using a trehalose auxotrophic strain, we have demonstrated that trehalose depletion abolishes de novo
180 MA biosynthesis, likely due to intermediates accumulating on Pks13. In addition, we have established that
181 Pks13 is highly specific, and only uses trehalose to release β-keto-mycolates from its thioesterase domain.
182 Furthermore, we have found that CmrA is unable to reduce β-keto-mycolates on Pks13 itself in cells, a process
183 that occurs only after the formation of TMM*k*. Our study has recapitulated the final steps in the MA biosynthetic
184 pathway with native substrates, and the details therein, revealing why the specific disaccharide trehalose is
185 essential in mycobacteria.

186 Our findings that trehalose is a highly specific acceptor for mycolates on Pks13 differ from an earlier study
187 in vitro[11]. One key factor that may account for this difference is the use of short mycolate analogs in that study,
188 compared to the tracking of native substrates accumulating on Pks13 here, both in cells and in vitro. The
189 thioesterase domain in polyketide synthases (PKSs) often serves as a chain length regulator or a proof-reading
190 enzyme: in the case of shorter products formed on the acyl carrier protein domain, the thioesterase domain may
191 remove the acyl chain to prevent blockage of the synthetic cascade[29]. We therefore reason that the use of shorter
192 mycolate analogs may have rendered Pks13 more promiscuous in the choice of the sugar acceptor. Furthermore,
193 it is conceivable that high specificity for trehalose as the acceptor for mycolates is important for streamlining
194 downstream processes, at least for transport. Specifically, the exquisite synthesis of TMM circumvents the need
195 for multiple redundant transporters to accommodate different hydrophilic sugar headgroups during flipping
196 across the IM, and/or transport to the OM[30].

197 Mycobacteria leverage multiple PKSs for the biosynthesis of complex lipids to build their unique OM[31].
198 Among them, Pks13 is essential for mycobacterial growth[9] and has been revealed as the target of a few hit and
199 lead compounds[32-34]. It had not been established whether the reduction of ketomycolates by CmrA happens

200 before or after the release and esterification by trehalose in the TMM biosynthesis pathway. Cells lacking CmrA
201 still produce TMM*k*[10], indicating that ketomycolates can be released from Pks13 by endogenous trehalose.
202 Notably, we have now demonstrated that cells depleted of trehalose accumulate ketomycolates on Pks13,
203 revealing that endogenous CmrA cannot reduce this precursor. We have further shown that CmrA-mediated
204 reduction would proceed after the formation of TMM*k* in vitro, establishing that reduction of ketomycolates
205 only takes place after release from Pks13 by trehalose. Such post-release reduction contrasts from most other
206 PKSs where keto-reduction steps occur before final release[35]. Given that β-keto-acids can undergo spontaneous
207 decarboxylation reactions, we speculate that preventing reduction of ketomycolates in the absence of trehalose
208 may provide an avenue for degradation and re-assimilation, especially in the context of corynebacteria where
209 MAs are non-essential[36].

210 We have shown that trehalose is required for de novo MA biosynthesis, due to accumulation of
211 intermediates on Pks13 without trehalose. However, it is not clear how such inhibition results in a
212 transcriptional response that up-regulates *acpS-fas*, *fabD-acpM-kasA-kasB-accD6* and *accD4-pks13-fadD32*
213 operons, which likely accounts for the high α'- to α-MA ratio when trehalose was re-supplemented.
214 Interestingly, these transcriptomic changes are highly similar to those reported for drugs inhibiting FAS-I or II
215 but not other envelope pathways[25], suggesting that the changes were elicited specifically by lack of mature
216 mycolates. While it is clear that cells depleted of trehalose exhibit general envelope stress, evidenced by up-
217 regulation of the *iniBAC* operon[26], we postulate that mycobacteria may have yet-to-be-identified mechanisms
218 to sense mycolates in the cell, so as to counteract defects in overall envelope assembly. This would be analogous
219 to how Gram-negative bacteria deploy various LPS sensing mechanisms to ensure proper OM assembly[37].

