



**HAL**  
open science

## Supramolecular organization and dynamics of mannosylated phosphatidylinositol lipids in the mycobacterial plasma membrane

Chelsea Brown, Robin Corey, Axelle Grélard, Ya Gao, Yeol Kyo Choi,  
Emanuel Luna, Martine Gilleron, Nicolas Destainville, Jérôme Nigou, Antoine  
Loquet, et al.

### ► To cite this version:

Chelsea Brown, Robin Corey, Axelle Grélard, Ya Gao, Yeol Kyo Choi, et al.. Supramolecular organization and dynamics of mannosylated phosphatidylinositol lipids in the mycobacterial plasma membrane. *Proceedings of the National Academy of Sciences of the United States of America*, 2023, 120 (5), pp.e2212755120. 10.1073/pnas.2212755120 . hal-04037054

**HAL Id: hal-04037054**

**<https://hal.science/hal-04037054v1>**

Submitted on 23 Oct 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Supramolecular Organisation and Dynamics of**  
2 **Mannosylated Phosphatidylinositol Lipids in the Mycobacterial Plasma**  
3 **Membrane**

4 *Chelsea M. Brown*<sup>1</sup>, *Robin A. Corey*<sup>2</sup>, *Axelle Grélard*<sup>3</sup>, *Ya Gao*<sup>4,5</sup>, *Yeol Kyo Choi*<sup>5</sup>, *Emanuel Luna*<sup>5</sup>,  
5 *Martine Gilleron*<sup>6</sup>, *Nicolas Destainville*<sup>7</sup>, *Jérôme Nigou*<sup>6</sup>, *Antoine Loquet*<sup>3</sup>, *Elizabeth Fullam*<sup>1</sup>, *Wonpil*  
6 *Im*<sup>5,\*</sup>, *Phillip J. Stansfeld*<sup>1,8,\*+</sup>, *Matthieu Chavent*<sup>6,\*+</sup>

7  
8 <sup>1</sup> School of Life Sciences,  
9 University of Warwick,  
10 Coventry,  
11 CV4 7AL,  
12 UK

13  
14 <sup>2</sup> Department of Biochemistry,  
15 University of Oxford,  
16 Oxford,  
17 UK

18  
19 <sup>3</sup> Université de Bordeaux,  
20 CBMN (UMR5248),  
21 CNRS, IPB,  
22 Institut Européen de Chimie et Biologie,  
23 F-33600 Pessac,  
24 France

25  
26 <sup>4</sup> School of Mathematics, Physics and Statistics,  
27 Shanghai University of Engineering Science,  
28 Shanghai 201620,  
29 China

30  
31 <sup>5</sup> Department of Biological Sciences,  
32 Department of Chemistry,  
33 Department of Bioengineering,  
34 Lehigh University,  
35 Pennsylvania 18015,  
36 USA

37  
38 <sup>6</sup> Institut de Pharmacologie et Biologie Structurale (IPBS),  
39 Université de Toulouse, CNRS,  
40 Université Toulouse III – Paul Sabatier,

41 31400, Toulouse,

42 France

43

44 <sup>7</sup>Laboratoire de Physique Théorique,

45 Université de Toulouse, CNRS,

46 UPS, Toulouse,

47 France

48

49 <sup>8</sup>Department of Chemistry,

50 University of Warwick,

51 Coventry,

52 CV4 7AL,

53 UK

54

55 + equal contribution

56

57 \*to whom correspondence should be addressed:

58 e-mail: wonpil@lehigh.edu

59 phone: +1 (0) 610-758-4524

60

61 e-mail: phillip.stansfeld@warwick.ac.uk

62 phone: +44 (0) 2476523864

63

64 e-mail: matthieu.chavent@ipbs.fr

65 phone: +33 (0) 5-61-17-59-00

66

67 **Abstract**

68 *Mycobacterium tuberculosis* (*Mtb*) is the causative agent of tuberculosis (TB), a  
69 disease that claims ~1.6 million lives annually. The current treatment regime is long  
70 and expensive, and missed doses contribute to drug resistance. Therefore,  
71 development of new anti-TB drugs remains one of the highest public health priorities.  
72 *Mtb* has evolved a complex cell envelope that represents a formidable barrier to  
73 antibiotics. The *Mtb* cell envelop consists of four distinct layers enriched for *Mtb*  
74 specific lipids and glycans. Although the outer membrane, comprised of mycolic acid  
75 esters, has been extensively studied, less is known about the plasma membrane,  
76 which also plays a critical role in impacting antibiotic efficacy. The *Mtb* plasma  
77 membrane has a unique lipid composition, with mannosylated phosphatidylinositol  
78 lipids (phosphatidyl-*myo*inositol mannosides, PIMs) comprising more than 50% of the  
79 lipids. However, the role of PIMs in the structure and function of the membrane  
80 remains elusive. Here, we used multiscale molecular dynamics (MD) simulations to  
81 understand the structure-function relationship of the PIM lipid family and decipher how  
82 they self-organize to shape the biophysical properties of mycobacterial plasma  
83 membranes. We assess both symmetric and asymmetric assemblies of the *Mtb*  
84 plasma membrane, and compare this with residue distributions of *Mtb* integral  
85 membrane protein structures. To further validate the model, we tested known anti-TB  
86 drugs and demonstrated that our models agree with experimental results. Thus, our  
87 work sheds new light on the organization of the mycobacterial plasma membrane. This  
88 paves the way for future studies on antibiotic development and understanding *Mtb*  
89 membrane protein function.

90

91 **Significance statement**

92 Understanding the biophysical properties of mycobacterial membranes is of crucial  
93 interest, especially in the context of drug discovery, but there is no realistic model of  
94 mycobacterial membranes. In this work, we have developed models of inner  
95 membrane of mycobacteria mainly constituted of mannosylated phosphatidylinositol  
96 lipids. Our results provide significant and unexpected insights into the molecular  
97 details of mycobacterial membranes. Especially, they highlight how recently  
98 determined proteins can accommodate into this specific membrane and how its lipid  
99 composition can affect antibiotics diffusion.

100

## 101 **Introduction**

102 Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (*Mtb*). In 2021 alone,  
103 there were an estimated 10.6 million new *Mtb* infections, leading to 1.6 million deaths  
104 (1). Thus, *Mtb* is one of the world's leading infectious killers, despite the availability of  
105 both a treatment regime and vaccine. The current course of antibiotics for drug  
106 susceptible TB cases can last as long as 6 months and consists of four drugs given in  
107 combination (2). This is not only expensive and demanding for the patient, but also  
108 encourages non-compliance that contributes towards the rise in multi-drug resistant  
109 and extremely drug-resistant TB (3). It is obvious that new treatments and a better  
110 vaccine are needed to meet the World Health Organization's 'End TB Strategy'. Their  
111 plan aims to reduce TB-related deaths by 90% by 2030, and thereby curtail the  
112 enormous public health cost caused by TB (4). The COVID-19 pandemic has undone  
113 some of the progress that had been made in the treatment of TB as fewer people were  
114 able to be diagnosed or access medication (5), which might result in a worldwide surge  
115 of untreatable cases. Thus, there is a pressing need for innovative research into the  
116 mechanisms of *Mtb* virulence and its ability to survive within the host for extended  
117 periods to help develop alternative intervention strategies.

118 One issue in anti-TB drug discovery is the complexity of the mycobacterial cell  
119 envelope (6, 7). This cell envelope consists of an array of lipids contributing to both  
120 hydrophobic and polar regions of various thicknesses and densities, making it  
121 extremely challenging to predict how molecules will cross this barrier and enter the  
122 cell. The *Mtb* cell envelope has four distinct layers: the outer layer (or the  
123 *mycomembrane*) comprised of mycolic acid esters and other complex lipids (such as  
124 phthiocerol dimycoserates), an arabinogalactan-peptidoglycan layer, the  
125 periplasmic space and the inner membrane, or the plasma membrane (8). The  
126 mycobacterial plasma membrane plays a key role in controlling nutrient/antibiotic  
127 uptake and contains important membrane proteins that are targets for antitubercular  
128 drugs (9), such as SQ109 that inhibits the transporter MmpL3 (10). The membrane  
129 composition is also known to change during different growth stages of the bacteria (7)  
130 and may modulate drug and membrane protein diffusion. Additionally, recent studies  
131 showed that *Mtb* plasma membrane organization, such as the formation of functional  
132 membrane microdomains (11, 12), can affect the survival ability of mycobacteria.  
133 Therefore, understanding the molecular organization and dynamics of the *Mtb* plasma  
134 membrane is essential for developing effective drug candidates.

135 The mycobacterial plasma membrane is composed of a variety of lipids and  
136 glycolipids, the most abundant being (**Figure 1A**): cardiolipin (CL),  
137 phosphatidylethanolamine (PE), phosphatidyl-*myo*inositol (PI), trehalose  
138 monomycolate (TMM) and phosphatidyl-*myo*inositol mannosides (PIMs) (13), with the  
139 PIMs accounting for over half the dry weight of the plasma membrane lipids (13). PIM<sub>2</sub>  
140 lipids are comprised of a modified PI core decorated with two mannose residues and  
141 one acyl chain (**Figure 1B**). Furthermore, additional modifications of an acyl group  
142 and up to 4 mannose sugars can be added to the core headgroup, further diversifying  
143 the PIM structure (14, 15) (**Figure 1B** and **SI Figure 1**). It is still unclear if the plasma  
144 membrane is symmetric or asymmetric (16). In one widely cited and accepted study,  
145 it is proposed to be asymmetric (13), with Ac<sub>2</sub>PIM<sub>2</sub> being the dominant species in the  
146 cytoplasmic leaflet accompanied by AcPIM<sub>2</sub>, while the periplasmic leaflet is more  
147 varied, containing AcPIM<sub>2</sub>, AcPIM<sub>6</sub>, Ac<sub>2</sub>PIM<sub>6</sub>, CL, PI, PE and TMM (6, 13, 17, 18).  
148 PIM<sub>6</sub> lipids can be further modified to LM and LAM that make up the bulk of the  
149 periplasmic space (19). Additionally, lipids have been shown to modulate membrane  
150 protein function (20, 21). However, due to the complexity of the mycobacterial cell  
151 envelope, the dynamics and properties of the plasma membrane is extremely difficult  
152 to probe experimentally.

153 Over the last decade, molecular dynamics (MD) simulations have emerged as  
154 a powerful strategy for studying structural and functional aspects of biomembranes  
155 (22, 23). However, at the time of writing, there are no complete models of the  
156 mycobacterial plasma membrane that capture a possible asymmetry and the diverse  
157 range of lipids (24). This limits our ability to understand *Mtb* plasma membrane biology,  
158 model drug-lipid interactions - important for anti-TB drug discovery efforts - and  
159 simulate mycobacterial membrane proteins in a native environment. Therefore, it is  
160 essential to develop models of the major phospholipids of the mycobacterial plasma  
161 membrane for application in MD simulations.

162 Herein, multiscale simulations were used to analyze the structure-function  
163 relationship of the four main PIM lipids (AcPIM<sub>2</sub>, Ac<sub>2</sub>PIM<sub>2</sub>, AcPIM<sub>6</sub> and Ac<sub>2</sub>PIM<sub>6</sub>) found  
164 in the mycobacterial membrane. An asymmetric bilayer containing these lipids was  
165 assembled and simulated, showing the stability of this composition. To highlight the  
166 robustness of our approach, additional simulations representing (in total) two different  
167 asymmetric growth stages as well as symmetric membrane configurations were  
168 performed. Systematic analysis of the distribution of *Mtb* protein residues in contact

169 with lipids reveals further indications that this membrane could possibly be  
170 asymmetric. We have also modelled how antibiotics, used to treat TB infections,  
171 diffuse through the membrane and interact with membrane proteins and demonstrated  
172 that our models agree with experimental results (25). Overall, we have developed  
173 robust possible representations of the mycobacterial plasma membrane that will  
174 enable studies of membrane dynamics, lipid interactions with integral membrane  
175 proteins, and diffusion of antibiotics across this barrier.

