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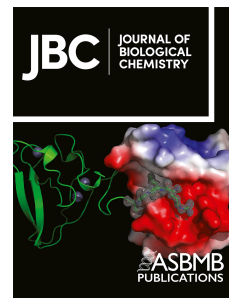
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1 **A dTDP-L-Rhamnose 4-epimerase required for glycopeptidolipid biosynthesis**
2 **in *Mycobacterium abscessus***
3

4 John Jairo Aguilera-Correa¹, Fangyu Wei^{3,§}, Louis-David Leclercq^{4,5,§}, Yara Tasrini^{1,§}, Edukondalu
5 Mullapudi⁶, Wassim Daher^{1,2}, Kazuki Nakajima⁷, Stéphane Canaan⁸, Jean-Louis Herrmann⁵,
6 Matthias Wilmanns^{6,9}, Yann Guérardel^{4,7}, Liuqing Wen³, and Laurent Kremer^{1,2*}
7

8 ¹Centre National de la Recherche Scientifique UMR 9004, Institut de Recherche en Infectiologie
9 de Montpellier (IRIM), Université de Montpellier, 1919 route de Mende, 34293, Montpellier,
10 France.

11 ²INSERM, IRIM, 34293 Montpellier, France.

12 ³Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Chinese Academy of
13 Sciences, 555 Zuchongzhi Rd, 201203, Shanghai, China

14 ⁴Univ. Lille, CNRS, UMR 8576 - UGSF - Unité de Glycobiologie Structurale et Fonctionnelle, F-
15 59000 Lille, France.

16 ⁵Université Paris-Saclay, UVSQ, Inserm, Infection et inflammation, 78180, Montigny-Le-
17 Bretonneux, France.

18 ⁶European Molecular Biology Laboratory, Hamburg Unit, Notkestrasse 85, 22607 Hamburg,
19 Germany.

20 ⁷Institute for Glyco-core Research (iGCORE), Gifu University, Gifu, Japan

21 ⁸Aix-Marseille Univ, CNRS, LISM, IMM FR3479, Marseille, France.

22 ⁹University Medical Center Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany.
23
24

25 [§]Equal contribution

26 *Corresponding author: Laurent Kremer; Tel: (+33) 4 34 35 94 47; E-mail:
27 laurent.kremer@irim.cnrs.fr

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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_{al}ose e_{pimerase}, 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 β -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- α -L-Talose (6-d-Tal) and on
89 the alaninol linked with an α -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 Ni²⁺-coated beads, which was assayed by incubation with NADP⁺ 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]⁻ 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 (δ 5.17 ppm), H2 (δ 4.05 ppm), H3 (δ 3.82 ppm)
161 and H5 (δ 3.75 ppm) of 6-d-Tal. The signal of H-4 proton (δ 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⁺. 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 α -helices from each protomer (residues 88-110 and 180-199), creating a four- α -helix bundle
187 (**Figure 3B**). The overall architecture includes an N-terminal NAD⁺ 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 α -helix, long loop and a pair of antiparallel β -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⁺, is highly conserved
199 among SDR family enzymes (44). Structural alignment with WbpP confirmed that majority of the
200 NAD⁺ 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 Ni²⁺ 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⁺ 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 (Δtle) 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 Δtle formed R,
229 corded colonies (**Figure 4A**). Complementation of Δtle ($\Delta tle::C$), 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 Δtle 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 Δtle
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, Δtle ,
240 and $\Delta tle::C$ strains. Δtle 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 Δtle , 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 Δtle is likely defective in surface-associated cell wall
247 components. The Δtle 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 Δtle 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. Δtle 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 Δtle), tigecycline (MIC of 0.5 $\mu\text{g}/\text{mL}$ for the wild-type strain to 1 $\mu\text{g}/\text{mL}$
256 for Δtle), 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 Δtle), clarithromycin (MIC of 8 $\mu\text{g}/\text{mL}$ for the wild-type strain and 4 $\mu\text{g}/\text{mL}$ for Δtle) or rifabutin
258 (MIC of 64 $\mu\text{g}/\text{mL}$ for the wild-type strain and 32 $\mu\text{g}/\text{mL}$ for Δtle).

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, Δtle 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 Δtle 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 Δtle 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 Δtle 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, Δtle , $\Delta tle::C$ (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 Δtle 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 Δtle , 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 Δtle
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 Δtle 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 Δtle 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 α -L-

413 rhamnopyranosyl-(1→3)- α -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^T (S) and CIP104536^T (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^{OADC}) or
435 on Middlebrook 7H10 agar (BD) containing 10% OADC (7H10^{OADC}) 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 (OD₆₀₀) 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⁺, 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[®]-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(D₂O) containing 10 mg of dTDP-L-Rha, 5 mg of NADP⁺, 5 mg of disodium
481 hydrogen phosphate (Na₂HPO₄), 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⁺, 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[®]-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 μ 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⁺ 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^T (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^{OADC} supplemented with 250 μ 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^{OADC} with 50 μ 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 KH₂PO₄, 500 mg/L NaCl, 2
531 g/L Na₂HPO₄, 1 g/L NH₄Cl, 200 µM CaCl₂, 2 mM MgSO₄, 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^{OADC} to
550 reach an initial OD_{595nm} 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^{OADC} and streaked on 7H10^{OADC}.
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^{OADC}, 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_{600nm}
562 of 0.5 (~1.5×10⁸ CFU/mL). White black, polycarbonate membranes (diameter, 13 mm; pore size
563 0.2 µm, Whatman, Merck, Darmstadt, Germany) were placed on 7H10^{OADC} 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_{600nm} = 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^{OADC} 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_{595nm} (A₀) 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_{595nm} of 200 µL in triplicate was
590 measured using a multimode microplate reader. Subsequently, the aqueous phase (A₁) 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^{OADC} agar plates for 72 hrs
632 and 7H9^{OADC} 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 NaBH₄ 10 mg/mL in 2 M NH₄ 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 CaCl₂, 0.33 mM MgSO₄). The transgenic reporter line
677 Tg(*mpx:eGFP*)^{il14} (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 ± 250 CFU of either the R, S, Δtle , 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^{OADC}. 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 μ L of PBS supplemented
690 with 0.025% of tyloxapol. Lysates were sonicated for 30 s after adding 50 μ L of PBS supplemented
691 with 2% of Triton-X-100. Serial dilutions were plated on LB agar plates containing rifampicin (15
692 μ g/mL) and kanamycin (250 μ 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 and 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⁺. 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⁺ 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]⁻ at *m/z* 547.1. **Lower panel:** MS² fragmentation
 980 spectra of standard dTDP-6-d-Tal and the enzymatic reaction product (*). **(D)** ¹H NMR spectra of
 981 dTDP-L-Rha and NADP⁺; *MAB_4111c* with dTDP-L-Rha and NADP⁺ after 120 min;
 982 *MAB_4111c_Y181A* with dTDP-L-Rha and NADP⁺ 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⁺.

