

1 **Surface-Shaving Proteomics of *Mycobacterium marinum* Identifies**  
2 **Biofilm Subtype-Specific Changes Affecting Virulence, Tolerance and**  
3 **Persistence**

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28 **Running title: *Mycobacterium marinum* cell-surface proteome profiling**

29

30 **ABSTRACT (250 words)**

31 The complex cell wall and biofilm matrix (ECM) act as key barriers to antibiotics in  
32 mycobacteria. Here, the ECM-proteins of *Mycobacterium marinum* ATCC927, a non-  
33 tuberculous mycobacterial model, was monitored over three months by label-free  
34 proteomics and compared with cell-surface proteins on planktonic cells to uncover  
35 pathways leading to virulence, tolerance, and persistence. We show that ATCC927 forms  
36 pellicle-type (PBFs) and submerged-type (SBFs) biofilms after two weeks and two days of  
37 growth, respectively, and that the increased CelA1 synthesis in this strain prevents biofilm  
38 formation and leads to reduced rifampicin tolerance. The proteomic data suggests that  
39 specific changes in mycolic acid synthesis (cord factor), Esx1-secretion, and cell-wall  
40 adhesins explain the appearance of PBFs as ribbon-like cords and SBFs as lichen-like  
41 structures. A subpopulation of cells resisting the  $64 \times$  MIC rifampicin (persisters) were  
42 detected in both biofilm subtypes, and already in one-week-old SBFs. The key forces  
43 boosting their development could include subtype-dependent changes in asymmetric cell  
44 division, cell wall biogenesis, tricarboxylic acid/glyoxylate cycle activities, and  
45 energy/redox/iron metabolisms. The effect of varying ambient oxygen tensions on each  
46 cell type and non-classical protein secretion are likely factors explaining majority of the  
47 subtype-specific changes. The proteomic findings also imply that Esx1-type protein  
48 secretion is more efficient in PL and PBF cells, while SBF may prefer both the Esx5- and  
49 non-classical pathways to control virulence and prolonged viability/persistence. In  
50 conclusion, this study reports a first proteomic insight into aging mycobacterial biofilm-  
51 ECMs and indicates biofilm subtype-dependent mechanisms conferring increased  
52 adaptive potential and virulence on non-tuberculous mycobacteria.

53

54 **IMPORTANCE (88 words)**

55 Mycobacteria are naturally resilient and mycobacterial infections are notoriously difficult to  
56 treat with antibiotics, with biofilm formation being the main factor complicating the  
57 successful treatment of TB. The present study shows that non-tuberculous *Mycobacterium*  
58 *marinum* ATCC927 forms submerged- and pellicle-type biofilms with lichen- and ribbon-  
59 like structures, respectively, as well as persister cells under the same conditions. We show  
60 that both biofilm subtypes differ in terms of virulence-, tolerance- and persistence-  
61 conferring activities, highlighting the fact that both subtypes should be targeted to  
62 maximize the power of antimycobacterial treatment therapies.

63

64 **INTRODUCTION (5735 words)**

65 Tuberculosis (TB) remains a major global health issue, with approximately 10 million new  
66 cases and 1.4 million deaths in 2019 (1). The causative agent, *Mycobacterium*  
67 *tuberculosis* (Mtb), is carried by an estimated one quarter of the human population as a  
68 latent infection, which has a 5–10% lifetime risk of developing into TB disease. In addition,  
69 the emergence of drug-resistant Mtb strains continues to be a public health threat, with  
70 about half a million new cases in 2019. Even in the case of drug-sensitive Mtb strains, the  
71 first line antibiotic treatment requires the use of four antimicrobials over a course of at least  
72 six months (WHO 2020). Moreover, despite successful treatment, the recurrence of TB  
73 carries a substantial risk, especially among immunocompromised patients (2, 3). The  
74 heterogeneity of the standard treatment outcome is also evident in PET-CT images  
75 showing non-resolving and active lesions and the presence of Mtb mRNA in sputum  
76 samples. This suggests that a significant proportion of patients generate viable  
77 mycobacteria in their lungs even after clinically curative antibiotic treatment (4). In a rabbit  
78 TB model, it was further shown that the caseum of granulomas contains Mtb that are

79 highly tolerant to most anti-TB drugs (5). The complex mycobacterial cell wall, involving  
80 capsule and outer/inner membranes connected by a dense mycolyl-arabinogalactan-  
81 peptidoglycan with high lipid levels, is the main barrier that protects the bacterial cells  
82 against drugs (6). While the mechanisms leading to drug tolerance in TB have remained  
83 poorly understood, biofilm formation was recently indicated as one of the strategies to  
84 increase viability, tolerance and persistence (7-10).

85 Biofilm formation is defined as adherent growth within self-produced extracellular  
86 matrix/ECM consisting of proteins, polysaccharides, and DNA/RNA, and it is the strategy  
87 bacteria use to escape the effects of antibiotics and host defense systems (11-13).  
88 Mycobacteria use phenotypically distinct biofilm subtypes for growth, which genetically and  
89 physiologically differ from the planktonic-type growth. These include (i) floating/pellicle-  
90 type biofilms (PBFs) at the air-liquid interface having an ECM rich in free mycolic acids  
91 (MAs) and with a frequent cord/ribbon-like appearance, while (ii) submerged-type biofilms  
92 (SBFs) show adherent growth on a solid substratum (11, 14-16). The capsule layer plays a  
93 vital role in triggering biofilm growth in mycobacteria, as cells cultured in the presence of  
94 Tween-80 (non-ionic surfactant) has been shown to detach the capsule layer and prevent  
95 the biofilm formation (17). Thus, this labile layer forming the first molecular interaction with  
96 the host/environment is likely to involve key factors contributing to persistence/adaptation  
97 and search of anti-TB targets. Although several studies on mycobacteria have pinpointed  
98 cellular pathways and proteins that affect the capsule/cell wall and the biofilm formation (9,  
99 14, 17-25), systematic investigation of the factors that directly interact with the surrounding  
100 environment is necessary to be able to maximize the power of antimycobacterial treatment  
101 therapies.

102 *Mycobacterium marinum* (Mmr) has proven to be an excellent alternative model pathogen  
103 for slow-growing Mtb, as it allows for the investigation of TB-like chronic and latent

104 infections in its natural host, the zebrafish (26-29). Cultured mycobacterial biofilms have  
105 been used to understand resilient bacterial phenotypes emerging in mycobacterial  
106 infections. However, the distinct phenotypic profiles associated with PBFs and SBFs,  
107 including marker proteins discriminating the two biofilm subtypes have remained poorly  
108 understood. To shed light on the specific attributes linking these biologically different  
109 biofilm subtypes to their phenotypes, we first cultured Mmr strain ATCC927 to create in  
110 vitro biofilms. These biofilms were then imaged using widefield deconvolution microscopy  
111 (WDeM) to investigate temporal effects on the biofilm architectures. Label-free quantitative  
112 (LFQ) proteomics was next used to uncover the ECM-proteome dynamics in maturing Mmr  
113 biofilms and to identify the cell surface proteins (proteome) on Mmr grown on Tween-80, a  
114 detergent known to prevent cells from clumping and forming a biofilm (17). The key  
115 proteome findings were validated by gene overexpression studies to indicate cellulose-  
116 dependent biofilm formation as well as biofilm killing assays to confirm the formation of  
117 persister cells in both biofilm subtypes. To the best of our knowledge, this is the first study  
118 monitoring mycobacterial ECM-proteomes over three months' time as well as protein and  
119 morphological phenotypic markers for distinguishing defined biofilm subtypes.

120

## 121 **RESULTS**

### 122 **SBFs and PBFs show distinct morphological characteristics**

123 The kinetics of development and maturation as well as the morphology of mycobacterial  
124 PBFs and SBFs has been reported to differ substantially (8). Here, we first show that that  
125 Mmr forms PBFs at the air-liquid interphase and SBFs attached onto the bottom of the  
126 culture well under the same physiological in vitro conditions after two weeks of growth  
127 **(Fig. 1SA)**. The SBF subtype develops earlier (visible already after two days of culture)  
128 than the PBF, which was not clearly distinguishable before two weeks of growth. Next, we

129 investigated the three-dimensional morphology of Mmr biofilms in more detail by culturing  
130 Mmr cells, carrying the pTEC27 plasmid with the tdTomato fluorescent marker gene (29),  
131 for two and three weeks to produce PBFs and SBFs, and analyzing the biofilms by  
132 widefield deconvolution microscopy (WDeM). **Figure 1** shows that Mmr forms organized,  
133 three-dimensional structures with distinctive, subtype-specific morphological features. For  
134 the SBF, the structures displayed a lichen- or moss-like appearance, having tens of  
135 microns high feature structures rising from the biofilm base after two weeks (**Fig. 1B**). In  
136 comparison, the morphology of the PBF subtype was very different by the first time point,  
137 showing flat, ribbon-like structures without any protruding structures (**Fig. 1C**). Defined,  
138 extensive structures in all dimensions, although less dense compared to those detected at  
139 the two-week-time point, were found for both biofilm subtypes also after three weeks of  
140 growth.

141

#### 142 **Submerged biofilms exhibit the greatest ECM-proteome diversity**

143 As the phenotypic profiles of PBFs and SBFs are clearly different, their ECM-proteomes  
144 were next quantitatively monitored and compared during the development and maturation  
145 stages. To this end, the PBF and SBF cells at the points in **Figure 2A** were subjected to  
146 trypsin/Lys-C digestion as well as LC-MS/MS-based protein identification and LFQ (all  
147 data available via PRIDE with identifier PXD02010). Logarithmic state planktonic cells  
148 (PL\_log), representing single-cell cultures, were obtained by growing the Mmr strain in the  
149 presence of Tween-80. The quality of each data set was high: 84.7% of all proteins were  
150 identified with at least three or more matching peptides, with an average sequence  
151 coverage of approximately 31% and only 11% of proteins were categorized as single-  
152 peptide-hits. In addition, a broad overlap in protein identifications was detected within the  
153 four biological replica samples; 41-89 % of the proteins were shared by each replicate,

154 with the two-week PBF and the three-week SBF showing the highest variation between  
155 replicates (**Fig. S1B**). **Table S1** lists the proteins detected in at least two out of four replica  
156 samples. An outlier replicate associated with one of the SBF identification replica sets at  
157 the three-week-timepoint was excluded from subsequent data analyses. The number of  
158 detected proteins was 1132, 1957, and 2133 for the PL, PBF and SBF cells, respectively.

159

### 160 **Cytoplasmic protein export/release is most efficient in submerged biofilms**

161 **Figure S2A** shows the distribution of all identified proteins according to their predicted  
162 secretion motif (Sec/SPII, TatP/SPI, LIPO/SPII, type VII secretion/T7SS, SecretomeP) and  
163 the number of transmembrane spanning domains (TMDs). The most notable differences  
164 were detected for membrane proteins with six to ten TMDs as well as in the number of  
165 cytoplasmic proteins. Nearly two-fold more TMD proteins were detected in the PL than the  
166 biofilm cells. In contrast, two-fold more cytoplasmic proteins predicted to be exported out  
167 of the cells via a non-classical route (SecretomeP) were identified from the biofilm-ECMs  
168 ( $n$ , 300) in comparison to the PL cells ( $n$ , 150). For many of these proteins, a secondary  
169 function as a moonlighting protein (30) could be indicated (**Table S1**). In addition, over  
170 900, 1600 and 1800 cytoplasmic proteins identified on the PL, PBF, and SBF cells,  
171 respectively, contained no motifs for classical or non-classical secretion and were  
172 assigned here as “Others” (**Table S1**).

173

### 174 **Most significant protein abundance changes specific to planktonic and biofilm cells**

175 The Venn diagram in **Figure S2B** indicates the highest number of specifically identified  
176 proteins on the SBFs ( $n$ , 173) and the lowest on the PBFs ( $n$ , 16), while no unique  
177 identifications were detected for the PL cells. The uniquely detected proteins with the  
178 highest raw intensity values included a signal transduction associated serine/threonine-

179 protein kinase (PknL), a LGFP-repeat protein specific to SBFs, and a  $\beta$ -1,3-endoglucanase  
180 and bacterioferritin BfrA specific to PBFs (**Table S2**). The proteins detected with the  
181 highest intensity values and only in the biofilm-ECMs included an error-prone polymerase  
182 DinB, a preprotein sec-translocase subunit YajC, a cytochrome P-450 monooxygenase, a  
183 PE family immunogen and a signal transduction-related adenylate cyclase involved in  
184 cyclic di-AMP biosynthesis (**Table S2**).

