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Antibiotic resistance and host immune evasion in *Staphylococcus aureus* mediated by a novel metabolic adaptation

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

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37 *Classification:* Biological Sciences/Microbiology
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.

\body

Introduction

Staphylococcus aureus is one of the most important human bacterial pathogens, with a worldwide distribution and an ability to cause infection of almost any human tissue (1). Effective treatment of staphylococcal infections has been hampered by the emergence of antibiotic resistance, leading to increased reliance on last-line antibiotics such as daptomycin (2). Daptomycin is a cyclic lipopeptide antibiotic that interacts with bacterial cell membranes but the precise mechanisms of action remain elusive (3). Notably, human infections caused by daptomycin-resistant *S. aureus* have been associated with persistent and complicated infections (2, 4). We and others have recently shown that clinically-derived daptomycin-resistant isolates caused persistent infections in non-mammalian and murine septicaemia models (5, 6), raising the question about the correlation between daptomycin resistance, immune evasion and bacterial survival *in vivo* (5, 6).

Bacteria have evolved highly conserved mechanisms mediating adaptation and maintenance of membrane integrity to defend against host microbicidal peptides (7, 8). Mutations in genes related to phospholipid biosynthesis are consistently reported in Gram-positive bacteria resistant to daptomycin (4). The most abundant phospholipid found in Gram-positive bacterial membranes, including staphylococcal membranes, is phosphatidylglycerol (PG). PG can be converted to cardiolipin (CL) and lysyl-phosphatidylglycerol (L-PG) by the enzymes cardiolipin synthase (Cls) and multiple peptide resistance factor (MprF), respectively (9-11). Gain-of-function mutations in MprF have been associated with daptomycin resistance (8, 9, 12). Most human bacterial pathogens have a Cls homolog that catalyzes condensation of two PG molecules to yield one CL and one glycerol molecule (13). In *S. aureus*, there are two *cls* genes, with

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

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

To assess the functional impact of the reduced daptomycin susceptibility with the *cls2* mutations, we performed daptomycin treatment assays (Fig. 1B). Daptomycin concentrations (2 μg/ml and 4 μg/ml) were chosen to mimic free daptomycin concentration observed in deep tissues and bones under standard human dosing (20, 21). Using a concentration of 2 μg/ml, we showed that daptomycin was rapidly bactericidal (≥ 3 $\log_{10}$ reduction in colony forming units [CFU]) against all strains over 8 hours, however significant regrowth occurred back to the starting inocula by 24 hours for A8819$_{Cls2T33N}$ and A8819$_{Cls2L52F}$ (Fig. 1B). In contrast, killing to undetectable levels was seen for the daptomycin-susceptible parent strain (A8819) and the T33N repaired strain (A8819$_{Cls2T33NN33T}$). Similar findings were observed with daptomycin concentrations up to 4 μg/ml (Fig. S1). Daptomycin treatment failure with A8819$_{Cls2T33N}$ and A8819$_{Cls2L52F}$ directly correlated with progressive resistance, with an MIC of daptomycin increasing up to 4 μg/ml, suggesting that additional adaptation occurred under daptomycin exposure (Fig. 1C). Together, these data show that as seen in patients, the observed *cls2* point mutations genetically predisposed *S. aureus* to resistance evolution and therapeutic failure under antibiotic selection.

Amino acid substitutions in *S. aureus* cardiolipin synthase enhance cardiolipin biosynthesis and alter bacterial membrane phospholipid composition

To determine the impact of the clinically derived *cls2* point mutations on membrane phospholipid composition, we extracted total lipids from the daptomycin-susceptible clinical strain (A8819), the three *cls2* point mutants (A8819$_{Cls2T33N}$, A8819$_{Cls2A23V}$, A8819$_{Cls2L52F}$) and the repaired strain (A8819$_{Cls2T33NN33T}$). We first assessed lipid profiles using thin-layer chromatography (Fig. S2A), which showed that relative to

A8819, the three *cls2* point mutants had an increase in membrane CL content and a reduction in PG, with no change in L-PG. A time-course lipid analysis over bacterial growth phases supported these findings (Fig. S2B, C). The concentration of individual phospholipid species was then quantified using liquid chromatography coupled with mass spectrometry (LC-MS). Consistent with the TLC analysis, the percentage of CL among total phospholipids increased significantly in the three *cls2* point mutants compared to A8819, whereas the PG content decreased significantly (Fig. 2A). None of the *cls2* mutations impacted on L-PG. Overall, the membrane phospholipid changes were most pronounced for $A8819_{Cls2T33N}$ (Fig. 2A), which paralleled the magnitude of daptomycin resistance. Importantly, as seen with daptomycin susceptibility, repair of the T33N mutation restored the membrane phospholipid profile back to wild-type levels (Fig. 2B and Fig. S2D). The increase in CL in the *cls2* point mutants was not secondary to an increase in *cls2* transcription or Cls2 membrane quantity, and Cls2 membrane localization was unchanged compared to A8819 (Fig. S3A-C).

