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2 **Growth-Environment Dependent Modulation of *Staphylococcus aureus* Branched-Chain to**
3 **Straight-Chain Fatty Acid Ratio and Incorporation of Unsaturated Fatty Acids**

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Abstract

The fatty acid composition of membrane glycerolipids is a major determinant of *Staphylococcus aureus* membrane biophysical properties that impacts key factors in cell physiology including susceptibility to membrane active antimicrobials, pathogenesis, and response to environmental stress. The fatty acids of *S. aureus* are considered to be a mixture of branched-chain fatty acids (BCFAs), which increase membrane fluidity, and straight-chain fatty acids (SCFAs) that decrease it. The balance of BCFAs and SCFAs in strains USA300 and SH1000 was affected considerably by differences in the conventional laboratory medium in which the strains were grown with media such as Mueller-Hinton broth and Luria broth resulting in high BCFAs and low SCFAs, whereas growth in Tryptic Soy Broth and Brain-Heart Infusion broth led to reduction in BCFAs and an increase in SCFAs. Straight-chain unsaturated fatty acids (SCUFAs) were not detected. However, when the organism was grown *ex vivo* in serum, the fatty acid composition was radically different with SCUFAs, which increase membrane fluidity, making up a substantial proportion of the total (<25%) with SCFAs (>37%) and BCFAs (>36%) making up the rest. Staphyloxanthin, an additional major membrane lipid component unique to *S. aureus*, tended to be greater in content in cells with high BCFAs or SCUFAs. Cells with high staphyloxanthin content had a lower membrane fluidity that was attributed to increased production of staphyloxanthin. *S. aureus* saves energy and carbon by utilizing host fatty acids for part of its total fatty acids when growing in serum. The fatty acid composition of *in vitro* grown *S. aureus* is likely to be a poor reflection of the fatty acid composition and biophysical properties of the membrane when the organism is growing in an infection in view of the role of SCUFAs in staphylococcal membrane composition and virulence.

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47 **Introduction**

48 *Staphylococcus aureus* is a worldwide significant pathogen in the hospital and the
49 community. Antibiotic resistance has developed in waves [1] such that we now have methicillin-
50 resistant *S. aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA) and vancomycin-
51 intermediate *S. aureus* (VISA) [2, 3]. Given the threat of multiply antibiotic-resistant *S. aureus*,
52 various aspects of staphylococcal biology including pathogenicity, antibiotic resistance, and
53 physiology are currently being investigated intensively, in part to support the search for novel
54 anti-staphylococcal agents.

55 The bacterial cytoplasmic membrane forms an essential barrier to the cell and is
56 composed of a glycerolipid bilayer with associated protein molecules, and is a critical
57 determinant of cell physiology. The biophysical properties of the membrane are to a large extent
58 determined by the fatty acyl residues of membrane phospholipids and glycolipids [4, 5]. The
59 lipid acyl chains influence membrane viscosity/fluidity, and impact the ability of bacteria to
60 adapt to changing environments, the passive permeability of hydrophobic molecules, active
61 transport, and the function of membrane-associated proteins [4-6]. Additionally, membrane fatty
62 acid composition has a major influence on bacterial pathogenesis, critical virulence factor
63 expression [7], and broader aspects of bacterial physiology [8].

64 *S. aureus* membrane fatty acids are generally considered to be a mixture of branched-
65 chain fatty acids (BCFAs) and straight-chain fatty acids (SCFAs) [9-11], and for a
66 comprehensive review of earlier literature see [12]. Typically, *S. aureus* contains about 65%
67 BCFAs and 35% SCFAs. In *S. aureus* the major BCFAs are odd-numbered iso and anteiso fatty
68 acids with one methyl group at the penultimate and antepenultimate positions of the fatty acid

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69 chains, respectively (Fig. 1). BCFAs have lower melting points than equivalent SCFAs and cause
70 model phospholipids to have lower phase transition temperatures [13], and disrupt the close
71 packing of fatty acyl chains [14, 15]. The membrane lipid composition of *S. aureus* is further
72 complicated by the presence of staphyloxanthin, a triterpenoid carotenoid with a C30 chain with
73 the chemical name of α-D-glucopyranosyl-1-*O*-(4,4'-diaponeurosporen-4-oate)-6-*O* (12-
74 methyltetradecanoate) [16] (Fig. 1). Staphyloxanthin, as a polar carotenoid, is expected to have a
75 significant influence on membrane properties with the expectation that it rigidifies the membrane
76 [17], and Bramkamp and Lopez [18] have suggested that staphyloxanthin is a critical component
77 of lipid rafts in *S. aureus* incorporating the organizing protein flotillin. Staphyloxanthin has
78 drawn considerable attention in recent years as a possible virulence factor by detoxifying
79 reactive oxygen species produced by phagocytic cells [19, 20], and as a potential target for
80 antistaphylococcal chemotherapy [21].

81 **Fig 1. Structures of major fatty acids and staphyloxanthin of the *S. aureus* cell membrane.**

82 In our laboratory, we are interested in the mechanisms of action of and resistance to novel
83 and existing anti-staphylococcal antimicrobials [22-24]. Because much antibiotic work employs
84 Mueller-Hinton (MH) medium, [25] we had occasion to determine the fatty acid composition of
85 a *S. aureus* strain grown in this medium. The analysis was carried out using the MIDI microbial
86 identification system (Sherlock 4.5 microbial identification system; Microbial ID, Newark, DE,
87 USA), [26]. We were taken aback when the fatty acid profile came back showing a very high
88 percentage (84.1%) of BCFAs, and the organism was not even identified by MIDI as a *S. aureus*
89 strain. In a previous study where we grew *S. aureus* in BHI broth we found that 63.5% of the
90 fatty acids were BCFAs, and 32.4% were SCFAs [10]. This is a much more typically observed

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91 balance between BCFAs and SCFAs in previous studies of the fatty acid composition of *S.*
92 *aureus* [9- 12].

