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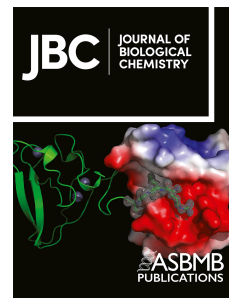
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1 **A dTDP-L-Rhamnose 4-epimerase required for glycopeptidolipid biosynthesis**  
2 **in *Mycobacterium abscessus***  
3

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28 **Running title:** Tle epimerase is required for glycopeptidolipid synthesis

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30 epimerase, morphotype, virulence, zebrafish.  
31

32 **ABSTRACT**

33 *Mycobacterium abscessus* causes severe lung infections in cystic fibrosis patients and exhibits  
34 smooth (S) or rough (R) morphotypes. Disruption of glycopeptidolipid (GPL) production results in  
35 the S-to-R transition but the underlying molecular mechanisms of this transition remain  
36 incompletely understood. Herein, we characterized MAB\_4111c in relation to GPL synthesis and  
37 investigated the effects of *MAB\_4111c* deletion in *M. abscessus* pathogenicity. An enzymatic  
38 assay indicated that MAB\_4111c, also designated Tle for T<sub>al</sub>ose e<sub>pimerase</sub>, is converting dTDP-  
39 L-Rhamnose into dTDP-6-deoxy-L-Talose. A *tle* deletion mutant was constructed in the S variant  
40 of *M. abscessus* and relative areas of Rhamnose and 6-deoxy-Talose and their methylated forms  
41 expressed as ratios of total monosaccharides, showed an altered GPL profile lacking 6-deoxy-  
42 Talose. Thus, Tle provides dTDP-6-deoxy-L-Talose, subsequently used by the glycosyltransferase  
43 Gtf1 to transfer 6-deoxy-Talose to the GPL backbone. Strikingly, the *tle* mutant exhibited a R  
44 morphotype, showed impaired sliding motility and biofilm formation, and these phenotypes  
45 were rescued upon functional complementation. Moreover, deletion of *tle* in *M. abscessus*  
46 results in increased pathogenicity and killing in zebrafish embryos. Together, our results  
47 underscore the importance of the dTDP-L-Rhamnose 4-epimerase activity in GPL biosynthesis  
48 and in influencing *M. abscessus* virulence.

49

50 **INTRODUCTION**

51

52 *M. abscessus* is an emerging opportunistic pathogen causing a variety of human infections that  
53 usually involve the skin and subjacent soft tissues, but also severe lung infections in patients with  
54 various chronic lung disorders, such as bronchiectasis and chronic obstructive pulmonary disease  
55 or cystic fibrosis (1, 2). This mycobacterium is intrinsically resistant to  $\beta$ -lactams, tetracyclines,  
56 aminoglycosides, and macrolides, which are routinely used to treat Gram-negative and Gram-  
57 positive bacterial infections (3), rendering treatments extremely challenging (4). Like other non-  
58 tuberculous mycobacteria (NTMs), *M. abscessus* has shown the ability to develop biofilms,  
59 structured bacterial aggregates enclosed in a self-produced polymeric matrix where multiple and  
60 complex socio-microbiological relationships reign, in the environment, water distribution  
61 systems (5, 6), hospital equipment (5, 6), human tissues, e.g., lungs (7), and medical devices (8,  
62 9). This rapidly-growing mycobacterium can be found as smooth (S) or rough (R) variants and  
63 these morphotypes rely mainly on the production of high (in S) or low (in R) levels of  
64 glycopeptidolipids (GPLs), respectively (10–12). Genome sequencing identified multiple indels in  
65 the *gpl* locus of the R strain as compared to the S strain (13) mainly in *mgs1* and *mgs2*, encoding  
66 non-ribosomal peptide synthases responsible for the biosynthesis of the GPL peptide backbone,  
67 and in *mmpL4a/mmpL4b*, encoding membrane proteins involved in GPL transport (13–15). Thus,  
68 these genetic changes in the *gpl* locus result in the irreversible loss of the GPL and are directly  
69 responsible for the R colony morphology (13).

70 GPLs are found in the outer leaflet of numerous NTMs (16). These lipids are pivotal for  
71 aggregation, biofilm development, motility, interaction with host cells and pathogenesis (16–22).  
72 Both S and R forms are isolated from sputums of patients with chronic lung infections but while  
73 the S form is thought to represent the environmental variant of *M. abscessus* (which infects and  
74 colonizes the lungs), the R form emerges from the S form within the infected host (18, 23, 24).  
75 Several studies support the enhanced pathogenesis of the R form over the S form, the former  
76 being associated with more persisting and aggressive infections in addition to the decline of the  
77 lung functions (18, 25–28). In the context of infection with *M. abscessus*, the zebrafish model has  
78 deserved considerable interest as it was used to help defining the interactions between *M.*  
79 *abscessus* and the innate immune response (27, 29). In particular, zebrafish embryos are killed  
80 by the *M. abscessus* R infection, as evidenced by intense bacterial proliferation, production of  
81 serpentine cords, abscesses and inflammation (27, 30). These features are in agreement with

82 epidemiological surveys, which support the prominence of the R variant in patients with severe  
83 lung infections and chronic airway colonization in cystic fibrosis patients (18, 24). Overall, this  
84 highlights the clinical relevance of morphological distinctions between S and R morphotypes and  
85 importance to understand the S-to-R transition at a molecular level (13).

86 The general GPL structure consists of a mixture of 3-hydroxy and 3-methoxy C28-30 fatty acids  
87 amidated by a tripeptide-amino-alcohol (D-Phe-D-*allo*-Thr-D-Ala-L-alaninol) (10). This  
88 lipopeptide core is glycosylated on the *allo*-Thr linked with a 6-deoxy- $\alpha$ -L-Talose (6-d-Tal) and on  
89 the alaninol linked with an  $\alpha$ -L-Rhamnose (Rha) (10). Some GPLs can be di-glycosylated  
90 containing a 3,4-di-*O*-acetylated 6-d-Tal and a 3,4-di-*O*-methylated or 2,3,4-tri-*O*-methylated Rha  
91 (31–34). We previously demonstrated that the glycosyltransferases Gtf1, Gtf2 and Gtf3, encoded  
92 within the *gpl* locus, transfer the 6-d-Tal, the first Rha and the second Rha to the lipopeptide  
93 core, respectively (35). Importantly, in a *gtf1* deletion mutant, the synthesis of GPL derivatives  
94 lacking 6-d-Tal leads to a R morphotype, mycobacterial cording and increased virulence and  
95 pathogenicity in zebrafish (35). This illustrates the importance of Gtf1 in modelling the GPL  
96 structure and in influencing *M. abscessus* virulence, although the functional and structural  
97 characterization of this enzyme remains to be established. In addition, the enzyme/pathway that  
98 provides dTDP-6-d-Tal, the substrate used by Gtf1 to talosylate GPL, remains unknown.

99 In mycobacteria, L-Rha is synthesized *via* the essential Rml pathway that generates dTDP-L-  
100 Rha from Glc-1-phosphate (36). This pathway is composed of four enzymes - RmlA (MAB\_4113),  
101 RmlB (MAB\_3779), RmlC (MAB\_3780), and RmlD (MAB\_3613c) - encoded by four genes that are  
102 not in close vicinity in the *M. abscessus* genome, except for *rmlB* and *rmlC*. Besides being a  
103 component of GPLs, Rha can be found in other glycolipids, such as phenolic glycolipids (37) and  
104 is present in all mycobacteria as being part of the linker disaccharide (Rha-N-acetyl-glucosaminyl-  
105 phosphate) unit that attaches the cell wall arabinogalactan (AG) to the peptidoglycan (PG) layer  
106 (36). In contrast to Rha, 6-d-Tal is an infrequent deoxyhexose found only in bacteria, essentially  
107 in the cell wall and capsules (38). The dTDP-6-d-Tal is the activated form of 6-d-Tal and it is  
108 synthesized from Glc-1-phosphate and dTTP partially or entirely *via* the Rml pathway. In  
109 *Haemophilus actinomycetemcomitans* NCTC9710 serotype C (partial synthesis *via* the Rml  
110 pathway), the first three enzymes, RmlA-C, are identical to those required to synthesize dTDP-L-  
111 Rha. The fourth enzyme, a dTDP-6-deoxy-L-talose 4-dehydrogenase (Tll) is also a dTDP-4-keto-6-  
112 deoxy-L-mannose reductase such as RmlD, but the stereoselectivity of Tll regulates dTDP-4-keto-  
113 6-deoxy-L-mannose, which is reduced to dTDP-6-deoxy-L-Talose (39). In *Burkholderia*

114 *thailandensis* (complete synthesis *via* the Rml pathway), a dTDP-L-Rha 4-epimerase, WbiB,  
115 converts dTDP-L-Rha into dTDP-6-d-Tal (40). Interestingly, in *M. abscessus*, a paralogous *rmlB*  
116 gene (*MAB\_4111c*) is present in the *gpl* locus (31) and *in silico* predictions suggest that  
117 *MAB\_4111c* encodes a putative epimerase/dehydratase (41).

118       Herein, we focused on *MAB\_4111c* to further advance our understanding of the synthesis of  
119 the dTDP-6-d-Tal substrate of Gtf1 and the presence of 6-d-Tal in the GPL of *M. abscessus*. We  
120 also aimed to study the consequences of *MAB\_4111c* deletion on bacteriological features and  
121 disease pathology in this emerging pathogen in zebrafish. Bioinformatic and genetic studies were  
122 also performed to determine the distribution and prevalence of *MAB\_4111c* orthologous genes  
123 in other NTMs.

124 **RESULTS**

125

126 **MAB\_4111c encodes a putative dTDP-L-Rhamnose 4-epimerase**

127 The S variant of *M. abscessus* synthesizes both diglycosylated GPL (GPL-2a) and triglycosylated  
128 GPL (GPL-3), containing one and two partially *O*-methylated Rha residues, respectively, as well as  
129 a di-*O*-acetyl 6-d-Tal residue (**Figure 1A**). While most proteins encoded by the *gpl* cluster and  
130 involved in the biosynthesis and transport of GPL have been characterized to date, the enzyme  
131 catalyzing the interconversion between dTDP-Rha and dTDP-6-d-Tal in mycobacteria remains  
132 enigmatic. *MAB\_4111c* is located between *gtf3*, encoding the rhamnosyltransferase which adds  
133 the second Rha to generate GPL-3 (35), and *atf2*, encoding an acetyltransferase which acetylates  
134 the 6-d-Tal (34) (**Figure 1B**). Bioinformatics analysis suggests that *MAB\_4111c* may code for an  
135 enzyme with epimerase activity, in agreement with a BLAST analysis which showed that  
136 *MAB\_4111c* shares 54% identity with WbiB, a dTDP-Rha 4-epimerase in *Burkholderia*  
137 *thailandensis*, the first enzyme reported to catalyze the interconversion between dTDP-Rha and  
138 dTDP-6-d-Tal (40). This analysis also shows that the putative catalytic Tyr residue (Y181) as well  
139 as other residues participating in the binding of the NADH cofactor and dTDP-Rha substrate are  
140 fully conserved in *MAB\_4111c* (**Figure 1C**).

141

142 **MAB\_4111c converts dTDP-Rhamnose into dTDP-6-deoxy-Talose**

143 To experimentally validate the function of *MAB\_4111c* as a dTDP-Rha 4-epimerase, a His-tagged  
144 version of the protein was produced in *Escherichia coli*. Initial attempts to express and purify  
145 soluble *MAB\_4111c* for biochemical and structural analyses yielded insufficient protein  
146 quantities. Thus, we generated a *MAB\_4111c*-enriched soluble fraction obtained after partial  
147 purification on Ni<sup>2+</sup>-coated beads, which was assayed by incubation with NADP<sup>+</sup> and dTDP-Rha  
148 and monitored for the production of dTDP-6-d-Tal (**Figure 2A**). The HPLC profile shows the  
149 presence of a weak signal with a retention time (RT) similar to the one of the dTDP-6-d-Tal  
150 standard (**Figure 2B, upper panel**). The nature of the observed weak signal was assessed by  
151 LC/MS analysis in multiple reaction monitoring (MRM) mode (**Figure 2C**). As shown in the upper  
152 panel, the use of specific precursor ion [M-H]<sup>-</sup> at *m/z* 547.1, characteristic of dTDP-dHex, showed  
153 two signals with RT similar to dTDP-Rha and dTDP-6-d-Tal. Fragmentation of the minor signal  
154 produced a MS/MS spectrum identical to standard dTDP-6-d-Tal, demonstrating that the minor  
155 signal corresponds to dTDP-6-d-Tal (**Figure 2C, lower panel**). Finally, NMR analysis of the total



156 reaction mixture showed signals characteristic of dTDP-6-d-Tal, along with signals attributed to  
157 the substrate dTDP-Rha, further demonstrating that MAB\_4111c transforms dTDP-Rha into  
158 dTDP-6-d-Tal (**Figure 2D**). Specifically, weak proton signals at 5.17, 4.05, 3.82, and 3.75 ppm are  
159 characteristic signals of dTDP-6-d-Tal. According to the NMR spectrum of the dTDP-6-d-Tal  
160 standard, proton signals were elucidated as H1 ( $\delta$ 5.17 ppm), H2 ( $\delta$ 4.05 ppm), H3 ( $\delta$ 3.82 ppm)  
161 and H5 ( $\delta$ 3.75 ppm) of 6-d-Tal. The signal of H-4 proton ( $\delta$ 3.65 ppm) in 6-d-Tal is overlapped with  
162 H-4 proton signal of Rha. Kinetic analysis indicated that MAB\_4111c could convert dTDP-Rha into  
163 dTDP-6-d-Tal with a 3-4% conversion rate within an hour, reaching a 7% conversion rate after  
164 300 min of incubation (**Figure 2E**). To address the specificity of the reaction, a similar assay was  
165 set up by replacing dTDP-Rha by dTDP-Glc in the reaction mixture, where no dTDP-6-d-Tal was  
166 detected (**Figure 2B, lower panel**). In addition, we also assayed the reverse reaction using dTDP-  
167 6-d-Tal as a substrate and observed the formation of dTDP-Rha after 60 min of reaction (data not  
168 shown), thus confirming that MAB\_4111c catalyzes both reactions.