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, Δtle 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 Δtle .** **(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 Δtle 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 Δtle 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 Δtle . **(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 Δtle in a zebrafish embryo model of infection.**

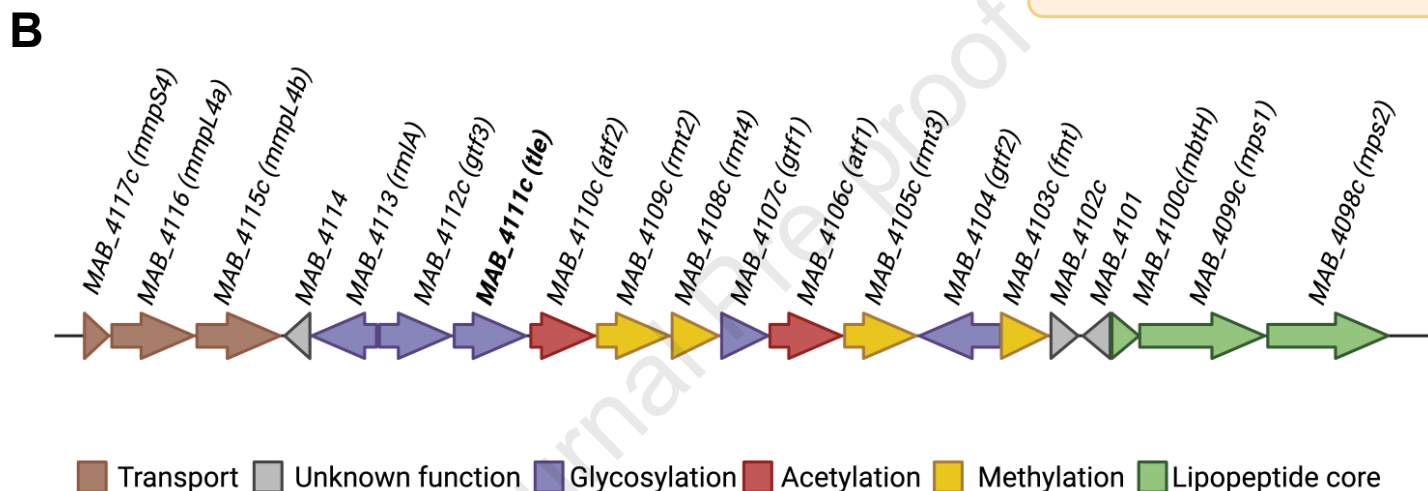
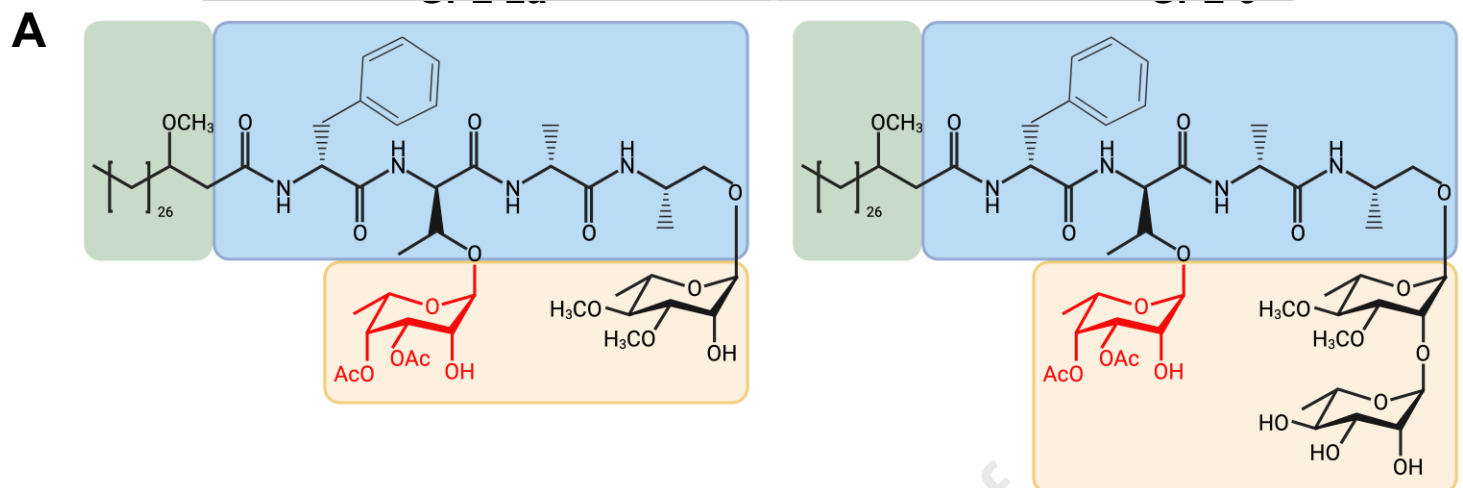
1041 **(A)** Schematic overview of *in vivo* experiments. **(B)** Survival of infected embryos with the R strain
 1042 (275 ± 27 CFU), the S strain (280 ± 36 CFU), Δtle (264 ± 33 CFU) and $\Delta tle::C$ (281 ± 47 CFU). The graph
 1043 shows the result of 4 pooled independent experiments. Embryos were 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 Log₁₀ 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

