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1 **_Streptococcus pneumoniae_, _S. pyogenes_, and _S. agalactiae_ membrane**
2 **phospholipid remodeling in response to human serum**
3
4
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11
12 **Running title: Phospholipid remodeling in pathogenic streptococci**
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20
21

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22 **Abstract**

23 *Streptococcus pneumoniae*, *S. pyogenes* (Group A *Streptococcus*; GAS), and *S.*
24 *agalactiae* (Group B *Streptococcus*; GBS) are major etiological agents of diseases in
25 humans. The cellular membrane, a crucial site in host-pathogen interactions, is poorly
26 characterized in streptococci. Moreover, little is known about whether or how
27 environmental conditions influence their lipid compositions. Using normal phase liquid
28 chromatography coupled with electrospray ionization mass spectrometry, we
29 characterized the phospholipids and glycolipids of *S. pneumoniae*, GAS, and GBS in
30 routine undefined laboratory medium, streptococcal defined medium, and, in order to
31 mimic the host environment, defined medium supplemented with human serum. In human
32 serum-supplemented medium, all three streptococcal species synthesize
33 phosphatidylcholine (PC), a zwitterionic phospholipid commonly found in eukaryotes but
34 relatively rare in bacteria. We previously reported that *S. pneumoniae* utilizes the
35 glycerophosphocholine (GPC) biosynthetic pathway to synthesize PC. Through substrate
36 tracing experiments, we confirm that GAS and GBS scavenge lysoPC, a major metabolite
37 in human serum, thereby using an abbreviated GPC pathway for PC biosynthesis.
38 Furthermore, we found that plasmanyl-PC is uniquely present in the GBS membrane
39 during growth with human serum, suggesting GBS possesses unusual membrane
40 biochemical or biophysical properties. In summary, we report cellular lipid remodeling by
41 the major pathogenic streptococci in response to metabolites present in human serum.

42

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43 **Introduction**

44 Streptococci are Gram-positive bacteria that natively colonize humans in niches including
45 the oral cavity, the nasopharynx, and the genitourinary tract (1). Three species of
46 streptococci, *Streptococcus pneumoniae*, *S. pyogenes* (Group A *Streptococcus*; GAS),
47 and *S. agalactiae* (Group B *Streptococcus*; GBS), are considered major human
48 pathogens, resulting in over 1.5 million deaths each year around the world (2,3). These
49 pathogens cause a wide range of diseases of varying severity in all age groups, including
50 streptococcal pharyngitis, soft tissue infections, pneumonia, meningitis, bacteremia, and
51 necrotizing fasciitis (3–10).
52
53 The surface of the streptococcal cell is a critical interface for host interactions. Many
54 studies into the pathogenicity of streptococci have focused on virulence factors including
55 secreted proteins, surface polymers and adhesins, and mechanisms for sensing the
56 extracellular environment (11–13). In contrast, little is known about the cellular membrane
57 composition and dynamics of streptococci. Earlier studies mainly utilized thin layer
58 chromatography (TLC) or column separation coupled with paper chromatography to study
59 membrane lipids extracted from cultures in routine laboratory medium (14–19). However,
60 these methods lack molecular specificity and sensitivity for the comprehensive
61 characterization of membrane lipids (20). The phospholipids phosphatidylglycerol (PG)
62 and cardiolipin (CL), and the glycolipids monohexosyldiacylglycerol (MHDAG) and
63 dihexosyldiacylglycerol (DHDAG) were previously detected in all three streptococcal
64 species (14–19,21–23).
65

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66 We recently utilized normal phase liquid chromatography coupled with electrospray
67 ionization mass spectrometry (NPLC-ESI/MS) to characterize the lipid membranes of the
68 Mitis group streptococcal species *S. pneumoniae*, *S. mitis*, and *S. oralis*, and described
69 for the first time the presence of the zwitterionic phospholipid phosphatidylcholine (PC) in
70 these organisms (24,25). We further showed that the biosynthesis of PC in these
71 organisms occurs through the rare glycerophosphocholine (GPC) pathway which relies
72 upon scavenging of GPC and/or lysophosphatidylcholine (lysoPC) from the growth
73 medium (24).
74
75 Here, we utilized NPLC-ESI/MS to characterize the membrane lipids of the three major
76 streptococcal pathogens. To mimic the human host environment and to assess the effects
77 of human serum on the membrane lipid compositions, the streptococci were cultured in
78 defined medium with or without human serum supplementation. We show that: 1) human
79 serum provides the substrates required for *S. pneumoniae* to synthesize PC; 2) the GAS
80 and GBS scavenge the human metabolite lysoPC to synthesize PC via an abbreviated
81 GPC pathway; and, 3) plasmanyl-PC (pPC) is uniquely present in the GBS membrane
82 during culture with human serum.
83

84 **Material and Methods**

85 **Bacterial strains, media, and growth conditions**

86 Culture media and bacterial strains used in this study are shown in Table 1 and Table S1,
87 respectively. Routine laboratory culture conditions for each species were 37°C for GBS,
88 and 37°C and 5% CO_2 for *S. pneumoniae* and GAS. Rich culture media used were Todd-

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89 Hewitt Broth for GBS and Todd-Hewitt Broth supplemented with yeast extract at 0.5% w/v
90 for *S. pneumoniae* and 0.2% w/v for GAS. Streptococcal chemically defined medium (26)
91 was diluted from stock as described (27) with 1% glucose, for GAS and GBS. For *S.*
92 *pneumoniae*, defined medium was supplemented with 0.5 mM choline (25). Where
93 appropriate, defined medium was supplemented with 5% v/v human serum (Sigma-
94 Aldrich), 100 µM GPC (Sigma-Aldrich), or 100 µM lysoPC 20:0 (Avanti Polar Lipids). See
95 Supplemental Text S1 for growth curve methodology.
96
97 **GAS and GBS culture for lipidomic analysis**
98 Single colonies of GAS and GBS were inoculated into 15 mL rich medium and incubated
99 overnight as described above, until pelleting and storage for lipidomic analysis described
100 below. For defined medium analysis, single colonies were cultured in defined medium
101 overnight, diluted into 15 mL pre-warmed defined medium supplemented with or without
102 5% v/v human serum to an OD_{600nm} of 0.05, and incubated for 8 h before pelleting.
103 Cultures were performed in biological triplicate.
104
105 ***S. pneumoniae* culture for lipidomic analysis**
106 Single colonies of *S. pneumoniae* were inoculated into 6 mL rich medium, serially diluted,
107 and incubated overnight as described. Cultures in early exponential phase were
108 inoculated into 15 mL pre-warmed rich medium and cultured until early stationary phase.
109 Overnight defined medium cultures were inoculated directly from freezer stocks and
110 serially diluted for overnight growth. Early exponential phase cultures were inoculated into