169 Overall, these results suggest that MAB\_4111c is an epimerase that specifically converts dTDP-  
170 Rha into dTDP-6-d-Tal *in vitro*. Given its activity, we propose to rename the protein Tle for Talose  
171 epimerase, based on a previous nomenclature used to designate a similar enzyme in  
172 *Acinetobacter baumannii* (42).

173

### 174 **Structural prediction of Tle**

175 Since we could not produce high yields of Tle for structural analysis, we employed AlphaFold 3  
176 for structural predictions (43). We first screened the Protein Data Bank (PDB) for experimentally  
177 determined epimerase structures, most of which are dimeric. Sequence identity between Tle and  
178 available epimerase structures ranged from approximately 23-32%. Despite low sequence  
179 identity, epimerases typically exhibit high structural homology. Using AlphaFold 3, we generated  
180 a dimeric structure of Tle in the presence of NAD<sup>+</sup>. The prediction covered more than 90% of the  
181 structure with a very high predicted local distance difference test (pLDDT) score (**Figure 3A**). The  
182 oligomeric state prediction yielded predicted template modelling (pTM) and interface predicted  
183 template modelling (ipTM) values of 0.91 and 0.92, respectively, indicating that the predicted  
184 structure can be used with confidence for further analysis. The structure reveals a classic Short-  
185 Chain Dehydrogenase/Reductase (SDR) fold (44) (**Figure 3B**). The dimeric interface is formed by  
186 two  $\alpha$ -helices from each protomer (residues 88-110 and 180-199), creating a four- $\alpha$ -helix bundle  
187 (**Figure 3B**). The overall architecture includes an N-terminal NAD<sup>+</sup> binding domain, a typical

188 Rosmann fold and a C-terminal substrate-binding domain (**Figure 3B**). Comparison of the  
189 AlphaFold-generated Tle structure with the known *Pseudomonas aeruginosa* UDP-N-  
190 acetylglucosamine 4-epimerase WbpP (PDB code:1SB8) epimerase structure yielded a root mean  
191 square deviation (RMSD) of approximately 1.37 Å (**Figure 3B**), suggesting a high degree of  
192 structural similarity. WbpP was used because it had one of the best RMSD scores in our analysis.  
193 Notably, Tle includes an extended secondary structure element from residues 128 to 165,  
194 characterized by an additional  $\alpha$ -helix, long loop and a pair of antiparallel  $\beta$ -strands. This feature  
195 contrasts with WbpP, which has a shorter loop in the corresponding region, highlighting a  
196 distinctive structural variation in Tle (**Figure 3B**).

197 We next compared the AlphaFold-predicted structure of Tle with the known WbpP structure  
198 to identify the key residues involved in catalysis. The binding site for NAD<sup>+</sup>, is highly conserved  
199 among SDR family enzymes (44). Structural alignment with WbpP confirmed that majority of the  
200 NAD<sup>+</sup> binding residues are conserved in Tle (**Figure S1A**). However, some differences were noted:  
201 V30, H32, and H36 in Tle correspond to N48, A50, and G52 in WbpP (**Figure S1B**). The catalytic  
202 triad, which is typically represented as SYK in the SDR family (44–46) is conserved in Tle as S121,  
203 Y181, and K185 (**Figure S1B**). No activity was observed when replacing the wild-type enzyme by  
204 the Tle-Y181A mutant in the assay (**Figure 2B and 2D**), thus indicating that introducing a Y181A  
205 change leads to a catalytically inactive enzyme. We also assessed the activity of Tle mutated  
206 proteins in which the conserved residues S121, N210, R249 (**Figure 1C and S1B**) were substituted  
207 by alanines. As for the His-tagged wild-type protein, the different mutated variants were enriched  
208 by affinity chromatography on Ni<sup>2+</sup> beads and the amount of each protein visualized by Western  
209 blotting using anti-His antibodies (**Figure S2A**). The relative concentration of each protein was  
210 determined by comparing the grayscale of the bands using ImageJ and adjusted so that the same  
211 quantity of each protein was added to the assay in the presence of NADP<sup>+</sup> and dTDP-6-d-Tal.  
212 **Figure S2B** shows that none of these mutated proteins catalysed the formation of dTDP-6-d-Tal,  
213 confirming the importance of S121, N210, and R249 in the activity of Tle.

214 Overall, these data validate the AlphaFold model and supports the structural similarity  
215 between WbpP and Tle.

216

### 217 **Bacteriological characterization of a *tle* deletion mutant in *M. abscessus***

218 To investigate the contribution and biological functions of the dTDP-L-Rha 4-epimerase in *M.*  
219 *abscessus*, *tle* was deleted in the GPL-producing S variant of *M. abscessus*, using a unmarked

220 deletion method (47) (**Figure S3A**). PCR/sequencing performed on the parental and mutant strain  
221 ( $\Delta tle$ ) using the primers listed in **Table S1** confirmed the proper genotype of the mutant (**Figure**  
222 **S3B**). Genetic complementation of the mutant was done through specific integration at the *attB*  
223 chromosomal site (48) of a copy of *tle* fused to a GFP-tag at the 3'-end and placed under the  
224 control of the endogenous promoter. Probing the crude lysates from the various strains using  
225 anti-GFP antibodies revealed a single band of the expected size, corresponding to the Tle-GFP  
226 fusion protein, validating complementation of the mutant (**Figure S3C**).

227 A previous work had shown that deletion of *gtf1* in *M. abscessus* S was associated with R  
228 morphotype on agar plates (35). Observation of individual colonies showed that  $\Delta tle$  formed R,  
229 corded colonies (**Figure 4A**). Complementation of  $\Delta tle$  ( $\Delta tle::C$ ), rescued the S morphotype  
230 (**Figure 4A**). The growth of all strains in liquid culture describes a logistic growth ( $R^2 > 0.9$  for all of  
231 them) (**Figure 4B and Table S4**). The growth rate of  $\Delta tle$  was higher than that of the R strain  
232 ( $p < 0.0001$ ), while the one of  $\Delta tle::C$  was similar to the growth rate of the parental S strain. This  
233 indicates that deletion of *tle* slightly impacts on the replication rate of *M. abscessus* in planktonic  
234 culture. However, additional transmission electron microscopy observations failed to show  
235 significant changes regarding the size and ultrastructural organization of the cell wall in  $\Delta tle$   
236 (**Figure S4**). Partitioning of mycobacterial pellets between hexadecane and aqueous buffer is  
237 used as a quantitative marker of cell surface hydrophobicity in mycobacteria to associate low  
238 pathogenicity with reduced hydrophobicity (49). To address whether deletion of *tle* affects  
239 surface hydrophobicity, aqueous hexadecane-buffer partitioning was applied to the S, R,  $\Delta tle$ ,  
240 and  $\Delta tle::C$  strains.  $\Delta tle$  appeared more hydrophobic than the parental S strain, in agreement  
241 with its R morphotype while the complemented strain displayed a hydrophobicity level similar to  
242 the parental S progenitor, correlating with its smoother morphological aspect (**Figure 4C**).  
243 Consistent with the ability of the *M. abscessus* S variants to slide from the centre towards the  
244 periphery of the plates (11, 28),  $\Delta tle::C$ , but neither R nor  $\Delta tle$ , showed a higher sliding motility  
245 (**Figure 4D and 4E**). Since sliding is dependent on the bacterial surface properties and interactions  
246 with the substrate, this suggests that  $\Delta tle$  is likely defective in surface-associated cell wall  
247 components. The  $\Delta tle$  colony biofilm was intermediate between the S and R biofilms (**Figure 4F**),  
248 being broader and higher than the S biofilm (**Figure 4F, 4G and S5**). In addition, the number of  
249 CFUs per membrane (**Figure 4H**), the colony volume (**Figure 4I**) and density of the biofilm (**Figure**  
250 **4J**) were very similar between the R and  $\Delta tle$  strains.  $\Delta tle::C$  showed no difference with the S  
251 progenitor.

252 We next investigated whether *tle* deletion affects drug susceptibility of *M. abscessus* towards  
253 a large panel of clinically used antibiotics.  $\Delta tle$  showed only very slight changes in its antibiotype  
254 **(Table S5)**, for instance a slight increase against amikacin (MIC of 16  $\mu\text{g}/\text{mL}$  for the wild-type  
255 strain and 32  $\mu\text{g}/\text{mL}$  for  $\Delta tle$ ), tigecycline (MIC of 0.5  $\mu\text{g}/\text{mL}$  for the wild-type strain to 1  $\mu\text{g}/\text{mL}$   
256 for  $\Delta tle$ ), and a slight reduction against imipenem (MIC of 64  $\mu\text{g}/\text{mL}$  for the wild-type to 32  $\mu\text{g}/\text{mL}$   
257 for  $\Delta tle$ ), clarithromycin (MIC of 8  $\mu\text{g}/\text{mL}$  for the wild-type strain and 4  $\mu\text{g}/\text{mL}$  for  $\Delta tle$ ) or rifabutin  
258 (MIC of 64  $\mu\text{g}/\text{mL}$  for the wild-type strain and 32  $\mu\text{g}/\text{mL}$  for  $\Delta tle$ ).

259

### 260 **Deletion of *tle* leads to the production of GPL lacking 6-d-Talose**

261 We next evaluated the impact of *tle* deletion on the glycolipid content extracted from lyophilized  
262 mycobacteria. Analysis of the apolar lipid fraction by TLC shows that trehalose 6,6'-dimycolate  
263 (TDM) remains equally produced in all tested strains **(Figure 5A)**. Analysis of the polar lipid  
264 fractions indicates that, except in the R strain lacking GPLs (33, 35), GPLs are produced at high  
265 levels in all strains **(Figure 5A, left panel)**. However, while the parental S strain and  $\Delta tle::C$   
266 displayed an identical GPL profile,  $\Delta tle$  exhibited lower migrating GPLs, resembling to those  
267 produced by  $\Delta gtf1$  **(Figure 5A right panel and S6A)**, a previously characterized mutant lacking  
268 the Gtf1 glycosyltransferase, which adds 6-d-Tal to the peptide backbone (35). Mild alkaline  
269 hydrolysis deacetylated polar lipids from S and  $\Delta tle::C$ , as shown by the lower  $R_f$  of NaOH-treated  
270 compared to untreated ones GPL **(Figure 5B right panel)**. In contrast, saponification did not  
271 influence the GPL pattern of  $\Delta tle$  and  $\Delta gtf1$  **(Figure 5B right panel and S6C)**, suggesting that they  
272 are not acetylated, as previously demonstrated for  $\Delta gtf1$  (35). Mass spectrometry analysis of  
273 native polar lipids from S and  $\Delta tle::C$  showed ions at  $m/z$  1258/1286 and 1404/1432 **(Figure 5C)**,  
274 previously identified as GPL-2a and GPL-3 **(Figure 1A)** (33, 34). After saponification, M-84 u.m.a  
275 ions at  $m/z$  1173/1201 and 1319/1347 were detected, corresponding to dGPL-2a and dGPL-3,  
276 respectively, in agreement with the loss of the two acetyl groups substituting 6-d-Tal **(Figure 5C**  
277 **and S6C-D)**. MS spectra of native and saponified polar lipids isolated from  $\Delta tle$  and  $\Delta gtf1$ , all  
278 showed an intense ion at  $m/z$  1187 accompanied with 1013/1041 pattern **(Figure 5C and S6C-D)**,  
279 previously attributed to the unnatural isomers GPL-2b and GPL-1b, which are devoid of 6-d-Tal  
280 (35). The absence of the 6-d-Tal was further confirmed by the monosaccharide composition  
281 analysis of the polar lipids, which demonstrates the lack of 6-d-Tal in  $\Delta tle$  and  $\Delta gtf1$  and its  
282 restoration in  $\Delta tle::C$  **(Figure 5D and S6E)**.

