nature microbiology

LETTERS

PUBLISHED: 24 OCTOBER 2016 | VOLUME: 2 | ARTICLE NUMBER: 16194

Staphylococcus aureus inactivates daptomycin by releasing membrane phospholipids

Vera Pader, Sanika Hakim, Kimberley L. Painter, Sivaramesh Wigneshweraraj, Thomas B. Clarke and Andrew M. Edwards*

Daptomycin is a bactericidal antibiotic of last resort for serious infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA)[1,2]. Although resistance is rare, treatment failure can occur in more than 20% of cases[3,4] and so there is a pressing need to identify and mitigate factors that contribute to poor therapeutic outcomes. Here, we show that loss of the Agr quorum-sensing system, which frequently occurs in clinical isolates, enhances *S. aureus* survival during daptomycin treatment. Wild-type *S. aureus* was killed rapidly by daptomycin, but Agr-defective mutants survived antibiotic exposure by releasing membrane phospholipids, which bound and inactivated the antibiotic. Although wild-type bacteria also released phospholipid in response to daptomycin, Agr-triggered secretion of small cytolytic toxins, known as phenol soluble modulins, prevented antibiotic inactivation. Phospholipid shedding by *S. aureus* occurred via an active process and was inhibited by the β-lactam antibiotic oxacillin, which slowed inactivation of daptomycin and enhanced bacterial killing. In conclusion, *S. aureus* possesses a transient defence mechanism that protects against daptomycin, which can be compromised by Agr-triggered toxin production or an existing therapeutic antibiotic.

Staphylococcus aureus encodes multiple virulence factors, many of which are controlled by Agr (refs 5,6), a quorum-sensing system encoded by a four-gene operon (*agrBDCA*) and a gene encoding a regulatory RNA (RNAIII). However, invasive *S. aureus* infections often give rise to Agr-defective mutants, typically involving *agrA* or *agrC*, which are hypothesized to provide a selective advantage in the presence of antibiotics[7–14]. To test this hypothesis, we determined the killing kinetics of wild-type *S. aureus* or *agr* mutants by clinically relevant antibiotics.

Agr status did not affect the rate of staphylococcal killing by vancomycin, oxacillin or gentamicin (Supplementary Figs 1 and 2). By contrast, although wild-type *S. aureus* was killed by daptomycin, loss of quorum-sensing components of Agr (AgrA or AgrC) enabled *S. aureus* strains USA300 or SH1000 to survive in the presence of daptomycin during the first 8 h of exposure (Fig. 1a,b). A mutant lacking the regulatory RNAIII component of *agr* was killed as efficiently as the wild type (Fig. 1a), as were Δ*agrA* or Δ*agrC* mutants complemented with the relevant genes on plasmids (Supplementary Fig. 3). After the initial period of killing, colony-forming unit (c.f.u.) counts of both wild-type and *agr*-mutant *S. aureus* recovered to similar levels by 24 h, without the acquisition of resistance, explaining why all strains had identical daptomycin minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values (Fig. 1c, Supplementary Table 1 and Supplementary Fig. 4). This biphasic killing and subsequent recovery profile is similar to several previously reported daptomycin killing assays, although the contribution of Agr to this phenomenon was unknown[15–17].

In addition to *agr*-deletion mutants, clinical isolates with dysfunctional Agr systems were also less susceptible to daptomycin than strains with functional Agr, indicating a potential role in treatment failure (Supplementary Fig. 5). To test this, mice were inoculated via the intraperitoneal route[18] and then treated with daptomycin. Similar to results reported previously[19], daptomycin treatment reduced the size of the wild-type population 15-fold by 8 h when compared with mice treated with PBS alone (Fig. 1d). By contrast, daptomycin did not significantly decrease the size of the Δ*agrA* mutant population (Fig. 1d). Therefore, the loss of Agr activity confers a selective advantage to *S. aureus* during daptomycin exposure, resulting in treatment failure.

Staphylococcal infections are often caused by mixed populations of *agr* mutants and wild-type bacteria[8,10–12] and we therefore explored how this affected the daptomycin susceptibility of the whole population. Using various ratios of wild-type and Δ*agrA* mutant *S. aureus*, we found that larger *agr*-mutant subpopulations resulted in greater survival of both the Agr-defective strain and the wild type (Fig. 1e and Supplementary Fig. 6). Similarly, we found that the presence of the Δ*agrA* mutant in the peritoneal cavity of mice protected wild-type bacteria from daptomycin (Fig. 1f), providing additional evidence that *agr*-mutants can promote daptomycin treatment failure.

To understand the mechanism by which *S. aureus agr* mutants survived daptomycin exposure, we determined the antibiotic activity in spent culture supernatants. After 8 h incubation with wild-type USA300, daptomycin activity was reduced 50%, although this was still sufficient for bacterial killing (Fig. 1g,h and Supplementary Fig. 7). However, there was a rapid and total loss of daptomycin activity in cultures of the Δ*agrA* mutant and the antibiotic was no longer bactericidal (Fig. 1g,h). In keeping with this, culture supernatant from the USA300 Δ*agrA* mutant exposed to daptomycin protected wild-type bacteria from subsequent daptomycin challenge both *in vitro* and *in vivo* (Supplementary Fig. 8). Similar findings were obtained with *S. aureus* SH1000, where the Δ*agrA* mutant inactivated daptomycin within 4 h, whereas this did not occur until 8 h in the wild-type (Supplementary Fig. 9). These experiments indicated that daptomycin was inactivated by a secreted factor, which we confirmed by showing that the supernatant from the Δ*agrA* mutant exposed to daptomycin inactivated a subsequent dose of the antibiotic, whereas supernatant from bacteria not exposed to the antibiotic did not (Supplementary Fig. 10).

To identify the secreted daptomycin inactivator we examined culture supernatant from the Δ*agrA* mutant and found that its capacity to inactivate the antibiotic was not sensitive to heat or proteases, suggesting an enzyme-independent process (Fig. 2a). Daptomycin targets phosphatidylglycerol (PG), the most abundant lipid in the staphylococcal membrane, triggering membrane

MRC Centre for Molecular Bacteriology and Infection, Imperial College London, Armstrong Road, London SW7 2AZ, UK. *e-mail: a.edwards@imperial.ac.uk