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111 defined medium supplemented with or without 5% v/v human serum at an OD_{600nm} of 0.05
112 and incubated for 8 h until pelleting. Cultures were performed in biological triplicate.
113
114 **GPC and lysoPC supplementation experiments**
115 Overnight defined medium cultures of GAS and GBS were diluted to a starting OD_{600nm}
116 0.05 in 15 mL defined medium supplemented with either 100 μM GPC or 100 μM lysoPC
117 (20:0) and incubated for 8 h as described above. Cultures were pelleted and stored for
118 lipidomic analysis. Cultures were performed in biological triplicate.
119
120 **Lipidomics**
121 Acidic Bligh-Dyer lipid extractions were performed as described (25,28). Normal-phase
122 LC-ESI/MS was performed on an Agilent 1200 quaternary LC system equipped with an
123 Ascentis silica high-performance liquid chromatography (HPLC) column (5 μm; 25 cm by
124 2.1 mm; Sigma-Aldrich) as described previously (25,28,29). See Supplemental Text S1
125 for more information.
126
127 **Results**
128 **Pathogenic streptococci remodel their membrane phospholipid composition in**
129 **response to human serum**
130 The major phospholipids detected by normal-phase LC-ESI/MS for each streptococcal
131 species and strain used in this study, when cultured in the rich, undefined laboratory
132 medium Todd-Hewitt Broth (supplemented with yeast extract where appropriate), and in
133 streptococcal defined medium supplemented with or without 5% v/v human serum, are

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134 shown in Table 1. The biosynthetic pathways for these lipids are shown in Figure 1.
135 Growth curves for streptococci cultured with or without 5% v/v human serum are shown
136 in Supplemental Figure S1A-F. Human serum supplementation did not significantly alter
137 the growth of the bacteria, except during late exponential and early stationary phase of
138 *S. pneumoniae* D39 (Supplemental Figure S1A).
139
140 The lipid profile of *S. pneumoniae* cultured in Todd-Hewitt Broth supplemented with 0.5%
141 w/v yeast extract consists of the major anionic phospholipids PG and CL, the zwitterionic
142 phospholipid PC, and the glycolipids MHDAG and DHDAG (25). Figure 2A (panel 1)
143 displays the positive ESI mass spectrum of PC (appearing at the retention time 19-20.5
144 min) in the membrane of *S. pneumoniae*. We previously determined that Todd-Hewitt
145 Broth contains GPC, a substrate utilized by *S. pneumoniae* to synthesize PC (25). A
146 similar phospholipid profile is observed for *S. pneumoniae* cultured in defined medium,
147 except that PC is absent, due to the lack of GPC and lysoPC substrates in the medium
148 (25). The total ion chromatogram (TIC) (Supplemental Figure S2) and mass spectrum
149 (MS) of retention time 19-20.5 min of *S. pneumoniae* D39 grown in defined medium
150 lacking human serum are shown in Figure 2A (panel 2), and no PC is present. However,
151 *S. pneumoniae* synthesizes PC when defined medium is supplemented with 5% v/v
152 human serum (Supplemental Figure S2). Figure 2A (panel 3) shows the ESI mass
153 spectrum of the major PC species. These data demonstrate that human serum provides
154 the substrates required for PC biosynthesis via the GPC pathway in *S. pneumoniae*.
155

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156 The lipid profile of GAS cultured in Todd-Hewitt Broth supplemented with 0.2% w/v yeast
157 extract is similar to that of *S. pneumoniae*, with the exception that PC is not detected
158 (Table 3.1, Figure 2B [panel 1]). The lipid profile remains unchanged in defined medium,
159 and no PC is detected (Figure 2B [panel 2] and Supplemental Figure S2). Interestingly,
160 PC is observed in the membrane of GAS when defined medium is supplemented with 5%
161 v/v human serum, indicating that human serum, but not Todd-Hewitt Broth or defined
162 medium, provide substrates required for PC biosynthesis (Supplemental Figure S2). The
163 ESI/MS of the major PC species identified in the GAS membrane is shown in
164 Supplemental Figure S3. The chemical structure of PC (16:0/18:2) (with the M^+ ion at
165 *m/z* 758) is shown in Supplemental Figure S3A and the supporting tandem mass
166 spectrometry (MS/MS) fragmentation of *m/z* 758 is shown in Supplemental Figure S3B.
167
168 The lipid profile of GBS cultured in Todd-Hewitt Broth consists of MHDAG, DHDAG, PG,
169 and CL, as well as the aminoacylated PG molecule lysyl-PG (Lys-PG), and a recently
170 described novel, cationic glycolipid, Lysyl-Glucosyl-DAG (LGD) (Table 1, Supplemental
171 Figure S2) (23). The lipidomic profile of the GBS is unchanged when cultured in defined
172 medium (Table 1). Like GAS, the GBS do not synthesize PC when cultured in Todd-Hewitt
173 Broth or defined medium (Figure 2C [panel 1 and panel 2]). Also, like GAS, GBS
174 synthesize PC when defined medium is supplemented with 5% v/v human serum (Figure
175 2C [panel 3], Supplemental Figure S2). However, the ESI mass spectrum (Figure 2C
176 [panel 3]) indicates a modified PC molecule that co-elutes off the column at the same time
177 as Lys-PG. The modified PC molecule was determined to be the plasmalogen, plasmanyl-
178 PC (pPC) (Supplemental Figure S3), in which the *sn*-1 acyl chain linkage is an ether bond,