220 Finally, we have also been able to show that TDM is not essential for in vitro mycobacterial growth. While
221 other studies have alluded to this idea, it was never before possible to remove TDM completely[38,39]; this
222 limitation may also impact the interpretation of in vivo studies that ascribed TDM as a key virulence factor and

223 immune response modulator[40,41]. A trehalose auxotrophic strain supplemented with 6-deoxy-trehalose analogs
224 may thus be useful for future studies on TDM in vivo. Our study provides valuable insights into the formation
225 of MA, TMM and TDM, and serves as a foundation for the development of new anti-mycobacterial drugs,
226 especially against Pks13.
227

228 **Supporting Information**

229 - Detailed materials and methods.
230 - Supplementary Figures 1–4 and Supplementary Table 1

231

232 **Acknowledgments**

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241

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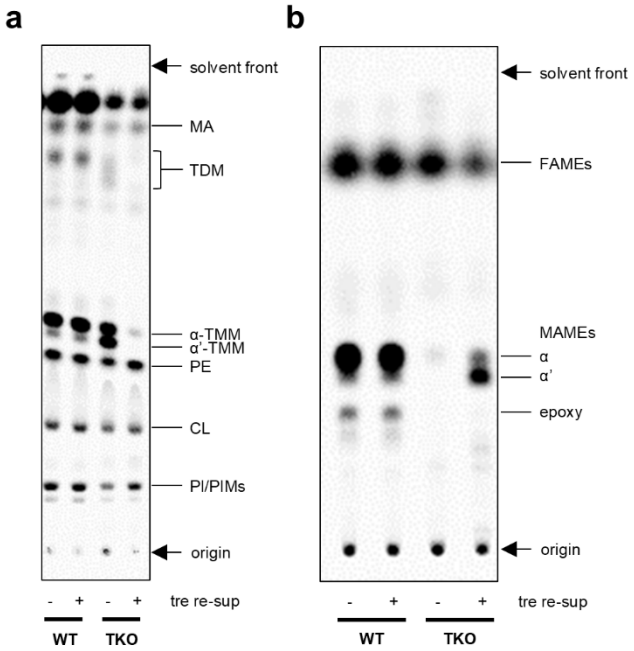


**Figure 1. Trehalose is required for TMM formation and de novo MA biosynthesis. (a)** TLC analysis of [$^{14}$C]-labeled lipids extracted from indicated *M. smegmatis* cells, with (+) or without (-) trehalose re-supplementation (tre re-sup). Developing solvent: chloroform:methanol:water 30:8:1. **(b)** TLC analysis of [$^{14}$C]-labeled fatty acids liberated from indicated strains, esterified as methyl esters. Developing solvent: three times in hexane:ethyl acetate 95:5. TKO cells were depleted of trehalose prior to labelling and lipid extraction. For samples on the same TLC plate, the same amount of radioactivity was loaded. The TLC plates were visualized by phosphor imaging. CL, cardiolipin; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIMs, phosphatidylinositol mannosides; FAME, fatty acid methyl ester; MAME, mycolic acid methyl ester.