176

177

## 178 **Methods**

179

### 180 *Building the coarse-grained lipid parameters*

181 The CG models of the lipids were parametrized for the newly released MARTINI 3  
182 force field (26) and generated using the protocol described for small molecules (27)  
183 on the MARTINI website ([http://cgmartini.nl/index.php/martini-3-](http://cgmartini.nl/index.php/martini-3-tutorials/parameterizing-a-new-small-molecule)  
184 [tutorials/parameterizing-a-new-small-molecule](http://cgmartini.nl/index.php/martini-3-tutorials/parameterizing-a-new-small-molecule)). The bead types and mapping to the  
185 PIM molecules were performed manually, comparing with the recently published CG  
186 model of PI (28). Atoms were grouped according to functional groups, in sets of 3-5  
187 non-hydrogen atoms. The CG mapping of  $Ac_xPIM_x$  is shown in **Figures 1B,C**. The  
188 parameter files that describe the bonds, constraints, and angles were assembled  
189 based on the previously described data (26, 28, 29) as an initial estimate.

190 Simulations of each lipid were set up using a modified version of *insane.py*  
191 python script (30), embedding one copy of a PIM lipid in a 10 x 10 nm<sup>2</sup>  
192 phosphatidylcholine (PC) bilayer. The system was solvated with MARTINI water (26)  
193 and neutralized with 150 mM NaCl, followed by minimization using the steepest  
194 descents algorithm. The system was then simulated for 3  $\mu$ s using a timestep of 20 fs  
195 at 310 K. The lipids, solvent and ions were temperature coupled separately. The  
196 velocity rescale (31) and Parrinello-Rahman (32) coupling methods were used with  
197 the time constants  $\tau_T = 1.0$  ps and  $\tau_p = 12.0$  ps for temperature and pressure,  
198 respectively. Simulations were run using GROMACS version 2021.3 (33). The  
199 reaction-field algorithm (34) was used for electrostatics interactions with a cut-off of  
200 1.1 nm. A single cut-off of 1.1 nm was used for the van der Waals interaction. Five  
201 repeats were performed for each lipid (**SI Figure 2**).

202

### 203 *Generating atomistic data*

204 Parameters for the atomistic (AT) Ac<sub>x</sub>PIM<sub>x</sub> lipids were generated using the CHARMM  
205 force field (35). The CHARMM-GUI (36) server was used to set up the lipid systems  
206 for GROMACS with the CHARMM36m force field (37, 38). One PIM lipid was  
207 embedded in an 8 x 8 nm<sup>2</sup> PC bilayer. The system was solvated with 150 mM NaCl  
208 and minimized and equilibrated as per the CHARMM-GUI Membrane Builder workflow  
209 (39). The system was further minimized using the steepest descents. Simulations  
210 were run for 2 μs using a timestep of 2 fs at 310 K. The lipids, solvent and ions were  
211 temperature coupled separately. The velocity rescale (31) and C-rescale coupling  
212 methods were used with the time constants  $\tau_T = 0.1$  ps and  $\tau_p = 1.0$  ps for temperature  
213 and pressure, respectively. Simulations were run using GROMACS version 2021.3  
214 (33). The particle mesh Ewald (PME) (40) method was used for electrostatic  
215 interactions with a cut-off of 1.2 nm. A single cut-off of 1.2 nm was used for the van  
216 der Waals interaction. Three repeats were performed for each lipid (**SI Figure 2**).

217

### 218 *Refining coarse-grained parameters*

219 The AT and CG representations of the four main PIM lipids are shown in **Figure 1C**.  
220 The distributions of the distances and angles were measured using the *gmx* tools  
221 (*distance*, *gangle* and *analyze*). For the AT simulations, the atoms were grouped  
222 according to their mapping and its center of geometry was used for calculations. The  
223 values for each bond/constraint and angle were iteratively refined based on the  
224 comparison of probability distribution, and the results are summarized in **SI Figures**  
225 **3-8**. When there was agreement between the AT and CG data, the solvent accessible  
226 surface area was measured using the *gmx sasa* tool to verify that the models behaved  
227 the same (**SI Figure 8C**). Diffusion, shape of the lipids, clustering of ions, aggregation  
228 and interaction with other lipids were also measured on a single lipid level (**SI Methods**  
229 and **SI Figures 9-19**). As typical for MARTINI (41), dihedral terms are not defined in  
230 the parameters.

231

### 232 *Measuring area per lipid*

233 Before setting up the complete bilayer system, the area per lipid was measured. The  
234 simulations were assembled as described above for CG, using a homogenous  
235 Ac<sub>x</sub>PIM<sub>x</sub> membrane (**SI Figure 2**). The area of XY-dimension was measured using  
236 *gmx energy* and then dividing the area by the number of lipids in one leaflet over the



237 course of the trajectory, and the final values were extracted using *gmx analyze*, as per  
238 the protocol described on the MARTINI website ([http://cgmartini.nl/index.php/tutorials-](http://cgmartini.nl/index.php/tutorials-general-introduction-gmx5/bilayers-gmx5#Area-per-lipid)  
239 [general-introduction-gmx5/bilayers-gmx5#Area-per-lipid](http://cgmartini.nl/index.php/tutorials-general-introduction-gmx5/bilayers-gmx5#Area-per-lipid)).

240

#### 241 *Bilayer composition and set-up*

242 The ratio of lipids in each bilayer was obtained by using their molecular weight and the  
243 previously reported dry masses (13) of the individual lipids, and the exact calculations  
244 can be seen in **SI Figure 20**. Since specific apolar lipids were not named, they were  
245 not included in this study. TMM was not included as there is no available refined AT  
246 model, the behavior of the mycolic acid is predicted to be complicated (42), and there  
247 is a relatively small proportion of TMM predicted to be present in the plasma  
248 membrane. The final composition of the plasma membrane is shown in **Figure 1A**.

249 The PI and PE lipids in mycobacteria are slightly different in structure to the  
250 corresponding *E. coli* lipids described in the MARTINI force field (26, 28): a methyl  
251 group replaces the alkene found in one of the acyl chains (**SI Figure 1**). Before  
252 assembling the membrane, the parameters for these lipids were modified by adapting  
253 the refined Ac<sub>2</sub>PIM<sub>2</sub> acyl tail parameters and changing the existing tail. The CL  
254 parameters were transferred over from the MARTINI 3 beta force field (21).

255 Simulations of the bilayer were set up using a modified version of *insane.py*  
256 python script (30) using the composition shown in **Figure 1A**, where the area per lipid  
257 for the periplasmic membrane was set to 0.92 nm<sup>2</sup> and the cytoplasmic leaflet set to  
258 1.13 nm<sup>2</sup>. The systems were then treated the same as described for the initial CG  
259 systems with added equilibration steps as per the Membrane Builder workflow (39)  
260 before the production simulation. There were two types of system assembled to  
261 represent the asymmetric membrane previously mentioned (13). The first one had an  
262 initial simulation box size of 20 x 20 x 15 nm<sup>3</sup> and one repeat was performed for 10 μs  
263 at 290 K, 300 K, 310 K, 320 K and 350 K (**Figure 2A**). The second one had an initial  
264 box size of 50 x 50 x 15 nm<sup>3</sup> and a single repeat was performed for 10 μs at 310 K  
265 (**Figure 2C** and **SI Figure 2**). The number of lipids in each system is summarized in  
266 **SI Figure 21**.

267 To test how the membrane properties change with differing compositions, the  
268 same assembly procedure was followed to set up a symmetric membrane and an  
269 'PIM-enriched' membrane (**SI Figure 22**), with one repeat for each in a simulation box  
270 of 20 x 20 x 15 nm<sup>3</sup>, and another in a box of 50 x 50 x 15 nm<sup>3</sup>, both at 310 K (**SI Figure**

271 **2).** Another membrane to capture an experimentally extracted membrane was  
272 simulated for 10  $\mu\text{s}$  in a  $20 \times 20 \times 20 \text{ nm}^3$  simulation box at 310 K. The number of lipids  
273 in each system is summarized in **SI Figure 21** and their compositions in **SI Figure 22**

274

### 275 *Behavior of the membrane*

276 To calculate the bending rigidities, the strategy proposed by Fowler *et al.* (43) to  
277 extract the bending modulus from CG simulations was followed. The membrane  
278 midplane position in Monge representation was determined by extracting the  
279 coordinates of all CG beads at the extremities of lipid fatty acid chains. Using a built-  
280 in function of the Mathematica software package, the positions were interpolated to  
281 get a smooth function before Fourier-transforming with a fast Fourier transform  
282 algorithm. From the so-obtained Fourier modes, the spectral density (or power  
283 spectrum) was estimated for a tensionless membrane:

$$284 \quad S(q) = L^2 k_B T \left( 1/(\kappa q^4) + 1/(\sigma_p r q^2) \right)$$

285 where  $L^2$  is the projected area in the  $(x, y)$  plane,  $k_B T$  is the thermal energy and  
286  $\sigma_p r \sim 0.1 \text{ J/m}^2$  is the tension associated with lipid protrusions at the nanometer scale.  
287  $S(q)q^4$  was plotted as a function of  $q$  and fit it with a second order polynomial  $P(q) =$   
288  $a + bq^2$ , from which estimates of  $\kappa$  (and  $\sigma_p r$  if needed) were obtained (**SI Figure 23**).

289 The error bars are standard deviations provided by the fitting function in Mathematica.

290 The density of each constituent was measured using *gmx density* (**Figure 2B**).  
291 Diffusion, bilayer thickness and number of neighbors was measured using LiPyphilic  
292 (44) (**Figures 2D,E, SI Methods** and **SI Figures 24-27**). The xy-positions of single  
293 lipids were tracked with PLUMED (45) (**Figure 2E**). Area per lipid for the membrane  
294 was calculated using FATSLIM (46) (**Figure 2F**). Plots were created using Matplotlib  
295 (47).

296

### 297 *Analysis of the residue distribution in membrane proteins*

298 All predicted protein structures from *Mtb* (taxonomy id:83332) and *E. coli* (taxonomy  
299 id:83333) labelled as transmembrane on UniProt (48) were downloaded from the  
300 AlphaFold database (49), totaling 729 for *Mtb* and 1,229 for *E. coli*. Alphafold was used  
301 to obtain a workable dataset of structures, expanding upon the number of  
302 experimentally determined *Mtb* and *E. coli* membrane protein structures available from  
303 the PDB. Each of 1,958 membrane protein structures was orientated in a membrane

304 using memembed (50) with the *-n in* flag, then converted to CG using martinize2 (51)  
305 and inserted into either the asymmetrical mycobacterial (see **Figure 1A**) or *E. coli*  
306 membrane (75% PE, 15% PG, 10% CL) using a modified version of the *insane.py*  
307 python script (30). Each system was simulated for 10 ns. The residues within 8 Å of  
308 the lipids were selected and the density over the z-coordinate plotted (**Figure 3, SI**  
309 **Figure 28**).

