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# Membrane adaptation in the hyperthermophilic archaeon *Pyrococcus furiosus* relies upon a novel strategy involving glycerol monoalkyl glycerol tetraether lipids

Maxime Tourte, Philippe Schaeffer, Vincent Grossi, Philippe M Oger

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1 **Membrane adaptation in the hyperthermophilic archaeon *Pyrococcus furiosus***  
2 **relies upon a novel strategy involving glycerol monoalkyl glycerol tetraether**  
3 **lipids**

4  
5 Maxime Tourte<sup>1,2</sup>, Philippe Schaeffer<sup>3</sup>, Vincent Grossi<sup>4</sup>, Philippe M. Oger<sup>2\*</sup>

6 ORCID IDs: 0000-0001-5089-7416 (MT); 0000-0003-0618-0834 (PS); 0000-0001-6263-3813 (VG); 0000-  
7 0001-6298-6870 (PMO)

8 <sup>1</sup> Univ Lyon, Univ. Lyon 1, CNRS, UMR 5240, F-69622, Villeurbanne, France

9 <sup>2</sup> Univ Lyon, INSA Lyon, CNRS, UMR 5240, F-69621, Villeurbanne, France

10 <sup>3</sup> Univ. Strasbourg, CNRS, UMR 7177, F-67000 Strasbourg, France

11 <sup>4</sup> Univ Lyon, Univ. Lyon 1, CNRS, ENSL, UJM, UMR 5276 LGL-TPE, F-69622 Villeurbanne, France

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15 **Corresponding author**

16 \*Philippe M. Oger, E-mail: philippe.oger@insa-lyon.fr, Tel: 0033 04 72 43 36 01

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19 **Running title**

20 Membrane adaptation in *Pyrococcus furiosus*

21 **Keywords**

22 Archaeal membrane lipids, extremophiles, *Pyrococcus*, stress response, tetraethers

## 23 **Abstract**

24 Microbes preserve membrane functionality under fluctuating environmental conditions by modulating their  
25 membrane lipid composition. Although several studies have documented membrane adaptations in Archaea,  
26 the influence of most biotic and abiotic factors on archaeal lipid compositions remains underexplored. Here,  
27 we studied the influence of temperature, pH, salinity, the presence/absence of elemental sulfur, the carbon  
28 source, and the genetic background on the core lipid composition of the hyperthermophilic neutrophilic  
29 marine archaeon *Pyrococcus furiosus*. Every growth parameter tested affected the core lipid composition to  
30 some extent, the carbon source and the genetic background having the greatest influence. Surprisingly, *P.*  
31 *furiosus* appeared to only marginally rely on the two major responses implemented by Archaea, i.e., the  
32 regulation of the ratio of diether to tetraether lipids and that of the number of cyclopentane rings in  
33 tetraethers. Instead, this species increased the ratio of glycerol monoalkyl glycerol tetraethers (GMGT, aka.  
34 H-shaped tetraethers) to glycerol dialkyl glycerol tetraethers (GDGT) in response to decreasing temperature  
35 and pH and increasing salinity, thus providing for the first time evidence of adaptive functions for GMGT.  
36 Besides *P. furiosus*, numerous other species synthesize significant proportions of GMGT, which suggests  
37 that this unprecedented adaptive strategy might be common in Archaea.

38

## 39 **Significance statement**

40 We describe here the membrane adaptive strategies the hyperthermophilic, neutrophilic, and marine model  
41 archaeon *Pyrococcus furiosus* implements in response to one of the largest sets of environmental stressors  
42 tested to date, including temperature, pH, salinity, presence/absence of elemental sulfur, carbon source, and  
43 genetic background. In contrast to the other archaea investigated so far, which response mainly involves the  
44 modulation of their diether/tetraether ratio and/or of their average number of cyclopentane rings, *P. furiosus*  
45 regulates its monoalkyl (so called H-shaped) to dialkyl tetraether ratio. Our study thus provides for the first  
46 time evidence of adaptive functions of archaeal monoalkyl tetraethers towards low temperature and pH and  
47 high salinity.

## 48 **Introduction**

49 Membranes are essential compartments that control the cell inward and outward fluxes and support  
50 bioenergetic processes. These functions are however impeded by environmental conditions that microbes  
51 have to face in nature. To ensure proper membrane physicochemical properties and thus preserve cellular  
52 integrity and functions under contrasting conditions, Bacteria, Eukarya, and Archaea modulate their lipid  
53 compositions (Ernst *et al.*, 2016).

54 One of the most diagnostic features of Archaea compared to Bacteria and Eukarya is the unique structure  
55 of their membrane lipids. Indeed, instead of the lipids built upon straight fatty-acyl chains ester-bound to a  
56 glycerol backbone in a *sn*-1,2 configuration commonly found in Bacteria and Eukarya, Archaea synthesize  
57 lipids with polyisoprenoid alkyl chains that are ether-bound to a glycerol in a *sn*-2,3 configuration (De Rosa  
58 and Gambacorta, 1988). In addition to these typical bilayer-forming lipids, hereafter referred to as diether  
59 lipids, most Archaea are also capable of synthesizing membrane-spanning lipids, hereafter referred to as  
60 tetraether lipids, that generate monolayer membranes (De Rosa and Gambacorta, 1988). Recent  
61 technological advances in lipidomics further enlarged the diversity of lipid structures Archaea are able to  
62 synthesize, which now includes mono- and dialkyl glycerol diethers (MGD and DGD) with C<sub>20</sub> and/or C<sub>25</sub>  
63 alkyl chains (De Rosa *et al.*, 1986), glycerol mono-, di-, and trialkyl glycerol tetraethers (GMGT aka. H-  
64 GDGT, GDGT, and GTGT, respectively; (Morii *et al.*, 1998; Knappy *et al.*, 2011)), di- and tetraether lipids  
65 with hydroxylated and/or unsaturated isoprenoid chains (Gambacorta *et al.*, 1993; Nichols *et al.*, 2004), and  
66 tetraether lipids with glycerol, butanetriol, pentanetriol, and nonitol backbones (Becker *et al.*, 2016) (Figure  
67 1). Besides this variety of lipid skeletons, or core lipids, Archaea also synthesize diverse polar head groups  
68 which mostly resemble those of Bacteria and Eukarya, i.e., phospho- and glyco-lipids deriving from sugars  
69 (glycerol, inositol, glucose, *N*-acetylhexosamine) (Jensen *et al.*, 2015), aminoacids (serine, ethanolamine)  
70 (Koga *et al.*, 1993), or combinations of both (Koga *et al.*, 1993) (Figure 1).

71 Archaea are the main inhabitants of the most severe environments on Earth, would it be due to  
72 temperature, pH, salinity, or hydrostatic pressure (Schleper *et al.*, 1995; Takai *et al.*, 2008; Birrien *et al.*,  
73 2011), and this tolerance to multiple extreme conditions has been associated with the peculiar structure of

74 their lipids. Indeed, polyisoprenoid alkyl chains provide greater membrane packing and impermeability  
75 compared to fatty acyl chains (Komatsu and Chong, 1998), while ether bonds are chemically and thermally  
76 more resistant than ester linkages (Baba *et al.*, 1999). Additionally, monolayer membranes generated by  
77 membrane-spanning tetraether lipids further enhance rigidity and impermeability compared to archaeal  
78 bilayers (Chong, 2010). While these physicochemical properties of classic diether and tetraether lipids  
79 rationalize the tolerance of Archaea to extreme conditions, the adaptive function and the behavior of  
80 membranes built upon the variety of lipids they can synthesize remain largely uncharted. Several membrane  
81 responses to abiotic stresses have nonetheless been evidenced in Archaea. The major membrane adaptation  
82 strategy in species producing a mixture of both di- and tetraether lipids consists in modulating the  
83 diether/tetraether ratio with changing growth conditions, which is congruent with the observation that  
84 monolayer-forming tetraether lipids tend to increase membrane packing, and thus stability, impermeability  
85 and rigidity compared to bilayer-forming diether lipids (Chong, 2010). In a pioneer work, Macalady and  
86 colleagues indeed reported increased proportions of tetraethers in archaeal species with lower pH optima  
87 (Macalady *et al.*, 2004). While such adjustments of the lipid compositions to optimal growth conditions do  
88 relate to adaptation, they do on a long time scale and are often not congruent with short-term adaptations –  
89 i.e., modifications of the lipid compositions in response to varying growth conditions – that were considered  
90 here. Decreased diether/tetraether ratios were observed in response to increasing temperature, decreasing  
91 hydrostatic pressure, or decreasing pH in a variety of Archaea (Sprott *et al.*, 1991; Lai *et al.*, 2008; Matsuno  
92 *et al.*, 2009; Cario *et al.*, 2015; Jensen *et al.*, 2015). In contrast, the membrane response of species producing  
93 only tetraether lipids involves the fine regulation of the number of unsaturations – in the form of  
94 cyclopentane rings – present along the hydrophobic alkyl chains in response to temperature and pH  
95 (Shimada *et al.*, 2008; Elling *et al.*, 2014; Jensen *et al.*, 2015; Bale *et al.*, 2019), according to the rationale  
96 that a higher number of rings induces a more compact membrane and, hence, enhances stability,  
97 impermeability and rigidity (Gliozzi *et al.*, 1983; Gabriel and Chong, 2000). However, whereas Archaea  
98 living at lower pH do tend to have higher numbers of rings (long term), there are examples of strains  
99 decreasing their ring index in response to decreased pH (short term; see for instance *Thermoplasma*

100 *acidophilum* in (Shimada *et al.*, 2008)). In a similar manner, the membrane homeostasis of species at the  
101 opposite side of the lipid spectrum, i.e., producing exclusively diether lipids, also involves the regulation of  
102 the lipid unsaturation levels – here in the form of double bonds (Nichols *et al.*, 2004; Gibson *et al.*, 2005;  
103 Dawson *et al.*, 2012). For instance, the psychrophilic archaeon *Methanococoides burtonii* increases its  
104 relative proportions of unsaturated diether lipids in response to decreased temperature (Nichols *et al.*, 2004),  
105 in a way reminiscent of the typical adaptive strategy employed by Eukaryotes and Bacteria (Grossi *et al.*,  
106 2010; Ernst *et al.*, 2016). Last, in addition to typical diether and tetraether lipids, the hyperthermophilic  
107 methanogen *Methanocaldococcus jannaschii* synthesizes an intriguing macrocyclic diether lipid in which  
108 the two phytanyl chains are covalently linked *via* a C-C bond, hereafter referred to as cMGD (Figure 1;  
109 Comita *et al.*, 1984)). This covalent bond reduces the lateral motion of the cMGD and increases membrane  
110 packing, stability and impermeability to solutes and protons (Dannenmuller *et al.*, 2000; Arakawa *et al.*,  
111 2001). The relative proportions of cMGD in *M. jannaschii* was thus naturally observed to increase in  
112 response to increased temperature (Sprott *et al.*, 1991). While all the aforementioned membrane responses  
113 concerned typical abiotic factors, Archaea were also demonstrated to modulate their lipid compositions in  
114 response to numerous (if not all) biotic parameters, e.g., carbon, phosphorous, and nitrogen sources and  
115 availability (Langworthy, 1977; Matsuno *et al.*, 2009; Elling *et al.*, 2014; Meador *et al.*, 2014; Feyhl-Buska  
116 *et al.*, 2016; Quehenberger *et al.*, 2020; Zhou *et al.*, 2020), although such reports remain scarce. The relative  
117 contribution of the polar moieties of the ether lipids in the membrane adaptation of Archaea remains in  
118 contrast underexplored.