185 Next, the log<sub>2</sub> transformed MaxLFQ data was subjected to pairwise comparisons to  
186 indicate statistically significant protein abundance changes (**Table S3**). **Figure 3** shows  
187 the greatest growth mode- and time-dependent fold-changes related for the PL vs. biofilms  
188 cells, PBF vs. SBF cells and each biofilm subtype at different time points. Comparison of  
189 the PL and both biofilm cells at their first timepoints of growth (PBF\_2w and SBF\_2d)  
190 indicated the most prominent changes for PPE-family proteins (e.g., PPE61) and enzymes  
191 involved in cell envelope biogenesis/metabolism (MurE, CwlM, cutinase and CelA1).  
192 Among these, the PPE61 immunogen was ca. 6000- and 1800-times more abundant on  
193 the PL compared to the PBF\_2w and SBF\_2d, respectively. CelA1, a  $\beta$ -1,4-  
194 cellobiohydrolase known to prevent biofilm growth in *M. smegmatis* and Mtb (11, 18, 19),  
195 was detected with 50- and 130-fold higher abundances on the PL compared to the PBFs  
196 at the one-week and the SBFs at the two-day timepoints, respectively (**Table S3**).

197 Comparison of the PBF\_2w and SBF\_2d cells indicated Esx1-associated virulence factors  
198 (i.e., EspF, EspA/EspE and ESAT-6) and PPE family immunogens as 30–130-fold more  
199 abundant on the PBF than the SBF cells, meanwhile tricarboxylic acid (TCA)/glyoxylate  
200 cycle-associated isocitrate lyase (ICL1) was over 200-fold more produced by the SBF than  
201 the PBF cells. After 12 weeks, the proteins more abundant in the SBF compared to the  
202 PBF included an LppP/LprE lipoprotein (ca. 16-fold), HemD involved in the synthesis of  
203 vitamin B12 (ca.15-fold), FadD29 contributing to the synthesis of phenolic glycolipids (~13-



204 fold),  $\beta$ -lactamase able to hydrolase  $\beta$ -lactam antibiotics (ca. 9-times) and ICL1 catalyzing  
205 the glyoxylate shunt-mediated activities (ca. 8-fold). More abundant proteins on the PBF at  
206 this time-point were identified as a polysaccharide (N-acetylmuramic acid, MurNAc)  
207 deacetylase PdaC (ca.15-fold) and a translocase subunit, SecE (ca.11-fold).  
208 In the PBF, an MPB64 immunogen, siderophore export accessory protein MmpS5, several  
209 Esx1-associated proteins (EspA/EspE, EspF) and adhesins (Ala-Pro-Ala rich protein APA  
210 and fibronectin binding protein FAP) displayed the most significant abundance decreases  
211 at the 12-week timepoint. In the SBFs, these proteins included a large-conductance  
212 mechanosensitive channel protein Msc, a membrane protein acting as the cells' safety  
213 valve to relieve osmotic pressure, arabinosyltransferases EmbA/EmbB, the Esx1  
214 associated EspA/EspE and the MycP1 protease. Proteins with the greatest abundance  
215 changes after 12 weeks in the SBFs included mammalian entry proteins (MCEs) and an  $\alpha$ -  
216 1,4-glucan:maltose-1-phosphate maltosyltransferase.

217

#### 218 **Decreased CelA1 synthesis is also required for biofilm formation in *M. marinum***

219 As our findings suggest that a lack of CelA could also promote the biofilm formation in  
220 Mmr, we tested this hypothesis by overexpressing the *celA1* gene in a Mmr strain  
221 equipped with pTEC27 with the tdTomato fluorescent marker (29). First, the *celA1*  
222 expression level in the transformed Mmr strain was confirmed by qPCR, indicating a ca.  
223 150-times higher *celA1* transcription compared to the control strain carrying an empty  
224 pTECV27 (**Fig. 4A**). Then we analyzed the morphology of both the SBFs and PBFs after  
225 two weeks using the CelA1-strain with WDeM. As seen in **Figure 4B**, the CelA1-strain  
226 showed altered morphology compared to the Mmr with pTEC27 (WT control strain). After  
227 two weeks of growth the CelA1-strain SBF showed a less defined/loss of the lichen-like  
228 morphology and lower total thickness when compared to the SBF control with pTEC27.

229 Similarly, the CelA1 overproduction in Mmr resulted in disrupted and fuzzy ribbon-like  
230 cords associated with PBF-type biofilm growth, as the PBF cells with pTEC27 had well  
231 defined and tight ribbon like structures.

232 CelA1 expression was recently linked with biofilm formation, antibiotic tolerance, and  
233 virulence in Mtb (9). Therefore, Mmr cells in planktonic and biofilm forms with/without the  
234 CelA1 overexpression were also exposed to rifampicin to determine the minimum  
235 inhibitory (MIC) and minimum bactericidal concentration (MBC) for this bactericidal first-  
236 line TB drug. **Figure 4C** shows that, in both the planktonic and biofilm cultures, CelA1  
237 overexpression decreases the MIC/MBC, with a clear impact on two-day-old and 4-day-old  
238 biofilms. These results indicate that CelA1 impedes biofilm formation and increases the  
239 susceptibility of the residing cells to rifampicin in Mmr.

240

#### 241 **Functional pathways specifically induced in planktonic and biofilm cells**

242 The LFQ proteomic data was next subjected to a PCA analysis for comparing growth  
243 mode- and time-dependent protein abundance patterns on the PL cells and aging biofilms.  
244 The PCA in **Figure 5A** shows clear clustering for each data set except for replicates  
245 associated with two-week-old PBF-proteomes, which show greater variation. PC1,  
246 separating the samples according to the growth mode, explains 39% of the total variation,  
247 while 24% (PC2) of the variation can be explained by the age of the culture. The two-day-  
248 old SBF-proteomes form a clearly distinguishable cluster, while the PL-proteomes and  
249 proteomes associated with the PBFs between the two- and four-week time-points show  
250 close clustering. Although the SBF- and PBF-proteomes differ greatly within the first four  
251 weeks of growth, these biofilm subtypes seem to undergo similar proteome changes  
252 during the later stages of growth, as proteomes of both subtypes clustered more closely at  
253 the 12-week timepoint. Notably, PBFs during the first weeks (two to three weeks) of growth

254 shared a more similar ECM-proteome with the PL cells than the SBFs under the same  
255 conditions.

256 Next, a multi-sample test (ANOVA) was conducted on the normalized LFQ intensity data to  
257 investigate growth mode-dependent proteome differences at time points between two days  
258 and three months. A dendrogram/ heatmap in **Figure 5B** shows hierarchically clustered  
259 co-abundance data for 690 proteins having a statistically significant abundance change in  
260 at least one of the conditions tested (**Table S4**). Six major clusters were clearly  
261 distinguished, among which cluster 1 ( $n$ , 375) and cluster 6 ( $n$ , 125) contained the greatest  
262 number of proteins, with higher abundances in one- to four-week-old SBFs (cluster 1) and  
263 two-day- to two-week-old SBFs (cluster 6), respectively. STRING (Search Tool for the  
264 Retrieval of Interacting Genes/Proteins) enrichment analyses performed on both clusters  
265 (**Table S5**) indicated the greatest changes for pathways coordinating cell envelope  
266 biogenesis/ metabolism, energy metabolism and protein secretion/export. **Figure 6A**  
267 shows a protein-protein interaction (PPI) network for cluster 1 proteins: **(i)** cytoplasmic  
268 proteins with a primary function in amino acid biosynthesis (*e.g.*, Gly, Asp, Tyr, Arg, His,  
269 Thr, Ser, Lys, Phe), purine/pyrimidine metabolism (*e.g.*, PyrG, PurD/L/H, GuaB) and stress  
270 response (HrcA, ClpC/X, DnaJ, HtpG, AhpC, SodC, RecA, Trx), **(ii)** proteins involved in  
271 cell-wall/outer layer and mycomembrane biogenesis/metabolism (*e.g.*, PknA/B, Weg31,  
272 CwsA, CwlM, PbpA1a, EmbA/B, KasA, DesA1/2, PpsA/B/D, PcaA, Fad enzymes), **(iii)**  
273 components of the respiratory electron transport chain (SDH, FMR, Qcr-complex) and ATP  
274 synthesis (F1F0 ATP synthase-complex), and **(vi)** proteins involved in iron  
275 storage/homeostasis (ferritin). The PPI network analysis on the cluster 6 proteins indicated  
276 the enrichment of metabolic activities related to translation (ribosomal proteins/r-proteins),  
277 stress response (GroEL/ES, GrpE, DnaK, TF, ClpB) and the TCA/glyoxylate cycle (*e.g.*,  
278 CitA, ICL1, FBA, GlcB) (**Fig. 6B**).

279 Clusters 2, 4 and 5 (*n*, 144) share co-abundance patterns, which indicate increased  
280 protein abundances during the first weeks of growth in the PBFs when compared to the  
281 SBFs. These contain virulence-, invasion- and viability/persistence-related proteins, such  
282 as EsxA/B, ESX-EspB/G/M/P/N, Esx5-secretion associated protease MycP, cutinase  
283 (Cut), a lysophospholipase (YtpA), endopeptidase (Lon), heparin binding hemagglutinin  
284 (HbhA), fibronectin binding (Apa), catalase-peroxidase (KatG), and mammalian entry  
285 proteins (MCEs). Cytoplasmic proteins were also detected in these clusters (e.g., ICL2,  
286 ACN, ENO, GapDH, GPD, Tpi, PGK, MDH, ClpP1/2, CpsA/D, Trp, Cys, Met, an 18-kDa  $\beta$ -  
287 CA) but their composition differs clearly from those in clusters 1 and 6. In addition, cluster  
288 2 contains virulence-associated ESAT-6-like proteins, TDM-cord factor synthesis  
289 associated Ag85A/C (mycolyltransferases), and an MPT64 immunogen with higher overall  
290 abundancies on the PL and PBF cells compared to the SBFs. The remaining cluster 3 (*n*,  
291 47) differs from the other five by proteins with its overall higher abundancies on the PL  
292 cells and/or on four- to 12-week-old PBFs when compared to the SBFs at the same  
293 timepoints. One of these was identified as a potential trehalase (A0A2Z5YJK7\_MYCMR),  
294 a glycoside hydrolase that catalyzes the conversion of trehalose to glucose, which had a  
295 high abundancy in four- and 12-week-old PBFs.

296 The protein identifications most relevant to biofilm growth and viability identifications are  
297 listed in **Table S6** according to their predicted functions. The major growth mode-  
298 dependent changes associate with the following five functional groups: **(i)** secretion  
299 mechanisms, virulence, and adherence; **(ii)** cell wall/membrane/lipid biogenesis and  
300 metabolism and biofilm formation; **(iii)** stress response; **(iv)** TCA/glyoxylate cycles and  
301 carbohydrate metabolism; and **(v)** maintaining redox balance and energy metabolism. An  
302 additional schematic model of the mycobacterial cell envelope in **Figure 7** illustrates the  
303 key proteome changes relevant to the PL-, SBF- and PBF-type growth in Mmr.

## 304 **Time-kill curve analysis for indicating persister cells in maturing biofilms**

305 As growth mode-dependent differences imply higher persistence/tolerance-associated  
306 activities in biofilms than in planktonic cultures, we next validated these findings by  
307 exposing both the planktonic and biofilm cells to rifampicin and monitored cell death using  
308 a time-kill curve analysis. This method enables the demonstration of an overall slower  
309 killing efficacy for tolerant populations or a bimodal time-kill curve that indicates the  
310 presence of a persistent bacterial subpopulation (31, 32).

311 First, we used a bacterial killing assay with bioluminescence as a readout to quantify the  
312 tolerance/persistence in the planktonic cultures and two-week-old biofilms. The planktonic  
313 and biofilm cells were treated with 400  $\mu\text{g mL}^{-1}$  rifampicin (64 x MIC, minimum inhibitory  
314 concentration), and the rate of bacterial killing was monitored for seven days. The use of  
315 bioluminescence as a readout for killing biofilm-associated bacteria was also assessed  
316 using an OD<sub>600</sub>-based method (**Fig. S3A**). The time-kill curve for the biofilm population  
317 was bimodal, showing the faster killing of a susceptible subpopulation followed by a slower  
318 killing of a persistent subpopulation of cells (**Fig. 8A**). These results indicate that Mmr  
319 biofilms harbor significantly more persister cells than logarithmic phase planktonic  
320 populations.

