1 **Antibiotic resistance and host immune evasion in *Staphylococcus aureus***

2 **mediated by a novel metabolic adaptation**

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31 **Short title: Lipid membrane evasion mechanism of *S. aureus***

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38

Abstract (196/250)

Staphylococcus aureus is a notorious human bacterial pathogen with considerable capacity to develop antibiotic resistance. We have observed that human infections caused by highly-drug resistant *S. aureus* are more prolonged, complicated and difficult to eradicate. Here, we describe a novel metabolic adaptation strategy used by clinical *S. aureus* strains that leads to resistance to the last-line antibiotic, daptomycin, and simultaneously impacts host innate immunity. This response was characterized by a change in anionic membrane phospholipid composition induced by point mutations in the phospholipid biosynthesis gene, *cls2*, encoding cardiolipin synthase. Single *cls2* point mutations were sufficient for daptomycin resistance, antibiotic treatment failure and persistent infection. These phenotypes were mediated by enhanced cardiolipin biosynthesis, leading to increased bacterial membrane cardiolipin and reduced phosphatidylglycerol. The changes in membrane phospholipid profile led to modifications in membrane structure that impaired daptomycin penetration and membrane disruption. The *cls2* point mutations also allowed *S. aureus* to evade neutrophil chemotaxis, mediated by the reduction in bacterial membrane phosphatidylglycerol, a previously undescribed bacterial-driven chemoattractant. Together, these data illustrate a novel metabolic strategy used by *S. aureus* to circumvent antibiotic and immune attack, and provide crucial insights into membrane-based therapeutic targeting of this troublesome pathogen.

Keywords: *S. aureus*, daptomycin, cardiolipin, phosphatidylglycerol, neutrophils

Significance Statement (109/120)

Staphylococcus aureus is one of the most significant human bacterial pathogens that has the capacity to cause serious infections and become highly resistant to antibiotics. In this study, we identified a metabolic adaptation mechanism used by *S. aureus* to simultaneously circumvent killing by one of the last-line anti-staphylococcal antibiotics, daptomycin, and attack from host innate immune cells. This process led to enhanced bacterial survival and was mediated by a change in bacterial membrane phospholipid composition sufficient to impair daptomycin membrane penetration and significantly impact neutrophil chemotactic responses. These results highlight the importance of bacterial membrane lipid adaptation in bacterial pathogenesis, and provide crucial insights into potentially novel therapeutic targeting.

72 \body

Introduction

74 *Staphylococcus aureus* is one of the most important human bacterial pathogens, with a

75 worldwide distribution and an ability to cause infection of almost any human tissue (1).

76 Effective treatment of staphylococcal infections has been hampered by the emergence

77 of antibiotic resistance, leading to increased reliance on last-line antibiotics such as

78 daptomycin (2). Daptomycin is a cyclic lipopeptide antibiotic that interacts with

79 bacterial cell membranes but the precise mechanisms of action remain elusive (3).

80 Notably, human infections caused by daptomycin-resistant *S. aureus* have been

81 associated with persistent and complicated infections (2, 4). We and others have

82 recently shown that clinically-derived daptomycin-resistant isolates caused persistent

83 infections in non-mammalian and murine septicaemia models (5, 6), raising the

84 question about the correlation between daptomycin resistance, immune evasion and

85 bacterial survival *in vivo* (5, 6).

86

87 Bacteria have evolved highly conserved mechanisms mediating adaptation and

88 maintenance of membrane integrity to defend against host microbicidal peptides (7, 8).

89 Mutations in genes related to phospholipid biosynthesis are consistently reported in

90 Gram-positive bacteria resistant to daptomycin (4). The most abundant phospholipid

91 found in Gram-positive bacterial membranes, including staphylococcal membranes, is

92 phosphatidylglycerol (PG). PG can be converted to cardiolipin (CL) and lysyl-

93 phosphatidylglycerol (L-PG) by the enzymes cardiolipin synthase (Cls) and multiple

94 peptide resistance factor (MprF), respectively (9-11). Gain-of-function mutations in

95 MprF have been associated with daptomycin resistance (8, 9, 12). Most human bacterial

96 pathogens have a Cls homolog that catalyzes condensation of two PG molecules to

97 yield one CL and one glycerol molecule (13). In *S. aureus*, there are two *cls* genes, with

5

98   *cls2* encoding the major CL synthase (10, 11). Thus far, the bacterial membrane

99   adaptation response to antibiotic and innate immune exposure in humans is poorly

100   understood.