119 *Pyrococcus furiosus* is a model archaeon belonging to the Thermococcales order that has been isolated  
120 from geothermally heated sediments on the coast of Vulcano Island, Italy (Fiala and Stetter, 1986), which  
121 are naturally subjected to contrasting conditions due to changes in tide and geothermal regimes (Rogers *et al.*  
122 *et al.*, 2007). The growth of *P. furiosus* occurs by fermentation of peptide and/or sugar mixtures under a large  
123 range of growth conditions, including temperature from 70 to 103 °C (optimal 98 °C), pH from 5 to 9  
124 (optimal 6.8), and salinity from 0.5 to 5 % *w/v* of NaCl (optimal 3 % NaCl), which allows the description  
125 of its adaptive strategy towards a large spectrum of biotic and abiotic parameters. In addition, preliminary

126 investigations of *P. furiosus* membrane content revealed a unique lipid composition (Figure S1). While it  
127 exhibits a limited diversity of polar head groups, i.e., phosphoinositol and a few derivatives (Spratt *et al.*,  
128 1997; Lobasso *et al.*, 2012; Tourte *et al.*, 2020a), those are attached to as many as 14 different core  
129 structures, namely DGD, GDGT with 0 to 4 cyclopentane rings (GDGT0-4, respectively), GTGT with 0 to  
130 2 cyclopentane rings (GTGT0-2, respectively), and GMGT with 0 to 4 cyclopentane rings (GMGT0-4)  
131 (Tourte *et al.*, 2020a) (Figure S1), paving the way for the elucidation of uncommon membrane adaptive  
132 strategies. Indeed, we show here that *P. furiosus* modulates the ratio of GMGT/GDGT and marginally  
133 regulates the number of cyclopentane rings instead of altering the diether/tetraether ratio as most other  
134 Archaea do, to respond to temperature, pH, salinity, the presence/absence of elemental sulfur, growth  
135 medium, and genetic background. In particular, the relative proportions of GMGT were significantly  
136 increased at low pH and high salinity, which highlights for the first time the adaptive functions of these  
137 intriguing core lipid structures to pH and salinity in a hyperthermophilic archaeon.

138

## 139 **Results**

### 140 ***Pyrococcus furiosus* displays a large core lipid diversity under contrasting growth conditions**

141 Although the hyperthermophilic archaeon *Pyrococcus furiosus* DSM3638 is able to grow over a wide  
142 range of conditions (Fiala and Stetter, 1986), only some of them allow growth yields ( $> 10^8$  cell ml<sup>-1</sup>) and  
143 rates ( $> 0.08$  h<sup>-1</sup>) compatible with lipid analysis. For instance, temperatures below 80 °C were not assessed  
144 despite *P. furiosus* being theoretically able to grow down to 70 °C. As numerous biotic parameters have  
145 been shown to impact lipid compositions (e.g., growth stage (Elling *et al.*, 2014)), growth was tightly  
146 monitored (refer to Table S1 for growth measurements under the conditions tested here) and cultures were  
147 all inoculated at a cell density of 10<sup>5</sup> cell ml<sup>-1</sup> and harvested in late log phase so as to limit the influence of  
148 other parameters on the lipid compositions observed.

149 *P. furiosus* DSM3638 was previously shown to produce at least 14 different core lipid structures  
150 (Figure S1) under optimal growth conditions, i.e., in Thermococcales rich medium (TRM) at 98 °C and pH  
151 6.8, with 3 % w/v NaCl and 10 g L<sup>-1</sup> of elemental sulfur (Tourte *et al.*, 2020a). Nine core structures, i.e.,

152 DGD, GDGT with up to 3 cyclopentane rings (GDGT0 to 3), GTGT without cyclopentane ring (GTGT0),  
153 and GMGT with up to 3 cyclopentane rings (GMGT0 to 3) were detected in all conditions tested and  
154 constituted the core set of core lipid structures, whereas the five remaining core lipids, i.e., GDGT with 4  
155 cyclopentane rings (GDGT4; up to 3.5 %), GTGT with 1 or 2 cyclopentane rings (GTGT1 and 2; up to 2.4  
156 and 1.0 %, respectively), and GMGT with 4 cyclopentane rings (GMGT4; up to 0.8 %), were only detected  
157 in low proportions under specific conditions (Table 1), and were thus considered as accessory or minor  
158 lipids.

### 159 **Impact of temperature on the core lipid composition of *P. furiosus***

160 The effect of growth temperature on the membrane core lipid composition of *P. furiosus* was  
161 investigated with cultures grown at 80, 85, 90, 98 ( $T_{opt}$ ), and 103 °C. The same core lipid structures, i.e.,  
162 DGD, GDGT0 to 3, GTGT0, and GMGT0 to 3 were detected under all temperatures tested, with the  
163 exception of GDGT4 which was detected only when *P. furiosus* was grown at 103 °C (Table 1, Figure 2A).  
164 To better account for the diversity of GDGT and GMGT structures, the proportions of all derivatives from  
165 each class of tetraether lipids (GDGT, GMGT) were summed and their diversity was represented by their  
166 average number of rings per molecule, or ring index (RI). Different RI were calculated to evaluate the  
167 incorporation of cyclopentane rings in the different tetraether populations, namely  $RI_{Tetraethers}$  which  
168 represents the average number of cyclopentane rings in all tetraethers, and  $RI_{GDGT}$  and  $RI_{GMGT}$  which  
169 represent the average number of cyclopentane rings in GDGT and GMGT, respectively (see methods for  
170 the calculation formulas).

171 Small differences were observed in the DGD and GTGT contents under the distinct growth  
172 temperatures tested. The relative proportions of DGD ranged from  $25.7 \pm 2.0$  % at 85 °C to  $33.6 \pm 4.4$  % at  
173 80 °C and those of GTGT0 from  $1.2 \pm 0.4$  % at 80 °C to  $4.2 \pm 0.5$  % at 90 °C (Table 1, Figures 2D and 2E).  
174 In contrast, the GDGT and GMGT contents showed much larger variations. The two lowest temperatures  
175 (80 and 85 °C) and the three highest (90, 98 and 103 °C) differed significantly from one another based on  
176 their GDGT (~25-30 % vs. ~50 %) and GMGT (~40 % vs. ~20-25 %) compositions (Table 1, Figures 2B  
177 and 2C), which would suggest the existence of a threshold temperature above which GMGT are substituted



178 by GDGT. This is best illustrated when representing the ratio between GMGT and GDGT (GMGT/GDGT)  
179 which dropped from  $1.78 \pm 0.67$  and  $1.51 \pm 0.25$  at 80 and 85°C to  $0.50 \pm 0.5$ ,  $0.43 \pm 0.04$  and  $0.57 \pm 0.10$   
180 at 90, 98, and 103 °C, respectively (Table 1, Figure 2G). The membrane response of *P. furiosus* DSM3638  
181 to temperature is further completed by variations of the  $RI_{GMGT}$  and  $RI_{GDGT}$ . Although all the different RI  
182 increased with temperature, they showed contrasting trends. Whereas  $RI_{GDGT}$  and  $RI_{Tetraethers}$  continuously  
183 increased from  $0.11 \pm 0.02$  and  $0.08 \pm 0.01$  at 85 °C to  $0.55 \pm 0.12$  and  $0.40 \pm 0.08$  at 103 °C, respectively  
184 (Table 1, Figure 2F),  $RI_{GMGT}$  values were constant at *ca.*  $0.06-0.10 \pm 0.01$  from 85 °C to 98°C, and only  
185 increased significantly at 103 °C to reach  $0.18 \pm 0.04$ .

186 Core lipid compositions and temperatures were correlated using the Spearman's coefficient  $\rho$  (Table  
187 2). Although a clear critical threshold can be drawn between 85 and 90 °C, no clear trend was observed  
188 between the GMGT/GDGT ratio and temperature. No significant correlation between DGD, GTGT, and  
189 GMGT proportions and temperature was observed, but GDGT, and especially cyclopentane ring-containing  
190 GDGT, were significantly positively correlated with temperatures ( $\rho = +0.90$ ,  $+1.00$ ,  $+1.00$  and  $+1.00$  for  
191 all GDGT, GDGT1, GDGT2 and GDGT3, respectively). As a consequence,  $RI_{GDGT}$  ( $\rho = +0.90$ ) and  
192  $RI_{Tetraethers}$  ( $\rho = +0.90$ ) were also significantly positively correlated with temperature. This was also valid for  
193  $RI_{GMGT}$  ( $\rho = +0.90$ ), although no clear trend could be highlighted between temperature and the proportion  
194 of GMGT with and without cyclopentane rings.

### 195 **Effect of pH on the core lipid composition of *P. furiosus***

196 Although *Pyrococcus furiosus* DSM3638 has been reported to be able to grow from pH 5.0 to 9.0,  
197 we could only assess its membrane adaptation to mild acidic pH (from 5.0 to 6.8) due to the instability of  
198 alkaline buffers at temperatures close to 100 °C. Under all the tested pH, the same set of core lipid structures,  
199 i.e., DGD, GDGT0 to 3, GTGT0, and GMGT0 to 3 was found (Table 1, Figure 3A). As seen for the  
200 temperature experiments, small differences in DGD and GTGT0 contents were observed, whereas the  
201 proportions of GDGT and GMGT showed much larger variations. Indeed, the relative proportions of DGD  
202 ranged from  $22.8 \pm 4.4$  % at pH 5.6 to  $35.6 \pm 5.2$  % at pH 6.2 and those of GTGT0 varied from  $1.5 \pm 0.6$  %  
203 at pH 6.4 to  $3.7 \pm 0.2$  % at pH 5.9 (Table 1, Figures 3D and 3E), whereas GDGT and GMGT showed relative

204 abundances ranging from  $22.5 \pm 3.5$  % and  $50.3 \pm 4.4$  % at pH 5.5 to  $60.8 \pm 15.7$  % to  $10.3 \pm 9.7$  % at pH  
205 6.4, respectively (Table 1, Figures 3B and 3C). In a manner similar to what was observed for temperature,  
206 two distinct groups could be delineated: cultures grown at pH 5.5, 5.6, and 5.9 had more GMGT than GDGT,  
207 and thus GMGT/GDGT ratios above 1 (i.e.,  $2.28 \pm 0.47$ ,  $1.23 \pm 0.14$ , and  $1.36 \pm 0.10$ , respectively), whereas  
208 cultures grown at higher pH, i.e. 6.2, 6.4, and 6.6, had more GDGT than GMGT and displayed  
209 GMGT/GDGT ratios below 1 (i.e.,  $0.77 \pm 0.57$ ,  $0.21 \pm 0.25$  and  $0.43 \pm 0.04$ , respectively; Table 1, Figure  
210 3G). In contrast to the response to temperature though, no clear trend could be observed in the RI values  
211 regardless of the class of tetraether (Table 1, Figure 3F). Altogether, these results suggest that pH mostly  
212 alters the GMGT/GDGT ratio in *P. furiosus*. This was further supported by the Spearman's correlations  
213 (Table 2): while the summed GDGT and GDGT0 relative proportions significantly increased with pH ( $\rho =$   
214  $+0.89$  for both), those of the summed GMGT and GMGT0 showed an opposite trend ( $\rho = -0.94$  for both),  
215 which resulted in a GMGT/GDGT ratio significantly negatively correlated with pH ( $\rho = -0.89$ ). In contrast,  
216 none of the DGD, GTGT, or ring-containing structures, and thus none of the RI, significantly correlated  
217 with pH.