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179 instead of the ester bond that is present in diacyl PC (Supplemental Figure S3A and S3C).
180 The identification of pPC is supported by MS/MS. Specifically, the *m/z* 482 ion in the
181 MS/MS spectrum of *m/z* 720 (Supplemental Figure S3D) is consistent with the pPC
182 structure, as compared to the *m/z* 496 ion in the MS/MS spectrum of *m/z* 758 for the
183 diacyl PC (Supplemental Figure S3B).
184
185 Taken together, these data demonstrate that major pathogenic streptococci remodel their
186 membrane lipid composition by synthesizing PC in response to metabolites present in
187 human serum. Furthermore, plasmanyl-PC is uniquely observed in the membrane of the
188 GBS during culture with human serum.
189
190 **GAS and GBS scavenge lysoPC to form PC**
191 Next, we sought to investigate if GAS and GBS utilize the GPC biosynthetic pathway
192 previously identified in the Mitis group streptococci (25). GAS and GBS strains were
193 cultured in defined medium supplemented with either 100 μM GPC or 100 μM lysoPC
194 (20:0), a non-natural acyl chain length in these species, and lipidomic analysis was
195 performed. These concentrations are physiologically relevant because lysoPC and GPC
196 are present in varying concentrations throughout the human body at ≤200 μM and ≤500
197 μM, respectively (30,31). The ESI mass spectra are shown in Figure 3. When GAS is
198 cultured in GPC-supplemented defined medium, very little PC is detected (Figure 3A). In
199 defined medium supplemented with lysoPC (20:0), a substantial amount of PC is detected
200 for GAS (Figure 3B). Similarly, when GBS is cultured in GPC-supplemented defined
201 medium, no PC is detected (Figure 3C), and robust levels of PC are observed when GBS

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202 are cultured in defined medium supplemented with lysoPC (20:0) (Figure 3D). Taken
203 together, these data identify lysoPC, not GPC, as the primary substrate scavenged by the
204 GAS and GBS to synthesize PC.
205
206 **Discussion**
207 The cellular membrane is a critical and dynamic surface of bacterial cells that plays a role
208 in many cellular processes yet is largely understudied in streptococci. In this study we
209 characterized the lipidome of the major streptococcal pathogens *S. pneumoniae*, GAS,
210 and GBS when cultured in a standard rich laboratory medium (Todd-Hewitt Broth), and a
211 defined medium supplemented with or without 5% v/v human serum. We show that all
212 three streptococcal species remodel their cellular membrane in response to human
213 metabolites, specifically, by synthesis of PC. To our knowledge, this is the first
214 identification of PC and the plasmalogen pPC in the GAS and GBS, respectively. PC has
215 been linked to virulence in certain bacteria (32,33). Streptococci may incorporate PC into
216 their membranes as a form of eukaryotic membrane mimicry to evade immune defense,
217 although this must be investigated further.
218
219 Human serum is a rich source of nutrients for streptococci. A major metabolite of human
220 serum is lysoPC (30,34). *S. pneumoniae* utilizes a full GPC pathway (25), scavenging
221 metabolites from human serum to synthesize PC. However, the GAS and GBS
222 predominantly synthesize PC using an abbreviated GPC pathway, by primarily
223 scavenging lysoPC from the exogenous environment. This is likely due to a missing or
224 poorly expressed GPC transporter and/or the inability to acylate GPC to form lysoPC. The

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225 ability of *S. pneumoniae* but not GAS or GBS to scavenge GPC could be capitalized upon
226 in comparative genomic and laboratory experiments to help identify the GPC transporter
227 in *S. pneumoniae*, which is currently unknown.
228
229 To our knowledge, the presence of the plasmalogen pPC in the GBS membrane has not
230 been described before. To date, plasmalogens have only been observed in strict
231 anaerobes such as *Clostridium* and *Bifidobacterium* (35–37). Biosynthesis of
232 plasmalogens in bacteria is poorly understood, with the first bacterial desaturase involved
233 in plasmenyl *sn*-1 vinyl ether bond formation and the first plasmalogen synthase, PlsAR,
234 in *Clostridium perfringens* only recently identified (38,39). Plasmalogen lipids are thought
235 to promote oxidative stress survival in animal cells (40,41) and may play a role in GBS
236 pathogenesis. pPC has been detected in human blood at ~50 μM in healthy adults
237 (30,34). There are two possible pathways for the presence of pPC in the membrane of
238 GBS during culturing with human serum. Either, 1) GBS scavenge the intermediate lyso-
239 pPC, adding the second acyl chain, and/or full pPC directly from human serum or 2) *de*
240 *novo* pPC synthesis is occurring. Our experimental design does not allow us to
241 discriminate which pathway is utilized. Given that GBS lack an identifiable homolog of the
242 *C. perfringens* PlsAR synthase, coupled with the lack of plasmalogen form of other
243 common lipids, the pPC we identify in the membrane of GBS is likely derived from the
244 plasmalogen lipids present in human serum. The origin and roles of pPC in GBS is a
245 subject of further investigation.
246

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247 Overall, this work demonstrates that culture medium can significantly alter the membrane
248 lipid composition of major human streptococcal pathogens. This supports the significance
249 of culturing pathogens in media that more closely represent the host environments in
250 which the pathogens are found. Further investigation into the biosynthesis and
251 mechanistic roles of PC in the membrane will likely provide novel insights into the
252 pathogen-host interactions and pathogenesis of these streptococcal species.
253

