

1 **Title**

2 The exploration of *Thermococcus barophilus* lipidome reveals the widest variety of phosphoglycolipids in
3 Thermococcales.

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14 **Running title**

15 The lipidome of *Thermococcus barophilus*

16 **Keywords**

17 Archaeal membrane lipids, *Thermococcus barophilus*, phosphoglycolipids, core lipids, diethers, tetraethers,
18 phosphatidylhexose, phosphatidyl inositol, UHPLC-MS, MALDI-FT-ICR-MS.

19 **Abbreviations**

20 IPL, intact polar lipid(s); CL, core lipid(s); MGD, monoalkyl glycerol diethers; DGD, dialkyl glycerol
21 diethers; PSGD, phytanylsesterterpanyl glycerol diethers; MeDGD, methylated DGD; GDGT, glycerol
22 dialkyl glycerol tetraethers; GTGT, glycerol trialkyl glycerol tetraethers; MGDG, monogalactosyl
23 diacylglycerol; DGDG, digalactosyl diacylglycerol; C₄₆-GTGT, GTGT containing 46 carbon atoms; PI,
24 phosphatidyl inositol; PHex, phosphatidyl hexose; PHexNAc, phosphatidyl N-acetylhexosamine;
25 PHexHex, glycosylated phosphatidyl hexose; PHexHexNH₂, hexosamine phosphatidyl hexose;
26 PHexHexNAc, N-acetylhexosamine phosphatidyl hexose; PHexHex2NAc, di-N-acetylhexosamine
27 phosphatidyl hexose; PHexHex+C₅H₈, glycosylated phosphatidyl hexose bearing an additional mass of 68;

28 CDP, cytidine diphosphate; PG, phosphatidyl glycerol; C₂₁-PC, phosphatidyl choline diacylglycerol with
29 two C₂₁ fatty acid chains; B&D, Bligh and Dyer; MeOH, methanol; DCM, dichloromethane; ACN,
30 acetonitrile; TFA, trifluoroacetic acid; FA, formic acid; NH₃, ammonium hydroxide or aqueous ammonia;
31 DHB, 2,3-dihydroxybenzoic acid; UHPLC, ultra-high performance liquid chromatography; Q-TOF,
32 quadrupole time of flight; QQQ, triple quadrupole; MS, mass spectrometry; ESI, electrospray ionization;
33 APCI, atmospheric-pressure chemical ionization; MALDI, matrix-assisted laser desorption/ionization; FT,
34 Fourier transform; ICR, ion cyclotron resonance.

35 **Abstract**

36 One of the most distinctive characteristics of Archaea is their unique lipids. While the general nature of
37 archaeal lipids has been linked to their tolerance to extreme conditions, little is known about the diversity
38 of lipidic structures Archaea are able to synthesize, which hinders the elucidation of the physicochemical
39 properties of their cell membrane. In an effort to widen the known lipid repertoire of the piezophilic and
40 hyperthermophilic model archaeon *Thermococcus barophilus*, we comprehensively characterized its intact
41 polar lipid (IPL), core lipid (CL), and polar head group compositions using a combination of cutting-edge
42 liquid chromatography and mass spectrometric ionization systems. We tentatively identified 82 different
43 IPLs based on five distinct CLs and 10 polar head group derivatives of phosphatidylhexoses, including
44 compounds reported here for the first time, e.g., di-N-acetylhexosamine phosphatidylhexose-bearing lipids.
45 Despite having extended the knowledge on the lipidome, our results also indicate that the majority of *T.*
46 *barophilus* lipids remain inaccessible to current analytical procedures and that improvements in lipid
47 extraction and analysis are still required. This expanded yet incomplete lipidome nonetheless opens new
48 avenues for understanding the physiology, physicochemical properties, and organization of the membrane
49 in this archaeon as well as other Archaea.

50 **Introduction**

51 Cell membranes provide dynamic physical boundaries between the inside and the outside worlds of
52 cells of the three domains of life, Eukarya, Bacteria and Archaea. While their primary function is to ensure
53 cellular integrity, biological membranes are much more than simple barriers: they regulate inwards and
54 outwards fluxes, support signal transduction, cell bioenergetics and cell-to-cell communications, control
55 cell shape, growth and division, and deform to generate, release and accept vesicles and other membrane
56 macrostructures. These two-dimensional matrices are composite mixtures of a myriad of both lipids and
57 proteins that are compositionally, functionally and structurally complex systems. In Eukarya, membranes
58 are laterally organized into nano- to microscopic domains with specific compositions, physicochemical
59 properties and functions formerly termed lipid rafts (1, 2). Such a membrane structuration is essential for
60 membrane-hosted cellular functions, as it facilitates the organization, assembly and regulation of
61 multimolecular protein complexes (3). Membrane order is primarily determined by membrane lipids'
62 tendency to phase separate (4). In Eukarya, membrane domains are thus specifically enriched in
63 sphingolipids and cholesterol, which trigger liquid-liquid phase separation from the rest of the membrane
64 (5, 6). However, other components and parameters have been proven essential for lateral structuration of
65 the membrane. For instance, specific proteins such as flotillins regulate membrane domain formation (7),
66 while the geometrical conformation of lipid polar head groups dictates their intermolecular interactions and
67 lateral distribution (8, 9). Although they do not synthesize cholesterol, membrane lateral organization has
68 been recently expanded to Bacteria (10), and multidimensional structuration was thus suggested to be a
69 fundamental feature of all biological membranes (11, 12).

70 The membrane lipids of Archaea are however structurally divergent from those found in Bacteria and
71 Eukarya. While the latter are typically composed of fatty-acyl ester linked to a glycerol backbone in *sn*-1,2
72 configuration, archaeal lipids are built upon isoprenoid cores that are ether linked to a *sn*-2,3 glycerol
73 backbone (13–15). As a result, archaeal membranes are more stable and less permeable than those of
74 Bacteria and Eukarya, enabling Archaea to withstand a variety of environmental conditions, ranging from
75 the mildest to the harshest known on Earth (16, 17). Archaeal diether lipids are composed of C₁₅ to C₂₅

76 hydrocarbon chains that form bilayer membranes whereas tetraether lipids contain C₄₀ side chains linked to
77 two glycerol moieties and thus form monolayer membranes. Archaeal core lipids display a diversity of
78 structures, which includes mono- and dialkyl glycerol diethers (MGD and DGD; (18)), glycerol mono-, di-
79 , and trialkyl glycerol tetraethers (GMGT, GDGT, and GTGT, respectively; (19)), di- and tetraethers with
80 hydroxylated, methylated, and unsaturated isoprenoid chains (20, 21), and tetraethers with glycerol,
81 butanetriol, and pentanetriol backbones (22). With phosphatidic- and glycosidic polar head groups deriving
82 from typical sugars, amino acids or combinations of both (23), archaeal polar head group diversity does not
83 fundamentally diverge from that of Bacteria and Eukarya. However, how these diverse archaeal lipids
84 organize into functional membranes and whether lateral organization similar to that of eukaryotic and
85 bacterial membranes exist in Archaea remain elusive.

86 *Thermococcus barophilus* is a hyperthermophilic (optimal growth temperature 85 °C) and piezophilic
87 (optimal growth pressure 40 MPa) archaeon that synthesizes both diether and tetraether lipids (24). The
88 presence of both types of lipids implies that parts of *T. barophilus* membrane are in the form of bilayers,
89 whereas others are monolayered, thus delineating membrane domains reminiscent of the eukaryotic and
90 bacterial membrane lateral structuration. Additionally, the insertion of apolar polyisoprenoids in the bilayer
91 midplane was shown to trigger lipid phase separation (25), suggesting that lateral organization is indeed
92 possible in archaeal membranes. This model, based solely on the relative proportions of the different lipid
93 classes in the membrane, does not account for lipid polar head groups whose charge, steric hindrance,
94 geometry, polarity and hydrophily are critical for lipid distribution and membrane surface properties,
95 stability, impermeability and functions (26–28). For instance, the average geometrical shape of lipids
96 controls the propensity of these lipids to form specific phases, structures and thus domains on small to large
97 scales (29). Resolving the exact spectrum of archaeal lipids is thus of paramount importance to grasp their
98 biological relevance, i.e., their physiological and adaptive functions, and to comprehend membrane
99 architecture and physicochemical properties in Archaea.

100 Although essential to comprehend membrane physiology, the structural diversity and distribution of
101 archaeal lipids remain poorly characterized, partly because classic extraction procedures may lead to the

102 preferential extraction of some classes of lipids over others (24, 30). Current data on archaeal lipids might
103 thus not represent the real diversity in the original samples. Estimation of the lipid yield per cell indeed
104 showed strong discrepancies with theoretical calculations of the total lipid content of different archaeal cells
105 (31–34). For instance, intact polar lipid (IPL) extraction on *Methanothermobacter thermautotrophicus*
106 yielded 0.038 to 0.26 fg IPL cell⁻¹ whereas the theoretical lipid yield per cell for this rod-shaped archaeon
107 (0.3 μm × 2.2 to 5.9 μm) is estimated to lie between 4.5 and 12.1 fg cell⁻¹ (14). Similarly, the estimated lipid
108 yield per cell for the coccus-shaped *Thermococcus kodakarensis* (1.1 to 1.3 μm) ranges from 7.5 to 10.8 fg
109 cell⁻¹ but IPL extraction only yielded 0.38 to 1.61 fg cell⁻¹ (35). In contrast, IPL extraction on the much
110 smaller rod-shaped archaeon *Nitrosopumilus maritimus* (0.2 μm × 0.5 to 0.9 μm) yielded similar lipid
111 quantities than the theoretical estimation (0.9 to 1.9 fg cell⁻¹ vs 0.9 to 1.5 fg cell⁻¹, respectively; (32)), which
112 suggests that archaeal lipid extraction efficiency might be impacted by physiological parameters, e.g., size
113 and geometry of the cells, presence and characteristics of the cell envelope, as well as the lipids themselves,
114 e.g., the nature of the polar head groups. Although cellular lipid contents were not estimated for *T.*
115 *barophilus*, similar major extraction defects were also highlighted for this archaeon. The first and only
116 characterization of the IPL signature of *T. barophilus* only reported phosphatidylinositol(PI)-DGD. In
117 agreement with this simple IPL composition, the acid methanolysis of the total lipid extract yielded
118 exclusively DGD (36). However, direct acid methanolysis of *T. barophilus* biomass revealed both a high
119 proportion of tetraethers and a drastic bias of extraction and analytical procedures towards diether-based
120 IPLs (24, 37). Most of *T. barophilus* IPLs and polar head groups thus remain uncharacterized, impeding the
121 understanding of its membrane physiology and organization.

122 In an effort to solve this missing IPL enigma, we comprehensively investigated *T. barophilus* IPL
123 and polar head group compositions and assayed its core lipid (CL) composition as a quality control of our
124 methodology. We report the identification of up to 82 saturated and unsaturated IPLs, including the major
125 PI-DGD, and the first characterization of several novel archaeal IPLs, notably phosphatidyl di-N-
126 acetylhexosamine diethers and a tetraether bearing a peculiar derivative of glycosylated
127 phosphatidylhexose. The unsuspectedly large IPL diversity of *T. barophilus* widens the Thermococcales

128 lipid repertoire and contributes further refinements of the proposed membrane architecture in Archaea.

129 **Material and methods**

130 **Microorganism and growth conditions**

131 *Thermococcus barophilus* strain MP was isolated from the 3,550 m deep Snake Pit hydrothermal vent,
132 on the Mid-Atlantic Ridge (36). The strain was obtained from the UBOCC (Université de Bretagne
133 Occidentale – type Culture Collection, France). Cultures were grown under strict anaerobiosis in a rich
134 medium established for *Thermococcales* (38), containing 3 % w/v NaCl and 10 g L⁻¹ elemental sulfur, at 85
135 °C, pH 6.8, and atmospheric pressure. The medium was reduced by adding Na₂S (0.1% w/v final) before
136 inoculation. Growth was monitored by counting with a Thoma cell counting chamber (depth 0.01 mm) using
137 a light microscope (life technologies EVOS® XL Core, × 400). Under these conditions, cell concentrations
138 of 2 × 10⁸ cells mL⁻¹ were routinely achieved.

139 Cells of 1-L cultures in late exponential phase were recovered by centrifugation (4000 × g, 45 min, 4
140 °C) and rinsed twice with an isotonic saline solution (3 % w/v NaCl). A significant amount of sulfur from
141 the growth medium was recovered alongside cells, and the cellular dried mass was thus not estimated. The
142 cell pellets were lyophilized overnight and kept at -80 °C until lipid extraction.

143 **IPL extraction and UHPLC-ESI-MS analysis**

144 IPLs were extracted using a modified Bligh and Dyer (B&D) procedure (39), as previously described
145 (40). Briefly, dried cells were extracted with a monophasic mixture of methanol/dichloromethane/purified
146 water (MeOH/DCM/H₂O; 1:2.6:0.16; v/v/v) using a sonication probe for 15 min. After centrifugation (2500
147 × g, 8 min), the supernatant was collected, and the extraction procedure was repeated twice. The
148 supernatants were pooled, dried under a N₂ stream, solubilized in MeOH/DCM (1:5; v/v; hereafter referred
149 to as total lipid extract; TLE), and kept at -20 °C until analysis. A significant amount of sulfur from the
150 growth medium was extracted alongside archaeal lipids, and the total lipid dry mass was thus not estimated.

151 IPLs separation was first performed with a hydrophilic interaction liquid chromatography (HILIC)
152 setting. IPLs were separated on a Waters Acquity UPLC BEH Amide 1.7 μm column (150 mm×2.1 mm,
153 Waters Corporation, Eschborn, Germany) maintained at 40 °C by ultra-high performance liquid
154 chromatography (UHPLC) using a Dionex UltiMate 3000RS UHPLC (ThermoFisher Scientific, Bremen,
155 Germany) instrument equipped with an auto-injector and a Chromeleon chromatography manager software

156 following the method described by Wörmer *et al.* (41). Di- and tetraether IPLs were eluted in the same run
157 with a flow rate of 0.4 mL min⁻¹, using the following linear gradient with A [acetonitrile(ACN):DCM:formic
158 acid(FA):ammonium hydroxide(NH₃) (75:25:0.01:0.01, v/v/v/v)] and B [MeOH:H₂O:FA:NH₃
159 (50:50:0.4:0.4, v/v/v/v)]: 99 % A (2.5 min isocratic) to 95 % A in 1.5 min, then to 75 % A in 18.5 min, and
160 finally to 60 % A in 4 min (1 min isocratic). Alternatively and when mentioned, IPLs separation was
161 performed with a reverse phase (RP) setting. IPLs were eluted on a Waters Acquity UPLC BEH C₁₈ 1.7 μm
162 column (150 mm×2.1 mm, Waters Corporation, Eschborn, Germany) maintained at 65 °C using the
163 following linear gradient as described by Wörmer *et al.* (41) with A [MeOH:H₂O:FA:NH₃ (85:15:0.04:0.1,
164 v/v/v/v)] and B [propan-2-ol:MeOH:FA:NH₃ (50:50:0.04:0.1, v/v/v/v)] at a flow rate of 0.4 mL min⁻¹: 100
165 % A (2 min isocratic) to 85 % A in 0.1 min, then to 15 % A in 18 min and finally to 0 % A in 0.1 min (8
166 min isocratic). 100 % A was eventually held isocratically for 7 min. Samples were thawed, dried under a N₂
167 stream, and dissolved in either MeOH/DCM (1:9; v/v) or MeOH/DCM (9:1; v/v) for HILIC and RP
168 separation, respectively. The injection volume was set to 10 μL.

169 Detection was achieved using a maXis quadrupole time-of-flight mass spectrometer (Q-ToF-MS,
170 Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) source operating in
171 positive mode. The ESI source was also operated in negative mode, although this did not provide further
172 information on the IPL composition. Only the conditions for the MS analysis in positive mode are thus
173 described here and were as follows: capillary voltage 4500 V, nebulizer gas pressure 0.8 bar, drying gas
174 (N₂) flow 4 L min⁻¹ at 200 °C, mass range *m/z* 100-2000. MS calibration was performed by a tuning mixture
175 solution (*m/z* 322.048, 622.029, 922.010, 1221.991, 1521.972, and 1821.952) introduced by loop-injection
176 near the end of a run and an internal lock mass throughout the entire run. MS² scans were automatically
177 obtained in data-dependent mode by fragmentation of the three to ten most abundant ions at each MS scan.

