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Lipidomic and Ultrastructural Characterization of Cell Envelope of *Staphylococcus aureus* Grown in the Presence of Human Serum

Running title: Effect of serum on *S. aureus* lipids and cell envelope

Kelly M. Hines^{a, b}, Gloria Alvarado^c, Xi Chen^d, Craig Gatto^c, Antje Pokorny^e, Francis Alonzo III^d, Brian J. Wilkinson^c, and Libin Xu^a

^a Department of Medicinal Chemistry, University of Washington, Seattle, Washington, 98195, USA.

^b Department of Chemistry, University of Georgia, Athens, Georgia, 30606, USA.

^c School of Biological Sciences, Illinois State University, Normal, Illinois, 61790, USA.

^d Department of Microbiology and Immunology, Stritch School of Medicine, Loyola University Chicago, Maywood, Illinois, 60153, USA

^e Department of Chemistry and Biochemistry, University of North Carolina Wilmington, Wilmington, North Carolina, 28403, USA.

Address correspondence to Brian J. Wilkinson at bjwilkin@ilstu.edu or Libin Xu at libinxu@uw.edu.

26 **ABSTRACT**

27 *Staphylococcus aureus* can incorporate exogenous straight-chain unsaturated and
28 saturated fatty acids (SCUFAs and SCFAs, respectively) to replace some of the normally
29 biosynthesized branched-chain fatty acids and SCFAs. In this study, the impact of human serum
30 on the *S. aureus* lipidome and cell envelope structure was comprehensively characterized. When
31 grown in the presence of 20% human serum, typical human serum lipids, such as cholesterol,
32 sphingomyelin, phosphatidylethanolamines, and phosphatidylcholines, were present in the total
33 lipid extracts. Mass spectrometry showed that SCUFAs were incorporated into all major *S.*
34 *aureus* lipid classes, *i.e.*, phosphatidylglycerols, lysyl-phosphatidylglycerols, cardiolipins, and
35 diglucosyldiacylglycerols. Heat-killed *S. aureus* retained much fewer serum lipids and failed to
36 incorporate SCUFAs, suggesting that association and incorporation of serum lipids with *S.*
37 *aureus* requires a living or non-denatured cell. Cytoplasmic membranes isolated from
38 lysostaphin-produced protoplasts of serum-grown cells retained serum lipids, but washing cells
39 with Triton X-100 removed most of them. Furthermore, electron microscopy studies showed that
40 serum-grown cells had thicker cell envelopes and associated material on the surface, which was
41 partially removed by Triton X-100 washing. To investigate which serum lipids were
42 preferentially hydrolyzed by *S. aureus* lipases for incorporation, we incubated individual serum
43 lipid classes with *S. aureus* and found that cholesteryl esters (CEs) and triglycerides (TGs) are
44 the major donors of the incorporated fatty acids. Further experiments using purified Geh lipase
45 confirmed CEs and TGs being the substrates of this enzyme. Thus, growth in the presence of
46 serum altered the nature of the cell surface with implications for interactions with the host.

47 **IMPORTANCE**

48 Comprehensive lipidomics of *S. aureus* grown in the presence of human serum suggests
49 human serum lipids can associate with the cell envelope without being truly integrated into the
50 lipid membrane. However, fatty acids-derived from human serum lipids, including unsaturated
51 fatty acids, can be incorporated into lipid classes that can be biosynthesized by *S. aureus* itself.
52 Cholesteryl esters and triglycerides are found to be the major source of incorporated fatty acids
53 upon hydrolysis by lipases. These findings have significant implications for the nature of the *S.*
54 *aureus* cell surface when grown *in vivo*. Changes in phospholipid and glycolipid abundances and
55 fatty acid composition could affect membrane biophysics and function and the activity of
56 membrane-targeting antimicrobials. Finally, the association of serum lipids with the cell envelope
57 has implications for the physicochemical nature of the cell surface and its interaction with host
58 defense systems.

59 **KEYWORDS**

60 Lipidomics, human serum lipids, fatty acid incorporation, lipid association, cell envelope
61 structure

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71 INTRODUCTION

72 *Staphylococcus aureus* is a major bacterial pathogen of great versatility capable of
73 infecting most organs and tissues in the body. Treatment of *S. aureus* infections is challenging due
74 to the development of resistance to multiple antibiotics. Mechanistic studies of *S. aureus*
75 pathogenesis have been an area of active investigation for several decades, but there is still a need
76 to understand the metabolic and structural properties of the pathogen *in vivo*, which are likely to
77 be different from those when grown *in vitro*. In order for a pathogenic bacterium to cause an
78 infection, it must utilize nutrients available in the infection site for replication (1). In a 1960 paper
79 entitled “The host as a growth medium”, E.D. Garber proposed that understanding the physiology
80 of the bacterium at the infection site was of fundamental importance (2). In recent years, several
81 studies have reported that *ex vivo* growth of *S. aureus* in body fluids such as blood, ocular fluids,
82 and nasal secretions, has profound impact on the characteristics of the organism and genes required
83 for growth in these environments (3-5).

84 One striking example of differences between *S. aureus* cells grown in conventional
85 artificial laboratory media versus cells grown in the presence of complex host biological materials
86 is in the fatty acid composition of the lipids of the organism. Branched-chain fatty acids (BCFAs)
87 and straight-chain saturated fatty acids (SCFAs) comprise the entirety of the fatty acid composition
88 of the organism in cells grown in laboratory media (6, 7). However, it has been increasingly
89 recognized that host fatty acids, including straight-chain unsaturated fatty acids (SCUFAs), are
90 utilized by pathogens and incorporated directly into phospholipid molecules, thereby saving the
91 energy and carbon costs of *de novo* fatty acid biosynthesis by the type II fatty acid synthesis
92 (FASII) pathway (8, 9). In *S. aureus*, the fatty acids are predominantly found ester-linked in the

93 polar lipids of the organism, with major phospholipid species being phosphatidylglycerol (PG),
94 lysyl-phosphatidylglycerol (LysylPG), and cardiolipin (CL), and major glycolipid species being
95 diglucosyldiacylglycerol (DGDG) and monoglucosyldiacylglycerol (MGDG) (7, 10, 11).

96 It is generally considered that *S. aureus* is unable to biosynthesize SCUFAs, and cells
97 grown in the presence of serum (6), liver extract (12), and human low-density lipoprotein (LDL)
98 and egg yolk LDL (13) have been shown to contain significant amounts of SCUFAs in their fatty
99 acid profiles. In addition, free fatty acids are incorporated into phospholipids from medium
100 supplemented with them (14, 15). Mass spectrometry (MS) analysis suggests that PG 33:1 is a
101 major phospholipid when *S. aureus* is grown in the presence of LDL, which is likely made up of
102 C18:1^{Δ9} (oleic acid) at position *sn*-1 and anteiso C15:0 at position *sn*-2 based on MS fragmentation
103 (13, 15). The major source of lipids in human serum is from LDL particles that contain cholesterol
104 esters, unesterified cholesterol, triglycerides, and phospholipids (16) (Fig. 1). *S. aureus* secretes at
105 least two lipases, *S. aureus* lipase 1 (Sal1) and glycerol ester hydrolase (Geh) (17-19), that release
106 fatty acids from lipids found in serum (6), and LDL (13). These free fatty acids are then
107 incorporated into *S. aureus* phospholipids and glycolipids through the FakA/B and PlsXY systems
108 (15, 20), with or without further elongation via the type II fatty acid synthesis (FASII) system (Fig.
109 1). The two-component fatty acid kinase system (FakA/B) produces fatty acyl-phosphate via FakA
110 that phosphorylates fatty acids bound to FakB1 or FakB2 binding proteins, which have preferential
111 specificities for SCFAs and SCUFAs, respectively (21). The resulting fatty acyl-phosphate is then
112 incorporated into phospholipids via PlsXY.

113 However, despite previous work on utilization of exogenous fatty acids by *S. aureus*,
114 several major questions remain. First, comprehensive lipidomic changes in the presence of
115 exogenous lipids have not been characterized as previous studies focus on total fatty acid

116 composition and only PGs. Second, the specific lipid classes in LDL or serum that serve as the
117 donors of fatty acids have not been identified. Third, whether intact human serum lipids can be
118 incorporated into the *S. aureus* membrane has not been investigated. Fourth, structural changes to
119 the cell envelope when *S. aureus* was grown in the presence of serum have not been characterized.
120 To answer these questions, we grew *S. aureus* in Tryptic Soy Broth (TSB) supplemented with 20%
121 human serum and carried out comprehensive lipidomic and electron microscopic analysis of these
122 cells. Growth of *S. aureus* in serum has the advantage of being able to mimic *in vivo* growth (22).
123 Oogai *et al.* have shown increased expression of multiple virulence factors in *S. aureus* grown in
124 serum (23). Supplementation of medium with blood or blood products for antimicrobial
125 susceptibility testing of fastidious pathogens is a common practice (24, 25). The lipid composition
126 of *S. aureus* has an impact on the interaction of the organism with the host's defense systems (26,
127 27).