### 311 *Antibiotic simulations*

312 Bedaquiline (BDQ) and isoniazid (ISZ) were mapped to CG using PyCGTOOL (52)  
313 following 200 ns AT simulations with parameters from CHARMM-GUI (53) (**Figure**  
314 **4A**). The pK<sub>a</sub> of the amine in BDQ is 8.91 (54), suggesting it will be protonated at  
315 physiological pH. This was considered when assigning the MARTINI 3 bead type  
316 (**Figure 4A**). To validate the use of the protonated state, the same simulations with a  
317 neutral BDQ molecule were performed and analyzed (**SI Figures 29A-C**). This was  
318 done by replacing the Q1 particle with a SC3 bead type (**Figure 4A**). In addition, the  
319 LogP for these antibiotics were calculated by perturbing the antibiotic in a box  
320 containing 878 MARTINI waters or 363 octanol and 32 waters. The Lennard-Jones  
321 parameters for each molecule were decoupled over 11 evenly spaced  $\lambda$  windows, for  
322 50 ns per window. Simulations were run with 20 fs time steps in the NPT ensemble  
323 with the V-rescale thermostat at 323 K and an isotropic Parrinello-Rahman pressure  
324 coupling. Free energies were then computed using *gmx bar* and converted to  
325 water/octanol partitioning constants. The values were then compared to data from the  
326 ALOGPS online server (55). Alongside this, *gmx sasa* was used to calculate the  
327 solvent accessible surface area for the antibiotics in both CG and AT. The calculations  
328 were performed with 4800 dots per sphere and a probe size of 0.4 nm. Modified van  
329 der Waals radii for CG beads of 0.264, 0.230, and 0.191 nm for R, S and T beads  
330 were used respectively. For the Connolly surface generation, the calculations used  
331 240 dots and a probe of 0.4 nm (**SI Figure 30**).

332 BDQ was first simulated with the mycobacterial membrane alone to confirm  
333 association with the membrane (**SI Figures 2,31**). The *Mtb* a- and c-subunits of F-  
334 ATPase, BDQ's target in the mycobacterial membrane, was modelled using  
335 SwissModel (56) based on a structure from *Mycobacterium smegmatis* (25) (PDB:  
336 7JGC, **Figure 4C** and **SI Figures 32A,B**). The sequence identity between *Mtb* and *M.*  
337 *smegmatis* for the F-ATPase was calculated using Clustal Omega (57) at 80% and

338 72% for c- and a-subunits, respectively. The system with the protein in the asymmetric  
339 mycobacterial membrane (see **Figure 1A**) was assembled using martinize2 (51),  
340 memembed (50) and the modified *insane.py* python script (30), with eight molecules  
341 of BDQ placed either in the periplasmic or cytoplasmic leaflet with five repeats in each  
342 membrane (**SI Figure 2**). Simulations were run for 10  $\mu$ s using the same setting as  
343 described above. The xyz-positions of BDQ, the lipids and the backbone beads were  
344 tracked over the course of the simulations with PLUMED (45) and plotted with  
345 Matplotlib (47), and the results are shown in **Figure 4D** and **SI Figures 32C,D, 33**.  
346 The density of each constituent was measured using *gmx density* and plotted using  
347 Matplotlib (47) and the results are shown in **Figure 4E** (with representative positions  
348 shown in **Figure 4F**). The interaction of BDQ with the protein was calculated using  
349 PyLipID (58) (**Figure 4G** and **SI Figures 34,35**). The same simulation set up was  
350 performed with a model *E. coli* membrane (75% PE, 15% PG, 10% CL) and with the  
351 symmetric mycobacterial membrane (**SI Figure 22A**). The z-position of BDQ is shown  
352 in **SI Figure 32** and **SI Figures 36,37**.

353

#### 354 *PMF calculations*

355 The potential of mean force (PMF) calculations for the F-ATPase-BDQ interaction  
356 were performed (59). First, a representative pose of BDQ bound to the protein was  
357 produced using PyLipID from equilibrium simulations and built into a 12 x 20 x 11 nm<sup>3</sup>  
358 asymmetric mycobacterial membrane (see **Figure 1A**) membrane and minimized and  
359 equilibrated, as described above. Light (50 kJ/mol/nm<sup>2</sup>) xy positional restraints were  
360 added to Ala 66 on three c-subunits to prevent the protein from rotating in the  
361 membrane. Following 50 ns of equilibration, the BDQ was steered away from the  
362 protein along the y axis at a rate of 1 nm/ns with a 1,000 kJ/mol/nm<sup>2</sup> restraint potential.  
363 Frames were extracted at 0.1 nm spacing along this coordinate to seed a total of 58 x  
364 1.5  $\mu$ s production simulations with a static 1,000 kJ/mol/nm<sup>2</sup> umbrella potential  
365 imposed to keep the system in the same position along the reaction coordinate. The  
366 PMF mdp input files can be downloaded at <https://github.com/chelsea-brown/PIM-lipids/tree/main/PMF-files>. The PMF profiles were then constructed using the weighted  
367 histogram analysis method in GROMACS (*gmx wham* (60, 61)), and employing 200  
368 rounds of Bayesian bootstrapping to report statistical accuracy (**Figure 4H**).

370 For the membrane crossing PMFs, BDQ or ISZ was placed free in the solvent  
371 phase, 7 nm away from the membrane periphery in either an asymmetric

372 mycobacterial membrane (see **Figure 1A**) or *E. coli* membrane. The drug was then  
373 steered towards and through the membrane and into the solvent phase on the other  
374 side. Windows were extracted, simulated (1  $\mu$ s for ISZ per window and 2  $\mu$ s per  
375 window for BDQ) and analyzed as described above (**Figure 4B** and **SI Figure 29D** for  
376 BDQ neutral). The -cycl option was imposed when running *gmx wham*.

377

## 378 **Results**

379

### 380 *PIM properties*

381 As an initial step towards developing an *in-silico* model of the mycobacterial plasma  
382 membrane, we first examined lipid properties in our CG and AT models. As shown in  
383 **SI Figures 3-8**, the probability distributions of bonds and angles from the CG and AT  
384 simulations align well, which illustrates that the behavior of the acyl chains is similar  
385 to other phospholipids (30). The Ac<sub>x</sub>PIM<sub>x</sub> aggregation in the AT and CG simulations is  
386 comparable and shows no permanent clustering. When lipids did come into contact,  
387 all areas of the molecule appear to play an equal role in the interactions (**SI Figures**  
388 **15,17**). The interactions were not dominated by the sugars, as was seen with the  
389 previous iteration of the MARTINI 2 force field (62), and the phospholipids present in  
390 the mycobacterial membrane did not have strong interactions with the PIM lipids (**SI**  
391 **Figure 18,19**). The PIM lipids show a higher affinity for ions than PC in CG simulations,  
392 but the effect is less apparent in AT simulations (**SI Figure 14**). While the ion  
393 concentrations were the same, the number of ions in the simulation box were different  
394 (an order of magnitude higher for CG), which could explain these results. Overall, CG  
395 simulations of these lipids behave similarly to AT models (interactions with ions as a  
396 minor exception), therefore opening the door to significantly longer simulations by  
397 decreasing the degrees of freedom in the system.

398 The area per lipid in CG for each species in a PC bilayer was found to be 0.93  
399 nm<sup>2</sup> (AcPIM<sub>2</sub>), 1.15 nm<sup>2</sup> (Ac<sub>2</sub>PIM<sub>2</sub>), 1.01 nm<sup>2</sup> (AcPIM<sub>6</sub>), 1.14 nm<sup>2</sup> (Ac<sub>2</sub>PIM<sub>6</sub>), 0.6 nm<sup>2</sup>  
400 (PI), 0.55 nm<sup>2</sup> (PE) and 1.25 nm<sup>2</sup> (CL). The diffusion coefficients for each PIM species  
401 in a PC bilayer at 310 K are as follows: 6.7 x 10<sup>-7</sup> cm<sup>2</sup>/s (AcPIM<sub>2</sub>), 1.1 x 10<sup>-7</sup> cm<sup>2</sup>/s  
402 (Ac<sub>2</sub>PIM<sub>2</sub>), 7.6 x 10<sup>-7</sup> cm<sup>2</sup>/s (AcPIM<sub>6</sub>), 6.5 x 10<sup>-7</sup> cm<sup>2</sup>/s (Ac<sub>2</sub>PIM<sub>6</sub>) (**SI Figure 9**). The  
403 difference between AcPIM<sub>x</sub> and Ac<sub>2</sub>PIM<sub>x</sub> shows the effect of the extra acyl tail in terms  
404 of how freely these lipids diffuse through the membrane.

405 The lipid shapes are approximately the same from AT to CG (**SI Figures 10-**  
406 **13**), which agrees with the comparison of the surface areas (**SI Figure 8C**) and the  
407 sugar-phosphate z-distances in Ac<sub>x</sub>PIM<sub>6</sub> (**SI Figures 5C,7C**). The additional mannose  
408 moieties project upwards away from the membrane in both sets of simulations.  
409 Interestingly, the lipid tail region is measured to occupy approximately the same  
410 amount of space with three or four acyl chains. This is likely due to the placement of  
411 the fourth tail, projecting downwards from the inositol sugar (as highlighted in red in  
412 **Figure 1B**) and hence inhabiting space close to the other acyl chains.

413 Taken together, the lipid properties in our CG model broadly replicate those  
414 seen with AT resolution. This provided the confidence to assemble these lipids into a  
415 complex bilayer.

416

#### 417 *Mycobacterial membrane biophysical properties*

418 Traditional MD simulations of bacterial membranes use symmetric bilayer  
419 representation. However, a study has shown that the mycobacterial plasma  
420 membrane could be an asymmetric bilayer, and we used the published composition  
421 of the mycobacterial plasma membrane (13) (**Figure 1A**) (excluding apolar lipids and  
422 TMM) to assemble an asymmetric bilayer (**Figures 2A,C**). This bilayer was found to  
423 be stable during 10 μs simulation (**SI Movie 1**), and in good agreement with the  
424 literature (8, 13). The thickness of the plasma membrane in our simulations was at 5.3  
425 ± 0.1 nm (**Figure 2B**), which is slightly narrower than values obtained from imaging  
426 studies (6.3 nm for *Mycobacterium bovis* and 7 nm for *Mycobacterium smegmatis*)  
427 (63-65). However, we did observe thicknesses of up to 7 nm in the density plot (**Figure**  
428 **2B**). *In vitro* culture conditions or growth stages of the bacteria (65) impact the  
429 thickness on the membrane and could explain any minor differences with our model.

430 To test the phase behavior of this membrane, we performed simulations at  
431 various temperatures ranging from 290 K to 350 K. For the whole range of  
432 temperatures, lipids were in the liquid phase and allowed to diffuse freely (**Figure 2E**  
433 and **SI Figure 24**). The calculated diffusion coefficients for each species in the  
434 periplasmic leaflet at 310 K were as follows: 9.9 × 10<sup>-8</sup> cm<sup>2</sup>/s (AcPIM<sub>2</sub>), 6.9 × 10<sup>-8</sup> cm<sup>2</sup>/s  
435 (AcPIM<sub>6</sub>), 1 × 10<sup>-7</sup> cm<sup>2</sup>/s (Ac<sub>2</sub>PIM<sub>6</sub>), 1.3 × 10<sup>-7</sup> cm<sup>2</sup>/s (PI), 1.5 × 10<sup>-7</sup> cm<sup>2</sup>/s (PE) and 1.2  
436 × 10<sup>-7</sup> cm<sup>2</sup>/s (CL). In the cytoplasmic leaflet, the values were 6.1 × 10<sup>-8</sup> cm<sup>2</sup>/s (AcPIM<sub>2</sub>)  
437 and 8.1 × 10<sup>-8</sup> cm<sup>2</sup>/s (Ac<sub>2</sub>PIM<sub>2</sub>). Compared to isolated PIMs in a PC membrane, the  
438 diffusion in this plasma membrane was roughly one order of magnitude slower.

