A tuneable minimal cell membrane reveals that two lipids suffice for life

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Abstract

All cells are encapsulated by a lipid membrane which facilitates the interaction between life and its environment. How life exploits the diverse mixtures of lipids that dictate membrane property and function has been experimentally challenging to address. We introduce an approach to tune and minimize lipidomes in *Mycoplasma mycoides* and the Minimal Cell (JCVI-Syn3A) revealing that a 2-component lipidome can support life. Systematically reintroducing phospholipid features demonstrated that acyl chain diversity is more critical for growth than head group diversity. By tuning lipid chirality, we explored the lipid divide between Archaea and the rest of life, showing that ancestral lipidomes could have been heterochiral. Our approach offers a tuneable minimal membrane system to explore the role of lipid complexity, opening new directions in bioengineering.

Introduction

Cell membranes are complex and responsive systems that serve to protect and mediate interactions of life with its environment. A large part of this molecular complexity is due to the diverse panel of lipids that make up the lipidome and which ultimately determine the form and function of the membrane. The complexity of cellular lipidomes can be staggering, from tens of unique structures in bacteria[1], to hundreds in eukaryotic organisms[2]. How life has evolved to utilize such complex mixtures of lipids to build cellular membranes remains an active area of exploration[3,4]. One approach to studying the role of lipidome complexity is to experimentally tune lipidome composition and observe effects on cell fitness (e.g. growth). A recent study in bacteria, for example, revealed the importance of homeoviscous adaptation for electron

30 transport by tuning acyl chain unsaturation in *Escherichia coli*[5]. However, for interpreting
31 lipidome flexibility, *E. coli*, like many bacteria, is complicated by the fact that it has multiple
32 membranes (e.g. inner and outer). Changes in a single membrane are obfuscated by whole cell
33 lipid extracts, and purifying specific membrane types is laborious, and can lead to substantial
34 experimental error due to varying purity. Gram positive organisms such as *Bacillus subtilis*, that
35 have only a single membrane are better models in this regard[6, 7]. However, traditional genetic
36 approaches to tuning lipidome composition have so far not afforded complete control over both
37 lipid class composition, and phospholipid acyl chain composition. Thus, a cellular model system
38 in which lipidome complexity can be reduced in a systematic fashion has not yet been
39 established.

40 Mycoplasma are a class of genomically simple bacterial pathogens possessing several
41 features that are promising as tunable living model membrane systems. Mycoplasmas have a
42 single plasma membrane, and lack a cell wall[8]. Therefore, their membrane composition and
43 biophysical properties can be examined in situ without the need for laborious membrane
44 isolation. Having evolved to a parasitic lifestyle, mycoplasmas have lost many pathways for
45 biomolecular synthesis, including most of their lipid synthesis pathways, which they take up from
46 their hosts, or the growth media[8, 9, 10, 11, 12]. This affords the possibility to control their lipidome
47 composition by controlling what lipids are provided in the media. Further, because of their
48 relatively small genomes, the number of components involved in managing lipidome composition
49 and membrane adaptation is within reach of being completely characterized and modeled[13].
50 Recently, a minimal cell, JCVI-Syn3.0, was engineered from *Mycoplasma mycoides subsp capri*
51 strain *GM12* by systematically removing genes from *M. mycoides* to achieve an organism where
52 every remaining gene is essential or quasi-essential[14]. To restore normal division and remove
53 pleomorphism, 19 genes were added back to JCVI-Syn3.0 to create JCVI-Syn3A*;* a regularly
54 dividing quasi minimal cell which offers a platform to study the role of lipids for the most
55 fundamental requirements of life in a minimal genetic system[15].

56 In this study we establish an approach to modulate lipidome composition and reduce its
57 complexity in *M. mycoides* and JCVI-Syn3A. By tuning the lipid composition of the growth medium
58 and introducing diether phospholipids we introduce an approach to bypass cellular lipid

remodeling, achieving a lipidome with only two lipids. Using these lipidomically minimal living membranes, we compare the relative importance of phospholipid head group vs. acyl chain complexity. Additionally, we observed profound effects resulting from subtle changes in the lipid chirality that distinguishes Archaea from the rest of life. By developing approaches to tune and minimize mycoplasma lipidome composition, we hope to introduce a new tool for deciphering the principles of living membranes and to introduce a new paradigm for understanding why life has evolved to utilize so many lipids.

Results

Mycoplasmas as minimal model membrane systems

Mycoplasma mycoides is a pathogen of mammals that has historically been used as a simple model membrane system. There are a number of reasons why *M. mycoides* makes a good model membrane system. First, its small genome (~1,100,000 bp) limits the complexity of its genetic regulation, and has allowed researchers to fully sequence and annotate the genome (although a number of genes still have unknown or only putative functional assignments)[8,11]. Second, as a pathogen, the primary source of lipids for *M. mycoides* is through environmental uptake from the host rather than synthesis[8, 9, 10, 11, 16]. In a laboratory setting using growth medium as the lipid source, this feature provides the experimenter with direct control over the lipid components available for synthesizing and maintaining the membrane; by adding or removing specific lipids from the growth medium of *M. mycoides*, the composition of the membrane can be altered. Third, *M. mycoides* has a single plasma membrane and no cell wall or organelles, making it easy to interrogate the membrane and ensuring that membrane targeting probes in *M. mycoides* are acting on the membrane and not another structure[8]. Fourth, and perhaps most importantly for this research, *M. mycoides* is unable to synthesize or alter fatty acid composition[8, 9, 10, 11]. This means that, while it can alter the acyl chain composition of its membrane lipids, it is limited in its ability to do so by the pool of fatty acids it has access to. These factors combine to make *M. mycoides* one of the model systems in which membrane remodeling is both the simplest and most controllable, and as such it is an excellent model system to study membrane remodeling in living organisms[17].

88

89 In *M. mycoides* membranes phospholipids, sterols, and free fatty acids are taken up from
90 the environment and either incorporated into the membrane or taken up into the cytoplasm (Fig.
91 1; 1)[8, 18, 19, 20]. *M. mycoides* can cleave acyl chains from exogenous phospholipids (Fig. 1; 2)[21, 22, 23]
92 When a pool of free fatty acids is present, *M. mycoides* can use those fatty acids to modify the
93 acyl chain composition of phospholipids taken up from the media, or can synthesize the
94 phosphotidylglycerol (PG) class of lipids which can in turn be modified and used to synthesize
95 cardiolipins (Fig. 1; 3, 4, 5)[8, 20, 21, 24, 25, 26, 27]. These newly synthesized or modified lipids can be
96 broken down to replenish the fatty acid pool, or reinserted into the membrane (Fig. 1; 6)[25]. With
97 the exception of the modification of PG headgroups to make cardiolipins, all lipid remodeling in
98 *M. mycoides* is acyl chain remodeling–that is, it relies on having access to a pool of free fatty acids
99 to modify the existing acyl chain composition of phospholipids or synthesize *de novo* PGs[8, 9].
100 Cholesterol is essential for growth of *M. mycoides* (Fig. 1; 8)[28, 29, 30]. For the first time, we report
101 the feeding of *M. mycoides* on a defined lipid diet which contains no exogenous source of free
102 fatty acids. When fed such a diet, *M. mycoides* is forced to rely completely on acyl chain
103 scavenging from exogenous phospholipids as its only source of acyl chains for which to remodel
104 its membrane lipidome.

105

106 ***Minimizing the lipidome***

107 Our first goal was to determine the minimal lipidome that can support growth of *M.*
108 *mycoides*. Sterols, preferably cholesterol, are required for growth, and must be included in any
109 lipid diet. *M. mycoides* can synthesize several phospholipids (e.g. PG and CL) when provided free
110 fatty acids[20, 29, 30]. Therefore, to minimize the phospholipid diversity, we removed free fatty acids
111 from the lipid diet and instead provided a single phospholipid, POPC, along with cholesterol,
112 yielding a minimal 2-component lipid diet. When transferred from growth medium containing
113 fetal bovine serum (FBS, a complex undefined lipid source), to the two-component lipid diet, cells
114 initially grew poorly and tended to aggregate in clumps (not shown). Thin layer chromatography
115 (TLC) of cell lipid extracts showed that, initially, PG and CL (both internally synthesized lipids)
116 disappeared, and only traces of SM remained, presumably carry over from growth on FBS (Fig.

117 2a). After adaptation, sphingomyelin was no longer visible, however, PG and CL reappeared along
118 with lyso-PC (a PC lipid with one of its acyl chains removed). Since *M. mycoides* cannot synthesize
119 phospholipids in the absence of fatty acids, these observations indicate that acyl chains were
120 being scavenged from POPC for the synthesis of PG and CL, presumably as a result of lipase
121 activity (Fig. 2a). A systematic analysis of PG and CL production on minimal lipid diets composed
122 of varying phospholipid species demonstrated that *M. mycoides* is capable of scavenging acyl
123 chains from a broad range of phospholipid head groups (Fig. S1, TableS1), demonstrating a robust
124 capacity for *M. mycoides* to procure acyl chains for internal PG and CL synthesis from nearly any
125 phospholipid source.

126 The capacity for *M. mycoides* to scavenge acyl chains from exogenous phospholipids
127 provided a hurdle to our goal of minimizing the lipidome. Shotgun mass spectrometry of cells
128 grown on the minimal 2-component lipid diet revealed 28 lipid structures generated from the
129 remodeling of POPC, and presence of phospholipids including PG, CL, PA, and DAG (Fig. 2b). Since
130 the source of acyl chains for internal phospholipid synthesis presumably came from exogenous
131 POPC, we reasoned that internal lipid synthesis could be eliminated by blocking acyl chain
132 scavenging. To do this, we replaced POPC with an analogous lipid containing ether-linked 16:0
133 and 18:1 hydrocarbon chains, which are inert to lipase activity[31, 32]. Following the transfer of cells
134 from FBS or minimal POPC-cholesterol diet to a minimal diether PC-diet, TLC analysis of lipid
135 extracts over several passages showed the disappearance of CL and PG, and presence of only two
136 bands corresponding to cholesterol and diether PC (Fig. 2c). Shotgun lipidomic analysis
137 demonstrated that cholesterol and diether PC accounted for greater than 99 mol% of the
138 detected lipids. The remaining very low abundance lipids are derived from impurities in the media
139 and the diether PC stock. It is unlikely that these trace lipids play a significant role physically or
140 chemically, however this highlights the value of establishing a defined growth medium for the
141 continued development of mycoplasmas as minimal model membrane systems[26]. Thus, by
142 introducing an enzymatically inert phospholipid, diether PC, to the minimal lipid diet, we could
143 achieve a minimal lipidome composed predominantly of only two lipid structures, cholesterol
144 and diether PC.
145

146 ***Tuning lipidome composition***

147 Minimizing the lipidome of *M. mycoides* to two lipids resulted in a two-fold decrease in
148 growth rates. We next asked which components of the lipidome are critical for growth. By feeding
149 cells with diether PC, we had reduced lipidome composition in several ways. First, we eliminated
150 the presence of internally synthesized phospholipids such as PG and CL. Therefore, minimizing
151 the diversity of phospholipid head groups could have contributed to impaired growth. Second,
152 we eliminated phospholipid hydrocarbon chain diversity by limiting the cell to one configuration
153 (16:0/18:1). Thus, it is also possible that restricting the diversity of phospholipid hydrocarbon
154 chain configurations could have impaired growth.