128 We demonstrated that serum-derived SCUFAs are clearly incorporated into all classes of
129 lipids found in *S. aureus*, among which total cardiolipin levels are drastically increased when
130 grown in the presence of serum. Interestingly, we found that serum lipids are associated with the
131 cell envelope, which were not removed by washing with 0.9% NaCl but were removed with Triton
132 X-100. Electron microscopy studies showed overall thickened cell envelope and loosely associated
133 materials on the surface that were partially removable by Triton X-100. Growth in the presence of
134 individual lipid classes indicated that cholesteryl esters and triglycerides are the major donors of
135 the fatty acids, which is supported by studies using recombinantly expressed Geh. These findings
136 have implications for the biological and surface properties of the organism growing *in vivo*.

137 RESULTS

138 ***S. aureus* grown in serum retains serum lipids.** The total extractable lipids from 1 liter of
139 0.9% NaCl washed cells represented about 4.6% of the dry weight of the cell, consistent with
140 expectations (28). However, we found that total extractable lipids more than doubled (10.2%)
141 when the cells were grown in the presence of 20% serum. Cells grown in the presence and absence
142 of serum were subjected to comprehensive lipidomic analysis using hydrophilic interaction liquid
143 chromatography-ion mobility-mass spectrometry (HILIC-IM-MS) (11, 29). The major lipid
144 species observed in *S. aureus* grown in TSB included DGDGs, PGs, plasmalogen PGs (pPGs), and
145 LysylPGs, as shown in the IM-extraction ion chromatogram (IM-XIC) in Fig. 2a. The retention
146 time at which CLs are typically observed is noted in Fig. 2a, but CLs were below the detection
147 limit for *S. aureus* grown in TSB (data not shown). Each class of lipids contained fully saturated
148 fatty acids with 31 to 35 total carbons, with the species containing 33 total carbons as the most
149 abundant species across all classes of diacyl glycerolipids (Fig. 3).

150 When *S. aureus* was grown in TSB supplemented with 20% human serum (TSB+Serum),
151 the lipid profile, as shown in Fig. 2b, contained a mixture of the typical *S. aureus* lipid classes and
152 lipids that are abundant in human serum (see Fig. S1 for lipid profile of clean TSB+Serum). The
153 glycerophospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are not
154 produced by *S. aureus*, nor are sphingomyelins (SMs) and cholesterol (Chol). Rather, these lipids
155 were retained by *S. aureus* from the culture through the harvesting and washing procedures.

156 ***S. aureus* grown in serum incorporates SCUFAs.** The panels of Fig. 3 present the
157 abundances of individual lipid species found in TSB only and TSB+Serum-grown *S. aureus*.
158 Although the levels of LysylPGs (Fig. 3c) were elevated overall in the TSB+Serum condition, little
159 to no differences were observed between TSB and TSB+Serum-grown *S. aureus* for the major
160 fully-saturated species of DGDGs (Fig. 3a) and PGs (Fig. 3b) synthesized by *S. aureus*. However,

161 *S. aureus* grown in TSB+Serum contained species of all major lipid classes with unsaturated fatty
162 acids (i.e., 32:1, 33:1, 34:1, and 35:1) that were absent from *S. aureus* grown in TSB only. Specific
163 fatty acid compositions were obtained by tandem MS (MS/MS) experiments as discussed below.

164 As odd-numbered carbon fatty acids are not typically observed in human serum, the
165 occurrence of lipids with odd numbers of total carbons and one degree of unsaturation (i.e., 33:1
166 and 35:1) strongly indicates the incorporation of a serum-derived unsaturated fatty acid into the
167 lipids of *S. aureus*. The presence of such fatty acyl compositions in the DGDG, LysylPG, and CL
168 species, which are not observed in human serum (see Fig. S1B), further strengthens the evidence
169 for this incorporation.

170 Although CLs were not detected in *S. aureus* grown in TSB only, CLs with one to three
171 degrees of unsaturation were present in the lipid profiles of *S. aureus* grown in TSB+Serum (Fig.
172 3d). The most abundant CL was CL 66:2 with 15:0 and 18:1 being the major fatty acids (see
173 Supplemental Material Excel S1), which was consistent with the high abundance of PG 33:1 in the
174 serum-grown *S. aureus*. These data indicate an enrichment of unsaturated CL species when *S.*
175 *aureus* is grown in human serum. In contrast, no CLs were detected in the lipid profile of
176 uninoculated TSB+Serum (see Fig. S1B).

177 Targeted MS/MS experiments were performed in negative ionization mode to confirm the
178 fatty acid compositions of the lipid species presumed to contain SCUFAs based on *m/z*. An
179 inventory of all the fatty acids observed for each lipid species in the data shown in Fig. 3, as well
180 as those lipid species not shown in the figure, can be found in the Supplemental Material Excel
181 S1. The most abundant fatty acyl composition across lipid species, containing 33 carbons and no
182 double bonds, was determined to contain octadecanoic acid (C18:0) and pentadecanoic acid
183 (C15:0). Based on the relative intensities of the two fatty acyl fragments, it is likely that 18:0

184 occupied the *sn-1* position on the glycerol backbone and 15:0 occupied the *sn-2* position because
185 fatty acyl at the *sn-2* position tends to fragment more easily (30). Using this same approach, it was
186 confirmed that the lipids with 33:1 and 33:2 fatty acyl compositions contained 15:0 with C18:1
187 and C18:2 fatty acids, respectively, while 34:1 contained a major component with 16:0 and 18:1
188 fatty acids and a minor component with 20:1 and 14:0.

189 **Heat-killed *S. aureus* do not incorporate SCUFAs into their lipids.** The above
190 experiments were repeated using heat-killed *S. aureus* in order to determine whether the
191 incorporation of SCUFAs and the retention of serum lipids were active or passive processes. Fig.
192 4 shows that the heat-killed *S. aureus* incubated in TSB+Serum did not contain the same levels of
193 odd-carbon lipids with a degree of unsaturation as did live *S. aureus* incubated under the same
194 conditions. The heat-kill reduced the levels of the endogenous lipid species as well, but to a much
195 lesser extent. Much lower amounts of serum-derived lipids, such as cholesterol, PCs, and SMs,
196 were observed from the heat-killed *S. aureus* compared to the live *S. aureus* when both were
197 incubated in serum-supplemented TSB. These results indicate that SCUFA incorporation is an
198 active process, presumably via the FakAB and PlsXY systems (20), and the retention of serum
199 lipids also requires a living or non-denatured cell.

200 **Cytoplasmic membranes isolated from TSB+Serum-grown cells retain serum lipids.**
201 Cytoplasmic membranes were isolated from *S. aureus* grown in TSB and TSB+Serum by digestion
202 of the cell wall using lysostaphin in hypertonic sucrose followed by osmotic lysis of the
203 protoplasts. Lipidomics was performed on washed cytoplasmic membranes. The lipid profile of
204 the isolated membrane from TSB-grown *S. aureus* (see Fig. S2B) was consistent with the lipid
205 profile observed for whole *S. aureus* (Fig. 2A). The cytoplasmic membrane isolated from *S. aureus*
206 grown in the presence of serum still retained a substantial amount of serum lipids, including PCs

207 and SMs (see Fig. S2A). The overall topography of the lipid profile was consistent with that of
208 whole *S. aureus* cells grown in the presence of serum (Fig. 2B).

209 **Serum lipids are mostly removable by Triton X-100 washing.** The nature of the retained
210 serum lipids was further evaluated using a more rigorous washing procedure prior to lipid
211 extraction. In the experiments above, pellets were washed with 0.9% NaCl solution prior to lipid
212 extraction. To test whether the serum lipids were simply associated with the surface of the
213 bacterium, collected *S. aureus* pellets were washed first with 0.9% NaCl, followed by a second
214 wash with the detergent Triton X-100 (1%) to remove passively associated lipid material from the
215 growth medium. Principle components analysis (PCA) of the resulting lipidomics data, shown in
216 Fig. 5a, reveals that the Triton X-100 wash had a greater effect on the lipid profiles of serum-
217 grown *S. aureus* than the *S. aureus* grown in TSB only. While PC1 clearly corresponds to the
218 differences between TSB+serum-grown and TSB-only-grown *S. aureus*, the differences due to the
219 NaCl and Triton X-100 washes are revealed on PC2. Along PC2, the separation between NaCl
220 versus Triton X-100 washes for TSB+Serum-grown cells is much larger than the separation
221 between the two washing conditions for TSB-only-grown cells. The two washing techniques had
222 no significant effect on the abundances of the natively synthesized *S. aureus* lipids nor the
223 incorporation of serum-derived SCUFAs into *S. aureus* lipids, as shown in Fig. 5b. However, the
224 serum-derived PCs observed when *S. aureus* was grown in serum were nearly completely
225 eliminated by the Triton X-100 washing (Fig. 5c).