439 AcPIM<sub>2</sub>, which is the only lipid in both leaflets, diffused ~40% slower in the cytoplasmic  
440 membrane compared with the periplasmic leaflet. Using a previously reported model  
441 of mammalian plasma membrane (22), the diffusion coefficients of PE and PI were  
442 calculated to be  $3.3 \times 10^{-7} \text{ cm}^2/\text{s}$  and  $2.8 \times 10^{-7} \text{ cm}^2/\text{s}$ , showing that the diffusion in the  
443 mammalian membrane is equivalent to that in the mycobacterial plasma membrane.  
444 The membrane stiffness of our mycobacterial membrane was significantly lower at  
445  $\kappa = 8.2 \text{ k}_B\text{T}$  compared to  $13.9 \text{ k}_B\text{T}$  and  $19.1 \text{ k}_B\text{T}$  for the mammalian and PC membrane  
446 models, respectively (**SI Figure 23**), highlighting a unique dynamic behavior for the  
447 mycobacterial plasma membrane.

448 Lipid clustering was moderate and membrane composition remained  
449 heterogenous over the course of each  $10 \mu\text{s}$  simulation (**Figure 2C**). This can also be  
450 seen in **Figure 2D** where the number of surrounding lipids of the same type for each  
451 species in the periplasmic membrane was roughly equivalent to that of any other lipid  
452 species. The distribution of the area per lipid in the membrane also suggests high  
453 heterogeneity (**Figure 2F**). The average area per lipid in the membrane over the  
454 course of the simulation was  $0.89 \text{ nm}^2$  and  $1.18 \text{ nm}^2$  for the periplasmic and  
455 cytoplasmic leaflets, respectively. Thus, the cytoplasmic leaflet appears to be a little  
456 denser than the periplasmic leaflet. This can be related to the packing of the four acyl  
457 chains of Ac<sub>2</sub>PIM<sub>2</sub> lipids present in high concentration in this leaflet (66). A movie for  
458 the change in area per lipid for each leaflet over the course of the simulation can be  
459 found in **SI Movies 2,3**.

460 As was seen for the individual lipids in CG the overall bilayer attracted ions;  
461 both Cl<sup>-</sup> and Na<sup>+</sup> concentration was much higher close to the bilayer, especially around  
462 the sugar head groups compared to bulk solution (**Figure 2B**). It has been shown that  
463 lipid-ion interactions can affect the biophysical properties of the membrane, such as  
464 fluidity and stiffness, as well as the structure, which could modify the interaction with  
465 proteins (67).

466 The mycobacterial plasma membrane has been reported to change at different  
467 growth stages (7, 68), but the study that originally proposed the composition was  
468 based solely on late-exponential-phase cells. To explore a wider range of possible  
469 membrane compositions, we designed an 'PIM-enriched' bilayer, where the amount  
470 of PIM lipids in the periplasmic leaflet was increased to reflect cells in rapid growth (7)  
471 (see compositions in **SI Figure 22**). In this case, the overall biophysical properties of  
472 that leaflet were very similar to those seen in **Figure 2**, but interestingly a change in

473 the diffusion and density of the cytoplasmic leaflet was observed (**SI Figures 26,27**).  
474 Increasing the proportion of lipids in the periplasmic leaflet that have 3 or 4 acyl chains  
475 could mean that the lipid tails make more inter-leaflet interactions and hence slow  
476 down diffusion and make the leaflet denser. This decrease in fluidity in the membrane  
477 could impact antibiotic diffusion through the membrane (13, 69) which would make the  
478 bacteria less susceptible to treatment at a time when it is most vulnerable.

479 We have also tested alternative plasma membrane compositions with a  
480 symmetrical configuration. First, we performed a model of a membrane recapitulating  
481 the composition being experimentally derived using a combination of mass  
482 spectrometry and NMR (see composition in **SI Figures 22,38**). This symmetrical  
483 configuration is stable, and the dynamics of PIM lipids in CG-MD simulations agreed  
484 with solid state NMR results (see **SI Figures 38,39**). This data shows that the acyl  
485 chains extremities are highly mobile, while the core of the acyl chains displayed a more  
486 rigid structure. Interestingly, the sugar moieties adopted an intermediate dynamic  
487 regime, as seen from experimental NMR results (**SI Figure 38E**) and MD simulations  
488 (**SI Figure 39B**). We then designed a model of symmetrical membrane system with a  
489 lipid composition that mimics *Mtb* membranes (see composition in **SI Figure 22A**).  
490 This membrane showed similar interactions of lipids compared to the asymmetric  
491 composition and also similar rates of diffusion (**SI Figures 25,27**). Clustering of ions  
492 around the sugar headgroups was still observed.

493 Taken together our observations suggest that PIM lipids play a critical role in  
494 the dynamic and structuration of mycobacterial plasma membrane. Both the  
495 asymmetric and symmetric configurations of the membrane were equally stable and  
496 our analyses show that both share similar dynamic properties. This work demonstrates  
497 the plausible arrangements of the plasma membrane and provides a mechanism to  
498 easily study new membrane compositions based on future experimental results.

499

#### 500 *Asymmetrical distribution of residues at the surface of Mtb membrane proteins*

501 The lipids of the plasma membrane do not exist in isolation, but rather in cooperation  
502 with membrane proteins. These proteins could provide insights into how this  
503 membrane exists natively (70). To explore this, we used the accurate, fast and reliable  
504 workflows of AlphaFold (49) and memembed (50), to insert all transmembrane  
505 proteins from *Mtb* and *E. coli* into their native-like membranes (**Figure 3A,B**). We then  
506 assessed the distribution of residues in proximity to the lipid bilayer (**Figure 3C**).



507 For *E. coli*, the distribution is as expected and similar to results previously  
508 shown (21). The asymmetry of Arg and Lys reflect the 'positive inside rule' (71-73)  
509 present in bacteria which aids in the correct insertion of the transmembrane helices.  
510 The symmetric nature of the lipid bilayer is otherwise reflected in the distribution of the  
511 residues (**Figure 3C, SI Figure 28**). The *Mtb* distribution of Arg also shows a strong  
512 'positive inside rule'. This is at a higher intensity than in *E. coli*, likely due to the low  
513 levels of Lys observed (**Figure 3C**). Furthermore, the decrease in the bulky Phe and  
514 Tyr residues (**Figure 3C**) in the cytoplasmic leaflet compared to the periplasmic leaflet  
515 agrees with the increased density seen in an asymmetric membrane (**Figure 2B** and  
516 **SI Figure 26B**).

517 These results add evidence to an asymmetric model previously proposed (13).  
518 In addition to validating the model proposed in this work, it could aid understanding of  
519 how mycobacterial membrane proteins differ from those found in other organisms and  
520 hence aid targeted therapies.

521

#### 522 *Asymmetric interaction of the membrane with antibiotics*

523 We were interested in testing how the organization of this membrane affects the  
524 behavior of other molecules, such as antibiotics. We tested two widely used anti-TB  
525 antibiotics: Isoniazid (ISZ) and Bedaquiline (BDQ) (**Figure 4A**). To validate the  
526 behavior of these small molecules as well as the bond lengths/angles being account  
527 for through PyCGTOOL (52) (**SI Figure 30**), the LogP and solvent accessible surface  
528 area were calculated for each and compared to predicted values and atomistic values,  
529 respectively. The values for LogP for neutral BDQ (5.92 for CG, 6.37 predicted) and  
530 ISZ (-1.10 for CG, -0.71 predicted) agree very well, as do the solvent accessible  
531 surface area values (BDQ values  $13.01 \pm 0.49 \text{ nm}^2$  for CG and  $13.69 \pm 1.11$  for AT;  
532 ISZ values  $7.48 \pm 0.14 \text{ nm}^2$  for CG and  $7.35 \pm 0.04$  for AT).

533 ISZ is a first line anti-TB treatment that targets InhA, a cytosolic enzyme that is  
534 essential for mycolic acid synthesis (74). BDQ on the other hand is a last line antibiotic  
535 that targets the membrane protein complex of the ATP synthase (75, 76). The PMF  
536 results for ISZ and BDQ show that the mycobacterial membrane behaves as expected  
537 regarding the passage of a small molecule through a lipid rich membrane, showing  
538 favorable interactions with the largely hydrophobic BDQ and unfavourable interactions  
539 with hydrophilic ISZ (**Figure 4B**). The positive charge present on BDQ could account  
540 for the sharp free energy barrier at the membrane midplane. To assess the effect of

541 the charge on the passage of BDQ since the protonation state is unknown, we  
542 modeled a neutral BDQ where the charged Q1 bead (see **Figure 4A**) was replaced  
543 by SC3. The PMF results with the neutral BDQ show a reduction in the energy barrier  
544 at this point (**SI Figure 29D**). We note that the passage of these drugs through an *E.*  
545 *coli* inner membrane is symmetric from the mid-plane of the membrane as expected.  
546 The interactions of BDQ with the periplasmic leaflet of the mycobacterial membrane  
547 were ~6 kJ/mol stronger than for the simple membrane and the mycobacterial  
548 cytoplasmic leaflet. This is possibly due to the presence of tetraacylated PIMs in the  
549 mycobacterial periplasmic leaflet. Roughly the same difference between the  
550 mycobacterial and simple membranes was observed for ISZ at the mid-membrane  
551 region, showing less favorable passage through the membrane. These results suggest  
552 that ISZ, a first-line TB drug, is unlikely to freely diffuse through the membrane, which  
553 is in contrast with the previously proposed uptake mechanism based on passive  
554 diffusion (77-79). Small differences were seen for ISZ interactions at the interfaces of  
555 the mycobacterial and simple membrane periplasmic membrane. This could be due to  
556 the presence of certain PIM lipids in this leaflet. In our simulations, when the BDQ  
557 starting position was in the bulk solvent, the drug quickly associated with the  
558 membrane, as shown in **SI Figure 31**, and displayed no strong preference for either  
559 leaflet in these simulations.

560 BDQ has been shown to target the mycobacterial ATP synthase (Rv1304-  
561 Rv1311), a membrane protein complex. A cryo-EM structure from *M. smegmatis*  
562 (PDB: 7JGC) shows multiple binding sites for BDQ at the interface of the c-subunits  
563 and the interface between the a- and c- subunits (25), where a highly negatively  
564 charged area is located (**SI Figure 32A,B**). Using the available cryo-EM data, we  
565 performed 10 simulations of 10  $\mu$ s each to examine how *Mtb* ATP synthase complex  
566 behaves in our asymmetric mycobacterial plasma membrane model. ATP synthase  
567 was stable in the membrane and that there were no significant perturbations of the  
568 bilayer by the protein. The lipids of the mycobacterial membrane did not show any  
569 strong interactions with the protein (**Figure 4D** and **SI Figure 33**), apart from CL that  
570 localizes in the a-subunit and around the c-ring (**Figure 4D**) in positions similar to  
571 those observed in a previous study (80). The exact values for CL occupancy of each  
572 residue are shown in **SI Figure 35A**. Minimal interactions between BDQ and the  
573 ATPase occurred when the drug started in the periplasmic leaflet; however, when BDQ  
574 was introduced in the cytoplasmic leaflet, significant occupation of the binding sites on

575 ATPase were observed (**Figures 4D-G** and **SI Figures 32C,D**). The interactions were  
576 reduced with the neutral BDQ model (**SI Figures 29A-C**). Of note, no such leaflet  
577 differences were observed when we tested the *E. coli* symmetric membrane model (**SI**  
578 **Figures 36C,D; SI Figure 37**), but with a symmetric mycobacterial membrane (**SI**  
579 **Figure 32**). This suggests that the lipid composition plays a crucial role in the  
580 regulation of antibiotic interactions.