93 A range of different media are used for cultivating *S. aureus* in studies from different
94 laboratories [27]. These are mostly complex media such as TSB, Brain Heart Infusion (BHI)
95 broth, MH broth, Luria-Bertani (LB) broth, and, much more rarely, defined media [11]. Ray et
96 al. [27] and Oogai et al [28] have pointed out that different media have major, but largely
97 unstudied and ignored, effects on the expression of selected target virulence and regulatory
98 genes. Although seemingly prosaic at first glance, issues of choice of strain and medium are
99 nevertheless critical considerations in staphylococcal research [29]. These authors, in their recent
100 protocol publication on the growth and laboratory maintenance of *S. aureus,* have suggested that
101 TSB and BHI media are the media of choice for staphylococcal research. In light of recent
102 literature in various microorganisms, it is becoming glaringly evident that environment has a
103 tremendous effect on the physiology of different pathogens; hence cells from *in vivo* are
104 drastically different from *in vitro* cultured ones. Such distinctions are likely important for
105 studying antimicrobial susceptibilities, drug resistances and pathogenesis.

106 We decided to carry out a systematic study of the impact of growth medium on the fatty
107 acid and carotenoid composition of *S. aureus* given the large potential impact of these
108 parameters on membrane biophysical properties and its further ramifications. The BCFA: SCFA
109 ratio was significantly impacted by the laboratory medium used, with media such as MH broth
110 encouraging high proportions of BCFAs. However, strikingly, when cells were grown in serum,
111 an *ex vivo* environment, the fatty acid composition changed radically, with straight-chain
112 unsaturated fatty acids (SCUFAs), which were not detected in cells grown in laboratory media,
113 making up a major proportion of the total fatty acids. Biosynthesized bacterial fatty acids are

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114 produced by fatty acid synthase II (FASII) [30]. However, fatty acid biosynthesis is expensive in
115 terms of energy and carbon, and many bacteria are able to incorporate extracellular fatty acids
116 into their membrane lipids to varying degrees [30]. This extreme plasticity of *S. aureus*
117 membrane lipid composition is undoubtedly important in determining membrane physical
118 structure and thereby the functional properties of the membrane. The alterations in the fatty acid
119 composition as a result of interactions of the pathogen with the host environment may be a
120 crucial factor in determining its fate in the host. Typically used laboratory media do not result in
121 a *S. aureus* membrane fatty acid composition that closely resembles the likely one of the
122 organism growing *in vivo* in a host.

123 **Materials and Methods**

124 **Bacterial strains and growth conditions**

125 The primary *S. aureus* strains studied were USA300 and SH1000. USA300 is a
126 community-acquired MRSA strain, and is a leading cause of aggressive cutaneous and systemic
127 infections in the USA [1, 31, 32]. This clinical MRSA also has a well-constructed diverse
128 transposon mutant library [33]. *S. aureus* strain SH1000, is an 8325-line strain that has been used
129 extensively in genetic and pathogenesis studies [34]. The laboratory media used were MH broth,
130 TSB and Luria Broth (LB) from Difco. For growth and fatty acid composition studies cultures of
131 *S. aureus* strains were grown at 37° C in 250 ml Erlenmeyer flasks containing each of the
132 different laboratory media with a flask–to-medium volume ratio of 6:1. Growth was monitored
133 by measuring the OD_{600} at intervals using a Beckman DU-65 spectrophotometer.

134 **Growth of *S. aureus* in serum**

135 Sterile fetal bovine serum of research grade was purchased from Atlanta Biologics, USA.
136 The aliquoted serum was incubated in a water bath at 56° C for 30 min to heat inactivate the

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137 complement system. *S. aureus* cells were grown for 24 hours in 50 ml of serum in a 250 ml flask
138 at 37°C with shaking at 200 rpm.
139

140 **Analysis of the membrane fatty acid composition of *S. aureus* grown in different media**

141 The cells grown in the different laboratory media conventionally used were harvested in
142 mid-exponential phase (OD_{600} 0.6), and after 24 hrs of growth in serum, by centrifugation at
143 3000 x g at 4° C for 15 minutes and the pellets were washed three times in cold distilled water.
144 The samples were then sent for fatty acid methyl ester (FAME) analysis whereby the fatty acids
145 in the bacterial cells (30-40 mg wet weight) were saponified, methylated, and extracted. The
146 resulting methyl ester mixtures were then separated using an Agilent 5890 dual-tower gas
147 chromatograph and the fatty acyl chains were analyzed and identified by the Midi microbial
148 identification system (Sherlock 4.5 microbial identification system) at Microbial ID, Inc.
149 (Newark, DE) [26]. The percentages of the different fatty acids reported in the tables are the
150 means of the values from three separate batches of cells under each condition. The standard
151 deviations are ±1.5 or less. Some minor fatty acids such as odd-numbered SCFAs are not
152 reported.

153 **Extraction and estimation of carotenoids**

154 For quantification of the carotenoid pigment in the *S. aureus* cells grown in different
155 media, the warm methanol extraction protocol was followed as described by Davis et al.
156 [35]. Cultures of *S. aureus* were harvested at mid-exponential phase and were washed with
157 cold water. The pellets were then extracted with warm (55ºC) methanol for 5 min. The
158 OD_{465} of the supernatant after centrifugation was measured using a Beckman DU 70
159 spectrophotometer. Determinations were carried out in triplicate.