283

## 284 **Distribution of *tle* orthologs in NTMs and other bacteria**

285 We identified and analyzed 107 proteins orthologous to *Tle* considering the inclusion and exclusion  
286 criteria described in the Materials and Methods. The neighbour-joining phylogenetic tree showed  
287 two clear clades related to mycobacterial genes and other bacterial species. Orthologous were  
288 found in at least 26 bacterial species belonging to the phyla Actinomycetota (genera  
289 *Nocardioides*, *Pseudonocardia*, *Desertimonas*, *Glaciibater*, *Curtobacterium*, and *Cryobacterium*)  
290 and Pseudomodota (genera *Burkholderia*, *Paraburkholderia*, *Xanthomonas*, *Rhizobium*, and  
291 *Chitinimonas*). Seventy-nine mycobacterial species, excluding *M. abscessus*, possess an  
292 orthologous gene to *tle* based on amino acid sequence analysis (**Figure 6A**). These included 42  
293 slowly-growing mycobacteria, one intermediately-growing mycobacterium and 36 rapidly-  
294 growing mycobacteria. This phylogenetic analysis indicates that *Tle*-related proteins can be found  
295 in bacteria but are over-represented in mycobacteria. It also suggests that these orthologs may  
296 have descended from a common ancestor and, therefore, that they can be viewed as orthologous  
297 members of the same protein family.

298 In mycobacteria, 6-d-Tal has been only described in GPLs so far. We next investigated the  
299 glycolipid profile, focusing on GPL from a set of slow-growing and rapidly-growing NTM clinical  
300 isolates, which are representative strains present in the phylogenetic tree (**Figure 6A**). TLC  
301 analysis of the apolar lipid fraction of all species from a broad panel showed an intense band  
302 around  $R_f$  0.43 that was attributed to TDM (**Figure S7A**). TLC analysis of polar lipids in  
303 chloroform/methanol/water (65:25:4, v/v/v) also showed phospho-*myo*-inositol mannosides  
304 (PIMs) in all species (**Figure S7B**). Subsequent TLC analysis using chloroform/methanol/water  
305 (90:10:1, v/v/v) as solvent revealed a wider lipid diversity, including GPL-like compounds that were  
306 differentially expressed (**Figure 6B**). Overall, of the fifteen strains tested, ten strains (marked +)  
307 showed bands corresponding to GPL, whereas five (marked –) did not. Some strains showed  
308 similar TLC patterns such as *M. avium* and *M. gilvum* or *M. genavense* and *M. lentiflavum*,  
309 suggesting that these pairs of strains have close GPL profiles. The other GPL-positive strains (*M.*  
310 *abscessus*, *M. aurum*, *M. goodii*, *M. simiae*, *M. moriokaense* and *M. paraffinicum*) showed distinct  
311 TLC patterns, indicating a large structural variability in their GPL content. As a control, we checked  
312 that the identified GPL-related compounds were still detected as lower  $R_f$  bands following  
313 saponification. In contrast, all bands disappeared in *M. branderi*, *M. chelonae*, *M. mageritense*,  
314 *M. malmoense* and *M. scrofulaceum* further confirming the absence of GPLs in these species  
315 (**Figure S8**). We further established by GM/FID and GC/MS the monosaccharides composition of

316 the native polar lipid fractions from all the species in order to confirm the species-specific  
317 expression of GPLs (**Figure 6C**). All ten identified GPL-positive species expressed a wide range of  
318 deoxyhexose residues, including 6-d-Tal and its methylated-analogues (2-*O*-methyl 6-d-Tal, 3-*O*-  
319 methyl 6-d-Tal), and Rha with methylated analogues (3-*O*-methyl Rha, 3,4 di-*O*-methyl Rha, 2,3,4  
320 tri-*O*-methyl Rha) in different ratios. The 6-d-Tal or its methylated forms were detected in all  
321 strains, whereas Rha or its methylated forms were detected in all strains except *M. paraffinicum*  
322 that exclusively expressed 6-d-Tal. The presence of 3-*O*-methyl 6-d-Tal in *M. genavense*, *M.*  
323 *lentiflavum*, *M. simiae* and *M. moriokaense* has already been observed in previous studies, in  
324 agreement with the present work. In contrast, the polar lipids of three of the five GPL-negative  
325 strains (*M. branderi*, *M. chelonae*, *M. mageritense*) did not show any traces of deoxyhexoses.  
326 Finally, *M. malmoense* and *M. scrofulaceum* showed low quantities of 6-d-Tal, Rha and 3-*O*-  
327 methyl Rha, suggesting that these two strains may synthesise deoxyhexose independently of GPL.  
328 Altogether, these results indicate the exclusive presence of 6-d-Tal in all GPL-like molecules in  
329 mycobacteria, albeit with traces found in *M. scrofulaceum*.

330

### 331 **Deletion of *tle* in *M. abscessus* results in increased pathogenicity in zebrafish**

332 *M. abscessus* S and R variants have very different infection outcomes when injected in zebrafish  
333 embryos (27, 30). While R leads to acute infection and high embryo mortality, S is less virulent  
334 and more associated with chronic persistent infection. Therefore, because we observed a R  
335 morphotype upon knocking-out *tle*, we wanted to use the zebrafish model to evaluate the effect  
336 of this mutation on the infection outcome. To do so, 30 hpf embryos were infected with  
337 approximately 250 CFU of either R, S,  $\Delta tle$ ,  $\Delta tle::C$  (all strains expressing a red fluorescent marker)  
338 or the PBS control, and monitored for embryo survival, bacterial burden and whole embryo  
339 imaging as outlined in **Figure 7A**. As expected, mortality of embryos injected with  $\Delta tle$  was much  
340 higher than the S wild-type progenitor (10% and 42%, respectively) and more like the R  
341 morphotype (40%) at 12 dpi (**Figure 7B**). Complementation was only partial, similar to what was  
342 observed *in vitro*, with 21% of embryo mortality. In agreement with the enhanced embryo  
343 mortality, bacterial burden was significantly higher in embryos infected with  $\Delta tle$ , to levels close  
344 to the R strain, compared to S progenitor while infection with  $\Delta tle::C$  led to bacterial loads  
345 comparable to those found with the S strain (**Figure 7C**). These results were confirmed by embryo  
346 imaging, where we can clearly see very small infection foci in embryos injected with S and the

347 complemented strain at 3 dpi (**Figure 7D**), while embryos injected with the mutant were similar  
348 to the R, with a higher number of infected foci as well as a bigger size.

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349 **DISCUSSION**

350

351 6-deoxy-Talose is a rare deoxyhexose found in Gram-negative, Gram-positive bacteria and  
352 mycobacteria as a component of the cell wall and capsule structures (10, 35, 38, 42). The  
353 activated nucleotide sugar form of 6-d-Tal is dTDP-6-d-Tal, which is synthesised from Glc-1-  
354 phosphate and dTTP *via* the Rml pathway. Until now, the origin of the dTDP-6-d-Tal used by Gtf1  
355 for talosylation of GPL remained unknown. Based on the structural predictions with validated  
356 epimerases and the development of an enzymatic assay, we demonstrate here that  
357 MAB\_4111c/Tle catalyses the epimerisation of dTDP-Rha to dTDP-6-d-Tal, an activity lost when  
358 the catalytic Y181 or substrate-binding residues are replaced with alanine. Since the  
359 epimerization to TDP-6-d-Tal appears only partial *in vitro*, it is possible that *in vivo*, the activity of  
360 Tle is coupled with and/or regulated by the cognate glycosyltransferase Gtf1, previously shown  
361 to attach the talose residue to the GPL peptidic backbone (35).

362 Genomic analyses further showed that *tle* orthologues are widely distributed in actinomycetes,  
363 and was found in approximately 42% of all mycobacterial species for which a genome sequence  
364 is available (52). Even though GPL from various species appear structurally different, our  
365 compositional analysis indicates that all contain 6-d-Tal (or 2-*O*-methyl-6-d-Tal or 3-*O*-methyl-6-  
366 d-Tal) and that these species all possess a *tle* gene. Conversely, a few species failed to produce  
367 GPLs despite the presence of *tle*, although it is very likely that these strains (at least some of  
368 them) do not produce GPL due to the presence of mutation in the *gpl* biosynthetic and transport  
369 gene cluster, as demonstrated previously in *M. abscessus* (13). *M. avium* represents another  
370 typical example of an NTM producing GPL which encodes a *tle* gene. Interestingly, the lack of 6-  
371 d-Tal attached to the *allo*-threonine in a R variant of *M. avium* MAC 104 has been previously  
372 reported (53), and the genetic lesion responsible for the loss of 6-d-Tal in this strain corresponded  
373 with the deletion of a genomic region containing *gtfA*, an ortholog of *gtf1* (54). This further  
374 supports the hypothesis that the loss of 6-d-Tal conditioning a R morphotype is not specific to *M.*  
375 *abscessus* but can be extrapolated to other NTMs.

376 To validate the structure-function relationship between Tle and 6-d-Tal-containing GPL, we  
377 generated a *tle* unmarked deletion in the *M. abscessus* S variant. A thorough lipid analysis clearly  
378 emphasized the absence of 6-d-Tal in the GPL of the mutant strain, while the presence of Rha  
379 residues remained intact. Previously, we characterized a *gtf1* mutant, which, similarly to the *tle*  
380 mutant, produced GPL lacking 6-d-Tal. Gtf1 uses dTDP-6-d-Tal produced by Tle, as a substrate to



381 glycosylate the D-*allo*-Thr residue of GPL. In addition to the loss of 6-d-Tal, both  $\Delta gtf1$  and  $\Delta tle$   
382 share in common other phenotypes. First, deletion of *tle* produces a virtually R morphotype,  
383 although it appeared creamy on plates, while deletion of *gtf1* generated colonies with a R and  
384 dry texture. Whereas the vast majority of clinical isolates of the R morphotype have mutations  
385 in the biosynthetic genes *mps1*, *mps2*, or transporter genes *mmpL4a/mmpL4b/mmpS4* (15), GPL  
386 glycosylation can also lead to colony morphological changes (10). This study shows that enzymes  
387 involved in the production of nucleotide-activated sugars can also lead to these morphological  
388 changes. However, it remains to be established whether mutations in *tle* are responsible for the  
389 acquisition of an R morphotype occurring during infection in the host. Second, while GPL are  
390 present in the outermost mycobacterial layer (10), their presence/absence or modifications in  
391 their structures can profoundly alter the surface properties of *M. abscessus*. Sliding motility may  
392 play an important role in surface colonisation by mycobacteria in the environment and in the  
393 host (35). The *tle* mutant showed considerably reduced sliding motility. Previous studies between  
394 S and R strains underscored the correlation between low GPL production and enhanced  
395 hydrophobicity with virulence (2, 10). As expected for a R variant, we found that  $\Delta tle$  was more  
396 hydrophobic than the parental S variant, as reported previously for the *gtf1* mutant (35). Third,  
397 the development of biofilms appears crucial for *M. abscessus* infections (55, 56). The deletion of  
398 *tle* altered the biofilm development of *M. abscessus*. Although most of the measured parameters  
399 (number of bacteria, colony volume and density) indicated that the *tle* mutant behaved similarly  
400 to the R morphotype, its colony shape was significantly different from that of the S and R  
401 morphotypes, as the colony height of the *tle* mutant was greater and colony diameter was less  
402 wide than that of the reference R strain. This emphasizes the importance of 6-d-Tal for shaping  
403 *M. abscessus* colony-biofilms. Herein, we also took advantage of using the zebrafish model of  
404 infection as a reliable and manageable model for revealing and comparing the difference in  
405 virulence between the parental S and the  $\Delta tle$  strains (27, 57). Our results are consistent with the  
406 previous findings obtained with  $\Delta gtf1$  (35) and indicate that, in the presence of the sole innate  
407 immunity, the loss of 6-d-Tal in GPL translates into increased pathogenesis and lethal infection in  
408 zebrafish embryos.

409 Overall, this study also emphasizes the biosynthetic interconnection between the  
410 arabinogalactan and GPL pathways in *M. abscessus*, as depicted in **Figure 8**. The mycobacterial  
411 cell wall consists of a mycolic acid layer that is bound to peptidoglycan *via* the polysaccharide  
412 arabinogalactan. Arabinogalactan is covalently bound to the peptidoglycan *via* the  $\alpha$ -L-

413 rhamnopyranosyl-(1→3)- $\alpha$ -D-N-acetylglucosaminosyl-1-phosphate unit (58). On the one hand,  
414 the donor dTDP-Rha, used by the rhamnosyltransferase WbbL to produce the rhamnosyl-  
415 containing linker unit (59), is synthesized by four enzymes (RmlA-D) beginning with dTPP and Glc-  
416 1-phosphate. TDP-Rha biosynthesis is essential for the growth of mycobacteria (60), prompting  
417 to the development of dTDP-Rha inhibitors for new tuberculosis therapeutics (61). On the other  
418 hand, the donor dTDP-Rha is also used by the rhamnosyltransferases Gtf2 and Gtf3, which add the  
419 first and second Rha to the GPL biosynthetic intermediates. That dTDP-Rha stands on the  
420 crossroad between the arabinogalactan and GPL pathways is further substantiated by its direct  
421 use by Tle, which converts dTDP-Rha into dTDP-6-d-Tal, which is itself used by Gtf1 to talosylate  
422 the rhamnosylated GPL intermediates. However, while arabinogalactan (and disaccharide linker  
423 unit) is fundamental to the structural integrity of the cell wall and required for mycobacterial  
424 viability, this is not the case for the GPLs, which are lacking in the R strains of *M. abscessus*.  
425 However, the loss of GPL has important consequences on *M. abscessus* pathogenesis and  
426 infection outcome, as shown in various animal models (26, 27, 30, 35) and in humans (18, 24).