101

102   Neutrophils form one of the most fundamental host innate immune effectors against

103   bacterial pathogens, including *S. aureus* (14). The clinical significance of neutrophils

104   is well illustrated by the predisposition to severe and recurrent staphylococcal infections

105   in patients with functional or quantitative neutrophil deficiencies (15). Neutrophil

106   recruitment to the site of infection caused by Gram-positive bacteria is often mediated

107   by bacterial-driven chemoattractants such as formylated peptides and phenol soluble

108   modulins, or by endogenous cytokines (eg. IL-8) released from host cells (16, 17). In

109   response, bacteria have evolved mechanisms that interfere with neutrophil chemotaxis,

110   including chemotaxis inhibitory protein of *S. aureus* (CHIPS) and formyl peptide

111   receptor-like 1 inhibitor (FLIPr) (18, 19). Deepening our understanding of how bacteria

112   evolve during human infection to simultaneously circumvent key innate immune

113   effectors and antibiotic selection pressure is crucial in our pursuit of novel therapeutic

114   strategies.

115

RESULTS

Mutations in *S. aureus* cardiolipin synthase lead to the evolution of daptomycin resistance and antimicrobial failure

We have previously collected and reported on *S. aureus* isolates from nine patients with bloodstream infection who were all treated with daptomycin (4). Samples were collected as soon as the infection was detected and later in infection when resistance to daptomycin and treatment failure was evident (4). All patients had persistent bacteremia and complicated infections involving heart valves, bone and joints, and deep soft-tissues (4). Whole genome sequencing of the nine paired samples identified non-synonymous point mutations in *cls2* in daptomycin-resistant isolates, which were tightly positioned in the two predicted N-terminal transmembrane domains encoded by *cls2*, resulting in A23V, T33N, L52F and F60S amino acid substitutions (Fig. 1A) (4). To study these nucleotide changes in *cls2*, independent of other mutations observed in the clinical daptomycin-resistant strains, allelic replacement experiments were performed to introduce the individual point mutations into a daptomycin-susceptible clinical isolate (A8819) producing A8819$_{Cls2A23V}$, A8819$_{Cls2T33N}$ and A8819$_{Cls2L52F}$ (Table S1). Several attempts to generate the F60S substitution were unsuccessful. Daptomycin susceptibility was most perturbed with the T33N mutation (A8819$_{Cls2T33N}$), which led to a minimum inhibitory concentration (MIC) of daptomycin similar to that observed in our clinical resistant isolates (MIC 2 μg/ml) compared to the susceptible parent strain (MIC 0.5 μg/ml) (Table S1). This magnitude rise in MIC of daptomycin has been associated with therapeutic failure and poor patient outcomes (2), confirming the significance of the increase. Chromosomal repair of the T33N mutation (A8819$_{Cls2T33NN33T}$) restored daptomycin susceptibility back to wild-type levels (MIC 0.5 μg/ml) (Table S1). L52F and A23V mutations also increased MICs of daptomycin

142   but to a lesser degree (up to 1 μg/ml) (Table S1).

143

144   To assess the functional impact of the reduced daptomycin susceptibility with the *cls2*

145   mutations, we performed daptomycin treatment assays (Fig. 1B). Daptomycin

146   concentrations (2 μg/ml and 4 μg/ml) were chosen to mimic free daptomycin

147   concentration observed in deep tissues and bones under standard human dosing (20,

148   21). Using a concentration of 2 μg/ml, we showed that daptomycin was rapidly

149   bactericidal (≥ 3 $\log_{10}$ reduction in colony forming units [CFU]) against all strains over

150   8 hours, however significant regrowth occurred back to the starting inocula by 24 hours

151   for A8819$_{Cls2T33N}$ and A8819$_{Cls2L52F}$ (Fig. 1B). In contrast, killing to undetectable levels

152   was seen for the daptomycin-susceptible parent strain (A8819) and the T33N repaired

153   strain (A8819$_{Cls2T33NN33T}$). Similar findings were observed with daptomycin

154   concentrations up to 4 μg/ml (Fig. S1). Daptomycin treatment failure with

155   A8819$_{Cls2T33N}$ and A8819$_{Cls2L52F}$ directly correlated with progressive resistance, with an

156   MIC of daptomycin increasing up to 4 μg/ml, suggesting that additional adaptation

157   occurred under daptomycin exposure (Fig. 1C). Together, these data show that as seen

158   in patients, the observed *cls2* point mutations genetically predisposed *S. aureus* to

159   resistance evolution and therapeutic failure under antibiotic selection.