321 Next, the development of persistence in the biofilms was monitored by killing two-day-,  
322 four-day-, and one-week-old biofilm cells with 64 x MIC rifampicin. Analysis of the time-kill  
323 curves showed that persistence increases gradually in the maturing biofilms, reaching a  
324 statistically significant increase in one-week-old biofilms compared to the planktonic cells  
325 ( $P = 0.0002$ ) (**Fig. 8B**). In untreated biofilms, the bioluminescence signal level continues to  
326 increase well past the one-week timepoint, showing that the biofilm-associated  
327 mycobacterial population is replicating and/or metabolically active at this stage (**Fig. S3B**).  
328 This indicates that increased persistence is not (mainly) caused by the induction of

329 dormancy or metabolic inactivity. According to our experimental settings, PBFs form later  
330 than SBFs and are visually detectable only after two weeks. Thus, this data shows that a  
331 substantial persister subpopulation develops in SBFs by the first week of biofilm  
332 development.

333 To test if the formation of persister cells differs between the two biofilm subtypes, PBFs  
334 and SBFs were collected separately and tested with the time-kill assay under 64 x MIC  
335 rifampicin. After seven days, the time-kill curves indicated no significant differences in the  
336 rate of persistence between the two-week-old pellicle and submerged biofilms ( $P = 0.51$ )  
337 (**Fig. 8C**). Thus, our results indicate that the proportion of persisters is greater in over one-  
338 week-old Mmr biofilms than in logarithmic planktonic cell populations, and that the biofilm-  
339 associated persistence increases over time.

340

## 341 **DISCUSSION**

### 342 **Mmr grows in morphologically distinct biofilm subtypes *in vitro***

343 A recent study confirmed that Mtb forms biofilm-like communities *in vivo*, which confers  
344 increased tolerance to rifampicin and thus provides an explanation for the chronic nature  
345 of TB (11). The present study shows that Mmr grows in two different biofilm subtypes, and  
346 that reduced CelA1 hydrolase activity is one of the main triggers of biofilm growth and  
347 increased tolerance to rifampicin in both biofilm subtypes. Studies on Mtb and *M.*  
348 *smegmatis* have demonstrated that cellulose filaments are vital structural constituents of  
349 mycobacterial biofilm-ECMs as well as essential for biofilm formation and the development  
350 of tolerance/persistence (9, 11, 18, 19). We also show that the Mmr biofilm subtypes show  
351 distinct morphologies, with SBFs containing lichen-like structures and PBFs consisting of  
352 ribbon-like cords under the same *in vitro* conditions. Biofilm growth accompanied by  
353 cording-like growth morphology is also reported for other mycobacteria and Mtb, in which

354 the surface interactions mediated by e.g., mycolic acids modulating the  
355 mycomembrane/capsule hydrophobicity (11, 33). The proteomic data presented here  
356 suggest that subtype-specific changes in cord-factor TDM-synthesis (mycolyltransferase  
357 Ag85), Esx1-secretion, phthiocerol dimycocerosate (PDIM) export (MmpL7), MA  
358 cyclopropanation (PcaA/Cma2), and lectin synthesis (33-37) may have affected the  
359 mycomembrane composition and thereby contributed to distinct biofilm growth  
360 morphologies in Mmr.

361

### 362 **Mmr may use membrane vesicles to deliver proteins in the biofilm-ECM**

363 The LFQ proteomics identified cytoplasmic proteins and proteins associated with the inner-  
364 /mycomembrane as the largest protein group in both the planktonic and biofilm cells.  
365 These findings are supported by studies identifying cytoplasmic proteins in the capsule of  
366 another Mmr strain (E11) and by showing that their number increases when mycobacterial  
367 cells grow in the biofilms, as demonstrated for *M. bovis* (17, 20). Membrane vesiculation is  
368 the most likely explanation for their presence on Mmr cells and within the biofilm-ECM, as  
369 several reports have demonstrated the presence of MVs on mycobacterial cells (38) as  
370 well as trapped in biofilm-ECMs in other bacteria (39). In addition, several of the  
371 cytoplasmic and inner-/mycomembrane-proteins detected here, including e.g., enzymes  
372 involved in cell wall synthesis and lipid/ fatty acid metabolism, were previously identified in  
373 MVs released by *Mycobacterium avium* 104 in response to starvation (40). Mycobacteria  
374 have been shown to form MVs from mycomembrane (mMV) during normal growth (cell  
375 lysis/death) and/or from inner-membrane (iMV) by blebbing in response to stress (e.g.,  
376 iron-limitation and anoxia) (38). This report supports the idea that the identified myco-  
377 /inner-membrane-proteins could have also entered the biofilm-ECMs by MVs in our study.  
378 We further propose that CwIM, a N-acetylmuramoyl-L-alanine amidase (41, 42), detected



379 in one-week-old SBFs, is involved in this process, as weakening the link between the  
380 mycomembrane and peptidoglycan has been suggested to stimulate MV blebbing in the  
381 mycobacteria (38). Taken together, these findings may explain why more cytoplasmic  
382 proteins were detected on this biofilm subtype, as the maturing biofilm cells grow under  
383 reduced oxygen tension and anoxia is one of the factors able to trigger the membrane  
384 vesiculation.

385 Bacterial MVs are involved in i.e., cell-cell communication, biofilm formation, virulence,  
386 antibiotic resistance, iron scavenging, nutrient acquisition and modulating the host immune  
387 system (43). We detected several cytoplasmic proteins involved in signal transduction  
388 (e.g., PknL specific to SBFs and an adenylate cyclase detected only in biofilm-ECMs) and  
389 enzymes involved in biofilm formation. GroEL1 and Fatty-Acid-Synthase system (FAS-I  
390 and FAS-II) enzymes were among the detected proteins that coordinate biofilm formation  
391 in mycobacteria. The GroEL1 chaperone is involved in the synthesis of mycolic acids  
392 (MAs) that eventually become inserted in the mycomembrane as trehalose dimycolates  
393 (TDM) and monomycolates (TMM) beneath the capsule (14, 21). This chaperone interacts  
394 with ketoacyl-ACP synthase KasA (FAS-II) to modulate the synthesis of short-chain MAs  
395 specifically during biofilm formation (21). A lack of GroEL1 has been reported to prevent  
396 the biofilm formation and to affect the biosynthesis and composition of MAs in  
397 *Mycobacterium bovis* BCG, whereas the GroEL1 deficiency blocks the formation of mature  
398 biofilms in *M. smegmatis* (21, 24). In addition, the overexpression of KasA and the  
399 inactivation of other FAS-II enzymes, such as enoyl-ACP reductase (InhA) and 3-oxoacyl-  
400 [acyl-carrier-protein] synthase 2 (KasB), have also been reported to prevent biofilm  
401 formation and formation of cords by reducing the cyclopropanation of MAs (14, 21, 25).  
402 Here, GroEL1, KasA and InhA were detected as more abundant in the SBFs, implying that



403 these enzymes could support the initial stages of SBF-type biofilm growth, as GroEL and  
404 KasA were detected with the highest abundancies already on the two-day-old SBFs.  
405 Although no cell lysis was seen during the sample preparation for proteomic analysis  
406 **(Table S7)**, we cannot exclude the possibility that some of the cytoplasmic or inner-/myco-  
407 membrane-proteins were released by autolysis during growth. In other Gram-positive  
408 bacteria, cytoplasmic proteins reach the extracellular space via regulated autolysis  
409 (involving autolysins/peptidoglycan hydrolases), and, as soon as the pH of the culture  
410 medium drops (due to the active metabolism of the growing cells), many of the released  
411 proteins show an enhanced ability to bind to the cell wall and biofilm-ECM structures (43-  
412 48). SBF cells are exposed to hypoxic conditions, and oxygen limitation acidifies the  
413 biofilm matrix (48), allowing for a more efficient interaction between the cytoplasmic  
414 proteins and biofilm-ECM structures. Thus, this could explain the presence of r-proteins as  
415 the largest cytoplasmic protein group already on two-day-old SBFs; the strong positive  
416 charge of these proteins has been proposed to mediate electrostatic interactions with  
417 anionic cell-surface components, which promotes cell aggregation and biofilm stabilization  
418 (48). Since the exposed mycomembranes with MAs as the major components create a  
419 condition stimulating an interaction with many cytoplasmic proteins, pH-dependent binding  
420 with the cell surface components could also explain why cytoplasmic proteins were  
421 detected on Mmr cells grown on Tween-80.

422

### 423 **Biofilm subtypes differ in terms of secreted virulence and adhesion factors**

424 The proteomics data indicated that the mycomembrane-associated PPE/PE family  
425 proteins were remarkably greater in number in the PL than in the PBFs or SBFs, indicating  
426 that Mmr in a single cell state could more readily interact with the host, and modulate the  
427 host immune response and/or nutrient transport (49, 50). PL cells were cultured in the

428 presence of Tween-80, which, in detaching the mycobacterial capsule (17), most likely  
429 helped identify these immunogens. Tween-80 can also induce alterations in the  
430 morphology, pathogenicity, and virulence of mycobacteria (51). For example, genes  
431 encoding lipases and cutinases have been shown to be significantly upregulated in Mtb in  
432 response to this nonionic surfactant. Our data is in line with this by showing that several  
433 lipases/cutinases, with a likely ability to hydrolyze Tween-80, were more abundant on PL  
434 cells compared to biofilms. As Tween-80 is considered to mimic a lipid rich milieu of  
435 macrophages (51), the detected PL-proteome changes here may reflect a metabolic  
436 adaption to conditions faced in vivo.

437 Our findings also suggest that Mmr uses different T7SS pathways in SBFs and PBFs for  
438 virulence and adherence. For example, the Esx1-secretion components and substrates  
439 (EsxA/B, EspB, EspF, EccA1, EspG1, EspH, EspL and MycP) were detected as more  
440 abundant in the PBFs, while those associated with the Esx5-type secretion were overall  
441 more abundant in the SBFs (Ecc, EspG, PPE/PE proteins). Both secretion pathways can  
442 contribute to virulence and subverting the host immune system in Mtb (52). The major  
443 subtype-dependent differences between the PBFs and SBFs were related to invasion and  
444 adherence, including the MCE proteins, fibronectin binding APA and HphA, which can  
445 modulate host cell signaling as well as aid adhesion or entry into host cells (53-55). All  
446 these proteins were significantly more produced on the PBFs than the SBFs, and, in the  
447 case of MCEs, may also involve MVs, as these adhesins are located on the inner  
448 membrane of the mycobacterial cell wall. HphA also has implications in promoting cell-cell  
449 aggregation in Mtb (56), suggesting that this adhesin could also contribute to cording  
450 during PBF-type growth.

451

452

## 453 **Biofilm subtypes use different tolerance- and persistence-conferring mechanisms**

454 Tolerance is defined as the extent of time that bacteria can survive in the presence of a  
455 high antibiotic concentration (31), whereas persisters are a subpopulation of  
456 phenotypically drug tolerant cells that do not grow in the presence of an antibiotic (32). We  
457 show that antibiotic killing of biofilm cells occurs at a significantly slower rate when  
458 compared to PL cells. The time-kill curve indicated the temporally increased formation  
459 of a persistent subpopulation with slower killing kinetics as well as the formation of  
460 persisters in SBFs already after one week. At this stage, Mmr biofilms remained  
461 metabolically active and replicating, indicating that persistence develops due to phenotypic  
462 differentiation during biofilm growth rather than via the induction of dormancy.