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160 **Measurement of the fluidity of the *S. aureus* membrane**

161 The fluidity of the cell membrane of the *S. aureus* strains grown in different media
162 were determined by anisotropic measurements using the fluorophore diphenylhexatriene
163 (DPH) following the protocol described previously [36]. Mid exponential phase cells grown in
164 respective media and serum were harvested and washed with cold sterile PBS (pH 7.5).The
165 pellets were then resuspended in PBS containing 2 µM DPH (Sigma, MO) to an OD_{600} of
166 about 0.3 and incubated at room temperature in the dark for 30 min. Fluorescence polarization
167 emitted by the fluorophore was measured using a PTI Model QM-4 Scanning
168 Spectrofluorometer at an excitation wavelength of 360 nm and emission wavelength of 426
169 nm. The experiments were performed with three separate fresh batches of cells and the Student
170 T-test of the mean polarization values was used to determine statistically significant
171 differences.

172 **Results**

173 **MH broth and LB increase the content of BCFAs and TSB and BHI broth increase the**
174 **content of SCFAs**

175 The fatty acid compositions of strain USA300 grown in different laboratory media are
176 shown in Table 1. Growth in MH broth and LB broth resulted in a high content of BCFAs-
177 80.9% and 77.2% respectively, whereas SCFAs were 19.1% and 22.8% respectively. However,
178 in TSB and BHI broth the BCFAs contents were lower at 51.7% and 51.5% respectively, and
179 SCFAs were increased to 48.3 and 48.5% respectively. In MH broth anteiso odd-numbered
180 fatty acids were the major fatty acids in the profile (59.8%), followed by even numbered
181 SCFAs (16.6%), iso odd-numbered fatty acids (15.8%), with iso even-numbered fatty acids
182 making up only a minor portion (4.7%). Anteiso C15:0 was the predominant fatty acid in the

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membrane lipids (39%). This particular fatty acid has a significant impact on fluidizing membranes [37, 38]. The anteiso fatty acids were significantly reduced in TSB-grown cells (29.3%). The major SCFAs in TSB-grown cells were C18:0 and C20:0 at 19.1% and 18.6% respectively. Overall, the fatty acid compositions were in line with many previous studies of *S. aureus* fatty acid composition [9-12], but we are unaware of previous studies that have identified this impact of medium on the proportions of BCFAs and SCFAs in the membrane.

Table 1. The membrane fatty acid profile of *S. aureus* USA300

% (wt/wt) of total fatty acids

Growth Medium	***Anteiso odd***				***Iso odd***				***Iso even***				***Straight Even***					***BCFA***	***SCFA***
	C15:0	**C17:0**	**C19:0**	**SUM**	**C15:0**	**C17:0**	**C19:0**	**SUM**	**C14:0**	**C16:0**	**C18:0**	**SUM**	**C14:0**	**C16:0**	**C18:0**	**C20:0**	**SUM**		
BHI	28.5	2.6	ND	31.1	12.2	1.8	ND	14	2.9	1.4	ND	4.3	3.3	7.9	21.5	13.4	46.1	51.5	48.5
TSB	26.9	2.4	ND	29.3	12.8	2	ND	14.8	3.7	1.8	ND	5.5	2.3	6.4	19.1	18.6	46.4	51.7	48.3
MHB	39	15	5.8	59.8	7.7	4.9	3.2	15.8	1	2.1	1.6	4.7	1.1	1.8	6.5	7.2	16.6	80.9	19.1
LB	36.5	11	2.4	49.9	13.3	6.6	2.6	22.5	1.4	2.2	1	4.6	ND	3	10	6.9	19.9	77.2	22.8

ND- Not detected

The results of a similar series of experiments with strain SH1000 are shown in Table 2. Overall, the proportion of BCFAs of this strain was higher than strain USA300. In strain SH1000 the BCFAs were higher than USA300 in all media- BHI 66.6%, TSB 68.5%, with particularly high contents in MH broth 90.2% and LB 89%. The proportion of SCFAs was correspondingly smaller in all cases compared to strain USA300. Anteiso fatty acids were the major class of fatty acids in all media, amongst which anteiso C15:0 was present in the highest amount in all cases. However, the same phenomenon was noted where MH broth and

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LB encouraged a high proportion of BCFAs, low SCFAs, and TSB and BHI had the opposite effects on fatty acid composition. Two additional media were studied with this strain. Both Tryptone broth [39] and defined medium [40] resulted in high BCFAs (80.4% and 85% respectively), and low SCFAs (19.7% and 15% respectively).

Table 2. The membrane fatty acid composition of *S. aureus* strain SH1000

% (wt/wt) of total fatty acid

Growth Medium	***Anteiso odd***				***Iso odd***				***Iso even***				***Straight Even***					***BCFA***	***SCFA***
	C15:0	**C17:0**	**C19:0**	**SUM**	**C15:0**	**C17:0**	**C19:0**	**SUM**	**C14:0**	**C16:0**	**C18:0**	**SUM**	**C14:0**	**C16:0**	**C18:0**	**C20:0**	**SUM**		
BHI	33.6	6.4	1	41	15.7	4.3	ND	20	2.6	3.1	ND	5.7	2.7	8.1	15.2	6.4	41	66.6	33.4
TSB	31.6	6	ND	37.6	18.7	5.2	1.2	25.1	2.2	2.7	ND	4.9	2	7.8	15.4	6.3	31.5	68.5	31.5
MHB	43.7	20.7	5.7	70.1	6.8	5.1	2.3	14.2	1.2	3.1	1.6	5.9	ND	1.4	4.2	2.3	7.9	90.2	9.8
LB	42	16.8	3.1	61.9	12.2	7.2	2.3	21.7	1.3	2.9	1.2	5.4	ND	2.4	6	2.6	11	89	11

ND- Not detected

The phenomenon of higher BCFAs in MH broth-grown cells and higher SCFAs in TSB grown cells was observed in 8 out of 9 strains tested which included MRSA, VISA and daptomycin decreased susceptibility strains (data not shown). This indicates that the phenomenon noted in strains USA300 and SH1000 also extends to other *S. aureus* strains.