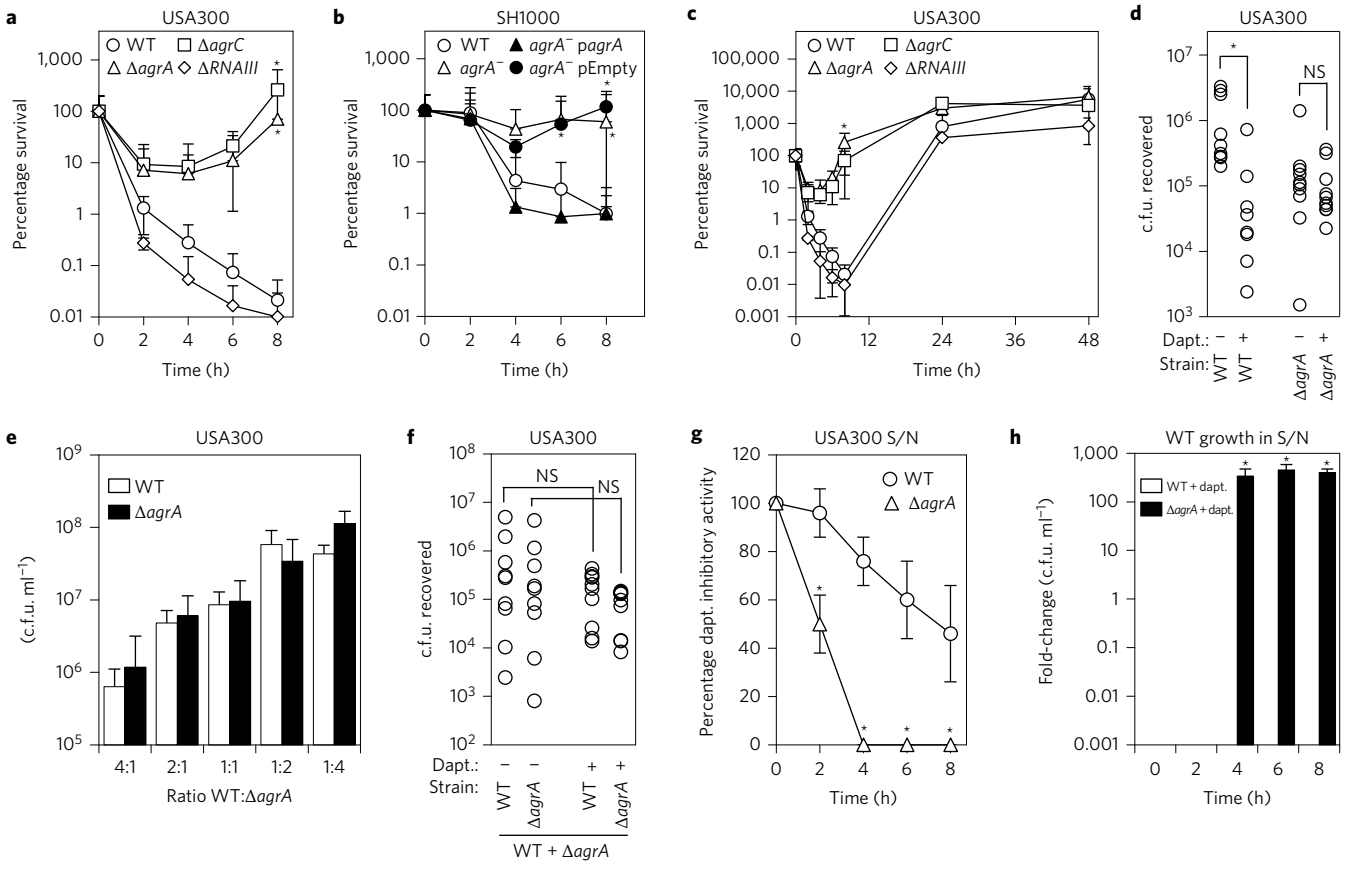

Figure 1 | Agr-defective *S. aureus* survives daptomycin exposure by inactivating the antibiotic. a,**b**, Survival of USA300 wild-type (WT) (**a**) or SH1000 WT (**b**) or *agr* mutants over 8 h in the presence of 20 μg ml^{-1} daptomycin ($n = 3$ in duplicate; for WT versus *agrA* or *agrC*, $^*P < 0.001$ at indicated time points). **c**, Survival of USA300 WT or *agr* mutants over 48 h in the presence of 20 μg ml^{-1} daptomycin ($n = 3$ in duplicate; for WT versus *agrA* or *agrC*, $^*P < 0.0001$ at indicated time points). **d**, c.f.u. counts of USA300 WT or Δ*agrA* mutant after 8 h in the peritoneal cavity of mice, treated with 20 μg ml^{-1} daptomycin (Dapt.) (+) or PBS only (–) ($^*P < 0.05$; NS $P > 0.05$; each circle represents a single mouse; for WT groups $n = 9$ and for Δ*agrA* groups $n = 10$). **e**, Survival of a mixed population of USA300 WT and Δ*agrA* mutant at various ratios after 8 h in the presence of 20 μg ml^{-1} daptomycin ($n = 3$ in duplicate). **f**, c.f.u. counts from the peritoneal cavity of mice infected with WT *S. aureus* or a 1:1 mixture of WT and Δ*agrA* mutant bacteria after 8 h of treatment with 20 μg ml^{-1} daptomycin (+, $n = 10$) or PBS only (–, $n = 9$) (NS, $P > 0.05$; each circle represents a single mouse). **g**,**h**, Daptomycin activity in culture supernatants (S/N) from WT or Δ*agrA* mutant exposed to the antibiotic using a zone of inhibition assay (**g**) or bactericidal assay (**h**) ($n = 3$ in duplicate; WT versus *agrA* supernatant, $^*P < 0.0001$ at indicated time points). Data in **a**–**c**,**g**,**h**, were analysed using a two-way ANOVA with Dunnett's post-hoc test. Data in **d**,**f** were analysed by Mann–Whitney test. Where shown, error bars represent the standard deviation of the mean.

blebbing without lysis[20–24]. We thus hypothesized that membrane phospholipids released into the extracellular space could bind and inactivate daptomycin, as has been demonstrated for pulmonary surfactant[25]. In support of this hypothesis, treatment of Δ*agrA* culture supernatant with phospholipases or Triton X-100 significantly impaired daptomycin inactivation (Fig. 2a). Analysis of culture supernatants revealed that daptomycin triggered the release of lipid from the Δ*agrA* mutant (Fig. 2b and Supplementary Fig. 11) and we confirmed the presence of membrane phospholipid using thin-layer chromatography (TLC, Supplementary Fig. 12). This identified PG as the most abundant lipid species (>65% total phospholipid), along with a small quantity of lysyl-PG (LPG) and cardiolipin (CL) (Supplementary Table 2 and Supplementary Fig. 12). At concentrations similar to those found in the culture supernatant, purified PG inactivated daptomycin in a dose-dependent manner (Fig. 2c). By contrast, LPG only inhibited daptomycin activity at supraphysiological concentrations and CL had no effect on the antibiotic (Fig. 2c). Furthermore, a mixture of PG, LPG and CL at the approximate ratio found in the membrane[24] efficiently inactivated daptomycin (Fig. 2d). In keeping with these findings, purified PG, and to a lesser extent LPG, protected *S. aureus* from daptomycin, whereas CL did not (Fig. 2e). Together, these data confirm that released membrane PG was responsible for inactivating the antibiotic.

Next, we investigated whether the presence of phospholipids in the supernatant occurs via an active process or was simply a consequence of membrane damage caused by the antibiotic. The presence of inhibitors of respiration, protein biosynthesis or lipid biosynthesis significantly reduced lipid release in response to daptomycin, as did reducing the incubation temperature to 4 °C (Fig. 2f). By contrast, inhibitors of protein biosynthesis or respiration did not block lipid released from bacteria due to damage caused by lysostaphin or high temperature (Supplementary Fig. 13). We therefore concluded that lipid released in response to daptomycin occurred via an active mechanism.

Previous work with *Escherichia coli* suggested that outer membrane vesicles provide protection against membrane-targeting antimicrobials[26]. However, most of the phospholipid released by *S. aureus* existed as monomers or small micelles and, although some of the released phospholipid was in the form of membrane vesicles, these contained very little daptomycin and were inefficient at inactivating the antibiotic (Supplementary Fig. 14).