254 **Conflicts of interest**

255 The author(s) declare that there are no conflicts of interest.
256

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266

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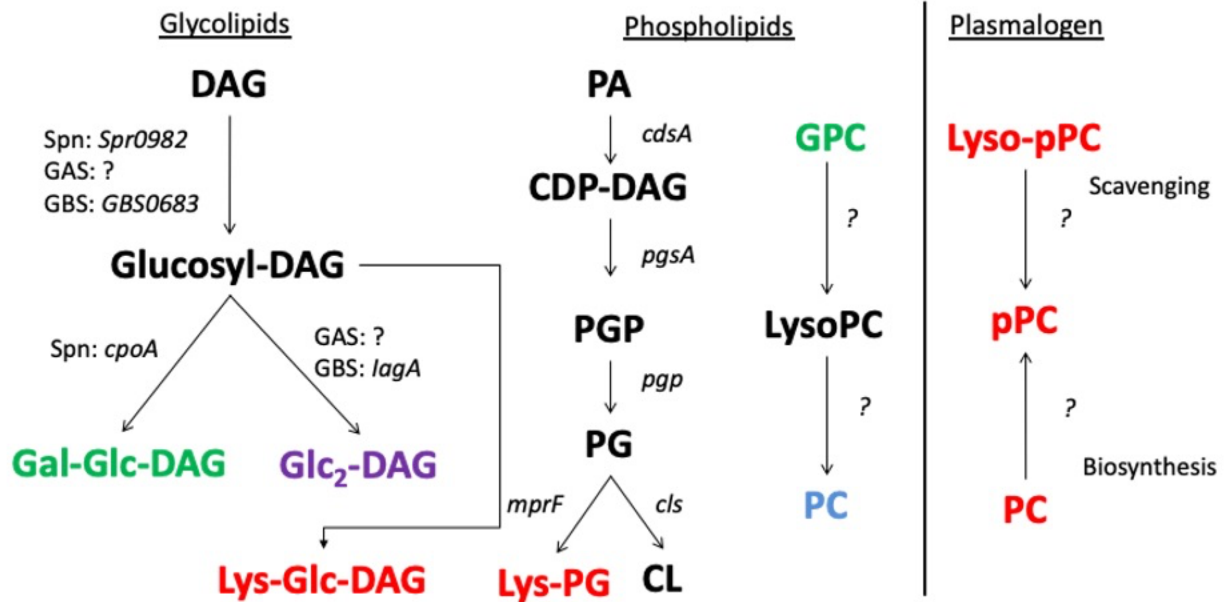

397

398 **Figure 1. Glycolipid and phospholipid biosynthesis pathways in *S. pneumoniae*,**
399 **GAS, and GBS.** Genes of known or predicted function in each pathway are indicated.
400 Lipids and substrates in black are common to all three species, in green are specific to *S.*
401 *pneumoniae* (Spn)*,* in red are specific to GBS, in blue are present in *S. pneumoniae* and
402 GAS, and in purple are present in both GAS and GBS. DAG, diacylglycerol; Glc_2-DAG,
403 diglucosyl-DAG; Gal-Glc-DAG, galactosyl-glucosyl-DAG; Lys-Glc-DAG, lysyl-glucosyl-
404 DAG (synthesized by *mprF* (23)); PA, phosphatidic acid; CDP-DAG, cytidine
405 diphosphate-DAG; PGP, PG-3-phosphate; PG, phosphatidylglycerol; Lys-PG, lysyl-
406 phosphatidylglycerol; CL, cardiolipin; GPC, glycerophosphocholine; lysoPC, lyso-
407 phosphatidylcholine; PC, phosphatidylcholine; Lyso-pPC, lyso-plasmanyl-PC; pPC,
408 plasmanyl-PC. “?” denotes unidentified genes. It is currently unknown whether GBS
409 scavenge lyso-pPC or pPC from human serum or if it is *de* novo synthesized from PC.
410 This figure combines lipids and genes described in literature (14-19,21-25,32,33) and
411 detected lipids from Table 1.

412

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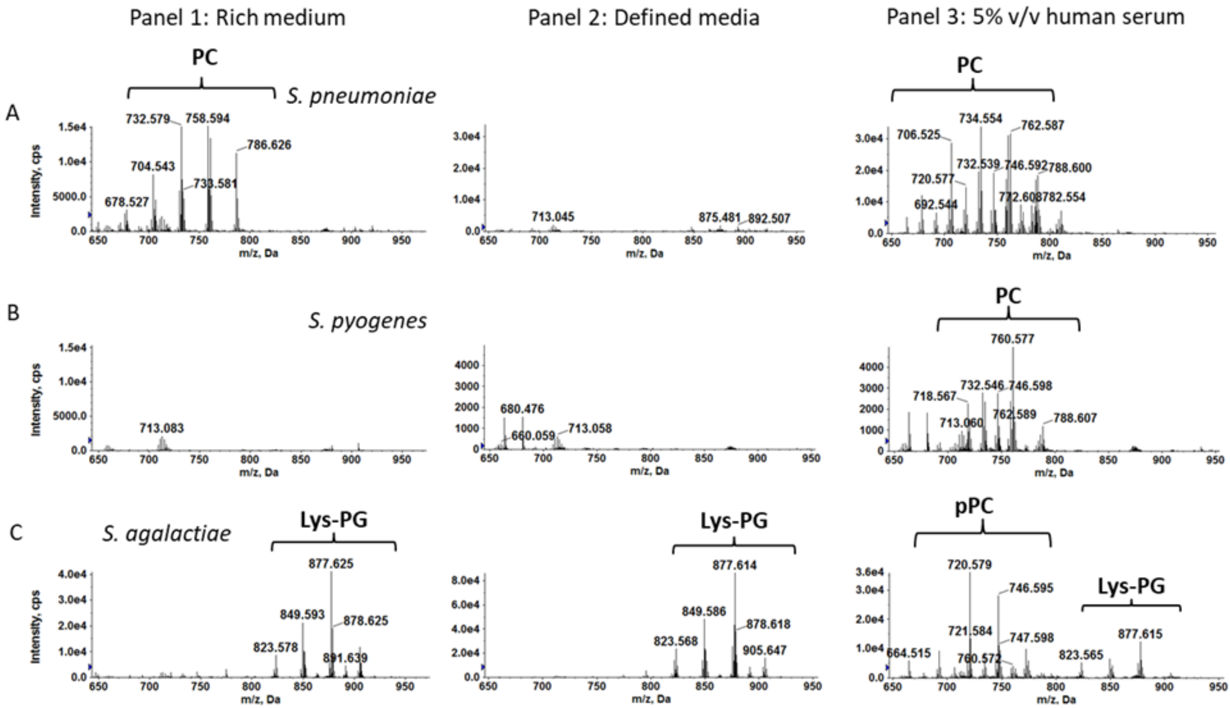