463 The proteomic findings suggest that Mmr could use both overlapping and subtype-specific  
464 mechanisms for increasing its tolerance and persistence, in which MVs or other non-  
465 classical routes for protein export may play a role. Here, most significant proteome  
466 changes related to cytoplasmic and inner-/mycomembrane-proteins and included  
467 enzymes/proteins involved in the TCA cycle and glyoxylate shunt, mycolic acid synthesis  
468 stress response, and energy and redox metabolisms. A recent transcriptome analysis of  
469 another non-tuberculous mycobacterial model, *Mycobacterium abscessus*, supports our  
470 findings; biofilm growth activated the glyoxylate shunt, redox metabolism and the MA  
471 synthesis-associated elongation and desaturation pathways. The TCA cycle associated  
472 enzyme CitA was recently reported to control the asymmetric cell division in *Caulobacter*  
473 *crescentus* (57). This process has been shown to lead to the formation of heterogenous  
474 cell populations in biofilms, macrophages, and granulomatous lesions also in mycobacteria  
475 (7, 58, 59). Here, our findings indicated the presence of this enzyme on one-week-old  
476 SBFs, suggesting that the asymmetric cell division occurs before the PBFs are formed.  
477 Moreover, arabinosyltransferases EmbA and EmbB, involved in the polymerization of the

478 arabinogalactan, were also detected with high abundances in SBFs by one week onward,  
479 suggesting that strengthening the arabinogalactan could further help residing cells,  
480 including the persisters, increase their tolerance to rifampicin, as demonstrated with Mtb  
481 persisters under hypoxia (60). Taken together, these findings strengthen the hypothesis  
482 that persisters are indeed formed in one-week-old SBFs, and supports the results obtained  
483 with the biofilm killing assay on the SBFs at this time point.

484 We also suggest that cells in PBFs use different TCA cycle enzymes, such as aconitase  
485 (ACN), malate dehydrogenase (MDH), enolase (ENO), and/or fructose-bisphosphate  
486 aldolase (FBA), to maintain long-term survival. In other gram-positive bacteria these  
487 enzymes belong to known moonlighting proteins with established secondary roles outside  
488 of the bacterial cell (e.g., adhesion)(30). In mycobacteria, these enzymes have been  
489 reported to contribute to increased viability or persistence (61-63). The associated  
490 glyoxylate shunt could also be involved (64), as isocitrate lyase 1 (ICL1) was detected as  
491 more abundant on the SBFs, implying that this enzyme could help residing cells increase  
492 their antioxidant defense and antibiotic tolerance (65). In contrast, ICL2 was produced  
493 more on the PBFs, which may help the cells to survive under starvation conditions when  
494 fatty acids are used as the primary carbon source (66). This is in line with the temporally  
495 increased production of diacylglycerol O-acyltransferase (Tgs1) in PBFs, which can  
496 promote the accumulation of triacylglycerols (TAGs); a process that has been considered  
497 a hallmark feature of persisting Mtb/latent TB and a long-term energy source for Mtb and  
498 have been found in substantial amounts in the mycobacterial cell wall (67, 68). The  
499 detection of trehalase as significantly more abundant in four- to 12-week-old PBFs,  
500 strengthens the idea that cells within this biofilm subtype suffer from nutrient stress and  
501 activate trehalose salvage/recycling to promote redox and energy homeostasis, as seen  
502 under carbon limitations in Mtb (69). These findings may also explain the detection of

503 proteases, chaperones, and assisting stress-proteins in high numbers in the biofilm-ECMs,  
504 including e.g., the proteases Clp/Lon and the cold-shock protein CpsD, with known  
505 implications in stringent response, persistence and/or post-antibiotic recovery (70-72).  
506 These proteins were detected here as more abundant in the PBFs than in the SBFs,  
507 implying that these pathways are preferred in PBFs to maintain viability.

508 A recent study comparing high numbers of persister Mtb mutants using genomics and  
509 transcriptomics indicated a significant upregulation of energy production pathways and  
510 pathways involved in redox reactions (oxidoreductase) (73). The ECM-proteome changes  
511 occurring during SBF-type growth are in line with this report, as the components of the  
512 respiratory electron transfer chain (cytochrome bc<sub>1</sub> complex, cytochrome c terminal  
513 oxidase, and F<sub>0</sub>F<sub>1</sub>-ATPase synthase) were detected as more abundant on the SBFs  
514 facing more hypoxic conditions than the PBFs. Our findings also agree with previous  
515 reports showing that the electron transfer chain is essential for maintaining ATP  
516 homeostasis and the viability of nonreplicating/persistent Mtb cells under hypoxia (74-76).  
517 In addition, we show that both redox and iron metabolism could also play a biofilm  
518 subtype-specific role in helping the cells cope with hypoxia/aeration-related stress (77);  
519 several oxidoreductases, thioredoxin and a superoxide dismutase (SOD) were overall  
520 more abundant in the SBFs, and a catalase-peroxidase (KatG) and alkyl  
521 hydroxyperoxidases (AhpCF) as more abundant in the PBFs. These enzymes have been  
522 shown to protect Mtb against oxidative stress by the reduction of superoxide radicals into  
523 less toxic intermediates for inhibiting autophagy, apoptosis and cellular damage (78). Iron  
524 storing proteins ferritin (BfrB) and bacterioferritin (BfrA) can confer increased redox  
525 resistance on Mtb and protect the cells against oxidative stress and hypoxia, respectively  
526 (79). Here, these iron storing proteins displayed biofilm subtype-specific abundance

527 changes, implying that SBFs could rely on BfrB to cope with hypoxia and PBFs on BrfA to  
528 help cells grow at the air–liquid interface.

529

## 530 **CONCLUSIONS**

531 The present study reports an in-depth view of ECM-proteome changes occurring in Mmr  
532 ATCC927 during biofilm growth in vitro from two days to three months. We show that this  
533 non-tuberculous mycobacterial model forms SBFs already after two days, whereas the  
534 formation of detectable PBFs was observed after two weeks of growth in the absence of  
535 Tween-80. Both biofilm subtypes were formed physically under the same conditions with  
536 clearly distinct growth morphologies: SBFs with lichen-like structures and PBFs with  
537 ribbon-like cords. We show that the reduced CelA1-mediated cellulose hydrolysis is  
538 necessary to establish proper biofilm growth, growth morphology and increased tolerance  
539 to rifampicin for both biofilm subtypes. The formation of persisters in both biofilm subtypes  
540 and increased tolerance was further confirmed by the newly established bioluminescence-  
541 based time-kill assay, which provides an effective tool for quantifying tolerance and  
542 persistence in Mmr. The proteomic findings imply that subtype-dependent changes in the  
543 MA synthesis and modification, Esx1-type secretion, and the production of specific  
544 adhesins were the major drivers of distinct biofilm growth morphologies. We also propose  
545 that pathways associated with MA biosynthesis, development of tolerance/persistence and  
546 oxidative/redox stress are differentially used in PBFs and SBFs to maintain prolonged  
547 viability. Possible explanations for these differences include the different oxygen tensions  
548 encountered by the biofilm subtypes, differences in membrane vesiculation activities  
549 and/or other non-classical pathways for protein export. Taken together, this is the first  
550 study reporting on ECM-proteome dynamics in maturing mycobacterial biofilms and  
551 predicting biofilm subtype-specific changes in cell-cell communication, biofilm matrix

552 formation, virulence, and tolerance/persistence. This is also the first time that the kinetics  
553 of persistence have been explicitly measured from mycobacterial biofilms.

554

## 555 **MATERIALS AND METHODS**

### 556 **Preparing bacterial cells for surface proteomics**

557 *Mycobacterium marinum* (ATCC 927) with the pTEC27 plasmid expressing the red  
558 fluorescent protein tdTomato (Addgene #30182, <http://n2t.net/addgene:30182>) (29) was  
559 pre-cultured on Middlebrook 7H10 plates with 10% (v/v) Oleic Albumin Dextrose Catalase  
560 (OADC) enrichment (Fisher Scientific, NH, USA) and 0.5% (v/v) glycerol at 29 °C for one  
561 week. For planktonic cultures, an inoculum of Mmr was transferred into a Middlebrook 7H9  
562 medium supplemented with 10% (v/v) ADC (Fisher Scientific, NH, USA), 0.2% (v/v)  
563 glycerol, and 0.2% (v/v) Tween-80 (Sigma-Aldrich, MO, USA), and the cells were cultured  
564 at 29 °C in cell culture flasks with filter caps. After three days of incubation the cell cultures  
565 were diluted to obtain an OD<sub>600</sub> of 0.042 and the dilutions were cultured for an additional  
566 2 days at +29 °C until harvesting. For the biofilm cultures, a Middlebrook 7H9 medium with  
567 the ADC growth supplement but without Tween-80 or glycerol was used. The inoculum  
568 was cultured for three days at +29 °C until the OD<sub>600</sub> reached 0.45. The cell cultures were  
569 diluted 1:40, and the dilutions were divided into 10 ml aliquots. The cap of each tube was  
570 sealed with Parafilm M® laboratory wrapping film, and the cultures were incubated at +29  
571 °C. Planktonic and biofilm cell samples (SBFs and PBFs separately) were collected at the  
572 time points indicated in **Figure 2A**. All the cultures were performed in quadruplicates.  
573 Planktonic cells were harvested by centrifugation (3 min, 5 000g, +4 °C) and the pelleted  
574 cells were suspended gently in ice-cold buffer (100 mM Bis-Tris, pH 6.5) to remove  
575 interfering/non-specifically bound proteins. This step prevents the detachment/removal of  
576 cytoplasmic moonlighters bound to the cell surfaces/biofilm-ECM (43-46, 79, 80). The



577 PBFs were collected with an inoculation loop, the extra medium was removed by pipetting  
578 to avoid cross-biofilm type contamination, and the SBFs were harvested by  
579 pipetting/scraping. The PBFs and SBFs were collected in separate Eppendorf tubes the  
580 ice-cold buffer (100 mM Bis-Tris, pH 6.5). Cells (planktonic and biofilm cultures) were  
581 pelleted by centrifugation (3 min, 5 000g, +4 °C) and the washed cells were suspended  
582 gently in 95 µL of 100 mM TEAB (triethylammonium bicarbonate, pH 8.5) for the enzymatic  
583 shaving reaction.

584

### 585 **Trypsin/Lys-C shaving of planktonic and biofilm cells**

586 Peptides from cell-surface/biofilm-ECM-associated proteins were released via a  
587 Trypsin/Lys-C mix (Promega) at a final concentration of 50 ng µL<sup>-1</sup>, and the digestions  
588 were incubated at 37 °C for 20 minutes. The method was validated by counting the  
589 number of colonies formed on the planktonic/single and biofilm cells treated with/without  
590 the enzyme mix (**Table S7**). The released peptides and the enzymes were recovered by  
591 filtration through a 0.2 µm acetate membrane (Costar® Spin-X Centrifuge Tube Filter,  
592 Corning Inc., Corning, NY, US) by centrifugation (8000g, 3 min, 20 °C). Flow-troughs were  
593 incubated for 16 hours at 37 °C. The concentration of released peptides in each sample  
594 was measured with a NanoDrop2000 spectrophotometer (Thermo Scientific). Digestions  
595 were terminated with 0.6% (v/v) trifluoroacetic acid (TFA) (Sigma Aldrich) and the peptides  
596 were purified using ZipTip C18 (Millipore) according to the manufacturer's instructions and  
597 dried using a miVac centrifugal vacuum concentrator (GeneVac).

598

### 599 **LC-MS/MS analysis**

600 The peptides were dissolved in 0.1% (v/v) formic acid (FA) and analyzed with nanoLC-  
601 MS/MS using an Easy-nLC 1000 nano-LC system (Thermo Scientific) coupled with a



602 quadrupole Orbitrap mass spectrometer (Q Exactive<sup>TM</sup>, ThermoElectron, Bremen,  
603 Germany) as previously reported (80). The obtained MS raw data was processed via  
604 MaxQuant software (version v.1.6.1.0) with the built-in search engine, Andromeda (81,  
605 82), using a protein database comprising all 5,564 Mmr protein sequences (Uniprot  
606 proteome: up000257451, genome accession: PEDF01000000) both forward and reverse.  
607 Carbamidomethyl (C) was set as a fixed and methionine oxidation was set as a variable  
608 modification. Tolerance was set to 20 ppm in the first search and 4.5 ppm in the main  
609 search. Trypsin without the proline restriction enzyme option and with two allowed  
610 miscleavages was used. The minimal unique plus+ razor peptide number was set to 1; the  
611 FDR was set to 0.01 (1%) for peptide and protein identification; and LFQ with default  
612 settings was used. The mass spectrometry proteomics was deposited in the  
613 ProteomeXchange Consortium via the PRIDE (83) partner repository with the dataset  
614 identifier PXD02010.