160

161   **Amino acid substitutions in *S. aureus* cardiolipin synthase enhance cardiolipin**

162   **biosynthesis and alter bacterial membrane phospholipid composition**

163   To determine the impact of the clinically derived *cls2* point mutations on membrane

164   phospholipid composition, we extracted total lipids from the daptomycin-susceptible

165   clinical strain (A8819), the three *cls2* point mutants (A8819$_{Cls2T33N}$, A8819$_{Cls2A23V}$,

166   A8819$_{Cls2L52F}$) and the repaired strain (A8819$_{Cls2T33NN33T}$). We first assessed lipid

167   profiles using thin-layer chromatography (Fig. S2A), which showed that relative to

168   A8819, the three *cls2* point mutants had an increase in membrane CL content and a

169   reduction in PG, with no change in L-PG. A time-course lipid analysis over bacterial

170   growth phases supported these findings (Fig. S2B, C). The concentration of individual

171   phospholipid species was then quantified using liquid chromatography coupled with

172   mass spectrometry (LC-MS). Consistent with the TLC analysis, the percentage of CL

173   among total phospholipids increased significantly in the three *cls2* point mutants

174   compared to A8819, whereas the PG content decreased significantly (Fig. 2A). None

175   of the *cls2* mutations impacted on L-PG. Overall, the membrane phospholipid changes

176   were most pronounced for A8819$_{Cls2T33N}$ (Fig. 2A), which paralleled the magnitude of

177   daptomycin resistance. Importantly, as seen with daptomycin susceptibility, repair of

178   the T33N mutation restored the membrane phospholipid profile back to wild-type levels

179   (Fig. 2B and Fig. S2D). The increase in CL in the *cls2* point mutants was not secondary

180   to an increase in *cls2* transcription or Cls2 membrane quantity, and Cls2 membrane

181   localization was unchanged compared to A8819 (Fig. S3A-C).

182

183   To test whether the *cls2* mutations increased cardiolipin synthesis activity *in vivo*,

184   bacteria were metabolically labelled with ^{13}C-glycerol, which is incorporated into the

185   glycerol backbone and head group of PG and CL (Fig. S4A-C). The formation of

186   complex PG or CL mass isotopomers (+3, +6 and +9) by LC-MS can then be used to

187   measure *de novo* biosynthesis of these phospholipids (Fig. S4A-C). Compared to wild-

188   type, we showed that CL biosynthesis significantly increased over time in our T33N

189   mutant strain (Fig. 2C) and this was mirrored by a reduction in PG biosynthesis (Fig.

190   2D). Given that PG is both a metabolic end-product and precursor for CL, these results

191   suggest that CL is synthesized from a sub pool of PG in the mutant to account for the

192   different degree of ^{13}C-labelling. No difference in biosynthesis of L-PG was observed

193   (Fig. 2E). Together, these data confirm that clinically relevant Cls2 amino acid

194　substitutions caused enhanced membrane cardiolipin biosynthesis that was proportional

195　to the degree of antibiotic resistance.

196

197　**Changes in *S. aureus* membrane composition blocks daptomycin mediated lipid**

198　**extraction, membrane penetration and disruption**

199　To assess the impact of Cls2-mediated lipid changes on daptomycin-membrane

200　interactions, we reconstituted Gram-positive bacterial bilayer membranes immobilised

201　on a planar surface for characterization by neutron reflectometry (NR) (22, 23) (Fig.

202　S5A). The bilayers were incubated with isotopic solvents (D_2O and H_2O) and the NR

203　profiles of wild-type and $A8819_{Cls2T33N}$ were modelled simultaneously to analyze the

204　bilayer structures (Fig. 3A-B). The cardiolipin-rich membrane caused by the Cls2 T33N

205　amino acid substitution was thicker compared to the wild-type membrane (57.8 ± 3.7

206　Å versus 49.4 ± 3.3 Å, respectively) (Fig. S5B, Table S2-3, Fig. 3C-D). Despite the

207　overall differences in membrane thickness, the cell surface charge was similar, and

208　using super-resolution microscopy of cell surface bound fluorescent daptomycin, we

209　showed that the level and distribution of daptomycin binding on $A8819_{Cls2T33N}$ cell

210　surface was similar to A8819 (Fig. S6A-D). These data support the hypothesis that

211　structural modification of the membrane impairs daptomycin penetration and

212　membrane disruption rather than initial binding.