226 **Electron microscope studies reveal more cell clumping, associated surface material**
227 **partially removable by Triton X-100, and thicker cell envelopes in serum-grown cells.**

228 Transmission and scanning electron microscopy analysis was carried out to examine the
229 effect of serum on cell envelope structure (Fig. 6). In SEM images, *S. aureus* cells grown in the

230 presence of serum are seen clumped together compared to cells grown in TSB only, which are
231 more dispersed (Fig. 6A). Clumping of cells grown in TSB+Serum is consistent with observations
232 made while handling bacterial pellets, where pellets were much harder to resuspend compared to
233 TSB only-grown cells. Additionally, serum-grown cells display a textured cell surface unlike the
234 smooth surface seen in TSB only-grown cells. TEM analysis revealed more detailed changes to
235 the cell wall of serum-grown cells (Fig. 6B). TSB+Serum-grown cells appear to display a thicker
236 cell wall and large protrusions with irregular shapes on the cell surface while TSB-grown cells
237 again display a relative smooth cell surface. Materials at the protrusions appear to be partially
238 removed through washing with 0.1% Triton X-100, suggesting some of these materials are
239 associated with the cell wall. Quantitative analysis of overall cell wall thickness including the
240 protrusion support the visual conclusions (Fig. 6C). Cell walls of TSB+Serum-grown cells are
241 thicker than those of TSB only-grown cells regardless of washing conditions although there does
242 not appear to be a difference between NaCl and Triton X-100 washed cells.

243 ***Sources of serum FAs for incorporation into S. aureus lipids.*** Serum is a complex mixture
244 containing several classes of lipids that includes cholesteryl esters (CEs), triglycerides (TGs), and
245 phospholipids (16). To evaluate which of these lipids may provide fatty acid substrates for
246 incorporation into *S. aureus* lipids, bacteria were grown in TSB supplemented with 0.1 mM of
247 oleic acid, cholesteryl oleate and linoleate (CEs), and extracts of PEs and PCs from chicken egg
248 in ethanol. The lipid profiles resulting from growth of *S. aureus* with free oleic acid and the
249 cholesteryl oleate/cholesteryl linoleate mixture were highly similar, as indicated by the tight cluster
250 of these two sample groups in the PCA plot (Fig.7A). The ethanol treatment alone appears to
251 increase the amount of PG extracted (Fig. 7B), but the overall effect on the lipid profile was small
252 enough that the TSB+EtOH and TSB only samples are grouped closely in the PCA along with the

253 egg PC- treated group. The treatment with the intact phospholipids PEs and PCs did not lead to
254 any significant incorporation of SCUFAs into the lipids of *S. aureus*. The PE and PC extracts used
255 in this study indeed contain significant amount of SCUFAs (PE: 18% 18:1 and 14% 18:2; PC: 32%
256 18:1 and 17% 18:2), suggesting they are not readily available or not good substrates of the secreted
257 lipases under this growth condition.

258 The dramatically increased abundance of CLs with multiple degrees of unsaturation
259 observed in the serum-grown *S. aureus* was recapitulated with the growth of *S. aureus* in TSB
260 supplemented with oleic acid and CEs (Fig. 7C). Oleic acid and CE supplementation also resulted
261 in the incorporation of oleate and linoleate into the major lipid classes of *S. aureus*, including PGs,
262 LysylPG (Fig. 7D) and DGDGs (Figure 7E). Additional targeted tandem mass spectrometry was
263 performed to confirm the fatty acid compositions of the lipid species presented in Fig. 7 as
264 18:2/15:0, 18:1/15:0 and 18:0/15:0, respectively (see Supplemental Material Excel S1). In a
265 separate experiment, *S. aureus* grown in the presence of tri-oleate glyceride (TG 18:1/18:1/18:1)
266 and tri-linoleate glyceride (TG 18:2/18:2/18:2) yielded similar results, including the high
267 abundance of unsaturated CL species (see Fig. S3).

268 Evidence of *in vivo* elongation of oleic and linoleic acids into C20:1 and C20:2 fatty acids
269 was also observed in *S. aureus* grown in TSB supplemented with oleic acid, CEs, and TGs. Figure
270 S4 shows *S. aureus* PG, DGDG and LysylPG species with fatty acyl compositions of 35:0, 35:1
271 and 35:2 from *S. aureus* grown in lipid supplemented TSB. Elevated levels of 35:1 lipid species
272 were observed from growth in the presence of oleic acid CEs and TGs. Elevated levels of 35:2
273 lipid species were observed in TG- and cholesteryl ester-grown *S. aureus*. Tandem MS of each
274 lipid species individually identified the exact fatty acyl compositions for the lipids shown in Fig.
275 S4. While the 35:0 lipid species contained 20:0 and 15:0 fatty acids, the 35:1 and 35:2 species

276 contained 15:0 with 20:1 and 20:2 fatty acids, respectively (see Supplemental Material Excel S1).
277 As no 20:1 and 20:2 fatty acids were supplemented into the TSB, the presence of these lipid species
278 in *S. aureus* grown in the presence of 18:1 and 18:2 fatty acyl lipids indicated that these fatty acids
279 were elongated prior to incorporation into diacylglycerolipids.

280 While oleic acid is a free fatty acid that is readily available for uptake and incorporation,
281 the CEs and TGs contain esterified fatty acids that must undergo hydrolysis in order to generate
282 free fatty acids. Geh is a lipase secreted by *S. aureus* with specificity for long-chain fatty acids. To
283 evaluate the potential of Geh to generate free fatty acids, standards of CEs, TGs, PC, and PEs
284 containing oleic or linoleic acids were incubated with purified Geh. Fig. 8 shows the abundances
285 of free fatty acids in the supernatants following the incubation of Geh with lipid standards. As seen
286 in the figure, despite a consistently high background level of oleic acid, CEs, PC, PE, and TG
287 containing oleic acid yielded levels of free oleic acid higher than the background level taken from
288 lipids that did not contain oleic acid. On the other hand, higher levels of free linoleic acid were
289 only observed from incubation of Geh with CE, PC, and TG containing linoleic acid. The
290 observation of PCs and PEs being substrates of Geh *in vitro*, but not donors of fatty acids *in vivo*,
291 may be due to the different incubation conditions with the former being in 1x PBS with 10%
292 isopropanol while the latter being in TSB with less than 1% ethanol.

293

294 **DISCUSSION**

295 **Increased overall lipid content in *S. aureus* grown in the presence of human serum.**

296 In 1971, Rédei et al. reported total extractable lipids comprised 20% of the dry weight of the
297 organism grown in broth supplemented with 20% human serum (31). In this study, we found that
298 the total extractable lipids of serum-grown *S. aureus* more than doubled (10.2% vs. 4.6%)

299 compared to cells grown in TSB alone. This large increase in lipid content in the presence of
300 human serum suggest host-derived lipids could be associated with or incorporated into *S. aureus*
301 cell envelope.

302 **Incorporation of host fatty acids into *S. aureus* lipids.** We have previously shown that
303 SCUFAs became about 25% of the total fatty acid profile of *S. aureus* grown in 100% total bovine
304 serum (6). Delekta et al. grew *S. aureus* in the presence of human LDL and analyzed the PG species
305 produced under these conditions by mass spectrometry (13). PG species containing C16:1, C18:1,
306 C18:2, and C20:1 were observed. The most abundant PG species were PG 33:1, 35:1, and 36:2.
307 Gruss and co-workers found that addition of exogenous fatty acids promotes resistance to FASII
308 antibiotics by *S. aureus* and selection of resistant strains that bypass FASII inhibition (12, 32). The
309 same group showed that exogenous fatty acids could occupy both the *sn*-1 and *sn*-2 positions of
310 PG when cells were grown in Brain Heart Infusion broth supplemented with C14:0, C16:0, and
311 C18:1, or serum (33). This seemingly disproves the essentiality of the requirement for
312 biosynthesized fatty acid anteiso C15:0 at the *sn*-2 position (15, 34) and undermines the viability
313 of inhibitors of the FASII pathway as useful therapeutic agents (33). In this work, we also observed
314 lipid species containing no C15:0, such as PG 32:1 (18:1/14:0), PG 34:1 (18:1/16:0 and 20:1/14:0),
315 and PG 36:1 (18:1/18:0, 20:1/16:0, and 22:1/14:0), which supports the notion that anteiso C15:0
316 is not essential. Furthermore, we observed incorporation of SCUFA into all major classes of lipids
317 that can be synthesized by *S. aureus* (Fig. 3, 7, and S3) and that SCUFAs can undergo elongation
318 within *S. aureus* (Fig. S4), suggesting that host-derived fatty acids can fully participate in all fatty
319 acid (FASII) and glycerolipid metabolic pathways.