427 **EXPERIMENTAL PROCEDURES**

428

429 **Mycobacterial strains, growth conditions, and reagents**

430 *M. abscessus* CIP104536<sup>T</sup> (S) and CIP104536<sup>T</sup> (R) strains used in this study were previously  
431 sequenced and their genomic differences identified and characterized (13, 62). All bacterial  
432 strains and plasmids used for this study are listed in **Table S2** and **Table S3**, respectively.  
433 Mycobacteria were grown in Middlebrook 7H9 broth (BD, New Jersey, USA) supplemented with  
434 0.025% tyloxapol and 10% oleic acid- albumin-dextrose-catalase enrichment (OADC) (7H9<sup>OADC</sup>) or  
435 on Middlebrook 7H10 agar (BD) containing 10% OADC (7H10<sup>OADC</sup>) at 37 °C, with antibiotics when  
436 required. A Bio-Rad Gene pulser (25 µF, 2500 V, 800 Ω) was used to transform electrocompetent  
437 mycobacteria. After transformation, strains carrying the pMV306 (48) derivatives were selected  
438 on 250 µg/mL kanamycin.

439

440 **Expression of Tle in *E. coli***

441 *MAB\_4111c* (*tle*) was PCR-amplified using *M. abscessus* genomic DNA using the specific primers  
442 (**Table S1**, primers 9-10) and Q5 polymerase. The amplicons were cloned into pET30 restricted  
443 with KpnI and EcoRI, enabling to introduce the genes in frame with the six-histidine tag. The  
444 resulting pET30-*tle* was used for site-directed mutagenesis of specific amino acids involved in the  
445 active site (Y181, primers 11-12) or the substrate-binding sites (S121, primers 13-14; N210,  
446 primers 15-16; R249, primers 17-18) using the QuikChange II Site-Directed Mutagenesis Kit  
447 (Agilent) and based on structural similarity with the *B. thailandensis* dTDP-L-Rha 4-epimerase  
448 (UniProt ID: Q2SYH7 · WBIB\_BURTA). The pET30 constructs containing the native or mutated *tle*  
449 genes were used to transform *Escherichia coli* strain BL21 Star DE3 (Life Technologies). Cultures  
450 were grown in terrific broth medium containing 50 µg/mL kanamycin at 37 °C under agitation  
451 until an optical density at 600 nm (OD<sub>600</sub>) of 0.7-0.9 was achieved. Cultures were placed at 4 °C  
452 for 30 min before addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and an  
453 overnight incubation at 16 °C under agitation. Bacteria were collected by centrifugation and  
454 pellets were resuspended in lysis buffer (50 mM Tris-HCl pH 8, 400 mM NaCl, 5% glycerol,  
455 supplemented with 10 mg/mL of NP-40, 78 µg/mL of dithiothreitol, 1 pill of cOComplete tablets,  
456 Mini EDTA-free, EASYpac per 50 mL, 2.175 mg/mL of imidazole and 1mg/mL of lysozyme)  
457 previously chilled on ice. Bacteria were lysed by sonication and the lysate clarified by  
458 centrifugation (12,000 rpm at 4°C for 30 min), incubated overnight at 4 °C with nickel beads (Ni

459 Sepharose 6 Fast Flow, GE Healthcare), washed using washing buffer I (50 mM Tris-HCl pH 8, 350  
460 mM NaCl, 5% glycerol, 78 µg/mL of dithiothreitol, 1 pill of cOmplete tablets, Mini EDTA-free,  
461 EASYpac per 50 mL, and 1.3 mg/mL of imidazole) and in washing buffer II (50 mM Tris-HCl pH 8,  
462 200 mM NaCl, 5% glycerol, 78 µg/mL of dithiothreitol, 1 pill of cOmple tablets, Mini EDAT-free,  
463 EASYpac per 50 mL and 1.3 mg/mL of imidazole). Proteins were eluted in elution buffer (50 mM  
464 Tris pH=7, 100 mM NaCl, and 5% glycerol, and 17 mg/mL of imidazole) and then dialyzed  
465 overnight at 4 °C in dialysis buffer (50 mM Tris-HCl pH7, 200 mM NaCl, and 5% glycerol).

466

#### 467 **Enzymatic activity assay**

468 A 50 µL reaction mixture (in 50 mM of PBS buffer pH 7.5) containing 10 mM of dTDP-L-Rha, 1 mM  
469 of NADP<sup>+</sup>, and Nickel-beads eluted fractions enriched with the various MAB\_4111c protein  
470 variants (0.42 µg/µL MAB\_4111c, 0.31 µg/µL MAB\_4111c-Y181A, 0.86 µg/µL MAB\_4111c-S121A,  
471 0.70 µg/µL MAB\_4111c-N210A, 0.75 µg/µL MAB\_4111c-R249A, and 0.69 µg/µL MAB\_4111c-  
472 R249A/N210A/S121A) was incubated at 37 °C for 1 hr. The reaction was stopped by diluting the  
473 reaction with cooled buffer of acetonitrile/100 mM aqueous ammonium acetate pH 4.5 (60%  
474 acetonitrile). As a control reaction, a similar assay was performed by replacing dTDP-L-Rha by 10  
475 mM of dTDP-D-Glc. The diluted solution was analyzed by HPLC equipped with UV detector at 254  
476 nm using ZIC<sup>®</sup>-cHILIC column. The column was eluted at 40 °C with acetonitrile/100 mM aqueous  
477 ammonium acetate pH 4.5 (60% acetonitrile) at a flow rate of 0.6 mL/min. The enzyme activity  
478 was determined by the conversion of the product (Yield = [dTDP-6-deoxy-L-Tal]/([dTDP-6-deoxy-  
479 L-Tal] +[dTDP-L-Rha])). To analyse the activity by NMR, a reaction mixture consisting of 500 µL  
480 deuterium oxide(D<sub>2</sub>O) containing 10 mg of dTDP-L-Rha, 5 mg of NADP<sup>+</sup>, 5 mg of disodium  
481 hydrogen phosphate (Na<sub>2</sub>HPO<sub>4</sub>), and enriched MAB\_4111c protein variants (0.14 µg/µL  
482 MAB\_4111c, 0.13 µg/µL MAB\_4111c-Y181A) was incubated at 37 °C for 5 hrs.

483 For the time-curve analysis, a 100 µL reaction mixture (in 50 mM of PBS buffer, pH 7.5) containing  
484 10 mM of dTDP-L-Rha, 1 mM of NADP<sup>+</sup>, and 0.42 µg/µL of MAB\_4111c was incubated at 37 °C for  
485 different time point. The reaction was stopped by diluting the reaction with cooled buffer of  
486 acetonitrile/100 mM aqueous ammonium acetate pH 4.5 (60% acetonitrile). The diluted solution  
487 was analyzed by HPLC equipped with UV detector at 254 nm using ZIC<sup>®</sup>-cHILIC column. The  
488 column was eluted at 40 °C with acetonitrile/100 mM aqueous ammonium acetate pH 4.5 (60%  
489 acetonitrile) at a flow rate of 0.6 mL/min. All dTDP-activated sugar nucleotides were produced  
490 enzymatically, as reported previously (63).

491 LC-MS/MS analysis was performed on a triple quadrupole mass spectrometer LCMS8060XS  
492 (Shimadzu, Kyoto, Japan) coupled with a Nexcera system (Shimadzu, Kyoto, Japan).  
493 Chromatography was performed on a ZIC-cHILIC column as previously described (2.1 mm i.d. x  
494 150 mm, 1.7  $\mu$ m; SeQuant, Sweden) (64, 65). The mobile phases were: (A) 20 mM acetate buffer  
495 (pH 4.5) containing 90 % acetonitrile, and (B) the same buffer. The elution gradient was as  
496 follows: 12-18 % linear gradient of buffer B for 12 min; 18-25 % linear gradient of buffer B for 5  
497 min; 25-55 % buffer B for 5 min; and 55 % buffer B for 5 min. The flow rate was maintained at 0.2  
498 mL/min. Analysis of dTDP-rhamnose and dTDP-d-Tal was performed in the multiple reaction  
499 monitoring mode using the specific precursor ion  $[M-H]^-$  at  $m/z$  547.1 and product ion pairs at  
500  $m/z$  321.1. Product ion scanning was also conducted by MS/MS analysis of the precursor ion  $[M-$   
501  $H]^-$  at  $m/z$  547.1.

502

### 503 **Structural modeling of Tle using AlphaFold 3**

504 The dimeric structure of Tle was predicted using AlphaFold 3 (43). Two identical sequences of  
505 Tle, each comprising 353 amino acids, along with two copies of the NAD<sup>+</sup> ligand, were input into  
506 the AlphaFold 3 web server (<https://alphafoldserver.com/>). The server produced five model  
507 outputs. The model with the highest predicted local-distance difference test (pLDDT) score was  
508 selected for further analysis. The pLDDT score ranges from 0 to 100, where 0 indicates the lowest  
509 confidence and 100 the highest confidence in the structural prediction. Additionally, for  
510 multimeric targets, AlphaFold 3 provides predicted template modelling (pTM) and interface  
511 predicted template modelling (ipTM) scores, both ranging from 0 to 1, indicating the confidence  
512 in the predicted oligomeric state of the protein.

513

### 514 **Deletion of *tle* and complementation in *M. abscessus***

515 The deletion mutant was generated in the S morphotype of the reference strain CIP104536<sup>T</sup> (62)  
516 using the suicide vector pUX1-*katG* by double homologous recombination (47). Briefly, the  
517 upstream and downstream gene regions were PCR-amplified using the primers listed in **Table S1**  
518 and ligated into the PacI/NheI-linearized pUX1-*katG*. After transformation, bacteria were  
519 selected on 7H10<sup>OADC</sup> supplemented with 250  $\mu$ g/mL kanamycin with a visual screening of red  
520 fluorescent colonies, which have undergone the first homologous recombination. The second  
521 homologous recombination event was induced by isoniazid counter-selection and selected on  
522 7H10<sup>OADC</sup> with 50  $\mu$ g/mL isoniazid and screening for non-fluorescent colonies and susceptibility

523 to kanamycin (35, 47). Complementation was performed by introducing a complementation  
524 plasmid generated using the integrative pMV306 (**Table S3**). Genes in fusion with a super folder  
525 green fluorescent protein (sfGFP)-tagging sequence were amplified by PCR under the control of  
526 the endogenous promoter. Proper gene deletion and all plasmids were verified by PCR and DNA  
527 sequencing.

528

### 529 **Western blotting**

530 Fifty millilitres of cultures were grown in minimal salt medium (1 g/L KH<sub>2</sub>PO<sub>4</sub>, 500 mg/L NaCl, 2  
531 g/L Na<sub>2</sub>HPO<sub>4</sub>, 1 g/L NH<sub>4</sub>Cl, 200 µM CaCl<sub>2</sub>, 2 mM MgSO<sub>4</sub>, 5% glycerol, and 0.02% tyloxapol). The  
532 bacterial pellet was washed twice with PBS supplemented with 0.025% tyloxapol (v/v) and  
533 resuspended in PBS supplemented with cComplete™ protease inhibitors cocktail (Roche, Sigma  
534 Aldrich) and disrupted by bead beating using 1-mm diameter glass beads and a Mixer Mill MM  
535 301 (Retsch, Germany) for two pulses of 3 min at 30 Hz. Protein concentration was determined  
536 using the BCA Protein Assay Reagent kit (Thermo Fisher Scientific), according to the  
537 manufacturer's instructions. Equal amounts of proteins (20 µg) were separated by 12% SDS-  
538 PAGE, transferred onto a nitrocellulose membrane, probed for 1 hr with either mouse anti-GFP  
539 (dilution 1:1000; Sigma) (antibodies-online GmbH, Aachen, Germany) or mouse anti-Antigen 85  
540 (dilution 1:20; loading control) antibodies. Membranes were washed with PBS supplemented  
541 with 0.02% Tween20 (w/v) and incubated for 45 min with goat anti-mouse antibody conjugated  
542 to HRP (dilution 1:5000; Abcam). Bands were revealed using a SuperSignal West Femto  
543 (ThermoFisher Scientific) and a ChemiDoc MP system (Bio-Rad laboratories). For detection of the  
544 MAB\_4111c variants produced in *E. coli*, the membrane was probed with anti-His mouse  
545 monoclonal antibodies (dilution 1:1000) and incubated with HRP-labeled goat anti-mouse  
546 IgG(H+L) (dilution 1:2000).

547

### 548 **Growth, colony morphology, biofilm formation and sliding motility**

549 Growth curves were established based on cultures grown for 72 hrs and diluted in 7H9<sup>OADC</sup> to  
550 reach an initial OD<sub>595nm</sub> of 0.05 and dispensed in flat-bottom 96-well microtiter plates (200 µL per  
551 well). Plates were statically incubated at 37 °C and measurements were taken on a daily basis  
552 using a spectrophotometer multimode microplate reader (Tecan Spark 10M, Tecan Group Ltd.,  
553 Switzerland).