178 Mass spectra were visualized and analyzed using the Bruker Data Analysis software by comparing
179 the parent molecular ion masses (occurring as H⁺, NH₄⁺ or Na⁺ adducts) and the characteristic fragmentation
180 patterns with previously described ones (35, 42, 43). For quantification, 2 ng of a phosphatidylcholine C₂₁-
181 diacylglycerol (C₂₁-PC) standard were added to the sample prior to injection. The response factors of

182 bacterial mono- and digalactosyl diacylglycerols (MGDG and DGDG, respectively) relative to the injection
183 standard C₂₁-PC were used to approximate those of the detected IPLs. Calibration curves were established
184 by injecting two times a standard solution consisting of C₂₁-PC, MGDG and DGDG in six different
185 concentrations ranging from 0.001 to 30 ng μL^{-1} . Detection was achieved only at concentrations higher than
186 0.1 ng μL^{-1} . Under our analytical conditions, MGDG and DGDG showed molecular response factors of 0.58
187 and 0.21 relative to C₂₁-PC, respectively. Different response factors are to be expected in ESI-MS, notably
188 for lipids bearing distinct polar head groups (e.g. hexose vs. hexosamine), but the same response factors
189 were applied for all IPLs bearing the same number of sugar residues in their polar head group, i.e., 0.58 for
190 all mono- and 0.21 for all diglycosylated lipids, respectively. IPL relative abundances were determined in
191 positive mode by integration of the peak area on the extraction ion mass chromatograms with a width of
192 0.02 Da corresponding to the protonated, ammoniated and sodiated adducts, and subsequent correction using
193 the corresponding response factor.

194 **CL extraction and UHPLC-APCI-MS analysis**

195 In order to exhaustively analyze the CL composition of *T. barophilus*, polar head groups were removed
196 by acid methanolysis (1 N HCl in MeOH at 70 °C for 16 h) of the biomass (total CLs) as described by
197 Becker *et al* (44). The hydrolyzed lipids were extracted with a monophasic mixture of MeOH/DCM (1:5;
198 v/v) using a sonication probe for 15 min. After centrifugation (2500 \times g, 8 min), the supernatant was
199 collected in a separatory funnel and the extraction procedure was repeated twice. CLs were partitioned into
200 the organic phase following addition of Milli-Q water, and the aqueous phase was subsequently washed
201 three times with an equal amount of DCM. The organic phases were collected, pooled, and subsequently
202 washed three times with an equal amount of Milli-Q water. The solvent of the resulting CL extracts was
203 evaporated under a N₂ stream and the extracts were resolubilized in *n*-hexane/propan-2-ol (99.5:0.5; v/v).
204 The same procedure was applied to the TLE (CLs from IPLs) to evaluate our IPL extraction method.

205 CLs were separated on two coupled Waters Acquity UPLC BEH Amide 1.7 μm columns (150 mm \times 2.1
206 mm, Waters Corporation, Eschborn, Germany) maintained at 50 °C using a Dionex UltiMate 3000RS
207 UHPLC (ThermoFisher Scientific, Bremen, Germany) instrument equipped with an auto-injector and a

208 Chromeleon chromatography manager software. The injection volume was set to 10 μL . Di- and tetraether
209 CLs were eluted in the same run using a linear gradient with *n*-hexane and *n*-hexane/propan-2-ol (9:1; *v/v*)
210 at a flow rate of 0.5 mL min^{-1} , as described by Becker *et al.* (44).

211 Detection was achieved using a maXis Q-ToF-MS (Bruker Daltonics, Bremen, Germany) equipped
212 with an atmospheric pressure chemical ionization (APCI) source operating in positive mode. The conditions
213 for the MS analyses were as in Becker *et al.* (44): nebulizer gas pressure 5 bar, corona discharge current
214 3500 nA, drying gas (N_2) flow 8 L min^{-1} at 160 $^\circ\text{C}$, vaporizer 400 $^\circ\text{C}$, mass range m/z 150-2000. MS
215 calibration and MS^2 scans were performed as described above.

216 Mass spectra were visualized and analyzed on a Bruker Data Analysis software using parent
217 molecular ion masses (occurring exclusively as H^+ adducts) and characteristic fragmentation patterns (45).
218 For quantification, 2 ng of a C_{46} analogue of GTGT (C_{46} -GTGT) were added to the sample prior injection.
219 To determine the response factors of the detected core structures, calibration curves were established by
220 injecting two times a standard solution consisting of C_{46} -GTGT, GDGT with no cyclopentane ring (GDGT0)
221 and DGD in 5 different concentrations ranging from 0.001 to 10 $\text{ng } \mu\text{L}^{-1}$. GDGT0 was detected only at
222 concentrations higher than 0.1 $\text{ng } \mu\text{L}^{-1}$, whereas C_{46} -GTGT and DGD were detected at all concentrations.
223 Under our analytical conditions, DGD and GDGT0 showed relative response factors of 0.42 and 0.57
224 relative to C_{46} -GTGT, respectively. In the absence of a measured response factor for the different archaeal
225 core lipids, we assumed the same response factor as DGD for all diethers and that of GDGT0 for all
226 tetraethers. CL relative abundances were determined by integration of the peak area on the extracted ion
227 mass chromatograms with 0.1 Da width corresponding to the protonated adducts, and subsequent correction
228 by the corresponding response factor.

229 **Isolation of *T. barophilus* major IPLs**

230 In order to further characterize and validate the structural diversity of *T. barophilus* lipids, its major
231 IPLs were isolated using the aforementioned HILIC UHPLC method. 50 % of a TLE were dried under a N_2
232 stream and resolubilized in 10 μL of MeOH/DCM (1:9; *v/v*) for injection. Collecting vials were placed at
233 the exit of the chromatography column, and 7 fractions corresponding to *T. barophilus* well-separated major

234 IPLs were manually collected (F1, 11.5-12.5 min; F2, 13.3-14.0 min; F3, 14.5-15.5 min; F4, 15.7-16.4 min;
235 F5, 17.9-18.2 min; F6, 18.4-19.0 min; F7, 23.0-27.0 min). Collected fractions were dried under a N₂ stream,
236 resolubilized in 100 µL of MeOH/DCM (9:1; v/v), and their purity was assayed using the aforementioned
237 RP UHPLC-MS method. Injection volume was set to 10 µL. Detection was achieved as described in the
238 previous section.

239 **IPL cleavage and UHPLC-ESI-QQQ-MS analysis of the hexose-based polar head groups**

240 In order to characterize the hexose-based polar head groups of *T. barophilus*, both the biomass and
241 individual IPL fractions were cleaved by acid hydrolysis (30 % trifluoroacetic acid (TFA) in H₂O at 70 °C
242 for 16 h) to release the monosaccharide(s) from IPLs. The reaction was stopped by drying the sample under
243 a stream of N₂ and the remaining TFA was removed by washing three times with DCM. Hydrolysates were
244 solubilized in ACN/H₂O (95:5, v/v), and CLs were extracted upon addition of hexane while
245 monosaccharides remained in the ACN/H₂O phase.

246 CLs were separated and analyzed using the aforementioned UHPLC-APCI-MS system.

247 Monosaccharides were separated on a Waters Acquity UPLC BEH Amide 1.7 µm column (150 mm×2.1
248 mm, Waters Corporation, Eschborn, Germany) maintained at 60 °C using a Dionex UltiMate 3000RS
249 UHPLC (ThermoFisher Scientific, Bremen, Germany). Samples were dissolved in ACN/H₂O (95:5, v/v)
250 and the injection volume was set to 10 µL. All hexose-based polar head groups were eluted in the same run
251 using the linear gradient described by Lowenthal *et al.* (46) with A [0.1 % NH₃ in H₂O] and B [0.1 % NH₃
252 in ACN] at a flow rate of 0.2 mL min⁻¹: 5 % A (3 min isocratic) to 10 % A in 22 min, then to 40 % A in 3
253 min (7 min isocratic), and finally to 5 % A in 2 min.. Detection was achieved by scheduled multiple reaction
254 monitoring (sMRM) on a QTRAP 4500 triple quadrupole MS (ABSciEX, Darmstadt, Germany) equipped
255 with an ESI source operating in positive mode. Source conditions were as follows: curtain gas (CUR)
256 pressure 30 psi , ion source gas 1 (GS1) pressure 40 psi, ion source gas 2 (GS2) pressure 30 psi, ion spray
257 (IS) voltage 4500 V, capillary temperature (TEM) 400 °C. The MRM method was established by direct
258 infusion of 14 carbohydrates (Table S1) and consisted of 20 different transitions, with two transitions for
259 each carbohydrate type.

260 The different sugar-based head groups were quantified by external calibration. Linear calibration curves
261 were established for a wide variety of sugar derivatives by injecting two times standard solutions containing
262 β -D-allopyranose (Alp), β -D-fructopyranose (Fru), α -D-glucopyranose (Glc), β -D-galactopyranose (Gal),
263 α -D-mannopyranose (Man), β -D-xylofuranose (Xyl), α -L-arabinopyranose (Ara), α -D-lyxopyranose (Lyx),
264 D-glucosamine (GlcNH₂), N-acetyl-D-glucosamine (GlcNAc), myo-inositol (Ino) and D-saccharose (Sac)
265 in 12 different concentrations ranging from 0.005 to 1000 μ M. Alp and Fru and Xyl and Ara peaks could
266 not be distinguished and were integrated as two single peaks (Alp/Fru and Xyl/Ara, respectively). Every
267 standard was detected in concentration as low as 0.05 μ M, with the exception of Sac which was not
268 identified below 5 μ M.

269 **Extraction-free analysis via MALDI-FT-ICR-MS**

270 To further investigate the IPL diversity of *T. barophilus*, and particularly that of tetraether-based
271 lipids, matrix-assisted laser desorption/ionization coupled with Fourier transform ion cyclotron resonance
272 mass spectrometry (MALDI-FT-ICR-MS) was used directly on the biomass.

273 *T. barophilus* dried cell pellets were resuspended in 1 mL of Milli-Q water and centrifuged (500 \times g, 1
274 min) to remove as much sulfur as possible. Supernatants were then decanted by centrifugation (15000 \times g,
275 15 min) and cells were resuspended in Milli-Q water. 10 mg of 2,3-dihydroxybenzoic acid (DHB) was
276 dissolved in 1 mL of H₂O/ACN (3:7, v/v) containing 1 % of TFA and used as matrix. The cell suspension
277 and the matrix solution were mixed (1:1; v/v) and 1 μ L was spotted and evaporated on a ground steel MALDI
278 target plate.

279 The analysis was carried out on a 7T solariX XR FT-ICR-MS coupled to a DUAL source with a
280 Smartbeam II laser (Bruker Daltonics, Bremen, Germany). The FT-ICR-MS was operated in positive serial
281 mode, with data being acquired over the mass range m/z 600-3000. Instrument settings were optimized for
282 larger molecules ($m/z > 1000$). Each scan was generated by accumulating the ions of 25 laser shots at 40 %
283 laser power and a frequency of 2000 shots min⁻¹. External calibration was performed with a standard peptide
284 mixture (Bruker Daltonics). An internal lock mass calibration was applied using the sodiated adduct of PI-
285 GDGT0-PI (m/z 1808.343).

286 **Results**

287 ***Thermococcus barophilus* exhibits a diverse membrane lipid composition**

288 Our B&D extracts analyzed with the UHPLC-MS procedure yielded very low cellular lipid content
289 (0.12 fg cell⁻¹, as calculated from cell counts and lipid abundance). The combined analyses of (1) the TLE
290 and (2) the polar head groups and core structures separated from purified major lipids nevertheless revealed
291 a diverse membrane composition for *T. barophilus* (for structures, refer to Figure 1). All IPLs identified in
292 *T. barophilus* TLE showed ions diagnostic of archaeal phosphoglycolipids (for instance, ions at $m/z = 733.6$
293 and 453.3; Figure S1), whereas no glycolipids were detected.

294 The TLE of *T. barophilus* appeared to be dominated by compound II (Figure 2, Table 1), whose
295 molecular mass ($[M+H]^+$ at $m/z = 895.704$) and fragmentation pattern (for instance, ions at $m/z = 615.4$ and
296 733.6; Figure S2) corresponded to a DGD bearing a phosphatidylhexose head group. The molecular mass
297 and fragmentation pattern of the minor compound III (Figure S2) appeared identical to compound II but the
298 difference in retention times (13.7 vs. 15.0 min for compounds III and II, respectively) indicated distinct
299 hexose isomers as polar head groups of II and III. The analysis of the purified fractions revealed 100 % of
300 Ino and 98 % of DGD for fraction F3 (98 % of compound II) and 100 % of Glc and 100 % of DGD for
301 fraction F2 (containing only III; Table 2), respectively. This confirmed II to be a PI-DGD and III its Glc
302 isomer PGlc-DGD. Compound IV was another major IPL of *T. barophilus* TLE (Figure 2, Table 1). Its
303 molecular mass ($[M+H]^+$ at $m/z = 936.713$), retention time (12.2 min), fragmentation pattern (for instance,
304 ions at $m/z = 138.1$ and 204.1; Figure S2), and the presence of 52 % of Glc and 48 % of GlcNAc and of 100
305 % of DGD in fraction F1 (containing only IV) allowed to identify as a PGlcNAc-DGD (Table 2). The
306 fragmentation pattern of IV did not allow to determine the exact position of the N-acetylation.

307 Compounds XIII and XVII had retention times almost identical to compound II (14.8 and 14.9 min,
308 respectively; Figure 2) but higher molecular masses ($[M+H]^+$ at $m/z = 965.778$ and 909.690, respectively),
309 and fragmentation patterns that suggested these compounds to be a phytanylsesterterpanyl glycerol diether
310 (PSGD) and a methylated DGD (MeDGD) bearing phosphatidylhexose head groups (for instance, ions at
311 $m/z = 723.8$ and 649.7, respectively; Figure S3). The analysis of fraction F3 containing compounds II, XIII

312 and XVII showed exclusively Ino and 98 % of DGD and 2 % of PSGD (Table 2). The structures of PI-DGD
313 II and PI-PSGD XIII were thus confirmed while compound XVII was only suggested to correspond to PI-
314 MeDGD.

315 Compounds V and VI, which differed from one another by slightly less than one mass unit ($[M+H]^+$
316 at $m/z = 1057.754$ and 1056.774 , respectively) and showed similar fragmentation patterns (for instance, ions
317 at $m/z = 777.5$ and 776.5 , respectively; Figure S4), were identified as DGD bearing a glycosylated
318 phosphatidylhexose head group and its hexosamine derivative in which one hydroxyl group is replaced by
319 an amino group, respectively. The acid hydrolysis of the fractions containing V and VI (F6 and F5,
320 respectively) yielded only Glc and DGD with traces of PSGD (Table 2). The presence of only Glc and the
321 absence of disaccharides in both F5 and F6 suggested that the hydrolytic conditions were adequate to cleave
322 off the glycosidic bond between the two sugar moieties, and that the structure of V was probably PGlcGlc-
323 DGD. No hexosamine was detected in F5, impeding further characterization of compound VI, which we
324 therefore tentatively assigned to PGlcHexNH₂-DGD. Similarly to V and VI, compounds XIV and XV also
325 differed from one another by slightly less than one mass unit ($[M+H]^+$ at $m/z = 1127.827$ and 1126.842 ,
326 respectively) and were present in fractions F6 and F5, respectively. Their molecular masses, shifted upwards
327 by 70 mass units compared with PGlcGlc-DGD V and PGlcHexNH₂-DGD VI, retention times (18.5 and
328 17.8 min, respectively), fragmentation patterns (for instance, ions at $m/z = 685.5$; Figure S5), and the
329 presence of traces of PSGD in F6 and F5 (Table 2) suggested compounds XIV and XV to correspond to
330 PGlcGlc-PSGD and PGlcHexNH₂-PSGD, respectively. Similarly to compound VI, compounds VII, VIII,
331 IX, and X ($[M+H]^+$ at $m/z = 1098.781$, 1139.806 , 1125.815 , and 1124.831 , respectively) could be associated
332 to derivatives of PGlcGlc-DGD V (Table 1, Figures S4, S6, and S7). The comparison of the fragmentation
333 patterns of VII and VIII with those of PGlcNAc-DGD IV and PGlcGlc-DGD V, and notably the detection
334 of ions at $m/z = 138.1$ and 818.5 and at 245.1 and 859.5 (Figures S4 and S6), suggested the former to
335 correspond to DGDs bearing mono- (PHexHexNAc-DGD VII) and di-N-acetylated glycosylated
336 phosphatidylhexose (PHexHex2NAc-DGD VIII), respectively. The exact nature of the sugar moieties and
337 the positions of the N-acetylations could not be further resolved. Compounds IX and X showed molecular

338 masses ($[M+H]^+$ at $m/z = 1125.816$ and 1124.827 , respectively) shifted upwards by 68 mass units compared
339 with PGlcGlc-DGD V and PGlcHexNH₂-DGD VI, respectively, and displayed similar fragmentation
340 patterns (for instance ions at $m/z = 845.5$ and 844.5 ; Figures S4 and S7). Although the nature of this increase
341 in 68 Da could not be determined, we suggested it to be an isoprene unit (C₅H₈). The acid hydrolysis of
342 fraction F4 (93 % of IX) released a majority of DGD and exclusively Glc (Table 2), and compound IX was
343 thus assigned the partially solved structure PGlcHex+C₅H₈-DGD. Compound X was detected in very low
344 amount (5.0×10^{-6} fg cell⁻¹; Table 1), which hinders its complete characterization. Although no Glc was
345 detected in F3 containing X, its similarity to both PGlcHexNH₂-DGD VI and PGlcHex+C₅H₈-DGD IX
346 (Figures S4 and S7) suggested it to correspond to PGlcHexNH₂+C₅H₈. Other minor compounds with masses
347 shifted upwards by either 68 or 70 Da were detected, e.g., a compound at 15.8 min with $[M+H]^+$ at $m/z =$
348 1195.886 could correspond to PGlcGlc-PSGD with 68 additional Da, i.e., PGlcGlc+C₅H₈-PSGD. Their low
349 abundances and the absence of MS² spectra however prevented to solve their exact structures.