213

214　Using NR, we then characterised the impact of the observed membrane changes on

215　daptomycin interactions. Daptomycin treatment at 2, 4 and 8 µg/ml significantly shifted

216　the fringe of the NR curves of the wild-type membranes in a concentration dependent

217　manner (Fig. 3A. arrows), indicating extraction and solubilisation of the membrane by

218　daptomycin. In contrast, there was a less pronounced change in reflectivity for the

219　cardiolipin-rich, daptomycin-resistant $A8819_{Cls2T33N}$ membrane (Fig. 3B). Analysis of

the NR curves showed that at a daptomycin concentration of 2 μg/ml, the lipid volume

fraction of the wild-type membrane was reduced, and no daptomycin was seen within

the membrane consistent with a lipid extraction mechanism (24) (Table 1, Table S4).

At a higher daptomycin concentration (4 μg/ml), antibiotic membrane penetration was

evident, extending through the bilayer and causing dislocation of acyl-chains (Table 1,

Table S4). At the highest concentration (8 μg/ml), the membrane was completely

solvated by daptomycin, with a substantial decrease in the lipid volume fraction from

86.5% to 26.6% (Table 1, Table S4). For the thicker, cardiolipin rich, $A8819_{Cls2T33N}$

membrane, only mild lipid extraction was observed at 2, 4 and 8 μg/ml with the lipid

volume fraction only reducing from 78.5% to 62.8% (Table1, Table S5). The

$A8819_{Cls2T33N}$ membrane resisted daptomycin penetration and remained intact (Table 1,

Table S5).

To further define the daptomycin-membrane interaction, we then used small-angle

neutron scattering (SANS), which provided information on the daptomycin aggregation

characteristics within the bilayer membranes under physiologically relevant conditions

(25). Consistent with our NR data, at a clinically relevant daptomycin concentration of

4 μg/ml, we observed daptomycin penetrate and form aggregates straddling the bilayer

membrane for wild-type (A8819) but not for the $A8819_{Cls2T33N}$ membranes (Fig. 3E,

and F). These data also concur with our bacterial killing data, which showed complete

bacterial killing for wild-type A8819 but therapeutic failure toward $A8819_{Cls2T33N}$ (Fig.

S1). Using established core-shell and hollow-cylinder models to characterise the

daptomycin aggregates (25), it was estimated that daptomycin constituted a 28 ± 0.86

Å radius spherical micelle structure within the membrane bilayer (Fig. S7). Taken

together, these results show that *S. aureus* adapts during treatment with daptomycin in

human infections by increasing its cardiolipin membrane content leading to a thicker

246 membrane that resists daptomycin lipid extraction, and membrane penetration and

247 disruption, promoting bacterial survival.

248

249 **Cls2 amino acid substitutions impair neutrophil recruitment *in vivo* and promote**

250 **bacterial survival**

251 Apart from impairing daptomycin-membrane interactions, we hypothesised that the

252 altered bacterial membrane phospholipid profile caused by *cls2* point mutations may

253 impact on *S. aureus* – host interactions. Neutrophils are the dominant innate immune

254 cell for controlling *S. aureus* infection (14, 15). To interrogate neutrophil behaviour *in*

255 *vivo* and in real-time with high resolution, we utilised the vertebrate zebrafish (*Danio*

256 *rerio*) model system (26). In common with humans, zebrafish have cellular and soluble

257 immune arms, and complex tissue environments that enable a mechanistic

258 understanding of human infectious diseases (26, 27). To assess neutrophil trafficking

259 and recruitment to a localized soft tissue staphylococcal infection, we used transgenic

260 embryos with red fluorescent neutrophils (Tg[*lyz*:dsRed]) (28) that were infected into

261 the somatic muscle with GFP-expressing *S. aureus* (Fig. 4A). Notably, neutrophil

262 recruitment to the localized infection site was significantly compromised for infection

263 with the three *cls2* point mutants compared to infection by A8819 after 6 hours post-

264 infection (Fig. 4B,C). Mutation repair for the T33N strain (A8819$_{Cls2T33NN33T}$) was