554 Colony morphology was assessed from 72 hrs cultures in 7H9<sup>OADC</sup> and streaked on 7H10<sup>OADC</sup>.  
555 Plates were incubated at 37 °C for 5 days and photographed using a Zeiss microscope equipped  
556 with a Zeiss Plan Neo Fluor Z13/0.25 FWD objective and an Axiocam503 monochrome camera  
557 (Zeiss, Jena, Germany). Images were processed using ZEN 2 (Blue Edition).

558 *M. abscessus* biofilm formation was performed following a modification of the colony-biofilm  
559 model previously described (66). Briefly, *M. abscessus* cultures were grown in Middlebrook  
560 7H9<sup>OADC</sup>, 0.2% glycerol and tyloxapol (0.025%) at 37 °C and 80 rpm for 72 hrs. Cultures were  
561 centrifuged at 3,500 rpm for 5 min, washed two times with PBS and then diluted to an OD<sub>600nm</sub>  
562 of 0.5 (~1.5×10<sup>8</sup> CFU/mL). White black, polycarbonate membranes (diameter, 13 mm; pore size  
563 0.2 µm, Whatman, Merck, Darmstadt, Germany) were placed on 7H10<sup>OADC</sup> and inoculated with  
564 10 µL of the bacterial suspension. The membrane-supported biofilms were statically incubated  
565 for 5 days at 37 °C. Afterwards, the membrane-supported biofilms were imaged and processed  
566 using a ZEISS Axio Zoom V16 binocular equipped with a lighting device (Zeiss HXP 200C Zeiss,  
567 Germany at 7× zoom, detecting the grey level. On biofilm fluorescence pictures, a straight line  
568 was drawn (covering all the diameter of the biofilm-colony) and grey-level intensity (GLI) of each  
569 pixel as retrieved to draw GLI spectra using ZEN 2 software (Blue Edition). The colony volume was  
570 calculated by using the half volume of an ellipsoid: Colony volume =  $2/3 \times \pi \times \text{GLIM} \times (\text{CD}/2)^2$ , where  
571 GLIM is the GLI maximum and CD is the colony diameter in pixels. After taking pictures, each  
572 membrane-supported biofilm was processed for quantifying the number of CFU/membrane.  
573 Colony-biofilm density was estimated as a coefficient resulting of CFU/membrane and the colony  
574 volume.

575 Sliding motility of each strain was evaluated using a modified methodology previously described  
576 (28). For each strain, 10 µl of 24 hrs cultures (OD<sub>600nm</sub> = 0.9–1.0) were dropped in the centre of  
577 well from a 6-well containing 7H9 broth with 0.3% agar and without adding any carbon source.  
578 The sliding distance was evaluated and measured after incubation for 10 days at 37 °C under  
579 humidified conditions. This experiment was performed by using three biological replicates and  
580 two technical replicates.

581

## 582 **Hydrophobicity assay**

583 Mycobacterial surface hydrophobicity was determined following the methodologies developed  
584 previously (49). Each strain was seeded on 7H9<sup>OADC</sup> at 37 °C for 72 hrs, and then some colonies  
585 were resuspended and sonicated in 5 mL of 0.9% NaCl in PYREX® 16 × 100 mm disposable round-



586 bottom threaded culture tubes (Corning) before measuring the OD<sub>595nm</sub> (A<sub>0</sub>) of 200 µL in triplicate  
587 was measured using spectrophotometer multimode microplate reader (Tecan Spark 10M).  
588 Subsequently, 2 mL of hexadecane (Sigma Aldrich) was added to glass tubes, and the mixtures  
589 were vortexed for 30 s. After phase separation (~20 min), the OD<sub>595nm</sub> of 200 µL in triplicate was  
590 measured using a multimode microplate reader. Subsequently, the aqueous phase (A<sub>1</sub>) was  
591 measured again and compared to the organic phase. The percentage of cell surface  
592 hydrophobicity (H) of each strain immersed in the organic hexadecane phase was calculated  
593 using the equation  $H (\%) = [(A_0 - A_1)/A_0] \times 100$ . This experiment was performed by using three  
594 biological replicates and three technical replicates.

595

### 596 **Transmission electron microscopy**

597 72-hrs cultures were washed with PBS, immersed overnight at 4 °C in PHEM buffer containing  
598 2.5% glutaraldehyde (pH 7.4), rinsed in PHEM buffer and post-fixed in 0.5% osmium acid and  
599 0.8% potassium ferricyanide trihydrate for 2 hrs at room temperature and in the dark. After  
600 washing twice with PHEM buffer, mycobacteria were dehydrated in a graded series of ethanol  
601 solutions (30-100%) and embedded in EmBed 812 using an Automated Microwave Tissue  
602 Processor for electron microscopy (Leica). Seventy nanometre sections were cut at different  
603 levels of each block/stem using a microtome (Leica-Reichert Ultracut E, Leica), counterstained in  
604 70% ethanol with 1.5% uranyl acetate and lead citrate and observed using a Tecnai F20 TEM at  
605 120KV.

606

### 607 **Drug susceptibility testing**

608 Antimicrobial susceptibility was tested using broth microdilution following the European  
609 Committee on Antimicrobial Susceptibility Testing (EUCAST) (67). For this purpose, 96-well  
610 RAPMYCOI Sensititre™ titration plates (Thermo Fisher Scientific, Massachusetts, USA) were used  
611 following the manufacturer's recommendations.

612

### 613 **Phylogenetic analysis of *tle* orthologous genes**

614 Ortholog genes of *tle* in the genus *Mycobacterium* were found using BLASTp (68) against the NCBI  
615 non-redundant (nr) database by limiting the search to mycobacteria (taxid:85007) with a full  
616 bacterial scientific name registered and extending the maximum target sequences up to 1,000.  
617 In case of having more than one time the same mycobacterial species name, only the one with



618 the highest identity percentage was considered. All the proteins related to the Rml pathway were  
619 excluded from the analysis. Orthologs of *t1e* in other bacteria (non-mycobacteria) were found in  
620 a manner similar to that described above but excluding the search to Mycobacteria (taxid:85007)  
621 and limiting the maximum target sequences to 100. The Molecular Evolutionary Genetics Analysis  
622 (MEGA) version 11 software (69) was used to generate a ClustalW multiple sequence alignment  
623 of all the sequence and to construct Neighbour-Joining tree. The obtained tree was redrawn using  
624 TreeViewer Version 2.1.0 (70) and coloured with Inkscape (71). To structurally validate the  
625 findings from the phylogenetic tree, we analysed the presence of GPL and their glycosidic  
626 composition in 14 clinical NTM isolates identified with Genotype Mycobacterium CM or AS (Hain  
627 Lifescience, Nehren, Germany) from the Department of Clinical Microbiology of the University  
628 Hospital Fundación Jiménez Díaz (Madrid, Spain).

629

### 630 **Lipid extraction and analysis**

631 Rapid-growing and slow-growing mycobacteria were grown on 7H10<sup>OADC</sup> agar plates for 72 hrs  
632 and 7H9<sup>OADC</sup> broth for at least 14 days. After growing, pellets were collected and lyophilized, then  
633 25 to 50 mg of bacterial pellets were subjected to apolar lipid extractions using petroleum ether  
634 (72). GPL were extracted from the polar fraction using chloroform/methanol/0.3% NaCl (9/10/3,  
635 v/v/v) and then with chloroform/methanol/0.3% NaCl (5:10:4, v/v/v). The combined solvent  
636 extracts were mixed for 5 min with chloroform and 0.3% NaCl (1:1, v/v) and centrifuged at 3,000  
637 g for 5 min to separate the lower organic phase from the aqueous phase. The upper aqueous  
638 layer was discarded, and the lower organic phase was evaporated under a stream of nitrogen  
639 and resuspended in chloroform/methanol (2:1, v/v) for TLC analysis or itol-acetates derivation.

640 *Thin-layer chromatography (TLC) analysis.* Between 50-150 µg of each extract were spotted  
641 along 5 mm lane on Silica gel 60 F254 plates (Merck). GPL were separated at 4 °C using  
642 chloroform/methanol/water (90:10:1, v/v/v). More polar lipids were separated using  
643 chloroform/methanol/water (65:25:4, v/v/v). Glycolipids were revealed by spraying the plates  
644 with orcinol in 20% sulfuric acid and charring.

645 *Glycolipids saponification.* Five hundred µg of the polar lipid fraction were dried under nitrogen.  
646 Then, 1 mL of 0.1 M sodium hydroxide in chloroform/methanol (1:1, v/v) was added, and heated  
647 overnight at 37 °C. One mL of butanol and 1 mL of water were added and the mixture vortexed  
648 for 1 min. After centrifugation for 30 s, the upper butanolic phase was dried under a nitrogen  
649 stream and dissolved in 300 µL of chloroform/methanol (2:1, v/v).

650 *Itol-acetates derivation.* After addition of 1 mL of 4 M TFA the sample was heated for 4 hrs at 100  
651 °C, dried and desiccated overnight. The monosaccharides were reduced for 4 hrs at room  
652 temperature in 500 µL NaBH<sub>4</sub> 10 mg/mL in 2 M NH<sub>4</sub> and stopped with concentrated glacial acetic  
653 acid. Samples were dried at 55 °C under a nitrogen stream by co-distillation with methanol five  
654 times, dessicated overnight. Samples were peracetylated in 500 µL anhydride acetic for 4 hrs at  
655 80 °C. The reaction products were washed five times with chloroform/water (1:1, v/v). The  
656 chloroform-rich phase was then filtered, dried, and dissolved in 500 µL chloroform. For GC-FID,  
657 1 µL of itol-acetate derivatives was injected in splitless mode with an automatic sampler on a  
658 Solgel 1 MS 30 m × 0.25 mm × 0.25 µm capillary column with the following gradient temperature:  
659 120 to 230 °C at 3 °C/min, then to 270 °C at 10 °C/min. Compounds were detected either with a  
660 flame ionization detector or a single quadrupole on a HP7820 or 5975N (Agilent Technologies).  
661 Previously determined retention times of differentially methylated Rha and unmodified 6-d-Tal  
662 were used for identification (33) while electronic impact mass spectrometry was used for  
663 methylated 6-d-Tal determination.

664 *MALDI-TOF mass spectrometry.* Before spotting 1 µL on the MALDI plate, 10 µL of 20 mg/mL  
665 dihydroxybenzoïc acid (DHB) in chloroform/methanol (1:2, v/v) were mixed with 5 µL of the  
666 sample extract in chloroform/methanol (2:1, v/v). MS positive and negative spectra were  
667 acquired on an Axima Resonance (Shimadzu, Kyoto, Japan) in reflectron mode.

668  
669 **Zebrafish maintenance**

670 Zebrafish (*Danio rerio*) were kept and handled in compliance with the guidelines of the European  
671 Union for handling laboratory animals and approved by the Direction Sanitaire et Vétérinaire de  
672 l'Hérault for the ZEFIX-CRBM zebrafish facility (Montpellier) (registration number C-34-172-39).  
673 All experiments were approved by Le Ministère de l'Enseignement Supérieur et de la Recherche  
674 under the reference APAFIS#24406-2020022815234677 V3. Eggs were obtained by natural  
675 spawning, quickly bleached, and incubated at 28.5 °C in Petri dishes containing E3 medium (5  
676 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl<sub>2</sub>, 0.33 mM MgSO<sub>4</sub>). The transgenic reporter line  
677 Tg(*mpx:eGFP*)<sup>il14</sup> (73) was used in this study.

678  
679 **Zebrafish infection**

680 At 24 hrs post-fertilization (hpf), embryos were enzymatically dechorionated using 1mg/mL of  
681 pronase (stock of 5mg/mL diluted in E3) and placed in 60 mm Petri dishes containing E3 at 28.5

682 °C. Microinjection was performed as previously described (74). Embryos were injected in the  
683 caudal vein with  $\pm 250$  CFU of either the R, S,  $\Delta tle$ , carrying the pMV306-mScarlet,  $\Delta tle::C$   
684 carrying the pTEC27-tdTomato or the PBS control. Injected embryos were then rinsed twice and  
685 transferred individually in 48-well plates containing E3 and randomized for survival assays and  
686 CFU counts. Inoculum was checked by microinjecting a PBS drop and plating it on 7H10<sup>OADC</sup>. For  
687 survival assay, embryos were monitored every 24 hrs for 12 days, and marked as dead in the  
688 absence of a heartbeat. Bacterial burden was determined at 2dpi, 3dpi and 5dpi as following:  
689 embryos were individually manually disrupted with pellet pestles in 50  $\mu$ L of PBS supplemented  
690 with 0.025% of tyloxapol. Lysates were sonicated for 30 s after adding 50  $\mu$ L of PBS supplemented  
691 with 2% of Triton-X-100. Serial dilutions were plated on LB agar plates containing rifampicin (15  
692  $\mu$ g/mL) and kanamycin (250  $\mu$ g/mL) and incubated for 4 days at 37 °C.

693

#### 694 **Zebrafish microscopy**

695 For zebrafish imaging, embryos were anesthetized with 0.02% buffered MS222 and immobilized  
696 with 0.5% low melting agarose in a lateral position. Images were acquired with an EVOS™ M7000  
697 Imaging System at 3dpi and 5dpi with the Olympus™ 2X Objective PlanApo 0.08 NA and the EVOS  
698 10x Objective 0.3NA using the EVOS™ Light Cube, GFP 2.0. After imaging, embryos were rinsed  
699 and transferred back the 48-well plate with fresh E3. Image processing was done using ZEISS  
700 ZEN3.7 software.