#### 218 **Effect of salinity on the core lipid composition of *P. furiosus***

219 We tested the membrane response of *P. furiosus* strain DSM3638 to salinity covering from 1 to 4  
220 % w/v of NaCl, as salinities outside this range resulted in extremely low growth yields. Cultures grown  
221 under all salinities tested exhibited the same set of core lipids, i.e., DGD, GDGT0 to 3, GTGT0 and GMGT0  
222 to 2 (Table 1, Figure 4A). As for temperature and pH, small differences in the DGD and GTGT0 contents  
223 were observed, the relative proportions of DGD ranging from  $27.0 \pm 1.5$  % at 4 % NaCl to  $39.3 \pm 9.0$  % at  
224 3 % NaCl and those of GTGT0 from  $1.1 \pm 0.3$  % at 4 % NaCl to  $2.9 \pm 0.4$  % at 2 % NaCl (Table 1, Figures  
225 4D and 4E). Only the GTGT0 content at 4 % NaCl appeared significantly distinctive (*ca.* 1.1 % vs. above  
226 2.0 %). Similarly to the aforementioned stresses, the relative abundances of GDGT and GMGT were more  
227 affected by changes in salinity and ranged from  $35.6 \pm 9.4$  % and  $27.1 \pm 7.4$  % at 1 % NaCl to  $17.3 \pm 4.2$  %  
228 and  $54.6 \pm 3.6$  % at 4 % NaCl, respectively (Table 1, Figures 4B and 4C). The GMGT/GDGT ratio thus  
229 increased with increasing salinity, with values ranging from  $0.83 \pm 0.37$  at 1 % NaCl to  $3.30 \pm 0.92$  at 4 %

230 NaCl, respectively (Table 1, Figure 4G). In contrast to pH and temperature, increasing salinity tended to  
231 decrease the  $RI_{GDGT}$ ,  $RI_{GMGT}$  and  $RI_{Tetraethers}$ , with values ranging from  $0.31 \pm 0.04$ ,  $0.15 \pm 0.01$ , and  $0.23 \pm$   
232  $0.02$  at 1 % NaCl to  $0.13 \pm 0.04$ ,  $0.05 \pm 0.02$ , and  $0.07 \pm 0.03$  at 4 % NaCl, respectively (Table 1, Figure  
233 4F). The summed GMGT and GMGT0 ( $\rho = +1.00$  for both) and the GMGT/GDGT ratio ( $\rho = +1.00$ ) were  
234 significantly positively correlated with the % NaCl, whereas significant negative correlations were observed  
235 between the % NaCl and GDGT1 to 3 and  $RI_{GDGT}$ ,  $RI_{GMGT}$  and  $RI_{Tetraethers}$  ( $\rho = -1.00$  for all) (Table 2). Thus,  
236 the major alterations triggered by salinity on the core lipid composition of *P. furiosus* appeared to be the  
237 fine tuning of the GMGT/GDGT ratio and of the number of cyclopentane rings.

### 238 **Impact of the presence/absence of elemental sulfur on the core lipid composition of *P. furiosus***

239 Like other Thermococcales, *P. furiosus* uses sulfur to detoxify  $H_2$ , a major by-product of its  
240 heterotrophic metabolism (Chou *et al.*, 2007). Although elevated concentrations of  $H_2$  in closed cultures are  
241 toxic for some Thermococcales, significant growth of *P. furiosus* can be achieved without the addition of  
242 sulfur (Fiala and Stetter, 1986). The absence of elemental sulfur nonetheless requires a lag time for  
243 adaptation, which suggests that the lack of elemental sulfur is perceived as a stress (Table S1). Here, we  
244 investigated the influence of the presence or absence of elemental sulfur on *P. furiosus* core lipid  
245 composition by assessing two concentrations: 0 (-S) and  $10 \text{ g L}^{-1}$  (saturation level; +S). Under such growth  
246 conditions, the same core lipid structures, i.e., DGD, GDGT0 to 3, GTGT0 and GMGT0 to 2, could be  
247 identified (Table 1, Figure S2A). In contrast to all the other parameters tested, the core lipid compositions  
248 were not significantly different in the presence or absence of elemental sulfur. For instance, growth with  
249 sulfur resulted in relative proportions of DGD, GDGT, and GMGT of  $39.3 \pm 9.0$ ,  $14.9 \pm 4.0$ , and  $43.6 \pm 5.8$   
250 %, respectively, whereas those after growth without sulfur were of  $34.1 \pm 7.8$ ,  $16.8 \pm 5.2$ , and  $48.6 \pm 8.7$  %,   
251 respectively (Table 1, Figure S2B-D). Similarly, no significant differences were observed for the  
252 GMGT/GDGT ratio and the RI values (Table 1, Figure S2F-G). Only the relative proportions of GTGT0  
253 were significantly different between the two conditions, i.e.,  $2.2 \pm 0.9$  with and  $0.5 \pm 0.1$  % without  
254 elemental sulfur (Table 1, Figure S2E).

255 **Impact of the carbon source on the core lipid composition of *P. furiosus***

256 It is well established in Bacteria and Eukaryotes that the carbon source greatly impacts membrane  
257 lipid compositions (Vinçon-Laugier *et al.*, 2016). We tested the impact of the switch from a proteinaceous  
258 carbon source in TRM medium to a reducing sugar, namely cellobiose, in defined cellobiose (DC) medium  
259 on the lipid composition of *P. furiosus* DSM3638. Surprisingly, in addition to the core lipids typically  
260 synthesized by the strain, i.e., DGD, GDGT0 to 4, GTGT0, and GMGT0 to 3, novel core structures, i.e.,  
261 GTGT1 and 2, and GMGT4, were identified during growth in DC medium (Table 1, Figure 5A). Despite  
262 these additional core lipid structures, the relative abundances of DGD ( $47.6 \pm 22.2$  vs.  $39.3 \pm 9.0$ ), summed  
263 GDGT ( $14.9 \pm 4.0$  vs.  $20.2 \pm 8.2$ ), and summed GMGT ( $43.6 \pm 5.8$  vs.  $25.9 \pm 12.3$ ) were not significantly  
264 different between DC and TRM media, respectively (Table 1, Figure 5B-D). When grown in DC medium,  
265 *P. furiosus* nonetheless harbored significantly higher proportions of GDGT1 to 4 and of GMGT1 to 4, which  
266 resulted in much higher values for  $RI_{GDGT}$ ,  $RI_{GMGT}$ , and  $RI_{Tetraethers}$ , i.e.,  $2.10 \pm 0.32$ ,  $1.46 \pm 0.40$ , and  $1.61 \pm$   
267  $0.34$  in DC compared to  $0.29 \pm 0.04$ ,  $0.13 \pm 0.02$ , and  $0.16 \pm 0.03$  in TRM, respectively (Table 1, Figure  
268 5F). In contrast to any other growth condition tested here, the nature of the growth medium impacted the  
269 total proportions of GTGT, which was significantly higher in DC ( $6.3 \pm 1.7$ ) than in TRM medium ( $2.3 \pm$   
270  $0.9$ ), notably due to the presence of GTGT1 ( $2.4 \pm 1.0$ ) and GTGT2 ( $1.0 \pm 0.4$ ) that were not detected in  
271 TRM medium (Table 1, Figure 5E). Similarly to all the RI calculated previously,  $RI_{GTGT}$  was thus much  
272 higher in DC than in TRM medium ( $0.69 \pm 0.12$  vs.  $0$ ; Table 1, Figure 5F). The major core lipid composition  
273 changes triggered by the switch in carbon source from protein to sugar thus appeared mostly restricted to  
274 cyclopentane ring-containing tetraethers.

275 **Lipid composition of the quasi-isogenic *P. furiosus* strain COM1**

276 We tested whether small genetic modifications could induce variations in the lipid compositions of  
277 near isogenic strains of *P. furiosus*, i.e., the wild type strain DSM3638 and the genetically tractable COM1  
278 strain which is a *pyrF*-deleted derivative of DSM3638 (Bridger *et al.*, 2012). As expected, the two isolates  
279 exhibited the same set of core lipids structures, i.e., DGD, GDGT0 to 3, GTGT0, and GMGT0 to 3, but in  
280 surprisingly different proportions (Table 1, Figure 6A). Indeed, strains COM1 and DSM3638 showed

281 notably different GDGT ( $75.2 \pm 10.8\%$  vs.  $14.9 \pm 4.0\%$ ) and GMGT ( $1.4 \pm 0.2\%$  vs.  $43.6 \pm 5.8\%$ ) contents,  
282 which resulted in very contrasting GMGT/GDGT ratios ( $0.02 \pm 0.01$  vs.  $2.99 \pm 0.37$ ; Table 1, Figures 6B,  
283 6C and 6G). The higher total proportion of GDGT observed for strain COM1 reflects a significant increase  
284 in the abundance of all ring-containing GDGT structures, including GDGT4, a core structure that was not  
285 detected in strain DSM3638 under optimal growth conditions (Table 1, Figure 6B). Although strain COM1  
286 exhibited minor proportions of GMGT, ring-containing structures also appeared in comparatively higher  
287 proportions. The  $RI_{GDGT}$ ,  $RI_{GMGT}$ , and  $RI_{Tetraethers}$  were consequently higher for COM1, with values of  $0.80$   
288  $\pm 0.11$ ,  $0.37 \pm 0.07$ , and  $0.76 \pm 0.12$  for strain COM1 compared to  $0.29 \pm 0.04$ ,  $0.13 \pm 0.02$ , and  $0.16 \pm 0.03$   
289 for strain DSM3638, respectively (Table 1, Figure 6F).

290

## 291 Discussion

### 292 The membrane response of *Pyrococcus furiosus* follows a particular strategy involving the alteration 293 of the GMGT/GDGT ratio

294 The 14 different core lipid structures identified in the present study in the hyperthermophilic and  
295 neutrophilic marine archaeon *Pyrococcus furiosus* are in accordance with those reported previously (Tourte  
296 *et al.*, 2020a), which included DGD, GTGT0 to 2, GDGT0 to 4 and GMGT0 to 4 (Figure S1). GTGT2 and  
297 GMGT2 to 4 were only sporadically reported in Archaea (see for instance Knappy *et al.*, 2011; Bauersachs  
298 *et al.*, 2015 and references therein), but were regularly detected in *P. furiosus*, confirming the very peculiar  
299 core lipid composition of this archaeon compared to closely related Thermococcales and to other Archaea  
300 (Tourte *et al.*, 2020b). Here, we examined the role of these peculiar lipids in the stress response of *P.*  
301 *furiosus*, and showed that all but the presence/absence of sulfur in the growth medium affected the core lipid  
302 composition of *P. furiosus* to some extent.

303 In contrast to other archaea producing both di- and tetraether lipids (Lai *et al.*, 2008; Matsuno *et al.*,  
304 2009; Cario *et al.*, 2015), we have surprisingly found no evidence for a regulation of the diether/tetraether  
305 ratio in *P. furiosus*. In addition, although a trend seems to exist between the RI and some of the stressors  
306 tested, the variations in the number of rings per molecule remain very limited (often below 0.2), and do not

307 reach what is observed for instance in thermoacidophiles, which RI values usually vary by *ca.* 0.5-1 unit  
308 over the range of the environmental stressor (Shimada *et al.*, 2008; Feyhl-Buska *et al.*, 2016). *P. furiosus*  
309 thus appears to rely on an uncommon and specific membrane adaptation strategy which involves the  
310 modification of the ratio of two tetraether core lipid classes, namely glycerol mono- and dialkyl glycerol  
311 tetraethers (GMGT and GDGT, respectively).