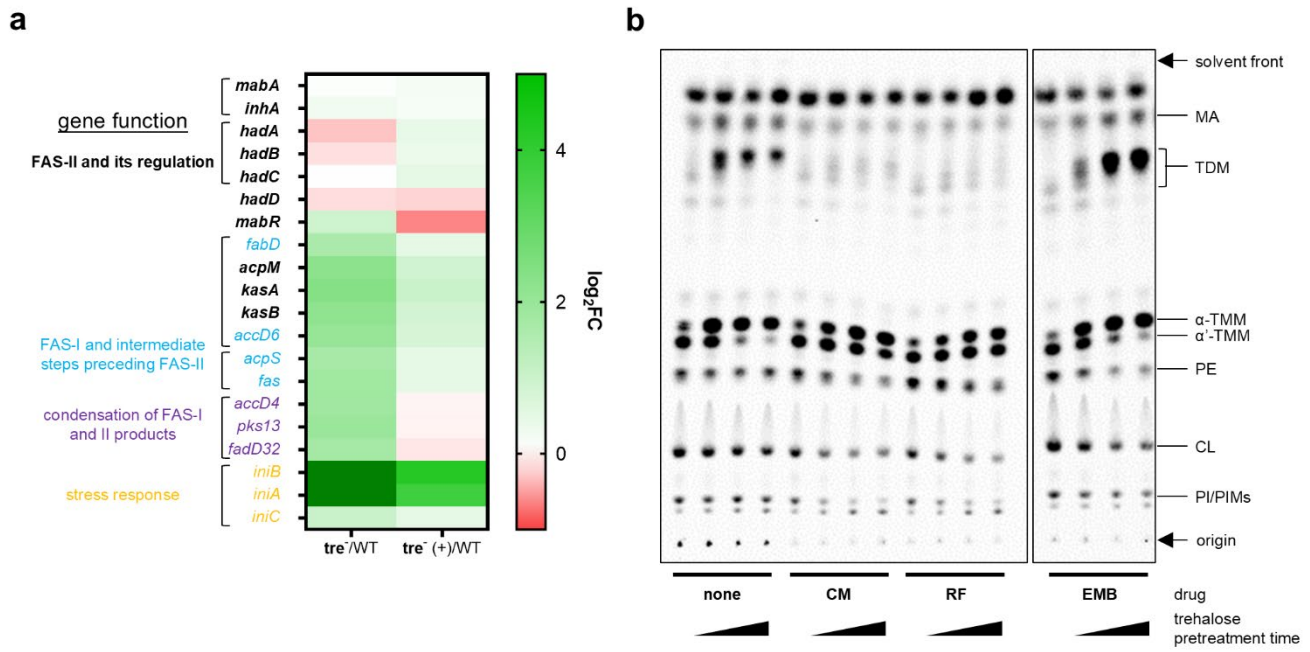


**Figure 2. Trehalose-depleted TKO cells are in a distinct metabolic state. (a)** Heat maps representing changes in transcript levels of indicated genes, either involved in MA metabolism or reportedly induced by FAS inhibitors, when comparing **tre**$^-$ cells (TKO with trehalose depletion) or **tre**$^-$ cells with trehalose re-supplementation (4h, +) to WT cells. Three biological replicates were used for each condition. FC, fold change. Dark green color on the heat map (*iniA* and *iniB* in the **tre**$^-$/WT column) indicates $\log_2$FC > 4. Genes in the same bracket are in the same operon. **(b)** TLC analysis of [$^{14}$C]-labeled lipids extracted from **tre**$^-$ cells pretreated with 20 μM trehalose in the presence of different drugs for extended periods of time (0, 1, 2.5 or 4 h) before radiolabeling with [$^{14}$C]-acetate. Developing solvent: chloroform:methanol:water 30:8:1. The same amount of radioactivity was loaded for each sample. The TLC plate was visualized by phosphor imaging. Chloramphenicol (CM), rifampicin (RF) and ethambutol (EMB) inhibit translation, transcription and AG biosynthesis, respectively.