265 sufficient to restore neutrophil recruitment back to wild-type (A8819) infection levels

266 (Fig. 4D). This attenuated neutrophil response impacted bacterial clearance, with more

267 persistent infection observed with A8819$_{Cls2T33N}$ compared to the wild-type and

268 repaired strains (Fig. 4E). To further validate the observed neutrophil migration findings

269 in zebrafish, we assessed human neutrophil migration within an ex-vivo assay. As

270 shown in Fig. 4F, significantly less human neutrophil recruitment was observed with

271 A8819$_{Cls2T33N}$ compared to the wild-type strain. Mutation repair of the T33N mutant

272   (A8819$_{Cls2T33NN33T}$) caused similar neutrophil recruitment as wild-type (Fig. 4F). These

273   results indicated that the Cls2 amino acid substitutions equipped *S. aureus* with the

274   ability to circumvent neutrophil recognition and response, leading to a persistent

275   infection as observed in patients and animal models (2, 5, 6).

276

277   **Compositional changes of membrane phospholipids resulted in reduced**

278   **neutrophil recruitment in zebrafish**

279   To investigate the contribution of the *S. aureus* bacterial membrane to neutrophil

280   evasion, total lipids were extracted from each of the *cls2* mutant and the daptomycin-

281   susceptible parent (A8819) and repaired strains. Liposomes were then processed and

282   injected into the otic vesicle of zebrafish (Fig. 4A, white circle). The otic vesicle is

283   normally devoid of leukocytes and is relatively confined, preventing dispersion of the

284   liposomes (29). As shown in Fig. 5A, and similar to that seen with infection of live,

285   whole bacterial cells (Fig. 4B-D), the liposomes from the three *cls2* point mutants were

286   significantly attenuated in inducing neutrophil recruitment compared to liposomes from

287   A8819 and the T33N repaired strain (A8819$_{Cls2T33NN33T}$), suggesting that altered anionic

288   phospholipid composition may be responsible for the immune evasion. To determine

289   the phospholipid driver of this *S. aureus* evasion response, purified PG and CL that

290   were free of DNA, peptidoglycan, wall-teichoic acid, and lipoteichoic acid from A8819

291   cells (Fig. S8) were processed to form liposomes. Injection of the PG- and CL-specific

292   liposomes into the otic-vesicle of zebrafish embryos showed that PG liposomes induced

293   substantial neutrophil recruitment whereas the recruitment of neutrophils by CL

294   liposomes was comparable to the PBS control (Fig. 5B,C). These data suggest that PG

295   is the major bacterial-mediated phospholipid driver of neutrophil chemoattraction, and

296   the *S. aureus* adaptation response to daptomycin represents a novel bacterial membrane

13

297   based stealth strategy to simultaneously evade an antibiotic and a key innate immune

298   effector cell to promote survival within a host.

299

DISCUSSION

300 *S. aureus* has evolved a wealth of strategies to optimise its survival in various host

301 niches and under nocuous selection pressures (30). Here, we report a new metabolic

302

303 resistance mechanism used by *S. aureus* to prevent membrane lysis by the last line anti-

304 staphylococcal antibiotic, daptomycin. Single amino acid substitutions in cardiolipin

305 synthase 2 led to a significant increase in the bacterial membrane CL/PG ratios due to

306 enhanced cardiolipin biosynthesis, which led to a thicker membrane that resisted

307 daptomycin penetration and membrane disruption. Intriguingly, this adaption also led

308 to immune evasion. Specifically, we showed that membrane PG acted as a bacterial-

309 driven neutrophil chemoattractant and, in the context of *cls2* point mutations, reduced

310 PG membrane content led to less neutrophil trafficking to a localized site of infection

311 and prolonged bacterial survival.

312

313 Recent studies on the interactions between daptomycin and bacterial membranes have

314 used indirect methodological approaches such as measuring permeability loss of

315 liposomes exposed to daptomycin using a fluorescence assay, and membrane systems

316 that are less relevant to Gram-positive bacteria, particularly using phosphatidylcholine

317 (PC) and not L-PG (31). It was shown that the addition of cardiolipin to 10% of total

318 lipids (using a PC/PG membrane system) was sufficient to prevent daptomycin

319 interaction with membranes (31), however we have clearly shown that our daptomycin

320 susceptible clinical strain (A8819) already has a greater percent of cardiolipin than 10%

321 (Fig. 2A), suggesting the possibility of discordance between clinically relevant