615

## 616 **Proteome statistics and bioinformatics**

617 The identified Mmr proteins were manually curated by characterizing hypothetical and  
618 tentatively annotated proteins with the aid of the Basic Local Alignment Search Tool  
619 (BLAST) program from the National Center for Biotechnology Information (NCBI) (84-86),  
620 combined with CDD/SPARCLE conserved domain identification (87) and SmartBLAST  
621 (UniProt) searches. General protein functions were annotated using the Gene Ontology  
622 (GO) database (88). Isoelectric points (pIs) and molecular weights (MWs) for the identified  
623 proteins were predicted using EMBOSS Pepstats (89) at [https://www.ebi.ac.](https://www.ebi.ac.uk/Tools/seqstats/emboss_pepstats/)  
624 [uk/Tools/seqstats/emboss\\_pepstats/](https://www.ebi.ac.uk/Tools/seqstats/emboss_pepstats/). The presence of possible protein secretion motifs  
625 (classical and nonclassical) for all the predicted and identified proteins was obtained with  
626 SignalP4.1 (90) (<http://www.cbs.dtu.dk/services/SignalP/>) and SecretomeP 2.0/SecP (91)

627 (<http://www.cbs.dtu.dk/services/SecretomeP/>). The presence of transmembrane spanning  
628 domains/helices (TMDs) was determined with the TMHMM Server v. 2.0 at <http://www.cbs.dtu.dk/services/TMHMM/> (92, 93) for the identified proteins.  
629  
630 For indicating statistically significant abundance changes the log<sub>2</sub>-transformed LFQ data  
631 was analyzed in Perseus v.1.6.2.3 (94) using a Student's *t*-test with permutation-based  
632 FDR adjustment. For the multivariate analyses, the missing values were replaced by  
633 imputed values from the normal distribution (width = 0.3, down shift = 1.8) and then  
634 normalized (z-score) prior to ANOVA for multi-sample testing (S0 set to 0.1 and a  
635 permutation-based FDR of 5%) and hierarchical clustering/PCA. STRING Protein  
636 Interaction Network and Functional Enrichment Analyses (GO, KEGG, InterPro, Pham)  
637 were studied using the STRING database v. 11 (95). Interaction scores were set to high  
638 (0.700) confidence, and the interacting proteins were clustered using Markov clustering  
639 (MCL) with the inflation parameter set to 4.0–6.0. Functional enrichments were statistically  
640 assessed with both rank- and gene set-based approaches (FDR of 0.05).

641

#### 642 **Creation of the CelA1 overexpression construct in Mmr**

643 The Mmr CelA1 overexpression strain was created by ordering the MMAR\_0107 open  
644 reading frame in the pUC57 vector with appropriate restriction sites from GenScript and  
645 subcloning the construct into the pTEC27 vector (AddGene) (29), which carries the red  
646 fluorescent protein tdTomato. The sequence of the plasmid was confirmed by sequencing.  
647 The resulting plasmid was transformed into an electrocompetent Mmr ATCC927 strain.  
648 Transformants were selected on Middlebrook 7H10 agar plates containing 10% (v/v)  
649 OADC enrichment, 0.5% (v/v) glycerol and 75 µg mL<sup>-1</sup> hygromycin.

650

651

652 **RNA and DNA extractions**

653 For RNA and DNA extractions, the *CelA1* overexpression strain and Mmr were precultured  
654 on MiddleBrook 7H10 plates and transferred into the Middlebrook 7H9 medium described  
655 above (75  $\mu\text{g mL}^{-1}$  hygromycin for the *CelA1* strain). After three days, the bacterial cells  
656 were harvested, pelleted, and homogenized in TRI Reagent (Thermo Fisher Scientific, NH,  
657 USA) with ceramic beads using the PowerLyzer24 (Mobio, CA, USA). After  
658 homogenization, the samples were sonicated for nine minutes and the RNA and DNA  
659 were extracted according to the manufacturer's instructions.

660

661 ***CelA1* expression and the quantification of mycobacterial loads by qPCR**

662 Prior to qPCR analysis, RNA was reverse transcribed into cDNA with a Reverse  
663 Transcription kit (Fluidigm, CA, USA) according to the manufacturer's instructions. *CelA1*  
664 expression was measured using soFast EvaGreen Supermix with the Low ROX qPCR kit  
665 (Bio-Rad, CA, USA) and the CFX96 qPCR system (Bio-Rad, CA, USA). The primers used  
666 for *CelA1* were: (forward: 5'- ACACTCCGCAGTCCTACT-3' and reverse: 5'- TAGAGCGTC  
667 AGAATCGGC-3'). The number of mycobacterial cells in the sample was quantified using  
668 the SensiFAST SYBR No-ROX qPCR kit (Bioline, London, UK) on bacterial DNA  
669 according to the manufacturer's instructions. The primers used for Mmr quantification were  
670 targeted against 16S-23S, locus AB548718 (forward: 5'- CACCACGAGAAACTCCAA-3'  
671 and reverse: 5'- CACCACGAGAAACTCCAA-3'). Each bacterial quantification qPCR  
672 run included a standard curve of the known amounts of Mmr DNA. The mycobacterial cell  
673 number in each sample was used to normalize the *CelA1* expression.

674

675

676

## 677 **Widefield deconvolution microscopy (WDeM) of Mmr biofilms**

678 PBFs and SBFs formed by Mmr with pTEC27 (WT), expressing the red fluorescent protein  
679 tdTomato (29), and Mmr overexpressing *CelA1* were prepared as follows. Briefly, the cells  
680 were incubated at 29 °C and the surface-attached cells were imaged at seven, 14 and 21  
681 days after dilution. In situ imaging of the SBFs was conducted with Nikon FN1 upright  
682 epifluorescence microscope equipped with 20x/0.8 dry objective, Hamamatsu ORCA-  
683 Flash4.0 V3 Digital CMOS camera and CoolLED pE-4000 light source. tdTomato was  
684 excited with 550 nm LED and fluorescence was collected with 617/73 band-pass emission  
685 filter. Image stack were collected with 2µm intervals (x-y pixel size 325 nm). The data was  
686 deconvolved with Huygens Essential deconvolution software (SVI, Amsterdam,  
687 Netherlands) using 200 iteration limit, signal-to-noise ratio of 30 and quality threshold of  
688 0.01.

689

## 690 **Biofilm tolerance assays**

691 The role of *CelA1* overexpression in the antibiotic tolerance of Mmr was assessed as  
692 follows. First, the *CelA1* overexpression strain and pTEC27 control strain were cultured  
693 for one week on 7H10 plates (10% OADC, 0.5% glycerol + 75 µg mL<sup>-1</sup> hygromycin) and  
694 then transferred in a Middlebrook 7H9 medium supplemented with 10% ADC and 75 µg  
695 mL<sup>-1</sup> hygromycin) at an OD<sub>600</sub> of 0.1 to initiate biofilm growth. Aliquots of bacterial  
696 suspension (192 µl of per well) were added to 96-well-plates in triplicate, sealed with  
697 parafilm and incubated at +29 °C in the dark. Planktonic cultures grown in the presence of  
698 0.2% glycerol were used as controls. Eight µl of antibiotics per well was added two, four,  
699 seven and 14 days after the start of the liquid culture. The final antibiotic concentrations  
700 used were 400, 100, 25 and 6.25 µg mL<sup>-1</sup> for the Rifampicin TOKU-E solution. Untreated  
701 wells were used as controls. Ten µl per sample was plated on 7H9 plates (10% OADC, 75

702  $\mu\text{g mL}^{-1}$  hygromycin) one week after the addition of antibiotics. The plates were incubated  
703 at +29 °C for seven to nine days and the colonies were counted.

704

### 705 **Biofilm persistence assays**

706 Mmr (ATCC 927) with a bioluminescence cassette in the pMV306hsp+LuxG13 plasmid  
707 was used for antibiotic tolerance assays. pMV306hsp+LuxG13 was provided by Brian  
708 Robertson and Siouxsie Wiles (Addgene plasmid #26161; <http://n2t.net/addgene:26161>).  
709 To measure the kinetics of bacterial killing, the bioluminescent Mmr strain was first  
710 cultured on Middlebrook 7H10 agar (Sigma-Aldrich) supplemented with 0.5% (v/v) glycerol  
711 (Sigma-Aldrich) and 10% (v/v) OADC enrichment (BD, Becton Dickinson) at 29 °C for  
712 seven days in the dark. To initiate biofilm formation, the Mmr cells were suspended in  
713 Middlebrook 7H9 broth (Sigma-Aldrich) supplemented with 10% (v/v) ADC enrichment  
714 (BD, Becton Dickinson) at a starting  $\text{OD}_{600}$  of 0.1. Planktonic cultures were prepared in the  
715 same way except that the medium contained 0.2% (v/v) glycerol (Sigma-Aldrich) and 0.2%  
716 (v/v) Tween-80 (Sigma-Aldrich). Bacterial suspensions (192  $\mu\text{L}$  per well in triplicates) were  
717 divided on to white 96-well plates (Perkin Elmer). The biofilm cultures were sealed with  
718 laboratory film and incubated at 29 °C in the dark to the desired ages. Rifampicin solution  
719 (TOKU-E) in water at a final concentration of 400  $\mu\text{g mL}^{-1}$  corresponding to 64 x MIC  
720 (minimum inhibitory concentration) was added to the bacterial suspensions and incubated  
721 for seven days at 29 °C in the dark. The bioluminescence signal was measured with an  
722 EnVision plate reader (Perkin Elmer) as a readout for bacterial survival three times for  
723 three seconds per well daily from a white 96-well plate for seven days. The background  
724 signal from media only wells was first subtracted from the sample wells and an average of  
725 the three measurements normalized with the starting bioluminescence signal was used to

726 draw time-kill curves of the bacterial population in the biofilms at different maturation  
727 stages.

728 To compare the level of persistence/tolerance in the PBFs and SBFs, Mmr was cultured in  
729 a total volume of 10ml at the starting OD<sub>600</sub> value of 0.1. After two weeks, the biofilms were  
730 collected separately from the tubes by lifting the pellicle with a 1- $\mu$ L inoculation loop  
731 coupled with careful pipetting. The pellicle and submerged biofilms were centrifuged at  
732 10,000g for three minutes, the supernatants were collected, and the wet weight of the  
733 bacterial mass was measured. The bacterial cells were suspended into previously  
734 collected spent media at the concentration of 15 mg mL<sup>-1</sup>, vortexed briefly, and divided on  
735 white 96-well plates (Perkin Elmer) with 192  $\mu$ L of cell suspension per well in triplicate.  
736 Eight  $\mu$ L of TOKU-E solution at a final concentration of 400  $\mu$ g mL<sup>-1</sup> were pipetted on the  
737 bacterial suspension. Liquid cultures were incubated for seven days at 29 °C in the dark  
738 and the bioluminescence signal was measured daily with an EnVision plate reader (Perkin  
739 Elmer) three times for three seconds per well. The background signal from the media-only  
740 wells was first subtracted from the sample wells, and an average of the three  
741 measurements was normalized with the starting bioluminescence signal measured just  
742 before adding the rifampicin. The statistical significance of the differences between the  
743 time-kill curves was tested with a log-rank test using Prism5 software (GraphPad).

744

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1050

## 1051 **LEGENDS for figures and tables**

1052 **Figure 1.** Mmr biofilms show distinct growth morphologies after two-weeks of growth.  
1053 SBFs grow with lichen-like structures, whereas PBFs have a ribbon-like cords morphology,  
1054 which becomes more defined with maturation (after 3 weeks). The WDeM images are  
1055 maximum- intensity projections of one-, two-, and three-week-old biofilms (a) together with  
1056 an image where Z-position is color-coded (b). X/Y scale bar corresponds to 10  $\mu\text{m}$  and  
1057 frame interval is 2 $\mu\text{m}$ .

1058 **Figure 2. (A)** A workflow depicting the conditions and time points used for preparing the  
1059 planktonic and biofilm cells of Mmr. Grey arrows indicate sampling time points for pellicle  
1060 (PBF) and submerged (SBF) biofilms. (B) Workflow used for identification of surface  
1061 proteins associated with planktonic (PL\_log) cells, PBFs and SBFs. Marker proteins were  
1062 identified by comparing the raw intensity data, statistically significant protein abundance  
1063 changes by pairwise comparisons of the log<sub>2</sub> converted LFQ data and the protein co-  
1064 abundance patterns by subjecting the LFQ data to imputation and z-score normalization.  
1065 STRING and pathway enrichment analyses were conducted on the selected heat-map  
1066 clusters and necessary phenotypic assays to validate the key proteome differences.