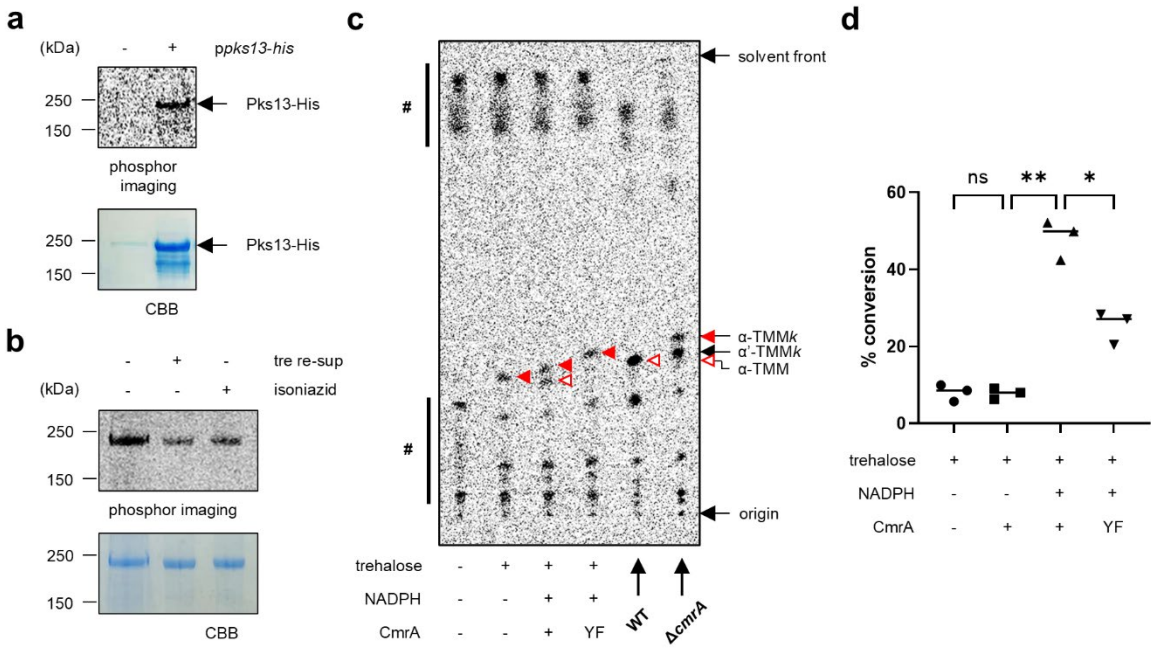


**Figure 3. Unreduced mycolates accumulate on Pks13 upon trehalose depletion. (a)** and **(b)** SDS-PAGE analyses of Pks13-His affinity purified from **tre**$^-$ cells harboring empty vector or pJEB402-*pks13-his*$_8$ grown in the presence of [$^{14}$C]-acetate. The gel was visualized by phosphor imaging (top) and Coomassie brilliant blue staining (CBB; bottom). tre re-sup, trehalose re-supplementation. In **(b)**, trehalose (tre re-sup) or isoniazid was added together with [$^{14}$C]-acetate. **(c)** TLC analysis of lipids extracted from affinity purified Pks13-His subjected to in vitro mycolate release by trehalose and reduction by CmrA. The top and bottom spots (#) on the TLC other than TMM*k* and TMM are present even in the absence of trehalose and are likely non-specific lipids bound to TALON resin used for affinity purification of the protein. Developing solvent: chloroform:methanol:water 30:8:1. The TLC plate was visualized by phosphor imaging. **(d)** Quantification of the conversion of trehalose mono-β-ketomycolate (TMM*k*) to TMM in different conditions. Each data point (mean ± SD) represents the results from three biological replicates. YF, CmrA containing a Y157F mutation. ns, not significant; *, $p < 0.05$; **, $p < 0.01$.