312 GMGT are a particular type of tetraether lipids exhibiting a covalent C-C bond between the two C<sub>40</sub>  
313 alkyl chains (Morii *et al.*, 1998) and can represent a significant proportion of membrane lipids in numerous  
314 archaea, such as *Methanothermus fervidus* (31 %; Morii *et al.*, 1998), *Ignisphaera aggregans* (39 %;  
315 Knappy *et al.*, 2011), *Methanothermococcus okinawensis* (15 %; Baumann *et al.*, 2018), and a few  
316 Thermococcales (e.g., 50 % in *Thermococcus waiotapuensis*, 34 % in *P. horikoshii*, and *ca.* 15 % in *T. celer*  
317 and *T. guaymasensis*; Sugai *et al.*, 2004; Tourte *et al.*, 2020b). Since all these archaea are  
318 (hyper)thermophiles and since the abundance of GMGT was observed to positively correlate with the mean  
319 annual air temperature in peats (Naafs *et al.*, 2018), GMGT were first associated with the adaptation to  
320 increased temperature. Although the role of GMGT in terms of membrane structure and properties remains  
321 uncharacterized, the presence of a covalent C-C bond between the two alkyl chains suggests that the free  
322 motion of the GMGT molecule will be strongly reduced, thus resulting in increased membrane stability and  
323 impermeability compared to GDGT in a manner similar to that of cMGD *vs.* DGD (Sprott *et al.*, 1991;  
324 Dannenmuller *et al.*, 2000; Arakawa *et al.*, 2001). Building on this hypothesis, GMGT were then proposed  
325 to partake in adaptation of Archaea to high temperature, but also to high salinity and low pH (Schouten *et*  
326 *al.*, 2008a; Knappy *et al.*, 2011). However, to date, there has been no demonstration of the role of GMGT  
327 in the membrane response of Archaea to any stressor, which thus remains elusive. While GMGT did appear  
328 to have a role in adaptation to temperature in *P. furiosus*, their proportion relative to GDGT decrease when  
329 the strain was grown at high temperatures, e.g.,  $43.4 \pm 3.1$  % at 85 °C *vs.*  $20.8 \pm 2.0$  % at 98 °C (Figure 2G,  
330 Table 2), which is opposite to the trend previously reported for these compounds. Such variations are also  
331 antagonistic with the proportion of cMGD relative to DGD in *M. jannaschii*, which rose with increasing

332 temperature, e.g., 12 % at 44 °C to *ca.* 45 % at 65 °C (Sprott *et al.*, 1991). The relative amounts of bilayer-  
333 forming cMGD in *M. jannaschii* and monolayer-forming GMGT in *P. furiosus* being opposite in response  
334 to growth temperature clearly indicate that the membranes behave in fundamentally distinct ways under  
335 temperature stress. Further characterization of their physicochemical properties is now sorely required to  
336 elucidate the features of the membranes they create and to rationalize their adaptive functions in response  
337 to temperature.

338 In contrast to temperature, relatively little is known about the adaptive strategies Archaea implement  
339 in response to pH and salinity stress outside of thermoacidophiles and extreme halophiles, respectively. One  
340 would nonetheless expect the membrane impermeability to ions and water to increase with osmotic or proton  
341 pressures in order to ensure proper cellular functioning and integrity. No cMGD has been detected so far in  
342 halophilic archaea, which instead shield their membrane with negatively charged polar head groups in order  
343 to preserve membrane impermeability under extreme salt conditions (Tenchov *et al.*, 2006; Kellermann *et*  
344 *al.*, 2016). Our procedure for core lipid extraction resulting in the excision of the polar head groups, such a  
345 strategy could not be investigated here. The adaptation to pH of thermoacidophilic archaea to pH  
346 implementing a modulation of the number of cyclopentane rings in tetraether lipids (Shimada *et al.*, 2008;  
347 Feyhl-Buska *et al.*, 2016) could on the other hand be assessed here. No significant modification of the  
348 number of cyclopentane rings was observed in the tetraether pool of *P. furiosus* with changing pH or salinity  
349 (Table 1, Figure 3F and Figure 4F). However, as aforementioned, the covalent C-C bond between the two  
350 alkyl chains in GMGT is supposed to provide a more efficient barrier to solutes and water than the classic  
351 DGD and GDGT, respectively. One would thus expect an increase of the GMGT/GDGT ratio with  
352 increasing salinity and decreasing pH, which was exactly what was observed for *P. furiosus* (Figure 3G and  
353 Figure 4G). These results suggest that, at least for *P. furiosus*, GMGT are essential lipids for the membrane  
354 adaptation to salinity and pH, and could for instance help maintaining proper membrane permeability to  
355 solutes and protons under stressful conditions. Such an adaptive function again contrasts with environmental  
356 data, for which a positive correlation between pH and the relative abundance of GMGT has been observed

357 in both low temperature (peats, < 20 °C; Naafs *et al.*, 2018) and high temperature (terrestrial hydrothermal  
358 vents, > 50 °C; Jia *et al.*, 2014) settings. Elucidating the adaptive functions of GMGT in response to pH and  
359 salinity in other archaeal species, such as *Ignisphaera aggregans*, a freshwater neutrophilic  
360 hyperthermophile (Knappy *et al.*, 2011), or *Aciduliprofundum boonei*, a marine acidophilic  
361 hyperthermophile (Schouten *et al.*, 2008), is now essential to determine whether the mechanisms observed  
362 in *P. furiosus* are specific to this marine neutrophilic hyperthermophile or shared between ecologically and  
363 phylogenetically distant archaea.

364 **The regulation of the number of cyclopentane rings in membrane lipids is a minor component of the**  
365 **adaptive response in *Pyrococcus furiosus***

366 Tetraether lipids form monolayer membranes that are more rigid and impermeable than typical  
367 bilayer membranes (Chong, 2010). The presence of one to eight cyclopentane rings further enhances the  
368 packing of the monolayer (Gliozzi *et al.*, 1983; Gabriel and Chong, 2000; Chong, 2010), thus reducing  
369 proton and solute permeability and maintaining membrane stability at high temperatures. Ring-containing  
370 GDGT were initially identified in thermoacidophilic archaea, such as *Sulfolobus acidocaldarius* (De Rosa  
371 *et al.*, 1983) or *Thermoplasma acidophilum* (Shimada *et al.*, 2002), although these compounds were more  
372 recently demonstrated to be also vastly distributed within mesophilic archaea, such as Thaumarchaeota  
373 (e.g., Schouten *et al.*, 2008b; Elling *et al.*, 2017). Several studies have shown that the membrane adaptive  
374 response to temperature triggers an increase of the RI<sub>GDGT</sub> in thermoacidophiles, such as *Acidilobus*  
375 *sulfurireducens* (+0.6 cycle from 65 °C to 81 °C; Boyd *et al.*, 2011), but also in mesophiles, such as  
376 *Nitrosopumilus maritimus* (+0.7 cycle from 22 °C to 28 °C; Elling *et al.*, 2015), suggesting that the  
377 regulation of the RI<sub>GDGT</sub> in Archaea is a common membrane adaptation strategy to increased temperature  
378 (Uda *et al.*, 2004; Feyhl-Buska *et al.*, 2016; Bale *et al.*, 2019). Modulations of the RI<sub>GDGT</sub> have also been  
379 observed in thermoacidophiles grown at varying pH (Boyd *et al.*, 2013; Feyhl-Buska *et al.*, 2016). However,  
380 whether and how these variations contribute to the membrane response to pH remains unclear as the RI<sub>GDGT</sub>  
381 decreases with pH for some species (-0.6 cycles from pH 3.0 to pH 5.0 in the case of *Acidilobus*  
382 *sulfurireducens*; Boyd *et al.*, 2011) while it increases with pH for some others (e.g., +1.1 cycles from pH



383 1.2 to pH 3.0 for *Thermoplasma acidophilum*; Shimada *et al.*, 2008), thus challenging the predictions based  
384 on the physicochemical properties of these lipids. To our knowledge, only one study investigated the  
385 variations of the  $RI_{GDGT}$  in response to varying salinity and reported no significant modification with the  
386 environmental stressor (Elling *et al.*, 2015).

387 In *P. furiosus*, RI values were also significantly positively correlated with temperature (Table 2 and  
388 Figure 2F). The temperature of 103 °C was notably the only one at which we could detect GDGT4 (Figure  
389 2B). Interestingly, the  $RI_{GMGT}$  followed the same trend (Figure 2F), suggesting that the regulation of the RI  
390 in response to temperature is independent of the tetraether core structure. In contrast to mesophilic  
391 neutrophiles which do not modulate their  $RI_{GDGT}$  in response to pH (Elling *et al.*, 2015), *P. furiosus*  
392 significantly increased the  $RI_{GDGT}$  with increasing pH (Figure 3F, Tables 1 and 2). In contrast, both  $RI_{GDGT}$   
393 and  $RI_{GMGT}$  varied significantly only at the highest salinity tested (Figure 4F), suggesting that the decrease  
394 of the RI in response to increasing salinity could be triggered only above a salinity threshold. Despite these  
395 observations, the low RI in *P. furiosus* (< 0.6 under all the conditions tested) as compared to that of other  
396 archaea (generally > 1) indicates that the regulation of the average number of cyclopentane rings in tetraether  
397 lipids may be negligible in the adaptive strategy of *P. furiosus*.

### 398 **Diether lipids are not involved in membrane adaptation in *Pyrococcus furiosus***

399 Diether lipids form bilayer membranes that are more fluid and permeable than their tetraether-based  
400 monolayer counterparts (Chong, 2010). They thus play a major role in the membrane adaptation in Archaea  
401 capable of synthesizing both diether and tetraether lipids (Sprott *et al.*, 1991; Matsuno *et al.*, 2009; Cario *et*  
402 *al.*, 2015). While some variations of the DGD relative abundances did occur in *P. furiosus* (Table 1), no  
403 significant differences nor correlations with temperature or any other parameter tested were observed in the  
404 present study (Table 2), suggesting that, in contrast to other Thermococcales, the modulation of the  
405 diether/tetraether ratio might not be part of the adaptive response of *P. furiosus*. This however does not  
406 necessarily preclude the existence of adaptive functions for diether lipids in *P. furiosus*. Indeed, we  
407 previously reported that in Thermococcales, diether lipids can harbor up to seven different polar head groups  
408 of variable size and polarity in contrast to tetraether lipids which all harbor two phosphoinositol headgroups

409 (Tourte, *et al.*, 2020a). Although no adaptive response could be detected for diether core lipids in the case  
410 of *P. furiosus*, the possibility that diether lipids could respond through the alteration of their polar head  
411 groups cannot be ruled out. Regardless, the presence of relatively large proportions of diether lipids in *P.*  
412 *furiosus* is puzzling since the stability of diether lipid-based membranes at its optimal growth temperature  
413 of 98 °C remains questionable, and suggests that diether lipids could harbor other important physiological  
414 functions. They may for instance only exist in mixture with tetraether lipids, i.e., diether lipids would be  
415 dispersed throughout a monolayer membrane, and, given their very divergent properties, act as membrane  
416 fluidizing agents in *P. furiosus*. However, no data about the spatial distribution of both types of lipids in the  
417 archaeal membrane have been reported to date, and these functions of diether lipids in the membrane of *P.*  
418 *furiosus* thus remain hypothetical.