1067 **Figure 3.** The most significant protein abundance fold changes between the indicated cell  
1068 types at selected timepoints. The log<sub>2</sub> transformed LFQ data was analyzed  
1069 using Student's t-test with permutation-based FDR adjustment. In the first two top panels,  
1070 the fold-change x 1000.

1071 **Figure 4. (A)** Comparison of relative transcript abundance for *celA1* between the Mmr-  
1072 *CelA1* overexpression strain and the Mmr control strain with pTEC27 (WT). The *CelA1*  
1073 overexpression levels were normalized to the expression level of *CelA1* in the WT control.  
1074 The data is obtained from two technical replicates from two different bacterial clones. The  
1075 bars represent the standard deviation. *CelA1* expression is normalized with the amount of  
1076 Mmr DNA in each sample. **(B)** *CelA1* overexpression disrupts the biofilm development,  
1077 and the formation of the subtype-specific growth morphologies. The WDeM images are  
1078 maximum intensity projections of the two-week-old Mmr control biofilms (pTEC27, WT)  
1079 and the Mmr-*CelA1* cultures (a), together with color-coded by Z-position images (b). Scale  
1080 bars 100  $\mu\text{m}$  and frame interval is 2 $\mu\text{m}$ . **(C)** The MIC/MBC of rifampicin is reduced in both  
1081 the PL and biofilms formed with the *CelA1* overexpressing strain compared to the Mmr  
1082 control cultures (PTEC27, WT). Rifampicin was added to the liquid cultures two, four,  
1083 seven and 14 days after the start of the culture. Ten  $\mu\text{l}$  per sample (in triplicate) was plated  
1084 seven days after the addition of rifampicin and CFUs were counted seven days thereafter.  
1085 100 CFU per sample was used as the cut-off limit for bacterial growth. The experiment  
1086 was carried out three times. The figure shows a representative experiment. -, = no growth,  
1087 + = bacterial growth , UNT= untreated

1088 **Figure 5. (A)** A PCA analysis of all detected proteins (based on LFQ intensities excluding  
1089 one SBF\_3w outlier) with PC1 and PC2 indicating growth mode- and time point-dependent  
1090 changes. **(B)** Hierarchical clustering of proteins (complete linkage; n, 690) with significantly  
1091 changed expression profiles. Color intensity: red and green indicate higher and lower  
1092 protein abundances, respectively.

1093 **Figure 6. (A)** A PPI network analysis on cluster 1 proteins (Fig. 5B) with higher  
1094 abundancies on SBFs between two and four weeks. No. nodes, 368; no. edges, 3256; PPI  
1095 enrichment  $P < 1.0\text{e-}16$ . **(B)** A PPI network analysis on cluster six proteins with higher

1096 abundancies on SBFs between two days and 2 weeks. Proteins were clustered using MCL  
1097 with the inflation parameter set to 4.0 (cluster 6) and 6.0 (cluster 1). No. nodes, 155; no.  
1098 edges, 3024; PPI enrichment  $P < 1.0e-16$ . Circles indicate the most enriched protein  
1099 interactions.

1100 **Figure 7.** A schematic model of the Mmr cell envelope with key protein abundance  
1101 changes specific to PL, PBF and SBF cells. Colored arrows pointing up/down refer to  
1102 protein abundances/abundance changes within the indicated cell sample types (green, PL;  
1103 blue, PBFs; red, SBFs). MA, mycolic acids; cMAs, cyclopropanated mycolic acids;  
1104 TDM/TMM, trehalose-6,6-dimycolate/trehalose monomycolate; PDIM/PGL, phthiocerol  
1105 dimycoceros- ates/phenolic glycolipids. C, cytoplasm; IM, inner membrane; PP,  
1106 periplasmic space; AG, arabinogalactan; PG, peptidoglycan; MM, mycomembrane; ECM,  
1107 extracellular matrix.

1108 **Figure 8.** The proportion of persistent bacterial cells increases in Mmr biofilms. Time-kill  
1109 curve analysis was performed by culturing biofilms from two days to two weeks and  
1110 treating the bacteria with  $400 \mu\text{g mL}^{-1}$  rifampicin. The killing kinetics were monitored for  
1111 seven days by measuring the bioluminescence signal produced by Lux-Mmr daily. **(A)**  
1112 Logarithmic growth phase planktonic and 2-week-old biofilm Mmr were treated with  $400 \mu\text{g}$   
1113  $\text{mL}^{-1}$  of rifampicin. The time-kill curves of the planktonic and biofilm-associated bacteria  
1114 were significantly different ( $P < 0.0034$ , log-rank test). The means and SEMs of three  
1115 biological replicates are shown. **(B)** In biofilms, persistence increases over time and is  
1116 significantly higher after one week compared to planktonic bacteria ( $P = 0.0002$ , log-rank  
1117 test). Planktonic culture and two-day-old biofilm show similar killing curves. Means and  
1118 SEMs of three biological replicates are shown. **(C)** Two-week-old PBFs and SBFs were  
1119 tested separately for persistence. The two different biofilm-types show no difference in

1120 their persistence levels ( $P = 0.51$ , log-rank test). Means and SEMs of three biological  
1121 replicates are shown.

1122

## 1123 SUPPLEMENTAL MATERIAL

1124 **Figure S1. (A)** Pellicle-type (PBF) and submerged (SBF) biofilms after culturing for two  
1125 weeks (left) and 12 weeks (right). **(B)** Distribution of overlapping protein identifications  
1126 within four replica samples from planktonic cell surfaces and biofilm-ECMs. PL\_log,  
1127 logarithmic planktonic cells; SBF, submerged type biofilms; PBF, pellicle type biofilms.

1128 **Figure S2. (A)** Distribution of identified proteins in terms of their predicted secretion motifs  
1129 and the number of predicted TMDs. PL\_log, logarithmic planktonic cells; PBF\_all and  
1130 SBF\_all, all identified proteins from pellicle and submerged biofilm matrices, respectively.  
1131 Other, proteins without any known motifs for classical or non-classical secretion. **(B)** Venn  
1132 diagrams indicating the core and marker proteomes within all identifications (planktonic  
1133 and biofilms) at each time point.

1134 **Figure S3. (A)** The bioluminescence-based readout of the biofilm killing assay was  
1135 validated using the  $OD_{600}$ -method to monitor bacterial growth at varying time points of  
1136 growth, showing similar killing kinetics as observed with the bioluminescence  
1137 measurements of the same samples. Here, the four-day-old biofilm and two-day-old  
1138 planktonic cells were treated with  $200 \mu\text{g mL}^{-1}$  rifampicin. The mean of three biological  
1139 replicates of the  $OD_{600}$ -based assay is shown. **(B)** Mmr growth is accompanied by  
1140 increased bioluminescence values in maturing biofilms without the antibiotic treatment,  
1141 even at timepoints of over one week of biofilm culture. The bioluminescence was  
1142 measured three times/three seconds using EnVision equipment (Perkin Elmer), and the  
1143 mean of relative light units (RLUs) per one second was calculated. Means and SEMs of  
1144 three biological replicates are shown.

1145 **Table S1.** List of all proteins identified on Mmr cells during different growth modes. The  
1146 colored cells refer to the average (log<sub>2</sub>) raw intensity values for proteins detected in at  
1147 least two replica samples. Cells in grey, protein was not detected. PL\_log, logarithmic  
1148 planktonic cells; PBF, pellicle type biofilm cells; SBF, submerged type biofilm cells.

1149 **Table S2.** Proteins specific to pellicle biofilms (PBFs), submerged biofilms (SBFs) and to  
1150 both biofilms that lack an identifiable counterpart on planktonic cell surfaces. Proteins  
1151 detected in each replica samples are shown. Color gradient bar refers to log<sub>2</sub>-transformed  
1152 raw intensity values (avr., n ≥ 2): blue, low abundance; yellow, high abundance; PBF,  
1153 pellicle biofilm; SBF, submerged biofilm.

1154 **Table S3.** Log<sub>2</sub>-transformed MaxLFQ data with minimum of two valid identifications (out of  
1155 four) in at least one group, and statistically significant protein abundance changes between  
1156 the PL and PBF\_2, PL and SBF-2d, PBF\_2w\_SBF\_2d, PBF\_12w and SBF\_12w, PBF\_4w  
1157 and 12w and SBF\_4w and 12w.

1158 **Table S4.** Statistically significant protein abundance changes within planktonic cell  
1159 surfaces and biofilm-ECMs. Colored cells in column "Cluster" correspond to those used in  
1160 the heat-map (Fig. 5). Significant changes were calculated using a multiple-sample test  
1161 (ANOVA model, FDR < 0.05, S0 = 0.1). Color intensity code bar below, blue - low  
1162 abundance; yellow - high abundance.

1163 **Table S5.** Functional enrichment analysis (GO, KEGG, InterPro, Pham) on cluster 1, 2 and  
1164 6 proteins (Figure 5) were studied using the STRING database v. 11 with both the rank-  
1165 and gene set-based approaches (FDR of 0.05).

1166 **Table S6.** Key proteome changes within the planktonic cell surfaces and biofilm matrices  
1167 at different time points of growth. Gradient bar, normalized identification intensity values  
1168 (avg. ≥ 3).



1169 **Table S7.** Colony ability of non-shaved and enzymatically shaved biofilm and planktonic  
1170 cells. Biofilm cells were cultured for two weeks and planktonic cells for two days, as  
1171 described in material and methods. Cells in three biological replicates were suspended in  
1172 trypsin/Lys-C digestion buffer and then divided into two aliquots; the first aliquot was taken  
1173 as the non-shaved cell control containing only the digestion buffer, and the second aliquot  
1174 of cells was treated with the trypsin/Lys-C enzyme. After 20 min incubation at 37 °C, the  
1175 cell suspensions were suspended gently in PBS containing 0.2% Tween80 (v/v) and  
1176 serially diluted cells were spotted in four technical replicates (10 uL each) on an agar plate.  
1177 After one week cultivation at 37 °C, the colonies were calculated and compared under  
1178 different conditions. nd, colonies could not be counted due to the presence of cell  
1179 aggregates.