581 Furthermore, the interactions of the charged BDQ we observed matched well  
582 with what was previously seen in the cryo-EM structure (25), with an occupancy of  
583 ~30% of the simulation time on some subunits (**Figure 4G**). The occupancy value  
584 averaged over all subunits is shown in **SI Figure 35B**. The simulations were able to  
585 identify all three types of sites reported previously (25). Most interactions were seen  
586 through the leading site (46% of the time,  $K_{off} = 5.2 \mu s^{-1}$ ) followed by the lagging site  
587 (22% of the time,  $K_{off} = 7.6 \mu s^{-1}$ ) and finally further interactions around the rotor (36%  
588 of the time,  $K_{off} = 1.2 \mu s^{-1}$ , **SI Figure 34**). For the leading site, the PMF calculations  
589 confirm this as a binding site, giving a moderate energy well of approximately 7 kJ/mol  
590 (**Figure 4H**) and making it equivalent to a typical cholesterol-protein binding interaction  
591 (81). Tracking the z-position of the central bead from the antibiotic over the course of  
592 the unbiased simulations with the protein shows that the binding sites are occupied to  
593 some extent by each of the drug molecules and some are flipped to the outer leaflet  
594 from the plasma leaflet, as shown in **SI Figure 32C**.

595 These studies illustrate that our asymmetrical mycobacterial plasma  
596 membrane model can be combined with studies of antibiotics, as well as with studies  
597 of membrane proteins and their interactions with lipids and/or antibiotics. Taken  
598 together, our simulations suggest that BDQ must enter the target protein from the  
599 cytoplasmic side, which agrees with resistance mechanisms against BDQ that involve  
600 upregulation of MmpL5, a drug efflux pump (82, 83). Importantly, an *E. coli* membrane  
601 could not capture this feature, highlighting that the mycobacterial membrane model is  
602 a more accurate and functionally relevant representation of the *Mtb* plasma  
603 membrane.

604

## 605 **Discussion**

606 Here, we provide models for lipids constituting the mycobacterial plasma membrane  
607 focused on the PIM lipids, as they represent more than 50% of the total lipid content  
608 in *Mtb*. Thus, understanding how these lipids behave on an individual level and as a

609 constituent of a membrane could provide key insight into the intrinsic resistance of *Mtb*  
610 to antibiotics.

611 In this CG model, clustering of ions around these lipids was observed at both  
612 the single lipid and bilayer level, probing how this affects the biophysical properties of  
613 the bilayer and whether this could be exploited for treatment of TB is an interesting  
614 area for future research. The lipids did not cluster together excessively over the  
615 timescales studied and all diffused well through the membrane. This confirmed that  
616 the mycobacterial cell envelope is dynamic, which could potentially be an important  
617 insight into how this cell wall functions. A low membrane bending rigidity compared to  
618 a PC and eukaryotic plasma membrane is interesting and could suggest the  
619 importance of other cell envelope components in maintaining the shape of the cell.

620 The simulations confirmed that our asymmetric plasma membrane model is  
621 stable with a composition of over 50% PIM lipids. However, considering that this  
622 arrangement has not been experimentally validated, a symmetric and 'enriched PIM'  
623 asymmetric membrane was also shown to share similar biophysical properties. The  
624 high proportion of PIM lipids resulted in some unique properties, most notably lower  
625 membrane stiffness, which may have important implications for membrane  
626 microdomain formation, drug penetration, and integral membrane protein behavior. In  
627 this context, the integral membrane proteins from *Mtb* are of interest for the  
628 development of new antibiotics for TB (9), and we show evidence that our membrane  
629 model can be used to simulate proteins in a native lipid environment to determine any  
630 key lipid interactions, as illustrated by our study of antibiotic BDQ and its target,  
631 ATPase. In addition, an overall comparison of plasma membrane protein residues  
632 found in contact with the lipid bilayer revealed differences between *Mtb* and *E. coli*,  
633 supporting the asymmetric nature of the mycobacterial plasma membrane model. This  
634 potentially hints at topological differences between proteins in mycobacteria and those  
635 within other bacteria. If this is the case, currently unknown proteins which maintain the  
636 asymmetry might be present (such as those identified in animal cells (84)), and would  
637 constitute interesting drug targets to explore.

638 Potential limitations of our model stem from the reduced type of lipids we  
639 included in this initial development. Thus, future studies will focus on expanding the  
640 model to include apolar lipids (such as triglycerides (17)), TMM and LM/LAM.  
641 Moreover, future simulations with proteins and probing protein/lipid interactions with  
642 atomistic resolution could help further elucidate the role of these complex lipids.

643 In conclusion, our study is a starting point for building an entire mycobacterial  
644 cell envelope, as done for gram-negative bacteria (85). Other mycobacterial lipids  
645 have already been parameterized (86), and combining them with the model described  
646 here has the potential to significantly advance the field that has been lacking  
647 experimental strategies for plasma membrane investigations. Additionally, the  
648 mycobacterial cell envelope is known to change at different growth stages during its  
649 lifecycle (7, 68, 87, 88) and in response to different environments (69). With our model  
650 and set-up methods, as shown with the multiple compositions simulated, it will be  
651 possible to understand drug permeability or protein behavior at different stages of  
652 infection. Taken together, integrating our mycobacterial plasma membrane model into  
653 broader studies of this pathogen, its pathogen/host interactions, as well as into anti-  
654 TB drug discovery and development has a potential to reveal new functional insights  
655 and yield improved therapies.

656

657 Data availability: <https://github.com/pstansfeld/PIM-lipids>

658 Atomistic systems and CG-membrane set-up can be performed using respectively  
659 CHARMM-GUI bilayer builder ( <https://charmm-gui.org/input/membrane.bilayer> ) and  
660 CHARMM-GUI MARTINI bilayer Maker (   
661 <https://charmm-gui.org/?doc=input/martini.bilayer> )

662

### 663 **Author contributions**

664 C.M.B., R.A.C., W.I., P.J.S. and M.C. designed research; C.M.B., R.A.C., Y.G., Y.K.C.  
665 and E.L. performed computational research; C.M.B., R.A.C. and N.D. analyzed  
666 computational data; A.G., M.G., J.N. and A.L. performed experimental research and  
667 analysis; C.M.B., R.A.C., A.L., P.J.S. and M.C. wrote the paper; J.N., A.L., E.F., W.I.,  
668 P.J.S. and M.C. obtained funding.

669

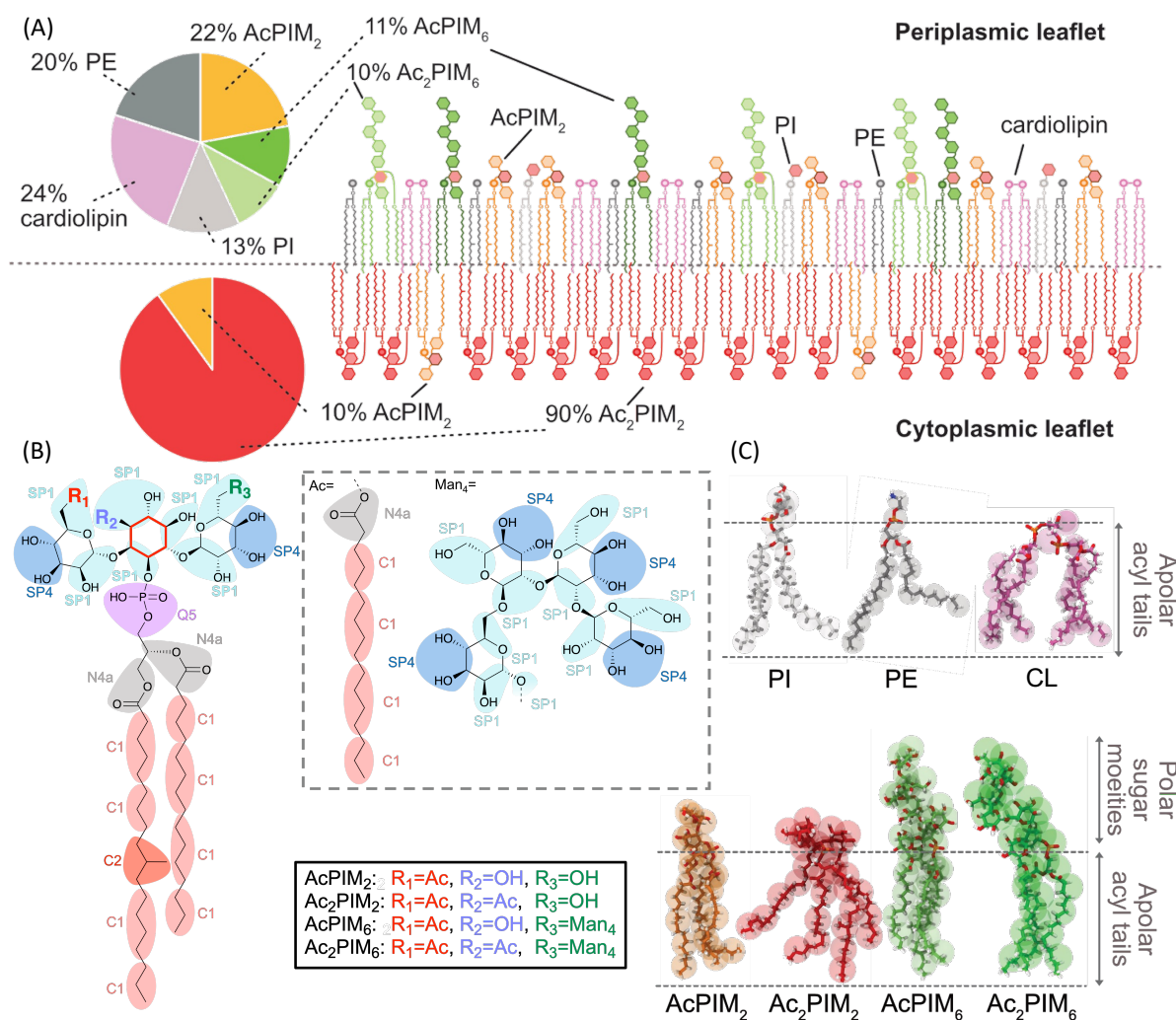
### 670 **Acknowledgements**

671 C. M. B. is supported by an MRC studentship (MR/N014294/1). R. A. C. is funded by Wellcome  
672 (208361/Z/17/Z). Research in P. J. S.'s lab is funded by Wellcome (208361/Z/17/Z), the MRC  
673 (MR/S009213/1) and BBSRC (BB/P01948X/1, BB/R002517/1 and BB/S003339/1). M. C. is  
674 supported by the CNRS-MITI grant "Modélisation du vivant" 2020. W. I. is funded by NSF  
675 (MCB-2111728). E.F is a Sir Henry Dale Fellow jointly funded by the Wellcome Trust and  
676 Royal Society (104193/Z/14/Z and 104193/Z/14/B). M. G. would like to acknowledge the

677 European Union's Horizon 2020 research and innovation program under grant agreement  
678 H2020-PHC-08-2014-643381, TBVAC2020. This work was granted access to the HPC  
679 resources of CALMIP supercomputing center (under the allocation 2021-17036) and TGCC  
680 Joliot-Curie supercomputer (under the GENCI allocation A0110712941). This project made  
681 use of time on ARCHER2 and JADE2 granted via the UK High-End Computing Consortium  
682 for Biomolecular Simulation, HECBioSim (<http://hecbiosim.ac.uk>), supported by EPSRC  
683 (grant no. EP/R029407/1). This project also used Athena and Sulis at HPC Midlands+, which  
684 were funded by the EPSRC on grants EP/P020232/1 and EP/T022108/1. This work has  
685 benefited from the facilities and expertise of the Biophysical and Structural Chemistry platform  
686 (BPCS) at IECB, CNRS UAR3033, INSERM US001, and Bordeaux University. We thank the  
687 University of Warwick Scientific Computing Research Technology Platform for computational  
688 access. We acknowledge Life Science Editors for proofreading the manuscript. We thank C.  
689 Cooper for fruitful discussions and M. Costic for comments on the manuscript.  
690