WBIB_BURTA/1-363	1	-----MSDVNASLVDGKKILVTGGAGFIGCAISERLAARASRYVMDNLH	45
MAB_4111c/1-353	1	-----MLISGGAGFIGSALSRLIQAGYDVAVMDVLH	32

WBIB_BURTA/1-363	46	PQIHANAVRPPVALHEKAELVVADVTDAGAWDALLSDFQPEIIHLAAETGTGQSLT	101
MAB_4111c/1-353	33	PQVHARGRAIDLPAVRLFTGDVTHAPDWDVLRLEFESQVVHLAAETGTAQSLS	87
		***	*****
WBIB_BURTA/1-363	102	EASRHALVNVVGTTRLTDAIVKHGIAVEHILLTSSRAVYEGEGAWQKADGTIVYPGQ	157
MAB_4111c/1-353	88	EATRHGSVNVVGTQLLDALSRSAVLPDQLVLAASSRAVYEGEGAWQSGAEVFP	142
		*	
WBIB_BURTA/1-363	158	RGRAQLEAAQWDFPG-----MTMLPSRADRTEPRPTSVYGATKLAQEHVLRASL	208
MAB_4111c/1-353	143	RSHAQLLAGEWDPQGPAGASATPLSSCADRTEPRPTNIYAGTKLAQEHLLASWAA	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	DDVDAALFAAVHTPATERTLDIGSGTATTIHELARKVADICAAPEPVVVGKFRDG	310
WBIB_BURTA/1-363	321	DVRHAACDLSLANLGWKPQWSL ERGIGELQTWIAQELDRKN	363
MAB_4111c/1-353	311	DVRAASCDIAPAVEGLGWSPKWTLEDGLHALLEWIDKDCPNRF	353

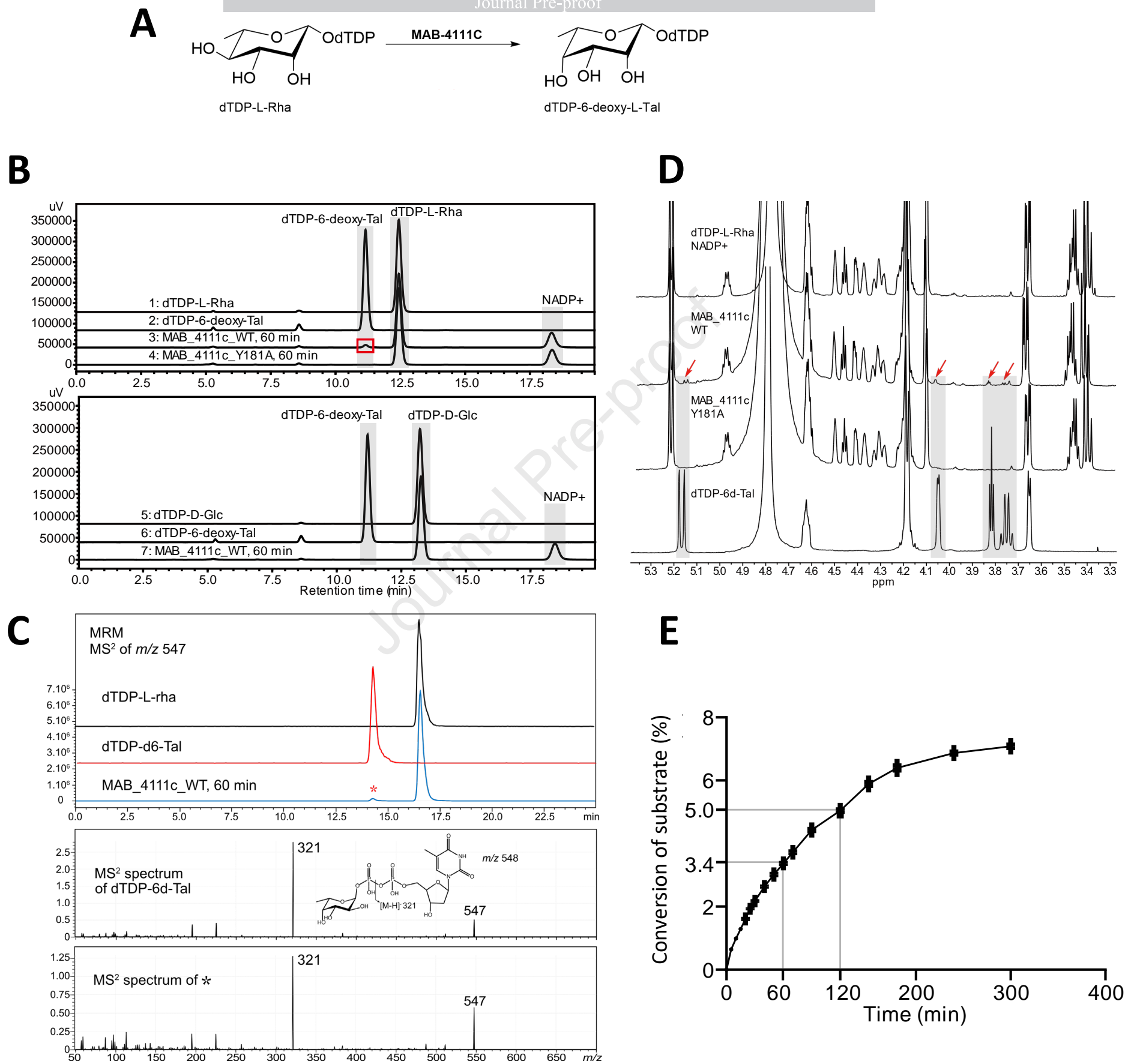
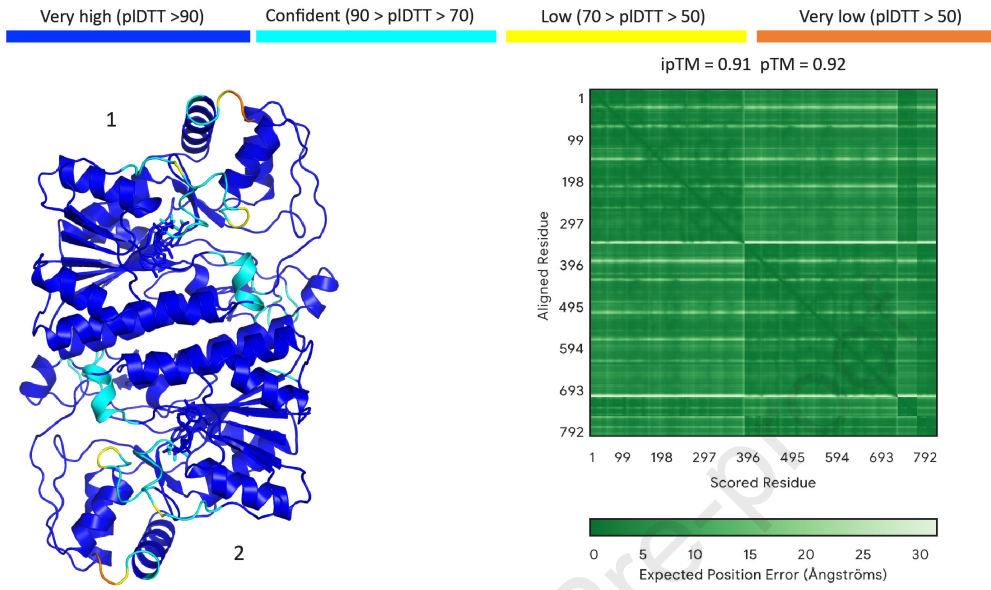
Figure 2