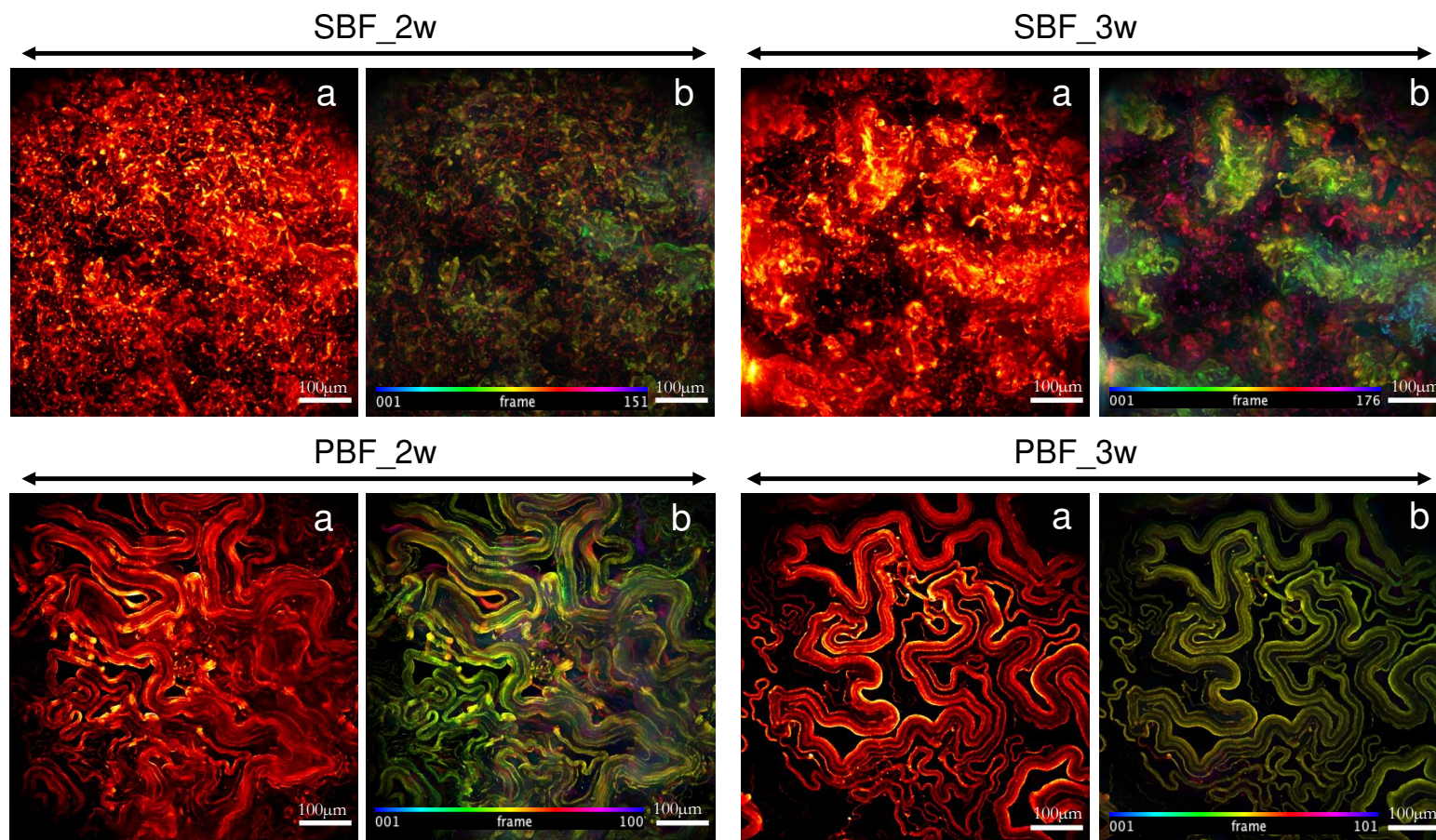
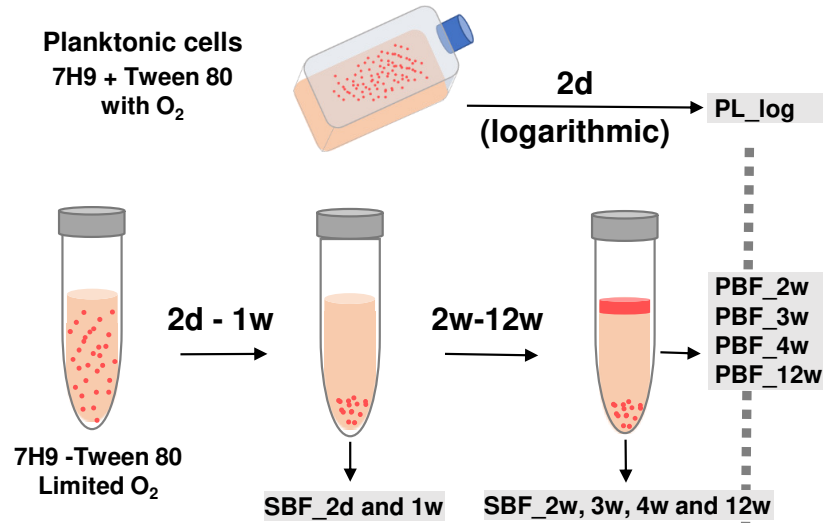


Fig. 1

Fig. 2

A



B

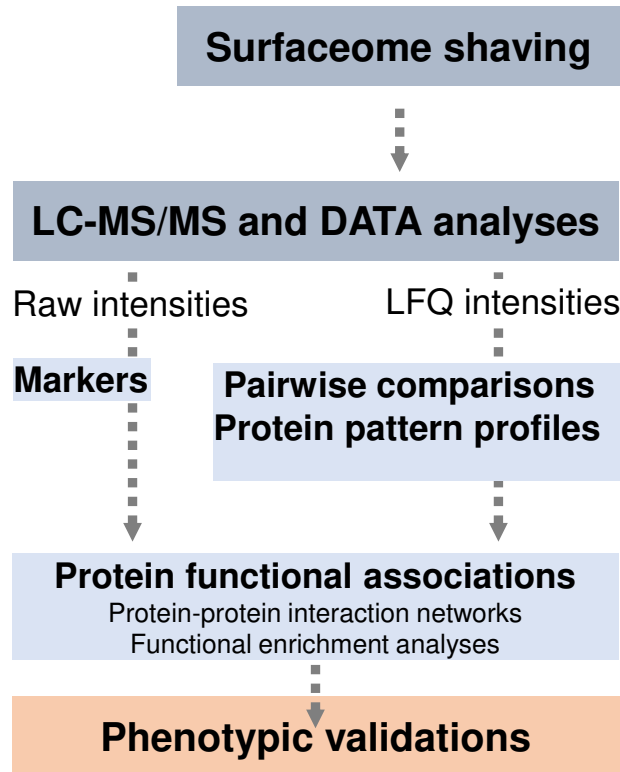


Fig. 3

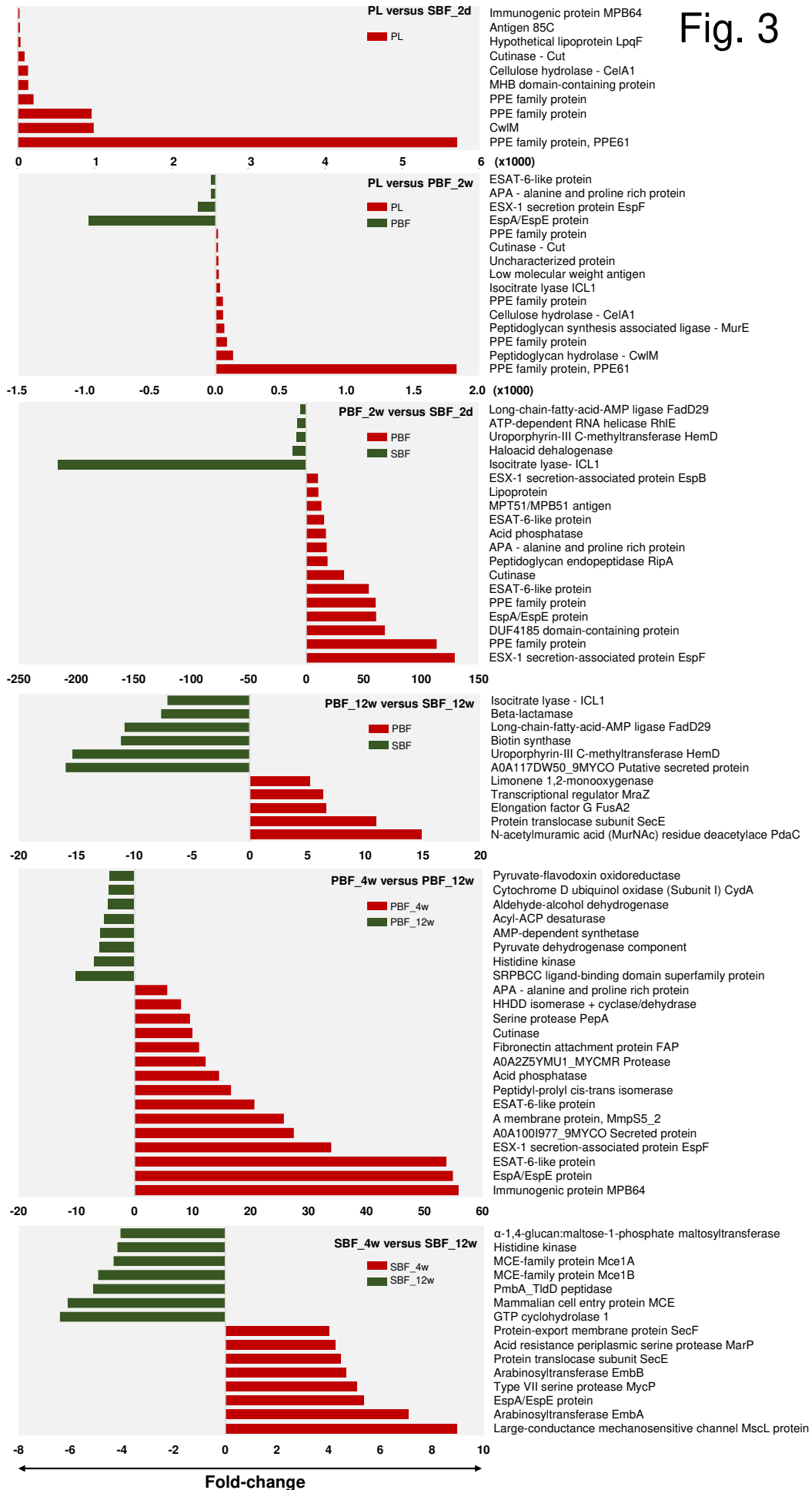
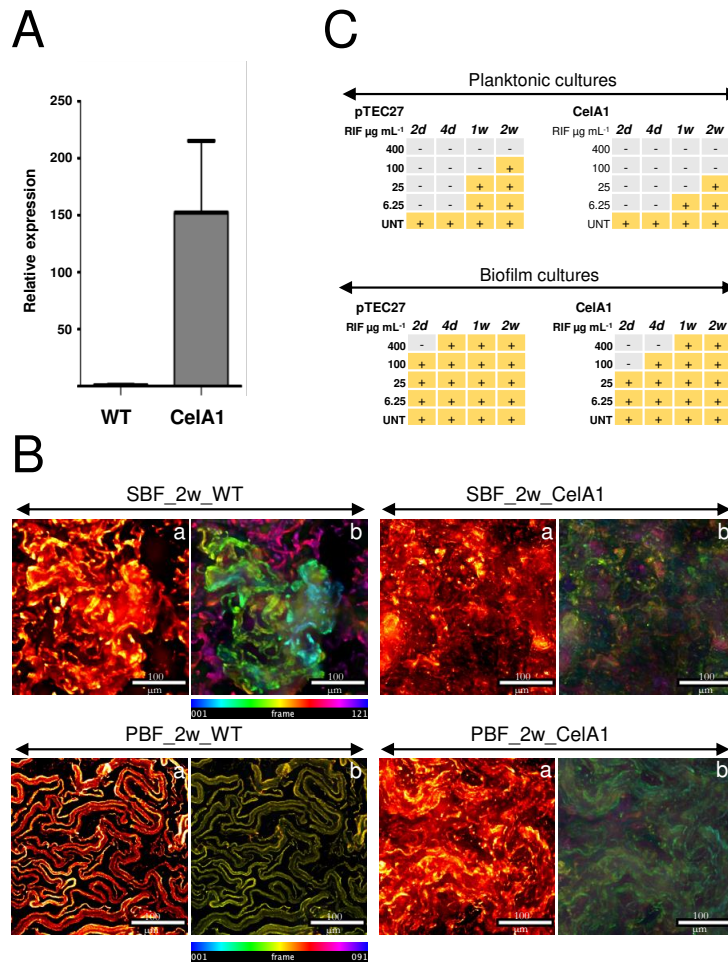