701

#### 702 **Statistical analyses**

703 All analyses were performed using R commander (75–76) and/or GraphPad Prism version 9.0.0  
704 for Windows (GraphPad Software). The normality of data was evaluated using a Shapiro-Wilk  
705 test. Descriptive data are cited as median and interquartile range in case of non-normal  
706 distribution for each of the variables were calculated. A non-parametric Kruskal-Wallis test was  
707 used to compared more than three groups and a Wilcoxon test was used to compared two  
708 groups. Zebrafish survival assays are represented in Kaplan-Meier graphs and analysed with a Log  
709 rank test. For determination of bacterial counts, CFU were  $\log_{10}$  transformed and the significance  
710 between multiple selected groups was determined using one-way ANOVA with Šidák's Multiple  
711 Comparisons test after validating the normality of the data.

712

713

**714 DATA AVAILABILITY**

715 All data are contained within the manuscript and the Supporting Information. The raw data can  
716 be share upon request.

717

**718 SUPPORTING INFORMATION**

719 This article contains supporting information.

720

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733

**734 CONFLICT OF INTEREST**

735 The authors report there are no competing interests to declare.

736

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960 **FIGURE LEGENDS**

961

962 **Figure 1. MAB\_4111c belongs to the GPL biosynthetic cluster and is a predicted epimerase. (A)**  
 963 Structure of diglycosylated (GPL-2a) and triglycosylated (GPL-3) glycopeptidolipids. The green,  
 964 blue and yellow shadings represent the lipid, peptide and glycosydic moieties of the GPL. Di-O-  
 965 acetyl 6-d-Tal is drawn in red. **(B)** *M. abscessus* GPL locus encoding enzymes involved in the  
 966 synthesis, modification, and transport of GPL. *MAB\_4111c* is indicated in bold. **(C)** Alignment of  
 967 the protein sequence of dTDP-L-Rha 4-epimerase from the *B. thailandensis* (ATCC 700388)  
 968 (*wbiB\_BURTA*) with that of *MAB\_4111c* from *M. abscessus*. The orange and blue shadings  
 969 represent the non-conserved and conserved amino acids, respectively. The red, green and  
 970 blue asterisks indicate residues involved in the putative NADH-binding site, the substrate-binding  
 971 site and the catalytic residue reported in *B. thailandensis*, respectively.

972

973 **Figure 2. Enzymatic activity of MAB\_4111c. (A)** Reaction catalyzed by *MAB\_4111c*. **(B) Upper**  
 974 **panel:** HPLC profile of the standards and products formed during the enzymatic reaction in the  
 975 presence of *MAB\_4111c* or *MAB\_4111c\_Y181A* with dTDP-Rha and NADP<sup>+</sup>. The incubation was  
 976 performed for 60 min. The product of the reaction is labeled with a red box. **Lower panel:** HPLC  
 977 profile of standard dTDP-Glc, standard dTDP-6-d-Tal and dTDP-Glc and NADP<sup>+</sup> incubated with  
 978 *MAB\_4111c* for 60 min. **(C) Upper panel:** LC/MS profile of the enzymatic reaction mixture in  
 979 MRM mode using the specific precursor ion [M-H]<sup>-</sup> at *m/z* 547.1. **Lower panel:** MS<sup>2</sup> fragmentation  
 980 spectra of standard dTDP-6-d-Tal and the enzymatic reaction product (\*). **(D)** <sup>1</sup>H NMR spectra of  
 981 dTDP-L-Rha and NADP<sup>+</sup>; *MAB\_4111c* with dTDP-L-Rha and NADP<sup>+</sup> after 120 min;  
 982 *MAB\_4111c\_Y181A* with dTDP-L-Rha and NADP<sup>+</sup> after 120 min; dTDP-6-d-Tal. The characteristic  
 983 peaks of dTDP-6-d-Tal are indicated with red arrows. **(E)** Kinetic analysis of the reaction catalyzed  
 984 by *MAB\_4111c* in the presence of dTDP-L-Rha and NADP<sup>+</sup>.

985

986 **Figure 3. Structural Prediction of Tle. (A)** Cartoon representation of the AlphaFold 3 predicted  
 987 dimeric structure of Tle. The structure is colored according to the pLDTT score range (blue=high  
 988 confidence, cyan=confidence, yellow=low confidence, orange=very low confidence). **(B) Left**  
 989 **panel:** cartoon representation of the predicted Tle homodimer, with each chain colored in green  
 990 and cyan. NAD is depicted as a stick model. **Right panel:** Alignment of the monomeric structure  
 991 of Tle with the WbpP crystal structure (Tle in cyan and WbpP in magenta).

992 **Figure 4. Deletion of *tle* in *M. abscessus* alters colony morphology, hydrophobicity, sliding**  
 993 **motility and biofilm-colony development. (A)** Knock-out of *tle* is associated with a switch from  
 994 a S to a R-like morphotype. The scale bar represents 1 mm. **(B)** The deletion of *tle* is associated  
 995 with a higher growth rate. The experiment was performed using two biological replicates with  
 996 six technical replicates per biological replicate. Deletion of *tle* modifies hydrophobicity **(C)** and  
 997 the sliding motility **(D-E)**. The hydrophobicity experiment was performed using three biological  
 998 replicates with three technical replicates per biological replicate. The sliding motility experiment  
 999 was performed using three biological replicates with two technical replicates per biological  
 1000 replicate. The black scale bar indicates 5 mm. **(F)** Representative 2- and 2.5-dimensional pictures  
 1001 of colony-biofilms on biofilm-supporting membranes after 5 days of incubation. The absence of  
 1002 *tle* alters the biofilm-colony growth **(F)** (the scale bar represents 2 mm) and the biofilm-colony  
 1003 profile **(G)** (green, dark grey, red and blue dots represent the profiles of S, R,  $\Delta tle$  and  $\Delta tle::C$ ,  
 1004 respectively). The CFUs per membrane are shown in **(H)**, the colony volume in **(I)**, and the colony  
 1005 density in **(J)**. This experiment was performed using five biological replicates. The error bar  
 1006 denotes the interquartile range. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ ; and \*\*\*:  $p < 0.001$ .

1007  
 1008 **Figure 5. Structural analysis of the GPL content in  $\Delta tle$ .** **(A)** TLC analysis representative from  
 1009 three independent experiments of native apolar (left) and polar (right) lipid extracts. Lipids were  
 1010 separated using chloroform/methanol/water (90:10:1, v/v/v) followed by spraying with orcinol  
 1011 and charring. GPL-1b and GPL-2b previously described (35) from  $\Delta tle$  display a lower  $R_f$  than wild-  
 1012 type GPL-2a and GPL-3, which are restored in  $\Delta tle::C$ . TDM: trehalose dimycolate; DAT: Di-*O*-acyl  
 1013 trehalose. **(B)** TLC analysis of native (NaOH -) and saponified (NaOH +) polar fractions. Lipids were  
 1014 separated using chloroform/methanol/water (90:10:1, v/v/v) followed by spraying with orcinol  
 1015 and charring. The  $R_f$  of GPL-2a and GPL-3 from  $\Delta tle$  are not modified as it is the case for S and  
 1016  $\Delta tle::C$ . **(C)** MALDI-TOF positive spectra of native polar lipid fraction (black, NaOH -) from the S  
 1017 and  $\Delta tle::C$  strains show ions for GPL-2a at  $m/z$  1257.9/1285.9 and for GPL-3 at  $m/z$  1403.9/1432.  
 1018 Spectra from saponified polar lipid fraction (red, NaOH +) show a 84 u.m.a decrease, to  
 1019 1173.4/1201.4 and 1319.3/1347.4, corresponding to deacetylated dGPL-2a and dGPL-3, as  
 1020 previously described (34). Conversely, ions for GPL-1b at  $m/z$  1013.8/1041.8 and from GPL-2b at  
 1021 1187.9 were intense in native and saponified polar extracts from  $\Delta tle$ . **(D)** Relative proportion of  
 1022 each monosaccharides was determined in the GPL-enriched saponified polar lipid fraction. 6-d-  
 1023 Tal could only be detected in S and  $\Delta tle::C$ .

1024 **Figure 6. Distribution of *tle* orthologues in NTM and correlation with the presence of 6-d-Tal.**

1025 **(A)** Phylogenetic tree reconstructed using already available *tle* sequences. Non-mycobacterial  
 1026 species and mycobacterial species are represented in pink and blue areas, respectively. Green,  
 1027 yellow and red dots label slowly-growing, intermediately-growing and rapid-growing  
 1028 mycobacteria, respectively. **(B)** TLC analysis of the polar lipid fraction of a selected panel of NTM  
 1029 clinical strains, representative from two independent experiments. Lipids were separated using  
 1030 chloroform/methanol/water (90:10:1, v/v/v) followed by spraying with orcinol and charring. +:  
 1031 presence of GPL-like components, confirmed by their detection after saponification **(Figure S8)**.  
 1032 -: absence of GPL-like components. **(C)** Deoxyhexoses composition analysis of polar lipid fractions  
 1033 separated with GC and GC-MS. Relative area of identified monosaccharides (n=4) show Rha  
 1034 residues in green and 6-d-Tal in blue. Identification of 6-d-Tal and Rha was confirmed by the  
 1035 retention time and fragmentation pattern of standards while *O*-methylated Rha was determined  
 1036 previously (33, 35). Identification of 2- and 3-*O*-methyl 6-d-Tal was deduced from the retention  
 1037 time and fragmentation pattern. Relative areas of Rha and 6-d-Tal and their methylated forms  
 1038 are expressed as ratios of total monosaccharides.

1039  
 1040 **Figure 7. Increased virulence and pathogenesis of  $\Delta tle$  in a zebrafish embryo model of infection.**

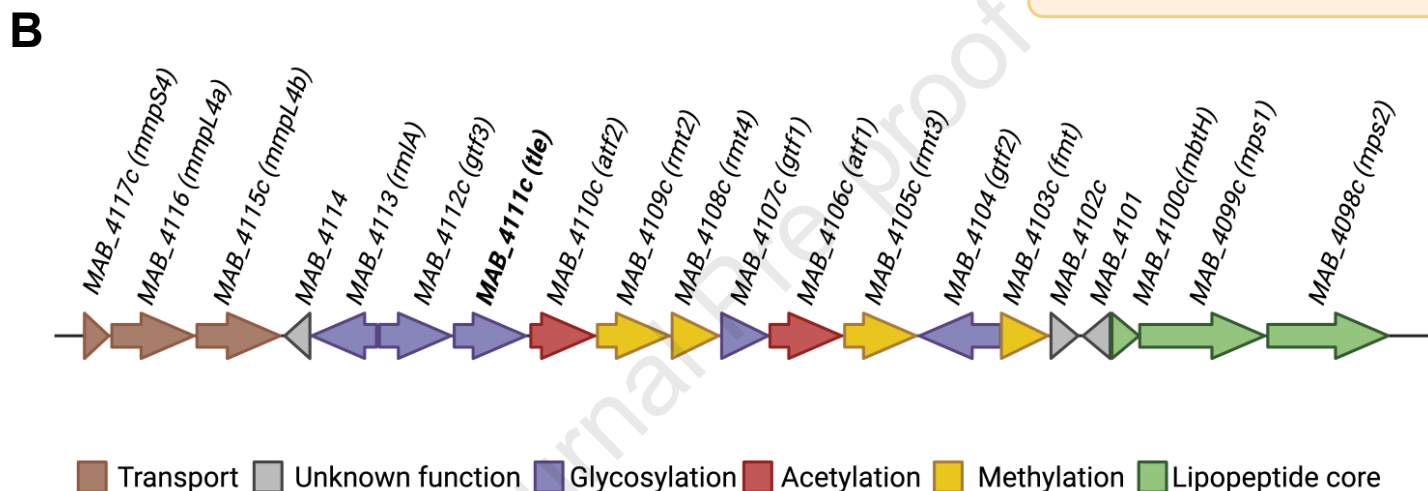
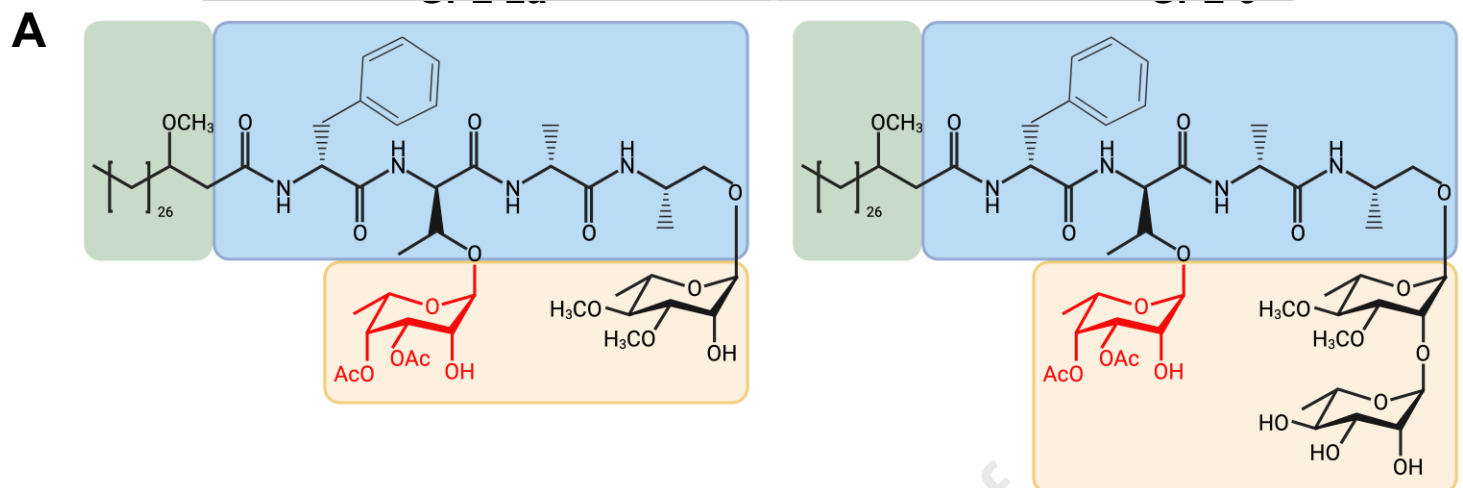
1041 **(A)** Schematic overview of *in vivo* experiments. **(B)** Survival of infected embryos with the R strain  
 1042 ( $275 \pm 27$  CFU), the S strain ( $280 \pm 36$  CFU),  $\Delta tle$  ( $264 \pm 33$  CFU) and  $\Delta tle::C$  ( $281 \pm 47$  CFU). The graph  
 1043 shows the result of 4 pooled independent experiments. Embryos were marked dead in the absence  
 1044 of a heartbeat. Statistical analysis was performed using Log-rank (Mantel-Cox) test. \*\*\*\*:  
 1045  $p < 0.0001$ ; \*\*:  $p < 0.01$ , ns: non-significant. **(C)** Bacterial burden from infected embryos at 2, 3 and  
 1046 5dpi. The graph shows the result of 3 pooled independent experiments. n=5 embryos per time-  
 1047 point per condition. CFU counts were Log<sub>10</sub> transformed. Statistical analysis was performed per  
 1048 time point using ordinary one-way ANOVA with Šídák's multiple comparisons test, after verifying  
 1049 the normality of the data. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$  \*\*\*\*:  $p < 0.0001$ . **(D)** Imaging of  
 1050 infected embryos at 3dpi. One zoomed representative image of the head is shown per condition.  
 1051 Scale bar, 0.2 mm.