We then determined why *S. aureus* lacking a functional Agr system, but not wild type, inactivated daptomycin. The simplest

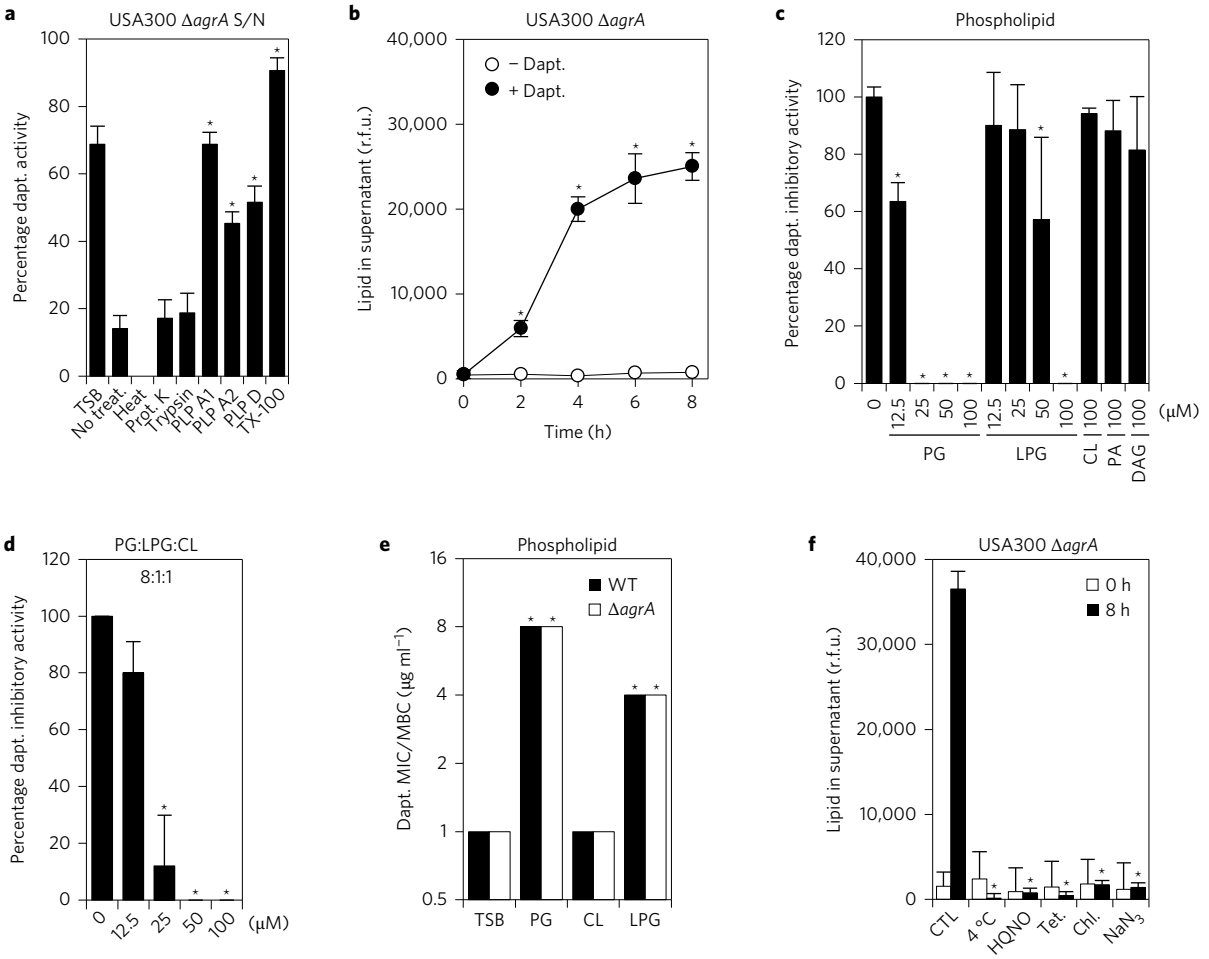

Figure 2 | Agr-defective *S. aureus* inactivates daptomycin by shedding membrane phospholipid. a, Daptomycin activity in TSB only (TSB) or culture supernatant from USA300 Δ*agrA* exposed to daptomycin and subsequently treated with heat (80 °C, 20 min), proteinase K (Prot. K, 10 μg ml^{-1}), trypsin (10 μg ml^{-1}), phospholipases (PLP) A1, A2 or D (each at 5 U ml^{-1}) or 0.1% Triton X-100 (TX-100) ($n = 4$, in duplicate; for untreated samples versus phospholipase or TX-100-treated samples, $^*P < 0.0001$). **b**, Lipid shedding from USA300 Δ*agrA* mutant exposed, or not, to 20 μg ml^{-1} daptomycin ($n = 3$ in duplicate; for daptomycin-treated versus untreated, $^*P < 0.0001$ at indicated time points). **c**, Activity of daptomycin after incubation with purified phosphatidylglycerol (PG), lysyl-phosphatidylglycerol (LPG), cardiolipin (CL), phosphatidic acid (PA) or diacylglycerol (DAG) at the indicated concentrations ($n = 3$ in duplicate; for TSB without lipid versus indicated lipids, $^*P < 0.0001$). **d**, Activity of daptomycin after incubation with a mixture of purified PG, LPG and CL (8:1:1 ratio), which approximates that found in the staphylococcal membrane ($n = 3$ in duplicate; for TSB without lipid versus indicated lipid concentrations, $^*P < 0.0001$). **e**, Daptomycin MIC/MBC of WT or Δ*agrA* mutant in the presence of 10 μM purified PG, CL or LPG ($n = 3$ in duplicate; data represent the median values, for TSB without lipid versus indicated lipids, $^*P < 0.0001$. **f**, Lipid shedding from USA300 Δ*agrA* mutant exposed to 20 μg ml^{-1} daptomycin at 37 °C (CTL), 4 °C, respiratory inhibitors 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) or NaN_3, or antibiotics tetracycline (Tet.) or chloramphenicol (Chl.) ($n = 3$ in duplicate; for TSB only (CTL) at 8 h versus TSB containing inhibitors or at 4 °C, $^*P < 0.0001$). Data in **a**,**c**,**d**,**e**,**f** were analysed using a one-way ANOVA with Tukey's post-hoc test. Data in **b** were analysed using a two-way ANOVA with Dunnett's post-hoc test. Where shown, error bars represent standard deviation of the mean.

explanation was that the wild type did not release lipids in response to daptomycin. However, exposure of wild-type *S. aureus* to daptomycin resulted in release of phospholipid at similar levels and composition to the Δ*agrA* mutant, demonstrating that the Agr quorum-sensing system does not influence lipid release (Supplementary Figs 12, 14 and 15 and Supplementary Table 2). Using pulldown assays, we discovered that daptomycin bound to phospholipid in culture supernatant from Δ*agrA* but not wild-type *S. aureus* (Supplementary Fig. 15). Because Agr triggers the secretion of numerous products[5,6], we hypothesized that these prevented the binding of daptomycin by phospholipid from wild-type bacteria. In support of this hypothesis, we discovered that spent culture supernatant from wild-type bacteria prevented Δ*agrA* bacteria from inactivating daptomycin (Supplementary Fig. 16). Further analysis determined that the agent responsible was heat-sensitive and passed through a 3 kDa cutoff filter, indicative of a small peptide (Supplementary Fig. 16). AgrA regulates the expression of seven small peptide cytolysins known as phenol soluble modulins (PSMs), categorized into alpha (PSMα1–4) or beta (PSMβ1,2) families[27,28]. Given the surfactant properties of PSMs, we hypothesized that they prevented the sequestration of daptomycin by phospholipids.