691 **Abbreviations**

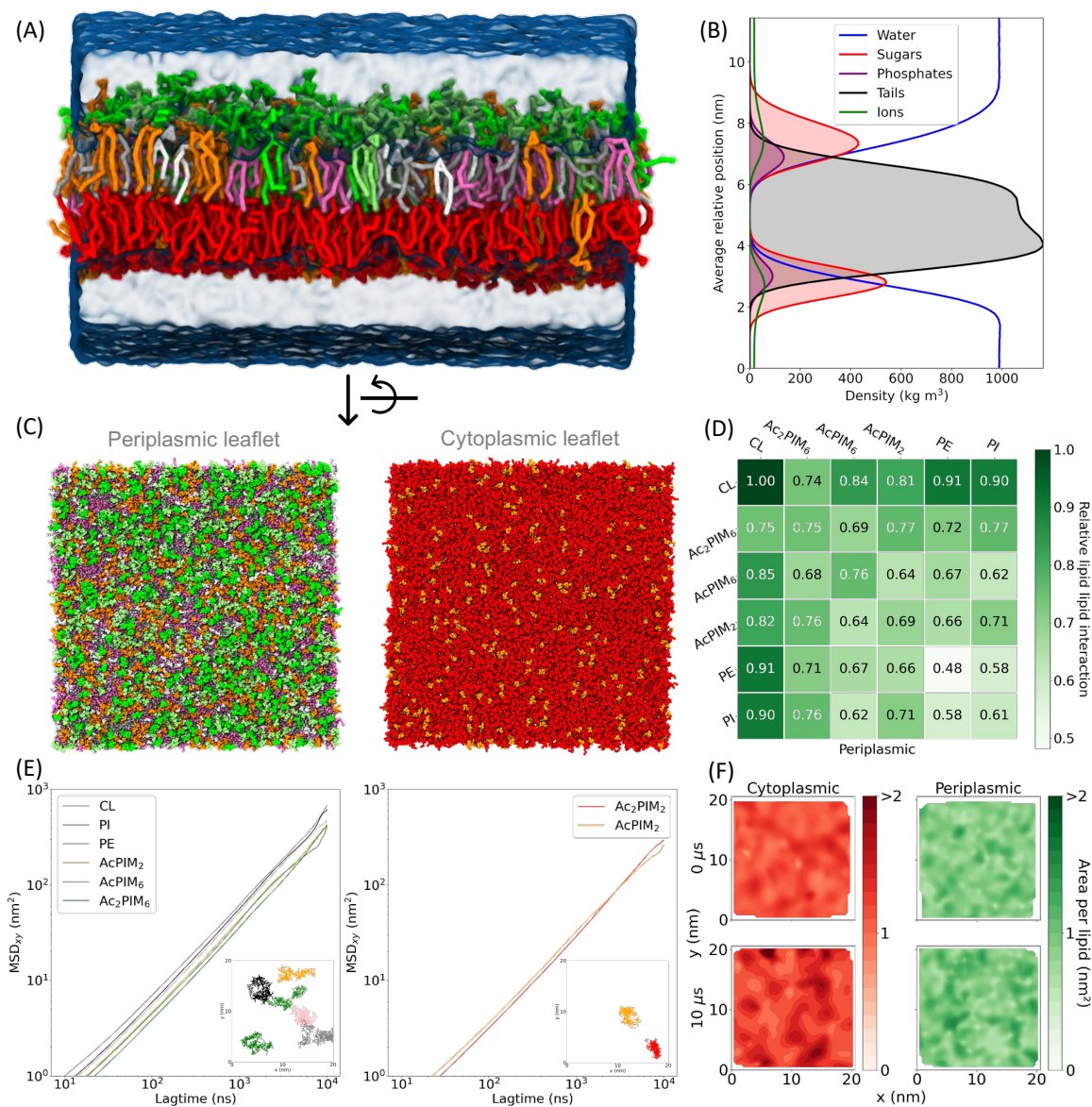
692	AT:	Atomistic
693	ATP:	Adenosine triphosphate
694	BDQ:	Bedaquiline
695	CG:	Coarse grained
696	CL:	Cardiolipin
697	ISZ:	Isoniazid
698	LAM:	Lipoarabinomannan
699	LM:	Lipomannan
700	MD:	Molecular dynamics
701	<i>Mtb</i> :	<i>Mycobacterium tuberculosis</i>
702	NMR:	Nuclear magnetic resonance
703	PC:	Phosphatidylcholine
704	PE:	Phosphatidylethanolamine
705	PG:	Phosphatidylglycerol
706	PI:	Phosphatidylinositol
707	PIMs:	Phosphatidyl- <i>myo</i> -inositol mannosides
708	PIPs:	Phosphatidylinositol phosphates
709	PME:	Particle mesh Ewald
710	PMF:	Potential of mean force
711	TB:	Tuberculosis
712	TMM:	Trehalose monomycolate
713	VdW:	van der Waal
714		



715  
 716  
 717  
 718  
 719  
 720  
 721  
 722

**Figure 1:** The structure of the mycobacterial lipids. **(A)** Schematic of an asymmetrical model of the mycobacterial plasma membrane and composition as previously defined (13). **(B)** Schematic of the core of the PIM lipids found in mycobacteria with the groupings for coarse-grained (CG) beads. The inositol core is highlighted in red. The bead types for MARTINI 3 are shown. **(C)** Overlay of the AT (sticks) and CG (spheres) models for each lipid, with chemical characteristics shown to the right.

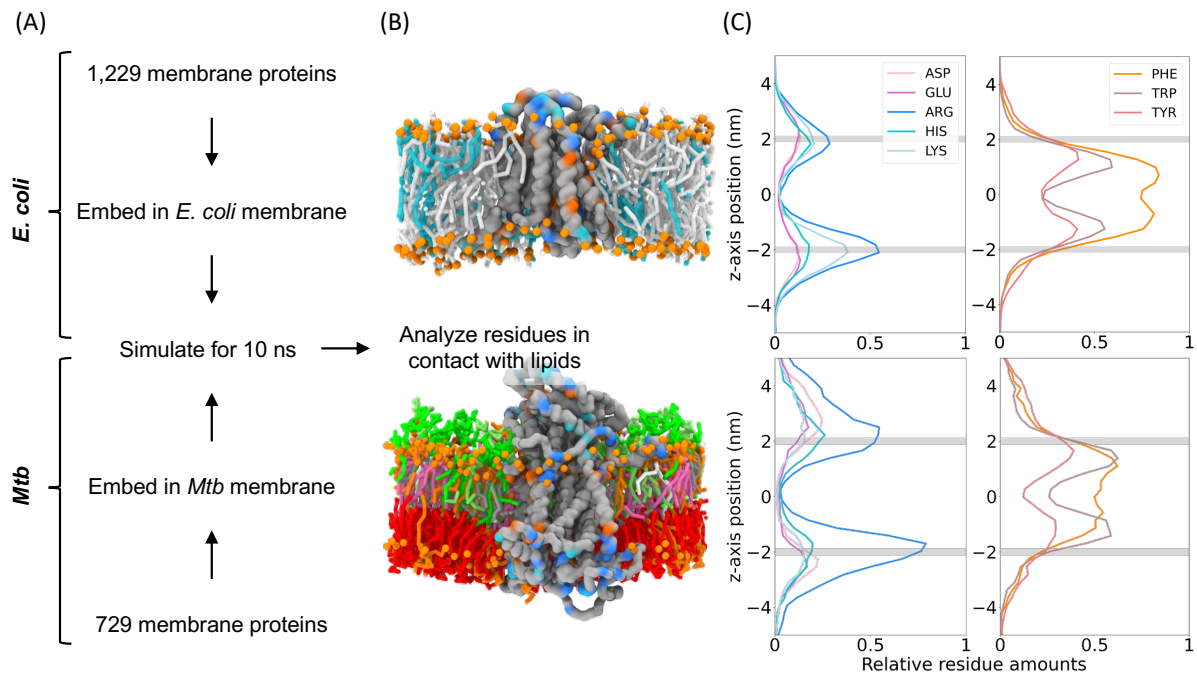




723

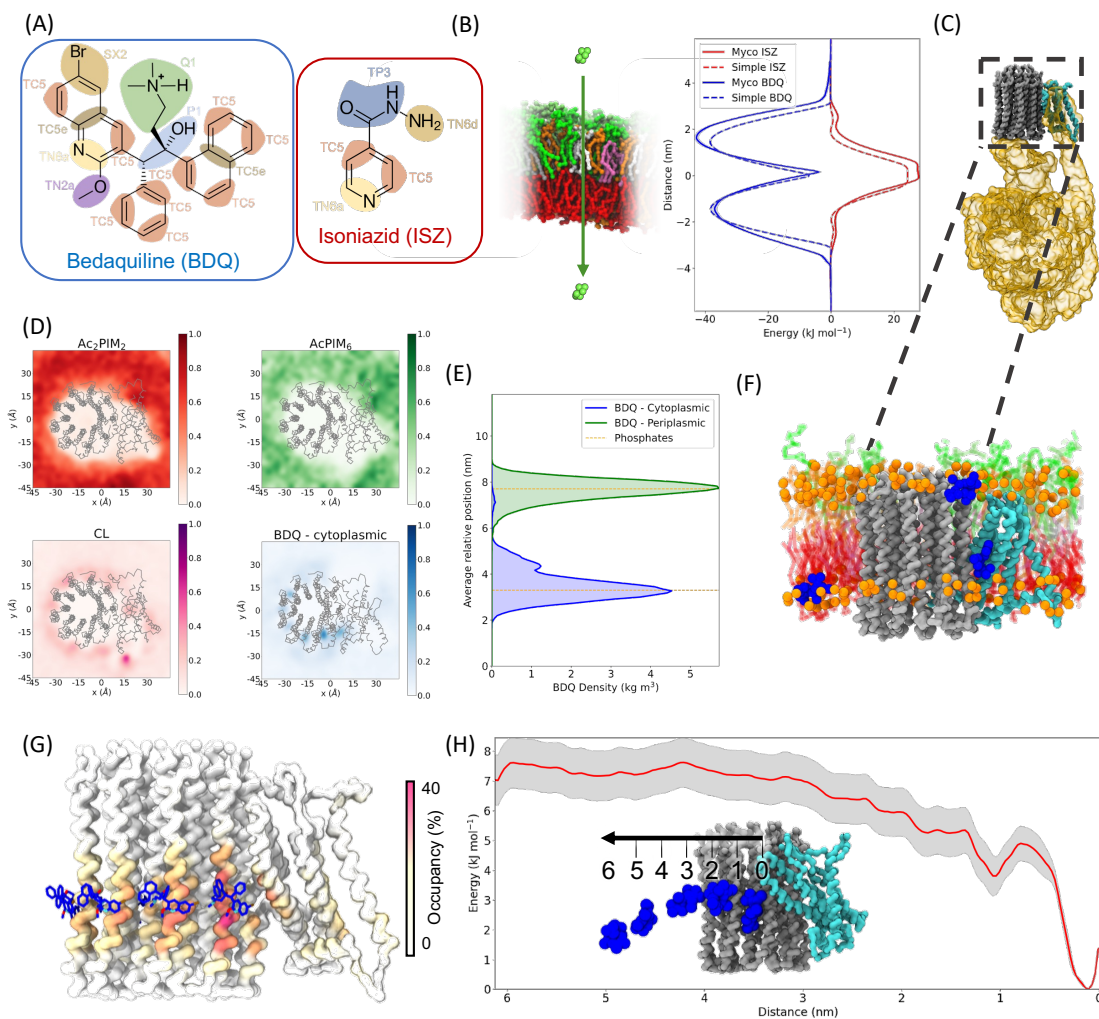
724 **Figure 2:** A mycobacterial membrane model. (A) Side view of the membrane with each lipid  
 725 type depicted in a different color as shown in **Figure 1A**. (B) A density plot showing the density  
 726 of water, sugar groups, phosphate groups, tail groups and ions over the simulation box. (C)  
 727 Snapshots of the periplasmic and cytoplasmic leaflets whose compositions are: AcPIM<sub>2</sub> 22%,  
 728 AcPIM<sub>6</sub> 11%, Ac<sub>2</sub>PIM<sub>6</sub> 10%, CL 24%, PE 20% and PI 13% (periplasmic leaflet) and AcPIM<sub>2</sub>  
 729 10% and Ac<sub>2</sub>PIM<sub>2</sub> 90% (cytoplasmic leaflet). The system size is 50 x 50 x 15 nm. (D) Relative  
 730 number of neighbors of each lipid type for the periplasmic membrane. (E) Mean squared  
 731 displacement (MSD<sub>xy</sub>) (nm<sup>2</sup>) as a function of lagtime (ns) for each lipid type in the periplasmic  
 732 and cytoplasmic leaflets. The inserts show the position of the phosphate group of each lipid  
 733 type over the last 500 ns of the simulation. (F) Contour plots of the area per lipid (nm<sup>2</sup>) in each  
 734 leaflet at the starting frame (upper) and final frame (lower) of the simulation. A darker color  
 735 indicates a larger area per lipid.