155 To test whether head group diversity was a factor in determining growth rate we provided
156 cells with lipid diets containing diether PG and a mixture of diether PG with diether PC. When
157 lipid extracts of cells grown on diether PG were analyzed by TLC, we observed bands
158 corresponding to both PG and CL, indicating that CL synthesis can proceed from diether PG (Fig.
159 3a). When grown on diether PG and PC, TLC analysis showed three bands corresponding to PC,
160 PG, and CL (Fig. 3b). Therefore, cells grown on diether PG generate lipidomes containing two
161 phospholipid head groups and when grown on diether PG and PC generate lipidomes with three
162 head phospholipid head groups. Surprisingly, growth rates for both diether PG and diether PG+PC
163 diets were lower than for diether PC alone (Fig. 3f). Further, growth of cells on POPC prior to
164 adaptation and ability to synthesize PG and CL, show only slightly higher growth rates than
165 diether PC diets, indicating that reduced growth is not solely due to the introduction of diether
166 phospholipids. Phospholipid headgroup diversity alone, therefore, is not sufficient to rescue
167 growth.

168 We next asked whether phospholipid acyl chain diversity could account for impaired
169 growth. To this end, we grew cells on a diet of cholesterol and two fatty acids (palmitate and
170 oleate) designated as '2FA'. On this diet, the phospholipidome is composed predominantly of PG
171 and CL (Fig. 3c) and cells can synthesize phospholipids with a variety of acyl chain configurations
172 (e.g. 16:0/18:1, 16:0/16:0, etc…). Thus, headgroup diversity is effectively two, comparable to
173 growth on diether PG, however, diversity of acyl chain configurations is higher. Growth rates on
174 the 2FA diet were more than twice growth on diether PG, and approached growth rates of cells

175 adapted to growth on POPC (Fig. 3f). Therefore, for a lipidome with two phospholipid
176 headgroups, increased diversity of acyl chain configurations rescues growth, demonstrating the
177 importance of acyl chains for growth.

178 Finally, we asked whether growth could also be rescued by providing the full suite of ~28
179 lipids in cells adapted to growth on POPC. It is possible, for example, that growth is impaired by
180 the disruption of internal phospholipid synthesis or remodeling pathways due to coupling of lipid
181 synthesis with cellular growth. To test this, we took advantage of the fact that when cells grown
182 on the minimal lipid diet are switched to a diet containing ester phospholipids (e.g. POPC +
183 cholesterol) there is a period of adaptation similar to that seen in Fig. 2a. After the first passage,
184 before adaptation, the cells do no yet undergo significant acyl chain scavenging, and the
185 membrane composition remains predominantly composed of the two lipids POPC and
186 cholesterol (Fig. 3d). This delayed adaptation to acyl chain scavenging allows us a brief window
187 to study simplified membranes composed of lipids *M. mycoides* could normally scavenge acyl
188 chains from. The simplified membranes only persist until *M. mycoides* is adapted to the new diet
189 after several passages. We thus transferred cells grown on the minimal diet (Diether PC +
190 cholesterol) to a lipid diet derived from lipid extracts of cells adapted to growth on POPC (Fig.
191 3e), and measured growth in the first passage, before cells adapted to scavenge acyl chains from
192 the dietary phospholipids. Growth rates on this transplanted lipidome diet resulted in a nearly
193 complete rescue to levels observed in POPC cells after adaptation (Fig. 3f). These results indicate
194 that internal phospholipid synthesis and remodeling is not required for optimal growth, and
195 demonstrates a proof-of-principle that functional lipidomes can be engineered and transplanted
196 to living membranes to support growth.

197

198 ***Minimizing the lipidome of the Minimal Cell***

199 JCVI-Syn3A is a synthetic cell created by the J. Craig Venter Institute (JCVI) by removing
200 every non-essential gene from *M. mycoides*[14]. To do this, Hutchison et al. conceptually divided
201 the genome of *M. mycoides* into 8 segments and systematically went through each, removing
202 genes to see which were essential or non-essential (Fig. 4a). As such, JCVI-Syn3A is an even
203 simpler (genetically) model system than *M. mycoides*, while still possessing all of the previously

204 expressed characteristics of *M. mycoides* that make it a valuable model membrane system[33]. In
205 this study we used a strain that expresses an additional gene for the fluorescent mCherry protein,
206 to create JCVI-Syn3A-*mCherry*[15, 34], which for simplicity we subsequently refer to as JCVI-Syn3A.
207 JCVI-Syn3A provides a good comparison organism to *M. mycoides*, and an experimental platform
208 to examine the role of lipidome composition in supporting the minimal requirements for life. We
209 therefore asked whether the JCVI-Syn3A lipidome could also be minimized.

210 Since JCVI-Syn3A has a truncated set of genes compared to *M. mycoides*, we first tested
211 whether they retained the capacity to scavenge acyl chains from exogenous phospholipids for
212 the internal synthesis of PG and CL. Unexpectedly we observed that JCVI-Syn3A could still
213 synthesize PC and CL when fed with a minimal 2-component diet of cholesterol and POPC, as well
214 as with a range of other phospholipid head groups (Fig. 4b). This suggests that an undiscovered
215 lipase remains in the JCVI-Syn3A genome. As a more minimal system, JCVI-Syn3A is a more fragile
216 organism, and not all cells for a given lipid diet survived the growth protocol. To not bias the
217 growth rates, these cells were removed from the growth rate analysis. An interesting feature was
218 noticed, however, that a much higher fraction of cells that could not undergo acyl chain
219 scavenging were non-viable than cells that could (Fig. 4b) further illustrating the decreased
220 fitness of cells unable to remodel acyl chain composition.

221 Next, we assayed growth of JCVI-Syn3A on a 2-component lipid diet of cholesterol +
222 diether PC (16:0/18:1). Growth for JCVI-Syn3A and *M. mycoides* on the Diether POPC +
223 Cholesterol diet are commensurate, but on the POPC + Cholesterol diet *M. mycoides* has
224 significantly improved growth (Fig. 4c). Shotgun lipidomic analysis confirmed that JCVI-Syn3A
225 cells grown on a 2-component diether PC + Cholesterol diet yielded a lipidome with over 99 mol%
226 composed of only two lipid species (Fig. 4d). As such, this work has developed the simplest known
227 living membrane in one of the simplest known living organisms, thereby extending the concept
228 of minimal life from the genome to the lipidome.

229

230 ***Tuning lipid chirality***

231 Having explored the minimal requirements for lipidome complexity in *M. mycoides* and
232 JCVI-Syn3A, we next sought to leverage these model systems to probe another fundamental

233 aspect of lipid biology: chirality. Glycerolipids (including phospholipids) have a chiral center in the
234 glycerol backbone leading to enantiomeric lipids that are mirror images of each other (Fig. 5a).
235 In bacteria and eukaryotes, glycerolipids are synthesized with acyl chains at the sn-1 and sn-2
236 positions, and the phosphate head group at the sn-3 position (Fig. 5a: G3P enantiomer).
237 Conversely, archaea synthesize glycerolipids with the phosphate at the sn-1 position and the acyl
238 chains at the sn-2 and sn-3 positions (G1P enantiomer)[35, 36]. Known as the 'Lipid Divide', this
239 difference in stereochemistry between lipids of archaea and the rest of life has long stood as an
240 unexplained enigma[37, 38, 39, 40]. Did a last common ancestor have membranes with both
241 enantiomers? What are the consequences of having a racemic mixture of phospholipids in a living
242 membrane? To date, no naturally occurring organism has been found that has comparable
243 amounts of both enantiomers in its membrane. Furthermore, experimentally modulating
244 phospholipid chirality in the lipidome through genetic approaches presents significant
245 challenges, as shown by the work of Caforio et al[41]. However, the ability of mycoplasma to uptake
246 exogenous lipids makes them an exceptionally well-suited model for unraveling this elusive
247 problem in membrane biology.

248 To establish whether *M. mycoides* and JCVI-Syn3A can grow on a G1P phospholipid
249 enantiomer, we prepared a lipid diet consisting of cholesterol and enantiomeric POPC (entPOPC),
250 as well as a racemic mixture of cholesterol and POPC:entPOPC (1:1 mol%). We compared the two
251 diets containing entPOPC against growth on cholesterol + POPC (Fig. 5b, 5c). Growth rates of both
252 organisms show that the introduction of entPOPC to the lipid diet results in impaired growth,
253 with the racemic diet yielding the most pronounced decrease in growth rate. Thus, we
254 demonstrate that cells are viable when fed enantiomeric phospholipids.

255 We next asked how the introduction of entPOPC affected the mechanical robustness and
256 permeability of the membrane. To assay membrane robustness, we measured sensitivity of cells
257 to hypoosmotic shock, by determining what fraction of cells are lysed following a shock. Since *M.*
258 *mycoides* and JCVI-Syn3A lack a cell wall or cytoskeleton, cell lysis during rapid hypoosmotic shock
259 is indicative of membrane rupture strength. Hypoosmotic sensitivity increased significantly for
260 cells grown on entPOPC diets, demonstrating that the enantiomeric lipids affect the mechanical
261 robustness of the membrane and, consequently, the whole cell (Fig. 5b, c). To assay permeability,

262 we measured the rate of permeation of fluorescein diacetate (FDA), a non-chiral molecule, across
263 the cell membrane[42, 43]. Membrane permeability, as measured by the permeability coefficient of
264 FDA increased significantly upon the introduction of enantiomeric lipids, but was the highest for
265 both *M. mycoides* and JCVI-Syn3A for the racemic lipid diet (Fig. 5b, c). When plotted against
266 growth rate, there is an apparent correlation of lower growth with higher permeability (Fig 5d,
267 e), possibly indicating membrane leakiness as one of the factors underlying impaired growth. The
268 found permeability coefficients were similar to those previously reported in mammalian cells[43].
269 Increased permeability and reduced membrane robustness could be due either to
270 changes in the property of the lipid bilayer, or through changes in lipid-protein interactions.
271 Previous work on phosphatidylcholine enantiomers in model membranes revealed that modest
272 changes in permeability to calcein occur in membranes composed of both enantiomers, in
273 particular scalemic (not 1:1) mixtures. However, such small changes would not account for the
274 large change in permeability we observe. To determine if introducing enantiomeric POPC
275 affected membrane bilayer robustness or permeability we reconstituted cellular lipid extracts
276 from *M. mycoides* into liposomes and evaluated C-Laurdan fluorescence (Fig. 6). The C-Laurdan
277 General Polarization index (GP) reports bilayer hydration, which is closely coupled with
278 permeability and mechanical robustness of the membrane[44, 45, 46]. Surprisingly, GP values did not
279 vary significantly across all three POPC diets, indicating that the lipid bilayer itself was not
280 disrupted by changes in chirality, and implicating an effect on lipid-protein interactions. Indeed,
281 several studies have demonstrated an effect of varying lipid chirality on lipid-peptide
282 interactions, and the permeability of membranes to chiral amino acids[47, 48]. Thus, disrupted lipid-
283 protein interactions are the most likely basis for the observed phenotypes, setting the stage for
284 future work employing *M. mycoides* and JCVI-Syn3A as model systems to explore the significance
285 of lipid chirality on lipid-protein interactions in a living membrane.
286