Mutants in both the USA300 and SH1000 backgrounds lacking PSMα cytolysins inactivated daptomycin and survived exposure to the antibiotic in a similar manner to *agr*-mutants (Fig. 3a,b and Supplementary Fig. 17). By contrast, PSMβ-defective mutants were impaired in daptomycin inactivation and were as susceptible to the antibiotic as the wild type, despite releasing similar levels of phospholipid to the Δ*psm*α mutant (Fig. 3a–c; Supplementary Fig. 11). Supplementation of the culture medium with a mixture of synthetic PSMα, but not PSMβ, peptides restored killing of Δ*psm*αβ mutants (Fig. 3d,e and Supplementary Fig. 18). Of all the

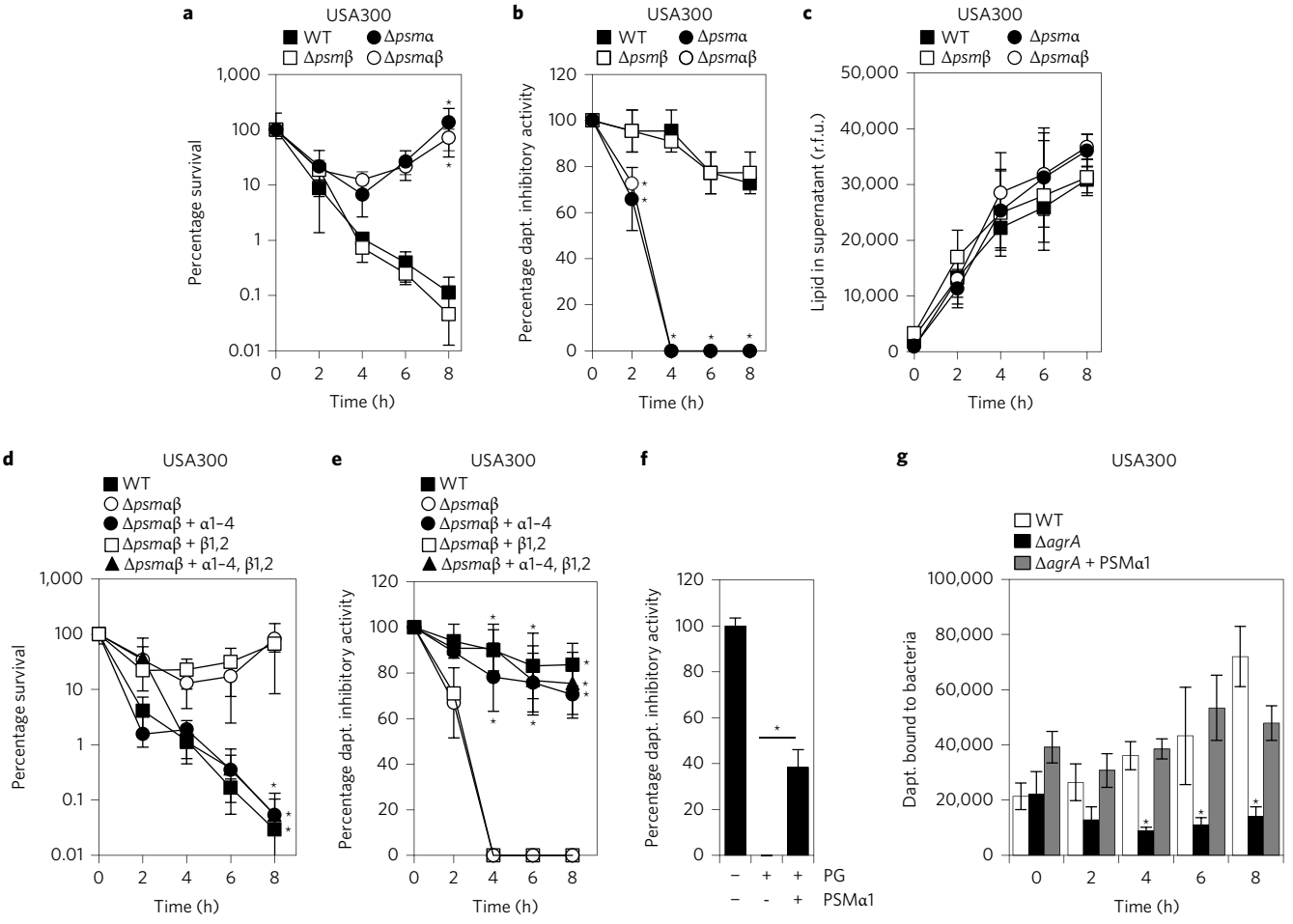

Figure 3 | Alpha phenol soluble modulins prevent daptomycin inactivation by *S. aureus*. **a**, Survival of USA300 WT or *psm* mutants during exposure to 20 μg ml^{-1} daptomycin. **b**, Activity of the antibiotic in associated culture supernatants. In both cases $n = 3$ in duplicate; for WT versus Δ*psm*α or Δ*psm*αβ, $^*P < 0.0001$ at indicated time points. In **b**, open circles are obscured by filled circles at 4, 6 and 8 h. **c**, Quantity of lipid shed from USA300 WT or *psm* mutants incubated with daptomycin in **a**. **d**, Survival of the USA300 *psm*αβ mutant in the absence or presence of mixtures of PSMα peptides (α1–4), PSMβ peptides (β1,2) or PSMα and PSMβ (α1–4, β1,2). The survival of WT *S. aureus* in the absence of peptides is included for comparison ($n = 3$ in duplicate; for Δ*psm*αβ without peptides versus Δ*psm*αβ with PSMα peptides, $^*P < 0.0001$ at the indicated time points). Filled triangles are obscured by filled squares and filled circles. **e**, Activity of daptomycin in culture supernatants from the experiment described in **d** ($n = 3$ in duplicate; for Δ*psm*αβ without peptides versus Δ*psm*αβ with PSMα peptides, $^*P < 0.0001$ at the indicated time points). **f**, Activity of daptomycin in the presence of 25 μM phosphatidylglycerol (PG) ± 20 μM PSMα1 ($n = 4$; for PG without PSMα1 versus PG with PSMα1, $P < 0.01$). **g**, Binding of BoDipy-labelled daptomycin to WT, Δ*agrA* mutant or Δ*agrA* mutant in the presence of PSMα1 ($n = 3$ in triplicate; for WT versus *agrA*, $^*P < 0.001$ at the indicated time points). Data in **a**–**e**,**g** were analysed using a two-way ANOVA with Dunnett's post-hoc test. Data in **f** were analysed using Student's *t*-test. Where shown, error bars represent standard deviation of the mean.