322 membranes. More recently, alteration of membrane curvature and diverting daptomycin

323 binding, or interference with fluid membrane microdomains have been proposed using

324 the model organism *Bacillus subtilis* or enterococcal strains (7, 32, 33). However, the

325 phospholipid membrane composition of these organisms is different to *S. aureus*, and

15

326 daptomycin-membrane interactions appear to be bacterial-species dependent. For

327 example, daptomycin has an irregular membrane binding pattern in *B. subtilis* and

328 *Enterococcus*, with a particular predilection for the division septum (7, 32, 33), whereas

329 we have observed universal distribution of daptomycin on *S. aureus* membranes (Fig.

330 S6C-D).

331

332 Here, we have reconstituted clinically relevant *S. aureus* membranes and have provided

333 the first direct structural analysis of daptomycin-membrane interactions. The scattering

334 of free neutrons by matter provides excellent structural detail and this experimental

335 approach has only recently been used to study biological membranes (25, 34). We used

336 unsaturated phospholipids with complex head group composition to reconstitute the

337 membranes, and the surface coverage achieved for the wild-type membranes was 86.5%

338 (Table 1), which is higher than what has been previously reported using unsaturated

339 phospholipids with similar techniques (35). Using SANS and NR, we identified three

340 distinct modes of action of daptomycin on the membrane. At lower concentrations (2

341 μg/ml), lipid extraction occurred and caused lesions in the bilayer membrane, whereas

342 at higher concentrations (4 μg/ml), daptomycin molecules penetrated into the bilayer

343 and formed organised micelles. At the highest concentrations (8 μg/ml), the bilayer was

344 completely solvated by daptomycin. The process of lipid extraction, penetration,

345 micelle formation and membrane lysis was inhibited in the context of the clinically

346 derived Cls2 amino acid substitution T33N, which coincided with daptomycin

347 resistance. Interestingly, the daptomycin molecules still attached to the outer leaflet of

348 the membrane in the A8819$_{Cls2T33N}$ strain, and this was corroborated with super-

349 resolution imaging of fluorescent daptomycin binding to bacterial cells, but the

350 daptomycin was functionally impaired due to the barrier created by the thicker CL-rich

351 membrane. This is an anionic phospholipid driven mechanism of daptomycin resistance

16

352 and is independent of the charge-based repulsion theory proposed for *mprF* mutations

353 that alter L-PG membrane content (4, 12).

354

355 Subversion of host immune surveillance contributes to persistent bacterial infections

356 (36). Given the importance of neutrophils in eliminating *S. aureus* (15), we utilized a

357 transgenic zebrafish line carrying red fluorescent neutrophils to characterize the

358 interactions between *S. aureus* and neutrophils *in vivo* and in real-time during acute

359 infection. We found that infection with the *S. aureus cls2* point mutants compromised

360 neutrophil recruitment and this was associated with a more prolonged bacterial burden

361 in the host. This compromised neutrophil migration was further confirmed using human

362 neutrophils. The purified PG and CL liposomes used for the neutrophil migration

363 studies were found to be free of DNA, peptidoglycan, lipoteichoic acids and wall-

364 teichoic acids (Fig. S8), suggesting that established bacterial driven neutrophil

365 chemoattractants for compromised neutrophil migration were not at play (37). We then

366 focused on the phospholipid membrane components and through injection of purified

367 CL and PG liposomes into zebrafish, established that PG was a driver for neutrophil

368 chemotaxis. This provided a mechanistic model whereby Cls2 amino acid substitutions

369 led to increased cardiolipin synthase activity and increased CL production that was

370 subsequently causing a reduction in PG. This change in lipid profile not only disturbed

371 daptomycin-membrane interactions, but also reduced neutrophil chemoattraction to a

372 localized site of infection, finally resulting in a more persistent infection, which has

373 been observed in patients and animal models without a clear explanation (2, 5, 6).

374 Future analyses are still required to investigate the generalization of bacterial PG in

375 inducing neutrophil migration and if PG acts directly as a chemotactic or indirectly via

376 activation of endogenous cells. In conclusion, we have characterised a bacterial

377 metabolic adaptation process that leads to simultaneous evasion of host immune and

378 antimicrobial attack, providing important insights for future therapeutic targeting for

379 this troublesome pathogen.