320 It is particularly worth noting that the proportion of CL of the total phospholipids was
321 drastically increased in cells grown in the presence of serum, oleic acid, and CEs (Figures 3 and

322 7) and these CLs contain at least one SCUFA. When grown in TSB only, no CL was detected
323 under the same condition, including CLs with fully saturated fatty acids. CL is synthesized by
324 condensation of two molecules of PG by CL synthase enzymes (35). The *cls2* gene encodes the
325 major CL synthase of the two in *S. aureus* (36, 37). Notably, all observed CLs in TSB+Serum-
326 grown cells contain at least one SCUFA, suggesting that PGs containing a SCUFA are
327 preferentially used as substrates of Cls2 over PGs containing fatty acids that are *de novo*
328 synthesized by *S. aureus*.

329 Increased membrane CL content has been shown to be involved in decreased susceptibility
330 to the important last-line anti-staphylococcal drug daptomycin. CL is a non-bilayer phospholipid
331 with a small head group and four fatty acyl chains, that typically organizes in microdomains at
332 high-curvature regions of the membrane, such as the sites of cell division and membrane fusion
333 (9, 38-40). Daptomycin was found to attract and cluster fluid lipids in the membrane, causing
334 membrane depolarization and delocalization of membrane proteins (41). Jiang et al. found some
335 clinical daptomycin-resistant mutants had gain-of-function mutations in *cls2*, leading to increased
336 CL content and decreased PG content, which then resulted in decreased daptomycin susceptibility
337 (42). Zhang et al. have found that CL renders liposomes impermeable to daptomycin and proposed
338 that this could be due to the prevention of flipping of the daptomycin to the inner leaflet of
339 liposomes (43). The CL enriched membrane was also thicker than wild type membrane and resisted
340 daptomycin lipid extraction, membrane penetration and disruption (43). In bilayer model systems,
341 inclusion of CL has been shown to lead to increased bilayer thickness and a stiffening of the
342 membrane, which correlates with decreases susceptibility to membrane lysis induced by helical
343 antimicrobial peptides (44, 45). Thus, increased content of CLs in *S. aureus* grown in a host

344 environment could result in decreased susceptibility to daptomycin and other antimicrobial
345 peptides.

346 **Association of serum lipids with the cell envelope of *S. aureus*.** TSB+Serum-grown *S.*
347 *aureus* cells retain all major serum lipids, but these lipids are mostly removable by washing with
348 Triton X-100. Furthermore, electron microscope images reveal that serum-grown cells have
349 thicker cell envelopes and associated materials on their surfaces that can be partially removed by
350 Triton X-100 washing. These observations suggest that serum lipids are associated with the cell
351 wall, either directly as liposomes through hydrogen-bonding between the polar lipid headgroup
352 and the cell wall or mediated by serum proteins, instead of being truly incorporated into the cell
353 membrane. Association of serum lipids with the cell significantly decreased in heat-killed cells,
354 suggesting the cell envelope must not be denatured for efficient association of the serum lipids.
355 When cytoplasmic membranes were isolated from lysostaphin-induced protoplasts from serum-
356 grown cells, the total lipid profile was very similar to that of NaCl-washed intact cells grown in
357 TSB+Serum medium. The fact that lysostaphin-induced protoplasts, but not Triton X-100-treated
358 cells, retain all serum lipids suggest that there is a secondary process in lysostaphin-treated cells
359 through which the serum lipids are incorporated into the membrane.

360 The incorporation of serum lipids to cell wall-removed *S. aureus* is not surprising as this
361 phenomenon has been observed in *S. aureus* L-forms. Bacterial L-forms are derived from typical
362 bacteria, often through treatment with cell wall-active antibiotics, and lack an organized cell wall,
363 yet they can proliferate in suitable media (46). Supplementation of medium with serum is often
364 used to grow L-forms. Interestingly, cholesterol, cholesteryl esters, and triglycerides (all serum
365 lipids) have been reported to be a component of the lipids of *S. aureus* L-forms although the content
366 of PCs, PEs, and SMs was not examined (47). Nishiyama and Yamaguchi reported electron

367 microscopic detection of complexes between the sterol-specific antibiotic filipin and cholesterol
368 in the membrane of staphylococcal L-forms (48). Thus, the presence of cholesterol in L-forms is
369 a precedent for our finding of this mammalian serum lipid in *S. aureus* cells and in their
370 membranes. Interestingly, L-forms were also reported to have double the CL content of parental
371 bacterial forms (47).

372 We cannot completely exclude the possibility that serum lipids, likely as small liposome
373 vesicles, could migrate through the cell wall and directly interact with the membrane. Lee et al.
374 show that extracellular vesicles produced from the cytoplasmic membrane of *S. aureus* can
375 traverse the cell wall (49). Extracellular vesicles, which are delimited by a lipid bilayer and cannot
376 replicate, are naturally released from the cells by many different organisms (50), including *S.*
377 *aureus*. Coelho et al. found that the composition of extracellular vesicles from the gram-positive
378 bacterial pathogen *Listeria monocytogenes*, grown in Brain Heart Infusion broth supplemented
379 with 10% bovine fetal serum, were enriched in PE, sphingolipids and triacylglycerols (51).
380 Although it is possible that serum lipids can cross the cell wall in the other direction and insert into
381 the membrane, the fact that Triton X-100 can effectively remove these lipids make this hypothesis
382 less likely.

383 **Cell surface and interaction with host defense systems.** Incorporation of SCUFAs into
384 *S. aureus* membrane has been shown to impact host-pathogen interactions. Lopez et al. showed
385 that incorporation of *cis* SCUFAs from the host into membrane phospholipids activated the type
386 VII secretion system for multiple virulence factors (26). On the other hand, Nguyen et al.
387 demonstrated that SCUFAs C16:1, C18:1, and C18:2 were taken up, elongated, and incorporated
388 into membrane phospholipids and the lipid moiety of lipoproteins. This led to an increased
389 recognition of the *S. aureus* by the innate immune system dependent on Toll-like receptor 2 (27).

390 However, it is also plausible the association of human serum-derived lipids with the cell envelope
391 could change the response by the host innate immune system, *i.e.*, the host material-decorated cells
392 could allow them to escape the immune system. Detailed composition, in addition to lipids, of the
393 associated materials and their effect on host immune system would be worth further investigation.

394

395 MATERIALS AND METHODS

396 **Bacterial strain and growth conditions.** The studies were carried out using *S. aureus*
397 strain JE2 derived from strain LAC USA300, a prominent community-acquired methicillin-
398 resistant *S. aureus* strain responsible for aggressive cutaneous and systemic infections in the USA
399 (52). The strain was grown in Tryptic Soy Broth (TSB) (BD Difco; Franklin Lakes, NJ), at 37°C
400 with shaking (200 rpm) in 50 mL medium in 250 mL Erlenmeyer flasks. For growth in the presence
401 of serum, TSB was supplemented with 20% heat-treated pooled gender human serum (BioIVT;
402 Hicksville, NY). Cultures were harvested by centrifugation (9,800 x g at 4°C for 5 min) and were
403 washed twice by resuspension and centrifugation in cold 0.9% NaCl. For treatment with lipid
404 standards TSB was supplemented with oleic acid (Sigma-Aldrich; St. Louis, MO), cholesteryl
405 oleate (Sigma-Aldrich; St. Louis, MO), cholesteryl linoleate (Sigma-Aldrich; St. Louis, MO),
406 triglycerides (NuChek Prep. Inc., Elysian, MN), phosphatidylcholine egg extract (Avanti Polar
407 Lipids; Alabaster, AB), and phosphatidylethanolamine egg extract (Avanti Polar Lipids;
408 Alabaster, AB) in ethanol to a final concentration of 0.1 mM in TSB. For washing experiments,
409 cells were initially washed twice in 0.9% NaCl followed by two washes in 0.1% vol/vol Triton X-
410 100. After Triton X-100 washing the cells were washed twice with 0.9% NaCl.