350 Apart from the ions typical of archaeal phospholipids (Figure S1), compound XI ($[M+H]^+$ at $m/z =$
351 1038.689) showed a fragmentation pattern completely distinct from the IPLs described above (for instance,
352 ions at $m/z = 324.1$ and 758.4 ; Figure S8) which suggested a head group distinct from typical hexoses.
353 Compound XI could indeed be attributed to a DGD bearing a diphosphatidyl cytidine head group, i.e., the
354 first intermediate in the pathway for polar head group fixation (47). The lack of relevant standard and the
355 low quantities of XI prevented further elucidation of its structure by the acid hydrolysis of fraction F6 (Table
356 2).

357 In addition to their fully saturated forms, minute amounts of PI-DGD II, PGlc-DGD III, PGlcNAc-
358 DGD IV, PGlcHexNH₂-DGD VI, and CDP-DGD XI were detected with one to eight unsaturations whereas
359 PGlcGlc- V, PHexHexNAc- VII, PGlcHex+C₅H₈- IX, and PHexHexNH₂+C₅H₈-DGD X were detected with
360 one to six unsaturations (Figure 2). Only the fully saturated forms were detected for the other diethers,
361 namely PHexHex2NAc-DGD VIII, PI-PSGD XIII, PGlcGlc-PSGD XIV, PGlcHexNH₂.PSGD XV and PI-
362 MeDGD XVII.

363 As various Glc derivatives were detected, we looked for putative IPLs bearing similar and additional
364 combinations of NH₂, NAc and/or C₅H₈ groups, but none were detected. Similarly, glycolipids and
365 phospholipids regularly found in Archaea, e.g., lipids bearing phosphatidylcholine (PC), phosphatidylserine
366 (PS) and phosphatidylethanolamine (PE), were searched for but not detected in *T. barophilus*.

367 The very few tetraether-based IPLs detected in *T. barophilus* TLE all gather in a poorly resolved
368 broad peak (Figure 2). The major component of this peak was compound XIX, whose molecular mass
369 ([M+H]⁺ at $m/z = 1786.360$), fragmentation pattern (for instance, ions at $m/z = 731.6$ and 1544.3 ; Figure S9)
370 and the acid hydrolysis of F7 (95 % of GDGT0, 43 % of Ino; Table 2) identified it as PI-GDGT0-PI. No
371 fragmentation pattern was obtained for compound XXIII, but its molecular mass ([M+H]⁺ at $m/z = 1788.376$)
372 shifted upwards by two mass units compared to XIX and the presence of 3 % of GTGT0 in F7 suggested
373 that it might correspond to PI-GTGT0-PI (Table 2; see further details in Supplementary text). F7 also
374 contained 57 % of a compound with the same fragmentation pattern as the Sac standard but with a slightly
375 distinct retention time, which thus suggested it to be another disaccharide (hereafter referred to as 2Hex).
376 The abundance of 2Hex in fraction F7 and the absence of tetraether-based IPLs with a 2Hex polar head
377 group in UHPLC-ESI-MS suggested that this fraction might contain other, unresolved tetraether-based IPLs.

378 In addition to this set of IPLs, we detected a series of polyprenyl derivatives in *T. barophilus* TLE
379 using our RP UHPLC method (Figure S11). These compounds represent a large family of membrane-bound
380 polyisoprenoids known as major lipid carriers for membrane protein glycosylation in all three domains of
381 life (48). Such compounds have been identified in a wide variety of archaea, in which the sugar residue can
382 be attached to either alcohol, mono-, or diphosphate end-groups of polyprenyls with six to 14 isoprene units
383 (49). Here, polyprenyl derivatives comprising 10 to 12 isoprene units and 1 to 5 unsaturations with only
384 monophosphate head groups were identified (Figure S11).

385 **PI-DGD dominates the total lipid extract of *T. barophilus***

386 The IPLs identified in *T. barophilus* TLE were quantified considering a response factor of 0.58 for
387 monoglycosidic (II, III, IV, XI, XIII, and XVII) and 0.21 for diglycosidic IPLs (V, VI, VII, VIII, IX, X,

388 XIV, XV, XIX, and XXIII) relative to the internal standard C₂₁-PC. Since unsaturated IPLs were detected
389 in minute amounts, only the saturated forms were quantified. *T. barophilus* TLE (0.12 fg cell⁻¹) was
390 overwhelmingly dominated by PI-DGD II (91 %, 0.11 fg cell⁻¹; Table 1), with a few other major IPLs, i.e.,
391 PGlcNAc-DGD IV, PGlcHexNH₂-DGD VI, PGlcHex+C₅H₈-DGD IX, and PGlcGlc-DGD V (ca. 8 %, 1.05
392 × 10⁻² fg cell⁻¹). The remaining minor IPLs detected in trace amounts or in too low abundances to be
393 quantified represented only 6.25 × 10⁻³ fg cell⁻¹ (ca. 1 % of the TLE mass). DGD-based IPLs represented
394 ca. 99 % of *T. barophilus* TLE, whereas tetraether-based IPLs were only recovered in trace amounts (Table
395 1).

396 To evaluate the efficiency of our extraction and analysis protocols, we performed several
397 calculations to approximate a putative total IPL composition of TLE and of the original biomass. The acid
398 methanolysis of the TLE provides reliable information on the IPL core lipid distribution (CLs from IPLs),
399 which was composed of ca. 80 % of diethers and 20 % of tetraethers (Table 3). To appraise the lipid content
400 per cell based on this CL composition, i.e., lipids without polar head group, an estimation of the nature and
401 relative proportions of *T. barophilus* polar head groups is however required. The dedicated analysis of the
402 polar head groups only allowed to access a few, low mass hexose derivatives (ca. 242 Da with a
403 phosphatidyl group). Although none were detected here, a larger range of polar head groups, including
404 smaller, e.g., PE (123 Da), and substantially larger ones, e.g., PHexHex2NAc (486 Da), sulfonotri- and
405 tetrahexoses (551 and 713 Da), is to be expected in archaea (50, 51). This suggests that the polar head group
406 can represent a non-negligible mass proportion of a given IPL, which we hypothesized to range from 0.2
407 (e.g., only small head groups like one and two PE for diether and tetraethers lipids, respectively) to 1 (e.g.,
408 only massive head groups like one and two phosphatidyltetrahexose for diether and tetraethers lipids,
409 respectively) times the mass of the core lipid. Considering putative low (1:0.2) and high (1:1) mass ratios
410 between the core lipid and the polar head group and the quantity of core lipids retrieved upon acid
411 methanolysis (Table 3), we estimated that our TLE actually contained from 0.1 to 0.2 fg of lipids per cells.
412 The 18 IPLs identified with UHPLC-MS (0.12 fg cell⁻¹) thus represented 50-100 % of the TLE (Figure 4).

413 To further estimate how close this lipid composition was from the real lipidome of *T. barophilus*,
414 we compared it with calculated and an experimentally-derived theoretical total lipid contents (refer to Figure
415 4 for a summary of these estimations). *T. barophilus* cells are coccoid with a cell diameter of 0.8 to 2.0 μm
416 (36) and covered in one or more dense proteinaceous surface layers like other Thermococcales (52). Using
417 the calculations described by Lipp *et al.* (31) with a membrane thickness of 5.5 nm and protein content of
418 70 %, our calculated theoretical total lipid content ranged between 3.0 and 20 fg cell⁻¹. On the other hand,
419 the direct acid methanolysis of *T. barophilus* biomass yielded its total core lipid content (total CLs), which
420 contained *ca.* 30 % of diethers and 70 % of tetraethers (Table 3). As for the estimation of the TLE content,
421 considering low and high mass ratios between the core lipid and the polar head group resulted in an
422 experimentally-derived theoretical total lipid content of *T. barophilus* ranging from 0.5 to 0.8 fg cell⁻¹. Our
423 TLE (0.1 to 0.2 fg cell⁻¹) thus represented *ca.* 12-40 % of *T. barophilus* lipidome (experimental-derived
424 theoretical lipid content; Figure 4).

425 **MALDI-FT-ICR-MS reveals novel tetraether-based IPLs in *T. barophilus***

426 MALDI-FT-ICR-MS allows for the direct ionization of a sample, without prior wet-chemical
427 treatment, and thus offers a valuable tool to explore archaeal IPLs that are unreachable by extraction-based
428 analytical procedures. The direct ionization of *T. barophilus* biomass using MALDI-FT-ICR-MS resulted
429 in clusters of peaks for each IPL (Figure 5), suggesting the presence of numerous isotopologues and singly
430 charged adducts, i.e., [M+H]⁺, [M+Na]⁺, [M+K]⁺, [M+2Na-H]⁺, [M+K+Na-H]⁺ and [M+3Na-2H]⁺. Lipids
431 were assigned based on the molecular masses of the different adducts detected, the monosodiated one being
432 always the most abundant. Under the optimal experimental conditions determined here (not shown), we
433 could detect the majority of *T. barophilus* most abundant diether-based IPLs identified in UHPLC-MS, i.e.,
434 PI-DGD II and/or PGlc-DGD III, PGlcGlc-DGD V and PGlcHex+C₅H₈-DGD IX, although some were not
435 observed, e.g., PGlcNAc-DGD VI. A special focus was paid to specifically target *T. barophilus* unidentified
436 tetraether lipid diversity, and PI-GDGT0-PI XIX and PI-GTGT0-PI XXIII indeed appeared in a seemingly
437 much higher abundance than that revealed by UHPLC-ESI-MS (Figure 5). Other, lower mass ion adducts
438 were detected, e.g., at $m/z = 1646.304$, 1566.339 and 1324.390 , and were assigned to sodiated adducts of

439 PI-GDGT0-P, PI-GDGT0 and GDGT0 XVIII, respectively. As those compounds were not identified in *T.*
440 *barophilus* TLE analyzed with UHPLC-ESI-MS, they were assumed to result from laser-induced partial
441 degradation of the much more abundant PI-GDGT0-PI XIX (Figure 5). Additionally, two putative
442 uncharacterized tetraether-based IPLs were observed, namely compounds XX and XXI. Compound XX
443 showed molecular ions at $m/z = 1948.451$, 1970.413 and 1992.396 that could be assigned to the $[M+H]^+$,
444 $[M+Na]^+$ and $[M+2Na-H]^+$ adducts of PHex-GDGT0-PHexHex (theoretical ions at $m/z = 1948.414$,
445 1970.396 and 1992.378 , respectively). Similarly, molecular ions of compound XXI at $m/z = 2016.483$,
446 2038.485 and 2060.468 were assigned to $[M+H]^+$, $[M+Na]^+$ and $[M+2Na-H]^+$ adducts of PHex-GDGT0-
447 PHexHex+C₅H₈ (theoretical ions at $m/z = 2016.476$, 2038.458 and 2060.440 , respectively). Despite a
448 relatively large gap between theoretical and observed masses which might result from very low amounts of
449 those compounds and the increasing absolute mass error with higher masses, these putative structures were
450 further supported by the detection of 2Hex polar head groups in fraction F7 corresponding to the tetraether
451 unresolved peak in UHPLC-MS (Table 2). Other tetraether-based IPLs bearing polar head groups similar to
452 those detected on the diether-based IPLs, such as (poly)N-acetylated hexosamine, were also screened for
453 but not detected. Scanning *T. barophilus* biomass for higher masses neither yielded other ions nor improved
454 the recovery of the newly identified IPLs (not shown).

455 Altogether, 18 saturated and 64 unsaturated IPLs were detected and tentatively identified in *T.*
456 *barophilus*. Structures of PI-DGD II, PGlc-DGD III, PGlcNAc-DGD IV, PGlcGlc-DGD V, PGlcHexNH₂-
457 DGD VI, PGlcHex+C₅H₈-DGD IX, PI-PSGD XIII, PGlcGlc-PSGD XIV, PGlcHexNH₂-PSGD XV, and PI-
458 GDGT0-PI XIX were validated by analyzing independently their polar head groups and core structures,
459 whereas those of PHexHexNAc-DGD VII, PHexHex2NAc-DGD VIII, CDP-DGD XI, and PI-MeDGD
460 XVII were determined based on their fragmentation patterns alone. In contrast, the structure of PI-GTGT0-
461 PI XXIII derived solely from the acid hydrolysis results, whereas those of PHex-GDGT0-PHexHex XX and
462 of PHex-GDGT0-PHexHex+C₅H₈ XXI resulted from the molecular masses detected by FT-ICR-MS.

463 **Discussion**

464 **Novel IPL structures were uncovered from the diverse lipid composition of *T. barophilus***

465 By means of UHPLC-MS and MALDI-FT-ICR-MS, 18 saturated and 64 unsaturated IPLs were
466 identified in *T. barophilus* (Table 1). Fourteen IPLs were based on diethers, i.e., DGD I, PSGD XII and
467 MeDGD XVI, and four on tetraethers, i.e., GDGT0 XVIII and GTGT0 XXII. Ten distinct polar head groups
468 were detected, among which three were derivatives of phosphatidylhexose (PI, PGlc, PGlcNAc), six of
469 glycosylated phosphatidylhexose (PGlcGlc, PGlcHexNH₂, PHexHexNAc, PHexHex2NAc,
470 PGlcHex+C₅H₈, PHexHexNH₂+C₅H₈), and one of nucleoside diphosphate (CDP; Table 1).

471 Similarly to numerous other archaea, and especially Thermococcales (35, 42, 53–55), PI-DGD II
472 was the dominant IPL of *T. barophilus*. Whereas PSGD-based IPLs were reported in numerous halophilic
473 archaea and a few methanogens (see for instance (22, 56–58)), this study reported for the first time the
474 presence of PSGD and MeDGD-based IPLs in Thermococcales and in hyperthermophilic archaea. In
475 addition to *T. barophilus*, PI-GTGT0-PI XXIII has been reported in *Thermococcus kodakarensis*,
476 *Pyrococcus furiosus* and *P. yayanosii* (40) and may be a common IPL to all Thermococcales, as the core
477 lipid GTGT0 XXII was reported in every Thermococcales investigated so far (37). Glc has been reported
478 repeatedly as a major sugar residue in archaeal glycolipids (see, for example, (59–63)), but only rarely in
479 phosphoglycolipids, i.e., in *Aeropyrum pernix* (64) and in *Thermococcus zilligi* (54), a close relative of *T.*
480 *barophilus*. Meador *et al.* (35) recently updated the IPL composition of *Thermococcus kodakarensis*, and
481 notably identified PHexNAc-DGD, PHexHex-DGD, PHexHexNH₂-DGD and PHexHexNH₂+C₅H₈-DGD,
482 with no further characterization of the polar head groups. To our knowledge, our detailed investigation of
483 the sugar residues is the first report of such a diversity of Glc derivatives as polar head groups of
484 phosphoglycolipids in Archaea, which were initially assumed to be mostly built upon Ino. This study also
485 reported for the first time mono- and diacetylated PHexHex-DGD VII and VIII as well as PHexHex+C₅H₈
486 as a GDGT0 polar head group in XXI. Although our results do not drastically contrast with the lipid
487 composition of other Thermococcales, they extend the known lipid diversity for this order of Archaea and

488 beyond, and places *T. barophilus* as a prime model for further investigation of the Thermococcales
489 membrane composition, organization and adaptation.