380

381 **Materials and Methods**

382 **Media and reagents**

383 Bacterial strains, plasmids, and oligonucleotides used in this study are described in

384 Table S6. *S. aureus* ~~and *Escherichia coli*~~ cells were cultured in heart infusion broth (HI)

385 (Oxoid) ~~and Luria-Bertani broth~~ with constant shaking at 37 ºC~~, respectively~~.

386

387 **Genetic manipulation**

388 The vector pIMAY and the *Escherichia.* coli* strain DC10B were used to genetically

389 manipulate *S. aureus* isolate ~~A8819~~ following the published protocol (SI Materials and

390 Methods) (38).

391

392 **Daptomycin susceptibility testing**

393 Broth microdilution MIC testing was performed based on guidelines by the Clinical

394 and laboratory standards institute (CLSI). Daptomycin MIC > 1 µg/ml is officially

395 termed daptomycin-nonsusceptible, but was termed daptomycin resistant throughout

396 the manuscript for clarity. Time-kill assays were performed with an initial bacterial

397 inoculum of 10^6 CFU/ml in Mueller-Hinton broth supplemented with 50 mg/litre

398 calcium and daptomycin. (SI Materials and Methods).

399

400 **Lipid analysis by mass spectrometry**

401 Lipids were extracted ~~and analysed using thin layer chromatography~~ following the

402 published protocol (10). Lipid species were processed and quantified according to

403 standard procedures (39). For *de novo* phospholipid biosynthesis, the metabolic

404 labelling was initiated by the addition of glycerol or [^{13}C]-glycerol (Sigma-Aldrich) to

405 1 mM after bacterial growth from optical density 600nm of 0.4 for 30 minutes at 37 ℃.

406 Data collected for lipid composition of PG, CL and L-PG with ^{13}C-glycerol

407 incorporation was analysed using Metaboanalyst (http://www.metaboanalyst.ca) (SI

408 Materials and Methods).

409

410 *S. aureus* **bilayer membrane formation and neutron reflectometry (NR)**

411 Deposition of the model membrane on the top of a SiO$_2$ surface was performed using a

412 custom built Langmuir-Blodgett trough (Nima Technology, Conventry, UK) following

413 published Langmuir–Blodgett (LB) and Langmuir–Schaefer (LS) procedures (22) (SI

414 Materials and Methods). Synthetic PG (18:1), CL (18:1) and L-PG (18:1) (Avanti Polar

415 Lipids, Inc) were mixed at the molar ratios of 69:12:19 and 23:60:17 to create A8819

416 and A8819$_{Cls2T33N}$ symmetric membrane bilayers, respectively.

417 Specular neutron reflection at solid-liquid interface was carried out on the Platypus

418 time-of-flight neutron reflectometer at the OPAL 20 MW Multi-purpose Research

419 Reactor, Lucas Heights, Australia. The final reflectivity (reflected intensity / incident

420 intensity) is presented as a function of momentum transfer. Analysis of the NR profiles

421 was performed using MOTOFIT followed by Monte Carlo Analysis to determine 95%

422 confidence intervals (40). (SI Materials and Methods).

423

424

425 **Small angle neuron scattering (SANS)**

426 Synthetic PG, CL and L-PG were mixed at the molar ratios of 69:12:19 and 23:60:17

427 for producing A8819 and A8819$_{Cls2T33N}$ membrane vesicles respectively. The samples

428 were measured using the small-angle neutron scattering instrument, Quokka, at

429 ANSTO. (SI Materials and Methods).

Zebrafish strains, maintenance and leukocyte enumeration

Wild-type Tübingen and Tg(*lyz*:DsRed)^{nz50} zebrafish (28) embryos were maintained in the Monash University Fish Core facility and infected with *S. aureus* cells according to standard protocols (27) (SI Materials and Methods).

In vitro **Transwell chemotaxis assay**

Human venous blood was collected from healthy volunteers for isolating neutrophils with the approval of the Monash University Human Research Ethics Committee. Chemotaxis assays were performed according to a previously published protocol (41) (SI Materials and Methods).

Liposome preparation

Staphylococcal PG or CL were extracted from TLC plates using chloroform/methanol/water (5:5:1) followed by centrifugation. Liposomes were prepared following an established protocol (42) (SI Materials and Methods).