287 **Discussion**

288 In this study, we introduce *M. mycoides* and the Minimal Cell JCVI-Syn3A as simple model
289 organisms with tunable lipidomes for studying the role of lipid complexity. By choosing a model
290 membrane system incapable of synthesizing or modifying fatty acids, and developing a set of

291 defined lipid diets for that system, we have demonstrated the creation of a platform in which the
292 lipidome can be tuned in terms of phospholipid head group, acyl chain composition or lipid
293 chirality. Using this platform, we created the simplest living membrane, and one incapable of
294 undergoing acyl chain remodeling. These tunable living membranes allowed us to quantitatively
295 examine the contribution of lipidomic features to the fitness of a minimal living system.
296
297 ***Lipid scavenging***
298 Our observations broadly demonstrate how lipidome composition is crucial even for
299 relatively simple microorganisms. This is anecdotally illustrated by the capacity we observe for
300 mycoplasma to produce a complex lipidome from a single exogenous phospholipid, which is an
301 impressive evolutionary adaptation to their pathogenic lifestyle. Indeed, this acyl chain
302 scavenging activity, which confounded our initial attempt to simplify the lipidome, suggests a
303 potential target for treating mycoplasma infection. Although mycoplasma can survive in pure
304 culture with a reduced lipidome, they may fare much worse in the context of a host immune
305 system. Furthermore, the ability for the Minimal Cell to grow with a lipidome of two lipids implies
306 the possibility for further minimization of the genome, through the deletion of pathways involved
307 in the scavenging of phospholipid acyl chains and the internal synthesis of phospholipids.
308
309 ***Minimal lipidomes***
310 A long-standing challenge in membrane biology has been to understand why life has
311 evolved such complex lipidomes, and to identify the essential features of lipidomes required for
312 optimal membrane function and cellular fitness. The simplest lipidome so far reported was from
313 a Gram-negative bacterium and was composed of 27 lipid species, excluding outer membrane
314 lipopolysaccharides that were not analyzed. By reducing the lipidome of *M. mycoides* and JCVI-
315 Syn3A down to 2 lipids, we show that a complex lipidome is not essential for life, but that two
316 lipids are far from optimal. By systematically reintroducing lipidomic features to cells with a
317 minimal lipidome, we were able to show that diversity of acyl chain configurations is more crucial
318 than diversity of phospholipid head groups. This observation is consistent with previous work
319 showing that reducing the head group diversity of *B. subtilis* had relatively little effect on

320 growth[49]. This is not to say that having a diversity of head groups is not important. There are well-
321 characterized examples of head group chemistry influencing bioactivity, for example the
322 dependence of bacterial peptide secretion machinery on phophatidylethanolamine[50]. We
323 previously showed how headgroup structure influences the biophysical effect of acyl chain
324 composition[4]. Thus, having the right phospholipid head group may be as important as having
325 diverse headgroups. However, acyl chain structure directly influences the properties of the
326 bilayer and the activity of integral membrane proteins. Indeed, the critical role of phospholipid
327 acyl chain unsaturation was recently demonstrated for the assembly and function of the nuclear
328 pore complex in Eukaryotes[51]. Our results demonstrate that the diversity of acyl chain
329 configurations is one of the minimal parameters required for a functional lipidome.
330 One factor limiting the minimization of the lipidome to one lipid is that cholesterol (or an
331 analogue) is required for growth, but also cannot form a bilayer alone. So, it is possible that even
332 one bilayer forming lipid could support life in a cell that has not evolved to require sterols. It is
333 also, however, possible that having a membrane reinforcing sterol or sterol analogue is critical
334 for building a stable cell membrane with only one major phospholipid. A mycoplasma-like
335 organism such as Mesoplasma that does not require sterols for growth would provide a means
336 to test this hypothesis[52].
337
338 ***Lipid chirality and the lipid divide***
339 The lipid divide represents another major problem in membrane biology. Homochirality
340 is a fundamental feature of biomolecular chemistry. Biomolecules exhibit enantioselectivity for
341 chirally compatible interaction partners. Consequently, life has evolved to rely on homochiral
342 molecules (e.g. L-amino acids and nucleic acids). An interesting twist is in the divide between the
343 chirality of phospholipids made by Archaea and the rest of life[35]. The divide raises questions
344 about whether the last universal common ancestor (LUCA) possessed a heterochiral lipidome, or
345 whether the divide happened after the divergence of Bacteria and Archaea[53, 54]. Further, current
346 theories that eukaryotes emerged from an Archaeal ancestor[55], create a conundrum in explaining
347 why Eukaryotes don't have Archaeal lipid enantiomers, and whether a gradual transition

348 involving heterochiral lipidomes could have occurred. At the crux of these conundrums is
349 whether heterochiral lipidomes can support stable membranes and optimal cellular fitness.
350 We took advantage of the tuneability of *M. mycoides* and JCVI-Syn3A lipidomes to
351 observe how heterochiral lipidomes influence the membrane and cellular growth. Previous work
352 in living systems has achieved lipidomes with a mixture of structurally diverse bacterial and
353 archaeal lipids that differed not only by chirality, but also in a variety of other ways[56]. A unique
354 feature of this study is that we were able to introduce two enantiomers of a single lipid structure
355 (POPC) into a living membrane. Our results demonstrate that a heterochiral lipidome results in a
356 leakier membrane and impaired cellular robustness and growth. In contrast, previous work in *E.*
357 *coli* indicated that heterochiral lipidomes did not affect growth[56]. E. coli has an outer membrane
358 and cell wall that might compensate for lack of stability caused by heterochirality. The lipids in
359 the E. coli study were not stereoisomers, but rather entirely different lipid structures (ether vs.
360 ester acyl chain linkages, isoprenoid tails vs. fatty acid tails). It is possible that these structural
361 differences somehow obscured the destabilizing effects of heterochirality. Ultimately, the basis
362 for these diverging observations will provide insight into the biological significance of lipid
363 chirality. Nonetheless, our work shows that in a minimal organism with a single membrane,
364 heterochiral lipidomes can support growth, but lead to impaired robustness and fitness. This
365 demonstrates that ancestral heterochiral lipidomes could have supported life.
366 The changes in membrane robustness and permeability that we observed do not seem to
367 be explained by changes in lipid order of pure lipid vesicles reconstituted from cell lipid extracts
368 of homo- and heterochiral lipidomes. This implies that lipid-protein interactions may be affected
369 by lipid chirality in ways that impair membrane robustness and function. Numerous in vitro
370 studies have demonstrated the effect of lipid chirality on lipid-protein interactions, consistent
371 with the possibility that the phenotypes we observed are rooted in perturbed lipid-protein
372 interactions. Looking forward, *M. mycoides* and JCVI-Syn3A will be excellent model system to
373 explore the functional consequences of lipid chirality on lipid-protein interactions.
374
375 ***Outlook***

376 Our approach to employ *M. mycoides* and JCVI-Syn3A as minimal living model membrane
377 systems paves a new path towards unraveling the role of lipidome diversity and complexity. Our
378 observations reveal that life does not require complex lipidomes. However, minimization comes
379 with clear trade-offs in cellular fitness. We further demonstrate the capacity of these model
380 systems to serve as chassis for exploring fundamental questions in membrane biology. The ability
381 of this system as a testing platform for lipid diets with a variety of features (including class and
382 acyl chain composition) makes it a useful and simple in vivo model for design-test-build
383 applications of membrane composition experiments. Furthermore, we demonstrated the ability
384 to force *M. mycoides* and JCVI-Syn3A to take up and incorporate enantiomeric lipids in their
385 membranes, the first time this has been shown in eukaryotic or prokaryotic organisms, and an
386 exciting first step to allow us to probe questions about the lipid divide and the role of chirality in
387 membrane stability and cell fitness. Overall, we have demonstrated the creation of a simple,
388 tunable, living, model membrane system that can be used as a novel platform for probing the
389 design principles of living membranes.
390

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401

402 ## Materials and Methods

403 Cell Culture:

404 *M. mycoides* and JCVI-Syn3A were grown in liquid culture at 37ºC on a modified SP4 growth
405 medium with Phenol Red[57]. The lipid source was provided either by Fetal Bovine Serum (FBS),
406 complex natural source of mammalian lipids, or by complexing lipids with delipidated bovine
407 serum albumin (BSA). Lipids were dissolved in ethanol and added to the medium at 37ºC

408 immediately before the addition of cells, in the concentrations given in Supplementary Table 2.
409 *M. mycoides* was passaged to new growth medium by adding 50 µL of cell culture at OD600 0.4-
410 0.8 into 7 mL of freshly prepared media in a T25 Flask (Stand., Vent. Cap) or 10mL of media in a
411 Duran 100mL glass flask. This corresponded to roughly one passage per day. JCVI-Syn3A was
412 passaged to new medium by adding 200 µL of cell culture at OD600 0.2-0.6 into 7 mL of freshly
413 prepared media. This corresponded to one passage every two to four days, depending on cell
414 growth rates. Cells were grown at 37ºC and 40RPM in a KuhnerShakerX incubator.
415
416 Growth Rates:
417 Metabolic Growth Index:
418 Due to differences in cell size and behavior between *M. mycoides* and JCVI-Syn3A, measuring
419 growth curves by relying on taking the absorbance at 600nm proved to be an unreliable method
420 to achieve growth estimates that were robustly comparable between the two organisms. Phenol
421 red is a pH indicator that is commonly used as a readout for growth in mycoplasma cell culture[58]
422 as it detects changes in pH as metabolic activity acidifies the culture media. To obtain the rate of
423 pH change over time cells were grown on the liquid handler system Biomek i7 Automated
424 Workstation, and the absorbance at 562 nm was recorded hourly to record changes in phenol
425 red absorbance as cell growth acidifies the media[13, 58, 59]. All absorbances were then plotted with
426 respect to time, and then fitted to the logistic function $Nt = K (1 + (K-No)/No) e^{-rt}$) where Nt is
427 population at time t, K is the carrying capacity, N0 is the initial population size, and r is the growth
428 rate; or, in this case, the rate of pH change. r was determined by calculating the slope of change
429 at the steepest point of the logarithmic curve, corresponding to mid exponential growth. To
430 validate the relevance of this method as a readout for cell growth, growth rates for a
431 representative set of *M. mycoides* diets were calculated using the absorbance of the cells at
432 600nm and were compared to the rates calculated with the phenol red method. The result was
433 a strong linear correlation between the two methods (Supplementary figure 5a). Representative
434 curves of the entire period of growth for *M. mycoides* on one diet and generated by the two
435 methods is also shown (Supplementary figure 5b).
436