419 **The carbon source and the genetic content strongly affect the lipid composition of *Pyrococcus***  
420 ***furiosus***

421 It is now well documented that Archaea regulate their lipid composition in response to various  
422 parameters besides temperature, pH, and salinity, e.g., carbon, phosphorous, and nitrogen sources and  
423 availability, growth rate, and oxygen content (Langworthy, 1977; Matsuno *et al.*, 2009; Elling *et al.*, 2014;  
424 Meador *et al.*, 2014; Feyhl-Buska *et al.*, 2016; Quehenberger *et al.*, 2020; Zhou *et al.*, 2020). Here, we also  
425 tested the impact of the carbon source by comparing growth in DC (disaccharides) and TRM (polypeptides)  
426 media. Growth in the DC medium boosted the synthesis of ring-containing tetraethers to such an extent that  
427 *P. furiosus* exhibited RI values similar to those observed for Thaumarchaeota and thermoacidophilic archaea  
428 (Figure 5F). Interestingly, this mimics the response of *S. acidocaldarius* grown under nutrient limitation  
429 (Bischof *et al.*, 2019), suggesting that the RI increase observed in *P. furiosus* grown in DC medium could  
430 similarly result from energy flux slowdown. Unfortunately, no single monosaccharide can support growth  
431 of *P. furiosus* and only disaccharides could be used as carbohydrate carbon source in DC medium,  
432 preventing the identification of the limiting reaction which might for instance be the cleavage of di- into  
433 monosaccharides or the breakdown of monosaccharides. The shift in RI observed in DC medium appeared  
434 much larger than those observed with salinity, pH, and temperature variations (Table 2), indicating that

435 metabolism rate limitation, which is very frequent in the natural environment, may be a greater  
436 environmental stressor for *P. furiosus* than any other parameter tested here, an observation congruent with  
437 that made in other archaea (Hurley *et al.*, 2016).

438         Additionally, we compared the lipid composition of two quasi-isogenic strains, i.e., the wild-type  
439 strain DSM3638 and its derivative strain COM1. Although strain COM1 differs from strain DSM3638 by  
440 limited genomic modifications (Bridger *et al.*, 2012), it exhibited a completely distinct core lipid  
441 composition. For instance, COM1 and DSM3638 showed significantly different GDGT and GMGT  
442 proportions (Figure 6A-C). In addition, as seen for the wild-type strain in DC medium, strain COM1 showed  
443 much higher RI than strain DSM3638 (Figure 6F). Modification of the genomic region near the gene  
444 responsible for cyclization could directly explain the increase of the RI of strain COM1. However, the  
445 genomic comparison of the two strains did not allow the identification of any noticeable change in the  
446 vicinity of gene PF0210, which is homologous to the recently identified two radical S-adenosylmethionine  
447 (SAM) proteins involved in GDGT cyclization (Grs) in *Sulfolobus acidocaldarius* (Zeng *et al.*, 2019) and  
448 might similarly be involved in the formation of the GMGT interchain C-C bond. These differences in RI  
449 might also result from uracil starvation as seen in *Sulfolobus acidocaldarius* (Bischof *et al.*, 2019), since  
450 strain COM1 is an uracil auxotroph while strain DSM3638 is an autotroph (Lipscomb *et al.*, 2011). Overall,  
451 it is highly probable that these notable differences in lipid composition result from the general disturbance  
452 of the cell regulation network triggered by the few chromosomal rearrangements present in COM1.  
453 Chromosome stability in Thermococcales and other Archaea is a highly debated issue. It is influenced by  
454 numerous intrinsic factors, e.g., the genomes of *Pyrococcus* species are highly rearranged (Zivanovic, 2002;  
455 Cossu *et al.*, 2015), or extrinsic factors, such as virus infection or mobile element insertion (Cossu *et al.*,  
456 2017), that could strongly affect the lipid composition of their host. Altogether, our results indicate that  
457 besides typical abiotic factors, numerous biotic factors also greatly impact the average number of  
458 cyclopentane rings and the overall lipid composition in Archaea.

459 **Material and methods**

460 **Microorganism and growth conditions**

461 *Pyrococcus furiosus* strain DSM3638 was purchased from the Deutsche Sammlung von  
462 Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). *Pyrococcus furiosus* strain COM1  
463 was kindly provided by the Adams lab (University of Georgia, Athens, Georgia, USA). It is a naturally  
464 competent derivative of DSM3638 that has been developed for genetic manipulations by deleting its *pyrF*  
465 gene to make it auxotroph for uracil (Lipscomb *et al.*, 2011; Farkas *et al.*, 2012). Strain COM1 exhibits  
466 several chromosomal rearrangements, deletions, insertions, and single base modifications compared to the  
467 type strain DSM3638 (Bridger *et al.*, 2012).

468 Cultures were routinely grown under optimal growth conditions, i.e., at 98 °C and pH 6.8, with 3 % w/v  
469 NaCl and 10 g L<sup>-1</sup> elemental sulfur, in a rich medium established for Thermococcales (TRM; Zeng *et al.*,  
470 2009), containing for 1 L: MgCl<sub>2</sub>, 5 g; peptone (Difco), 4 g; PIPES [piperazine-N,N'-bis(2-ethanesulfonic  
471 acid)], 3.3 g (10 mM); yeast extract (Difco), 1 g; KCl 0.7 g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.5 g; KH<sub>2</sub>PO<sub>4</sub> 50 mg; K<sub>2</sub>HPO<sub>4</sub> 50  
472 mg; NaBr 50 mg; CaCl<sub>2</sub> 20 mg; SrCl<sub>2</sub> 10 mg; FeCl<sub>3</sub> 4 mg; Na<sub>2</sub>WO<sub>4</sub> 3 mg and resazurin (Sigma Aldrich) 1  
473 mg. Alternatively, cultures were grown in DC medium (Lipscomb *et al.*, 2011), a defined medium with  
474 cellobiose as a carbon source at 2.8 % w/v NaCl and pH 6.8, containing for 1 L: MgSO<sub>4</sub>, 3.5 g; cellobiose  
475 (Alfa Aesar), 3.5 g; MgCl<sub>2</sub>, 2.7 g; cysteine-HCl (Sigma Aldrich) , 1 g; NaHCO<sub>3</sub>, 1 g; KCl, 0.3 g; NH<sub>4</sub>Cl,  
476 250 mg; CaCl<sub>2</sub>, 140 mg; KH<sub>2</sub>PO<sub>4</sub>, 140 mg; K<sub>2</sub>HPO<sub>4</sub>, 170 mg; Na<sub>2</sub>WO<sub>4</sub>, 0.3 mg, amino acid solution, 40 mL;  
477 vitamin solution, 5 mL; and trace mineral solution, 1 mL. Growth media were supplemented with uracil (20  
478 μM final concentration) for *P. furiosus* strain COM1. Strict anaerobiosis was ensured by addition of Na<sub>2</sub>S  
479 (0.1 % w/v final concentration) before inoculation.

480 We evaluated the membrane response of *P. furiosus* to the following parameters: temperature (80, 85,  
481 90, 98, and 103 °C), salinity (1, 2, 3, and 4 % w/v NaCl), presence/absence of elemental sulfur (0 and 10 g  
482 L<sup>-1</sup>), type of growth medium (TRM and DC) and genetic background (the wild-type strain DSM3638 vs.  
483 strain COM1). Growth of *P. furiosus* was first reported at pH values ranging from 5 to 9 using only 0.05 M  
484 of glycylglycine as buffer for pH ≥ 8.0 (Fiala and Stetter, 1986). In contrast, we could not maintain pH ≥

485 6.8 by adding up to 1 M of either glycylglycine, 2-amino-2-methyl-1-propanol (AMP), 2-Amino-2-methyl-  
486 1,3-propanediol (AMPD), 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO), 2-  
487 (cyclohexylamino)ethanesulfonic acid (CHES), or PIPES buffer, likely because of the production and  
488 excretion of organic acids during growth. Monitoring growth in alkaline cultures showed that growth  
489 essentially picked up only after pH was lowered to values close to the optimum. Thus, only endpoint pH  
490 values are reported here (5.5, 5.6, 5.9, 6.2, 6.4 and 6.6). Cultures were set up with 10 mM PIPES for pH  $\geq$   
491 6.0, and 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) for pH  $\leq$  6.0. Growth was monitored by  
492 counting with a Thoma cell counting chamber (depth 0.01 mm) under a light microscope (Thermo Fisher  
493 Scientific EVOS<sup>®</sup> XL Core 400 $\times$ , Waltham, MA, USA), and growth curves were established under each  
494 condition on at least three biological replicates (for evaluation of the growth under each condition, refer to  
495 Table S1).

#### 496 **Core lipid extraction and HPLC-MS analysis**

497 Cells of 250-mL cultures in late exponential phase were recovered by centrifugation (4000  $\times$  g, 45 min,  
498 4 °C) and rinsed twice with an isotonic saline solution. The cell pellets were lyophilized overnight and kept  
499 at -80 °C until lipid extraction. Extraction was performed on three biological replicates. Following acid  
500 hydrolysis of the dried cells (1.2 N HCl in methanol at 110 °C for 3 h), core lipids were extracted by filtration  
501 over celite using methanol/dichloromethane (1:1, *v/v*). The resulting solvent extracts were dried under  
502 reduced pressure, solubilized in *n*-heptane/isopropanol (99:1, *v/v*) and analyzed by high-performance liquid  
503 chromatography-mass spectrometry (HPLC-MS) using an HP 1100 series HPLC instrument equipped with  
504 an auto-injector and a Chemstation chromatography manager software connected to a Bruker Esquire  
505 3000<sup>Plus</sup> ion trap mass spectrometer, as described in (Tourte *et al.*, 2021). A standard solution containing  
506 core DGD and GDGT0 in a 2/1 molar ratio demonstrated a molar response factor of DGD *ca.* 10 times  
507 lower than that of GDGT0 under our analytical conditions. In the absence of a measured response factor for  
508 the different tetraether lipids, we assumed that all tetraethers have the same response factor as GDGT0. Core  
509 lipid relative abundances were determined by integration of the peak area on the mass chromatograms  
510 corresponding to the  $[M+H]^+$  ion of the different core lipids using a Bruker Data Analysis mass spectrometry

511 software (version 4.2), and the relative abundances of DGD relative to that of tetraethers were corrected by  
512 a factor of 10.

### 513 **Statistical analyses**

514 Statistical analyses were computed using the functions implemented within the base R core package  
515 (version 3.6.3; R Core Team, 2020). The weighted average number of rings per lipid molecule, or ring index  
516 (RI), was calculated as follows (Schouten *et al.*, 2007):

$$517 \text{ RI} = (\sum_{i \in [0;4]} i \times (\text{GDGT}_i + \text{GTGT}_i + \text{GMGT}_i)) / (\sum_{i \in [0;4]} (\text{GDGT}_i + \text{GTGT}_i + \text{GMGT}_i))$$

518 Data normality and homoscedasticity were assessed using the Shapiro-Wilk and Levene tests, respectively.  
519 Lipid relative abundances under each condition were compared using the Student t-test when there were  
520 only two independent groups of conditions, i.e., presence of sulfur, medium and strain. With more than two  
521 groups, comparisons were computed using one-way ANOVA and Tukey tests (normality and  
522 homoscedasticity of the data; % NaCl) or Kruskal-Wallis and Dunn tests (data not normally distributed and  
523 significantly different variances; temperature and pH). Correlations between the lipid proportions and  
524 temperature, pH, and salinity were assessed by the two-tailed probability associated with the Spearman  
525 correlation coefficient ( $\rho$ ). Differences and correlations with *P*-values below 0.05 were considered  
526 significant.