413

414 **Figure 2. PC and Lys-PG detection when streptococci are cultured in different**
415 **media.** Shown are representative positive ESI mass spectra obtained during the LC
416 retention time of 19 – 20.5 min indicating the presence or absence of PC, pPC, and Lys-
417 PG in the membranes of A) *S. pneumoniae* TIGR4, B) *S. pyogenes* (GAS) MGAS315,
418 and C) *S. agalactiae* (GBS) COH1 when cultured in: Panel 1, rich undefined medium;
419 Panel 2, defined medium; and Panel 3, defined medium supplemented 5% v/v human
420 serum. All cultures were performed in biological triplicate and representative spectra are
421 shown.
422

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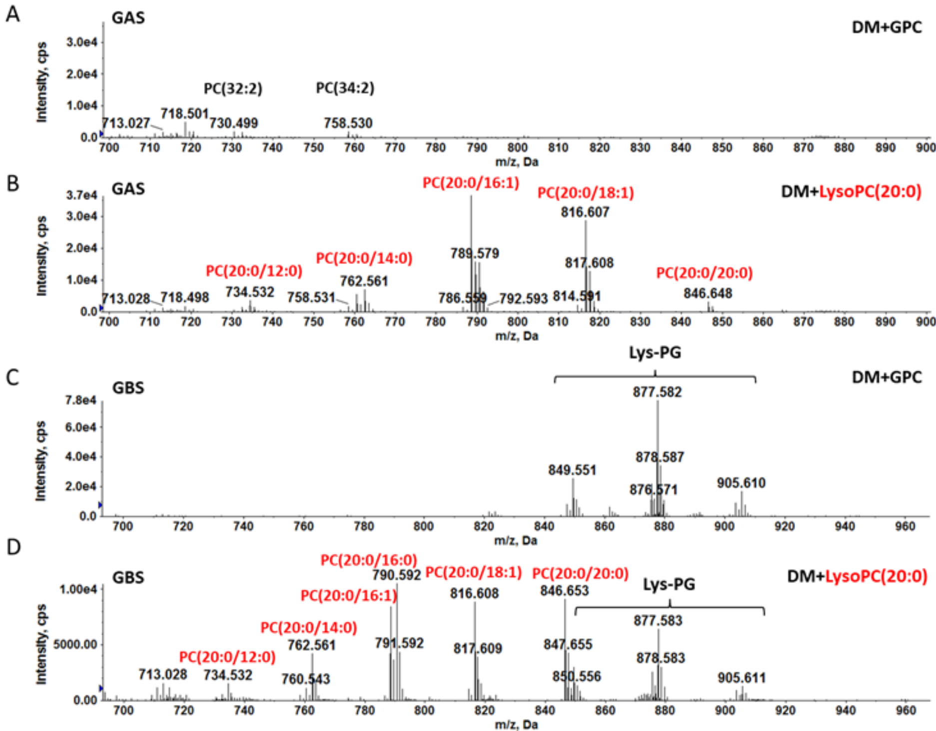

423
424 **Figure 3. LysoPC is scavenged by GAS and GBS to synthesize PC.** A) GAS strain
425 NZ131 cultured in defined medium supplemented with 100 µM GPC synthesizes a very
426 low level of PC. B) GAS strain NZ131 grown in defined medium supplemented with 100
427 µM lysoPC (20:0) synthesizes PC. C) No PC is detected for GBS strain COH1 cultured in
428 defined medium supplemented with 100 µM GPC. D) GBS strain COH1 cultured in
429 defined medium supplemented with 100 µM lysoPC (20:0) synthesizes PC. Cultures were
430 performed in biological triplicate and representative data are shown.
431

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Table 1. Major lipids detected in *S. pneumoniae*, GAS, and GBS.

Species	Strain	Medium[1]	Detection of[2]										
			DAG	MHDAG	DHDAG	PA	PG	CL	C_{55}-P	Lys-PG	LGD	PC	pPC
S. pneumoniae[3]	D39	THB+0.5Y	+	+	+	+	+	+	+	−	−	+	−
		DM	+	+	+	+	+	+	+	−	−	−	−
		DM-HS	+	+	+	+	+	+	+	−	−	+	−
	TIGR4	THB+0.5Y	+	+	+	+	+	+	+	−	−	+	−
		DM	+	+	+	+	+	+	+	−	−	−	−
		DM-HS	+	+	+	+	+	+	+	−	−	+	−
S. pyogenes (GAS)	NZ131	THB+0.2Y	+	+	+	+	+	+	+	−	−	−	−
		DM	+	+	+	+	+	+	+	−	−	−	−
		DM-HS	+	+	+	+	+	+	+	−	−	+	−
	MGAS 315	THB+0.2Y	+	+	+	+	+	+	+	−	−	−	−
		DM	+	+	+	+	+	+	+	−	−	−	−
		DM-HS	+	+	+	+	+	+	+	−	−	+	−
S. agalactiae (GBS)	COH1	THB	+	+	+	+	+	+	+	+	+	−	−
		DM	+	+	+	+	+	+	+	+	+	−	−
		DM-HS	+	+	+	+	+	+	+	+	+	−	+
	A909	THB	+	+	+	+	+	+	+	+	+	−	−
		DM	+	+	+	+	+	+	+	+	+	−	−
		DM-HS	+	+	+	+	+	+	+	+	+	−	+

[1]Todd-Hewitt Broth (THB); THB supplemented with 0.5% yeast extract (THB+0.5Y); THB supplemented with 0.2% yeast extract (THB+0.2Y); Streptococcal defined medium (DM), Streptococcal defined medium supplemented with 5% v/v human serum (DM-HS).