Figure 3

A



B

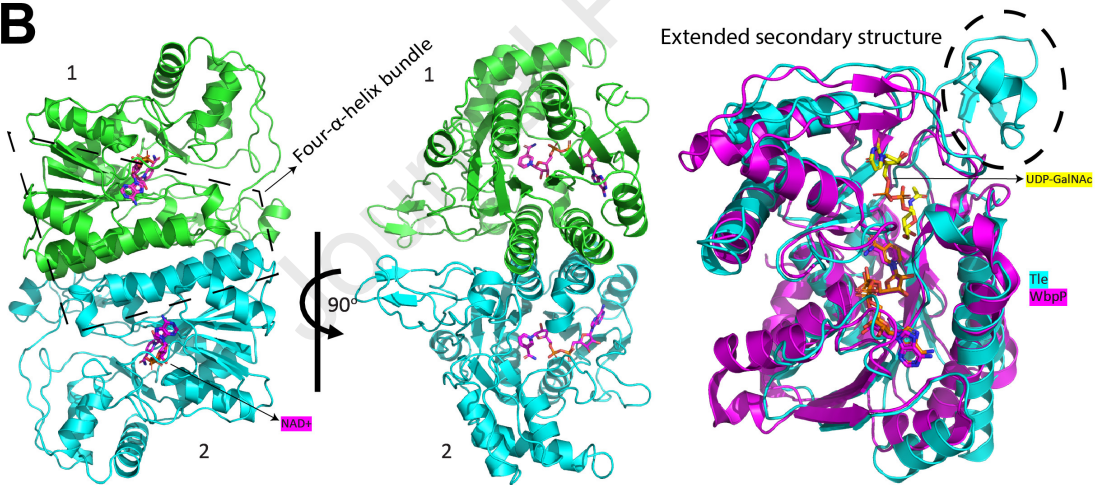


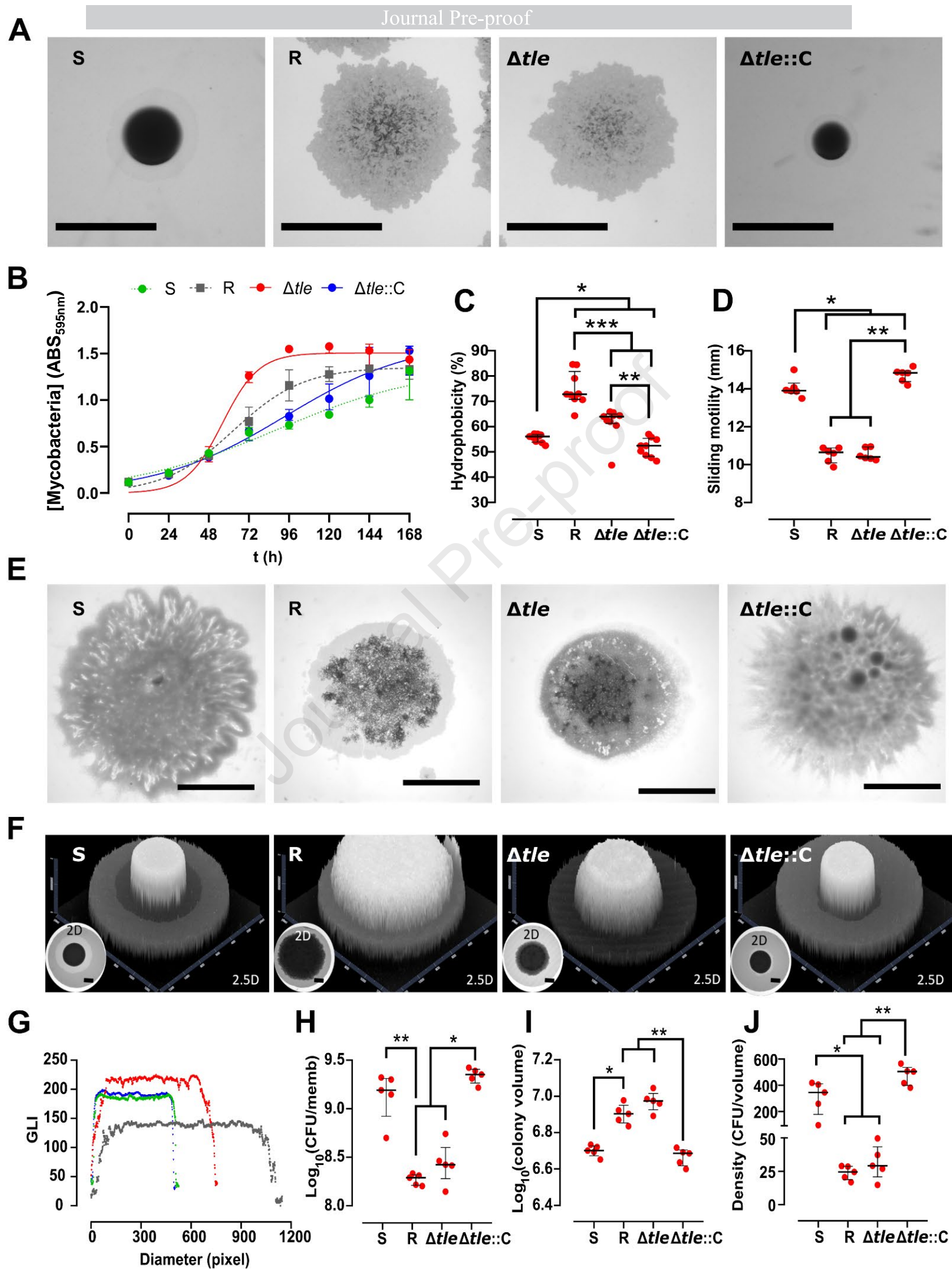