1052  
 1053 **Figure 8. The biosynthetic interconnections between the arabinogalactan and**  
 1054 **glycopeptidolipid pathways in *M. abscessus*.** dTDP-L-Rha is generated by the successive action  
 1055 of the Rml pathway enzymes, consisting of RmlA (MAB\_4113), RmlB (MAB\_3779), RmlC

1056 (MAB\_3780) and RmlD (MAB\_3613c). This dTDP-L-Rha is used by WbbL (MAB\_3612c) to produce  
1057 the Rha-N-acetyl-glucosaminyl-phosphate disaccharide unit that cross-links arabinogalactan to  
1058 peptidoglycan. In addition, dTDP-L-Rha is also used by Gtf2 (MAB\_4104) and Gtf3 (MAB\_4112c)  
1059 for GPL rhamnosylation (blue arrows). dTDP-6-d-Tal, produced from dTDP-L-Rha by the dTDP-  
1060 Rha-4-epimerase (MAB\_4111c/Tle) serves as the substrate for the talosyltransferase Gtf1 (green  
1061 arrows), which adds one 6-d-Tal unit to mono- or di-rhamnosylated GPL precursors. The  
1062 glycosylated GPLs (GPL-2a and GPL-3) are then transported across the inner membrane by the  
1063 MmpL4 complex (MAB\_4116c, MAB\_4115c, MAB\_4117c) and inserted into the outer leaflet of  
1064 the mycomembrane in the *M. abscessus* S variant.

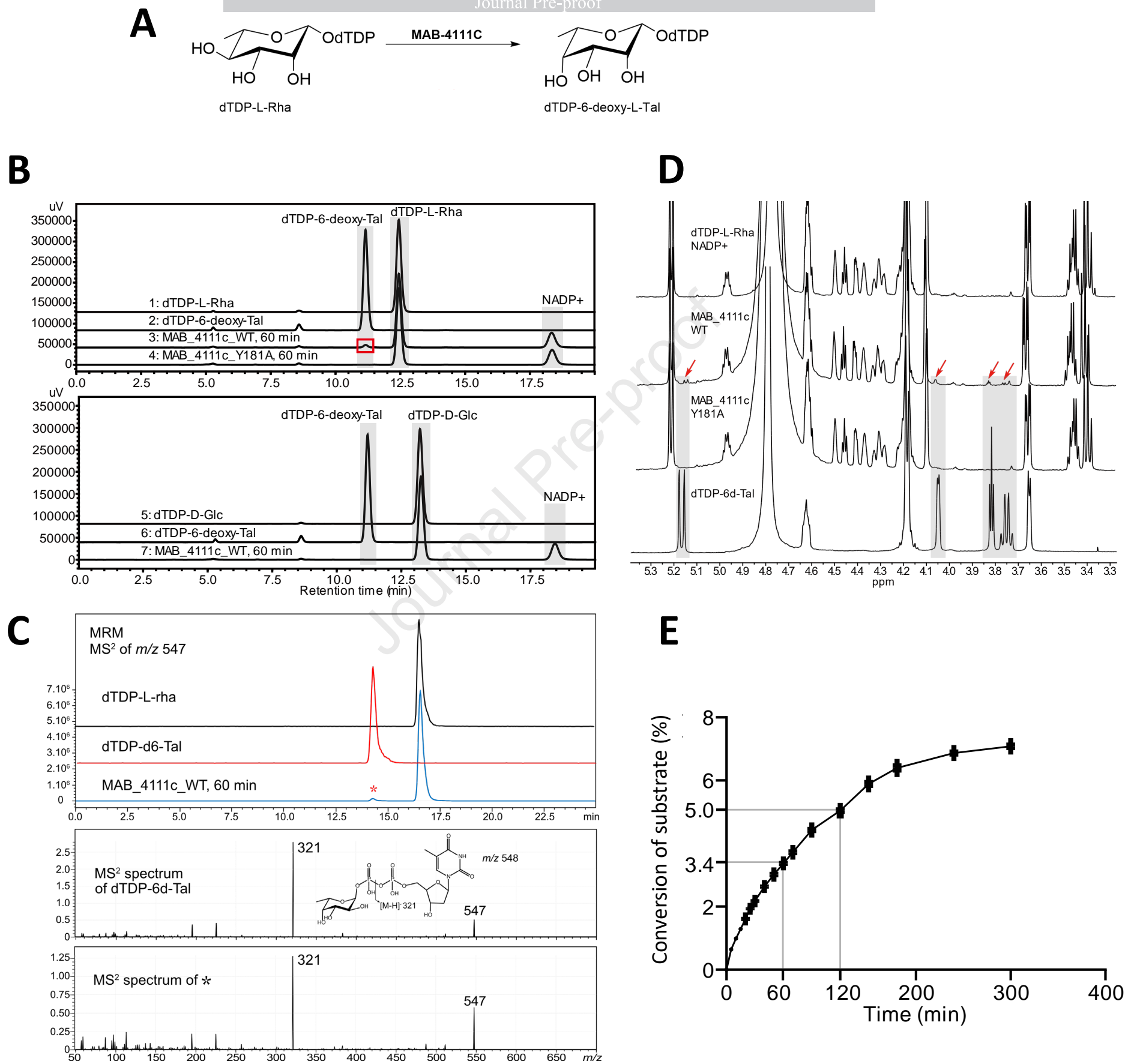
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# Figure 1



**C**

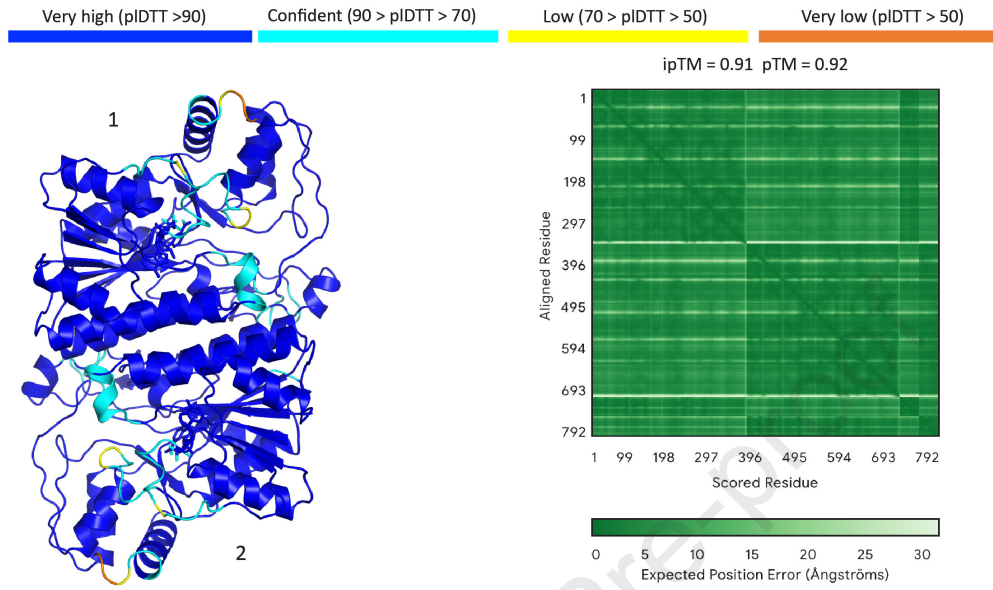
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		*****	
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MAB_4111c/1-353	33	PQVHARGRAIDLPA SVRLFTGDVTHAPDWDVLRLEFESQVVHLAAETGTAQSLS	87
		***	*****
WBIB_BURTA/1-363	102	EASRHALVNVVGTTRLTDAIVKHGIAVEHILLTSSRAVYEGEGAWQKADGTIVYPGQ	157
MAB_4111c/1-353	88	EATRHGSVNVVGTQLLDALSR SALVPDQLV LASSRAVYEGEGAWQSGAEVFYPRP	142
		*	
WBIB_BURTA/1-363	158	RGRAQLEAAQWDFPG-----MTMLPSRADRTEPRPTSVYGATKLAQEHVLRASWLA	208
MAB_4111c/1-353	143	RSHAQLLAG EWDPQGPAGASATPLSSCADRTEPRPTNIYAGTKLAQEHLLASWAAA	198
		*	*
WBIB_BURTA/1-363	209	TKTPLSILRLQNVYGPQSLTNSYTGIVALFSRLAREKKVIPLYEDGNVTRDFVSI	264
MAB_4111c/1-353	199	HDTNLSVLRQLQNVYGPQSLTNSYTGIVTLFARLAREKQALEVYEDGRIVRDFVFI	254
WBIB_BURTA/1-363	265	DDVADAI VATLAREPEALSLFDIGSGQATSILDMARIIAAHYGAPEPQVNGAFRDG	320
MAB_4111c/1-353	255	DDVDA LFAAVHTPATERTLDIGSGTATTIHELARKVADICAAPEPVVVGKFRDG	310
WBIB_BURTA/1-363	321	DVRHAACDLSLANLGWKPQWSL ERGIGELQTWIAQELDRKN	363
MAB_4111c/1-353	311	DVRAASCDIAPAVEGLGWSPKWTLEDGLHALLEWIDKDCPNRF	353