490 **A combination of UHPLC-MS and MALDI-FT-ICR-MS to elucidate *T. barophilus* IPL composition.**

491 Archaeal lipid extraction and fractionation have previously been demonstrated to be biased towards
492 certain lipid classes (24, 30, 65). The first and only description of *T. barophilus* intact polar lipids, which
493 reported exclusively PI-DGD II (36), undoubtedly suffered from such biases. The reevaluation of *T.*
494 *barophilus* CLs indeed showed an abundance of tetraether-based IPLs and demonstrated the impossibility
495 to exhaustively extract its IPLs with typical extraction procedures (24). While the reassessment of *T.*
496 *barophilus* IPLs in the present study did provide a greater insight into its diversity, including tetraether-
497 based IPLs (Table 1), we could only access 0.12 fg of lipid cell⁻¹. Although this remains in line with
498 observations made for other archaea, e.g., *T. barophilus* close relative *T. kodakarensis* (0.38 to 1.61 fg cell⁻¹
499 (35)), our results highlighted three limitations of the current procedure that can explain such a low yield.

500 First, we observed a large difference between *T. barophilus* calculated and experimentally-derived
501 theoretical lipid contents (3.0 to 20.0 vs. 0.5 to 0.8 fg cell⁻¹), which suggests that our calculations might be
502 far off *T. barophilus* real total lipid content. Indeed, even adding up the other lipid molecules *T. barophilus*
503 synthesizes and that are not visible after methanolysis of the biomass, i.e., polyprenyl phosphates (Figure
504 S10) and apolar polyisoprenoids (up to 1 % of the membrane content, (24)), to our experimentally-derived
505 theoretical total lipid content would not be enough to reach the calculated theoretical total lipid content. The
506 model for cell lipid content, initially built for bacterial cells and used here with rough estimates of *T.*
507 *barophilus* cell diameter and membrane lipid/protein ratio, thus requires revisions to better fit the archaeal
508 cell membrane. Although *T. barophilus* experimentally-derived theoretical total lipid content might also be
509 inaccurate, for instance due to other, unidentified polar head groups larger than the phosphatidylmono- and
510 di-hexoses considered here, it was used hereafter as a reference (Figure 4). Although closer to the total IPLs
511 detected, the experimentally-derived theoretical total lipid content still showed a large discrepancy with
512 what we could actually access with our B&D extraction (0.5 to 0.8 vs. 0.12 fg cell⁻¹), suggesting that other
513 biases might hinder the elucidation of *T. barophilus* entire lipidome.

514 We observed a major discrepancy between this experimentally-derived theoretical value and
515 extracted lipid contents (0.5 to 0.8 vs. 0.1 to 0.2 fg cell⁻¹; Figure 4), which indicates that most of *T.*
516 *barophilus* lipids are resistant to our extraction procedure. Our TLE contained only *ca.* 20 % of tetraethers
517 (Table 3), whereas they represented from 45 to 70 % of *T. barophilus* experimentally-derived theoretical
518 total lipid content in previous studies (24, 37) and here, respectively. Despite inconsistencies in the total
519 amount of tetraethers *T. barophilus* synthesizes that might stem from different growth or
520 extraction/analytical conditions, all three studies agree on a large discrepancy of diether/tetraether
521 distributions between the experimentally-derived theoretical total lipid content and the TLE. This suggests
522 that most of the IPLs not recovered are built upon tetraethers, and thus supports a major deficiency of our
523 extraction procedure in recovering tetraether-based IPLs.

524 We also highlighted a minor inconsistency between extracted and detected IPLs (0.1 to 0.2 vs. 0.12
525 fg cell⁻¹), which suggests that part of the IPLs that are indeed extracted might remain invisible to our
526 detection method. Despite being composed of up to 20 % of tetraethers (Table 3), *T. barophilus* TLE
527 displayed only two tetraether-based IPLs upon UHPLC-ESI-MS analysis, i.e., PI-GDGT0-PI XIX and PI-
528 GTGT0-PI XXIII, which represented less than 1 % of the TLE (Table 2). Although a fraction of tetraether-
529 based IPLs are indeed extracted and present in the TLE, these results suggest that they remain resistant to
530 detection by our analytical setup. Additionally, *T. barophilus* TLE was overwhelmingly dominated by PI-
531 bearing IPLs both in this study (90 %, Table 1, Figure 4) and that of Marteinson *et al.* (100 %) (36), while
532 neither glycolipids nor other polar head groups typically found in Archaea (e.g., PE, PS and PG, (15, 35,
533 50, 60, 66)) were observed. It is now widely accepted that physicochemical properties and physiological
534 and adaptive functions of biological membranes are governed by the structural diversity of both the alkyl
535 chains and the polar head groups found in the lipidome (67). One may thus speculate that a natural
536 membrane containing almost exclusively one polar head group might not be biologically functional. While
537 the absence of typical archaeal IPLs in *T. barophilus* might be linked to its particular membrane physiology
538 and/or environmental conditions, a functional membrane composed of > 90 % of a single IPL is hardly
539 conceivable, and *T. barophilus* membrane should theoretically contain other isomers and derivatives of

540 phosphatidyl(poly)hexoses not detected here to be functional. This therefore suggests that our UHPLC-ESI-
541 MS analytical procedure, and probably our B&D extraction method as well, might artificially enhance the
542 detection of PI-based over other phosphatidylhexose-derivative IPL populations. Altogether, our results thus
543 highlight two major shortcomings of our extraction and analytical procedure, i.e., preferential extraction
544 and detection of 1) diether-based and 2) PI-bearing IPLs, which resulted in a *T. barophilus* lipidome
545 artificially composed of almost exclusively PI-DGD II. However, our study shows that there is still much
546 to explore in the lipidome of *T. barophilus* and provides clues about the presence of other lipids such as
547 tetraethers with derivatives of phosphatidyl(poly)hexoses probably based on distinct sugar moieties.

548 In contrast to UHPLC-MS, MALDI-FT-ICR-MS of *T. barophilus* cell pellet revealed high levels of
549 several tetraether lipids when focusing on high m/z . For instance, PI-GDGT0-PI XIX appeared as one of *T.*
550 *barophilus* main IPLs with our MALDI-FT-ICR-MS settings (Figure 5). In addition to PI-GDGT0-PI XIX,
551 other low-mass tetraether derivatives such as PI-GDGT0-P, PI-GDGT0 and P-GDGT0 were identified
552 (Figure 5). PI-GDGT0 has been repeatedly reported in Archaea, including Thermococcales (35, 40, 68), but
553 almost always using rather destructive ionization methods (for instance, fast-atom bombardment in (68)).
554 Similarly, PI-GDGT0 was only detected here with MALDI-FT-ICR-MS under the highest laser power
555 setting (not shown) and not with our soft UHPLC-ESI-MS method (Figure 2). This suggests that our
556 MALDI-FT-ICR-MS procedure might alter tetraether lipids, preventing their detection as IPLs, and that the
557 aforementioned compounds could stem from laser-induced degradation rather than be true IPLs of *T.*
558 *barophilus*. MALDI-FT-ICR-MS nonetheless allowed to access previously unknown tetraether-based IPLs,
559 i.e., PHex-GDGT0-PHexHex XX and PHex-GDGT0-PHexHex+C₅H₈ XXI. Altogether, these results proved
560 MALDI-FT-ICR-MS to be a prime alternative to UHPLC-MS for exploring archaeal lipid diversity, and
561 especially tetraether-based IPLs. In the future of MALDI-FT-ICR-MS lipidomics, fine-tuning of the laser
562 parameters, including laser-based post-ionization (69), and of the matrix composition should help access an
563 even wider archaeal IPL diversity, although combination with UHPLC-MS remains necessary for lipid
564 quantitation and to elucidate the complete lipidome of Archaea.

565 **Insights into *T. barophilus* membrane organization.**

566 Most of the physical properties of archaeal lipids were based on the study of synthetic PE- and PC-
567 bearing lipids, and the absence of a comprehensive inventory of *T. barophilus*' polar head groups prevented
568 further understanding of its membrane physicochemical properties and organization. The detection of 82
569 IPLs however opens new avenues for understanding the membrane contribution and biological functions of
570 archaeal polar head groups as even the least abundant lipids were shown to ensure key cellular functions in
571 both Bacteria and Archaea (70, 71).

572 PI, present in PI-DGD II, PI-PSGD XIII, PI-MeDGD XVII, PI-GDGT0-PI XIX and PI-GTGT0-PI
573 XXII, was the most abundant polar head group in *T. barophilus* TLE (Table 1). Physical studies using
574 neutron and x-ray diffractions and NMR spectrometry demonstrated that PI extended deeply into the
575 aqueous environment in a slightly tilted configuration relative to the membrane surface normal (72–74).
576 The extension of PI away from the membrane surface favors intra- and intermolecular hydrogen bonds, thus
577 creating an extensive network that shields the membrane with a large electrostatic barrier preventing proton
578 and ion leakages (26, 72, 74). In addition, the direct projection of PI into the aqueous environment allows
579 for a maximum hydration of the inositol ring (74), which turns into a bulky hydrated head group. The high
580 volume of this bulky head group relative to that of the lipid alkyl chains enhances the conformational
581 freedom of the latter, which might eventually result into a looser packing and a higher water permeation in
582 model membranes containing PI (75). This packing defect generated by the PI head group, especially in the
583 tightly packed archaeal isoprenoid membranes, would in turn unlock more loading space for membrane
584 proteins and the higher water permeation would allow for solvent interactions essential to protein stability
585 within the membrane environment (75, 76). In contrast, the bulky head group creates a repulsive hydrated
586 layer that stabilizes the membrane by preventing deformation and membrane fusion (77, 78). The various
587 membrane macrostructures observed in Thermococcales, e.g., nanotubes and vesicles (79, 80), should
588 theoretically be greatly disfavored if their membrane was indeed composed exclusively of PI-based lipids
589 that prevent membrane remodeling. These results further confirm our assumption that PI might not
590 necessarily be the major head group in *T. barophilus* despite its overwhelming dominance in our extracts

591 (Table 1). In contrast, low proportions of PI would provide an enhanced fluidity in an otherwise tightly
592 packed archaeal membrane while preserving its impermeability.

593 In addition to inositol, glucose was found in a variety of polar head group derivatives that
594 represented *ca.* 8 % of *T. barophilus* TLE (Table 1). Due to the structural similarities between hexose
595 isomers, one might speculate that their effects on biological membranes would be comparable but synthetic
596 glycolipids bearing distinct hexose moieties showed different orientations relative to the membrane surface.
597 Glc nonetheless displayed an extended conformation similar to that of Ino (81), suggesting that it might
598 support membrane physicochemical properties analogous to those described above.

599 Stereochemical changes of a single hydroxyl group were shown to dramatically alter membrane
600 properties and stability (9, 82). Various derivatives of Glc bearing distinct additional groups were identified
601 in *T. barophilus* (Table 1), but their exact position on the hexose ring and the presence of different position
602 isomers could not be ascertained although they might support distinct functions. For instance, positions of
603 the phosphatidyl groups in phosphoinositides impacted their ionization properties, hydrogen bond networks
604 and thus their interactions with membrane proteins and lipids (83). No data are currently available on the
605 alterations of the lipid properties generated by the additional NAc, NH₂ and C₅H₈ moieties detected in *T.*
606 *barophilus*. However, an O-acetylation on the C-6 atom of the Glc ring of a fatty-acyl analogue of PGlc-
607 DGD III found in various bacterial and mammalian cells was demonstrated to change the immunogenic
608 properties of the IPL (84, 85), suggesting that NAc-bearing IPLs of *T. barophilus* might exert different
609 properties than their hydroxylated forms. Based on the polarity of these moieties, one might also speculate
610 that NAc and NH₂ would behave similarly than the regular hydroxyl group, whereas the apolar C₅H₈ could
611 cause dramatic changes in the orientation, hydration and interaction of the monosaccharide ring.

612 The polar moiety of lipids bearing diglycosides have been shown to extend away from the surface
613 and to generate intermolecular hydrogen bonds, hence conferring the diglycosidic lipids similar
614 physicochemical properties than those of monoglycosidic ones (86–88). In addition, the even higher relative
615 volume of the polar head group might result in further enhanced conformational freedom of the alkyl chain.

616 Derivatives of glycosylated phosphatidylhexose lipids, such as PHexHex, PHexHexNH₂ and
617 PHexHex+C₅H₈, have been detected in Bacteria, Eukarya and Archaea (35, 89). Although their biological
618 purpose remains elusive in Archaea, these lipid derivatives notably act as protein anchor in bacterial and
619 eukaryotic membranes (90), and similar functions might be expected for their archaeal counterparts.

620 Altogether, these results enhance our comprehension of the membrane structuration suggested for
621 *T. barophilus* and highlight putative biological functions for the different IPLs detected in this study.
622 Characterization of the physicochemical properties of synthetic or natural archaeal lipids with
623 phosphoglycosidic head groups remains nonetheless essential to precisely define the role of the diverse
624 archaeal lipid compositions in membrane physiology and organization.

625 **Conclusions**

626 We reassessed here the intact polar lipid, core lipid and lipid polar head group compositions of
627 *Thermococcus barophilus*, a model for membrane architecture and adaptation to extreme conditions in
628 Archaea. We unraveled the presence of at least 82 distinct membrane lipids, including a variety of core
629 structures, i.e., saturated and unsaturated DGD, MeDGD, PSGD, GDGT and GTGT, and the widest
630 diversity of polar head groups in Thermococcales known to date. Although not drastically different from
631 that of *T. barophilus* close relatives, the lipid composition reported here extends the known diversity of
632 phosphoglycosidic head groups known in Thermococcales. In agreement with previous investigations of *T.*
633 *barophilus* and other Thermococcales IPLs, the lipid diversity revealed here was overwhelmingly
634 dominated by PI-DGD. The low extraction yield, the excessive prevalence of PI-DGD, the low diversity of
635 polar head group moieties compared to bacterial and eukaryotic lipidomes (although high compared to other
636 archaeal lipidomes) and the CL released upon acid methanolysis demonstrated that a large portion of *T.*
637 *barophilus* lipidome still remains inaccessible to the employed extraction and analytical protocols.
638 Extraction-free analysis with MALDI-FT-ICR-MS allowed to access previously undetected tetraether-based
639 IPLs, and further improvements and developments of new methodologies might pave the way to the
640 discovery of completely new archaeal IPLs. Due to the isoprenoid alkyl chains of archaeal lipids, *T.*
641 *barophilus* membrane is tightly packed, but the addition of bulky phosphatidylhexose head groups might

642 provide relaxation while maintaining impermeability. Altogether, our results illustrate the complexity and
643 diversity of *T. barophilus* membrane structure and pose this species as a prime model to elucidate archaeal
644 membrane lipid diversity, properties and organization.

645 **Acknowledgments**

646 M.T. is supported by a Ph.D. grant from the French Ministry of Research and Technology. The authors
647 would like to thank the French National Research Agency for funding the ArchaeoMembranes project
648 (ANR-17-CE11-0012-01) and the CNRS Interdisciplinary program 'Origines' for funding the ReseArch
649 project. Research at MARUM was funded by Germany's Excellence Strategy (EXC-2077) project
650 390741603 "The Ocean Floor – Earth's Uncharted Interface".

651 **Author contributions**

652 Conceptualization, funding acquisition, project administration and supervision, K-U.H. and P.M.O.;
653 Formal analysis, M.T. and S.C.; Investigation, M.T., S.C., and L.W.; Methodology, M.T., S.C., J.S.L. and
654 L.W.; Visualization and writing – original draft, M.T.; Writing – Review and editing, M.T., S.C., L.W.,
655 J.S.L, K-U.H. and P.M.O.