PSMs, PSMα1 was most effective at preventing daptomycin inactivation by the USA300 Δ*psm*αβ mutant and was also able to prevent the inactivation of daptomycin by USA300 Δ*agrA* mutant and purified PG, most probably via its surfactant properties (Fig. 3f and Supplementary Fig. 18). To test whether PSMα1 bound to released phospholipid we measured the haemolytic activity of the toxin in the presence of shed membrane phospholipid, or purified PG. The presence of phospholipid completely inhibited the haemolytic activity of PSMα1, indicating direct binding of the cytolysin to the phospholipid (Supplementary Fig. 19). Based on these findings, we hypothesized that PSMα peptides enhanced daptomycin-mediated killing of *S. aureus* by preventing antibiotic sequestration by the released phospholipid. Using BoDipy-tagged daptomycin, we found that wild-type *S. aureus* steadily accumulated daptomycin over time (Fig. 3g). By contrast, binding of daptomycin to *S. aureus* Δ*agrA* decreased over time. In the presence of PSMα1 peptide, however, daptomycin bound strongly to the Δ*agrA* mutant, demonstrating that this cytolysin promoted binding of the antibiotic to bacteria, as observed for the wild type (Fig. 3g).

Daptomycin shares functional similarities with cationic antimicrobial peptides (CAMPs) and we therefore hypothesized that lipid shedding may also confer protection against these membrane-acting antimicrobials. We found that lipid shedding by the Δ*agrA* mutant was triggered by both nisin and melittin, which target the staphylococcal membrane, and that PG inactivated the antimicrobial peptides (Supplementary Fig. 20). These findings suggest that CAMPs may have provided a selection pressure for the evolution of the phospholipid-shedding response to membrane damage.

It has been reported previously that β-lactam antibiotics at sub-inhibitory concentrations promote daptomycin activity against wild-type *S. aureus* by increasing the binding of the lipopeptide to the bacterial membrane[29,30]. We decided, therefore, to determine if this combination therapy could prevent treatment failure of infection caused by the presence of *agr*-mutant *S. aureus*. A sub-inhibitory concentration of the β-lactam antibiotic oxacillin enhanced killing of the *S. aureus* Δ*agrA* mutant by daptomycin, by reducing the rate of lipid shedding and therefore prolonging the activity of the lipopeptide antibiotic (Fig. 4a–c). By contrast, oxacillin did

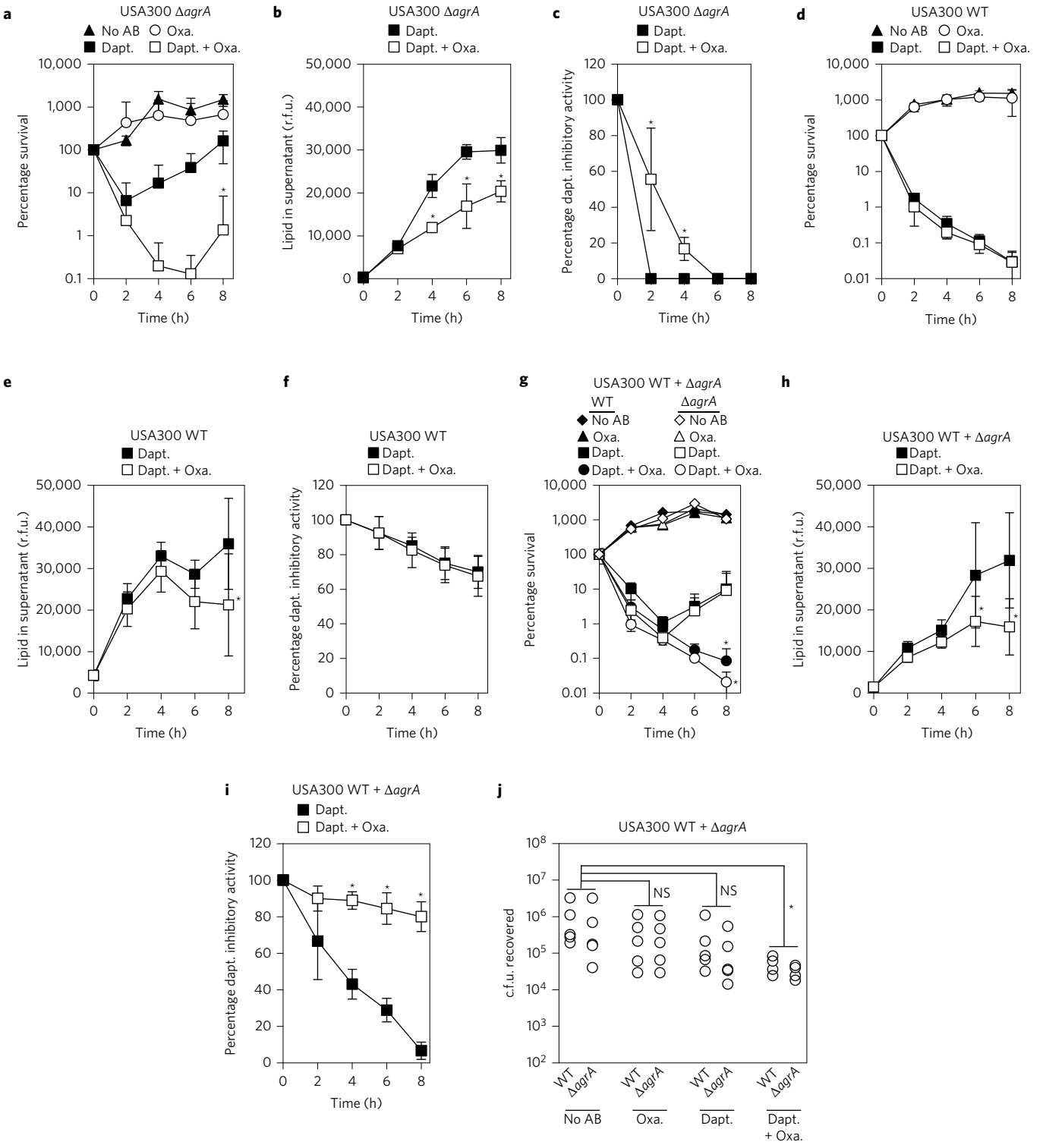

Figure 4 | Oxacillin prevents lipid shedding and daptomycin inactivation. a–c, USA300 Δ*agrA* was exposed to a sub-inhibitory concentration (0.25 μg ml^{-1}) of oxacillin (Oxa.), daptomycin (20 μg ml^{-1}) (Dapt.), both antibiotics (or neither) in combination and survival (**a**), lipid release (**b**) or daptomycin activity (**c**) were measured over 8 h ($n = 3$ in duplicate; for TSB containing daptomycin only versus daptomycin + oxacillin, $^*P < 0.001$ at the indicated time points). **d–f**, USA300 WT exposed to 0.25 μg ml^{-1} oxacillin, daptomycin (20 μg ml^{-1}), both antibiotics (or neither) in combination and survival (**d**), lipid release (**e**) or daptomycin activity (**f**) were measured after 8 h ($n = 3$ in duplicate; for TSB containing daptomycin only versus daptomycin + oxacillin $^*P < 0.05$ at the indicated time points). **g–i**, USA300 WT and Δ*agrA* at a 1:1 ratio were exposed to oxacillin, daptomycin, both antibiotics (or neither) in combination, and survival (**g**), lipid release (**h**) or daptomycin activity (**i**) were measured over 8 h ($n = 3$ in duplicate; for TSB containing daptomycin only versus daptomycin + oxacillin, $^*P < 0.0001$ at the indicated time points). **j**, c.f.u. counts from the peritoneal cavities of mice after 8 h treatment with oxacillin, daptomycin, neither or both antibiotics in combination ($^*P < 0.05$, NS $P > 0.05$; each circle represents a single mouse; for each group $n = 4$–5). Data in **a–j** were analysed by two-way ANOVA with Dunnett's post-hoc test. Where shown, error bars represent standard deviation of the mean.