437 Cellular growth data was obtained by growing cells on the liquid handler system Biomek i7
438 Automated Workstation with absorbance readings taken hourly at OD 562 nm to record changes
439 in phenol red absorbance as cell growth acidifies the media[13, 58, 59]. Growth rates were
440 determined by calculating the slope of change at the steepest point of the exponential growth
441 curve, corresponding to mid exponential growth.
442
443 Cell growth data measured by optical density at 600nm was obtained through hourly manual
444 measurements from cultures grown in 30mL in a Duran 100 mL glass flask at 37ºC using a DeNovix
445 Spectrophotometer (Supplementary figure 6a,b).
446
447 Lipid Extraction and Thin Layer Chromatography:
448 Lipids were extracted using a Bligh-Dyer lipid extraction protocol[60]. When appropriate, lipid
449 concentration was determined with a phosphate assay. TLC plates with Silica Gel and a 10x
450 concentrating zone were pre-washed with chloroform to remove debris and other artifacts, and
451 were then dried at 60ºC for > 30 minutes to ensure complete solvent evaporation. Lipid samples
452 were then loaded on the plate and were dried at 60ºC for > 10 minutes to again ensure complete
453 solvent evaporation. The plate was then run in a closed glass chamber using a running solution
454 of Chloroform:Methanol:Acetic Acid:Water (85:25:5:4), and were subsequently dried again at
455 60ºC for > 30 minutes. TLC plates were then rinsed with a 3% Copper acetate and 8% phosphoric
456 acid solution and heated by a handheld heat gun at 280ºC to char lipid spots.
457
458 Liposome Preparation: Lipids of a known concentration (either in stock solutions or extracted
459 from cells and validated with a phosphate assay) were prepared in a 2mL glass by having their
460 solvents evaporated overnight under a 10-17mbar vacuum. Lipids were then resuspended in
461 the appropriate volume of Liposome buffer (LiB) (10mM HEPES, 100 mM NaCl) to achieve 100mM
462 liposome solution and incubated for 30 minutes at 37ºC. Subsequently, liposomes were homogenized
463 with ten freeze thaw cycles (1 minute in liquid nitrogen followed by 5 minutes at 60C) and 7 extrusion
464 cycles in a Hamilton syringe setup through a 100nm filter.
465

466 Propidium Iodide Osmotic Shock Assay: Harvest 3X 0.4 ODU of cells with OD 0.2-0.4 (1 ODU
467 = 1 mL of cells at OD 1). Spin down in pre-warmed centrifuge at 5000g, 7minutes, 37ºC, slow
468 acceleration slow deceleration. Add 1 µL of 1mM Propidium Iodide dye to appropriate wells
469 on a black or clear bottom 96well plate. Aspirate medium and resuspend each cell pellet in
470 400 µL of either H2O or 5X diluted mycoplasma wash buffer (MWB) (original MWB: 20mM
471 HEPES, 200 mM NaCl, 1% W/V Glucose). Set up an extra tube with cells resuspended in 400 µL
472 H2O to be boiled as a positive control. Boil control tube in 95ºC thermoshaker at 1000 rpm
473 for 10 minutes. During that time, incubate all other tubes on the 37ºC thermoshaker at
474 600rpm. Add 100 µL of the cells in the appropriate buffer to each of their three analytical
475 wells on the 96 well plate. Incubate 30 minutes with shaking at 37ºC in a TECAN platereader
476 and subsequently measure the fluorescent signal at excitation 539nm emission 619nm.
477
478 Fluorescein Diacetate Permeability Assay: Harvest 3X 0.4 ODU of cells with OD 0.2-0.4 (1 ODU
479 = 1mL of cells at OD 1). Spin down in pre-warmed centrifuge at 5000g, 7 minutes, 37ºC, slow
480 acceleration slow deceleration. Wash 1x in MWB, resuspend in 400 µL MWB. Prepare a
481 Fluorescein Standard curve in plate: 0, 0.25 ,0.5, 1.2, 2 µM Fluorescein Add 100 µL
482 resuspended cells to proper wells (in triplicate), and 100 µL MWB to Fluorescein wells. Add
483 FDA to cell wells to achieve final FDA concentration of 5 µM. Incubate and measure on TECAN
484 (37ºC, 150 minutes, excitation 485 emission 525, measurements every 30 minutes). The
485 permeability coefficient (P) of FDA was calculated using Fick's Law ($Q = P*A*(C_{out} - Ci_n)$) where
486 Q is the flux across the membrane, and is a constant as the slope of fluorescein increase over
487 time was linear; A is the area of the membrane, calculated using the assumption that the
488 cross sectional area of POPC + Cholesterol in a bilayer is 45.1 $Å^2$ [61], the ratio of POPC :
489 Cholesterol is roughly 1:1, the area of POPC + cholesterol is equivalent to the area of
490 Enantiomeric POPC + Cholesterol, and the number of POPC molecules can be calculated from
491 the phosphate assay described below; C_{out} and C_{in} are the concentrations of FDA outside and
492 inside the cell (respectively); and Ci_n is 0 as FDA is immediately converted to fluorescein upon
493 entering the cell.

494

495 C-Laurdan General Polarization Assay: Incubate liposomes at 37ºc and 1000rpm for 10
496 minutes on a tabletop thermoshaker. While incubating, remove C-Laurdan from freezer and
497 let warm to temperature on bench. Add 1 µL C-Laurdan (Dilute 1mM stock 4x in EtOH. Add
498 1uL of diluted stock for final molarity 0.5 µM C-Laurdan) to 500 µL of liposomes. Incubate
499 samples at 37ºc and 1000rpm for 10 minutes on a tabletop thermoshaker.Add 100 µL of each
500 sample to a flat bottom 96 well plate well, repeating three times in order to achieve analytical
501 triplicates. Measure on TECAN (2 channel fluorescence reading with excitation at 385nm and
502 emission at 440 and 490 nm respectively). General polarization (GP) was calculated using the
503 formula: GP = (I440 - I490) / (I440 + I490) where I is the fluorescence emission intensity at the
504 respective wavelength.

505

506 Phosphate Assay: To estimate the concentration of phospholipids in a certain amount of cells,
507 the amount of phosphate in the lipid extraction of 1ODU of cells was measured. To measure
508 phosphate amount a modified version of the method of Chen et al. was used[62]. The lipid
509 extraction was added to glass pyrex tubes with both biological and analytical triplicates (3
510 vials per biological replicate), and the solvent was evaporated by a brief (<5 minute)
511 incubation at 200ºC. 50 µL of water were added to each sample. To prepare a standard curve of
512 phosphate amounts, an ICP Phosphorus standard was diluted in water to give 5, 10, 20, 50 and
513 100, and 200 nmols of phosphate. 500 µL of 70% perchloric acid was added to each vial and, after
514 brief vortexing, samples were incubated at 200ºC for 120 minutes. Tubes were cooled down in
515 ice water, and 1mL of 10% w/v ascorbic acid followed immediately by 1 mL of 2.5% w/v
516 ammonium heptamolybdate were added (with a brief vortex after adding each reagent). Samples
517 were incubated at 37ºC for 30 minutes, and then absorbance at 820nm was measured on a
518 TECAN plate reader by adding 200 µL of each tube to a clear bottom 96 well plate . A standard
519 curve of absorbance at 820 vs phosphate amount was calculated using the phosphate standard,
520 and lipid amount of each sample was calculated based on the absorbance value, standard curve,
521 and assumption that one phosphate molecule equals one phospholipid.

522

523 Lipidomic Analysis: Lipids extracted from cells using the aforementioned Bligh-Dyer protocol
524 were submitted to Lipotype for mass spectrometry based analysis[63]. The general procedure is
525 described in Sampaio et.al, 2011[64]. For the analysis, samples were spiked with internal lipid
526 standard mixture containing: cardiolipin 14:0/14:0/14:0/14:0, ceramide 18:1;2/17:0,
527 diacylglycerol 17:0/17:0, hexosylceramide 18:1;2/12:0, lyso-phosphatidate 17:0, lyso-
528 phosphatidylcholine 12:0, lyso-phosphatidylethanolamine 17:1, lyso-
529 phosphatidylglycerol 17:1, lyso-phosphatidylinositol 17:1, lyso-phosphatidylserine 17:1,
530 phosphatidate 17:0/17:0, phosphatidylcholine 17:0/17:0, diether phosphatidylcholine
531 16:0/18:1, phosphatidylethanolamine 17:0/17:0, phosphatidylglycerol 17:0/17:0,
532 phosphatidylinositol 16:0/16:0, phosphatidylserine 17:0/17:0, cholesterol ester 20:0,
533 sphingomyelin 18:1;2/12:0;0, triacylglycerol 17:0/17:0/17:0, and cholesterol D6. After
534 extraction, the organic phase was transferred to an infusion plate and dried in a speed
535 vacuum concentrator. It was then resuspended in 7.5 mM ammonium acetate in
536 chloroform/methanol/propanol (1:2:4, V:V:V) and a 33% ethanol solution of methylamine
537 in chloroform/methanol (0.003:5:1; V:V:V). All liquid handling steps were performed using
538 Hamilton Robotics STARlet robotic platform with the Anti Droplet Control feature for
539 organic solvents pipetting. Samples were analyzed by direct infusion on a QExactive
540 mass spectrometer (Thermo Scientific) equipped with a TriVersa NanoMate ion source
541 (Advion Biosciences). Samples were analyzed in both positive and negative ion modes
542 with a resolution of Rm/z=200=280000 for MS and Rm/z=200=17500 for MSMS
543 experiments, in a single acquisition. MSMS was triggered by an inclusion list
544 encompassing corresponding MS mass ranges scanned in 1 Da increments. Both MS
545 and MSMS data were combined to monitor CE, DAG and TAG ions as ammonium
546 adducts; PC, PC O-, as acetate adducts; and CL, PA, PE, PE O-, PG, PI and PS as
547 deprotonated anions. MS only was used to monitor LPA, LPE, LPE O-, LPI and LPS as
548 deprotonated anions; ceramide, hexosylceramide, sphingomyelin, LPC and LPC O- as
549 acetate adducts and cholesterol as an ammonium adduct of an acetylated derivative.