The fatty acid composition of *S. aureus* grown *ex vivo* in serum is radically different to those of the organism grown in laboratory media

It was of interest to try and get an idea of the fatty acid composition of *S. aureus* grown in vivo. Strain USA300 and SH1000 were grown in serum, which resulted in major changes in

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216 the fatty acid profile (Table 3). Total BCFAs were reduced to 37.5% in USA300 and 36.3 in
217 SH1000; SCFAs were at 37.8% in USA300 and 32.1% in SH1000, but 25% of the fatty acid
218 profile in the case of USA300 and 30.6% in SH1000 was accounted for by SCUFAs.
219 Strikingly, this type of fatty acid was not present in the profile of the organism when grown in
220 laboratory media. Interestingly, BCFAs and SCUFAs have similar effects in increasing fluidity
221 of the membrane [4].

222 **Table 3. The membrane fatty acid compositions of *S. aureus* USA300 and SH1000 grown *ex*
223 *vivo* in serum**

224 % (wt/wt) of total fatty acid

Membrane fatty acids		Strain USA300	Strain SH1000
Anteiso odd	C15:0	21	18.2
	C17:0	4	3
	Sum	25	21.2
Iso odd	C15:0	7.1	6
	C17:0	2	1.8
	Sum	9.1	7.8
Iso even	C14:0	1.7	2.4
	C16:0	1.7	1.6
	Sum	3.4	4
Straight even	C14:0	1.7	1.1
	C16:0	16.4	13.2
	C18:0	13.3	12.1
	C20:0	4.8	4.4
	Sum	36.2	30.8
Straight odd		1.6	1.3
Unsaturated fatty acids	C16:1Δ9	1.7	1.5
	C18:1Δ9	16	15.4
	C18:1Δ7	4.2	6.4
	C20:1Δ9	2.1	5
	C20:4Δ6,9,12,15	1	2.3
	Sum	25	30.6
BCFAs		37.5	36.3
SCFAs		37.8	32.1
SCUFAs		25	30.6

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226 **Carotenoid content of cells grown in different media**

227 Staphyloxanthin is another significant membrane component that might impact the
228 biophysical properties of the membrane. Accordingly, the carotenoid content of cells grown in
229 different media were determined and the results are shown in Fig. 2. Strain SH1000 cells
230 grown in MH broth had a much higher carotenoid content than cells grown in the other media.
231 The pellets of cells grown in this particular media were noticeably yellow. It is possible that
232 the carotenoid content rises to counterbalance the potentially high fluidity of MH broth-grown
233 cells with their high content of BCFAs, specifically mainly anteiso fatty acids. LB (high
234 BCFAs) and serum (high SCUFAs) - grown cells had higher carotenoid contents than TSB or
235 BHI broth –grown cells. In strain USA300 MHB- and serum-grown cells also had higher
236 carotenoid contents than did cells grown in BHI, TSB or LB. In general this strain was less
237 pigmented than strain SH1000.

238 **Fig 2. Influence of growth environment on the carotenoid content of *S. aureus.***

239 The strains, USA300 (black columns) and SH1000 (blue columns), were grown in different
240 growth media and the carotenoid was estimated after extraction by warm methanol.

241 **Membrane fluidity of *S. aureus* cells with different fatty acid compositions**

242 The membrane fluidity of cells of strain SH1000 grown in BHI broth, LB and TSB
243 were very similar (0.185-0.19) as shown in Fig. 3. The membranes of MH-broth and serum-
244 grown cells, 0.25 and 0.248 were significantly less fluid than cells grown in the other media.
245 Possibly the higher carotenoid contents of cells grown in MH broth and serum rigidifies the
246 membrane. Strain USA300 also showed a similar pattern of membrane fluidity in the different
247 growth media Fig. 3. The membrane fluidity of both strains was highest in cells grown in LB,
248 consistent with the high content of BCFAs. Furthermore, there was no accompanying increase
249 in staphyloxanthin content with its possible membrane rigidifying effect in contrast to MHB or

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250 serum-grown cells.

251 **Fig 3. Influence of growth environment on the membrane fluidity of *S. aureus* cells.**

252 The strains, USA300 (black columns) and SH1000 (blue columns), were grown in the different
253 media to mid exponential phase and membrane anisotropy was measured by fluorescence
254 polarization.

255 Discussion

256 From numerous studies over the past several decades of *S. aureus* grown *in vitro* in
257 various laboratory media it is considered that the membrane fatty acid composition of the
258 organism is a mixture of BCFA and SCFAs [9-12], and BCFAs have generally been found to
259 be predominant. Through study of a range of different conventional growth media, certain
260 media were found to encourage a higher proportion of BCFAs than others, whereas in some
261 media the proportion of SCFAs was increased. This may have significant physiological
262 ramifications given the opposing effects of BCFAs and SCFAs on membrane fluidity with
263 BCFAs fluidizing and SCFAs rigidifying the membrane [4]. However, there was a radical
264 change in the entire fatty acid composition when the organism was grown *ex vivo* in serum
265 with SCUFAs appearing in the profile in significant amounts accompanied with a decrease in
266 BCFA content.