not show synergy with daptomycin against wild-type *S. aureus*, despite reducing lipid release, presumably because the lipid-shedding mechanism is already compromised by PSM production (Fig. 4d–f). Similar to the Δ*agrA* mutant alone, daptomycin was significantly more effective against mixed populations of wild-type *S. aureus* and Δ*agrA* mutant in the presence of oxacillin both *in vitro* and in an *in vivo* model of invasive infection, suggesting that daptomycin/β-lactam combination therapy would reduce treatment failure due to *agr*-mutant *S. aureus* (Fig. 4g–j).

In conclusion, this work has identified membrane phospholipid shedding as a rapid and transient defence used by *S. aureus* against an antibiotic of last resort (Supplementary Fig. 21 and Supplementary Discussion). However, this defence mechanism is compromised by Agr-regulated toxins, providing a selective advantage to bacteria with *agr*-inactivating mutations during daptomycin exposure. Furthermore, there are several host factors that inhibit Agr activity[14], indicating that wild-type bacteria may successfully employ this defensive system during certain types of infection. To combat the sequestration of daptomycin by phospholipids, we describe a simple dual therapy approach of combining daptomycin with oxacillin. The presence of this β-lactam reduces the rate of lipid shedding, slows daptomycin inactivation and thereby reduces the likelihood of treatment failure.

Methods

Bacterial culture. The bacterial strains and plasmids used in this study are detailed in Supplementary Table 3. *S. aureus* was grown, unless otherwise stated, in tryptic soy broth (TSB) at 37 °C, with shaking (180 r.p.m.) Where required, culture medium was supplemented with kanamycin (50 μg ml^{-1}), chloramphenicol (10 μg ml^{-1}) or tetracycline (10 μg ml^{-1}). Staphylococcal c.f.u. were enumerated by serial dilution and plating onto tryptic soy agar (TSA). *E. coli* were grown in Luria broth at 37 °C, with shaking at 180 r.p.m. or on LB agar with ampicillin (100 μg ml^{-1}) as required.

Construction of *agr* mutants. Mutants deficient in *RNAIII*, *agrA* or *agrC* were generated in USA300 LAC with pIMAY as described previously[31,32]. Primers, detailed in Supplementary Table 4, were used to amplify DNA corresponding to approximately 500 bp up- and downstream of the coding regions to be deleted (Genbank accession no. CP000255). Primers were designed to include regions of homology that facilitated fusion of amplicons using the Gibson assembly kit (New England Bioscience). The resulting product was digested at the 5′ and 3′ ends using KpnI and SacI and ligated into similarly digested pIMAY, before transformation into *E. coli* DC10B. Constructs were recovered from DC10B using a Qiagen midiprep kit and transformed into electrocompetent *S. aureus* USA300 LAC (refs 31,32). Mutants were selected for using anhydrotetracycline, as described previously[31,32], on blood agar plates to identify *agr* mutants defective for haemolysis. This approach resulted in full, markerless deletion of *agrA* or *RNAIII*, and deletion of nucleotides 127–1119 from *agrC*, as confirmed by PCR using oligonucleotides labelled as 'Out Fwd' or 'Out Rev' in Supplementary Table 4 to amplify and sequence the mutated locus. DNA sequencing confirmed loss of relevant regions of DNA without the acquisition of mutations in flanking regions. Mutations in *agrA* or *agrC* were complemented using plasmid pCN34 containing the *agrA* or *agrC* gene under the control of the P2 promoter[33,34].

Determination of antibiotic MIC and MBC. MICs were determined using the broth microdilution protocol[35,36]. Stationary-phase *S. aureus* grown in TSB was adjusted to 5×10^5 c.f.u. ml^{-1} in fresh TSB or Muller–Hinton Broth (MHB), each containing $CaCl_2$ (50 μg ml^{-1}) and a range of concentrations of daptomycin. After static incubation at 37 °C in air for 18 h, the MIC was defined as the lowest concentration at which there was no visible growth[35,36]. MBC values were defined as the lowest concentration of antibiotic required for a >1,000-fold reduction in c.f.u. counts compared to the inoculum[37,38]. In some cases, MIC or MBC assays were done in TSB supplemented with 10 μM purified lipids.

Spent culture supernatant bactericidal activity assay. Bacterial cells were removed from spent culture supernatant by centrifugation (17,000*g*, 10 min) and filtration through a 0.2 μm filter. Wild-type USA300 was then added to the supernatant to a density of 5×10^5 c.f.u. ml^{-1} and incubated statically at 37 °C in air for 18 h. Bacterial c.f.u. were then determined by serial dilution and plating onto TSA.

Determination of antibiotic bactericidal activity. Stationary-phase bacteria were inoculated into 3 ml TSB containing antibiotics to ~10^8 c.f.u. ml^{-1}: daptomycin (20 μg ml^{-1}) with $CaCl_2$ (0.5 mM); gentamicin (10 μg ml^{-1}); vancomycin (10 μg ml^{-1}) or cloxacillin (1.25 μg ml^{-1}) or antimicrobial peptides nisin (20 μg ml^{-1}) or melittin (50 μg ml^{-1}). Cultures were subsequently incubated at 37 °C with shaking (180 r.p.m.) and bacterial viability determined by c.f.u. counts. Stationary-phase bacteria were used as this is when Agr is active *in vitro*. Most experiments used monocultures of strains, but some used mixtures of wild-type and *agr*-mutant bacteria, with the inoculum maintained at a total of 10^8 c.f.u. ml^{-1}. All concentrations used fall within previously described free serum concentrations from humans or animal models given clinically relevant therapeutic doses and were at least 2.5× MIC, which has been shown previously to be bactericidal for the antibiotics used[39–44].

In some assays the respiratory inhibitors 2-*n*-heptyl-4-hydroxyquinoline N-oxide (HQNO, 10 μg ml^{-1}) or sodium azide (0.05%) were included, or the protein synthesis inhibitors tetracycline (10 μg ml^{-1}) or chloramphenicol (20 μg ml^{-1}). Other assays included the β-lactam antibiotic oxacillin (0.25 μg ml^{-1}) or the lipid biosynthesis inhibitor platensimycin (7.5 μg ml^{-1}).

In several cases, the culture supernatant was recovered for analysis of daptomycin activity or lipid content. Bacteria were removed by centrifugation (17,000*g*, 5 min) and filtration through a 0.2 μm filter.

In other assays, bacteria were pelleted by centrifugation (17,000*g*, 5 min), the supernatant removed and the bacteria resuspended in an equal volume of filter sterilized spent culture supernatant from a different strain, before immediate use in assays.