550

551 Materials:

552 SP4 (For 1L):

553 PPLO (3.5g) (Becton, Dickinson, and Company product no. 255420)

554 Tryptone (10g) (Sigma product no 70169)

555 Peptone (5g) (Sigma product no 70176)

556 20% Glucose (25mL) (Ross product no. X997.2)

557 20% Yeastolate (10mL) (Becton, Dickinson, and Company product no. 255772)

558 15% Yeast Extract (35mL) (Roth product no. 2904.3)

559 70g/L BSA (85mL) (Sigma product no. A7030) or FBS (170mL) (Biowest Product no.
560 5181H-500)

561 400,000U/mL Penicillin G-sodium salt (2.5mL) (Roth product no. HP48.1)

562 10mg/mL L-Glutamate (5mL) (Roth product no. HN08.2)

563 Sodium Bicarbonate (1.04g) (Honeywell product no. 71630)

564 CMRL (4.9g) (US Biological Lifesciences product no. C5900)

565 Phenol Red (11mg) (Sigma product no. P3532)

566

567 Lipids:

568 Cholesterol (Avanti product no. 700100)

569 Diether POPC (Diether 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine) (Avanti
570 product no. 999983)

571 Diether DPPC (Diether 1-palmitoyl-2-palmitoyl-glycero-3-phosphocholine) (Avanti
572 product no. 999992)

573 Diether DOPC (Diether 1-oleoyl-2-oleoyl-glycero-3-phosphocholine) (Avanti
574 product no. 999991)

575 Diether POPG (Diether 1-palmitoyl-2-oleoyl-glycero-3-phosphotdylglycerol)
576 (Avanti product no. 999973)

577 POPC (1-palmitoyl-2-oleoyl-glycero-3-phosphocholine) (Avanti product no.
578 850457)

579 DPPC (1-palmitoyl-2-palmitoyl-glycero-3-phosphocholine) (Avanti product no.
580 850355)

581 DOPC (1-oleoyl-2-oleoyl-glycero-3-phosphocholine) (Avanti product no. 850375)
582 POPG (1-palmitoyl-2-oleoyl-glycero-3-phosphotdylglycerol) (Avanti product no.
583 840457)
584 DPPG (1-palmitoyl-2-palmitoyl-glycero-3-phosphoglycerol) (Avanti product no.
585 840455)
586 DOPG (1-oleoyl-2-oleoyl-glycero-3-phosphoglycerol) (Avanti product no. 850475)
587 POPE (1-palmitoyl-2-oleoyl-glycero-3-phosphoethanolamine) (Avanti product no.
588 850757)
589
590 DPPE (1-palmitoyl-2-palmitoyl-glycero-3-phosphoethanolamine) (Avanti product
591 no. 850705)
592 DOPE (1-oleoyl-2-oleoyl-glycero-3-phosphoethanolamine) (Avanti product no.
593 850725)
594 Oleic Acid (c*is*-9-Octadecenoic acid) (Sigma product no. 01383)
595 Palmitic Acid (1-Pentadecanecarboxylic acid) (Sigma P0500)
596 Enantiomeric POPC (3-palmitoyl-2-oleoyl-glycero-1-phosphocholine) (Avanti
597 product no. 850855)
598 16-0 Cardiolipin (1',3'-bis[1,2-dipalmitoyl-sn-glycero-3-phospho]-glycerol) (Avanti
599 product no. 710333)
600
601 Assays:
602 FDA (Sigma product no. F7378)
603 Fluorescein (Fluka product no. 28803)
604 Propidium Iodide (Sigma product no. 537059)
605 C-Laurdan (Stratech Scientific Ltd. product no. T0001-SFC-1)
606
607 Cells:
608 JCVI-Syn3A-*mCherry (*From Telesis Bio)
609 *Mycoplasma mycoides subspecies capri strain GM12 (*From Telesis Bio)

610

611 Machines:

612 TECAN

613 Biomek i7 Automated Workstation

614 DeNovix DS-11 FX +

615 PicoQuant FluoTime 300 High Performance Fluorescence Lifetime and Steady

616 State Spectrometer

617

618

619 **Citations**

620 1. van Meer, G., Voelker, D. R. & Feigenson, G. W. Membrane lipids: where they are and
621 how they behave. Nat. Rev. Mol. Cell Biol. 9, 112–124 (2008).
622 2. Membrane lipidome of an epithelial cell line.doi:10.1073/pnas.1019267108.
623 3. Klose, C. et al. Flexibility of a Eukaryotic Lipidome – Insights from Yeast Lipidomics. PLOS
624 ONE 7, e35063 (2012).
625 4. Chwastek, G. et al. Principles of Membrane Adaptation Revealed through Environmentally
626 Induced Bacterial Lipidome Remodeling. Cell Rep. 32, 108165 (2020).
627 5. Viscous control of cellular respiration by membrane lipid composition.
628 https://www.science.org/doi/10.1126/science.aat7925 doi:10.1126/science.aat7925.
629 6. Willdigg, J. R. & Helmann, J. D. Mini Review: Bacterial Membrane Composition and Its
630 Modulation in Response to Stress. Front. Mol. Biosci. 8, (2021).
631 7. Mascher, T., Margulis, N. G., Wang, T., Ye, R. W. & Helmann, J. D. Cell wall stress
632 responses in Bacillus subtilis: the regulatory network of the bacitracin stimulon. Mol. Microbiol.
633 50, 1591–1604 (2003).
634 8. Mycoplasma Cell Membranes. vol. 20 (Springer US, 1993).
635 9. Kornspan, J. D. & Rottem, S. The Phospholipid Profile of Mycoplasmas. J. Lipids 2012,
636 1–8 (2012).
637 10. McElhaney, R. N. Physical Studies of Lipid Organization and Dynamics in Mycoplasma
638 Membranes. in Mycoplasma Cell Membranes (eds. Rottem, S. & Kahane, I.) 53–108 (Springer
639 US, 1993). doi:10.1007/978-1-4615-2924-8_3.
640 11. Bittman, R. Mycoplasma Membrane Lipids Chemical Composition and Transbilayer
641 Distribution. in Mycoplasma Cell Membranes (eds. Rottem, S. & Kahane, I.) 29–52 (Springer US,
642 1993). doi:10.1007/978-1-4615-2924-8_2.
643 12. Razin S, Yogev D, Naot Y. Molecular biology and pathogenicity of mycoplasmas. Microbiol
644 Mol Biol Rev. 1998 Dec;62(4):1094-156. doi: 10.1128/MMBR.62.4.1094-1156.1998. PMID:
645 9841667; PMCID: PMC98941.
646 13. Gaspari, E. et al. Model-driven design allows growth of Mycoplasma pneumoniae on
647 serum-free media. Npj Syst. Biol. Appl. 6, 33 (2020).
648 14. Hutchison, C. A. et al. Design and synthesis of a minimal bacterial genome. Science 351,
649 aad6253 (2016).

15. James F. Pelletier, Lijie Sun, Kim S. Wise, Nacyra Assad-Garcia, Bogumil J. Karas, Thomas J. Deerinck, Mark H. Ellisman, Andreas Mershin, Neil Gershenfeld, Ray-Yuan Chuang, John I. Glass, Elizabeth A. Strychalski, Genetic requirements for cell division in a genomically minimal cell, Cell, Volume 184, Issue 9, 2021, https://doi.org/10.1016/j.cell.2021.03.008.

16. Rottem S, Yashouv J, Ne'eman Z, Razin S. Cholesterol in mycoplasma membranes. Composition, ultrastructure and biological properties of membranes from Mycoplasma mycoides var. capri cells adapted to grow with low cholesterol concentrations. Biochim Biophys Acta. 1973 Nov 16;323(4):495-508. doi: 10.1016/0005-2736(73)90158-2. PMID: 4357439.

17. Archer, D. B. Y. 1975. Modification of the Membrane Composition of *Mycoplasma mycoides* subsp. capri by the Growth Medium. Microbiology 88, 329–338.

18. Dahl J. Uptake of fatty acids by Mycoplasma capricolum. J Bacteriol. 1988 May;170(5):2022-6. doi: 10.1128/jb.170.5.2022-2026.1988. PMID: 2834319; PMCID: PMC211080.

19. Razin, S., Efrati, H., Kutner, S. & Rottem, S. Cholesterol and phospholipid uptake by mycoplasmas. Rev. Infect. Dis. 4 Suppl, S85-92 (1982).

20. Plackett, P. The Synthesis of Polar Lipids by Mycoplasma. Ann. N. Y. Acad. Sci. 143, 158–164 (1967).

21. Salman M, Rottem S. The cell membrane of Mycoplasma penetrans: lipid composition and phospholipase A1 activity. Biochim Biophys Acta. 1995 May 4;1235(2):369-77. doi: 10.1016/0005-2736(95)80026-c. PMID: 7756347.

22. Bhandari, S., Asnani, P.J. Characterization of phospholipase A2 of mycoplasma species. Folia Microbiol 34, 294–301 (1989). https://doi.org/10.1007/BF02814471

23. Rottem S, Adar L, Gross Z, Ne'eman Z, Davis PJ. Incorporation and modification of exogenous phosphatidylcholines by mycoplasmas. J Bacteriol. 1986 Jul;167(1):299-304. doi: 10.1128/jb.167.1.299-304.1986. PMID: 3087959; PMCID: PMC212875.

24. Rottem S. Membrane lipids of mycoplasmas. Biochim Biophys Acta. 1980 May 27;604(1):65-90. doi: 10.1016/0005-2736(80)90585-4. PMID: 6992872

25. Rottem S, Markowitz O. Membrane lipids of Mycoplasma gallisepticum: a disaturated phosphatidylcholine and a phosphatidylglycerol with an unusual positional distribution of fatty acids. Biochemistry. 1979 Jul 10;18(14):2930-5. doi: 10.1021/bi00581a002. PMID: 465448.

26. Thornburg ZR, Bianchi DM, Brier TA, Gilbert BR, Earnest TM, Melo MCR, Safronova N, Sáenz JP, Cook AT, Wise KS, Hutchison CA 3rd, Smith HO, Glass JI, Luthey-Schulten Z. Fundamental behaviors emerge from simulations of a living minimal cell. Cell. 2022 Jan 20;185(2):345-360.e28. doi: 10.1016/j.cell.2021.12.025. PMID: 35063075; PMCID: PMC9985924.

27. Gross Z, Rottem S. Lipid interconversions in aging Mycoplasma capricolum cultures. J Bacteriol. 1986 Sep;167(3):986-91. doi: 10.1128/jb.167.3.986-991.1986. PMID: 3745126; PMCID: PMC215969.

28. Shmuel Razin, Sterols in Mycoplasma Membranes, Current Topics in Membranes and Transport, Academic Press, Volume 17, 1982, Pages 183-205,https://doi.org/10.1016/S0070-2161(08)60311-1.

29. Dahl, J. The Role of Cholesterol in Mycoplasma Membranes. in Mycoplasma Cell Membranes (eds. Rottem, S. & Kahane, I.) 167–188 (Springer US, 1993). doi:10.1007/978-1-4615-2924-8_5.