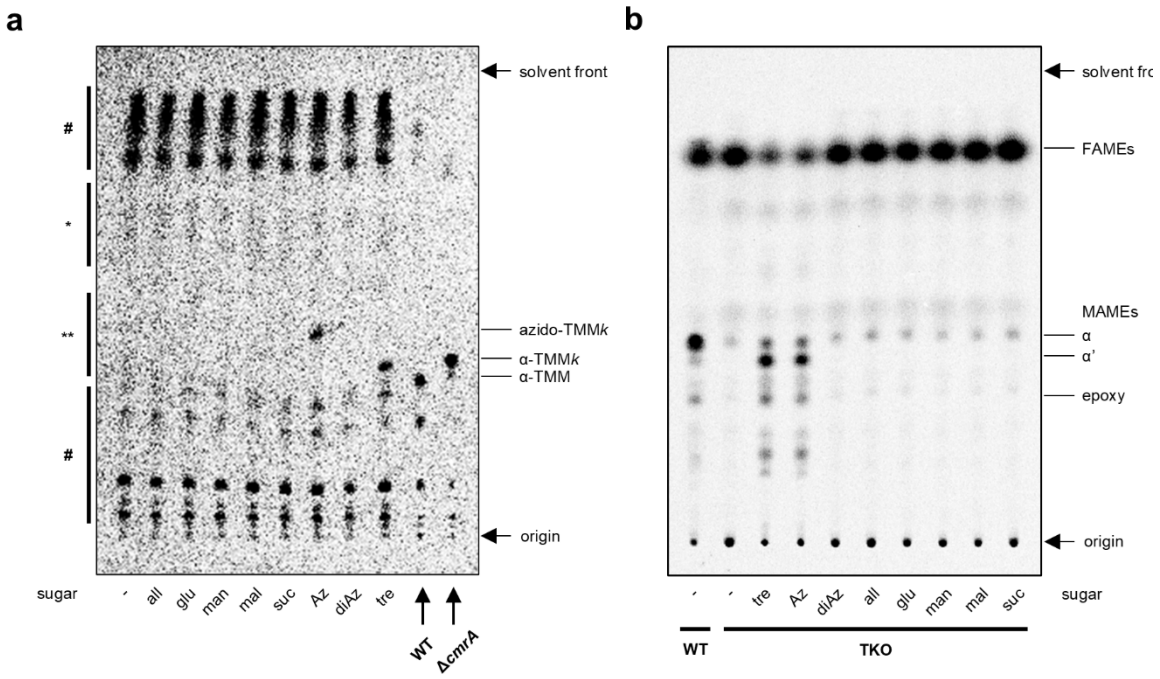


**Figure 4. Pks13 specifically uses trehalose to esterify MAs. (a)** TLC analysis of lipids extracted from the same amount of affinity purified Pks13-His incubated with the indicated sugar. Total extractable lipids from WT and Δ*cmrA* cells were used as reference for migration positions of TMM and TMM*k*, respectively. The top and bottom spots (#) on the TLC are likely non-specifically bound to TALON resin used for affinity purification of the protein. *, a region where monosaccharide monomycolates are expected to appear; **, a region where monosaccharide monomycolates are expected to appear. **(b)** TLC analysis of [$^{14}$C]-labeled total acyl methyl esters derived from **tre**$^{-}$ cells re-supplemented with the indicated sugar. Developing solvent: three times in hexane:ethyl acetate 95:5. The TLC plates were visualized by phosphor imaging. tre, trehalose; Az, 6-azido-trehalose; diAz, 6,6'-diazido-trehalose; all, allose; glu, glucose; man, mannose; mal, maltose; suc, sucrose.

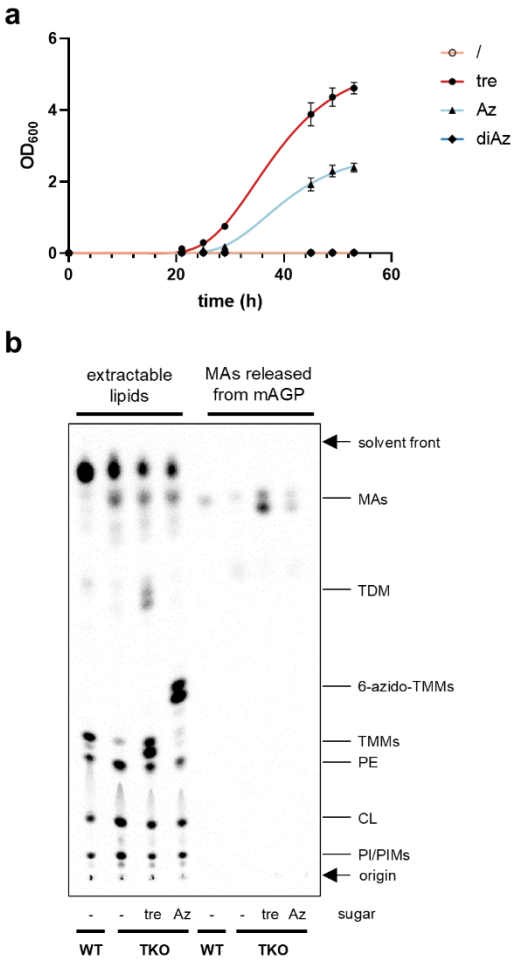


**Figure 5. TDM is not required for mycobacterial growth in vitro. (a)** Growth curves of TKO cells in the presence of the indicated trehalose analog. TKO cells were depleted of trehalose before subculture into fresh medium containing 5 μM trehalose (tre), 6-azido-trehalose (Az), or 6,6'-diazido-trehalose (diAz). Each data point (mean ± SD) represents the results from three biological replicates. **(b)** TLC analysis of [$^{14}$C]-labeled lipids extracted from trehalose-depleted cells re-supplemented with trehalose (tre) or 6-azido-trehalose (Az). TMMs and TDMs are fully extracted from cells, while MAs conjugated as mAGP were released by base hydrolysis. Developing solvent: chloroform:methanol:water 30:8:1. The same amount of radioactivity was loaded for each extractable lipid sample, while MAs released from mAGP were loaded in the same volume as extractable lipids from the same cells. The TLC plate was visualized by phosphor imaging.

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