Fig. 4



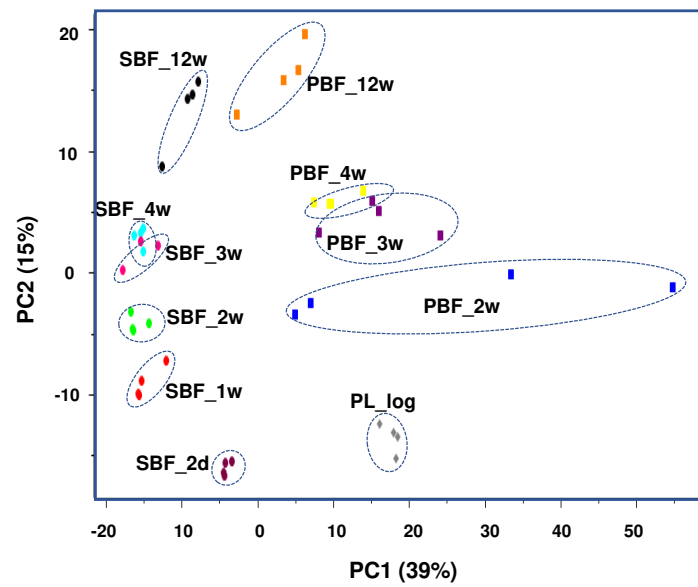
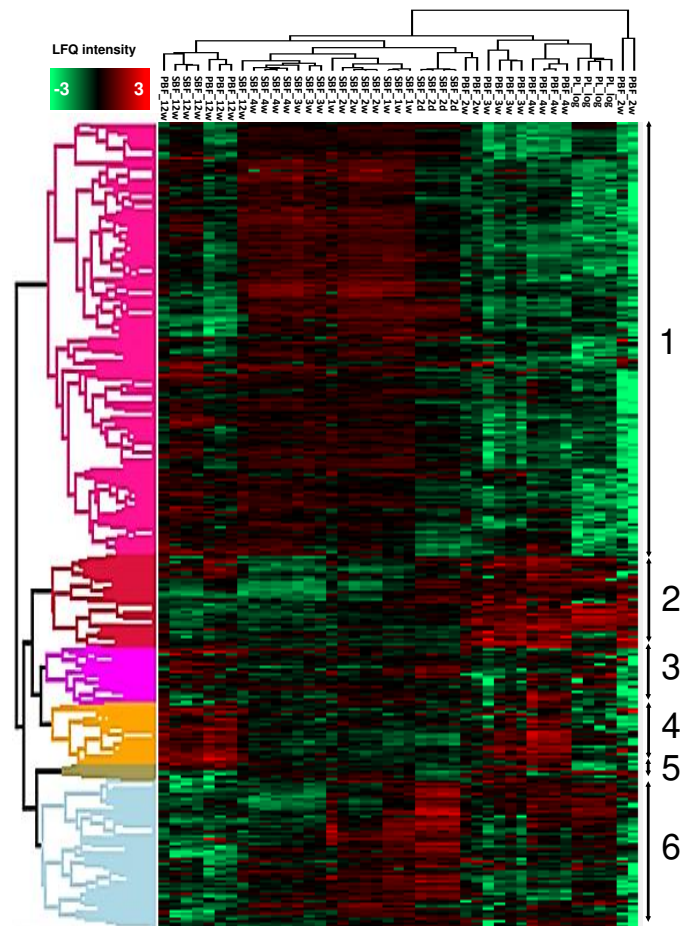
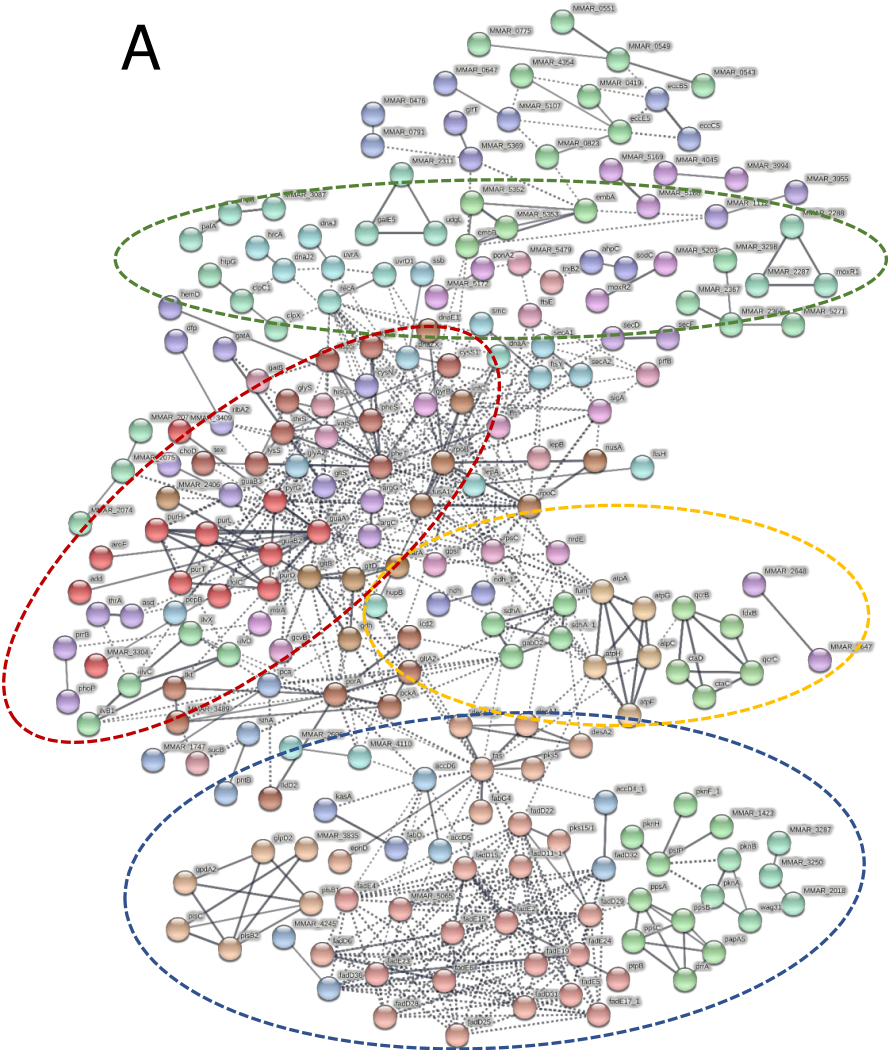
**A****B**

Fig. 5

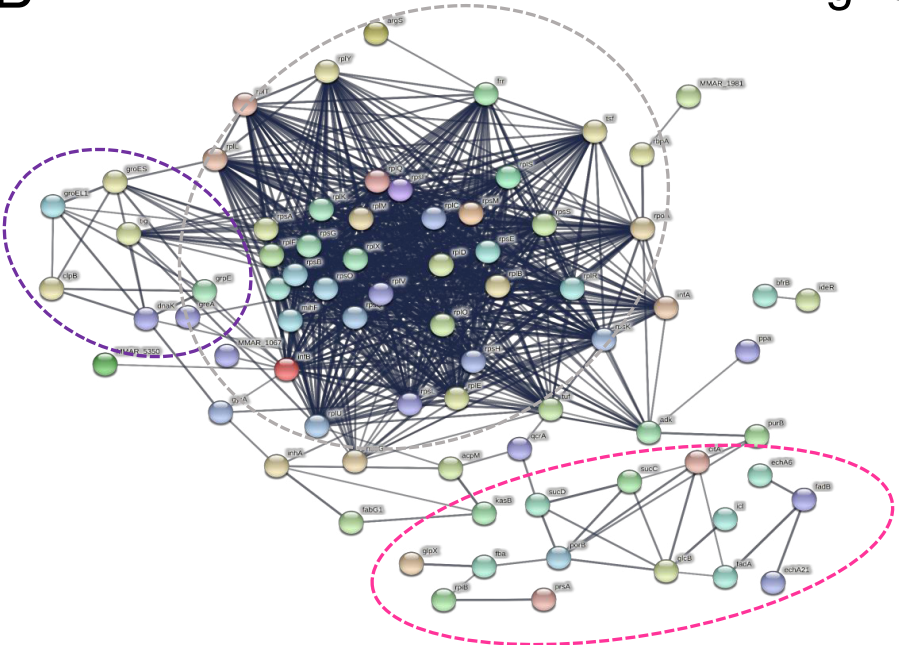


Fig. 6

A



B



- Amino acid biosynthesis
- Stress response
- Respiratory electron transfer and ATP synthesis
- Fatty acid biosynthesis and aerobic  $\beta$ -oxidative degradation fatty acids
- Translation (r-proteins)
- Glycolysis and TCA cycle



Fig. 7

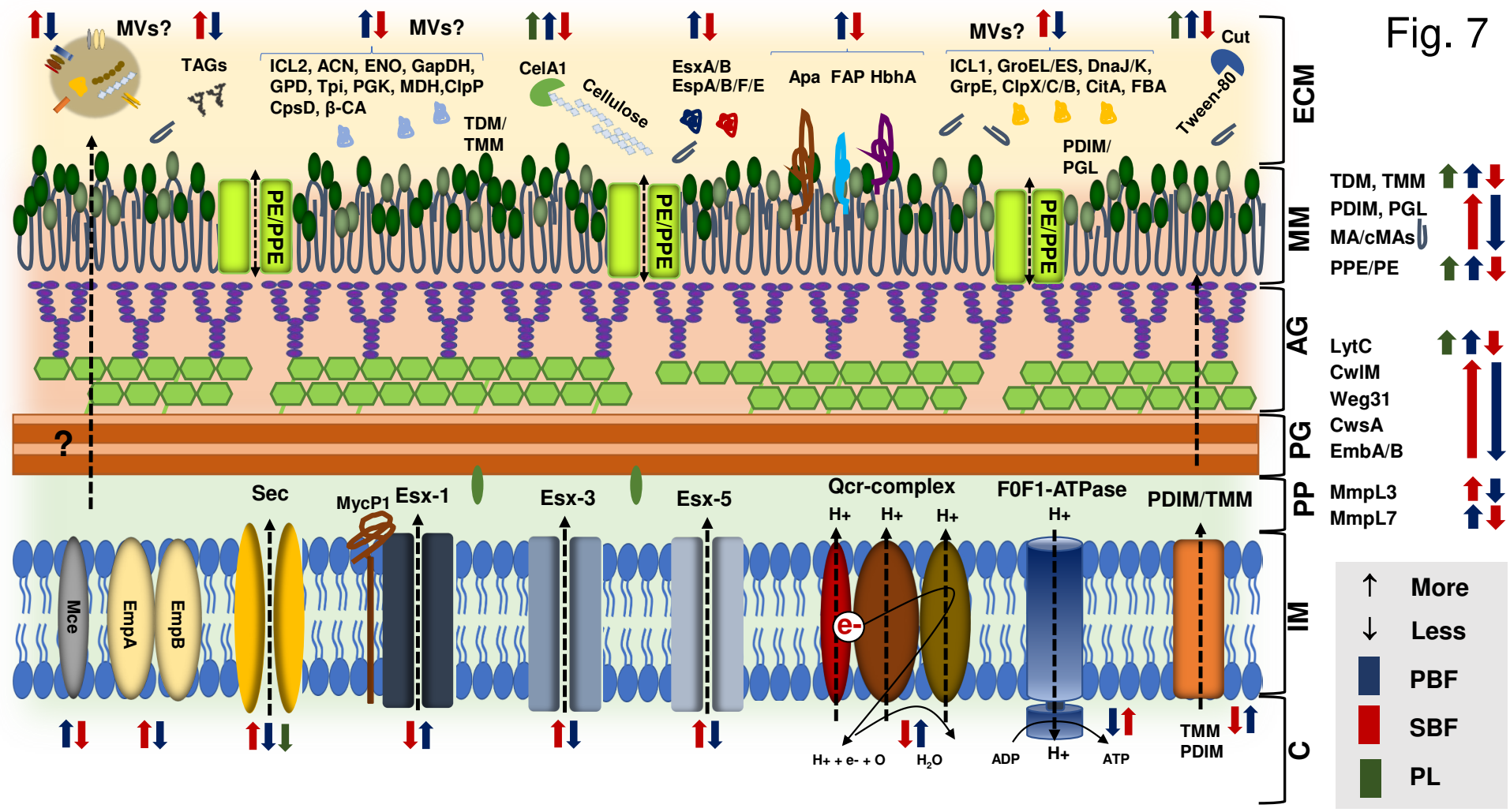
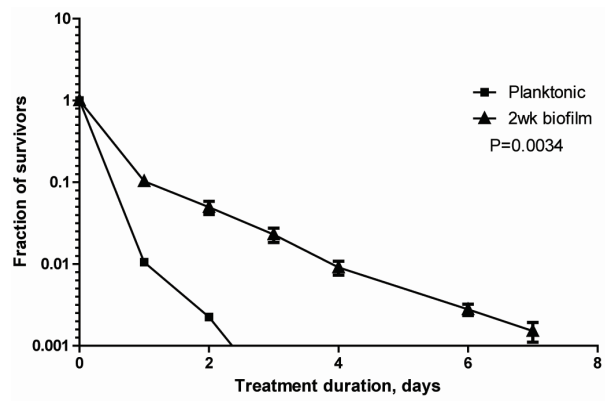
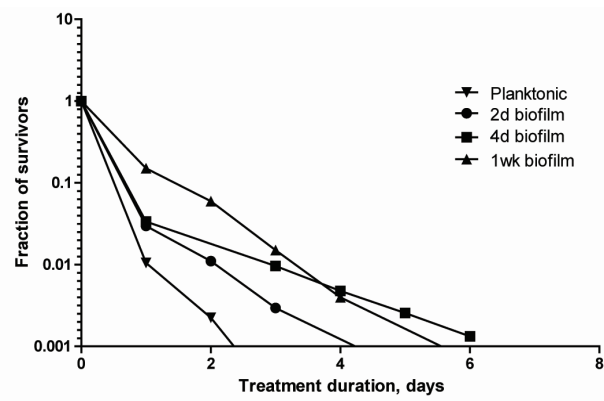


Fig. 8

A



B



C