267 It is useful to discuss our fatty acid compositional data in the context of what is known
268 about phospholipid biosynthesis and the positional distribution of fatty acids on the 1 and 2
269 carbon atoms of the glycerol residue (Fig. 4). Phosphatidic acid is a key intermediate in the
270 biosynthesis of the *S. aureus* phospholipids, which are phosphatidyl glycerol, cardiolipin and
271 lysyl-phosphatidyl glycerol [5]. Our current knowledge of the pathway of phospholipid
272 biosynthesis and the incorporation of exogenous and endogenous fatty acids is summarized in
273 Fig. 4 [41]. Phosphatidic acid (PtdOH), the universal precursor of phospholipids, is
274 synthesized by the stepwise acylation of *sn*-glycerol-3-phosphate first by PlsY that transfers a

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275 fatty acid to the 1-position from acyl phosphate. The 2-position is then acylated by PlsC
276 utilizing acyl-ACP. Acyl-ACP is produced by the FASII pathway and PlsX catalyses the
277 interconversion of acyl-ACP and acyl phosphate. Exogenous fatty acids readily penetrate the
278 membrane and are activated by a fatty acid kinase to produce acyl phosphate that can be
279 utilized by PlsY, or they can be converted to acyl-ACP for incorporation into the 2-position by
280 PlsC. Exogenous fatty acids can also be elongated by the FASII pathway. When *S. aureus* is
281 grown in medium that results in a high proportion of BCFAs the major phospholipid,
282 phosphatidyl glycerol (PtdGro), has, almost exclusively, anteiso C17:0 at position 1 and
283 anteiso C15:0 at position 2 [42]. Growth in the presence of oleic acid (C18:1Δ9) showed
284 anteiso C17:0 at position 1 was replaced by C18:1Δ9 and C20:1Δ11, whereas the anteiso
285 C15:0 at position 2 remained at about 50%. BCFAs are not present in serum and hence must be
286 biosynthesized from 2-methylbutyryl CoA, most likely produced from isoleucine.

287 **Fig 4. Pathway of phospholipid biosynthesis and the incorporation of exogenous and**
288 **endogenous fatty acids in *S. aureus.***

289 Phosphatidic acid (PtdOH), the universal precursor of phospholipids, is synthesized
290 by the stepwise acylation of *sn*-glycerol-3-phosphate first by PlsY that transfers a fatty acid to
291 the 1-position from acyl phosphate. The 2-position is then acylated by PlsC utilizing acyl-ACP.
292 Acyl-ACP is produced by the FASII pathway and PlsX catalyses the interconversion of acyl-
293 ACP and acyl phosphate. Exogenous fatty acids readily penetrate the membrane and are
294 activated by a fatty acid kinase (FakB1 for SCFAs and FakB2 for SCUFAs) to produce acyl
295 phosphate that can be utilized by PlsY, or that can be converted to acyl-ACP for incorporation
296 into the 2-position by PlsC. Exogenous fatty acids can also be elongated by the FASII pathway.
297 Figure modified from Parsons et al. [41].

298

299 **What determines the balance between BCFAs and SCFAs in cells grown in laboratory**
300 **media?**

301 MH medium leads to high proportion of BCFAs in the staphylococcal cells whereas
302 growth in TSB leads to an increase in the proportion of SCFAs. MH broth (Difco) is composed

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303 of beef extract powder (2 g/l), acid digest of caseine (17.5 g/l), and soluble starch (1.5 g/l). Thus,
304 by far the major medium component is acid digest of caseine, and this is expected to be high in
305 free amino acids. TSB (Difco) is composed of pancreatic digest of caseine (17 g/l), enzymatic
306 digest of soybean meal (3 g/l), dextrose (2.5 g/l), sodium chloride (5 g/l) and dipotassium
307 phosphate (2.5 g/l). The major components then of TSB are a mixture of peptides formed by
308 enzymatic digestion of caseine and soybean meal. Payne and Gilvarg [43] fractionated Bacto
309 Neopeptone using gel filtration. They found that peptides with a molecular weight below 650
310 represented about 25% of the mixture, and free amino acids were about 1 % of the entire
311 preparation. We believe that the free amino acids from the acid digest of casein can have a
312 dominant effect on the fatty acid composition.

313 *Listeria monocytogenes* is a Gram-positive bacterium with a very high (90%)
314 proportion of BCFAs in its cell membrane. The fatty acid composition of the organism grown in
315 defined medium not containing any branched-chain amino acids was readily modified by
316 exogenous isoleucine or leucine, which resulted in the fatty acid profile being dominated by
317 anteiso odd and iso odd fatty acids respectively [26]. *L. monocytogenes* can also obtain amino
318 acids by metabolism of peptides that are taken up [44] as can *S. aureus* [45]. It may be that
319 transport of free branched-chain amino acids results in higher pool levels than when they are
320 biosynthesized or produced through metabolism of transported peptides, giving them a dominant
321 effect on fatty acid composition. In *S. aureus* supplementation of medium with 2-methylbutyrate,
322 a precursor of anteiso fatty acids, significantly increased the content of anteiso C15:0 and C17:0
323 [10]. Mutants of *S. aureus* in the transporters of leucine and valine lacked odd and even
324 numbered fatty acids derived from these amino acids [46].