**Figure 2**

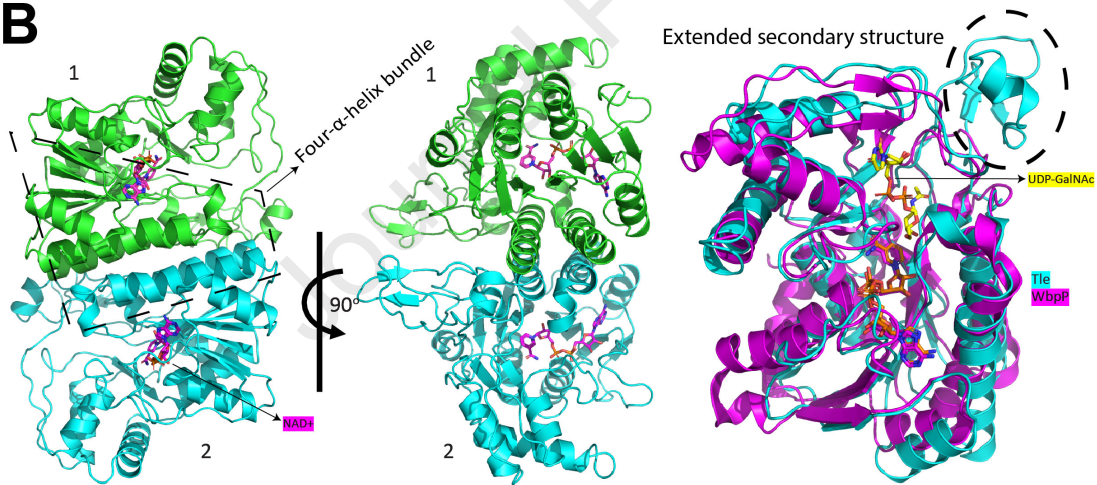


# Figure 3

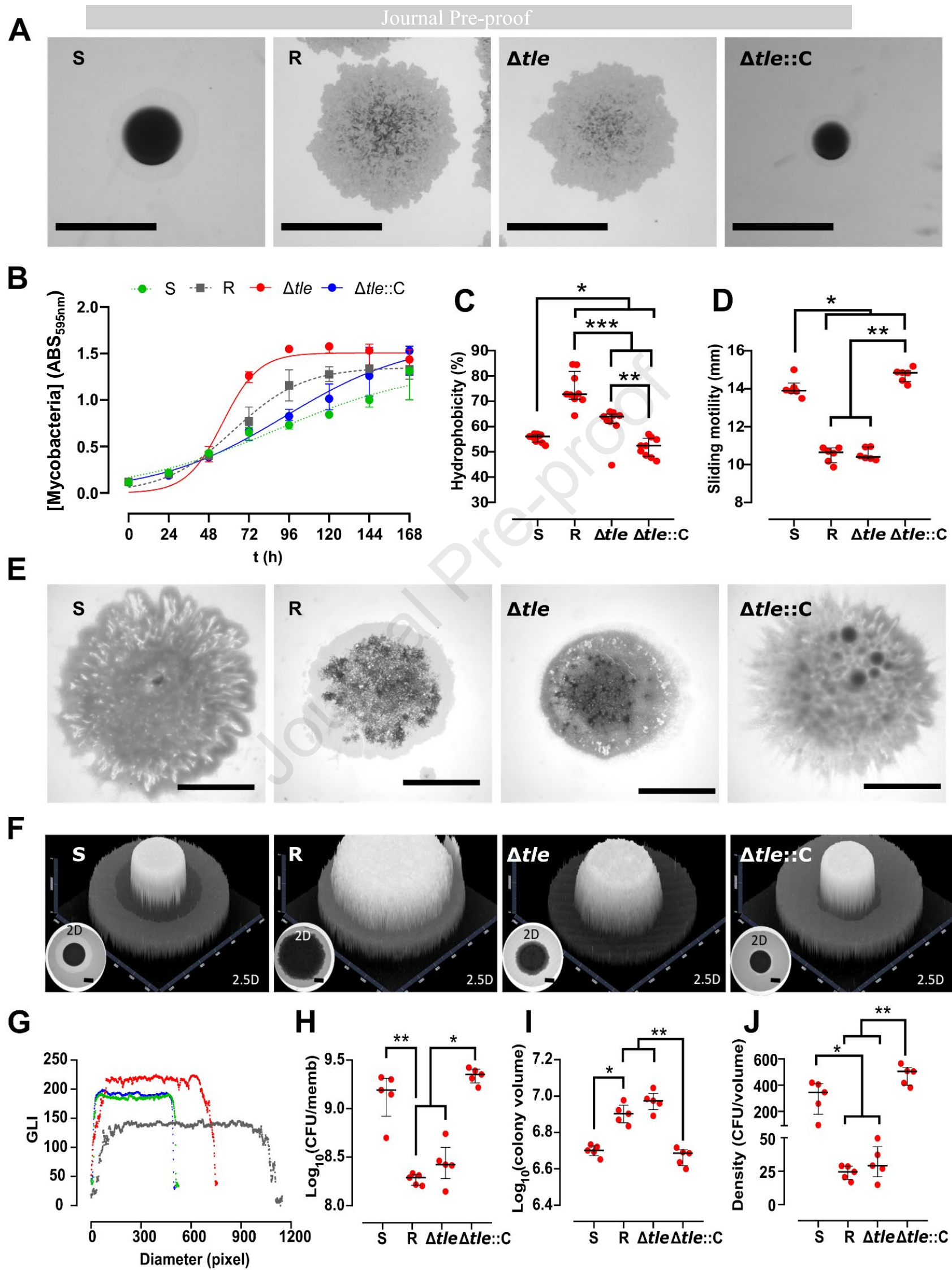
## A



## B





**Figure 4**



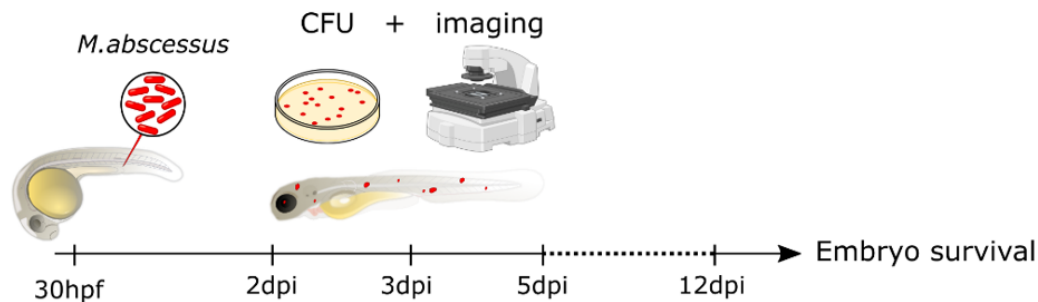




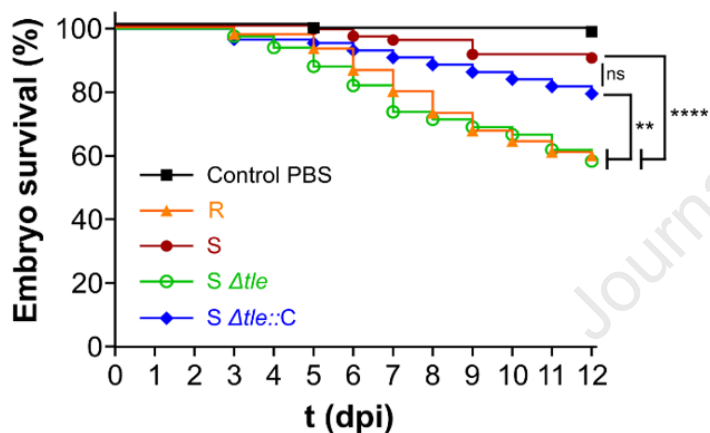
# Figure 7

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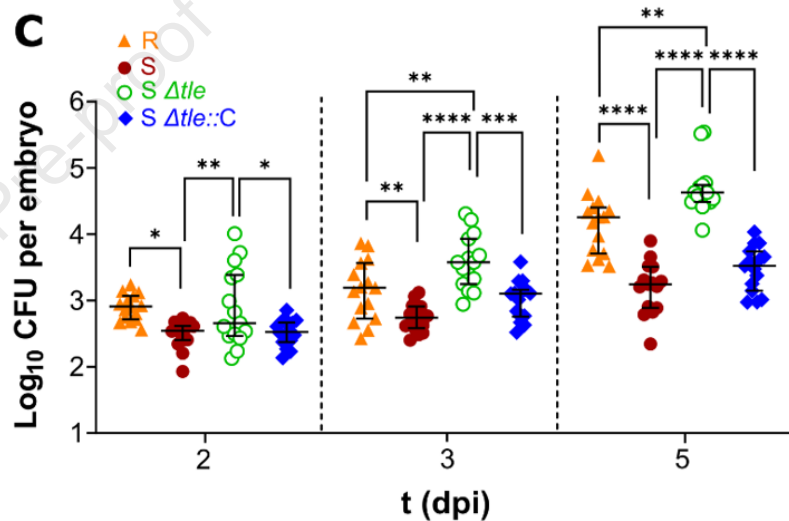
**A**



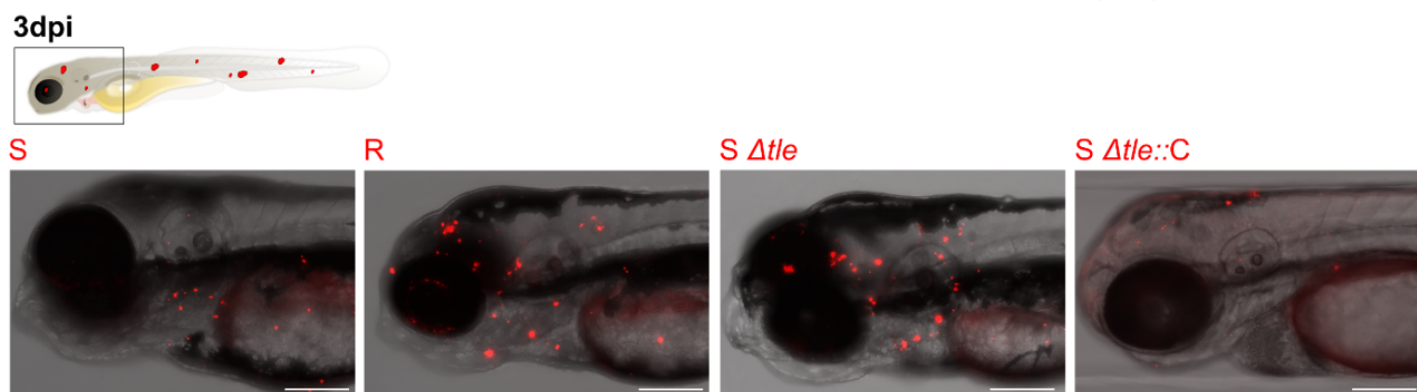
**B**



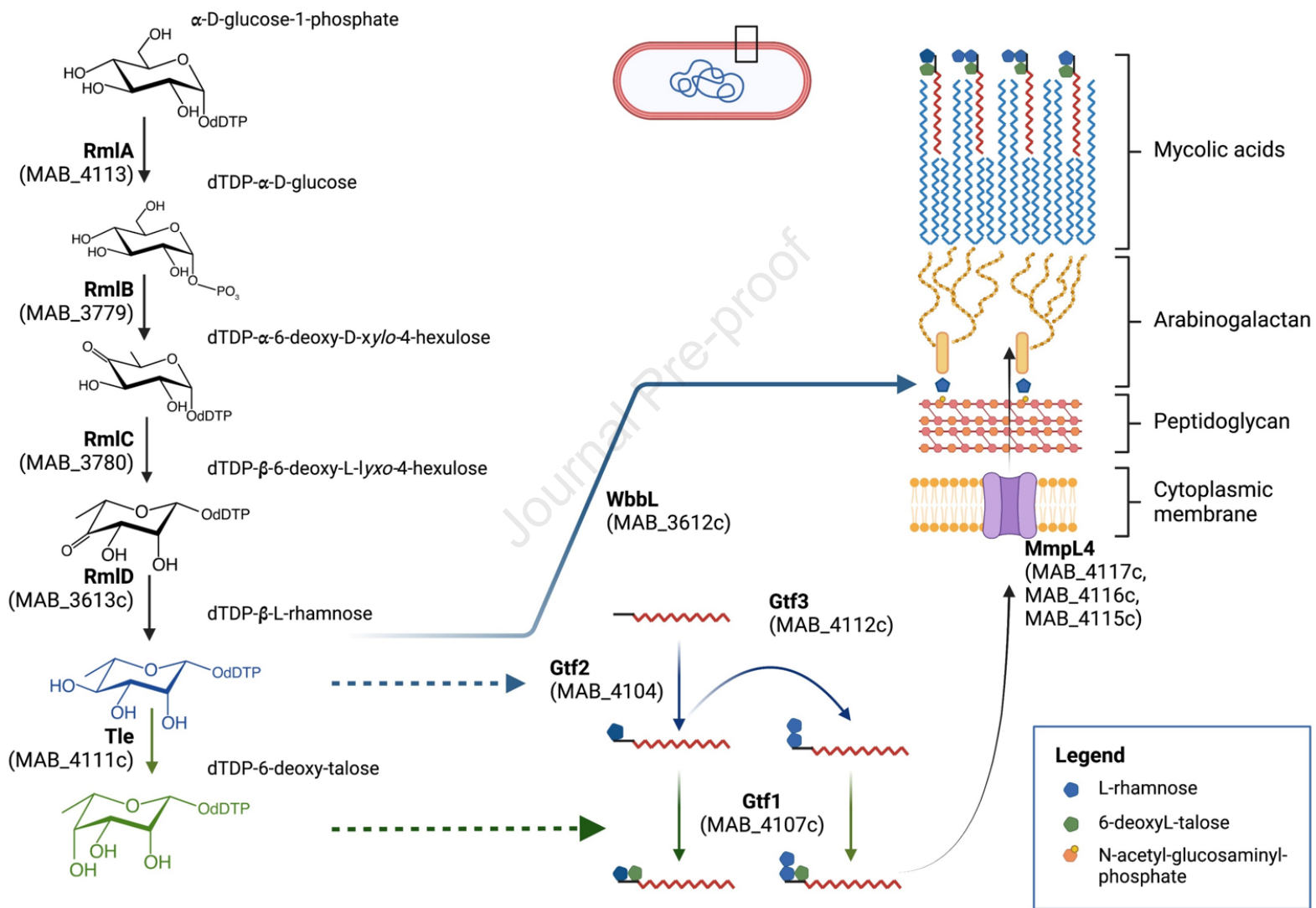
**C**



**D**



# Figure 8



**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The author is an Editorial Board Member/Editor-in-Chief/Associate Editor/Guest Editor for *[Journal name]* and was not involved in the editorial review or the decision to publish this article.

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