Figure 4

Figure 5

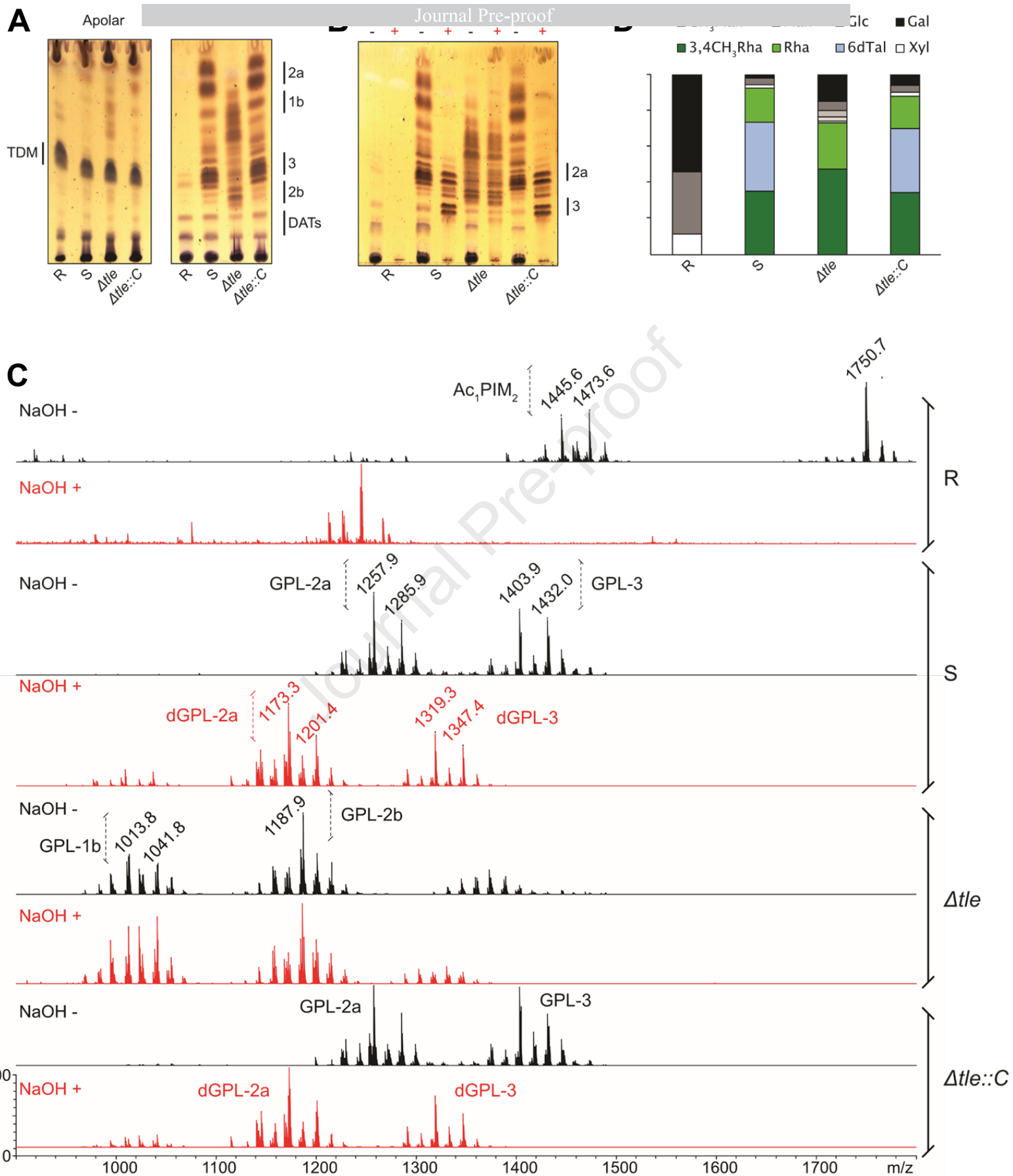