To test whether the *cls2* mutations increased cardiolipin synthesis activity *in vivo*, bacteria were metabolically labelled with ^{13}C-glycerol, which is incorporated into the glycerol backbone and head group of PG and CL (Fig. S4A-C). The formation of complex PG or CL mass isotopomers (+3, +6 and +9) by LC-MS can then be used to measure *de novo* biosynthesis of these phospholipids (Fig. S4A-C). Compared to wild-type, we showed that CL biosynthesis significantly increased over time in our T33N mutant strain (Fig. 2C) and this was mirrored by a reduction in PG biosynthesis (Fig. 2D). Given that PG is both a metabolic end-product and precursor for CL, these results suggest that CL is synthesized from a sub pool of PG in the mutant to account for the different degree of ^{13}C-labelling. No difference in biosynthesis of L-PG was observed (Fig. 2E). Together, these data confirm that clinically relevant Cls2 amino acid

substitutions caused enhanced membrane cardiolipin biosynthesis that was proportional to the degree of antibiotic resistance.

Changes in *S. aureus* membrane composition blocks daptomycin mediated lipid extraction, membrane penetration and disruption

To assess the impact of Cls2-mediated lipid changes on daptomycin-membrane interactions, we reconstituted Gram-positive bacterial bilayer membranes immobilised on a planar surface for characterization by neutron reflectometry (NR) (22, 23) (Fig. S5A). The bilayers were incubated with isotopic solvents (D_2O and H_2O) and the NR profiles of wild-type and A8819$_{Cls2T33N}$ were modelled simultaneously to analyze the bilayer structures (Fig. 3A-B). The cardiolipin-rich membrane caused by the Cls2 T33N amino acid substitution was thicker compared to the wild-type membrane (57.8 ± 3.7 Å versus 49.4 ± 3.3 Å, respectively) (Fig. S5B, Table S2-3, Fig. 3C-D). Despite the overall differences in membrane thickness, the cell surface charge was similar, and using super-resolution microscopy of cell surface bound fluorescent daptomycin, we showed that the level and distribution of daptomycin binding on A8819$_{Cls2T33N}$ cell surface was similar to A8819 (Fig. S6A-D). These data support the hypothesis that structural modification of the membrane impairs daptomycin penetration and membrane disruption rather than initial binding.

Using NR, we then characterised the impact of the observed membrane changes on daptomycin interactions. Daptomycin treatment at 2, 4 and 8 μg/ml significantly shifted the fringe of the NR curves of the wild-type membranes in a concentration dependent manner (Fig. 3A. arrows), indicating extraction and solubilisation of the membrane by daptomycin. In contrast, there was a less pronounced change in reflectivity for the cardiolipin-rich, daptomycin-resistant A8819$_{Cls2T33N}$ membrane (Fig. 3B). Analysis of

220 the NR curves showed that at a daptomycin concentration of 2 μg/ml, the lipid volume
221 fraction of the wild-type membrane was reduced, and no daptomycin was seen within
222 the membrane consistent with a lipid extraction mechanism (24) (Table 1, Table S4).
223 At a higher daptomycin concentration (4 μg/ml), antibiotic membrane penetration was
224 evident, extending through the bilayer and causing dislocation of acyl-chains (Table 1,
225 Table S4). At the highest concentration (8 μg/ml), the membrane was completely
226 solvated by daptomycin, with a substantial decrease in the lipid volume fraction from
227 86.5% to 26.6% (Table 1, Table S4). For the thicker, cardiolipin rich, A8819$_{Cls2T33N}$
228 membrane, only mild lipid extraction was observed at 2, 4 and 8 μg/ml with the lipid
229 volume fraction only reducing from 78.5% to 62.8% (Table1, Table S5). The
230 A8819$_{Cls2T33N}$ membrane resisted daptomycin penetration and remained intact (Table 1,
231 Table S5).
232
233 To further define the daptomycin-membrane interaction, we then used small-angle
234 neutron scattering (SANS), which provided information on the daptomycin aggregation
235 characteristics within the bilayer membranes under physiologically relevant conditions
236 (25). Consistent with our NR data, at a clinically relevant daptomycin concentration of
237 4 μg/ml, we observed daptomycin penetrate and form aggregates straddling the bilayer
238 membrane for wild-type (A8819) but not for the A8819$_{Cls2T33N}$ membranes (Fig. 3E,
239 and F). These data also concur with our bacterial killing data, which showed complete
240 bacterial killing for wild-type A8819 but therapeutic failure toward A8819$_{Cls2T33N}$ (Fig.
241 S1). Using established core-shell and hollow-cylinder models to characterise the
242 daptomycin aggregates (25), it was estimated that daptomycin constituted a 28 ± 0.86
243 Å radius spherical micelle structure within the membrane bilayer (Fig. S7). Taken
244 together, these results show that *S. aureus* adapts during treatment with daptomycin in
245 human infections by increasing its cardiolipin membrane content leading to a thicker

membrane that resists daptomycin lipid extraction, and membrane penetration and disruption, promoting bacterial survival.