656 **Competing interests**

657 The authors declare no conflicts of interest.

658 References

- 659 1. Kraft, M. L. 2013. Plasma membrane organization and function: moving past lipid rafts. *Mol. Biol. Cell.*
660 **24**: 2765–2768. [online] <https://www.molbiolcell.org/doi/10.1091/mbc.e13-03-0165>.
- 661 2. Nickels, J. D., M. D. Smith, R. J. Alsop, S. Himbert, A. Yahya, D. Cordner, P. Zolnierczuk, C. B.
662 Stanley, J. Katsaras, X. Cheng, and M. C. Rheinstädter. 2019. Lipid rafts: buffers of cell membrane
663 physical properties. *J. Phys. Chem. B.* **123**: 2050–2056. [online]
664 <https://pubs.acs.org/doi/10.1021/acs.jpcc.8b12126>.
- 665 3. Neumann, A. K., M. S. Itano, and K. Jacobson. 2010. Understanding lipid rafts and other related
666 membrane domains. *F1000 Biol. Rep.* **2**: 1–5. [online] <https://doi.org/10.3410/B2-31>.
- 667 4. Sonnino, S., and A. Prinetti. 2010. Lipids and membrane lateral organization. *Front. Physiol.* **1**: 1–9.
668 [online] <http://journal.frontiersin.org/article/10.3389/fphys.2010.00153/abstract>.
- 669 5. Wolf, C., K. Koumanov, B. Tenchov, and P. J. Quinn. 2001. Cholesterol favors phase separation of
670 sphingomyelin. *Biophys. Chem.* **89**: 163–172. [online]
671 <https://linkinghub.elsevier.com/retrieve/pii/S030146220000226X>.
- 672 6. Lindblom, G., G. Orädd, and A. Filippov. 2006. Lipid lateral diffusion in bilayers with
673 phosphatidylcholine, sphingomyelin and cholesterol. An NMR study of dynamics and lateral phase
674 separation. *Chem. Phys. Lipids.* **141**: 179–184.
- 675 7. García-Fernández, E., G. Koch, R. M. Wagner, A. Fekete, S. T. Stengel, J. Schneider, B. Mielich-Süss,
676 S. Geibel, S. M. Markert, C. Stigloher, and D. Lopez. 2017. Membrane microdomain disassembly
677 inhibits MRSA antibiotic resistance. *Cell.* **171**: 1354–1367.e20. [online]
678 <https://linkinghub.elsevier.com/retrieve/pii/S0092867417311996>.
- 679 8. Schütte, O. M., I. Mey, J. Enderlein, F. Savić, B. Geil, A. Janshoff, and C. Steinem. 2017. Size and
680 mobility of lipid domains tuned by geometrical constraints. *Proc. Natl. Acad. Sci.* **114**: E6064–
681 E6071. [online] <http://www.pnas.org/lookup/doi/10.1073/pnas.1704199114>.
- 682 9. Hinz, H. J., H. Kuttentreich, R. Meyer, M. Renner, R. Freund, R. Koynova, A. Boyanov, and B.
683 Tenchov. 1991. Stereochemistry and size of sugar head groups determine structure and phase
684 behavior of glycolipid membranes: densitometric, calorimetric, and x-ray studies. *Biochemistry.*
685 **30**: 5125–5138. [online] <https://pubs.acs.org/doi/abs/10.1021/bi00235a003>.
- 686 10. LaRocca, T. J., P. Pathak, S. Chiantia, A. Toledo, J. R. Silvius, J. L. Benach, and E. London. 2013.
687 Proving lipid rafts exist: Membrane domains in the prokaryote *Borrelia burgdorferi* have the same
688 properties as eukaryotic lipid rafts. *PLoS Pathog.* **9**: e1003353. [online]
689 <https://dx.plos.org/10.1371/journal.ppat.1003353>.
- 690 11. Lingwood, D., and K. Simons. 2010. Lipid rafts as a membrane-organizing principle. *Science (80-.).*
691 **327**: 46–50.
- 692 12. López, D., and R. Kolter. 2010. Functional microdomains in bacterial membranes. *Genes Dev.* **24**:
693 1893–1902.
- 694 13. De Rosa, M., and A. Gambacorta. 1988. The lipids of archaebacteria. *Prog. Lipid Res.* **27**: 153–175.
695 [online] [https://doi.org/10.1016/0163-7827\(88\)90011-2](https://doi.org/10.1016/0163-7827(88)90011-2).
- 696 14. Yoshinaga, M. Y., E. J. Gagen, L. WÄ¶rmer, N. K. Broda, T. B. Meador, J. Wendt, M. Thomm, and
697 K.-U. Hinrichs. 2015. *Methanothermobacter thermoautotrophicus* modulates its membrane lipids in
698 response to hydrogen and nutrient availability. *Front. Microbiol.* **6**: 1–9. [online]
699 <http://journal.frontiersin.org/article/10.3389/fmicb.2015.00005/abstract>.
- 700 15. Kamekura, M., and M. Kates. 1999. Structural diversity of membrane lipids in members of
701 *Halobacteriaceae*. *Biosci. Biotechnol. Biochem.* **63**: 969–972. [online]
702 <http://www.tandfonline.com/doi/full/10.1271/bbb.63.969>.
- 703 16. Baba, T., Y. Toshima, H. Minamikawa, M. Hato, K. Suzuki, and N. Kamo. 1999. Formation and
704 characterization of planar lipid bilayer membranes from synthetic phytanyl-chained glycolipids.
705 *Biochim. Biophys. Acta - Biomembr.* **1421**: 91–102. [online] [https://doi.org/10.1016/S0005-](https://doi.org/10.1016/S0005-2736(99)00114-5)
706 [2736\(99\)00114-5](https://doi.org/10.1016/S0005-2736(99)00114-5).
- 707 17. Komatsu, H., and P. L.-G. Chong. 1998. Low permeability of liposomal membranes composed of
708 bipolar tetraether lipids from thermoacidophilic archaebacterium *Sulfolobus acidocaldarius*.

- 709 *Biochemistry*. **37**: 107–115. [online] <https://pubs.acs.org/doi/10.1021/bi972163e>.
- 710 18. De Rosa, M., A. Gambacorta, and A. Gliozzi. 1986. Structure, biosynthesis, and physicochemical
711 properties of archaebacterial lipids. *Microbiol. Rev.* **50**: 70–80. [online]
712 <http://www.ncbi.nlm.nih.gov/pubmed/3083222>.
- 713 19. Knappy, C. S., C. E. M. Nunn, H. W. Morgan, and B. J. Keely. 2011. The major lipid cores of the
714 archaeon *Ignisphaera aggregans*: implications for the phylogeny and biosynthesis of glycerol
715 monoalkyl glycerol tetraether isoprenoid lipids. *Extremophiles*. **15**: 517–528. [online]
716 <http://link.springer.com/10.1007/s00792-011-0382-3>.
- 717 20. Nichols, D. S., M. R. Miller, N. W. Davies, A. Goodchild, M. Raftery, and R. Cavicchioli. 2004. Cold
718 adaptation in the Antarctic archaeon *Methanococcoides burtonii* involves membrane lipid
719 unsaturation. *J. Bacteriol.* **186**: 8508–8515. [online] <https://jb.asm.org/content/186/24/8508>.
- 720 21. Gambacorta, A., A. Trincone, B. Nicolaus, L. Lama, and M. De Rosa. 1993. Unique features of lipids
721 of Archaea. *Syst. Appl. Microbiol.* **16**: 518–527. [online]
722 <https://linkinghub.elsevier.com/retrieve/pii/S0723202011803218>.
- 723 22. Becker, K. W., F. J. Elling, M. Y. Yoshinaga, A. Söllinger, T. Urich, and K.-U. Hinrichs. 2016.
724 Unusual butane- and pentanetriol-based tetraether lipids in *Methanomassiliicoccus luminyensis*, a
725 representative of the seventh order of methanogens. *Appl. Environ. Microbiol.* **82**: 4505–4516.
726 [online] <http://aem.asm.org/lookup/doi/10.1128/AEM.00772-16>.
- 727 23. Koga, Y., M. Nishihara, H. Morii, and M. Akagawa-Matsushita. 1993. Ether polar lipids of
728 methanogenic bacteria: Structures, comparative aspects, and biosyntheses. *Microbiol. Rev.* **57**:
729 164–182.
- 730 24. Cario, A., V. Grossi, P. Schaeffer, and P. M. Oger. 2015. Membrane homeoviscous adaptation in the
731 piezo-hyperthermophilic archaeon *Thermococcus barophilus*. *Front. Microbiol.* **6**: 1–12. [online]
732 <http://journal.frontiersin.org/Article/10.3389/fmicb.2015.01152/abstract>.
- 733 25. Salvador-Castell, M., B. Demé, P. Oger, and J. Peters. 2020. Lipid phase separation induced by the
734 apolar polyisoprenoid squalane demonstrates its role in membrane domain formation in archaeal
735 membranes. *Langmuir*. **36**: 7375–7382. [online]
736 <https://pubs.acs.org/doi/10.1021/acs.langmuir.0c00901>.
- 737 26. Hinz, H. J., L. Six, K. P. Ruess, and M. Lieflander. 1985. Head-group contributions to bilayer
738 stability: monolayer and calorimetric studies on synthetic, stereochemically uniform glucolipids.
739 *Biochemistry*. **24**: 806–813. [online] <https://pubs.acs.org/doi/abs/10.1021/bi00324a041>.
- 740 27. Winter, R., and C. Jeworrek. 2009. Effect of pressure on membranes. *Soft Matter*. **5**: 3157. [online]
741 <http://xlink.rsc.org/?DOI=b901690b>.
- 742 28. Bagatolli, L. A., and O. G. Mouritsen. 2013. Is the fluid mosaic (and the accompanying raft
743 hypothesis) a suitable model to describe fundamental features of biological membranes? What may
744 be missing? *Front. Plant Sci.* **4**: 1–6. [online]
745 <http://journal.frontiersin.org/article/10.3389/fpls.2013.00457/abstract>.
- 746 29. Mouritsen, O. G. 2013. Physical chemistry of curvature and curvature stress in membranes. *Curr.*
747 *Phys. Chem.* **3**: 17–26. [online]
748 [http://www.eurekaselect.com/openurl/content.php?genre=article&issn=1877-
749 9468&volume=3&issue=1&spage=17](http://www.eurekaselect.com/openurl/content.php?genre=article&issn=1877-9468&volume=3&issue=1&spage=17).
- 750 30. Huguet, C., W. Martens-Habbena, H. Urakawa, D. A. Stahl, and A. E. Ingalls. 2010. Comparison of
751 extraction methods for quantitative analysis of core and intact polar glycerol dialkyl glycerol
752 tetraethers (GDGTs) in environmental samples. *Limnol. Oceanogr. Methods*. **8**: 127–145. [online]
753 <http://doi.wiley.com/10.4319/lom.2010.8.127>.
- 754 31. Lipp, J. S., Y. Morono, F. Inagaki, and K. U. Hinrichs. 2008. Significant contribution of Archaea to
755 extant biomass in marine subsurface sediments. *Nature*. **454**: 991–994.
- 756 32. Elling, F. J., M. Könneke, J. S. Lipp, K. W. Becker, E. J. Gagen, and K.-U. Hinrichs. 2014. Effects of
757 growth phase on the membrane lipid composition of the thaumarchaeon *Nitrosopumilus maritimus*
758 and their implications for archaeal lipid distributions in the marine environment. *Geochim.*
759 *Cosmochim. Acta*. **141**: 579–597. [online]

- 760 <https://linkinghub.elsevier.com/retrieve/pii/S0016703714004670>.
- 761 33. Schouten, S., A. Pitcher, E. C. Hopmans, L. Villanueva, J. van Bleijswijk, and J. S. Sinninghe Damsté.
762 2012. Intact polar and core glycerol dibiphytanyl glycerol tetraether lipids in the Arabian Sea
763 oxygen minimum zone: Selective preservation and degradation in the water column and
764 consequences for the TEX₈₆. *Geochim. Cosmochim. Acta*. **98**: 228–243. [online]
765 <https://linkinghub.elsevier.com/retrieve/pii/S0016703712002773>.
- 766 34. Sinninghe Damsté, J. S., W. I. C. Rijpstra, E. C. Hopmans, F. G. Prahl, S. G. Wakeham, and S.
767 Schouten. 2002. Distribution of membrane lipids of planktonic crenarchaeota in the Arabian Sea.
768 *Appl. Environ. Microbiol.* **68**: 2997–3002. [online] <https://aem.asm.org/content/68/6/2997>.
- 769 35. Meador, T. B., E. J. Gagen, M. E. Loscar, T. Goldhammer, M. Y. Yoshinaga, J. Wendt, M. Thomm,
770 and K.-U. Hinrichs. 2014. *Thermococcus kodakarensis* modulates its polar membrane lipids and
771 elemental composition according to growth stage and phosphate availability. *Front. Microbiol.* **5**:
772 1–13. [online] <http://journal.frontiersin.org/article/10.3389/fmicb.2014.00010/abstract>.
- 773 36. Marteinson, V. T., J. Birrien, A. Reysenbach, M. Vernet, D. Marie, A. Gambacorta, P. Messner, U. B.
774 Sleytr, and D. Prieur. 1999. *Thermococcus barophilus* sp. nov., a new barophilic and
775 hyperthermophilic archaeon isolated under high hydrostatic pressure from a deep-sea hydrothermal
776 vent. *Int. J. Syst. Evol. Microbiol.* **49**: 351–359. [online]
777 <https://www.microbiologyresearch.org/content/journal/ijsem/10.1099/00207713-49-2-351>.
- 778 37. Tourte, M., P. Schaeffer, V. Grossi, and P. M. Oger. 2020. Functionalized membrane domains: an
779 ancestral feature of Archaea? *Front. Microbiol.* **11**. [online]
780 <https://www.frontiersin.org/article/10.3389/fmicb.2020.00526/full>.
- 781 38. Zeng, X., J.-L. Birrien, Y. Fouquet, G. Cherkashov, M. Jebbar, J. Querellou, P. Oger, M.-A. Cambon-
782 Bonavita, X. Xiao, and D. Prieur. 2009. *Pyrococcus* CH1, an obligate piezophilic
783 hyperthermophile: extending the upper pressure-temperature limits for life. *ISME J.* **3**: 873–876.
784 [online] <http://www.nature.com/articles/ismej200921>.
- 785 39. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J.*
786 *Biochem. Physiol.* **37**: 911–917. [online] <http://www.nrcresearchpress.com/doi/10.1139/o59-099>.
- 787 40. Tourte, M., V. Kuentz, P. Schaeffer, V. Grossi, A. Cario, and P. M. Oger. 2020. Novel intact polar and
788 core lipid compositions in the *Pyrococcus* model species, *P. furiosus* and *P. yayanosii*, reveal the
789 largest lipid diversity amongst Thermococcales. *Biomolecules.* **10**: 830. [online]
790 <https://www.mdpi.com/2218-273X/10/6/830>.
- 791 41. Wörmer, L., J. S. Lipp, J. M. Schröder, and K.-U. Hinrichs. 2013. Application of two new LC–ESI–
792 MS methods for improved detection of intact polar lipids (IPLs) in environmental samples. *Org.*
793 *Geochem.* **59**: 10–21. [online] <https://linkinghub.elsevier.com/retrieve/pii/S0146638013000557>.
- 794 42. Lobasso, S., P. Lopalco, R. Angelini, R. Vitale, H. Huber, V. Müller, and A. Corcelli. 2012. Coupled
795 TLC and MALDI-TOF/MS analyses of the lipid extract of the hyperthermophilic archaeon
796 *Pyrococcus furiosus*. *Archaea.* **2012**: 1–10. [online]
797 <http://www.hindawi.com/journals/archaea/2012/957852/>.
- 798 43. Yoshinaga, M. Y., M. Y. Kellermann, P. E. Rossel, F. Schubotz, J. S. Lipp, and K.-U. Hinrichs. 2011.
799 Systematic fragmentation patterns of archaeal intact polar lipids by high-performance liquid
800 chromatography/electrospray ionization ion-trap mass spectrometry. *Rapid Commun. Mass*
801 *Spectrom.* **25**: 3563–3574. [online] <http://doi.wiley.com/10.1002/rcm.5251>.
- 802 44. Becker, K. W., J. S. Lipp, C. Zhu, X.-L. Liu, and K.-U. Hinrichs. 2013. An improved method for the
803 analysis of archaeal and bacterial ether core lipids. *Org. Geochem.* **61**: 34–44. [online]
804 <https://linkinghub.elsevier.com/retrieve/pii/S0146638013001174>.
- 805 45. Hopmans, E. C., S. Schouten, R. D. Pancost, M. T. J. van der Meer, and J. S. Sinninghe Damsté. 2000.
806 Analysis of intact tetraether lipids in archaeal cell material and sediments by high performance
807 liquid chromatography/atmospheric pressure chemical ionization mass spectrometry. *Rapid*
808 *Commun. Mass Spectrom.* **14**: 585–589. [online]
809 [https://onlinelibrary.wiley.com/doi/10.1002/\(SICI\)1097-0231\(20000415\)14:7%3C585::AID-RCM913%3E3.0.CO;2-N](https://onlinelibrary.wiley.com/doi/10.1002/(SICI)1097-0231(20000415)14:7%3C585::AID-RCM913%3E3.0.CO;2-N).
- 810