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325 Growth in media such as TSB and BHI lead to a higher proportions of SCFAs than
326 media such as MH broth, although SCUFAs were not detected. The origin of SCFAs is not clear
327 as to whether they originate from the medium or are biosynthesized. Typically in bacteria SCFAs
328 are biosynthesized from acetyl CoA via the activities of FabH. However, acetyl CoA was a poor
329 substrate for *S. aureus* FabH [47], whereas the enzyme had high activity for butyryl CoA raising
330 the possibility that butyrate is the primer for biosynthesis of SCFAs in *S. aureus*. It is also
331 possible that SCFAs that may be present in TSB and BHI may be utilized directly for fatty acid
332 elongation to the SCFAs in the membrane typical of growth in these media.

333 **The underappreciated ability of S. aureus to incorporate host fatty acids from serum**

334 A striking finding in our paper is that *S. aureus* has the capacity to incorporate large
335 proportions of SCFAs and SCUFAs when grown *ex vivo* in serum. Earlier reports of *S. aureus*
336 fatty acid composition have not reported significant amounts of SCUFAs in *S. aureus* [9-12].
337 Indeed it appears that *S. aureus* lacks the genes necessary to biosynthesize unsaturated fatty acids
338 [41]. However an early report by Altenbern [48] showed that inhibition of growth by the fatty
339 acid biosynthesis inhibitor cerulenin could be relieved by SCFAs or SCUFAs, implying *S.*
340 *aureus* had the ability to incorporate preformed fatty acids. Fatty acid compositional studies of
341 the cells were not reported though. Serum is lipid rich [49-51] and a comprehensive analysis of
342 the human serum metabolome including lipids has recently been published [52]. BCFAs are
343 present, if at all, in only very small amounts in serum. Bacterial pathogens typically have the
344 ability to incorporate host-derived fatty acids thereby saving carbon and energy since fatty acids
345 account for 95% of the energy requirement of phospholipid biosynthesis [30].

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346 The FASII pathway has been considered to be a promising pathway for inhibition
347 with antimicrobial drugs. The viability of FASII as a target for drug development was challenged
348 by Brinster et al. [53] especially for bacteria such as streptococci where all the lipid fatty acids
349 could be replaced by SCFAs and SCUFAs from serum. However, Parsons et al. [42] showed that
350 exogenous fatty acids could only replace about 50% of the phospholipid fatty acids in *S. aureus*
351 and concluded that FASII remained a viable drug target in this organism.

352 The relationship between *S. aureus* and long-chain SCUFAs and SCFAs is a complex
353 one. On one hand these fatty acids in the skin and other tissues form part of the innate defense
354 system of the host due to their antimicrobial activities [54-56]. Very closely related structures
355 can either be inhibitory to growth at low concentrations, or can have little effect on growth at
356 relatively high concentrations [39, 57-59]. For example C16:1Δ6 and C16:1Δ9 are highly
357 inhibitory whereas C18:1Δ9 and C18 are not inhibitory and are actually incorporated into the
358 phospholipids by this pathogen [39].

359 The enzyme fatty acid kinase (Fak) responsible for incorporation of extracellular fatty
360 acids into *S. aureus* phospholipids [41], is also a critical regulator of virulence factor expression
361 [60], and biofilm formation [61]. Fak phosphorylates extracellular fatty acids for incorporation
362 into *S. aureus* membrane phospholipids [41]. FakA is a protein with an ATP-binding domain that
363 interacts with FakB1 and FakB2 proteins that bind SCFAs and SCUFAs preferentially
364 respectively. Fatty acid kinase activity producing FakB (acyl-PO_4) was proposed to be involved
365 in the control of virulence gene expression. Interestingly FakB2 shows a high degree of
366 specificity for C18:1Δ9, a fatty acid not produced by *S. aureus*, and may act as a sensor for the
367 host environment via the abundant mammalian fatty acid C18:1Δ9 [41], which is subsequently
368 incorporated into the membrane lipids.

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369 Besides occurring in membrane phospholipids and glycolipids fatty acids are present
370 in lipoproteins at their N terminus in the form of an N-acyl-S-diacyl-glycerol cysteine residue
371 and an additional acyl group amide linked to the cysteine amino group [62]. It is estimated that
372 there are 50-70 lipoproteins in S. *aureus*, many of them involved in nutrient acquisition.
373 Additionally, lipoproteins contribute important microbe-associated molecular patterns that bind
374 to Toll-like receptors and activate innate host defense mechanisms. Recently, Nguyen et al. [63]
375 have shown that when *S. aureus* is fed SCUFAs they are incorporated into lipoproteins and the
376 cells have an increased toll-like receptor 2- dependent immune stimulating activity, which
377 enhances recognition by the immune defense system.

378 **Changes in staphyloxanthin in cells grown under different conditions with different**
379 **membrane fatty acid compositions**

380 The carotenoid staphyloxanthin is a unique *S. aureus* membrane component that
381 affects membrane permeability, defense against reactive oxygen species, and is a potential drug
382 target. It appeared that cells grown in media encouraging a high proportion of BCFAs or in
383 serum resulting in high SCUFAs, both of which would be expected to increase membrane
384 fluidity, tended to have higher staphyloxanthin contents. However, despite having high amounts
385 of fluidizing BCFAs or SCUFAs, cells grown in MH broth or serum had cellular membranes that
386 were significantly less fluid. Thus it may be inferred that the pigment staphyloxanthin could
387 actually be preventing the membrane from becoming hyper fluid under the particular growth
388 conditions which yield an unusually high amounts of BCFAs or SCUFAs. These conditions may
389 thus result in staphylococcal cells which have a better chance at surviving against oxidative
390 stress and host defense peptides [20]. However this relationship is likely to be complex in that
391 LB-grown cells that had high BCFAs did not have high carotenoid levels, and the phenomenon

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392 deserving of more detailed investigation. Interestingly, in the biosynthesis of staphyloxanthin,
393 the end step involves an esterification of the glucose moiety with the carboxyl group of anteiso
394 C15:0 by the activity of the enzyme acyltransferase CrtO [16]. Thus the availability of specific
395 fatty acid precursors and the lipid metabolism may play a significant role in pigment production.