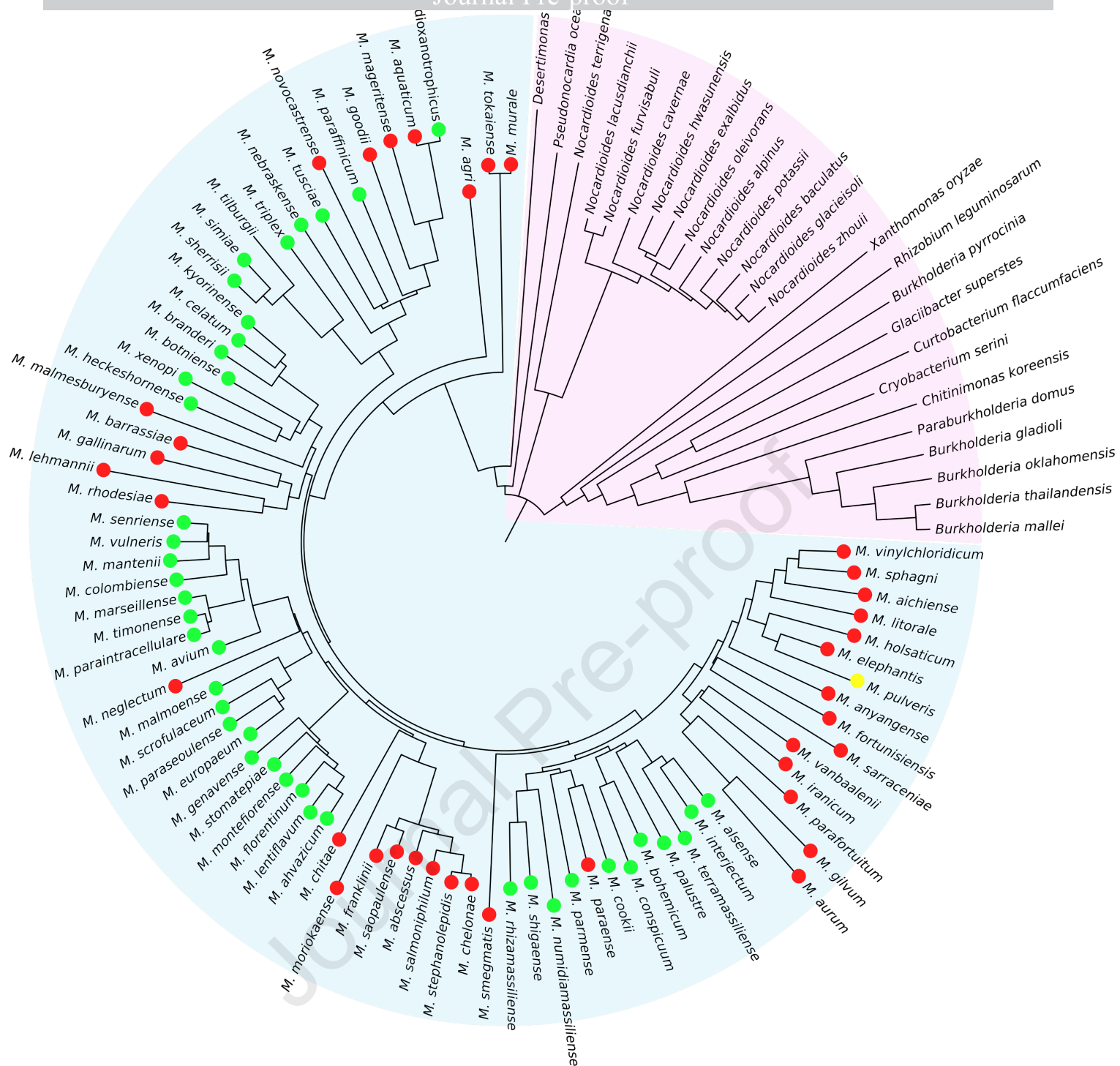
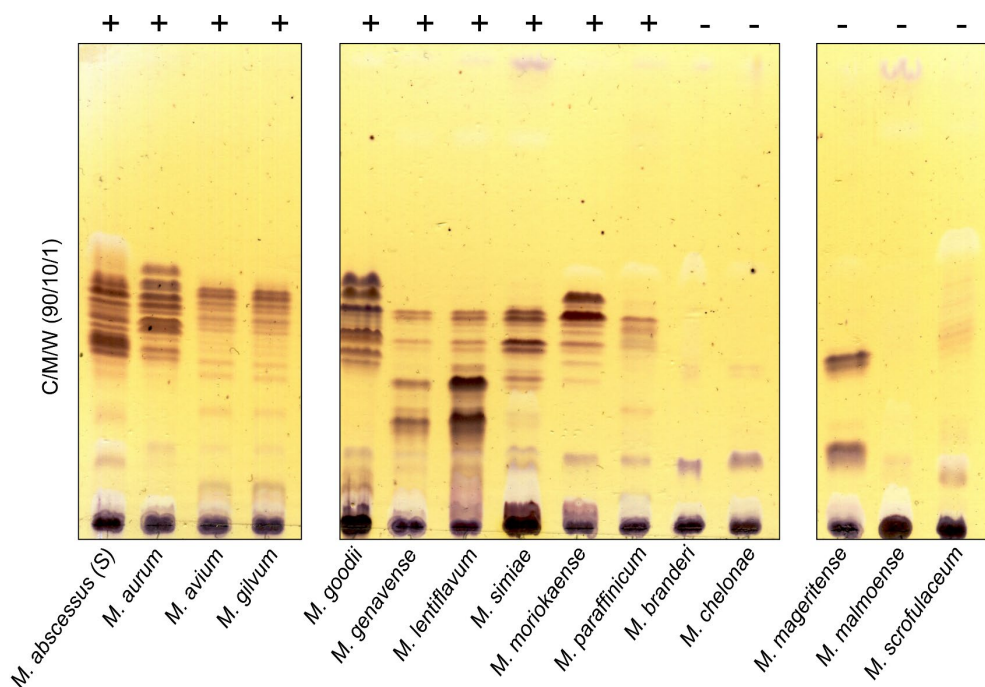