411 **Heat-killed cells.** Cells were grown in 50 mL TSB to an OD₆₀₀ of 1.0 and harvested and
412 washed once in 0.9% NaCl as described above. Washed cells were resuspended in 1 mL 0.9%

413 NaCl and incubated in a 56°C water bath for 30 mins. The heat-killed cells were then added to 50
414 mL of sterile TSB or TSB supplemented with 20% human serum and incubated at 37°C with
415 shaking (200 rpm) for one hour. Cells were harvested and washed twice in 0.9% NaCl before being
416 subjected to lipidomic analysis.

417 **Isolation of cytoplasmic membranes.** Cytoplasmic membranes were isolated from
418 lysostaphin-induced protoplasts as described by Wilkinson et al. (53). Briefly, washed cells were
419 resuspended in buffered hypertonic sucrose and cell walls were digested by lysostaphin treatment.
420 The protoplasts were recovered by centrifugation and lysed by resuspension in dilute buffer.
421 Cytoplasmic membranes were recovered by centrifugation and were washed in water.

422 **Lipidomic analysis.** Total lipids were extracted by the method of Bligh and Dyer (54).
423 Lipid extracts were dried under nitrogen and dissolved in 1:1 chloroform-methanol. Small aliquots
424 were diluted into 2:1 acetonitrile/methanol for analysis. Extracts were analyzed by hydrophilic
425 interaction chromatography (HILIC) coupled to an ion mobility-mass spectrometer (Synapt G2-Si
426 IM-MS; Waters Corp., Milford, MA) in positive and negative modes (11, 29). Data analysis was
427 performed with Progenesis QI (Nonlinear Dynamics; Waters Corp., Milford, MA) and lipid
428 abundances were normalized to bacterial dry weight (11).

429 **Transmission and scanning electron microscopy.** Samples were prepared for
430 transmission electron microscopy (TEM) using a modified high pressure freezing/freeze
431 substitution (HPF/FS) method as described by Hall et al. (55). Pelleted bacteria were loaded into
432 metal specimen carriers (2 mm diameter aluminum) coated with 1-hexadecene and frozen in an
433 HPM 010 high pressure freezer. Freeze substitution was performed in 2% OsO₄ (Electron
434 Microscopy Sciences; Hatfield, PA) and 0.1% uranyl acetate (Polysciences; Warrington, PA) in
435 2% H₂O and 98% acetone in an FS-8500 freeze substitution system. Samples warmed and washed

436 as described (55). Samples were infiltrated with 1:1 Polybed812 (Polysciences) resin:acetone for
437 24 hours, 2:1 resin:acetone for 36 hours, 100% resin for 24 hours, and then changed to fresh resin
438 for three days. All infiltration steps were conducted on an orbital shaker at room temperature.
439 Samples were then submerged into embedding molds with resin and hardener and baked at 60°C
440 for two days. 50-70 nm sections were collected using a PowerTome PC ultramicrotome with a
441 diamond knife and collected onto carbon coated copper slot grids. Sections were imaged with a
442 Phillips CM200 TEM. For scanning electron microscopy (SEM), samples were prepared using
443 liquid cultures by gently passing through and embedding in a 0.2 µm filter (Nuclepore). Filter-
444 embedded bacterial samples were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1
445 M sodium Cacodylate buffer at pH 7.4 for four hours on ice before being washed in buffer for 10
446 minutes with shaking. Samples were dehydrated in increasing ethanol concentrations three times
447 with 10 minutes of shaking each time until reaching 100% ethanol. Samples were then dried in a
448 Tousimis 931 critical point drier in 100% ethanol and coated with 6 nm gold-palladium. Images
449 were collected on a FEI Quanta FEG 450 ESEM.

450 **Expression and purification of Geh-6xHis in *E. coli* lysY/I^q.** C-terminally 6xHis-tagged
451 *S.aureus* Geh was expressed in *E. coli* lysY/I^q and purified as described previously (56).

452 **Incubation of lipids with Geh-6xHis.**

453 Individual lipid standards (1 mM stock in isopropanol; PCs and PEs from Avanti Polar Lipids,
454 CEs from Sigma-Aldrich Inc., and TGs from NuChek Prep.) were added to Geh-6xHis in 50 µL
455 1XPBS, resulting in a 100 µM:2 µM lipid:Geh-6xHis ratio. The reaction was incubated at 37°C
456 for 2 hours and then stopped by freezing at -80°C. Lipids were extracted and analyzed as described
457 above.

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465

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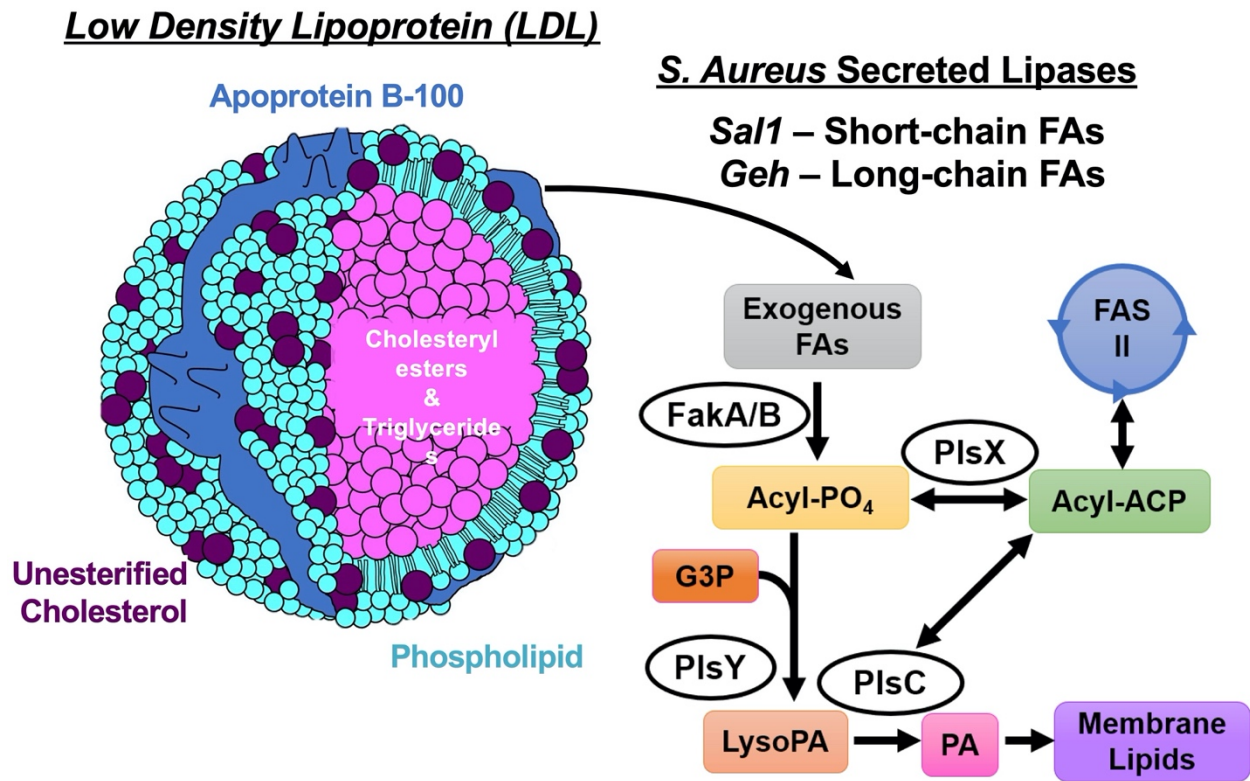
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635 FIGURES AND FIGURE LEGENDS