736



737

738 **Figure 3:** The asymmetry of the mycobacterial membrane. (A) A simplified workflow for the  
 739 lipid contacts analysis from all membrane proteins. (B) An example of a protein (*E. coli* protein  
 740 *MraY* UniProt id: P0A6W3 and *Mtb* transporter *MmpL3* UniProt id: P9WJV5) embedded in  
 741 either an *E. coli* or mycobacterial membrane. The orange spheres represent phosphates. For  
 742 *E. coli* membrane the cyan sticks show PG and the grey sticks PE. For mycobacterial  
 743 membrane, the lipid sticks are shown in the colors illustrated in **Figure 1A**. Selected residues  
 744 are colored according to the key in (C). (C) Graphs for *E. coli* and *Mtb* (top and bottom  
 745 respectively) showing the relative abundance of selected residues within 8 Å of lipids. The  
 746 gray lines show the position of the phosphates. The cytoplasmic region is shown by negative  
 747 z values, the periplasmic region by positive values.  
 748



749  
750

751 **Figure 4:** The behavior of the antibiotics with mycobacterial membrane and proteins. (A)  
 752 Chemical structures of BDQ and ISZ with the CG groupings overlaid and bead types shown.  
 753 (B) PMFs of the two antibiotics being pulled through either a mycobacterial or simple  
 754 membrane in the z-direction. BDQ is shown in blue and ISZ in red, with the mycobacterial  
 755 membrane results having a solid line and simple membrane having a dashed line. The error  
 756 is shown in grey. A schematic of the PMF is shown to the left. (C) Structure of *Mycobacterium*  
 757 *smegmatis* ATP synthase (PDB: 7JG5) with the c-subunits shown in grey, the a-subunit shown  
 758 in cyan, and the other components shown as a gold surface. (D) Density in the x and y  
 759 dimensions of selected lipids and BDQ when starting in the cytoplasmic leaflet relative to the  
 760 protein shown in grey. (E) Density of the phosphates (orange) and BDQ over the course of  
 761 the simulations where the antibiotic started in either the periplasmic leaflet (green) or the  
 762 cytoplasmic leaflet (blue). (F) Snapshot of a single simulation containing a *Mtb* ATPase model  
 763 and 8 x BDQ models showing the main positions BDQ occupied. Phosphates are shown in  
 764 orange, BDQ shown in blue, c-subunits are shown in grey, and the a-subunit is shown in cyan.  
 765 The lipid sticks are shown in the colors illustrated in **Figure 1A**. (G) Comparison of the highest  
 766 occupancy sites identified with PyLipID (surface) and BDQ from the Cryo-EM structure (PDB:  
 767 7JGC) (sticks). (H) PMF of BDQ moving through a mycobacterial plasma membrane with the  
 768 error shown in grey. A schematic of the PMF is shown as an insert.

769

770

771 **References**

772

- 773 1. WHO (2022) Global tuberculosis report 2022. (World Health Organization, Geneva).  
774 2. C. R. Horsburgh, C. E. Barry, C. Lange, Treatment of Tuberculosis. *New England Journal*  
775 *of Medicine* **373**, 2149-2160 (2015).  
776 3. WHO (2021) Global Tuberculosis Report 2021. (World Health Organization, Geneva).  
777 4. WHO (2022) The End TB Strategy.  
778 5. WHO (2021) 1.4 million with tuberculosis, lost out on treatment during first year of  
779 COVID-19.  
780 6. L. Chiaradia *et al.*, Dissecting the mycobacterial cell envelope and defining the  
781 composition of the native mycomembrane. *Scientific Reports* **7**, 12807 (2017).  
782 7. C. L. Dulberger, E. J. Rubin, C. C. Boutte, The mycobacterial cell envelope — a moving  
783 target. *Nature Reviews Microbiology* **18**, 47-59 (2020).  
784 8. S. M. Batt, D. E. Minnikin, G. S. Besra, The thick waxy coat of mycobacteria, a protective  
785 layer against antibiotics and the host's immune system. *Biochemical Journal* **477**,  
786 1983-2006 (2020).  
787 9. E. Fullam, R. J. Young, Physicochemical properties and Mycobacterium tuberculosis  
788 transporters: keys to efficacious antitubercular drugs? *RSC Medicinal Chemistry* **12**,  
789 43-56 (2021).  
790 10. K. A. Sacksteder, M. Protopopova, C. E. Barry, 3rd, K. Andries, C. A. Nacy, Discovery  
791 and development of SQ109: a new antitubercular drug with a novel mechanism of  
792 action. *Future Microbiol* **7**, 823-837 (2012).  
793 11. Y.-M. Boudehen *et al.*, Mycobacterial resistance to zinc poisoning requires assembly  
794 of P-ATPase-containing membrane metal efflux platforms. *Nature Communications*  
795 **13**, 4731 (2022).  
796 12. J. M. Hayashi *et al.*, Spatially distinct and metabolically active membrane domain in  
797 mycobacteria. *Proc Natl Acad Sci U S A* **113**, 5400-5405 (2016).  
798 13. R. Bansal-Mutalik, H. Nikaido, Mycobacterial outer membrane is a lipid bilayer and the  
799 inner membrane is unusually rich in diacyl phosphatidylinositol dimannosides.  
800 *Proceedings of the National Academy of Sciences* **111**, 4958-4963 (2014).  
801 14. M. Gilleron, V. F. J. Quesniaux, G. Puzo, Acylation State of the Phosphatidylinositol  
802 Hexamannosides from *Mycobacterium bovis* Bacillus Calmette Guérin and  
803 *Mycobacterium tuberculosis* H37Rv and Its Implication in Toll-like Receptor Response  
804 *Journal of Biological Chemistry* **278**, 29880-29889 (2003).  
805 15. M. Gilleron *et al.*, Acylation State of the Phosphatidylinositol Mannosides from  
806 *Mycobacterium bovis* Bacillus Calmette Guérin and Ability to Induce Granuloma and  
807 Recruit Natural Killer T Cells. *Journal of Biological Chemistry* **276**, 34896-34904 (2001).  
808 16. M. Daffé, H. Marrakchi, Unraveling the Structure of the Mycobacterial Envelope.  
809 *Microbiol Spectr* **7** (2019).  
810 17. M. Jackson, The mycobacterial cell envelope-lipids. *Cold Spring Harb Perspect Med* **4**,  
811 a021105 (2014).  
812 18. R. Kalscheuer *et al.*, The Mycobacterium tuberculosis capsule: a cell structure with key  
813 implications in pathogenesis. *Biochemical Journal* **476**, 1995-2016 (2019).  
814 19. M. E. Guerin, J. Korduláková, P. M. Alzari, P. J. Brennan, M. Jackson, Molecular Basis  
815 of Phosphatidyl-*myo*-inositol Mannoside Biosynthesis and Regulation in Mycobacteria  
816 *Journal of Biological Chemistry* **285**, 33577-33583 (2010).

- 817 20. V. Corradi *et al.*, Emerging Diversity in Lipid–Protein Interactions. *Chemical Reviews*  
818 **119**, 5775-5848 (2019).
- 819 21. R. A. Corey *et al.*, Identification and assessment of cardiolipin interactions with E. coli  
820 inner membrane proteins. *Sci Adv* **7** (2021).
- 821 22. H. I. Ingólfsson *et al.*, Lipid Organization of the Plasma Membrane. *Journal of the*  
822 *American Chemical Society* **136**, 14554-14559 (2014).
- 823 23. I. D. Pogozheva *et al.*, Comparative Molecular Dynamics Simulation Studies of Realistic  
824 Eukaryotic, Prokaryotic, and Archaeal Membranes. *Journal of Chemical Information*  
825 *and Modeling* **62**, 1036-1051 (2022).
- 826 24. P. Adhyapak *et al.*, Lipid Clustering in Mycobacterial Cell Envelope Layers Governs  
827 Spatially Resolved Solvation Dynamics. *Chemistry – An Asian Journal* **17**, e202200146  
828 (2022).
- 829 25. H. Guo *et al.*, Structure of mycobacterial ATP synthase bound to the tuberculosis drug  
830 bedaquiline. *Nature* **589**, 143-147 (2021).
- 831 26. P. C. T. Souza *et al.*, Martini 3: a general purpose force field for coarse-grained  
832 molecular dynamics. *Nature Methods* **18**, 382-388 (2021).
- 833 27. R. Alessandri *et al.*, Martini 3 Coarse-Grained Force Field: Small Molecules. *Advanced*  
834 *Theory and Simulations* **5**, 2100391 (2022).
- 835 28. L. Borges-Araújo, P. C. T. Souza, F. Fernandes, M. N. Melo, Improved Parameterization  
836 of Phosphatidylinositide Lipid Headgroups for the Martini 3 Coarse-Grain Force Field.  
837 *Journal of Chemical Theory and Computation* **18**, 357-373 (2022).
- 838 29. P. Banerjee, R. Lipowsky, M. Santer, Coarse-Grained Molecular Model for the  
839 Glycosylphosphatidylinositol Anchor with and without Protein. *Journal of Chemical*  
840 *Theory and Computation* **16**, 3889-3903 (2020).
- 841 30. T. A. Wassenaar, H. I. Ingólfsson, R. A. Böckmann, D. P. Tieleman, S. J. Marrink,  
842 Computational Lipidomics with insane: A Versatile Tool for Generating Custom  
843 Membranes for Molecular Simulations. *Journal of Chemical Theory and Computation*  
844 **11**, 2144-2155 (2015).
- 845 31. G. Bussi, D. Donadio, M. Parrinello, Canonical sampling through velocity rescaling. *J*  
846 *Chem Phys* **126**, 014101 (2007).
- 847 32. M. Parrinello, A. Rahman, Polymorphic transitions in single crystals: A new molecular  
848 dynamics method. **52:12** (1981).
- 849 33. M. J. Abraham *et al.*, GROMACS: High performance molecular simulations through  
850 multi-level parallelism from laptops to supercomputers. *SoftwareX* **1-2**, 19-25 (2015).
- 851 34. J. A. Barker, R. O. Watts, Monte Carlo studies of the dielectric properties of water-like  
852 models. *Molecular Physics* **26**, 789-792 (1973).
- 853 35. E. L. Wu, Y. Qi, K. C. Song, J. B. Klauda, W. Im, Preferred Orientations of  
854 Phosphoinositides in Bilayers and Their Implications in Protein Recognition  
855 Mechanisms. *The Journal of Physical Chemistry B* **118**, 4315-4325 (2014).
- 856 36. J. Lee *et al.*, CHARMM-GUI Membrane Builder for Complex Biological Membrane  
857 Simulations with Glycolipids and Lipoglycans. *Journal of Chemical Theory and*  
858 *Computation* **15**, 775-786 (2019).
- 859 37. K. Vanommeslaeghe *et al.*, CHARMM general force field: A force field for drug-like  
860 molecules compatible with the CHARMM all-atom additive biological force fields.  
861 *Journal of computational chemistry* **31**, 671-690 (2010).
- 862 38. J. Huang *et al.*, CHARMM36m: an improved force field for folded and intrinsically  
863 disordered proteins. *Nature Methods* **14**, 71-73 (2017).