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533

534 **Competing interests**

535 The authors declare no conflicts of interest.

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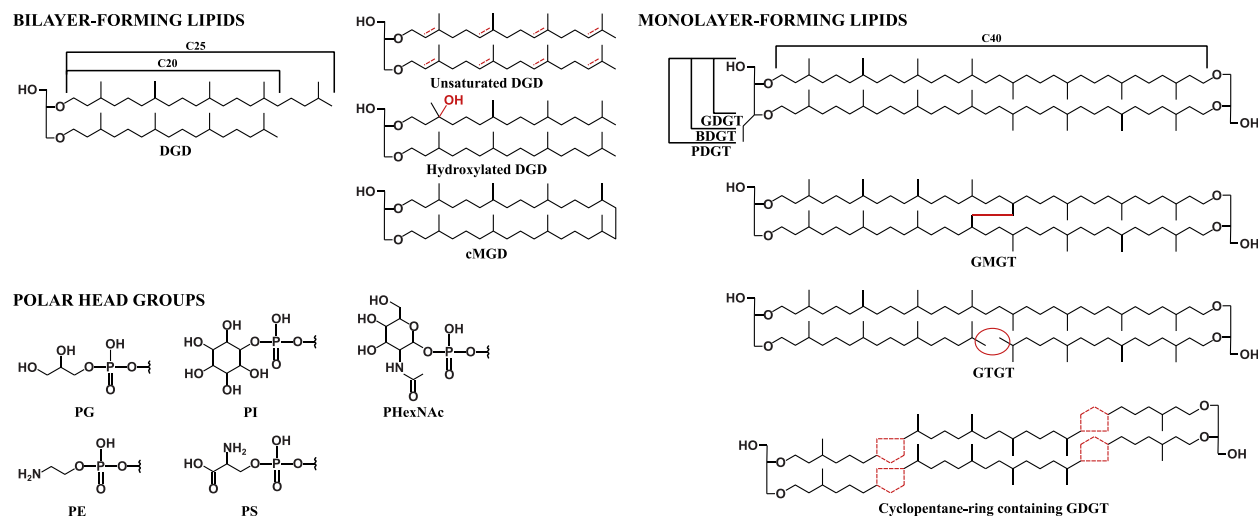
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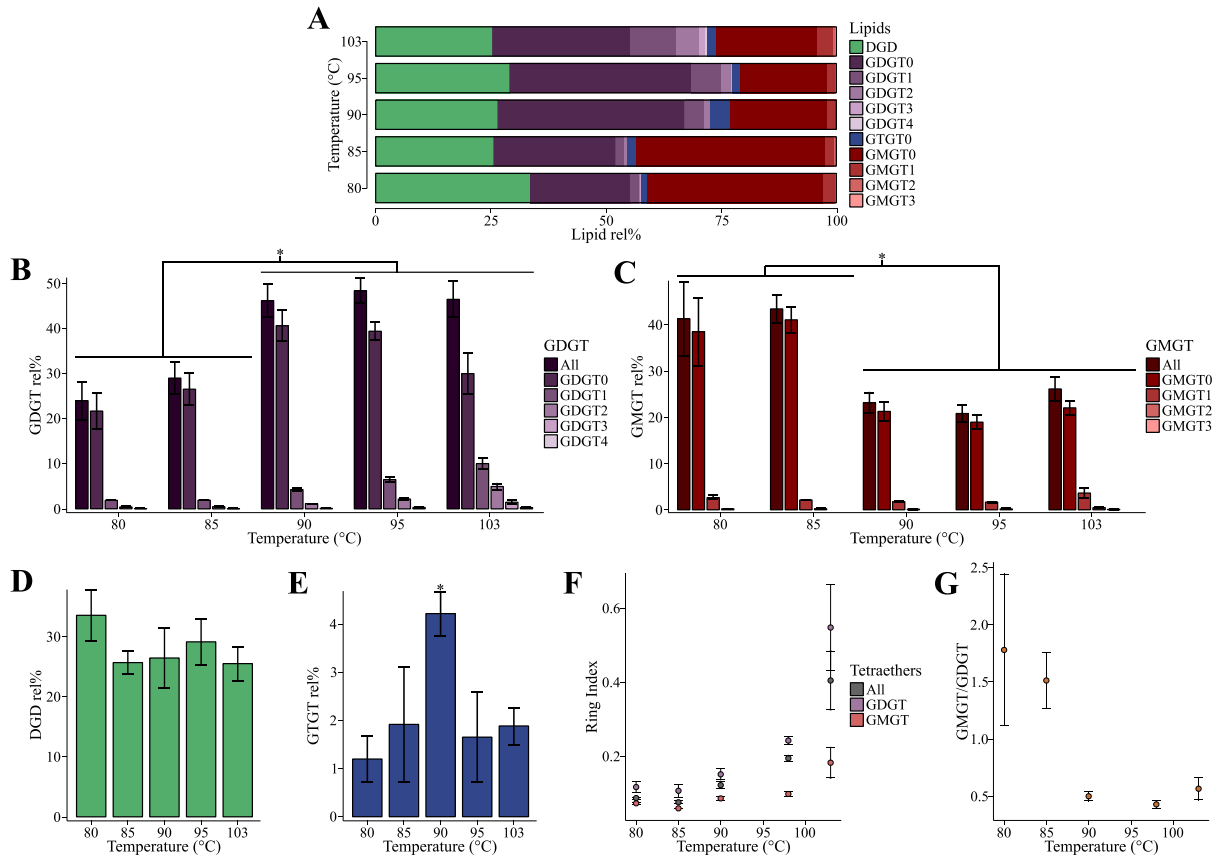
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- 737



739

740 **Figure 1: Archaeal membrane lipid diversity.**

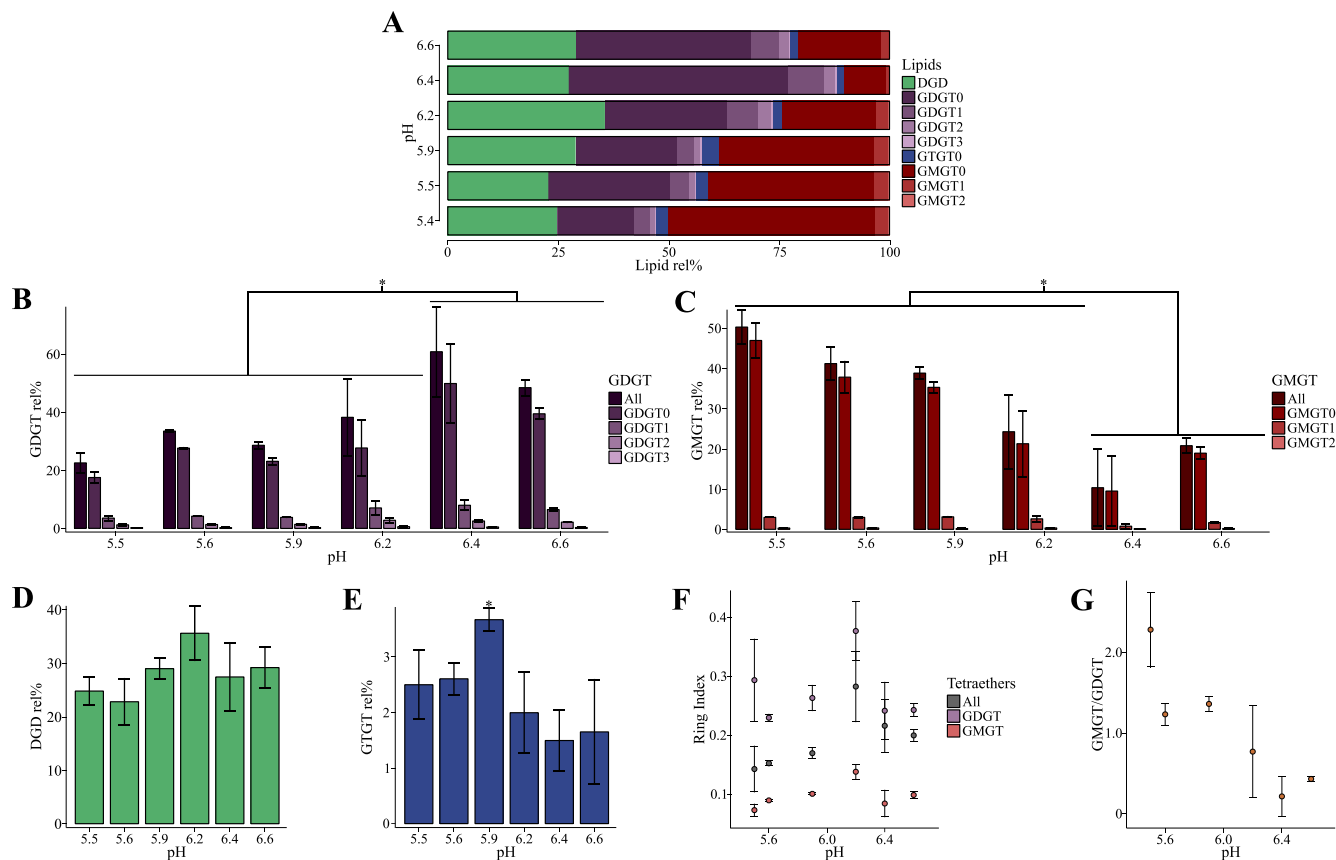
741 Core archaeal lipids include dialkyl glycerol diethers (DGD) with C<sub>20</sub> and/or C<sub>25</sub> isoprenoid alkyl chains, unsaturated, and hydroxylated DGD,  
 742 macrocylic monoalkyl glycerol diethers (cMGD), glycerol, butanetriol and pentanetriol dialkyl glycerol tetraethers (GDGT, BDGT, and PDGT),  
 743 glycerol monoalkyl glycerol tetraethers (GMGT), glycerol trialkyl glycerol tetraethers (GTGT), and tetraethers with 1 to 4 cyclopentane rings. Intact  
 744 polar lipids consist of di- and tetraether core lipids attached to polar head groups deriving from sugars, e.g., phosphatidylinositol (PI),  
 745 phosphatidylglycerol (PG), phosphatidyl-N-acetylhexosamine (PHexNAc), aminoacids, e.g., phosphoethanolamine (PE), and phosphatidylserine  
 746 (PS), or combinations of both.



748

749 **Figure 2: *Pyrococcus furiosus* responds to increasing temperature by increasing the average number**  
 750 **of cyclopentane rings.**

751 *P. furiosus* DSM3638 was grown under optimal conditions (TRM at 3 % w/v NaCl and pH 6.8 with 10 g L<sup>-1</sup>  
 752 elemental sulfur) at different temperatures (80, 85, 90, 98, and 103 °C). 98 °C represents the optimal  
 753 growth temperature. Error bars represent the standard deviation calculated on three biological replicates. \*  
 754 indicates temperatures with significantly different core lipid compositions. (A) Total core lipid compositions  
 755 under each temperature. (B) Influence of the temperature on GDGT relative proportions. GDGT (dark  
 756 purple) corresponds to the summed GDGT, regardless of the cyclopentane ring content. (C) Influence of the  
 757 temperature on GMT relative proportions. GMT (dark red) corresponds to the summed GMT,  
 758 regardless of the cyclopentane ring content. (D) Influence of the temperature on DGD relative abundance.  
 759 (E) Influence of the temperature on GTGT0 relative abundance. (F) Temperature dependence of the ring  
 760 index (RI) for all tetraethers (GDGT, GTGT, and GMT), GDGT and GMT (RI ± standard deviation).  
 761 (G) Temperature dependence of the GMT/GDGT ratio.