Daptomycin activity determination. A well of 10 mm was made in TSA plates containing 50 μg ml^{-1} $CaCl_2$ followed by the spreading of 50 μl stationary-phase wild-type USA300 LAC (~10^6 c.f.u. ml^{-1}) in TSB across the surface. The spread bacterial inoculum was allowed to air dry and the wells were filled with supernatant from cultures that had been processed by centrifugation (17,000*g*) and filter sterilization (0.2 μm filter) to remove bacterial cells. Plates were incubated for 16 h at 37 °C before the zone of growth inhibition around the well was measured at four perpendicular points. In some cases, fresh daptomycin was added to culture supernatants, incubated for 0–8 h at 37 °C, and antibiotic activity was determined as described above. In other experiments, culture supernatants were pretreated with heat (80 °C, 20 min) or Triton X-100 (0.1%), phospholipases (5 U ml^{-1}) or proteases (10 μg ml^{-1}) for 16 h at 37 °C before the addition of fresh daptomycin. In addition to culture supernatants, daptomycin activity was determined in the presence of purified lipids and/or PSMα1 peptide at the concentrations indicated in the figure. All experiments used the same batch of agar plates and a standard plot was generated for each experiment using a range of daptomycin concentrations, enabling the conversion of the size of the zone of inhibition into percentage daptomycin activity.

Murine infection model. Animal work was conducted in accordance with the Animals (Scientific Procedures) Act 1986 outlined by the UK Home Office regulations. Work was approved by the UK Home Office after ethical approval by the Imperial College Animal Welfare and Ethical Review Body (AWERB). Six- to eight-week-old female C57BL/6 mice (Charles River) were infected via the intraperitoneal route with 2.5×10^7 c.f.u. USA300 LAC or a 1:1 mixture of wild-type and Δ*agrA* mutant USA300 LAC (total of 2.5×10^7 c.f.u.). For the indicated experiment, wild-type bacteria were resuspended in spent culture supernatant from either wild-type or *agrA* bacteria exposed to daptomycin. Mice were then treated with 250 μl PBS containing daptomycin (40 μg ml^{-1}) and calcium (0.5 mM), with or without oxacillin (0.5 μg ml^{-1}), injected into the peritoneal cavity (that is, the daptomycin concentration within the peritoneal cavity was ~20 μg ml^{-1} and, where used, oxacillin was present at 0.25 μg ml^{-1}). Control groups were treated with PBS alone. Experiments were continued for 8 h before animals were humanely killed by cervical dislocation, and death was confirmed by severing the femoral artery. The peritoneal cavity was washed with PBS, and c.f.u. counts were determined on blood agar plates. Wild-type and Δ*agrA* mutant bacteria were differentiated on the basis of haemolytic activity. The size of the groups used was determined before undertaking the experiment using power analysis based on *in vitro* data[45]. Tubes containing the inocula and antibiotic solutions were blinded before commencing the experiment. Mice were randomly allocated to individual study group cages by animal husbandry technicians who were not involved in the study. Each group was randomly allocated to a treatment. In keeping with Home Office regulations, any animals that displayed two or more of the following signs were humanely killed by a Schedule 1 method and excluded from the study: reduced movement, hunched posture, shivering, dyspnoea or cyanosis or circling.

Bacterial growth and Agr activity assays. Bacteria were inoculated into the wells of black microtitre plates with clear bottoms containing 200 μl TSB to a concentration of ~10^7 c.f.u. ml^{-1} and incubated in a Polarstar Omega multiwell plate reader at 37 °C with shaking (500 r.p.m.)[31,34]. The plate reader was programmed to take readings of optical density at 600 nm (OD_{600}) every 30 min for 17 h. Simultaneously, green fluorescent protein (GFP) fluorescence from the *agr*-P3 promoter was measured using an excitation filter at 485 nm and an emission filter at 520 nm to generate values expressed as relative fluorescence units[31,34].

Haemolysis assay. Spent culture supernatants (1 ml) from stationary-phase bacteria were subjected to serial twofold dilution in fresh TSB before the addition of sheep blood to 2%. Supernatant/blood mixtures were incubated statically at 37 °C for 1 h before intact blood cells were removed by centrifugation. The degree of red cell lysis was determined by measuring absorbance at 450 nm, A_{450} (ref. 31). Blood incubated in TSB only served as a negative control, while TSB containing 0.1% Triton X-100 served as a positive control and was considered to represent 100% lysis.

Membrane lipid detection. *S. aureus* membrane lipid was detected and quantified using FM-4-64 (Life Technologies)[46]. Bacterial culture supernatants (200 µl) or other solutions were mixed with FM-4-64 to a final concentration of 5 µg ml^{-1}, and fluorescence was measured with a Tecan microplate reader using excitation at 565 nm and emission at 660 nm. Standard plots of purified phospholipids were used to determine the relative concentration of lipid in culture supernatants.

Daptomycin pulldown assays. Daptomycin was labelled with desthiobiotin (Pierce) as described in the manufacturer's instructions. Briefly, desthiobiotin (100 µl, 10 mM) was mixed with daptomycin (100 µl, 6 mM) and PBS to 1 ml before incubation at room temperature with gentle rocking for 1 h. The reaction mixture was then dialysed against H_2O at 4 °C to remove unbound desthiobiotin using a Float-A-Lyser G2 device (Spectrum Labs) with a molecular weight cutoff of 0.1 kDa. The antibiotic activity of the labelled daptomycin was confirmed using a MIC assay and time of flight mass spectroscopy analysis of labelled daptomycin was used to confirm labelling of the antibiotic (Supplementary Fig. 22). For the pulldown assay, spent culture supernatant (1 ml) was incubated with 20 µg ml^{-1} desthiobiotin-labelled daptomycin for 1 h at 37 °C with end-over-end mixing at 10 r.p.m. In some assays, 10 µM PSMα1 was included. Streptavidin-agarose beads (200 µl) were washed with PBS and added to the supernatant containing labelled daptomycin, and incubation continued for 1 h at 37 °C. Beads were then allowed to settle for 10 min before centrifugation at 500*g* for 10 min to pellet remaining beads in suspension. The supernatant was then removed and the pelleted beads washed with TSB. An additional round of centrifugation and a TSB wash were performed before the beads were resuspended in elution buffer (100 µl, 4 mM biotin) followed by incubation at 80 °C for 20 min. TSB was then added to the beads and elution buffer, mixed by pipetting, and beads were removed by centrifugation as described above. The supernatant was recovered and lipid content measured using FM-4-64 dye, as described above.

Measurement of cell-associated daptomycin. Daptomycin was labelled with BoDipy FL SE (D2184, Life Technologies). Daptomycin (50 µl, 50 mg ml^{-1}) was mixed with Bodipy (100 µl, 10 mg ml^{-1} in DMSO) and the reaction volume made to 1 ml with sodium bicarbonate (0.2 M pH 8.5) before incubation at 37 °C for 1 h. Unbound Bodipy was removed by dialysis as described above for desthiobiotin. Also as above, the antibiotic activity of the labelled daptomycin was confirmed using a MIC assay, and time of flight MS analysis of labelled daptomycin was used to confirm labelling of the antibiotic (Supplementary Fig. 22). Bacterial cells were incubated in TSB containing the labelled antibiotic for 0–8 h, as described for killing assays, before they were washed three times in TSB. BoDipy-labelled daptomycin bound to bacterial cells was quantified with a Tecan microplate reader using excitation at 502 nm and emission at 510 nm.