- 864 39. E. L. Wu *et al.*, CHARMM-GUI Membrane Builder toward realistic biological membrane  
865 simulations. *Journal of Computational Chemistry* **35**, 1997-2004 (2014).
- 866 40. U. Essmann *et al.*, A smooth particle mesh Ewald method. *The Journal of Chemical*  
867 *Physics* **103**, 8577-8593 (1995).
- 868 41. S.-J. Marrink (2022) Downloads: Lipids.
- 869 42. B. M. Groenewald W, Croft A, Marrink S-J., Molecular Dynamics of Mycolic Acid  
870 Monolayers. *ChemRxiv* (2019).
- 871 43. P. W. Fowler *et al.*, Membrane stiffness is modified by integral membrane proteins.  
872 *Soft Matter* **12**, 7792-7803 (2016).
- 873 44. P. Smith, C. D. Lorenz, LiPyphilic: A Python Toolkit for the Analysis of Lipid Membrane  
874 Simulations. *Journal of Chemical Theory and Computation* **17**, 5907-5919 (2021).
- 875 45. M. Bonomi *et al.*, Promoting transparency and reproducibility in enhanced molecular  
876 simulations. *Nature Methods* **16**, 670-673 (2019).
- 877 46. S. Buchoux, FATSLiM: a fast and robust software to analyze MD simulations of  
878 membranes. *Bioinformatics* **33**, 133-134 (2017).
- 879 47. J. D. Hunter, Matplotlib: A 2D Graphics Environment. *Computing in Science &*  
880 *Engineering* **9**, 90-95 (2007).
- 881 48. C. UniProt, UniProt: the universal protein knowledgebase in 2021. *Nucleic Acids Res*  
882 **49**, D480-D489 (2021).
- 883 49. J. Jumper *et al.*, Highly accurate protein structure prediction with AlphaFold. *Nature*  
884 **596**, 583-589 (2021).
- 885 50. T. Nugent, D. T. Jones, Membrane protein orientation and refinement using a  
886 knowledge-based statistical potential. *BMC Bioinformatics* **14**, 276 (2013).
- 887 51. P. C. Kroon (2020) Aggregate, automate, assemble. . (University of Groningen), pp 16-  
888 53.
- 889 52. J. A. Graham, J. W. Essex, S. Khalid, PyCGTOOL: Automated Generation of Coarse-  
890 Grained Molecular Dynamics Models from Atomistic Trajectories. *Journal of Chemical*  
891 *Information and Modeling* **57**, 650-656 (2017).
- 892 53. S. Kim *et al.*, CHARMM-GUI ligand reader and modeler for CHARMM force field  
893 generation of small molecules. *Journal of Computational Chemistry* **38**, 1879-1886  
894 (2017).
- 895 54. N. C. f. B. Information (2022) PubChem Compound Summary for CID 5388906,  
896 Bedaquiline. (National Center for Biotechnology Information).
- 897 55. I. V. Tetko *et al.*, Virtual Computational Chemistry Laboratory – Design and  
898 Description. *Journal of Computer-Aided Molecular Design* **19**, 453-463 (2005).
- 899 56. A. Waterhouse *et al.*, SWISS-MODEL: homology modelling of protein structures and  
900 complexes. *Nucleic Acids Research* **46**, W296-W303 (2018).
- 901 57. F. Sievers *et al.*, Fast, scalable generation of high-quality protein multiple sequence  
902 alignments using Clustal Omega. *Molecular Systems Biology* **7**, 539 (2011).
- 903 58. W. Song *et al.*, PyLipID: A Python Package for Analysis of Protein–Lipid Interactions  
904 from Molecular Dynamics Simulations. *Journal of Chemical Theory and Computation*  
905 **18**, 1188-1201 (2022).
- 906 59. R. A. Corey, O. N. Vickery, M. S. P. Sansom, P. J. Stansfeld, Insights into Membrane  
907 Protein–Lipid Interactions from Free Energy Calculations. *Journal of Chemical Theory*  
908 *and Computation* **15**, 5727-5736 (2019).

- 909 60. M. Souaille, B. t. Roux, Extension to the weighted histogram analysis method:  
910 combining umbrella sampling with free energy calculations. *Computer Physics*  
911 *Communications* **135**, 40-57 (2001).
- 912 61. J. S. Hub, B. L. de Groot, D. van der Spoel, g\_wham—A Free Weighted Histogram  
913 Analysis Implementation Including Robust Error and Autocorrelation Estimates.  
914 *Journal of Chemical Theory and Computation* **6**, 3713-3720 (2010).
- 915 62. P. S. Schmalhorst, F. Deluweit, R. Scherrers, C.-P. Heisenberg, M. Sikora, Overcoming  
916 the Limitations of the MARTINI Force Field in Simulations of Polysaccharides. *Journal*  
917 *of Chemical Theory and Computation* **13**, 5039-5053 (2017).
- 918 63. C. Hoffmann, A. Leis, M. Niederweis, J. M. Plitzko, H. Engelhardt, Disclosure of the  
919 mycobacterial outer membrane: Cryo-electron tomography and vitreous sections  
920 reveal the lipid bilayer structure. *Proceedings of the National Academy of Sciences*  
921 **105**, 3963-3967 (2008).
- 922 64. B. Zuber *et al.*, Direct visualization of the outer membrane of mycobacteria and  
923 corynebacteria in their native state. *J Bacteriol* **190**, 5672-5680 (2008).
- 924 65. P. Adhyapak *et al.*, Dynamical Organization of Compositionally Distinct Inner and  
925 Outer Membrane Lipids of Mycobacteria. *Biophysical Journal* **118**, 1279-1291 (2020).
- 926 66. A. V. Hughes *et al.*, Physical Properties of Bacterial Outer Membrane Models: Neutron  
927 Reflectometry & Molecular Simulation. *Biophysical Journal* **116**, 1095-1104 (2019).
- 928 67. R. Friedman, Membrane-Ion Interactions. *The Journal of membrane biology* **251**, 453-  
929 460 (2018).
- 930 68. B. Modak, S. Girkar, R. Narayan, S. Kapoor, Mycobacterial Membranes as Actionable  
931 Targets for Lipid-Centric Therapy in Tuberculosis. *Journal of Medicinal Chemistry* **65**,  
932 3046-3065 (2022).
- 933 69. P. P. Nguyen, T. Kado, M. Prithviraj, M. S. Siegrist, Y. S. Morita, Inositol acylation of  
934 phosphatidylinositol mannosides: a rapid mass response to membrane fluidization in  
935 mycobacteria. *Journal of Lipid Research* **63** (2022).
- 936 70. J. H. Lorent *et al.*, Plasma membranes are asymmetric in lipid unsaturation, packing  
937 and protein shape. *Nat Chem Biol* **16**, 644-652 (2020).
- 938 71. G. von Heijne, Net N-C charge imbalance may be important for signal sequence  
939 function in bacteria. *Journal of Molecular Biology* **192**, 287-290 (1986).
- 940 72. J. A. Baker, W.-C. Wong, B. Eisenhaber, J. Warwicker, F. Eisenhaber, Charged residues  
941 next to transmembrane regions revisited: “Positive-inside rule” is complemented by  
942 the “negative inside depletion/outside enrichment rule”. *BMC Biology* **15**, 66 (2017).
- 943 73. I. D. Pogozeva, S. Tristram-Nagle, H. I. Mosberg, A. L. Lomize, Structural adaptations  
944 of proteins to different biological membranes. *Biochimica et Biophysica Acta (BBA) -*  
945 *Biomembranes* **1828**, 2592-2608 (2013).
- 946 74. R. J. Anderson, P. W. Groundwater, A. Todd, A. J. Worsley, *Antibacterial agents :  
947 chemistry, mode of action, mechanisms of resistance and clinical applications* (Wiley-  
948 Blackwell, Chichester, 2012), pp. 378.
- 949 75. V. A. Dartois, E. J. Rubin, Anti-tuberculosis treatment strategies and drug  
950 development: challenges and priorities. *Nature Reviews Microbiology*  
951 10.1038/s41579-022-00731-y (2022).
- 952 76. A. Krah, G. Grüber, P. J. Bond, Binding properties of the anti-TB drugs bedaquiline and  
953 TBAJ-876 to a mycobacterial F-ATP synthase. *Current Research in Structural Biology* **4**,  
954 278-284 (2022).

- 955 77. F. Bardou, C. Raynaud, C. Ramos, M. A. Lan elle, G. Lan elle, Mechanism of isoniazid  
956 uptake in Mycobacterium tuberculosis. *Microbiology* **144**, 2539-2544 (1998).
- 957 78. A. N. Unissa, S. Subbian, L. E. Hanna, N. Selvakumar, Overview on mechanisms of  
958 isoniazid action and resistance in Mycobacterium tuberculosis. *Infection, Genetics and*  
959 *Evolution* **45**, 474-492 (2016).
- 960 79. M. Jackson *et al.*, Inactivation of the antigen 85C gene profoundly affects the mycolate  
961 content and alters the permeability of the Mycobacterium tuberculosis cell envelope.  
962 *Molecular Microbiology* **31**, 1573-1587 (1999).
- 963 80. A. L. Duncan, A. J. Robinson, J. E. Walker, Cardiolipin binds selectively but transiently  
964 to conserved lysine residues in the rotor of metazoan ATP synthases. *Proceedings of*  
965 *the National Academy of Sciences* **113**, 8687-8692 (2016).
- 966 81. R. A. Corey, P. J. Stansfeld, M. S. P. Sansom, The energetics of protein-lipid interactions  
967 as viewed by molecular simulations. *Biochem Soc Trans* **48**, 25-37 (2020).
- 968 82. J. Briffotiaux, W. Huang, X. Wang, B. Gicquel, MmpS5/MmpL5 as an efflux pump in  
969 Mycobacterium species. *Tuberculosis* **107**, 13-19 (2017).
- 970 83. G. Degiacomi *et al.*, In vitro Study of Bedaquiline Resistance in Mycobacterium  
971 tuberculosis Multi-Drug Resistant Clinical Isolates. *Frontiers in Microbiology* **11** (2020).
- 972 84. R. J. Clarke, K. R. Hossain, K. Cao, Physiological roles of transverse lipid asymmetry of  
973 animal membranes. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1862**,  
974 183382 (2020).
- 975 85. W. Im, S. Khalid, Molecular Simulations of Gram-Negative Bacterial Membranes Come  
976 of Age. *Annual Review of Physical Chemistry* **71**, 171-188 (2020).
- 977 86. J. Augenreich *et al.*, The conical shape of DIM lipids promotes *Mycobacterium*  
978 *tuberculosis* infection of macrophages. *Proceedings of the National Academy of*  
979 *Sciences* **116**, 25649-25658 (2019).
- 980 87. A. Queiroz, L. W. Riley, Bacterial immunostat: Mycobacterium tuberculosis lipids and  
981 their role in the host immune response. *Rev Soc Bras Med Trop* **50**, 9-18 (2017).
- 982 88. K. A. Abrahams, G. S. Besra, Synthesis and recycling of the mycobacterial cell envelope.  
983 *Current Opinion in Microbiology* **60**, 58-65 (2021).
- 984