- 811 46. Lowenthal, M. S., E. L. Kilpatrick, and K. W. Phinney. 2015. Separation of monosaccharides
812 hydrolyzed from glycoproteins without the need for derivatization. *Anal. Bioanal. Chem.* **407**:
813 5453–5462. [online] <http://link.springer.com/10.1007/s00216-015-8717-z>.
- 814 47. Morii, H., M. Nishihara, and Y. Koga. 2000. CTP:2,3-di-O-geranylgeranyl-sn-glycero-1-phosphate
815 cytidyltransferase in the methanogenic archaeon *Methanothermobacter thermoautotrophicus*. *J.*
816 *Biol. Chem.* **275**: 36568–36574.
- 817 48. Hartley, M. D., and B. Imperiali. 2012. At the membrane frontier: A prospectus on the remarkable
818 evolutionary conservation of polyprenols and polyprenyl-phosphates. *Arch. Biochem. Biophys.*
819 **517**: 83–97. [online] <http://dx.doi.org/10.1016/j.abb.2011.10.018>.
- 820 49. Salvador-Castell, M., M. Tourte, and P. M. Oger. 2019. In search for the membrane regulators of
821 Archaea. *Int. J. Mol. Sci.* **20**: 4434. [online] <https://www.mdpi.com/1422-0067/20/18/4434>.
- 822 50. Jensen, S. M., M. Brandl, A. H. Treusch, and C. S. Ejsing. 2015. Structural characterization of ether
823 lipids from the archaeon *Sulfolobus islandicus* by high-resolution shotgun lipidomics. *J. Mass*
824 *Spectrom.* **50**: 476–487. [online] <http://doi.wiley.com/10.1002/jms.3553>.
- 825 51. Kates, M. 1993. Biology of halophilic bacteria, Part II. *Experientia.* **49**: 1027–1036. [online]
826 <http://link.springer.com/10.1007/BF01929909>.
- 827 52. Kostyukova, A. S., G. M. Gongadze, Y. Y. Polosina, E. A. Bonch-Osmolovskaya, M. L.
828 Miroshnichenko, N. A. Chernyh, M. V. Obratsova, V. A. Svetlichny, P. Messner, U. B. Sleytr, S.
829 L’Haridon, C. Jeanthon, and D. Prieur. 1999. Investigation of structure and antigenic capacities of
830 Thermococcales cell envelopes and reclassification of “*Caldococcus litoralis*” Z-1301 as
831 *Thermococcus litoralis* Z-1301. *Extremophiles.* **3**: 239–246. [online]
832 <http://link.springer.com/10.1007/s007920050122>.
- 833 53. De Rosa, M., A. Gambacorta, A. Trincone, A. Basso, W. Zillig, and I. Holz. 1987. Lipids of
834 *Thermococcus celer*, a sulfur-reducing archaeobacterium: Structure and biosynthesis. *Syst. Appl.*
835 *Microbiol.* **9**: 1–5. [online] [http://dx.doi.org/10.1016/S0723-2020\(87\)80046-2](http://dx.doi.org/10.1016/S0723-2020(87)80046-2).
- 836 54. Lanzotti, V., A. Trincone, B. Nicolaus, W. Zillig, M. De Rosa, and A. Gambacorta. 1989. Complex
837 lipids of *Pyrococcus* and AN1, thermophilic members of archaeobacteria belonging to
838 Thermococcales. *Biochim. Biophys. Acta - Lipids Lipid Metab.* **1004**: 44–48. [online]
839 <https://linkinghub.elsevier.com/retrieve/pii/0005276089902105>.
- 840 55. González, J. M., Y. Masuchi, F. T. Robb, J. W. Ammerman, D. L. Maeder, M. Yanagibayashi, J.
841 Tamaoka, and C. Kato. 1998. *Pyrococcus horikoshii* sp. nov., a hyperthermophilic archaeon
842 isolated from a hydrothermal vent at the Okinawa Trough. *Extremophiles.* **2**: 123–130. [online]
843 <http://link.springer.com/10.1007/s007920050051>.
- 844 56. Bale, N. J., M. Palatinszky, W. I. C. Rijpstra, C. W. Herbold, M. Wagner, and J. S. Sinninghe Damsté.
845 2019. Membrane lipid composition of the moderately thermophilic ammonia-oxidizing archaeon
846 *Candidatus “Nitrosotenuis uzonensis”* at different growth temperatures. *Appl. Environ. Microbiol.*
847 **85**. [online] <http://aem.asm.org/lookup/doi/10.1128/AEM.01332-19>.
- 848 57. Hezayen, F. F., B. H. Rehm, B. J. Tindall, and A. Steinbüchel. 2001. Transfer of *Natrialba asiatica*
849 B1T to *Natrialba taiwanensis* sp. nov. and description of *Natrialba aegyptiaca* sp. nov., a novel
850 extremely halophilic, aerobic, non-pigmented member of the Archaea from Egypt that produces
851 extracellular p. *Int. J. Syst. Evol. Microbiol.* **51**: 1133–1142. [online]
852 <http://ijs.microbiologyresearch.org/content/journal/ijsem/10.1099/00207713-51-3-1133>.
- 853 58. Koga, Y., M. Akagawa-Matsushita, M. Ohga, and M. Nishihara. 1993. Taxonomic significance of the
854 distribution of component parts of polar ether lipids in methanogens. *Syst. Appl. Microbiol.* **16**:
855 342–351. [online] <https://linkinghub.elsevier.com/retrieve/pii/S072320201180264X>.
- 856 59. Ferrante, G., J. C. Richards, and G. D. Sprott. 1990. Structures of polar lipids from the thermophilic,
857 deep-sea archaeobacterium *Methanococcus jannaschii*. *Biochem. Cell Biol.* **68**: 274–283. [online]
858 <http://www.nrcresearchpress.com/doi/10.1139/o90-038>.
- 859 60. Trincone, A., B. Nicolaus, G. Palmieri, M. De Rosa, R. Huber, G. Huber, K. O. Stetter, and A.
860 Gambacorta. 1992. Distribution of complex and core lipids within new hyperthermophilic
861 members of the Archaea domain. *Syst. Appl. Microbiol.* **15**: 11–17. [online]

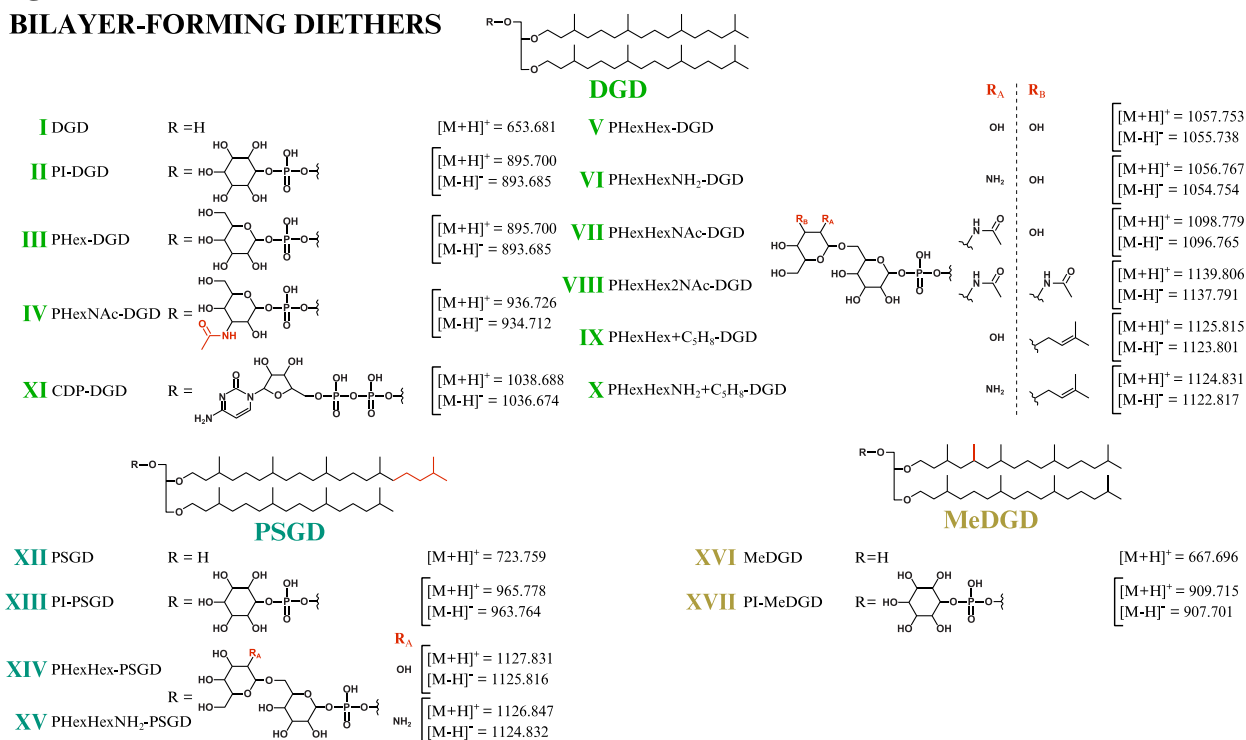
- 862 [http://dx.doi.org/10.1016/S0723-2020\(11\)80130-X](http://dx.doi.org/10.1016/S0723-2020(11)80130-X).
- 863 61. Lanzotti, V., M. De Rosa, A. Trincone, A. L. Basso, A. Gambacorta, and W. Zillig. 1987. Complex
864 lipids from *Desulfurococcus mobilis*, a sulfur-reducing archaeobacterium. *Biochim. Biophys. Acta -*
865 *Lipids Lipid Metab.* **922**: 95–102. [online]
866 <https://linkinghub.elsevier.com/retrieve/pii/0005276087901421>.
- 867 62. Koga, Y., H. Mori, M. Akagawa-Matsushita, and M. Ohga. 1998. Correlation of polar lipid
868 composition with 16S rRNA phylogeny in methanogens. Further analysis of lipid component parts.
869 *Biosci. Biotechnol. Biochem.* **62**: 230–236. [online]
870 <http://www.tandfonline.com/doi/full/10.1271/bbb.62.230>.
- 871 63. Cui, H.-L., Y.-Z. Mou, X. Yang, Y.-G. Zhou, H.-C. Liu, and P.-J. Zhou. 2012. *Halorubellus salinus*
872 gen. nov., sp. nov. and *Halorubellus litoreus* sp. nov., novel halophilic archaea isolated from a
873 marine solar saltern. *Syst. Appl. Microbiol.* **35**: 30–34. [online]
874 <http://dx.doi.org/10.1016/j.syapm.2011.08.001>.
- 875 64. Sako, Y., N. Nomura, A. Uchida, Y. Ishida, H. Morii, Y. KOGA, T. HOAKI, and T. MARUYAMA.
876 1996. *Aeropyrum pernix* gen. nov., sp. nov., a novel aerobic hyperthermophilic archaeon growing
877 at temperatures up to 100 °C. *Int. J. Syst. Bacteriol.* **46**: 1070–1077. [online]
878 <https://doi.org/10.1099/00207713-46-4-1070>.
- 879 65. Law, K. P., and C. L. Zhang. 2019. Current progress and future trends in mass spectrometry-based
880 archaeal lipidomics. *Org. Geochem.* **134**: 45–61. [online]
881 <https://linkinghub.elsevier.com/retrieve/pii/S0146638019300592>.
- 882 66. Koga, Y., M. Ohga, M. Nishihara, and H. Morii. 1987. Distribution of a diphytanyl ether analog of
883 phosphatidylserine and an ethanolamine-containing tetraether lipid in methanogenic bacteria. *Syst.*
884 *Appl. Microbiol.* **9**: 176–182. [online] [http://dx.doi.org/10.1016/S0723-2020\(87\)80019-X](http://dx.doi.org/10.1016/S0723-2020(87)80019-X).
- 885 67. Kučerka, N., F. A. Heberle, J. Pan, and J. Katsaras. 2015. Structural significance of lipid diversity as
886 studied by small angle neutron and X-ray scattering. *Membranes (Basel)*. **5**: 454–472.
- 887 68. Sprott, G. D., B. J. Agnew, and G. B. Patel. 1997. Structural features of ether lipids in the
888 archaeobacterial thermophiles *Pyrococcus furiosus*, *Methanopyrus kandleri*, *Methanothermus*
889 *fervidus*, and *Sulfolobus acidocaldarius*. *Can. J. Microbiol.* **43**: 467–476. [online]
890 <http://www.nrcresearchpress.com/doi/10.1139/m97-066>.
- 891 69. Ellis, S. R., M. R. L. Paine, G. B. Eijkel, J. K. Pauling, P. Husen, M. W. Jervelund, M. Hermansson,
892 C. S. Ejsing, and R. M. A. Heeren. 2018. Automated, parallel mass spectrometry imaging and
893 structural identification of lipids. *Nat. Methods*. **15**: 515–518. [online]
894 <http://www.nature.com/articles/s41592-018-0010-6>.
- 895 70. Kellermann, M. Y., M. Y. Yoshinaga, R. C. Valentine, L. Wörmer, and D. L. Valentine. 2016.
896 Important roles for membrane lipids in haloarchaeal bioenergetics. *Biochim. Biophys. Acta -*
897 *Biomembr.* **1858**: 2940–2956. [online]
898 <https://linkinghub.elsevier.com/retrieve/pii/S0005273616302814>.
- 899 71. Mileykovskaya, E., and W. Dowhan. 2009. Cardiolipin membrane domains in prokaryotes and
900 eukaryotes. *Biochim. Biophys. Acta - Biomembr.* **1788**: 2084–2091. [online]
901 <http://dx.doi.org/10.1016/j.bbamem.2009.04.003>.
- 902 72. McDaniel, R. V., and T. J. McIntosh. 1989. Neutron and X-ray diffraction structural analysis of
903 phosphatidylinositol bilayers. *Biochim. Biophys. Acta - Biomembr.* **983**: 241–246. [online]
904 <https://linkinghub.elsevier.com/retrieve/pii/0005273689902393>.
- 905 73. Hansbro, P. M., S. J. Byard, R. J. Bushby, P. J. H. Turnbull, N. Boden, M. R. Saunders, R. Novelli,
906 and D. G. Reid. 1992. The conformational behaviour of phosphatidylinositol in model membranes:
907 ²H-NMR studies. *Biochim. Biophys. Acta - Biomembr.* **1112**: 187–196. [online]
908 <https://linkinghub.elsevier.com/retrieve/pii/000527369290391X>.
- 909 74. Bradshaw, J. P., R. J. Bushby, C. C. D. Giles, and M. R. Saunders. 1999. Orientation of the headgroup
910 of phosphatidylinositol in a model biomembrane as determined by neutron diffraction.
911 *Biochemistry*. **38**: 8393–8401.
- 912 75. Peng, A., D. S. Pisal, A. Doty, and S. V. Balu-Iyer. 2012. Phosphatidylinositol induces fluid phase