[2]Abbreviations and denotations: + , detected; -, undetected; DAG, diacylglycerol; MHDAG, monohexosyldiacylglycerol; DHDAG, dihexosyldiacylglycerol; PA, phosphatidic acid; PG, phosphatidylglycerol; CL, cardiolipin; C_{55}-P, undecaprenyl phosphate; Lys-PG, lysyl-phosphatidylglycerol; LGD, lysyl-glucosyl-DAG; PC, phosphatidylcholine; pPC, plasmanyl-PC.

[3]*S. pneumoniae* lipid profiles in THB+0.5Y and defined medium were originally described in Joyce LR *et al* 2019 (25).

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1 **Supplemental Text, Figures, and Table**

2

3 ***Streptococcus pneumoniae*, *S. pyogenes*, and *S. agalactiae* membrane**

4 **phospholipid remodeling in response to human serum**

5

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7

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9 USA

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11

12

13 **Running title: Phospholipid remodeling in pathogenic streptococci**

14

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19 **Supplemental Text S1. Materials and Methods**

20 **Growth curves**

21 GAS and GBS were cultured overnight in defined medium and diluted to a starting
22 OD_{600nm} of 0.05 in 15 mL pre-warmed defined medium supplemented with or without 5%
23 v/v human serum. The OD_{600nm} was monitored manually every hour using a Thermo
24 Scientific Genesys 30 spectrophotometer. For *S. pneumoniae*, early exponential phase
25 defined medium overnight cultures, as described above, were diluted 1:50 into pre-
26 warmed defined medium supplemented with or without 5% v/v human serum. *S.*
27 *pneumoniae* cultures were incubated for 4 h before the OD_{600nm} was monitored every
28 hour. Growth curves were performed in biological triplicate. Repeated measures two-way
29 ANOVA with Bonferroni's multiple comparisons test was performed in GraphPad Prism
30 version 8 for Windows, GraphPad software, San Diego, California, USA,
31 www.graphpad.com.

32

33 **Acidic Bligh-Dyer extractions.**

34 Centrifugation was performed using a Sorvall RC6+ centrifuge. Cultures were pelleted at
35 4,280 x *g* for 5 min at room temperature. The supernatants were removed and stored at
36 -80°C until acidic Bligh-Dyer lipid extractions were performed as described (1,2). Briefly,
37 cell pellets were resuspended in 1X PBS (Sigma-Aldrich) and transferred to Coring Pyrex
38 glass tubes with PTFR-lined caps (VWR), followed by 1:2 vol:vol chloroform:methanol
39 addition. Single phase extractions were vortexed periodically and incubated at room
40 temperature for 15 minutes before 500 x *g* centrifugation for 10 min. A two-phase Bligh-
41 Dyer system was achieved by addition of 100 µl 37% HCL, 1 mL $CHCl_3$, and 900 µl of 1X
42 PBS, which was then vortexed and centrifuged for 5 min at 500 x *g.* The lower phase was

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43 removed to a new tube and dried under nitrogen before being stored at -80°C prior to
44 lipidomic analysis.
45
46 **Normal-Phase Liquid Chromatography-Electrospray Ionization/Mass Spectrometry**
47 **(LC-ESI/MS).**
48 Normal-phase LC-ESI/MS was performed on an Agilent 1200 quaternary LC system
49 equipped with an Ascentis silica high-performance liquid chromatography (HPLC) column
50 (5 µm; 25 cm by 2.1 mm; Sigma-Aldrich) as described previously (1–3). Briefly, mobile
51 phase A consisted of chloroform-methanol-aqueous ammonium hydroxide (800:195:5,
52 vol/vol), mobile phase B consisted of chloroform-methanol-water-aqueous ammonium
53 hydroxide (600: 340:50:5, vol/vol), and mobile phase C consisted of chloroform-methanol-
54 water-aqueous ammonium hydroxide (450:450:95:5, vol/vol/vol/vol). The elution program
55 consisted of the following: 100% mobile phase A was held isocratically for 2 min, then
56 linearly increased to 100% mobile phase B over 14 min, and held at 100% mobile phase
57 B for 11 min. The LC gradient was then changed to 100% mobile phase C over 3 min,
58 held at 100% mobile phase C for 3 min, and, finally, returned to 100% mobile phase A
59 over 0.5 min and held at 100% mobile phase A for 5 min. The LC eluent (with a total flow
60 rate of 300 ml/min) was introduced into the ESI source of a high-resolution TripleTOF5600
61 mass spectrometer (Sciex, Framingham, MA). Instrumental settings for positive-ion ESI
62 and MS/MS analysis of lipid species were as follows: IS = 5,000 V, CUR = 20 psi, GSI =
63 20 psi, DP = +55 V, and FP = +150V. The MS/MS analysis used nitrogen as the collision
64 gas. Data analysis was performed using Analyst TF1.5 software (Sciex, Framingham,
65 MA).