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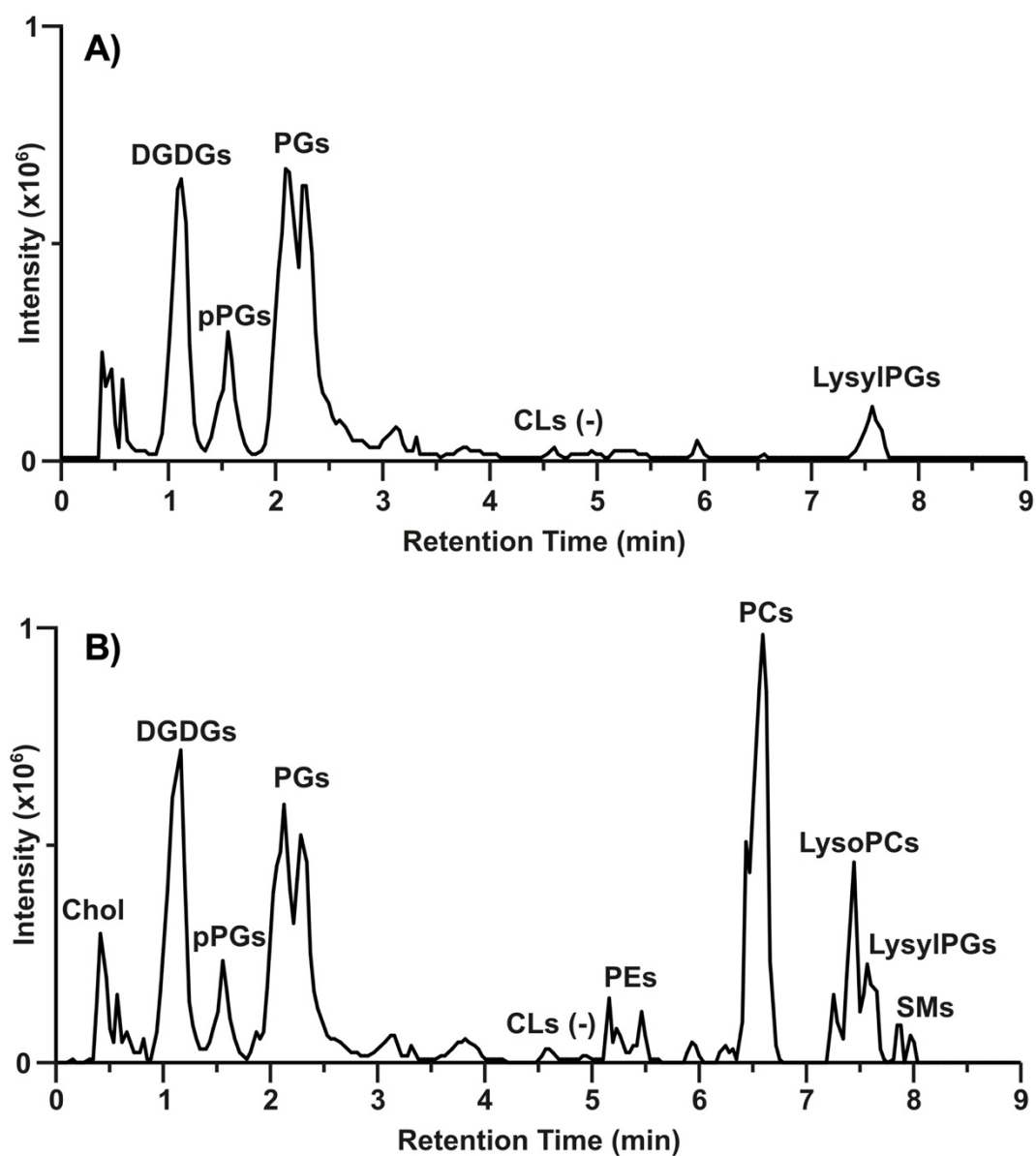
637 **Fig. 1.** The major source of lipids in human serum is from LDL particles that contain cholesteryl
638 esters, unesterified cholesterol, triglycerides and phospholipids. *S. aureus* secretes at least two
639 lipases, *Sal1* and *Geh*, that release free fatty acids from lipids found in serum and LDL. These
640 free fatty acids can be incorporated into *S. aureus* membrane lipids through the FakA/B and
641 PlsXY systems, with or without further elongation in type II fatty acid synthesis.

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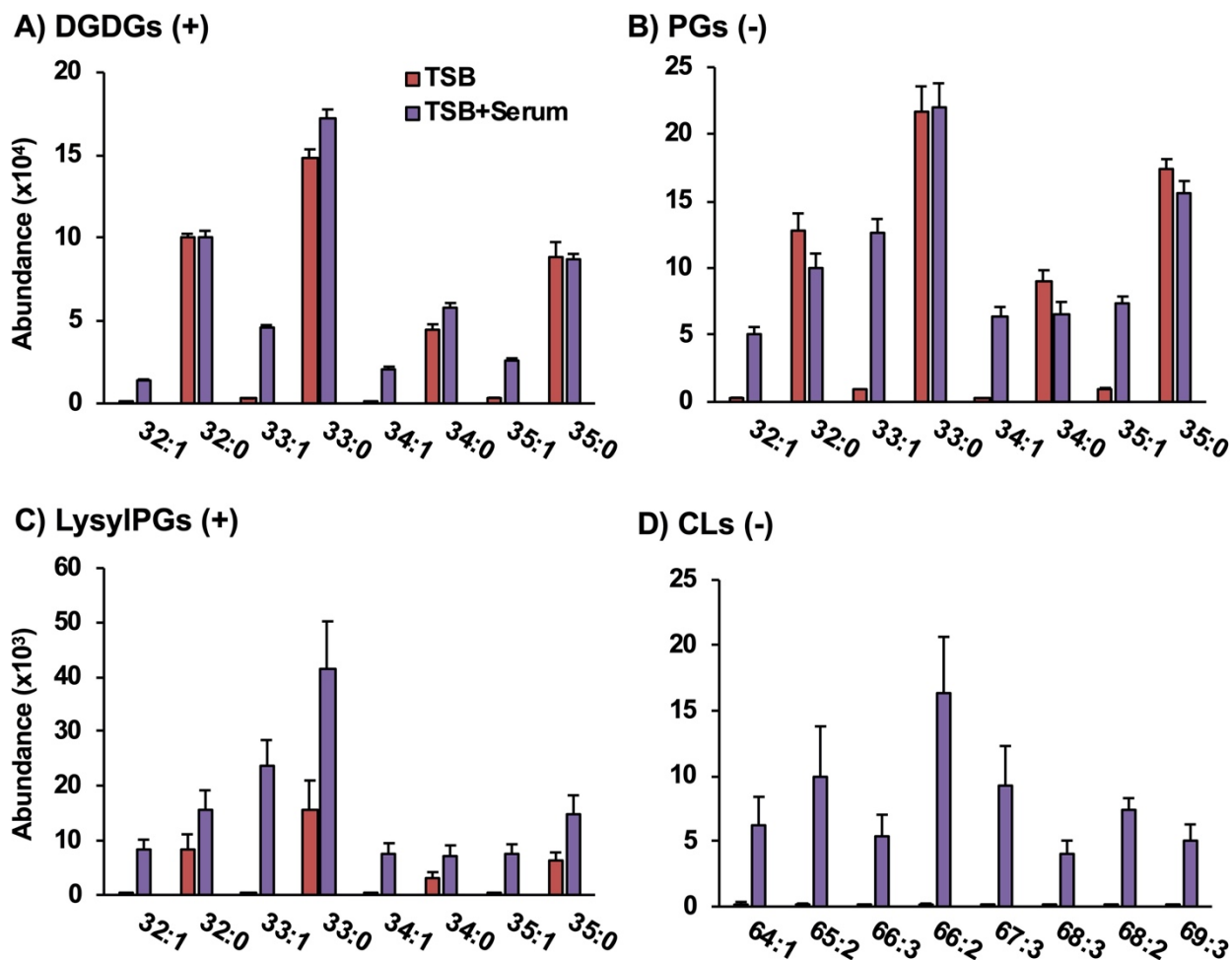
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647 **Fig. 2.** Lipid profiles of JE2 grown in A) TBS and B) TBS containing 20% human serum. Data
648 shown are ion mobility-extracted ion chromatograms from the positive ESI analysis.

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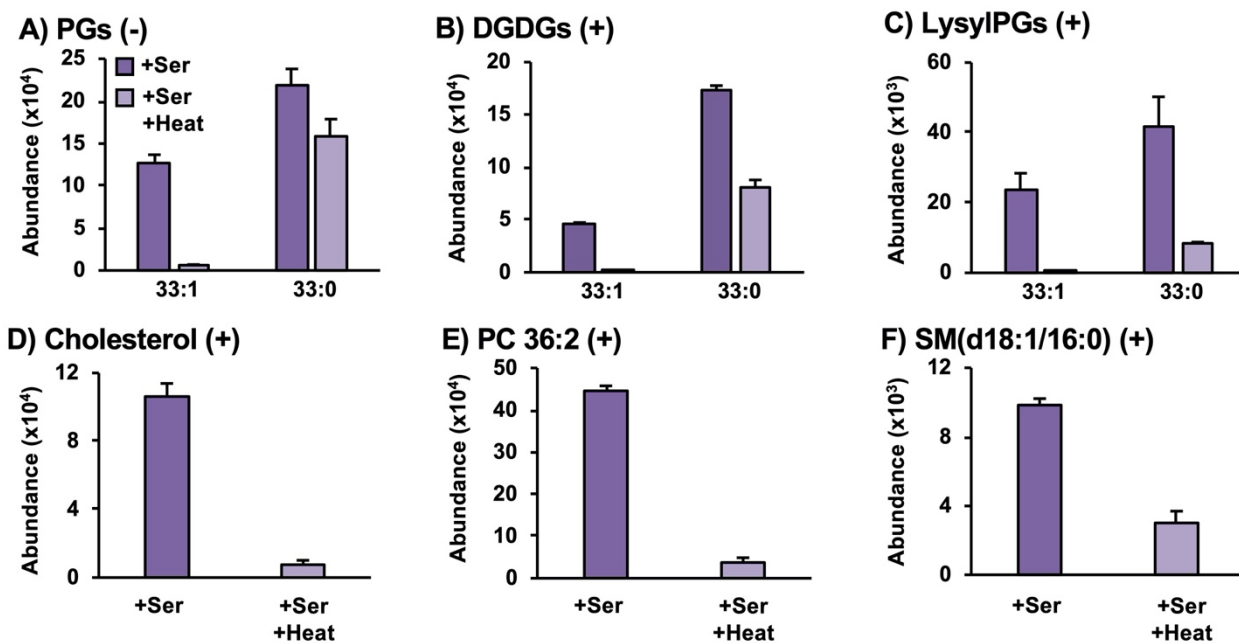
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653 **Fig. 3.** The presence of odd-carbon lipids with unsaturation are evidence that serum-derived
654 unsaturated fatty acids are incorporated into the A) DGDG, B) PG, C) LysylPG, and D) CL lipid
655 classes of *S. aureus*. Parentheses indicate that data is from positive (+) and negative (-) mode
656 ESI. N = 4 per group. Statistics and detailed fatty acid composition from MS/MS experiments
657 can be found in Supplemental Material Excel S1.