396 **Plasticity of *S. aureus* membrane lipid composition and its possible ramifications in**
397 **membrane biophysics and virulence**

398 Given the crucial role of the biophysics of the membrane in all aspects of cell
399 physiology, such radical changes in the membrane lipid profile can have significant but as yet
400 undocumented impacts on critical functional properties of cells such as virulence factor
401 production, susceptibilities to antimicrobials and tolerance of host defenses. It is important to
402 assess the biophysical and functional properties of the membranes of the cells with such radically
403 different fatty acid compositions. Although BCFAs and SCUFAs both increase membrane
404 fluidity, they do not yield cells with identical morphologies [14], or fitness for tolerating cold
405 stress [64]. Also a *S. aureus* fatty acid auxotroph created by inactivation of acetyl coenzyme A
406 carboxylase (*ΔaccD*) was not able to proliferate in mice, where it would have access to SCFAs
407 and SCUFAs [65]. Due to the ability of a pathogen to adapt and undergo dramatic alterations
408 when subjected to a host environment, there is a growing appreciation in the research community
409 for the fact that the properties of the organism grown *in vivo* are probably very different from
410 when it is grown *in vitro*. This distinction may have a huge impact on critical cellular attributes
411 controlling pathogenesis and resistance to antibiotics. Expression of virulence factors is
412 significantly different in serum-grown organisms [28], and there are global changes in gene
413 expression when *S. aureus* is grown in blood [66]. *S. aureus* grown in serum or blood will have

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414 different membrane lipid compositions than cells grown in laboratory media and this may have a
415 significant impact on the expression of virulence factors and pathogenesis of the organism.

416 We have demonstrated a hitherto poorly recognized growth environment-dependent
417 plasticity of *S. aureus* membrane lipid composition. The balance of BCFAs and SCFAs was
418 affected significantly by the variations in laboratory medium in which the organism grew.
419 SCUFAs became a major membrane fatty acid component when the organism was grown in
420 serum. These findings speak to the properties of pathogens grown *in vitro* versus *in vivo*. In 1960
421 Garber [67] considered the host as the growth medium and the importance of the properties of
422 the pathogen at the site of infection. There has been a renewed appreciation of this in recent
423 years [68]. Massey et al. [69] showed that *S. aureus* grown in peritoneal dialysate acquired a
424 protein coat. Krismer et al. [70] devised a synthetic nasal secretion medium for growth of *S.*
425 *aureus*. Tn-seq analysis has been used to identify genes essential for survival in infection models
426 versus rich medium. Citterio et al. [71] reported that the activities of antimicrobial peptides and
427 antibiotics were enhanced against various pathogenic bacteria by supplementation of the media
428 with blood plasma to mimic in vivo conditions. In order to replicate a membrane fatty acid
429 composition more closely resembling that of the bacteria growing *in vivo*, it may be desirable to
430 supplement laboratory media with SCFAs and SCUFAs.

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714

Figure 1

BCFA	Anteiso C15:0	
	Iso C15:0	
SCFA	C18:0 (Stearic acid)	
SCUFA	C18:1Δ9 (Oleic acid)	
Staphyloxanthin	β-D-glucopyranosyl 1-*O*-(4,4′-diaponeurosporen-4-oate)-6-*O*-(12-methyltetradecanoate)	