- 913 formation and packing defects in phosphatidylcholine model membranes. *Chem. Phys. Lipids*. **165**:
914 15–22. [online] <https://linkinghub.elsevier.com/retrieve/pii/S0009308411003355>.
- 915 76. Peng, A., R. M. Straubinger, and S. V. Balu-Iyer. 2010. Phosphatidylinositol containing lipidic
916 particles reduces immunogenicity and catabolism of Factor VIII in hemophilia A mice. *AAPS J.*
917 **12**: 473–481. [online] <http://link.springer.com/10.1208/s12248-010-9207-z>.
- 918 77. Sundler, R., and D. Papahadjopoulos. 1981. Control of membrane fusion by phospholipid head groups I.
919 Phosphatidate/phosphatidylinositol specificity. *Biochim. Biophys. Acta - Biomembr.* **649**: 743–750.
920 [online] <https://linkinghub.elsevier.com/retrieve/pii/0005273681901796>.
- 921 78. Kanduč, M., A. Schlaich, A. H. de Vries, J. Jouhet, E. Maréchal, B. Demé, R. R. Netz, and E.
922 Schneck. 2017. Tight cohesion between glycolipid membranes results from balanced water–
923 headgroup interactions. *Nat. Commun.* **8**: 14899. [online]
924 <http://www.nature.com/articles/ncomms14899>.
- 925 79. Marguet, E., M. Gaudin, E. Gaudiard, I. Fourquaux, S. le Blond du Plouy, I. Matsui, and P. Forterre.
926 2013. Membrane vesicles, nanopods and/or nanotubes produced by hyperthermophilic archaea of
927 the genus *Thermococcus*. *Biochem. Soc. Trans.* **41**: 436–442. [online]
928 [https://portlandpress.com/biochemsoctrans/article/41/1/436/68280/Membrane-vesicles-nanopods-](https://portlandpress.com/biochemsoctrans/article/41/1/436/68280/Membrane-vesicles-nanopods-and-or-nanotubes)
929 [and-or-nanotubes](https://portlandpress.com/biochemsoctrans/article/41/1/436/68280/Membrane-vesicles-nanopods-and-or-nanotubes).
- 930 80. Gorlas, A., E. Marguet, S. Gill, C. Geslin, J.-M. Guigner, F. Guyot, and P. Forterre. 2015. Sulfur
931 vesicles from Thermococcales: A possible role in sulfur detoxifying mechanisms. *Biochimie*. **118**:
932 356–364. [online] <https://linkinghub.elsevier.com/retrieve/pii/S0300908415002448>.
- 933 81. McDaniel, R. V. 1988. Neutron diffraction studies of digalactosyldiacylglycerol. *Biochim. Biophys.*
934 *Acta - Biomembr.* **940**: 158–164. [online]
935 <https://linkinghub.elsevier.com/retrieve/pii/000527368890020X>.
- 936 82. Róg, T., I. Vattulainen, A. Bunker, and M. Karttunen. 2007. Glycolipid membranes through atomistic
937 simulations: effect of glucose and galactose head groups on lipid bilayer properties. *J. Phys. Chem.*
938 *B.* **111**: 10146–10154. [online] <https://pubs.acs.org/doi/10.1021/jp0730895>.
- 939 83. Kooijman, E. E., K. E. King, M. Gangoda, and A. Gericke. 2009. Ionization properties of
940 phosphatidylinositol polyphosphates in mixed model membranes. *Biochemistry*. **48**: 9360–9371.
941 [online] <https://pubs.acs.org/doi/10.1021/bi9008616>.
- 942 84. Nagatsuka, Y., Y. Horibata, Y. Yamazaki, M. Kinoshita, Y. Shinoda, T. Hashikawa, H. Koshino, T.
943 Nakamura, and Y. Hirabayashi. 2006. Phosphatidylglucoside exists as a single molecular species
944 with saturated fatty acyl chains in developing astroglial membranes. *Biochemistry*. **45**: 8742–8750.
945 [online] <https://pubs.acs.org/doi/10.1021/bi0606546>.
- 946 85. Takahashi, H., T. Hayakawa, M. Murate, P. Greimel, Y. Nagatsuka, T. Kobayashi, and Y.
947 Hirabayashi. 2012. Phosphatidylglucoside: its structure, thermal behavior, and domain formation in
948 plasma membranes. *Chem. Phys. Lipids*. **165**: 197–206. [online]
949 <http://dx.doi.org/10.1016/j.chemphyslip.2011.12.010>.
- 950 86. Renou, J. P., J. B. Giziewicz, I. C. P. Smith, and H. C. Jarrell. 1989. Glycolipid membrane surface
951 structure: orientation, conformation, and motion of a disaccharide headgroup. *Biochemistry*. **28**:
952 1804–1814. [online] <https://pubs.acs.org/doi/abs/10.1021/bi00430a057>.
- 953 87. Jarrell, H. C., P. A. Jovall, J. B. Giziewicz, L. A. Turner, and I. C. P. Smith. 1987. Determination of
954 conformational properties of glycolipid head groups by deuterium NMR of oriented multibilayers.
955 *Biochemistry*. **26**: 1805–1811. [online] <https://pubs.acs.org/doi/abs/10.1021/bi00381a003>.
- 956 88. Polak, A., M. Tarek, M. Tomšič, J. Valant, N. P. Ulrih, A. Jamnik, P. Kramar, and D. Miklavčič. 2014.
957 Electroporation of archaeal lipid membranes using MD simulations. *Bioelectrochemistry*. **100**: 18–
958 26. [online] <https://linkinghub.elsevier.com/retrieve/pii/S1567539414000024>.
- 959 89. Nishihara, M., M. Utagawa, H. Akutsu, and Y. Koga. 1992. Archaea contain a novel diether
960 phosphoglycolipid with a polar head group identical to the conserved core of eucaryal glycosyl
961 phosphatidylinositol. *J. Biol. Chem.* **267**: 12432–5. [online]
962 <http://www.ncbi.nlm.nih.gov/pubmed/1535621>.
- 963 90. Low, M., and A. Saltiel. 1988. Structural and functional roles of glycosyl-phosphatidylinositol in

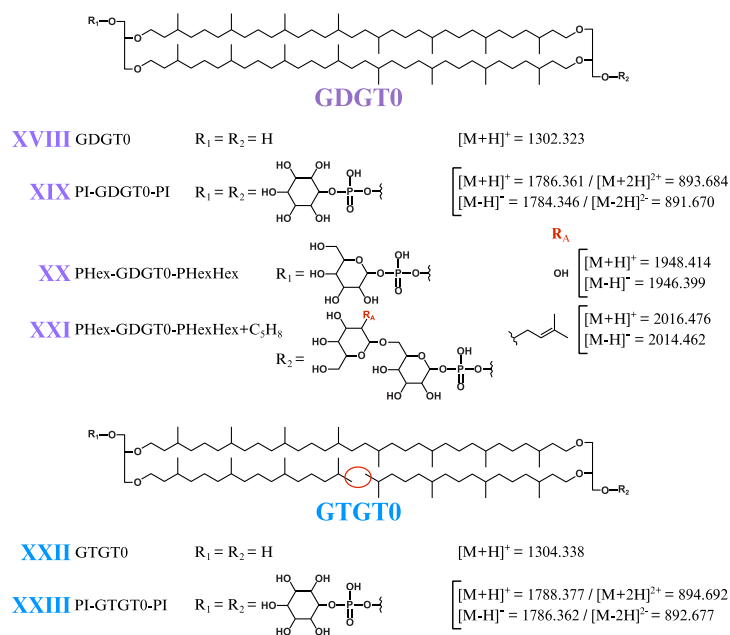
964 membranes. *Science* (80-.). **239**: 268–275. [online]
965 <https://www.sciencemag.org/lookup/doi/10.1126/science.3276003>.
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967

968 **Figures**

BILAYER-FORMING DIETHERS

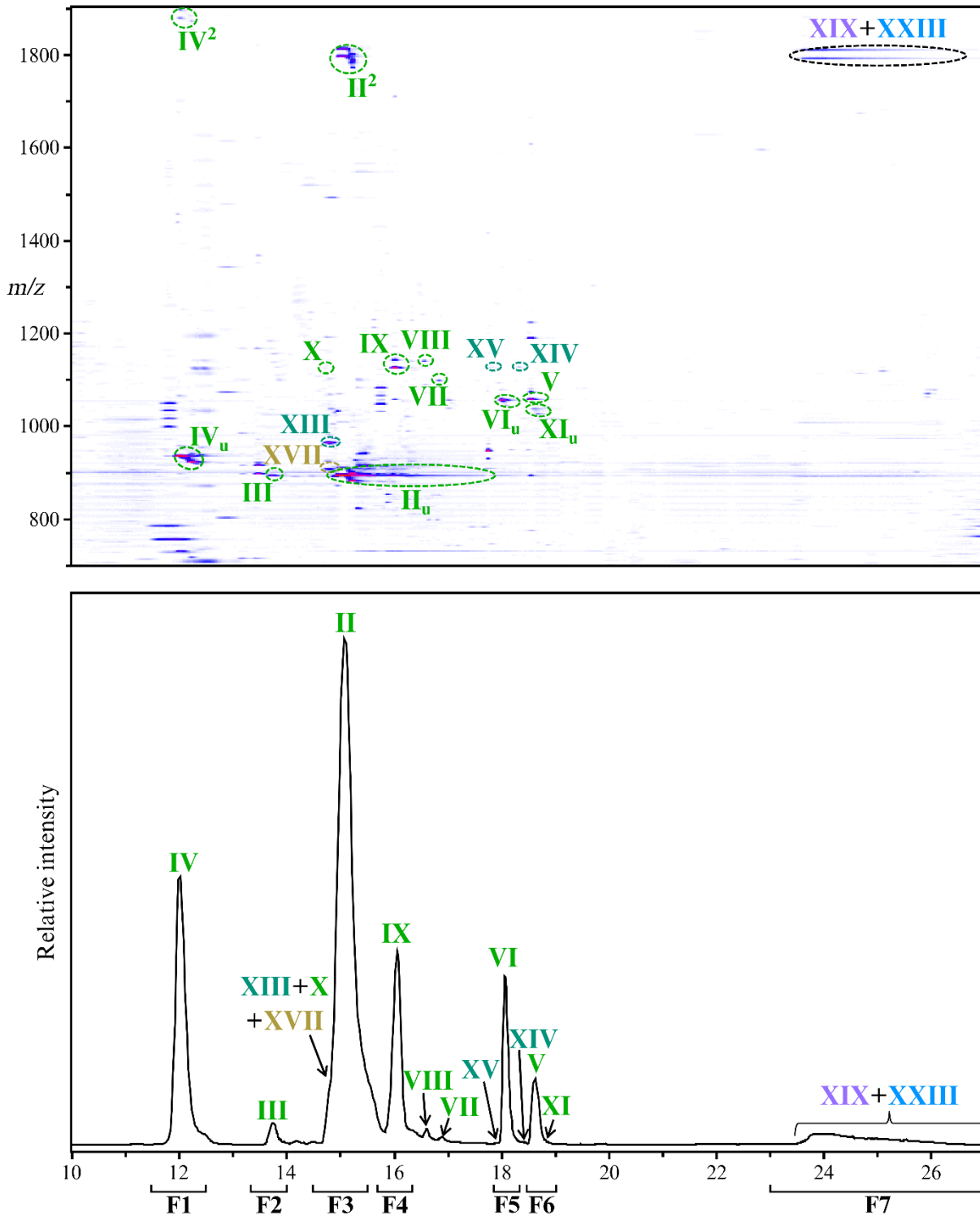


MONOLAYER-FORMING TETRAETHERS



969
 970 **Figure 1: Core and intact polar lipids of *Thermococcus barophilus*.**
 971 Short-hand nomenclature is indicated. The protonated, ammoniated, and sodiated adducts and only the
 972 deprotonated adducts were detected in positive and negative ion mode, respectively. Only the protonated
 973 and deprotonated ions are represented in the Figure. Core structures: diphytanyl glycerol diethers (DGD;

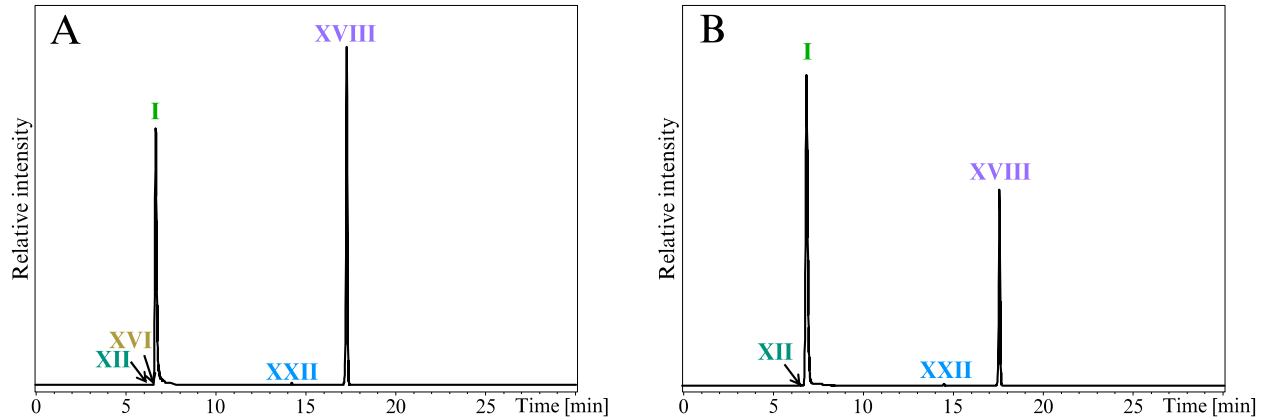
974 light green; I to XI), phytanylsesterterpanyl glycerol diethers (PSGD; dark green; XII to XV), DGD bearing
975 an additional methylation (MeDGD; yellow; XVI and XVII), glycerol dibiphytanyl (or dialkyl) glycerol
976 tetraethers with no cyclopentane ring (GDGT0; purple; XVIII to XXI) and glycerol biphytanyl diphytanyl
977 (or trialkyl) glycerol tetraethers with no cyclopentane ring (GTGT0; blue; XXII and XXIII). Note that II,
978 III, IV, VI and XI were detected with up to 8 unsaturations whereas V, VII, IX, X were detected with up to
979 6 unsaturations. No unsaturation was detected in the other core structures. Unsaturation are not represented.
980 Polar head groups: phosphatidylinositol (PI; II, XIII, XVII, XIX and XXIII), phosphatidylhexose (PHex;
981 III, XX and XXI), phosphatidyl-N-acetylhexosamine (PHexNAc; IV), glycosylated phosphatidylhexose
982 (PHexHex; V, XIV and XX), ammoniated PHexHex (PHexHexNH₂; VI and XV), one and two N-acetylated
983 PHexHex (PHexHexNAc and PHexHex2NAc; VII and VIII), PHexHex and PHexHexNH₂ bearing an
984 additional mass of 68 (PHexHex+C₅H₈ and PHexHexNH₂+C₅H₈; IX and XXI, and X) and cytidine
985 diphosphate (CDP; XI). Positions of additional methylation and of additional groups on the polar head
986 groups are drawn arbitrarily in the Figure.



987
988 **Figure 2: *Thermococcus barophilus* exhibits a large diversity of intact polar lipids.**
989 Intact polar lipids were detected in positive and negative ion mode. As no additional IPL could be identified
990 in the negative ion mode, only the density map and chromatogram obtained in positive ion mode are

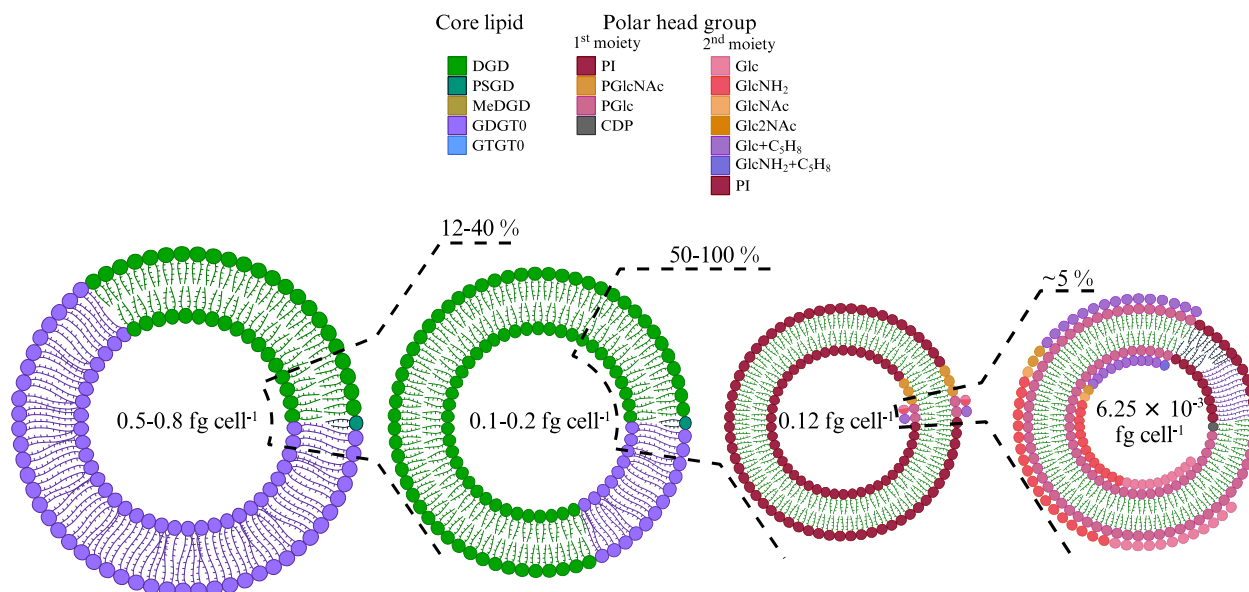
991 displayed (zoom in the 10-27 min, m/z 700-1900 window). Compounds detected with unsaturations and/or
992 diadducts are marked with _u and ², respectively. The UHPLC chromatogram was drawn by extracting the
993 following protonated ion masses with a mass deviation of ± 0.02 Da: 893.68, 894.70, 895.70, 909.72,
994 936.73, 965.78, 1038.69, 1056.77, 1057.75, 1098.78, 1124.83, 1125.82, 1126.85, 1127.83, 1139.81,
995 1786.36 and 1788.38. Refer to Figure 1, Table 1 and Figures S1-S9 for lipid structures and their respective
996 molecular masses. F1-F7 delineate the time range corresponding to each fraction collected to confirm the
997 structures of the identified lipids (refer to the Methods section).