Figure 6

A



B



C

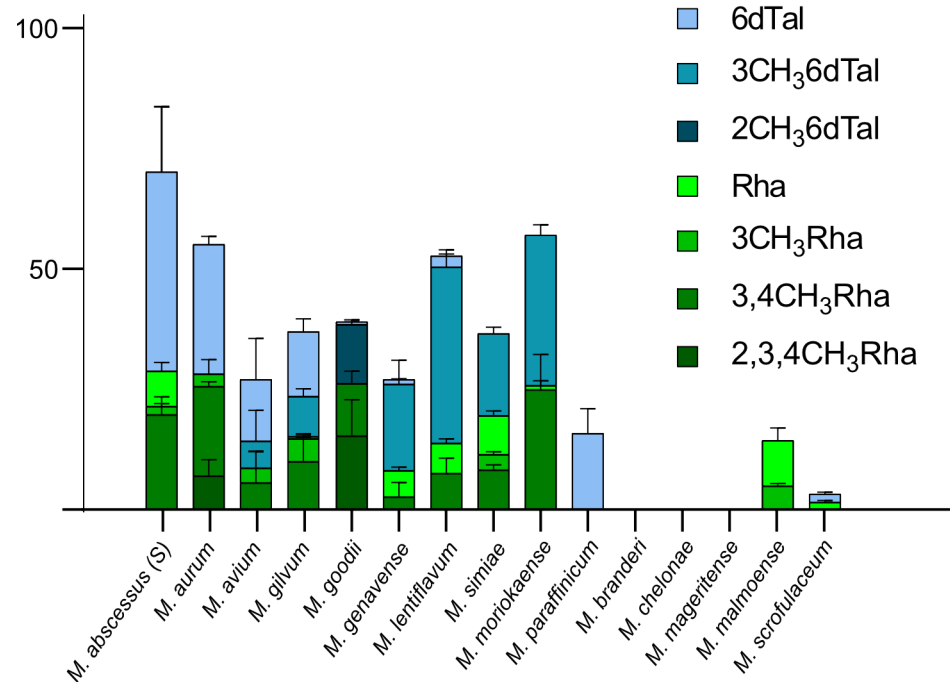
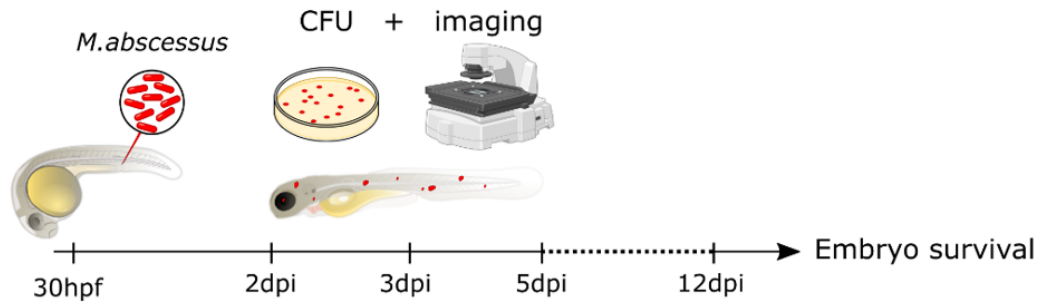


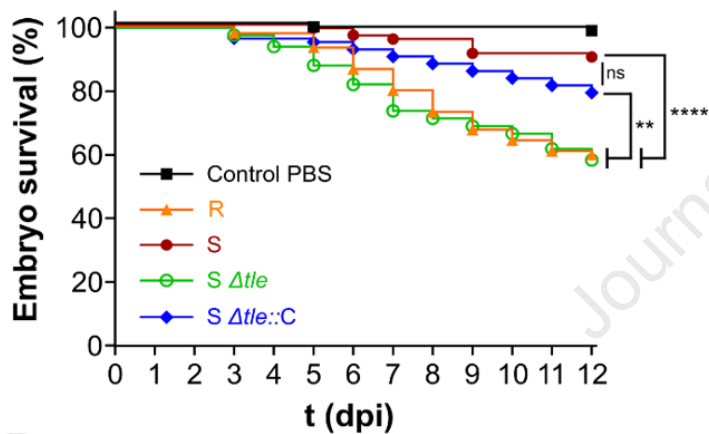
Figure 7

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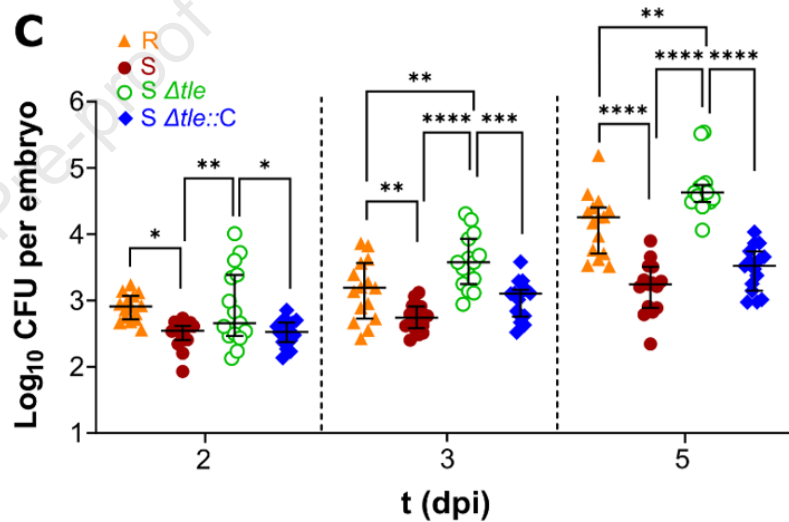
A