30. Razin, S., Efrati, H., Kutner, S. & Rottem, S. Cholesterol and phospholipid uptake by mycoplasmas. Rev. Infect. Dis. 4 Suppl, S85-92 (1982).
31. Rangholia, N., Leisner, T. M. & Holly, S. P. Bioactive Ether Lipids: Primordial Modulators of Cellular Signaling. Metabolites 11, 41 (2021).
32. Choquet, C. G., Patel, G. B., Beveridge, T. J. & Sprott, G. D. Stability of pressure-extruded liposomes made from archaeobacterial ether lipids. Appl. Microbiol. Biotechnol. 42, 375–384 (1994).
33. Breuer, M. et al. Essential metabolism for a minimal cell. eLife 8, e36842 (2019).
34. Sakai A, Deich CR, Nelissen FHT, Jonker AJ, Bittencourt DMC, Kempes CP, Wise KS, Heus HA, Huck WTS, Adamala KP, Glass JI. Traditional protocols and optimization methods lead to absent expression in a mycoplasma cell-free gene expression platform. Synth Biol (Oxf). 2022 May 21;7(1):ysac008. doi: 10.1093/synbio/ysac008. PMID: 35774105; PMCID: PMC9239315.
35. van Wolferen, M., Pulschen, A. A., Baum, B., Gribaldo, S. & Albers, S.-V. The cell biology of archaea. Nat. Microbiol. 7, 1744–1755 (2022).
36. Altamura, E. et al. Racemic Phospholipids for Origin of Life Studies. Symmetry 12, 1108 (2020).
37. Villanueva, L. et al. Bridging the membrane lipid divide: bacteria of the FCB group superphylum have the potential to synthesize archaeal ether lipids. ISME J. 15, 168–182 (2021).
38. Martin, H. S., Podolsky, K. A. & Devaraj, N. K. Probing the Role of Chirality in Phospholipid Membranes. ChemBioChem 22, 3148–3157 (2021).
39. Sojo, V. Why the Lipid Divide? Membrane Proteins as Drivers of the Split between the Lipids of the Three Domains of Life. BioEssays 41, 1800251 (2019).
40. Villanueva, L. Engineering E. coli to Have a Hybrid Archaeal/Bacterial Membrane. Trends Microbiol. 26, 559–560 (2018).
41. Caforio, A. et al. Converting Escherichia coli into an archaebacterium with a hybrid heterochiral membrane. Proc. Natl. Acad. Sci. 115, 3704–3709 (2018).
42. Rizk, S. et al. Functional diversity of isoprenoid lipids in Methylobacterium extorquens PA1. Mol. Microbiol. 116, 1064–1078 (2021).
43. Levental, K. R. et al. Lipidomic and biophysical homeostasis of mammalian membranes counteracts dietary lipid perturbations to maintain cellular fitness. Nat. Commun. 11, 1339 (2020).
44. Parasassi, T., De Stasio, G., Ravagnan, G., Rusch, R. M. & Gratton, E. Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of Laurdan fluorescence. Biophys. J. 60, 179–189 (1991).
45. Ma, Y., Benda, A., Kwiatek, J., Owen, D. M. & Gaus, K. Time-Resolved Laurdan Fluorescence Reveals Insights into Membrane Viscosity and Hydration Levels. Biophys. J. 115, 1498–1508 (2018).
46. Steinkühler, J., Sezgin, E., Urbančič, I. et al. Mechanical properties of plasma membrane vesicles correlate with lipid order, viscosity and cell density. Commun Biol 2, 337 (2019).
47. Lei Chen, Wen Si, Liang Zhang, Gangfeng Tang, Zhan-Ting Li, and Jun-Li Hou Journal of the American Chemical Society 2013 135 (6), 2152-2155
48. Takaaki Ishigami, Keishi Suga, and Hiroshi Umakoshi ACS Applied Materials & Interfaces 2015 7 (38), 21065-21072
49. Willdigg JR, Helmann JD. Mini Review: Bacterial Membrane Composition and Its Modulation in Response to Stress. Front Mol Biosci. 2021 May 11;8:634438.

50. van Klompenburg W, Paetzel M, de Jong JM, Dalbey RE, Demel RA, von Heijne G, de Kruijff B. Phosphatidylethanolamine mediates insertion of the catalytic domain of leader peptidase in membranes. FEBS Lett. 1998 Jul 10;431(1):75-9.

51. Romanauska, A., Köhler, A. Lipid saturation controls nuclear envelope function. Nat Cell Biol (2023). https://doi.org/10.1038/s41556-023-01207-8

52. Matteau D, Lachance JC, Grenier F, Gauthier S, Daubenspeck JM, Dybvig K, Garneau D, Knight TF, Jacques PÉ, Rodrigue S. Integrative characterization of the near-minimal bacterium Mesoplasma florum. Mol Syst Biol. 2020 Dec;16(12):e9844. doi: 10.15252/msb.20209844. PMID: 33331123; PMCID: PMC7745072.

53. Koga, Y. Early Evolution of Membrane Lipids: How did the Lipid Divide Occur?. J Mol Evol 72, 274–282 (2011).

54. Rie Matsumi, Haruyuki Atomi, Arnold J.M. Driessen, John van der Oost, Isoprenoid biosynthesis in Archaea – Biochemical and evolutionary implications, Research in Microbiology, Volume 162, Issue 1, 2011, Pages 39-52, ISSN 0923-2508

55. Koonin, E.V. Archaeal ancestors of eukaryotes: not so elusive any more. BMC Biol 13, 84 (2015).

56. Caforio A, Siliakus MF, Exterkate M, Jain S, Jumde VR, Andringa RLH, Kengen SWM, Minnaard AJ, Driessen AJM, van der Oost J. Converting Escherichia coli into an archaebacterium with a hybrid heterochiral membrane. Proc Natl Acad Sci U S A. 2018 Apr 3;115(14):3704-3709.

57. Fritz, B. A., Thomas, C. B., Van Ess, P. & Yuill, T. M. Comparison of a Modified Edward-Type Medium and a Modified SP4-Type Medium for Primary Isolation of Mycoplasma gallisepticum (MG) from Chickens Vaccinated with the F Strain of MG. Avian Dis. 35, 591–598 (1991).

58. Low, I. E. & Eaton, M. D. Replication of Mycoplasma pneumoniae in Broth Culture. J. Bacteriol. 89, 725–728 (1965).

59. MEUR, S. K., SIKDAR, A., SRIVASTAVA, N. C. & SRIVASTAVA, S. K. Rapid photometric assay of growth of *Mycoplasma mycoides* subsp. capri. J. Appl. Bacteriol. 66, 301–302 (1989).

60. Bligh, E. G. & Dyer, W. J. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917 (1959).

61. Leftin A, Molugu TR, Job C, Beyer K, Brown MF. Area per lipid and cholesterol interactions in membranes from separated local-field (13)C NMR spectroscopy. Biophys J. 2014 Nov 18;107(10):2274-86. doi: 10.1016/j.bpj.2014.07.044. PMID: 25418296; PMCID: PMC4241443.

62. Chen, P. S., Toriuaka, T. Y. and Warner, H. (1956). Microdetermination of phosphorus. Anal. Chew., 28. 1756-1758.

63. Ejsing CS, Sampaio JL, Surendranath V, Duchoslav E, Ekroos K, Klemm RW, Simons K, Shevchenko A. Global analysis of the yeast lipidome by quantitative shotgun mass spectrometry. Proc Natl Acad Sci U S A. 2009 Feb 17;106(7):2136-41. doi: 10.1073/pnas.0811700106. Epub 2009 Jan 27. PMID: 19174513; PMCID: PMC2650121.

64. Sampaio JL, Gerl MJ, Klose C, Ejsing CS, Beug H, Simons K, Shevchenko A. Membrane lipidome of an epithelial cell line. Proc Natl Acad Sci U S A. 2011 Feb 1;108(5):1903-7. doi: 10.1073/pnas.1019267108. Epub 2011 Jan 18. PMID: 21245337; PMCID: PMC3033259.

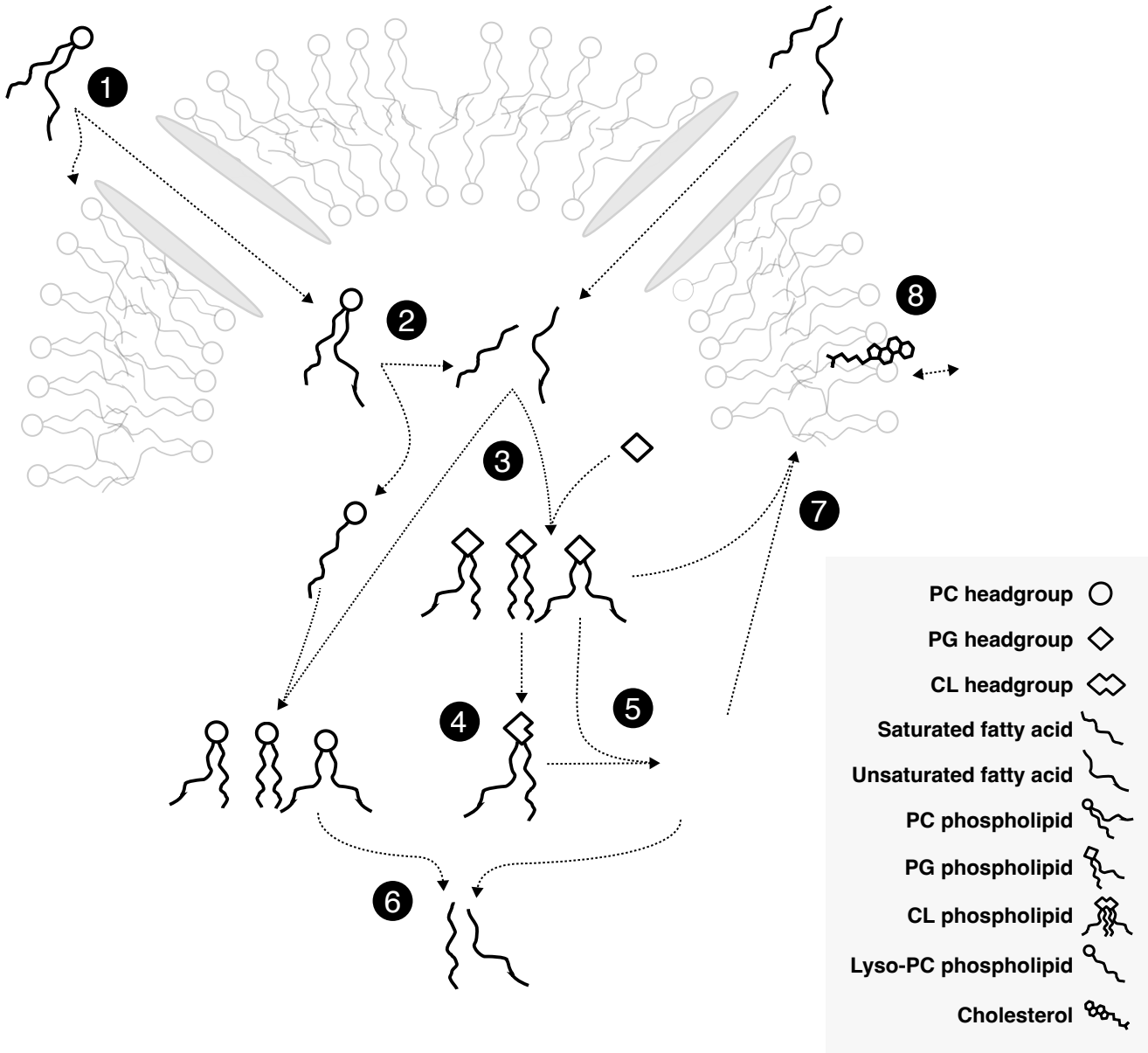
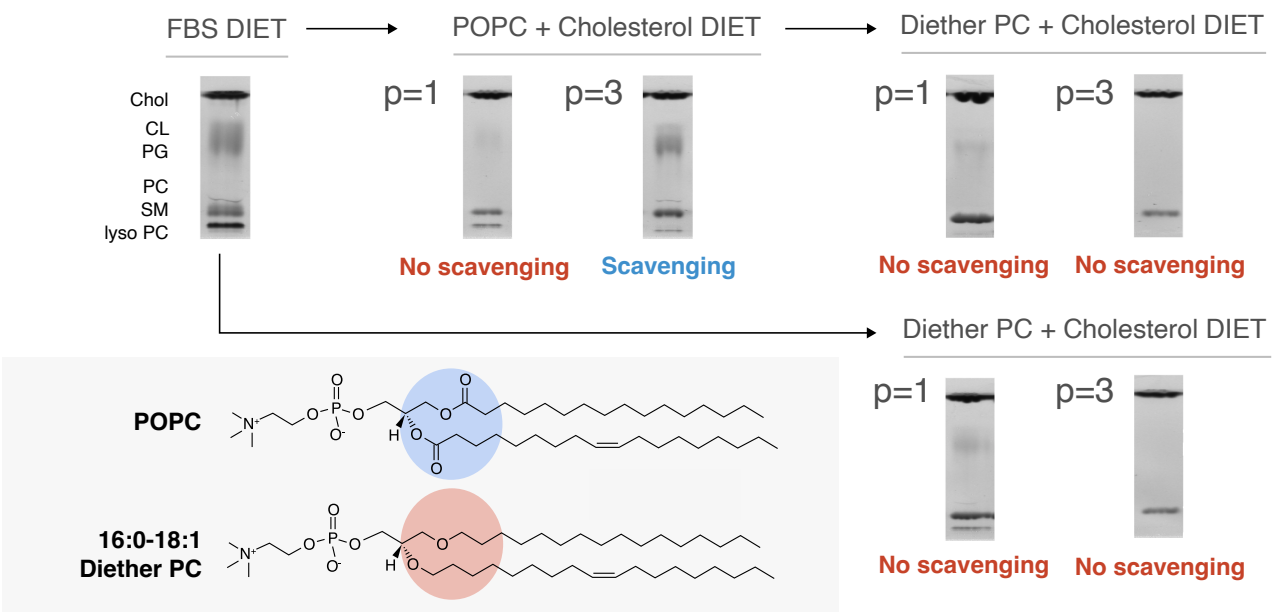
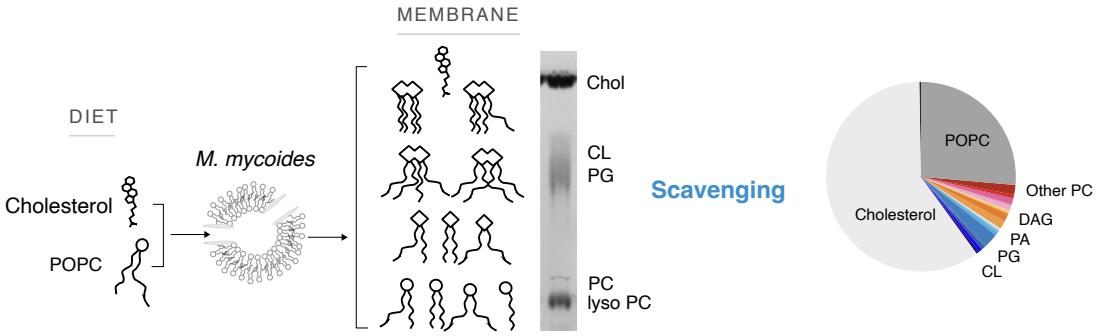