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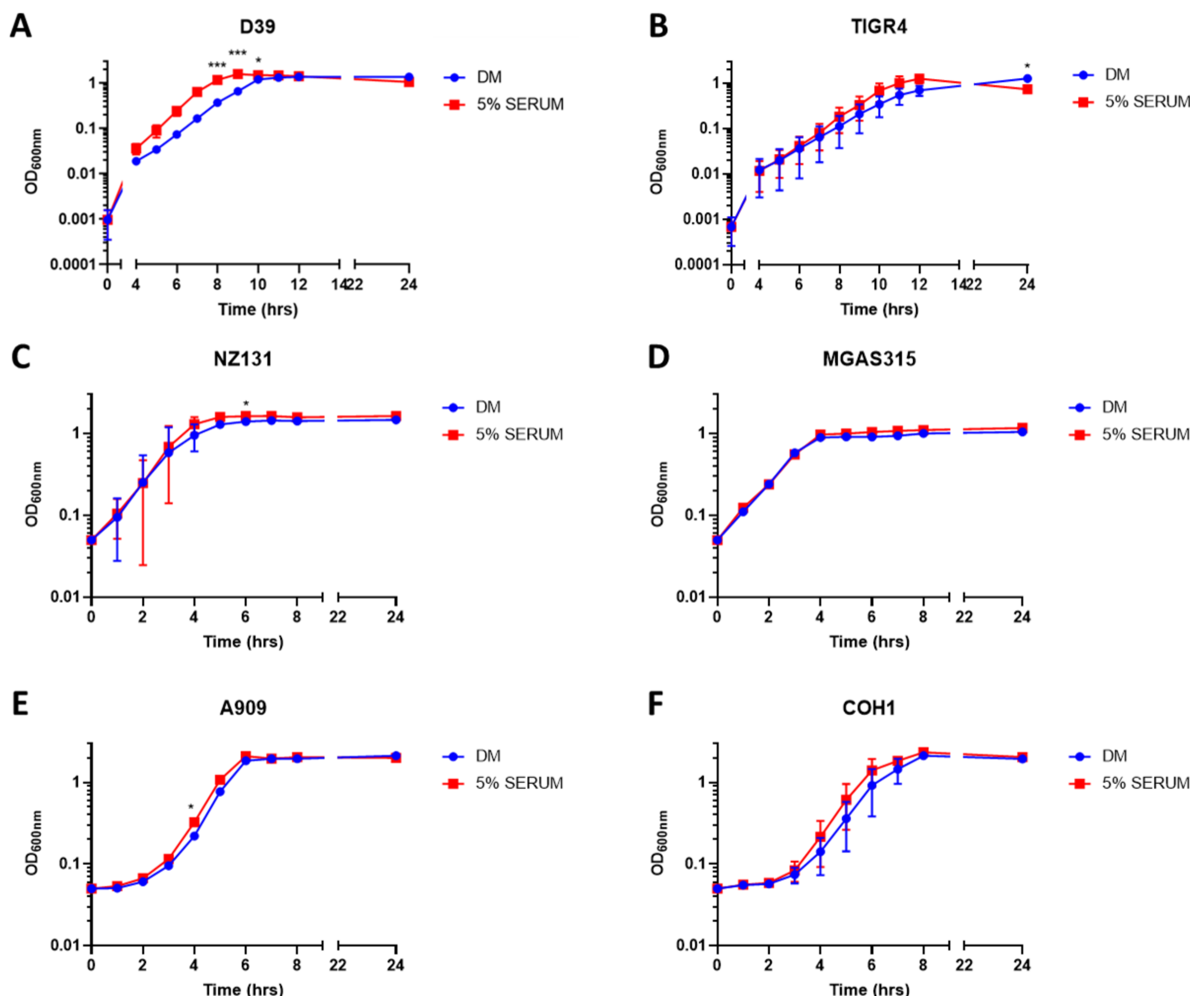

66

67 **Supplemental Figure S1. Growth characteristics of streptococci in the presence of**
68 **5% human serum.** Growth curves in defined medium (DM) are shown in blue and defined
69 medium supplemented 5% v/v human serum are shown in red. A) *S. pneumoniae* D39,
70 B) *S. pneumoniae* TIGR4, C) *S. pyogenes* NZ131, D) *S. pyogenes* MGAS315, E) *S.*
71 *agalactiae* A909, and F) *S. agalactiae* COH1. Manual OD_{600nm} readings were performed
72 every hour. *S. pneumoniae* cultures were grown for 4 h before manual readings were
73 performed. Growth curves were performed in biological triplicate. Mean and SD are
74 indicated. Statistical analysis: A-F) repeated measures two-way ANOVA with Bonferroni's
75 multiple comparisons test. * denotes p-value <0.05. *** denotes p-value <0.001.

76

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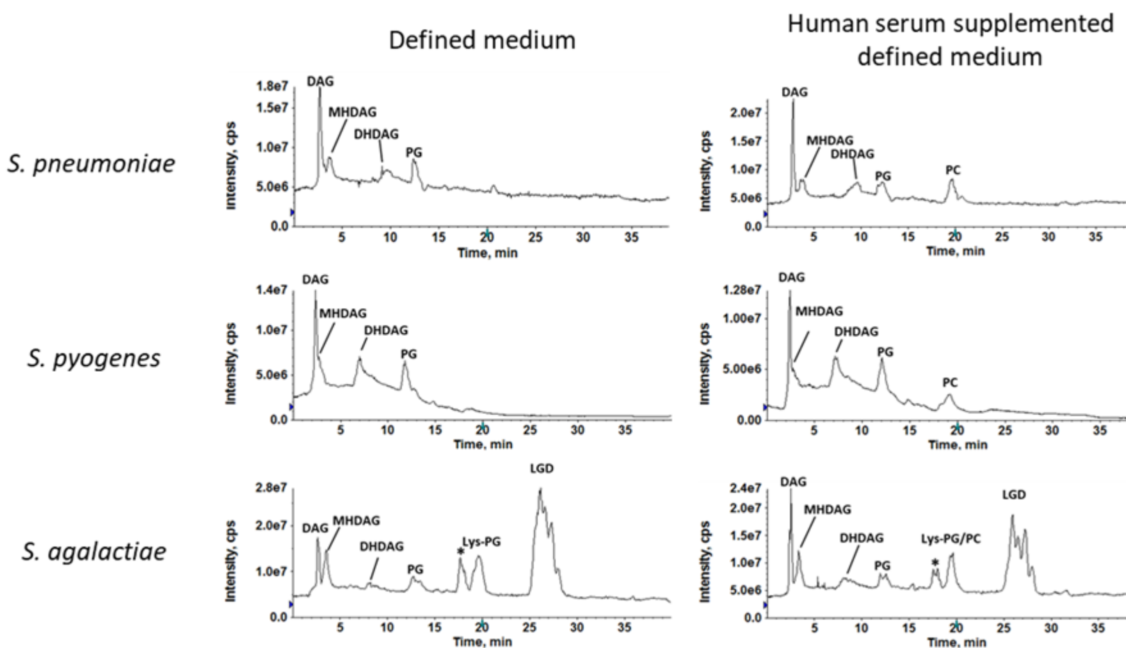