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662 **Fig. 4.** Heat-killed *S. aureus* lacks SCUFAs in A) PGs, B) DGDGs, and C) LysylPGs and retains
663 less amounts of serum lipids such as D) cholesterol, E) PC 36:2 and F) SM(d18:1/16:0). N = 3
664 per group. Statistics and detailed fatty acid composition from MS/MS experiments can be found
665 in Supplemental Material Excel S1.

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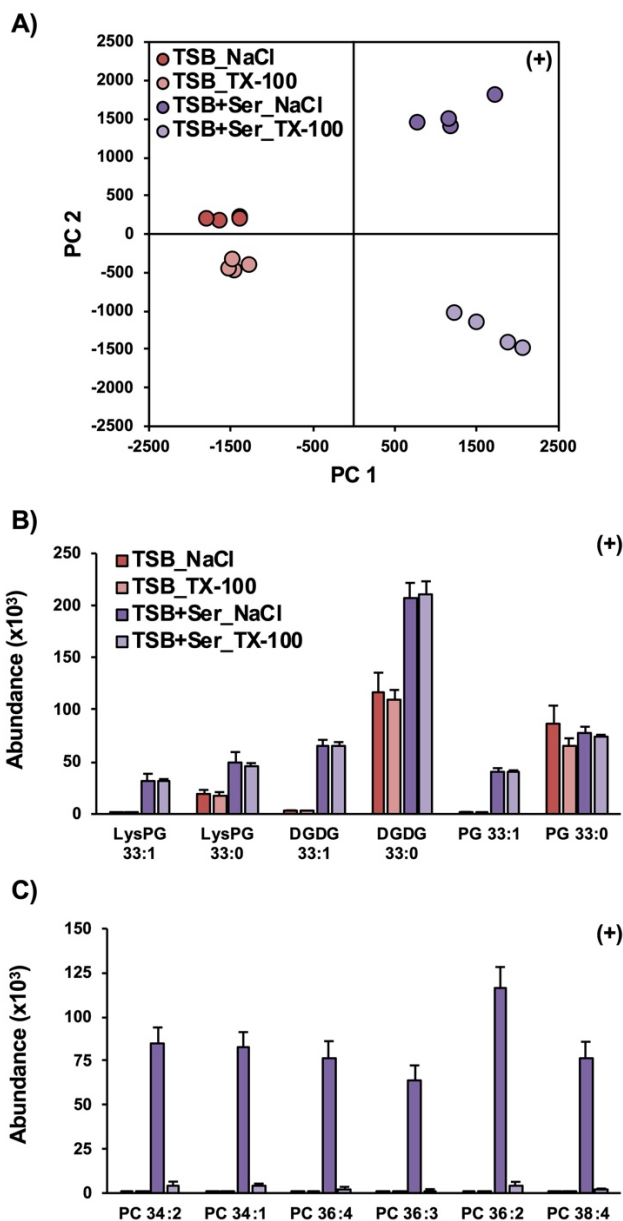
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675 **Fig. 5.** Principal component analysis of lipidomics data (A) reveals that washing pelleted *S.*

676 *aureus* with TX-100 prior to lipid extraction alters the lipid profile of *S. aureus* grown in TSB

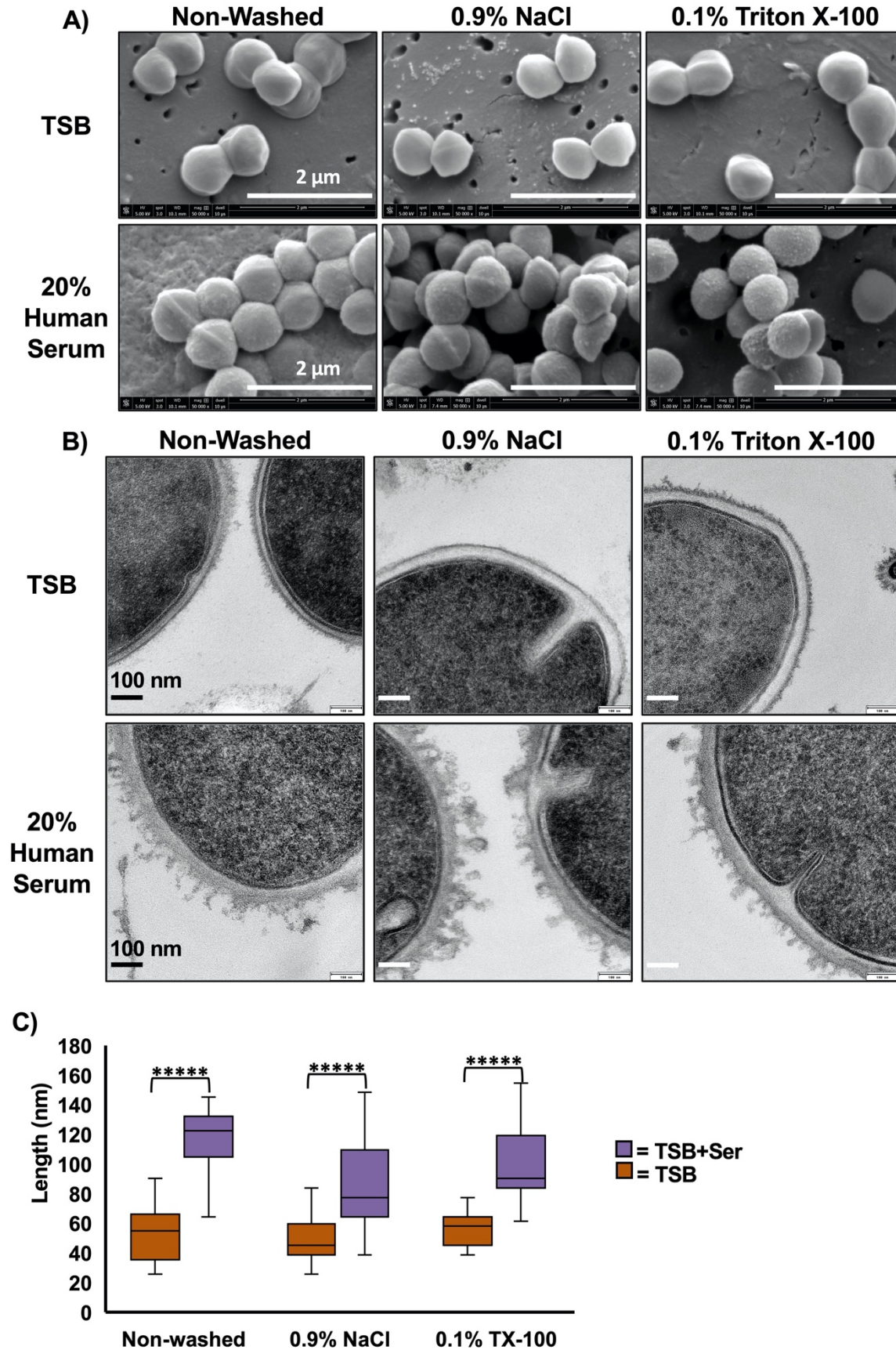
677 supplemented with serum but not *S. aureus* grown in TSB only. B) Washing with TX-100 has no

678 effect on the abundance of endogenous lipids or the incorporation of SCUFAs in serum-treated

679 *S. aureus*. C) Pellets from serum-grown *S. aureus* treated with TX-100 prior to lipid extraction

680 had significantly lower levels of serum lipids, such as PCs. N= 4 per group. Statistics and

681 detailed fatty acid composition from MS/MS experiments can be found in Excel S1.