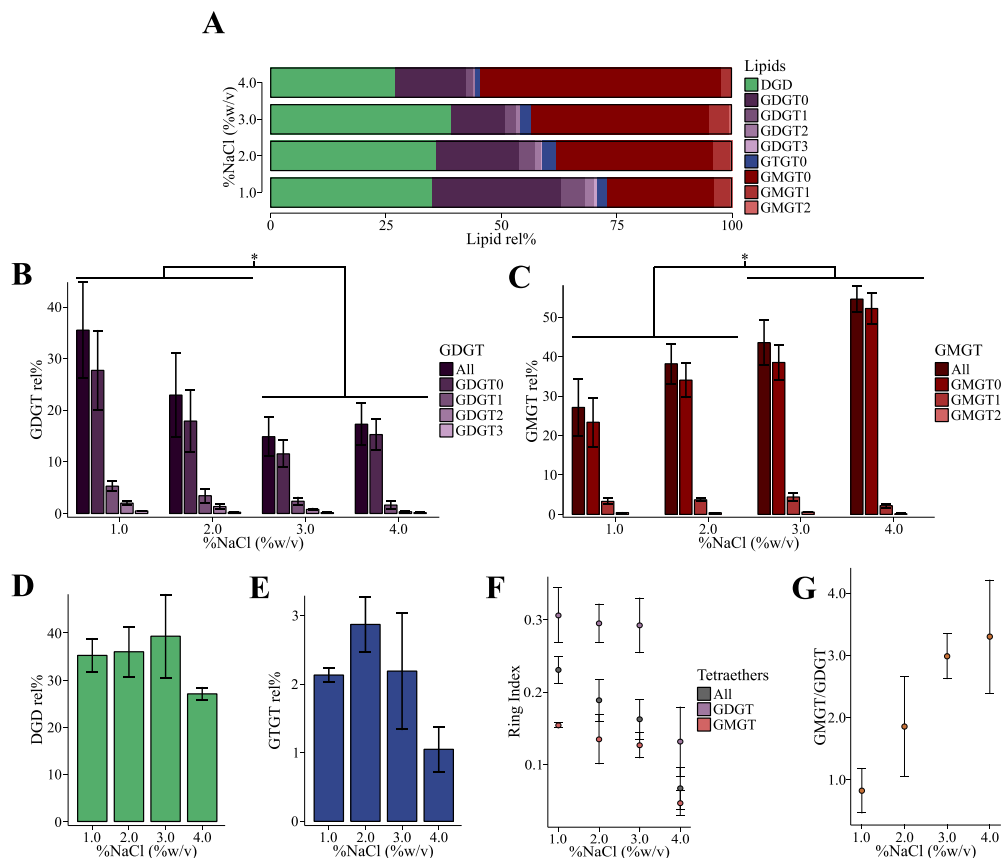


762

763 **Figure 3: *Pyrococcus furiosus* responds to sub-optimal pH by increasing the relative abundance of**  
 764 **GMGT.**

765 *P. furiosus* DSM3638 was grown under optimal conditions (TRM at 3 % w/v NaCl and 98 °C with 10 g L<sup>-1</sup>  
 766 elemental sulfur) at different pH (5.5, 5.6, 5.9, 6.2, 6.4 and 6.6). pH 6.6 represents the optimal growth pH.  
 767 Error bars represent the standard deviation calculated on three biological replicates. \* indicates pH with  
 768 significantly different core lipid compositions. (A) Total core lipid compositions under each pH. (B)  
 769 Influence of the pH on GDGT relative proportions. GDGT (dark purple) corresponds to the summed GDGT,  
 770 regardless of the cyclopentane ring content. (C) Influence of the pH on GMGT relative proportions. GMGT  
 771 (dark red) corresponds to the summed GMGT, regardless of the cyclopentane ring content. (D) Influence of  
 772 the pH on DGD relative abundance. (E) Influence of the pH on GTGT0 relative abundance. (F) pH  
 773 dependence of the ring index (RI) for all tetraethers (GDGT, GTGT, and GMGT), GDGT and GMGT (RI  
 774 ± standard deviation). (G) pH dependence of the GMGT/GDGT ratio.

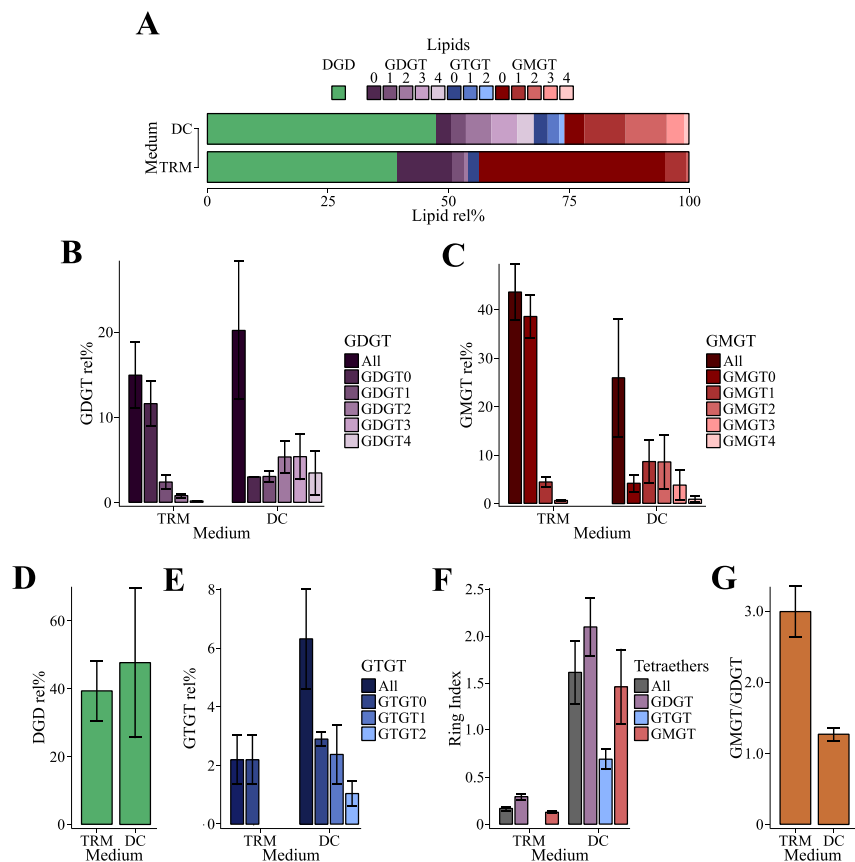




775

776 **Figure 4: *Pyrococcus furiosus* responds to increasing salinity by increasing the relative abundance of**  
 777 **GMGT and reducing the average number of cyclopentane rings.**

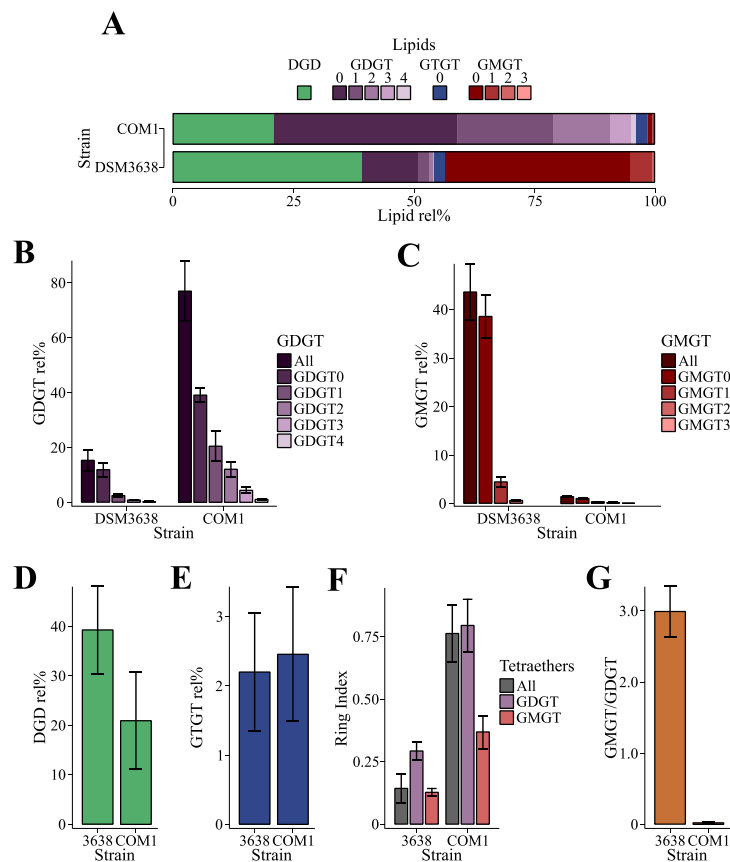
778 *P. furiosus* DSM3638 was grown under optimal conditions (TRM at 98 °C and pH 6.8 with 10 g L<sup>-1</sup>  
 779 elemental sulfur) at different salinities (1, 2, 3 and 4 % w/v NaCl). 3 % NaCl represents the optimal growth  
 780 salinity. Error bars represent the standard deviation calculated on three biological replicates. \* indicates %  
 781 NaCl with significantly different core lipid compositions. (A) Total core lipid compositions under each %  
 782 NaCl. (B) Influence of the salinity on GDGT relative proportions. GDGT (dark purple) corresponds to the  
 783 summed GDGT, regardless of the cyclopentane ring content. (C) Influence of the salinity on GMGT relative  
 784 proportions. GMGT (dark red) corresponds to the summed GMGT, regardless of the cyclopentane ring  
 785 content. (D) Influence of the salinity on DGD relative abundance. (E) Influence of the salinity on GTGT0  
 786 relative abundance. (F) Salinity dependence of the ring index (RI) for all tetraethers (GDGT, GTGT, and  
 787 GMGT), GDGT and GMGT (RI ± standard deviation). (G) Salinity dependence of the GMGT/GDGT ratio.



788

789 **Figure 5: Growth on sugars significantly increases the average number of cyclopentane rings in**  
 790 ***Pyrococcus furiosus*.**

791 *P. furiosus* DSM3638 was grown under optimal conditions (at 98 °C, pH 6.8 and 3 % w/v NaCl with 10 g  
 792 L<sup>-1</sup> elemental sulfur) in DC and TRM media. TRM represents the optimal growth condition. For details on  
 793 media compositions, refer to the Method section. Error bars represent the standard deviation calculated on  
 794 three biological replicates. (A) Total core lipid compositions under each medium condition. (B) Influence  
 795 of the growth medium on GDGT relative proportions. GDGT (dark purple) corresponds to the summed  
 796 GDGT, regardless of the cyclopentane ring content. (C) Influence of the growth medium on GMGT relative  
 797 proportions. GMGT (dark red) corresponds to the summed GMGT, regardless of the cyclopentane ring  
 798 content. (D) Influence of the growth medium on DGD relative abundance. (E) Influence of the growth  
 799 medium on GTGT0 relative abundance. (F) Growth medium dependence of the ring index (RI) for all  
 800 tetraethers (GDGT, GTGT, and GMGT), GDGT, GTGT and GMGT (RI ± standard deviation). (G) Growth  
 801 medium dependence of the GMGT/GDGT ratio.



802

803 **Figure 6: *Pyrococcus furiosus* strain COM1 shows a marked increase in GDGT content and average**  
 804 **number of cyclopentane rings compared to its wild-type parent strain DSM3638.**

805 Cells were grown under optimal conditions (TRM at 98 °C, pH 6.8 and 3 % w/v NaCl with 10 g L<sup>-1</sup> elemental  
 806 sulfur). Error bars represent the standard deviation calculated on three biological replicates. (A) Total core  
 807 lipid compositions for each strain. (B) GDGT relative abundance for each strain. GDGT (dark purple)  
 808 corresponds to the summed GDGT, regardless of the cyclopentane ring content. (C) GMGT relative  
 809 abundance for each strain. GMGT (dark red) corresponds to the summed GMGT, regardless of the  
 810 cyclopentane ring content. (D) DGD relative abundance for each strain. (E) GTGT relative abundance for  
 811 each strain. (F) Strain dependence of the ring index (RI) for all tetraethers (GDGT, GTGT, and GMGT),  
 812 GDGT, and GMGT (RI ± standard deviation). (G) Strain dependence of the GMGT/GDGT ratio.