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

Apart from impairing daptomycin-membrane interactions, we hypothesised that the altered bacterial membrane phospholipid profile caused by *cls2* point mutations may impact on *S. aureus* – host interactions. Neutrophils are the dominant innate immune cell for controlling *S. aureus* infection (14, 15). To interrogate neutrophil behaviour *in vivo* and in real-time with high resolution, we utilised the vertebrate zebrafish (*Danio rerio*) model system (26). In common with humans, zebrafish have cellular and soluble immune arms, and complex tissue environments that enable a mechanistic understanding of human infectious diseases (26, 27). To assess neutrophil trafficking and recruitment to a localized soft tissue staphylococcal infection, we used transgenic embryos with red fluorescent neutrophils (Tg[*lyz*:dsRed]) (28) that were infected into the somatic muscle with GFP-expressing *S. aureus* (Fig. 4A). Notably, neutrophil recruitment to the localized infection site was significantly compromised for infection with the three *cls2* point mutants compared to infection by A8819 after 6 hours post-infection (Fig. 4B,C). Mutation repair for the T33N strain ($A8819_{Cls2T33NN33T}$) was sufficient to restore neutrophil recruitment back to wild-type (A8819) infection levels (Fig. 4D). This attenuated neutrophil response impacted bacterial clearance, with more persistent infection observed with $A8819_{Cls2T33N}$ compared to the wild-type and repaired strains (Fig. 4E). To further validate the observed neutrophil migration findings in zebrafish, we assessed human neutrophil migration within an ex-vivo assay. As shown in Fig. 4F, significantly less human neutrophil recruitment was observed with $A8819_{Cls2T33N}$ compared to the wild-type strain. Mutation repair of the T33N mutant

(A8819$_{Cls2T33NN33T}$) caused similar neutrophil recruitment as wild-type (Fig. 4F). These results indicated that the Cls2 amino acid substitutions equipped *S. aureus* with the ability to circumvent neutrophil recognition and response, leading to a persistent infection as observed in patients and animal models (2, 5, 6).

Compositional changes of membrane phospholipids resulted in reduced neutrophil recruitment in zebrafish

To investigate the contribution of the *S. aureus* bacterial membrane to neutrophil evasion, total lipids were extracted from each of the *cls2* mutant and the daptomycin-susceptible parent (A8819) and repaired strains. Liposomes were then processed and injected into the otic vesicle of zebrafish (Fig. 4A, white circle). The otic vesicle is normally devoid of leukocytes and is relatively confined, preventing dispersion of the liposomes (29). As shown in Fig. 5A, and similar to that seen with infection of live, whole bacterial cells (Fig. 4B-D), the liposomes from the three *cls2* point mutants were significantly attenuated in inducing neutrophil recruitment compared to liposomes from A8819 and the T33N repaired strain (A8819$_{Cls2T33NN33T}$), suggesting that altered anionic phospholipid composition may be responsible for the immune evasion. To determine the phospholipid driver of this *S. aureus* evasion response, purified PG and CL that were free of DNA, peptidoglycan, wall-teichoic acid, and lipoteichoic acid from A8819 cells (Fig. S8) were processed to form liposomes. Injection of the PG- and CL-specific liposomes into the otic-vesicle of zebrafish embryos showed that PG liposomes induced substantial neutrophil recruitment whereas the recruitment of neutrophils by CL liposomes was comparable to the PBS control (Fig. 5B,C). These data suggest that PG is the major bacterial-mediated phospholipid driver of neutrophil chemoattraction, and the *S. aureus* adaptation response to daptomycin represents a novel bacterial membrane

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

S. aureus has evolved a wealth of strategies to optimise its survival in various host niches and under nocuous selection pressures (30). Here, we report a new metabolic resistance mechanism used by *S. aureus* to prevent membrane lysis by the last line anti-staphylococcal antibiotic, daptomycin. Single amino acid substitutions in cardiolipin synthase 2 led to a significant increase in the bacterial membrane CL/PG ratios due to enhanced cardiolipin biosynthesis, which led to a thicker membrane that resisted daptomycin penetration and membrane disruption. Intriguingly, this adaption also led to immune evasion. Specifically, we showed that membrane PG acted as a bacterial-driven neutrophil chemoattractant and, in the context of *cls2* point mutations, reduced PG membrane content led to less neutrophil trafficking to a localized site of infection and prolonged bacterial survival.