B



C



D

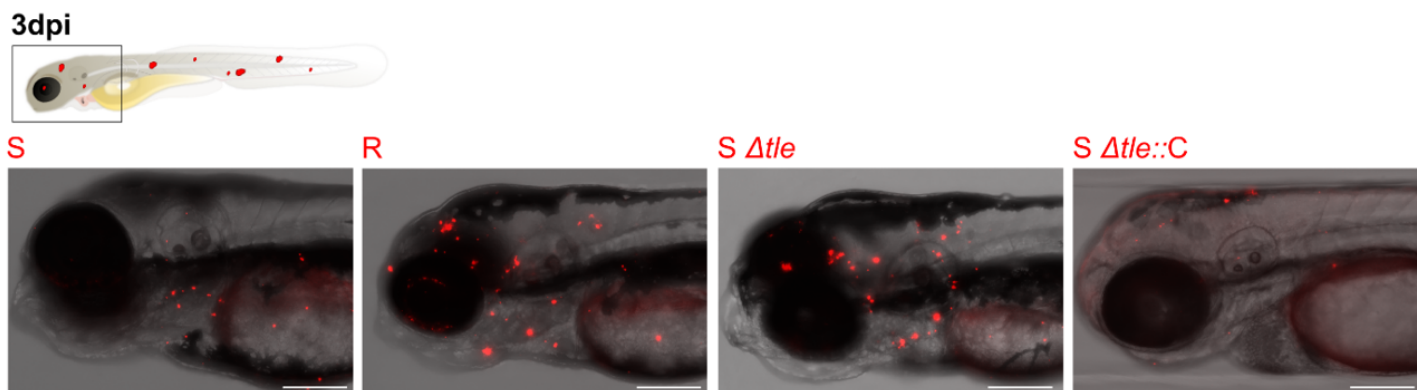
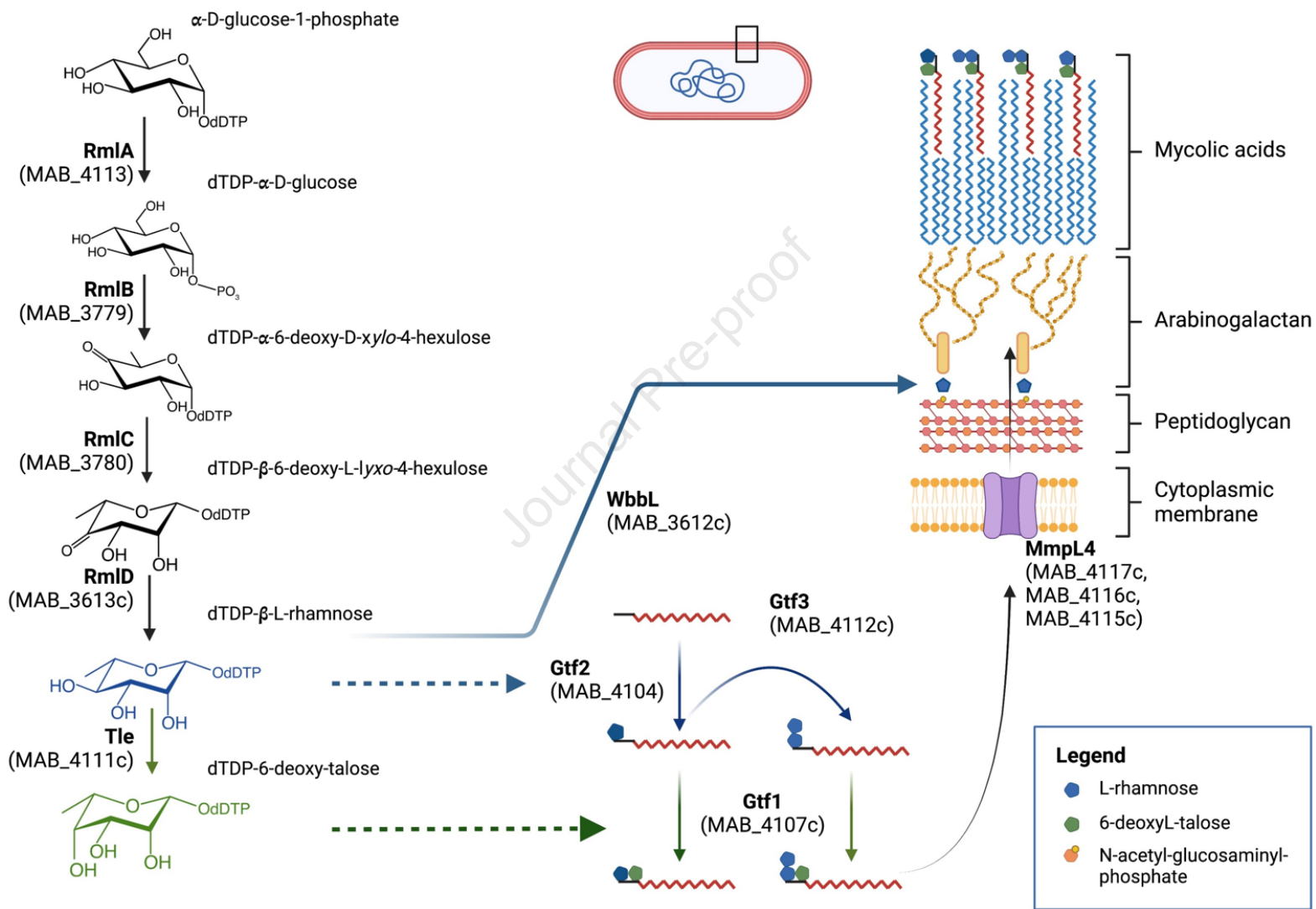


Figure 8



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.

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