77

78 **Supplemental Figure S2. Positive ion mode total ion chromatograms (TIC) of**
79 **streptococci grown in defined medium supplemented with or without 5% v/v human**
80 **serum.** Representative positive ion mode TIC for *S. pneumoniae* (top panels), *S.*
81 *pyogenes* (middle panels), and *S. agalactiae* (bottom panels), indicate relative abundance
82 of lipids present in the cell extract when streptococci are cultured in defined medium or
83 defined medium supplemented with 5% v/v human serum. Cultures were performed in
84 biological triplicate. Abbreviations: DAG, diacylglycerol; MHDAG,
85 monohexosyldiacylglycerol; DHDAG, dihexosyldiacylglycerol; PG, phosphatidylglycerol;
86 Lys-PG, lysyl-phosphatidylglycerol; LGD, lysyl-glucosyl-DAG; PC, phosphatidylcholine;
87 '*' Denotes methylcarbamate, an extraction artifact of LGD.

88

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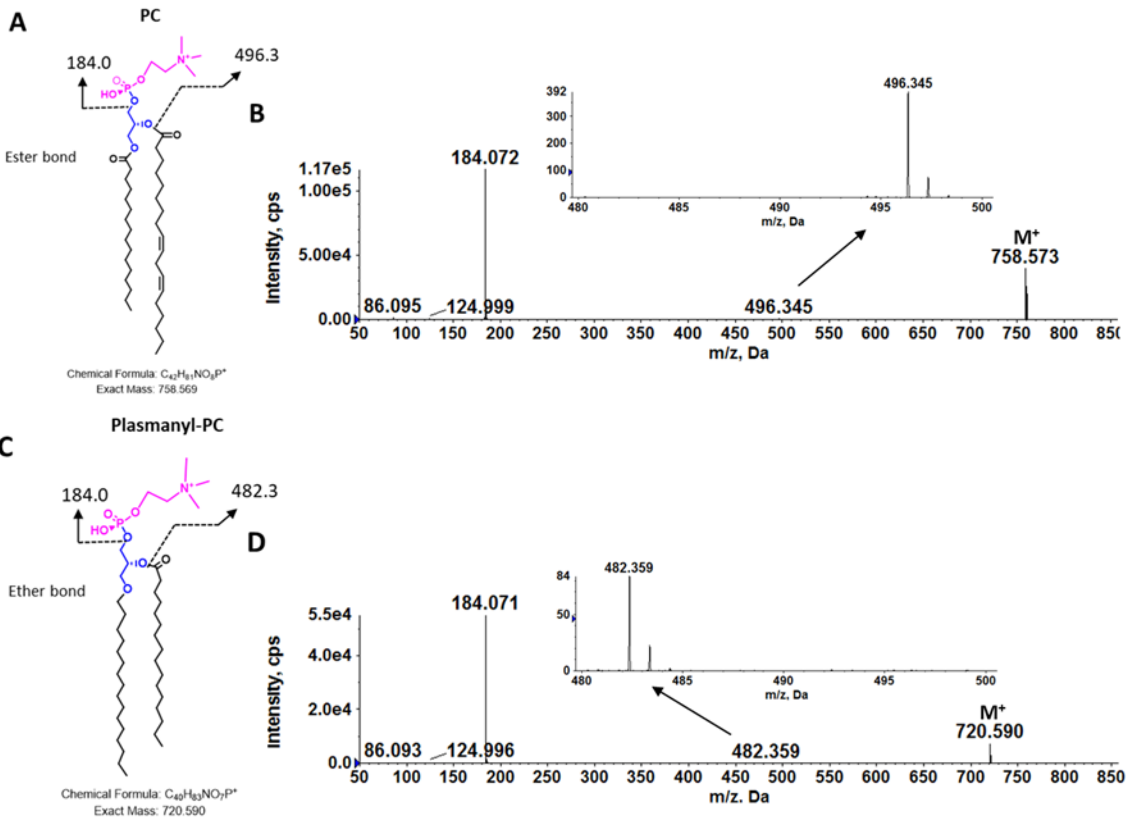

89

90 **Supplemental Figure S3. Confirmation of major PC and plasmanyl-PC species by**
91 **MS/MS fragmentation.** A) Chemical structure and fragmentation scheme of
92 PC(16:0/18:2), B) MS/MS of *m/z* 758 for PC(16:0/18:2), C) Chemical structure and
93 fragmentation scheme of pPC(16:0/16:0), and D) MS/MS of *m/z* 720 for pPC(16:0/16:0).
94 MS/MS fragmentation, specifically *m/z* 482 fragment, is consistent with the structure of
95 pPC present in the GBS.

96

97

98

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99 **Table S1. Strains used in this study.**

Organism	Strain	Description	Ref
S. pneumoniae	D39	Wild-type *S. pneumoniae* strain, capsule serotype 2. Obtained from Michael Federle, University of Illinois at Chicago	(4)
	ATCC BAA-334 (TIGR4)	Wild-type *S. pneumoniae* strain, capsule serotype 4	(5)
S. pyogenes (GAS)	ATCC BAA-595 (MGAS315)	Wild-type *S. pyogenes* strain, M3 serotype	(6)
	ATCC BAA-1633 (NZ131)	Wild-type *S. pyogenes* strain, M49 serotype	(7,8)
S. agalactiae (GBS)	ATCC BAA-1176 (COH1)	Wild-type *S. agalactiae* strain, serotype III	(9)
	ATCC BAA-1138 (A909)	Wild-type *S. agalactiae* strain, serotype Ia	(10)

100

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