Recent studies on the interactions between daptomycin and bacterial membranes have used indirect methodological approaches such as measuring permeability loss of liposomes exposed to daptomycin using a fluorescence assay, and membrane systems that are less relevant to Gram-positive bacteria, particularly using phosphatidylcholine (PC) and not L-PG (31). It was shown that the addition of cardiolipin to 10% of total lipids (using a PC/PG membrane system) was sufficient to prevent daptomycin interaction with membranes (31), however we have clearly shown that our daptomycin susceptible clinical strain (A8819) already has a greater percent of cardiolipin than 10% (Fig. 2A), suggesting the possibility of discordance between clinically relevant membranes. More recently, alteration of membrane curvature and diverting daptomycin binding, or interference with fluid membrane microdomains have been proposed using the model organism *Bacillus subtilis* or enterococcal strains (7, 32, 33). However, the phospholipid membrane composition of these organisms is different to *S. aureus*, and

daptomycin-membrane interactions appear to be bacterial-species dependent. For example, daptomycin has an irregular membrane binding pattern in *B. subtilis* and *Enterococcus*, with a particular predilection for the division septum (7, 32, 33), whereas we have observed universal distribution of daptomycin on *S. aureus* membranes (Fig. S6C-D).

Here, we have reconstituted clinically relevant *S. aureus* membranes and have provided the first direct structural analysis of daptomycin-membrane interactions. The scattering of free neutrons by matter provides excellent structural detail and this experimental approach has only recently been used to study biological membranes (25, 34). We used unsaturated phospholipids with complex head group composition to reconstitute the membranes, and the surface coverage achieved for the wild-type membranes was 86.5% (Table 1), which is higher than what has been previously reported using unsaturated phospholipids with similar techniques (35). Using SANS and NR, we identified three distinct modes of action of daptomycin on the membrane. At lower concentrations (2 μg/ml), lipid extraction occurred and caused lesions in the bilayer membrane, whereas at higher concentrations (4 μg/ml), daptomycin molecules penetrated into the bilayer and formed organised micelles. At the highest concentrations (8 μg/ml), the bilayer was completely solvated by daptomycin. The process of lipid extraction, penetration, micelle formation and membrane lysis was inhibited in the context of the clinically derived Cls2 amino acid substitution T33N, which coincided with daptomycin resistance. Interestingly, the daptomycin molecules still attached to the outer leaflet of the membrane in the $A8819_{Cls2T33N}$ strain, and this was corroborated with super-resolution imaging of fluorescent daptomycin binding to bacterial cells, but the daptomycin was functionally impaired due to the barrier created by the thicker CL-rich membrane. This is an anionic phospholipid driven mechanism of daptomycin resistance

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

antimicrobial attack, providing important insights for future therapeutic targeting for this troublesome pathogen.

Materials and Methods

Media and reagents

Bacterial strains, plasmids, and oligonucleotides used in this study are described in Table S6. *S. aureus* ~~and *Escherichia coli*~~ cells were cultured in heart infusion broth (HI) (Oxoid) ~~and Luria-Bertani broth~~ with constant shaking at 37 ºC~~, respectively~~.

Genetic manipulation

The vector pIMAY and the *Escherichia~~-~~ coli* strain DC10B were used to genetically manipulate *S. aureus* isolate ~~A8819~~ following the published protocol (SI Materials and Methods) (38).

Daptomycin susceptibility testing

Broth microdilution MIC testing was performed based on guidelines by the Clinical and laboratory standards institute (CLSI). Daptomycin MIC > 1 µg/ml is officially termed daptomycin-nonsusceptible, but was termed daptomycin resistant throughout the manuscript for clarity. Time-kill assays were performed with an initial bacterial inoculum of 10^6 CFU/ml in Mueller-Hinton broth supplemented with 50 mg/litre calcium and daptomycin. (SI Materials and Methods).

Lipid analysis by mass spectrometry

Lipids were extracted ~~and analysed using thin-layer chromatography~~ following the published protocol (10). Lipid species were processed and quantified according to 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 ºC.
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 ANSTO.
429 (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 ± 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 ± 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 (4μg/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 = 250μm. (*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 ± 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 emigrated neutrophils (*C*). For PBS, n = 10; PG, n = 17; CL, n = 17, pooled from three independent experiments. Error bars represent the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to PBS for (*A*) and (*C*), one-way ANOVA.