Figure 1: Membrane Remodeling in *M. Mycoides* is Dependent on Acyl Chain Scavenging. 1 *Mycoplasma mycoides* can take up phospholipids and free fatty acids from its environment.[8, 18, 19, 20] **2** Cleaving ester bonds from glycerophospholipids results in a pool of free fatty acids.[21, 22, 23] **3** With fatty acids, *M. mycoides* is able to synthesize Phosphatidylglycerols (PG) with various fatty acid compositions, as well as remodel other phospholipid classes.[8, 20, 21, 24, 25] **4** Cardiolipins can be synthesized from two PGs, starting with cleavage of PG headgroups.[26, 27] **5** *M. mycoides* can synthesize a diversity of Cardiolipins from PGs.[8, 24, 26, 27] **6** Cleaving acyl chains from remodeled and synthesized phospholipids replenishes the pool of free fatty acids.[25] **7** Glycerophospholipids are inserted into the membrane.[23] **8** Cholesterol is essential for *M. mycoides* growth.[28, 29, 30]

2a Adaptation of cells to a defined diet



2b Minimal diet: POPC + Cholesterol



2c Minimal diet: D.PC + Cholesterol

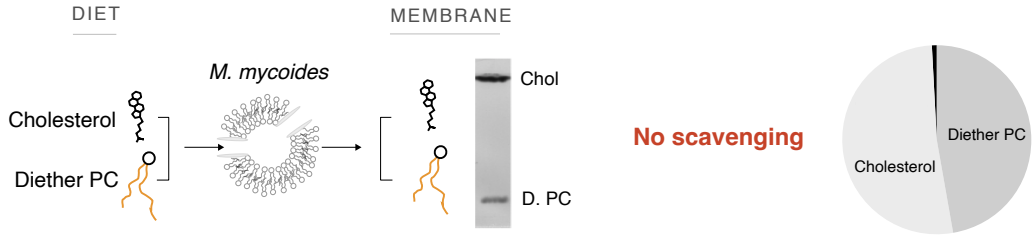


Figure 2: A Defined Lipid Diet Results in a Living Membrane with Two Lipids. **a** *M. mycoides* can adapt to defined lipid "diets" with diester or diether phospholipids; resulting in simpler membranes than when grown on a complex diet (e.g. FBS). Adaptation to new diets occours after 3 passages ($p > 3$). TLC aspect ratio modified for legibility; unmodified TLCs can be found in Figure S4. **b** *M. mycoides* can scavenge acyl chains from POPC, yielding a more complex membrane from a defined diet. Lipidomic analysis shows acyl chain scavenging leads to the synthesis of a diversity of lipids, resulting in a membrane with 28 lipids from a diet of only two. **c** 16:0-18:1 diether PC has ether-linked hydrocarbon chains that cannot be cleaved by *M. mycoides*, eliminating scavenging. Lipidomic analysis shows living cells with two lipids comprising 99.33 mol% of their lipidome.

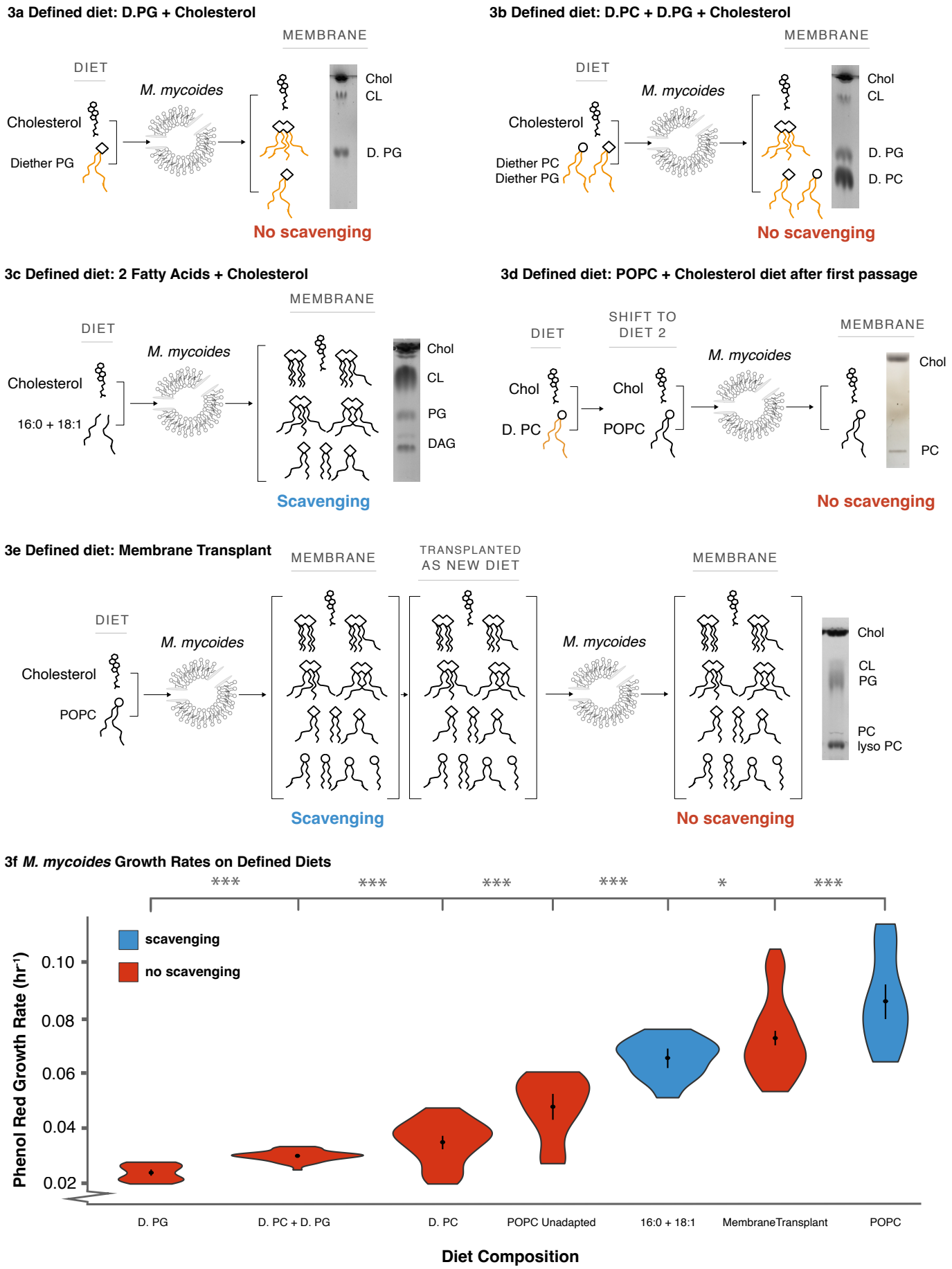
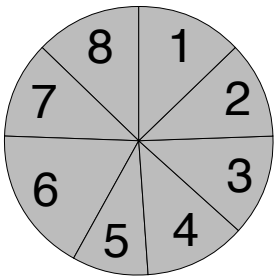


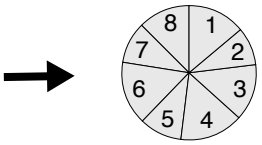
Figure 3: Living Membranes with Tunable Lipid Class and Acyl Chain Complexity. a 16:0-18:1 diether PG diets results in the synthesis of Cardiolipin with fully ether-linked hydrocarbon chains, increasing head group, but not acyl chain complexity. **b** A diet of both Diether PC and PG restores the headgroup, but not acyl chain, complexity of the POPC diet. **c** A diet of free fatty acids restores the acyl chain, but not head group, complexity of the POPC diet. **d** When switched from one diet to another, in this case from the D. PC diet to the POPC diet, there is an adaptation period before acyl chain scavenging occurs.**e** A diet derived from a total lipid extract of cells grown on the POPC diet (a "transplant" diet) restores the full complexity of the POPC. Growth on this transplanted diet was performed after the first passage ($p = 1$) to the new diet, before cells begin to scavenge acyl chains from exogenous phospholipids. **f** Reintroducing acyl chain complexity is more effective than headgroup complexity for rescuing growth (relative to cells adapted to a POPC diet), but, even when full complexity is restored, acyl chain scavenging capacity still improves growth.