Figure 2

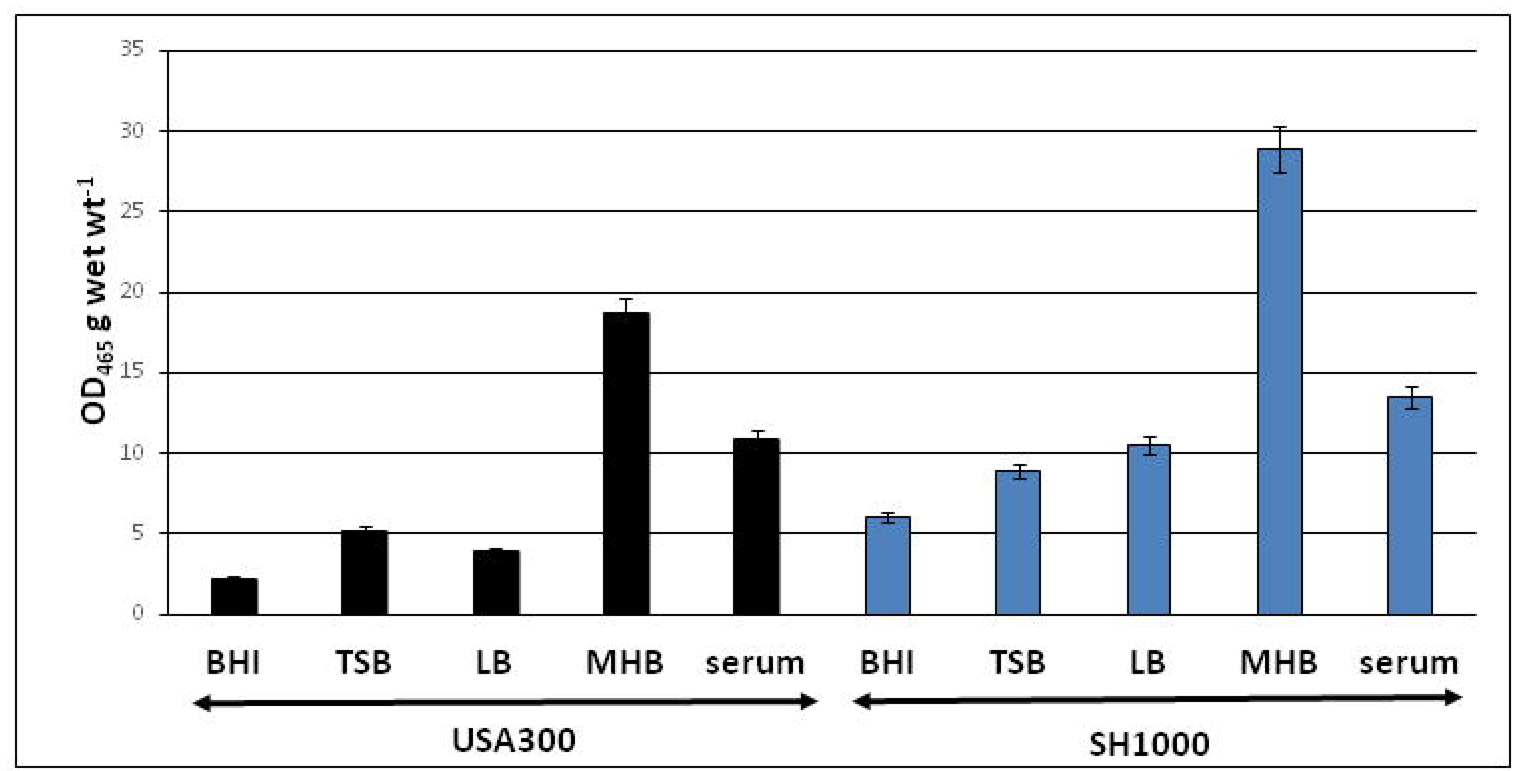

Figure 3

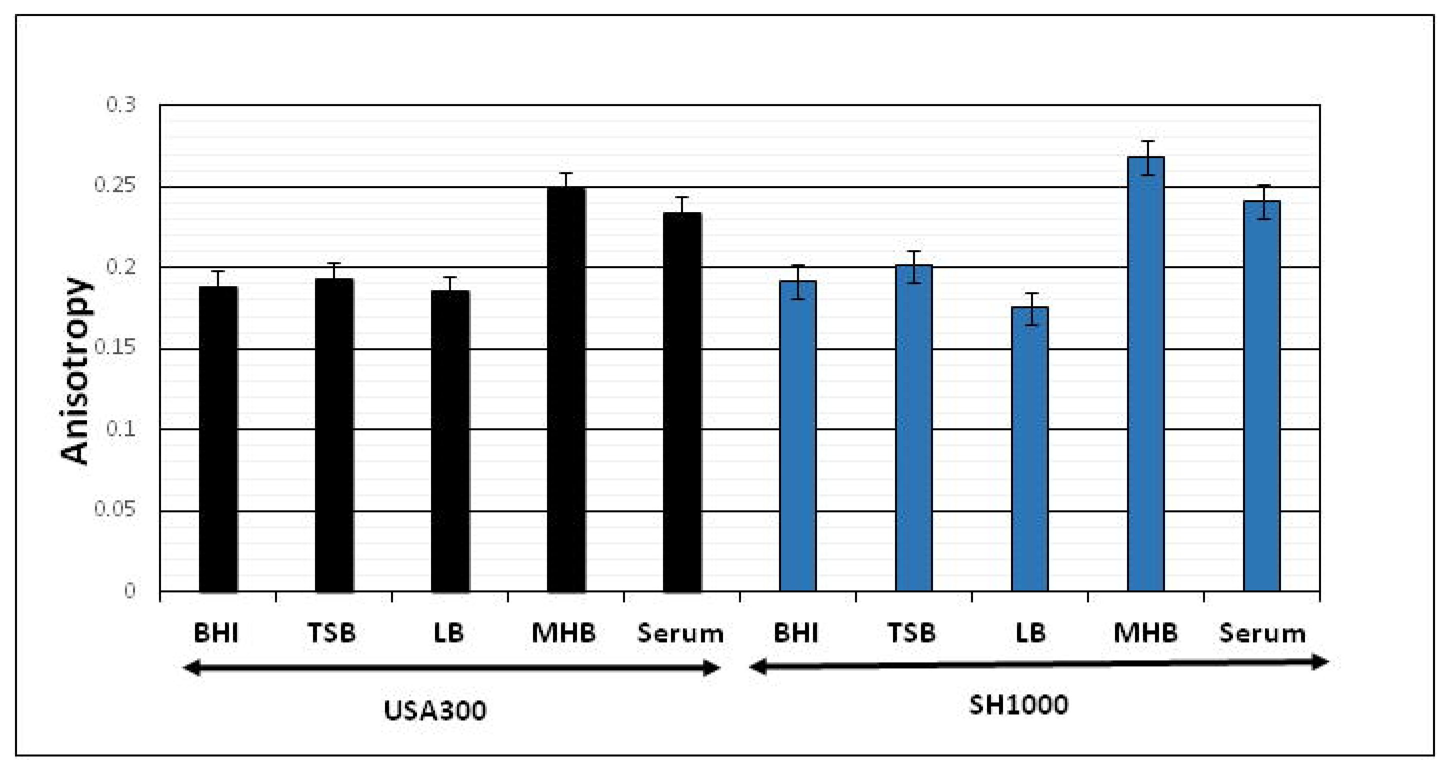

Figure 4