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Figure Legends

Fig. 1. Impact of Cls2 amino acid substitutions on daptomycin killing and resistance emergence. (*A*) The predicted amino acid change in Cls2 by individual *cls2* point mutations. N: amino-terminus. C: carboxyl-terminus. TM: transmembrane domain. PLD: phospholipase D domain. (*B*) Time-kill analyses showing quantitative bacterial counts during exposure to 2 µg/ml daptomycin. Dashed line indicates the detection limit. Comparison of the area under the curve between mutants and wild-type by one-way ANOVA (*$P < 0.05$, **$P < 0.01$). (*C*) Daptomycin MIC of staphylococcal cells at each time point from the time-kill analysis. Error bars represent mean $\pm$ SEM, three independent experiments.

Fig. 2. Impact of *cls2* point mutations on membrane phospholipid profiles. (*A*) The molar ratio of PG, CL, and L-PG among total phospholipids determined by LC-MS. (*B*) Repair of Cls2 T33N (T33NN33T) restored membrane phospholipid profiles to wild-type levels. Phospholipid biosynthesis was assessed after treatment with ^{13}C-glycerol. (*C*) The ratios of ^{13}C-CL, (*D*) ^{13}C-PG and (*E*) ^{13}C-L-PG (all isotopomers) among total lipid content were determined by LC-MS. Mean $\pm$ SD, three independent experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, one-way ANOVA. n.s. non-significant. Student's *t* test was used to compare T33N to WT (*C-E*).

Fig. 3. Daptomycin-membrane interactions. The neutron reflectivity profiles (symbols) and fits (lines) of A8819 (*A*) and A8819$_{Cls2T33N}$ (*B*) membrane models in D_2O and H_2O are shown. The NR profiles in H_2O are offset for clarity. The arrows indicate fringe shifts of NR profiles. The molecular models of reconstituted A8819 (*C*) and A8819$_{Cls2T33N}$ (*D*) membranes show a thicker, but more CL-rich membrane structure (with CL in yellow, PG in red, and L-PG in blue). Small angle neutron scattering

profiles measured for A8819 (*E*) and A8819$_{Cls2T33N}$ (*F*) membranes treated with and without daptomycin (4 μ g/ml). The arrow indicates the lipid bilayer whilst the star indicates the Bragg peak as the sign of micelle formation.

Fig. 4. Impact of Cls2 amino acid substitutions on neutrophil recruitment. (*A*) Schematic of a 48 h post-fertilization (hpf) zebrafish showing the *S. aureus* injection sites. Rectangle: somatic muscle. White circle: the otic vesicle. Scale bar = 250 μ m. (*B*) Representative images of localized sites of infection with *S. aureus* strains at 6 h post-infection. (*C*) The number of emigrated neutrophils to the localized site of infection. For WT, n = 22; A23V, n = 29; T33N, n = 34; L52F, n = 27, pooled from four independent experiments. (*D*) Neutrophil recruitment was restored to wild-type levels by the repaired strain A8819$_{Cls2T33NN33T}$. For PBS, n = 12; WT, n = 21; T33N, n = 22; T33NN33T, n = 23, pooled from three independent experiments. (*E*) The bacterial burden in zebrafish after a somatic muscle infection. Error bars represent mean + SEM, three independent experiments (***$P < 0.001$ compared to WT and T33NN33T, chi-square test for trend). (*F*) Human neutrophil recruitment was assessed using a Transwell system, with neutrophils and bacterial cells in the top and bottom wells respectively. Five independent experiments. For (*C*, *D*, and *F*), error bars represent mean $\pm$ SEM. *$P < 0.05$, ***$P < 0.001$ compared to WT, Kruskal-Wallis test.

Fig. 5. Impact of bacterial membrane phospholipids on neutrophil migration *in vivo*. (*A*) Quantification of emigrated neutrophils into the zebrafish otic vesicle at 6 h after injection of liposomes constituted from *S. aureus* membrane phospholipids. For PBS, n = 14; WT, n = 28; A23V, n = 26; T33N, n = 24; L52F, n = 24; T33NN33T, n = 17, pooled from four independent experiments. (*B*) Representative images of zebrafish otic vesicles (red circles) injected with purified PG or CL liposomes, with quantification of

643 emigrated neutrophils (*C*). For PBS, n = 10; PG, n = 17; CL, n = 17, pooled from three

644 independent experiments. Error bars represent the mean $\pm$ SEM. *$P < 0.05$, **$P < 0.01$

645 and ***$P < 0.001$ compared to PBS for (*A*) and (*C*), one-way ANOVA.

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