4a

Mycoplasma mycoides subsp. capri strain GM12 genome:
1,084,586 bp, 910 genes

Segment 1: 140,739 bp
2: 125,388 bp
3: 133,208 bp
4: 131,623 bp
5: 101,708 bp
6: 189,357 bp
7: 124,976 bp
8: 137,887 bp

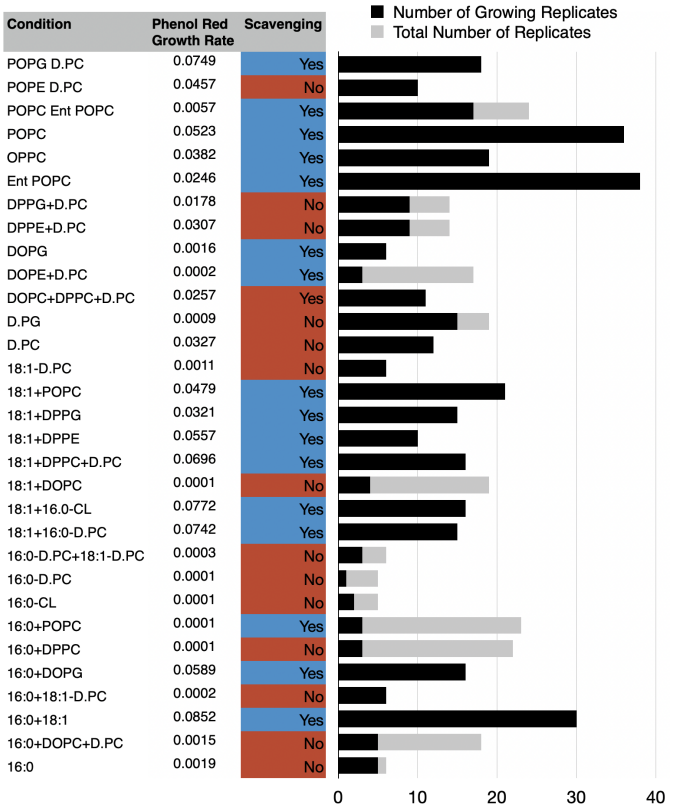




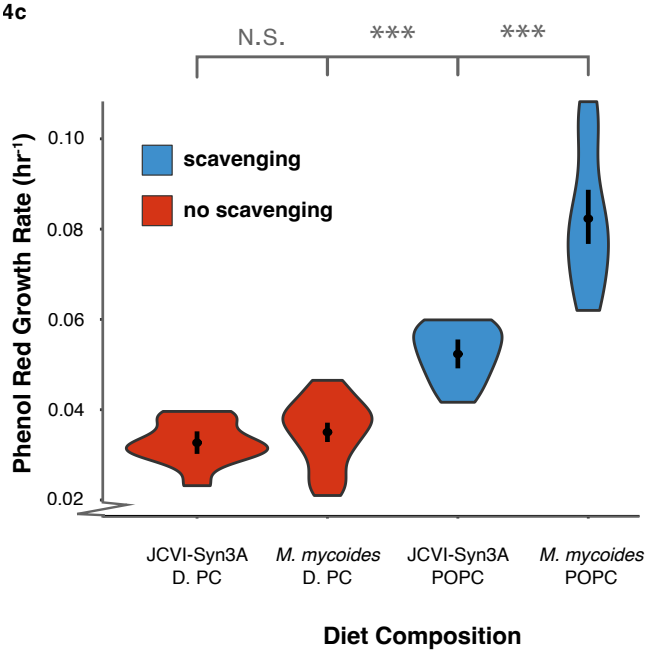
JCVI-Syn3A genome:
543,379 bp, 591 genes

Segment 1: 75,732 bp
2: 49,888 bp
3: 73,958 bp
4: 82,531 bp
5: 56,501 bp
6: 83,060 bp
7: 54,482 bp
8: 66,717 bp

4b



4c



4d

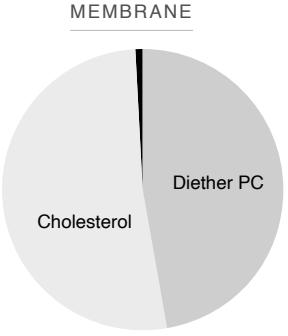


Figure 4:JCVI-Syn3A is a Simpler Cell with a More Fragile Membrane. **a** JCVI-Syn3A is minimal synthetic cell analog of *M. mycoides.* Adapted from Hutchison et. al, 2016. **b** Some JCVI- Syn3A replicates exhibit no growth on a number of lipid diets, especially where acyl chain scavenging is restricted. **c** JCVI-Syn3A grows significantly slower than *M. mycoides* on the same diets. **d** JCVI-Syn3A is viable grown on a Diether PC + Cholesterol diet with 99.16 mol% of the membrane comprised of only two lipids.

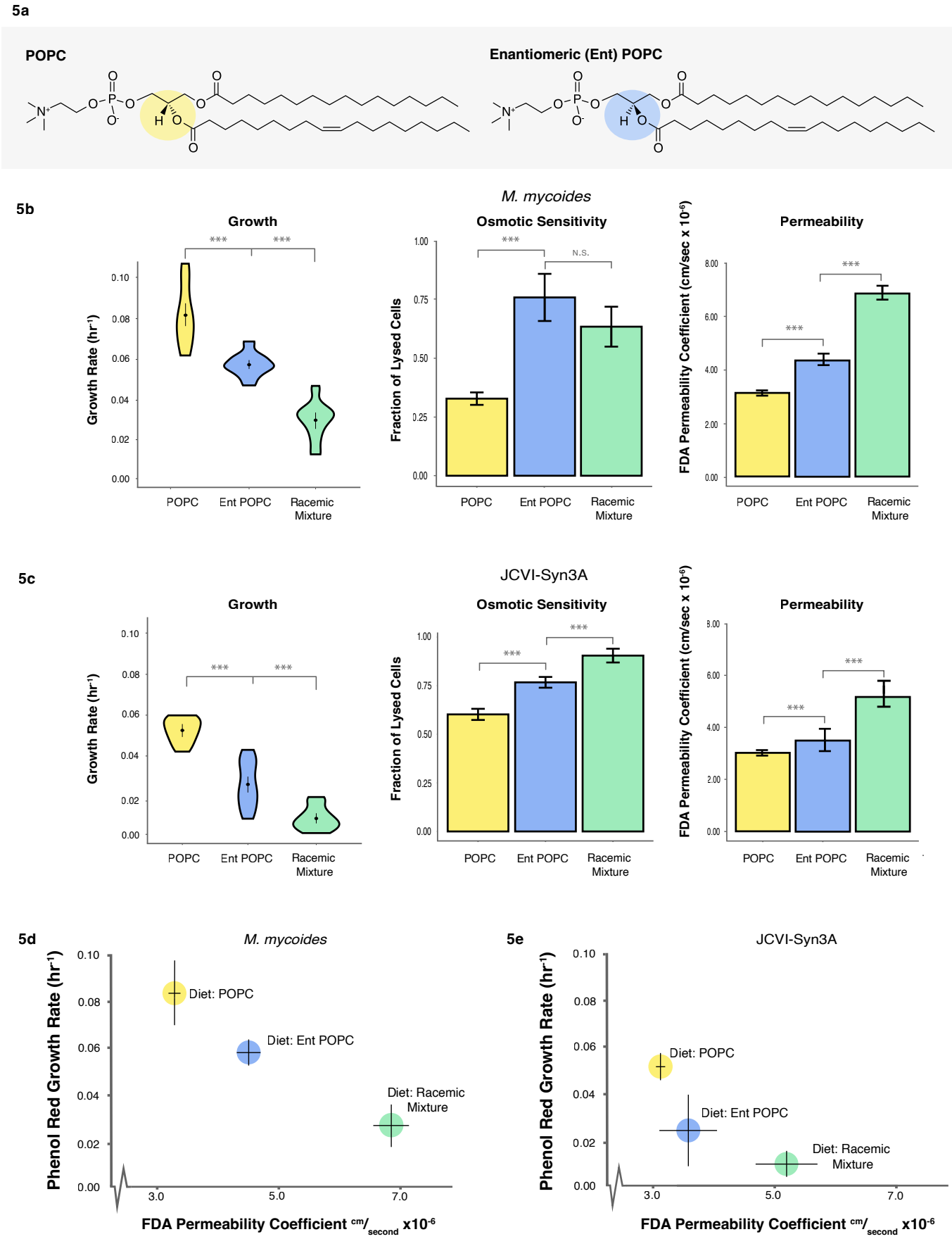


Figure 5: Enantiomeric Lipid Diets Negatively Affect Cell Growth and Membrane Properties. a Enantiomeric (Ent) POPC is a synthetic chiral POPC with the head group in the SN1 position, rather than SN3 position. **b** *M. mycoides* and **c** JCVI-Syn3A cells exhibit lower growth when grown on diets with enantiomeric POPC present; are more fragile to hypoosmotic shock when grown on diets with enantiomeric POPC present; and are more permeable to non-chiral fluorescein diacetate (FDA) when grown on diets with enantiomeric POPC present. **d** *M. mycoides* and **e** JCVI-Syn3A cell growth and membrane permeability are inversely correlated.

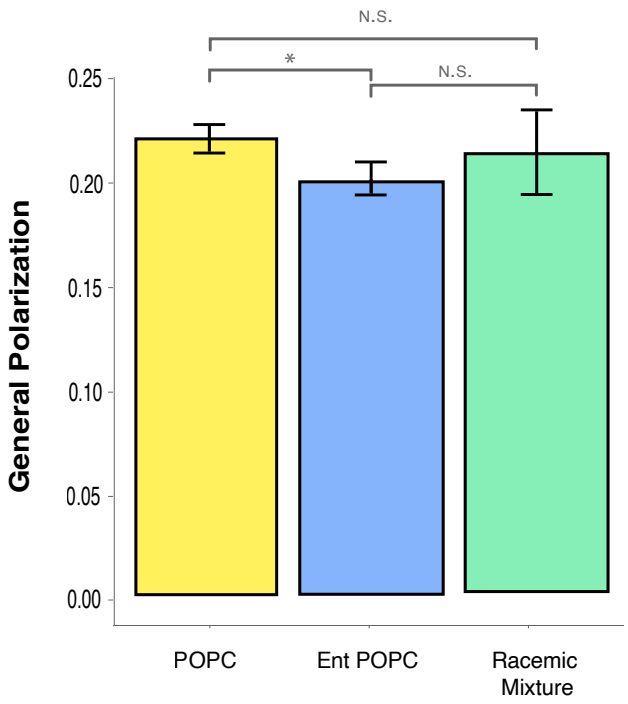


Figure 6: Enantiomeric Lipids Have no Significant Effect on Membrane Order. Lipid vesicles reconstituted from total lipid extracts of *M. mycoides* cells had a similar membrane order (measured as the general polarization of the membrane using C-laurdan) for all three diets with POPC enantiomers.