## 813 Tables

814 Table 1. Core lipid relative composition (%) of *Pyrococcus furiosus* as a function of growth variables

		Diethers*							Tetraethers*												
		DGD	GDGT	GDGT0	GDGT1	GDGT2	GDGT3	GDGT4	RI <sub>DGDT</sub>	GTGT	GTGT0	GTGT1	GTGT2	RI <sub>GTGT</sub>	GMGT	GMGT0	GMGT1	GMGT2	GMGT3	GMGT4	RI <sub>GMGT</sub>
Temp. (°C)	80	33.6 ± 4.4	24.0 ± 4.4	21.7 ± 4.2	1.9 ± 0.1	0.4 ± 0.0	Traces	ND	0.12 ± 0.02	1.1 ± 0.5	1.1 ± 0.5	ND	ND	0	41.3 ± 8.2	38.4 ± 7.5	2.6 ± 0.6	0.2 ± 0.0	ND	ND	0.07 ± 0.00
	85	25.7 ± 2.0	29.0 ± 3.6	26.6 ± 3.7	1.9 ± 0.1	0.5 ± 0.1	0.1 ± 0.0	ND	0.11 ± 0.02	1.9 ± 1.2	1.9 ± 1.2	ND	ND	0	43.4 ± 3.1	41.0 ± 2.9	2.1 ± 0.2	0.2 ± 0.0	ND	ND	0.06 ± 0.00
	90	26.4 ± 5.1	46.2 ± 3.8	40.7 ± 3.6	4.3 ± 0.5	1.2 ± 0.1	0.1 ± 0.0	ND	0.15 ± 0.02	4.2 ± 0.5	4.2 ± 0.5	ND	ND	0	23.1 ± 2.4	21.2 ± 2.1	1.7 ± 0.2	0.1 ± 0.0	ND	ND	0.09 ± 0.00
	98	29.1 ± 3.9	48.4 ± 3.0	39.4 ± 2.2	6.5 ± 0.7	2.2 ± 0.3	0.3 ± 0.0	ND	0.24 ± 0.01	1.6 ± 1.0	1.6 ± 1.0	ND	ND	0	20.8 ± 2.0	18.9 ± 1.7	1.7 ± 0.2	0.2 ± 0.0	ND	ND	0.10 ± 0.04
	103	35.5 ± 2.9	46.5 ± 4.2	30.0 ± 4.7	10.0 ± 1.4	5.0 ± 0.8	1.5 ± 0.6	0.3 ± 0.2	0.55 ± 0.12	1.9 ± 0.4	1.9 ± 0.4	ND	ND	0	26.1 ± 2.7	22.0 ± 1.7	3.6 ± 1.2	0.5 ± 0.1	0.1 ± 0.0	ND	0.18
pH	5.5	24.8 ± 2.8	22.5 ± 3.5	17.5 ± 2.2	3.5 ± 1.1	1.2 ± 0.5	0.2 ± 0.2	ND	0.29 ± 0.07	2.5 ± 0.6	2.5 ± 0.6	ND	ND	0	50.3 ± 4.4	46.9 ± 4.5	3.0 ± 0.1	0.3 ± 0.0	ND	ND	0.07 ± 0.01
	5.6	22.8 ± 4.4	33.5 ± 0.6	27.6 ± 0.4	4.2 ± 0.3	1.4 ± 0.1	0.3 ± 0.0	ND	0.23 ± 0.01	2.6 ± 0.3	2.6 ± 0.3	ND	ND	0	41.2 ± 4.3	37.8 ± 4.0	3.0 ± 0.3	0.3 ± 0.0	ND	ND	0.09 ± 0.00
	5.9	28.9 ± 2.1	28.6 ± 1.4	23.1 ± 1.5	3.8 ± 0.2	1.4 ± 0.1	0.3 ± 0.0	ND	0.26 ± 0.02	3.7 ± 0.2	3.7 ± 0.2	ND	ND	0	38.8 ± 1.6	35.3 ± 1.4	3.1 ± 0.2	0.4 ± 0.0	ND	ND	0.10 ± 0.00
	6.2	35.5 ± 5.2	38.2 ± 13.4	27.6 ± 9.8	7.1 ± 2.5	2.8 ± 1.1	0.6 ± 0.3	ND	0.38 ± 0.05	2.0 ± 0.7	2.0 ± 0.7	ND	ND	0	24.3 ± 9.3	21.3 ± 8.3	2.6 ± 0.9	0.3 ± 0.1	ND	ND	0.14 ± 0.01
	6.4	27.4 ± 6.5	60.8 ± 15.7	49.8 ± 13.8	8.0 ± 2.0	2.5 ± 0.7	0.5 ± 0.3	ND	0.24 ± 0.05	1.5 ± 0.6	1.5 ± 0.6	ND	ND	0	10.3 ± 9.7	9.5 ± 8.9	0.8 ± 0.8	0.1 ± 0.1	ND	ND	0.08 ± 0.02
	6.6	29.1 ± 3.9	48.4 ± 3.0	39.4 ± 2.2	6.5 ± 0.7	2.2 ± 0.3	0.3 ± 0.0	ND	0.24 ± 0.01	1.6 ± 1.0	1.6 ± 1.0	ND	ND	0	20.8 ± 2.0	18.9 ± 1.7	1.7 ± 0.2	0.2 ± 0.0	ND	ND	0.10 ± 0.01
	1	35.2 ± 3.7	35.6 ± 9.4	27.8 ± 7.8	5.3 ± 1.1	2.0 ± 0.5	0.5 ± 0.2	ND	0.31 ± 0.04	2.1 ± 0.1	2.1 ± 0.1	ND	ND	0	27.1 ± 7.4	23.4 ± 6.3	3.3 ± 1.0	0.5 ± 0.2	ND	ND	0.15 ± 0.01
%NaCl (w/v)	2	36.0 ± 5.4	23.0 ± 8.2	17.9 ± 6.1	3.4 ± 1.5	1.3 ± 0.6	0.2 ± 0.1	ND	0.30 ± 0.03	2.9 ± 0.4	2.9 ± 0.4	ND	ND	0	38.2 ± 5.2	30.3 ± 5.0	3.7 ± 0.6	0.4 ± 0.1	ND	ND	0.14 ± 0.03
	3	39.3 ± 9.0	14.9 ± 4.0	11.6 ± 2.7	2.4 ± 0.9	0.8 ± 0.3	0.2 ± 0.1	ND	0.29 ± 0.04	2.3 ± 0.9	2.3 ± 0.9	ND	ND	0	43.6 ± 5.8	38.5 ± 4.6	4.4 ± 1.1	0.6 ± 0.2	ND	ND	0.13 ± 0.02
	4	27.0 ± 1.5	17.3 ± 4.2	15.3 ± 3.1	1.6 ± 0.9	0.3 ± 0.3	Traces	ND	0.13 ± 0.05	1.1 ± 0.3	1.1 ± 0.3	ND	ND	0	54.6 ± 3.6	52.2 ± 4.2	2.2 ± 0.6	0.2 ± 0.1	ND	ND	0.05 ± 0.02
Sulfur	+S	39.3 ± 9.0	14.9 ± 4.0	11.6 ± 2.7	2.4 ± 0.9	0.8 ± 0.3	0.2 ± 0.1	ND	0.29 ± 0.04	2.3 ± 0.9	2.3 ± 0.9	ND	ND	0	43.6 ± 5.8	38.5 ± 4.6	4.4 ± 1.1	0.6 ± 0.2	ND	ND	0.13 ± 0.02
	-S	34.1 ± 7.8	16.8 ± 5.2	12.9 ± 3.5	2.8 ± 1.2	0.9 ± 0.4	0.2 ± 0.1	ND	0.31 ± 0.05	0.5 ± 0.2	0.5 ± 0.2	ND	ND	0	48.6 ± 8.7	40.8 ± 8.1	6.8 ± 0.7	1.0 ± 0.2	ND	ND	0.18 ± 0.03
Medium	TRM	39.3 ± 9.0	14.9 ± 4.0	11.6 ± 2.7	2.4 ± 0.9	0.8 ± 0.3	0.2 ± 0.1	ND	0.29 ± 0.04	2.3 ± 0.9	2.3 ± 0.9	ND	ND	0	43.6 ± 5.8	38.5 ± 4.6	4.4 ± 1.1	0.6 ± 0.2	ND	ND	0.13 ± 0.02
	DC	47.6 ± 22.2	20.2 ± 8.2	3.0 ± 0.1	3.0 ± 0.7	5.3 ± 1.9	5.4 ± 2.8	3.5 ± 2.7	2.10 ± 0.32	6.3 ± 1.7	2.9 ± 0.2	2.4 ± 1.0	1.0 ± 0.4	0.69 ± 0.12	25.9 ± 12.3	4.1 ± 1.9	8.6 ± 4.6	8.5 ± 5.7	3.8 ± 3.2	0.8 ± 0.7	1.46 ± 0.40
Strain	DSM3638	39.3 ± 9.0	14.9 ± 4.0	11.6 ± 2.7	2.4 ± 0.9	0.8 ± 0.3	0.2 ± 0.1	ND	0.29 ± 0.04	2.3 ± 0.9	2.3 ± 0.9	ND	ND	0	43.6 ± 5.8	38.5 ± 4.6	4.4 ± 1.1	0.6 ± 0.2	ND	ND	0.13 ± 0.02
	COM1	21.0 ± 10.0	75.2 ± 10.8	38.2 ± 2.6	19.9 ± 5.6	11.8 ± 2.9	4.2 ± 1.3	1.0 ± 0.3	0.80 ± 0.11	2.5 ± 1.0	2.5 ± 1.0	ND	ND	0	1.4 ± 0.2	1.0 ± 0.1	0.3 ± 0.1	0.1 ± 0.0	Traces	ND	0.37 ± 0.07

815 For each condition, values are the average of three biological replicates (relative % + standard deviation).

816 \* Relative proportions account for protonated adducts and were calculated using a response factor of 1/10 for DGD relative to tetraether lipids (refer to the Method section).

817 GDGT, GTGT and GMGT represent the cumulative proportions of the corresponding tetraethers. Ring Index (RI) represents the average number of cyclopentane rings per lipid and was calculated  
818 separately for each class of tetraethers (refer to the Method section).

819 Traces, &lt;0.1 %. ND: not detected.

820 For lipid structures, refer to Figure S1

821 **Table 2. Spearman correlation coefficient ( $\rho$ ) and P-values for individual core structures and total**  
 822 **GDGT, GTGT and GMGT as a function of temperature, pH and salinity.**

	Temperature ( $^{\circ}\text{C}$ )		pH		%NaCl (%w/v)	
	Spearman's $\rho$	P-value	Spearman's $\rho$	P-value	Spearman's $\rho$	P-value
DGD	-0.60		0.66		-0.2	
GDGT	0.90	*	0.89	*	-0.8	
GDGT0	0.60		0.89	*	-0.8	
GDGT1	1.00	****	0.77		-1	****
GDGT2	1.00	****	0.77		-1	****
GDGT3	1.00	****	0.77		-1	****
GDGT4	0.71		NA	NA	NA	NA
GTGT	0.2		-0.71		-0.4	
GTGT0	0.2		-0.71		-0.4	
GTGT1	NA	NA	NA	NA	NA	NA
GTGT2	NA	NA	NA	NA	NA	NA
GMGT	-0.60		-0.94	**	1	****
GMGT0	-0.60		-0.94	**	1	****
GMGT1	0		-0.71		-0.2	
GMGT2	0.20		-0.54		-0.4	
GMGT3	0.71		NA		NA	NA
GMGT4	NA	NA	NA		NA	NA

823 NA, not applicable because the core structure was not detected.

824 Only P-values  $\leq 0.05$  are indicated, as follows: < 0.0001, "\*\*\*\*"; 0.0001 to 0.001, "\*\*\*\*", 0.001 to 0.01, "\*\*\*" and 0.01 to 0.05, "\*\*".