998

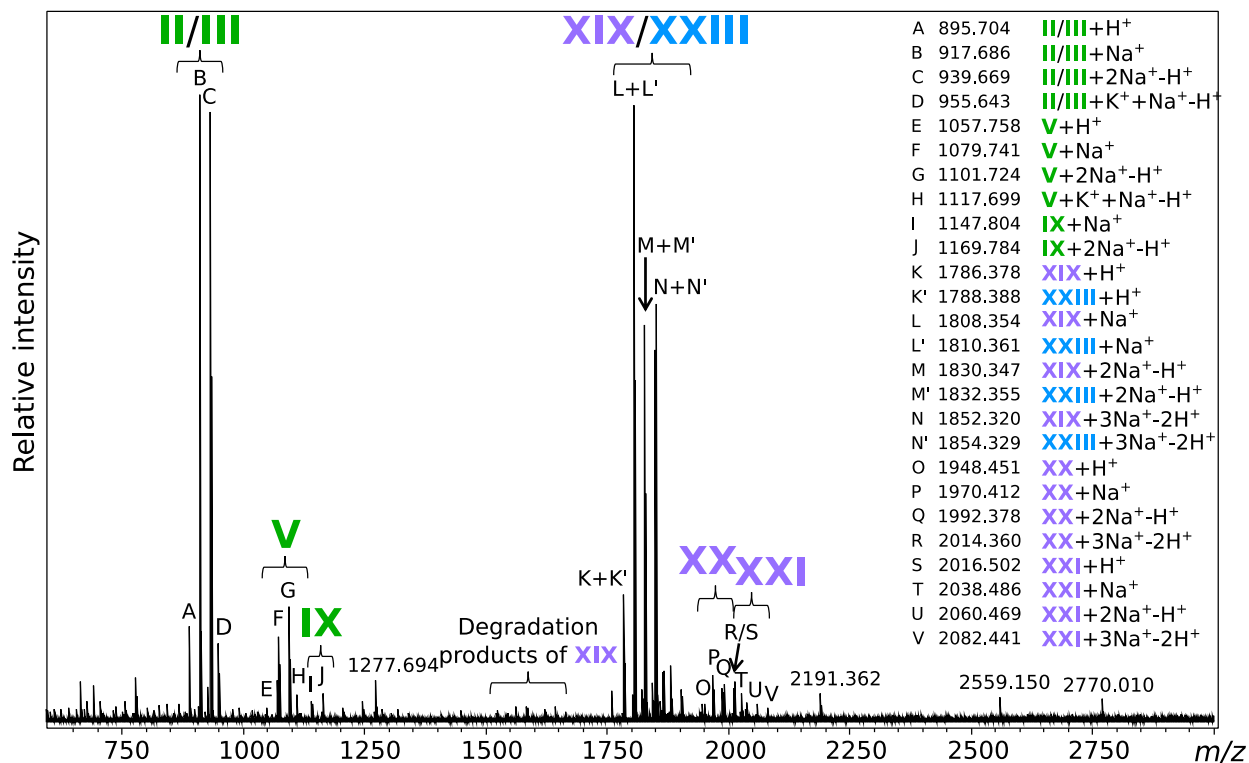


999 **Figure 3: Analysis of the core lipids highlights a significant discrepancy between *Thermococcus***
1000 ***barophilus* total and extracted lipids.**

1002 Total CLs (A) and CLs from IPLs (B) were recovered after methanolysis of the biomass and the TLE,
1003 respectively. Direct methanolysis and intact polar lipid extraction were both performed on the same amount
1004 of biomass. UHPLC chromatograms were drawn in positive mode by extracting the following protonated
1005 ions with a mass deviation of ± 0.1 Da: 653.68, 667.70, 723.76, 743.71, 1302.32, 1304.34. Refer to Figure
1006 1 for lipid structures.



1007 ESTIMATED TOTAL IPL
 1008 **Figure 4: The vast majority of *Thermococcus barophilus*' lipidome remains inaccessible.**
 1009 Pie chart representations of *Thermococcus barophilus* lipids at each extraction step. The direct acid
 1010 methanolysis of *T. barophilus* biomass yielded its total core lipids content, which contained *ca.* 30 % of
 1011 diethers (DGD, green; PSGD, dark green; MeDGD, dark yellow) and 70 % of tetraethers (GDGT0, purple;
 1012 GTGT0, blue; Table 3). Considering putative low and high mass polar head groups found in Archaea, the
 1013 direct acid methanolysis of the biomass yielded an experimentally-derived theoretical lipid content of 0.5
 1014 to 0.8 fg of lipids cell⁻¹. Acid methanolysis of the TLE allowed the determination of the extraction yield on
 1015 *T. barophilus* (0.1 to 0.2 fg cell⁻¹ considering putative low and high mass polar head groups, *ca.* 12-40 % of
 1016 the biomass' total lipids) and of the diether and tetraether distribution in extracted lipids (80/20; Table 3).
 1017 Although part of *T. barophilus* IPLs is indeed extracted, the majority of its IPLs, and especially tetraether-
 1018 based ones, remain resistant to extraction. 18 IPLs representing 50-100 % of these extracted lipids (0.12 fg
 1019 cell⁻¹) were identified with UHPLC-MS (Table 1). The putative discrepancy between extracted and
 1020 identified IPLs highlights only a partial detection of *T. barophilus* IPLs with our UHPLC-MS system.
 1021 Complex IPLs with at least two sugar residues (1st and 2nd polar head moiety) were recovered with an even
 1022 lower yield, i.e., 6.25×10^{-3} fg cell⁻¹, *ca.* 5 % of the IPLs identified (Table 1), thus showing that peculiar
 1023 polar head groups might be the main reason for the detection defect observed in *T. barophilus*.



1024
 1025 **Figure 5: Extraction-free analysis reveals novel tetraether-based intact polar lipids in *Thermococcus***
 1026 ***barophilus*.**
 1027 *m/z* detected for each marked peak and the putative corresponding lipid adduct are listed on the right side
 1028 of the figure. Masses similar to that expected from partial hydrolysis of tetraethers, e.g., PI-GDGT0-P, PI-
 1029 GDGT0 and P-GDGT0, are indicated as degradation products of PI-GDGT0-PI XIX. The masses of major
 1030 unidentified peaks are also displayed. Refer to Figure 1 for lipid structures.

1031 **Tables**1032 **Table 1. Intact polar lipid structures and lipid composition (absolute quantity, cellular abundance and molar relative %) of *Thermococcus***
1033 ***barophilus*.**

Lipid	Core	Head group	Acronym	Chemical formula	Theoretical [M+H] ⁺	Unsaturation	HPLC-ESI-MS					MALDI FT-ICR-MS
							RT (min)	Detected [M+H] ⁺	Absolute quantity (ng) ^a	Cellular abundance (fg cell ⁻¹) ^b	Molar relative % ^c	Detected [M+Na] ⁺
II	DGD	Phosphatidyl inositol	PI-DGD	C ₄₉ H ₉₉ O ₁₁ P	895.700	0-8	15.0	895.7042	21500	0.11	90.0	917.686
III	DGD	Phosphatidyl glucose	PGlc-DGD	C ₄₉ H ₉₉ O ₁₁ P	895.700	0-8	13.7	895.6879	115	5.8 × 10 ⁻⁴	Traces	917.686
IV	DGD	Phosphatidyl N-acetylglucosamine	PGlcNAc-DGD	C ₅₁ H ₁₀₃ O ₁₁ P	936.726	0-8	12.2	936.7133	1175	5.9 × 10 ⁻³	4.9	ND
V	DGD	Phosphatidyl glucose + glucose	PGlcGlc-DGD	C ₅₅ H ₁₀₉ O ₁₆ P	1057.753	0-6	18.6	1057.7536	230	1.2 × 10 ⁻³	Traces	1079.741
VI	DGD	Phosphatidyl glucose + hexosamine	PGlcHexNH ₂ -DGD	C ₅₅ H ₁₁₀ NO ₁₅ P	1056.769	0-8	18.0	1056.7736	305	1.5 × 10 ⁻³	1.3	ND
VII	DGD	Phosphatidyl hexose + N-acetylhexosamine	PHexHexNAc-DGD	C ₅₇ H ₁₁₂ NO ₁₆ P	1098.779	0-6	16.9	1098.7805	10	5.0 × 10 ⁻⁵	Traces	ND
VIII	DGD	Phosphatidyl hexose + di-N-acetylhexosamine	PHexHex2NAc-DGD	C ₅₉ H ₁₁₅ N ₂ O ₁₆ P	1139.806	0	16.6	1139.8062	20	1.0 × 10 ⁻⁴	Traces	ND
IX	DGD	Phosphatidyl glucose + hexose + C ₅ H ₈	PGlcHex+C ₅ H ₈ -DGD	C ₆₀ H ₁₁₇ O ₁₆ P	1125.815	0-6	16.0	1125.8164	370	1.9 × 10 ⁻³	1.5	1147.804
X	DGD	Phosphatidyl glucose + hexosamine + C ₅ H ₈	PGlcHexNH ₂ +C ₅ H ₈ -DGD	C ₆₀ H ₁₁₈ NO ₁₅ P	1124.831	0-6	14.7	1124.8270	1	5.0 × 10 ⁻⁶	Traces	ND
XI	DGD	Cytidine diphosphate	CDP-DGD	C ₅₂ H ₁₀₁ N ₅ O ₁₃ P ₂	1038.688	0-8	18.7	1038.6887	10	5.0 × 10 ⁻⁵	Traces	ND
XIII	PSGD	Phosphatidyl inositol	PI-PSGD	C ₅₄ H ₁₀₉ O ₁₁ P	965.778	0	14.8	965.7780	65	3.3 × 10 ⁻⁴	Traces	ND
XIV	PSGD	Phosphatidyl glucose + glucose	PGlcGlc-PSGD	C ₆₀ H ₁₁₉ O ₁₆ P	1127.831	0	18.5	1127.8274	4	2.0 × 10 ⁻⁵	Traces	ND
XV	PSGD	Phosphatidyl glucose + hexosamine	PGlcHexNH ₂ -PSGD	C ₆₀ H ₁₂₀ NO ₁₅ P	1126.847	0	17.8	1126.8424	3	1.5 × 10 ⁻⁵	Traces	ND
XVII	MeDGD	Phosphatidyl inositol	PI-MeDGD	C ₅₀ H ₁₀₁ O ₁₁ P	909.715	0	14.9	909.6897	NQ	NQ	NQ	ND
XIX	GDGT	Diphosphatidyl inositol	PI-GDGT0-PI	C ₉₈ H ₁₉₄ O ₂₂ P ₂	1786.361	0	23.4-26.6	1786.3595	100	5.0 × 10 ⁻⁴	Traces	1808.354
XX	GDGT	Phosphatidyl hexose + phosphatidyl hexose + hexose	PHex-GDGT0-PHexHex	C ₁₀₄ H ₂₀₄ O ₂₇ P ₂	1948.414	0	ND	ND	ND	ND	ND	1970.412

XXI	GDGT	Phosphatidyl hexose + phosphatidyl hexose + hexose + C ₅ H ₈	PHex-GDGT0-PHexHex+C ₅ H ₈	C ₁₀₉ H ₂₁₅ O ₂₇ P ₂	2016.476	0	ND	ND	ND	ND	ND	2038.486
XXIII	GTGT	Diphosphatidyl inositol	PI-GTGT0-PI	C ₉₈ H ₁₉₆ O ₂₂ P ₂	1788.377	0	23.4-26.6	1788.3763	NQ	NQ	NQ	1810.361

1034 Traces, <1 %

1035 ND, not detected; NQ, not quantified; DGD, dialkyl glycerol diethers; PSGD, phytalsesterterpanyl glycerol diethers; MeDGD, methylated DGD; GDGT, glycerol dialkyl glycerol tetraethers; GTGT, glycerol trialkyl glycerol tetraethers.

1037 ^a2 ng of the internal standard C₂₁-PC was injected to quantify the identified IPL. Quantities account for protonated, ammoniated and sodiated adducts of saturated IPLs in ESI positive mode and were calculated assuming a response factor of 0.58 for monoglycosidic IPLs and 0.21 for diglycosidic IPLs relative to the internal standard C₂₁-PC (refer to methods).

1040 ^bCalculated with an average cell number of 2.0 × 10⁸ cell mL⁻¹ (refer to methods).

1041 ^cMolar relative proportions were calculated from each IPL quantity weighted by their respective molar mass.

1042 **Table 2. Characteristics and distribution of the core structures and the polar head groups of purified**
 1043 **major lipids of *Thermococcus barophilus*.**

Fraction	Time range (min)	Expected IPL	Detected IPL (%) ^a	Core structure ^b					Polar head ^c									
				DGD I	PSGD XII	MeDGD XVI	GDGT0 XVIII	GTGT0 XXII	Pent	All/Fru	Man	Gal	Glc	Ino	2Hex	GlcNAc	AcidoHex	
F1	11.5-12.5	IV	IV (100)	100	ND	ND	ND	ND	ND	ND	ND	ND	ND	58	ND	ND	42	ND
F2	13.3-14.0	III	III (100)	100	ND	ND	ND	ND	ND	ND	ND	ND	ND	100	ND	ND	ND	ND
F3	14.5-15.5	II+X +XIII+XVII	II (98)+ XIII (2) +XVII (traces)+ X (traces)	98	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	100	ND	ND	ND
F4	15.7-16.4	IX	IX (93)+ II (7)+ V (traces) +XIII (traces)	100	Traces	ND	ND	ND	ND	ND	ND	ND	ND	100	ND	ND	ND	ND
F5	17.9-18.2	VI+XV	VI (95)+ IX (3)+ II (2) +XV (traces)	100	Traces	ND	ND	ND	ND	ND	ND	ND	ND	100	ND	ND	ND	ND
F6	18.4-19.0	V+XI+XIV	V (98)+ II (1)+ VI (1) +XIV (traces)	100	Traces	ND	ND	ND	ND	ND	ND	ND	ND	100	ND	ND	ND	ND
F7	23.0-27.0	XIX+XXIII	I (100)	2	ND	ND	95	3	ND	ND	ND	ND	ND	43	57	ND	ND	ND

1044 Traces, <1 %

1045 ND, not detected; DGD, dialkyl glycerol diethers; PSGD, phytanylsesterterpanyl glycerol diethers; MeDGD,
 1046 methylated DGD; GDGT0, glycerol dialkyl glycerol tetraethers with no cyclopentane ring; GTGT0, glycerol trialkyl
 1047 glycerol tetraethers with no cyclopentane ring; Pent, β -D-xylofuranose, α -L-arabinopyranose and α -D-lyxopyranose;
 1048 All/Fru, β -D-allopyranose and β -D-fructopyranose; Man, D-mannopyranose; Gal, β -D-galactopyranose; Glc, α -D-
 1049 glucopyranose; Ino, myo-inositol; 2Hex, dihexoses; GlcNAc, α -N-acetyl-D-glucosamine; AcidoHex, D-galacturonic
 1050 acid and D-glucuronic acid, D-galactosamine.

1051 ^aRelative proportions account for protonated, ammoniated and sodiated adducts of saturated IPLs in ESI positive mode
 1052 and were calculated assuming a response factor of 0.58 for monoglycosidic IPLs and 0.21 for diglycosidic IPLs relative
 1053 to the internal standard C₂₁-PC (refer to methods).

1054 ^bRelative proportions account for protonated adducts in APCI positive mode and were calculated assuming a response
 1055 factor of 0.42 for diethers and 0.57 for tetraethers relative to the internal standard C₄₆-GTGT, or of 0.74 for diethers
 1056 relative to tetraethers (refer to methods).

1057 ^cRelative proportions were calculated assuming the response factors determined for each sugar using a standard
 1058 solution (refer to methods).

1059 **Table 3. Core lipid composition of the biomass (totCLs) and of the total lipid extract (CLs from IPLs)**
 1060 **of *Thermococcus barophilus*.**

	DGD I			Diethers ^a			PSGD XII			GDGT0 XVIII			Tetraethers ^a			GTGT0 XXII		Total	
	μg	fg cell ⁻¹	Rel%	μg	fg cell ⁻¹	Rel%	μg	fg cell ⁻¹	Rel%	μg	fg cell ⁻¹	Rel%	μg	fg cell ⁻¹	Rel%	fg cell ⁻¹	D/T		
TotCLs	22.2	0.1	28	6.1 × 10 ⁻²	3.1 × 10 ⁻⁴	Traces	56.8	0.3	72	0.4	2.2 × 10 ⁻³	Traces				0.4	0.39		
CLs from IPLs	14.6	7.3 × 10 ⁻²	80	4.2 × 10 ⁻²	2.1 × 10 ⁻³	Traces	3.6	1.8 × 10 ⁻²	20	3.1 × 10 ⁻²	1.5 × 10 ⁻⁴	Traces				9.1 × 10 ⁻²	4.0		

1061 DGD, dialkyl glycerol diethers; PSGD, phytanylsesterterpanyl glycerol diethers; GDGT0, glycerol dialkyl glycerol
 1062 tetraethers with no cyclopentane ring; GTGT0, glycerol trialkyl glycerol tetraethers with no cyclopentane ring; D/T,
 1063 diethers over tetraethers ratio; Rel%, molar relative proportion; μg, absolute lipid quantity in μg; fg cell⁻¹, cellular lipid
 1064 abundance in fg cell⁻¹ calculated with an average cell number of 2.0 × 10⁸ cell mL⁻¹; ND, not detected.

1065 Traces, <1 %

1066 ^aRelative proportions account for protonated adducts in APCI positive mode and were calculated assuming a response
 1067 factor of 0.42 for diethers and 0.57 for tetraethers relative to the internal standard C₄₆-GTGT, or of 0.74 for diethers
 1068 relative to tetraethers (refer to methods).