683 **Fig. 6.** Electron microscopic analysis of *S. aureus* grown with and without human serum. (A)
684 SEM images reveal *S. aureus* grown in the presence of human serum leads to a textured cell
685 surface compared to the smooth cell surface of TSB-grown cells. (B) TEM images reveal that
686 cells grown in TSB+Serum display protrusions with irregular shapes, which can be partially
687 removed with Triton X-100 washing. (C) Quantitation of cell wall thickness reveals thicker cell
688 walls in cells grown in TSB+Serum than those grown in TSB only, but cell wall thickness does
689 not differ between washing with 0.9% NaCl or Triton X-100. $N \geq 21$ per group. *****, $p < 10^{-6}$.

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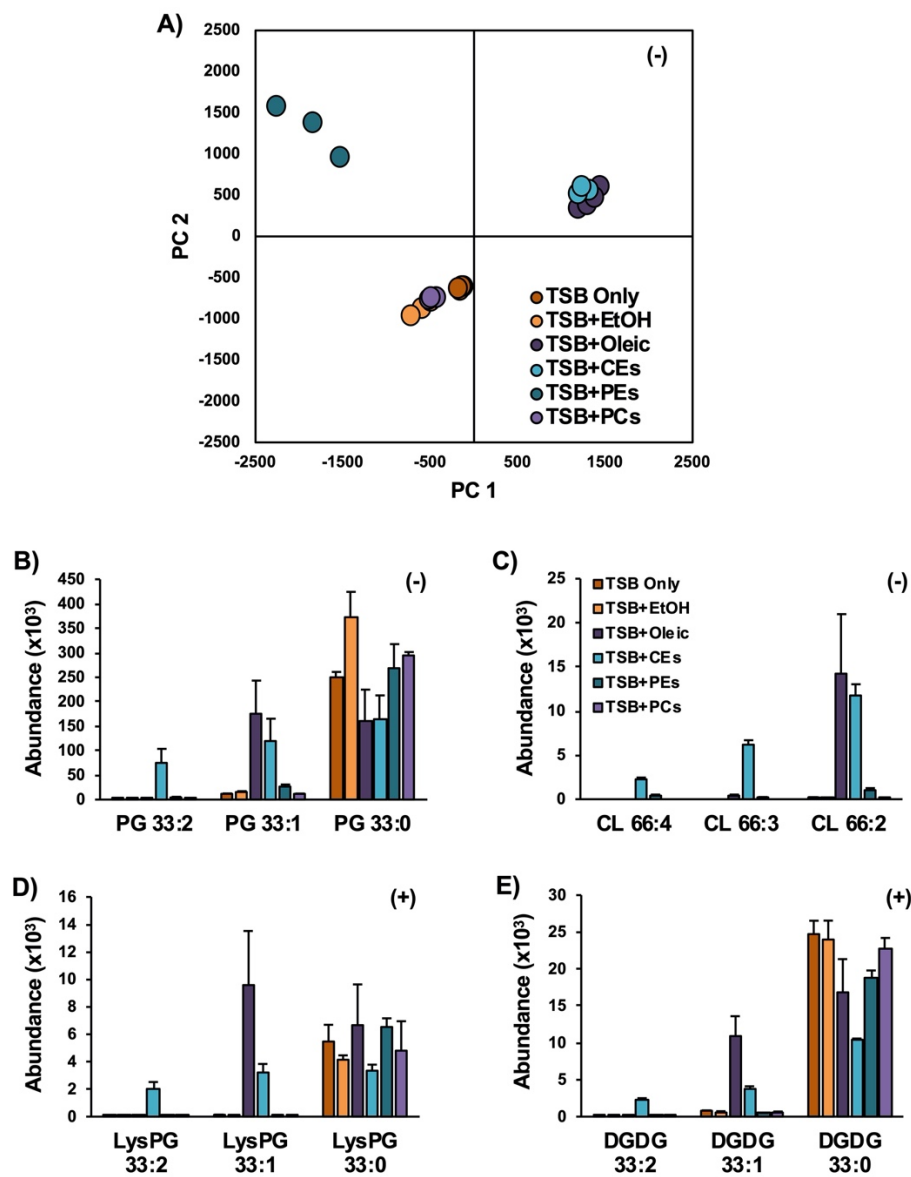
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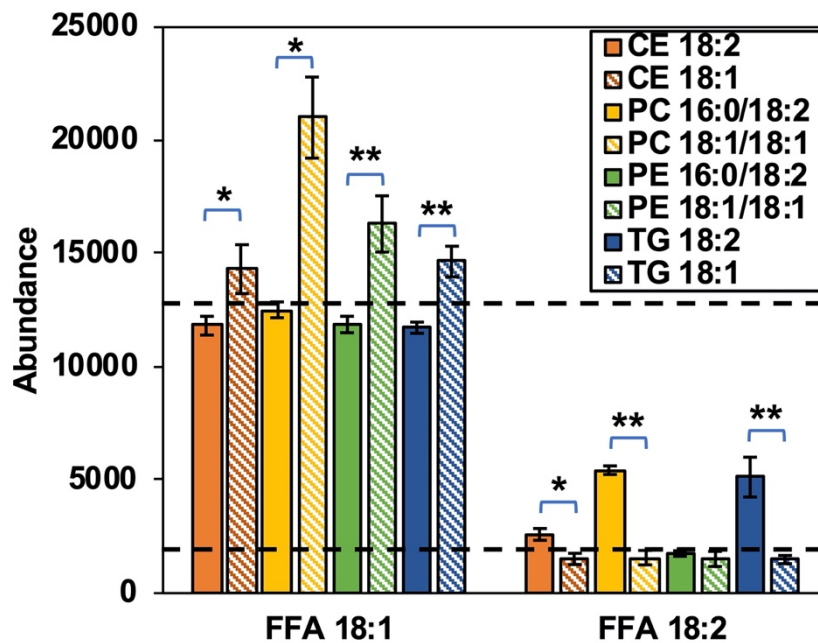
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703 **Fig. 7.** Incubations of *S. aureus* in TSB supplemented with lipid standards. A) PCA of the
 704 lipidomics data indicates that oleic acid and CEs, collectively, have similar effects on the lipid
 705 profiles of *S. aureus* relative to *S. aureus* grown in neat TSB or TSB with ethanol. The oleate and
 706 linoleate fatty acids from the oleic and CE-treated *S. aureus* are readily incorporated into B) PGs,
 707 C) CLs, D) LysylPGs, and E) DGDGs, whereas little-to-no incorporation was observed in the PE
 708 and PC-treated *S. aureus*. N = 3 per group. Statistics and detailed fatty acid composition from
 709 MS/MS experiments can be found in Excel S1.



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711 **Fig. 8.** The relative abundances of free oleic (FFA 18:1) and free linoleic (FFA 18:2) resulting
712 from the incubation of purified Geh with cholesteryl esters, phospholipids, and triglycerides
713 containing oleic and linoleic acids. N = 3 per group. Statistics were carried out using Student's *t*-
714 test. *, $p \leq 0.05$; **, $p \leq 0.01$.

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723 **LEGENDS for Supplemental Materials**

724 **Fig. S1.** Lipid profiles of clean A) TSB and B) TSB supplemented with 20% human serum. Data
725 shown are IM-XICs from positive ionization mode.

726 **Fig. S2.** Lipid profiles of isolated cytoplasmic membranes from *S. aureus* grown in A) TSB
727 supplemented with 20% human serum and B) TSB only. Data shown are IM-XICs from negative
728 ionization mode.

729 **Fig. S3.** Incubations of *S. aureus* in TSB supplemented with tri-oleate and tri-linoleate
730 triglycerides (TG). Oleic (18:1) and linoleic acid (18:2) were incorporated into A) PGs, B)
731 lysylPGs, C) DGDGs, and D) cardiolipins (CLs).

732 **Fig. S4.** Oleic and linoleic acids derived from cholesteryl esters and triglycerides can be
733 elongated by *S. aureus*. *S. aureus* lipid species PGs (A, B), DGDGs (C, D) and LysylPGs (E, F)
734 with 35 total carbons and one or two double bonds were observed when *S. aureus* was grown in
735 lipid supplemented TSB. Targeted MS/MS experiments revealed these lipids contained
736 pentadecanoic acid and eicosenoic (20:1) or eicosadienoic (20:2) acids.

737 **Excel S1.** Retention time, *m/z*, collision cross section, abundance, fold-changes, statistics, and
738 fatty acid composition obtained from MS/MS fragmentation of lipids observed in experiments
739 related to Figs. 3, 4, 5, 7, and S3.