Lipid extraction and thin-layer chromatography. Lipids were extracted from spent culture supernatants using a method based on that described by Bligh and Dyer[47]. Culture supernatants or TSB containing various purified lipid standards and daptomycin were extracted with one sample volume of chloroform:methanol (2:1, vol/vol). The samples were mixed by vortexing and then centrifuged (17,000*g*, 10 min) to separate the aqueous and organic phases. The aqueous phase was removed and the organic phase evaporated at room temperature. Samples were then resuspended in 100 µl of chloroform:methanol (2:1, vol/vol). Samples were then separated by one-dimensional TLC as described previously[48]. In brief, equal volumes of lipid extracts were spotted onto silica 60 F254 HPTLC plates (Merck) and migrated for 25 min with chloroform:methanol:ammonium hydroxide (30%) (65:30:4, by volume)[48]. TLC plates were allowed to dry by evaporation before lipids were detected using iodine vapour. Purified lipid standards included phosphatidylglycerol, cardiolipin, lysyl-phosphatidylglycerol, phosphatidic acid and phosphatidylethanolamine. All standards were purchased from Sigma-Aldrich with the exception of lysyl-phosphatidylglycerol (Avanti Polar Lipids). To ensure accurate comparison between spent culture supernatant and the lipid standards, purified lipids were added to TSB at 100 µM, and daptomycin (20 µg ml^{-1}) was added before the mixtures were subjected to the lipid extraction procedure.

The identity and relative proportions of lipids were determined as described previously[24,49]. Lipids were extracted from spent culture supernatant into chloroform:methanol (2:1 vol/vol) as described above. Samples were then subjected to two-dimensional TLC, first with chloroform:methanol:ammonium hydroxide (30%) (65:30:4, by volume) and then with chloroform:acetic acid:methanol:water (170:25:25:4, by volume). Plates were then dried and lipids visualized with iodine as described above. The identity of each spot was determined by reference to the migration pattern of purified phospholipid standards as described previously[49,50]. Each spot was scraped from the plate using a sterile scalpel and the quantity of phospholipid in each determined by digestion with 0.3 ml 70% perchloric acid at 150 °C for 3 h. The digested phospholipid was then incubated with a detection reagent (10% ascorbic acid, 2.5% ammonium molybdate, 5% perchloric acid at a 1:1:8 ratio by volume) at 37 °C for 2 h and quantification was carried out by measurement at 750 nm and reference to standards of known concentrations[49,50]. The concentration of each lipid was derived from quantification of lipid in the culture supernatant from USA300 WT or Δ*agrA* mutant using FM-4-64 and reference to a standard plot generated using purified phosphatidylglycerol.

Daptomycin resistance emergence assay. Bacterial strains were inoculated into TSB containing daptomycin (20 µg ml^{-1}) and $CaCl_2$ (0.5 mM) and survival over 8 h was measured as described in the section for the determination of antibiotic bactericidal activity. After 8 h, bacteria were pelleted by centrifugation (17,000*g*, 5 min), washed twice in TSB, and resuspended in 3 ml fresh TSB, before incubation at 37 °C with shaking (180 r.p.m.) for 16 h to the stationary phase. The activity of daptomycin in the culture supernatants was determined by a zone of inhibition assay (see section describing daptomycin activity determination). The bacterial populations were then inoculated once again into TSB containing daptomycin (20 µg ml^{-1}) and $CaCl_2$ (0.5 mM), and survival over 8 h was measured. We also determined the daptomycin MIC of recovered bacterial populations, as described above. At the end of the second period of daptomycin exposure, daptomycin activity was determined and bacteria were transferred into antibiotic-free TSB. This assay was repeated a third time. Measurements of bacterial survival, daptomycin activity and daptomycin MIC were made for all three rounds of daptomycin exposure.

Synthetic peptides. Synthetic peptides were generated by Peptide Protein Research Ltd according to previously described sequences, with formylated methione (fM) at the N termini[27]: PSMα1 fMGIIAGIIKVIKSLIEQFTGK; PSMα2 fMGIIAGIIKFIKGLIEKFTGK; PSMα3 fMEFVAKLFKFFKDLLGKFLGNN; PSMα4 fMAIVGTIIKIIKAIIDIFAK; PSMβ1 fMEGLFNAIKDTVTAAINNDGAKLGTSIVSIVENGVGLLGKLFGF; PSMβ2 fMTGLAEAIANTVQAAQQHDSVKLGTSIVDIVANGVGLLGKLFGF. In addition, a peptide composed of the reverse sequence of PSMα1 (PSM-rev; KGTFQEILSKIVKIIGAIIGM) was synthesized. Unless otherwise indicated, peptides were used in assays at 10 µM, based on concentrations described for stationary-phase cultures of USA300 (ref. 30). When used in combination, 10 µM of each peptide was used.

Statistical analyses. Unless otherwise mentioned, data are represented as the arithmetic mean average from three or more independent experiments and error bars represent the standard deviation of the mean. For multiple comparisons, data were analysed using a one-way analysis of variance (ANOVA) for data generated from a single time point, or two-way ANOVA for data collected from multiple time points. Post hoc tests were only used when the overall ANOVA *P* value was less than 0.05. Unless stated otherwise, for one-way ANOVA the Tukey post-hoc test was used and for two-way ANOVA Dunnett's or Sidak's multiple comparison tests were used as appropriate. For single comparisons, data were analysed using an unpaired, two-tailed Student's *t*-test. Where there were significant differences in values, these are described in the legend and indicated on the figure (by an asterisk). Non-significant differences are only highlighted for animal studies (they were omitted from other figures for clarity). For each experiment, *n* refers to the number of independent biological repeats, followed by the number of technical replicates in each case. c.f.u. counts from murine experiments are presented as the value obtained from each animal. These data were compared using Mann–Whitney or two-way ANOVA tests as indicated in figure legends, and median values were used for comparisons.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Received 12 January 2016; accepted 7 September 2016; published 24 October 2016

References

1. Steenbergen, J. N., Alder, J., Thorne, G. M. & Tally, F. P. Daptomycin: a lipopeptide antibiotic for the treatment of serious Gram-positive infections. *J. Antimicrob. Chemother.* **55,** 283–288 (2005).
2. Boucher, H., Miller, L. G. & Razonable, R. R. Serious infections caused by methicillin-resistant *Staphylococcus aureus*. *Clin. Infect. Dis.* **51,** S183–S197 (2010).
3. Stefani, S. *et al.* Insights and clinical perspectives of daptomycin resistance in *Staphylococcus aureus*: a review of the available evidence. *Int. J. Antimicrob. Agents.* **46,** 278–289 (2015).
4. Seaton, R. A. *et al.* Evaluation of effectiveness and safety of high-dose daptomycin: results from patients included in the European Cubicin® outcomes registry and experience. *Adv. Ther.* **12,** 1192–1205 (2015).
5. Novick, R. P. *et al.* Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *EMBO J.* **12,** 3967–3975 (1993).
6. Novick, R. P. & Geisinger, E. Quorum sensing in Staphylococci. *Annu. Rev. Genet.* **42,** 541–564 (2008).
7. Sakoulas, G. *et al.* Accessory gene regulator (*agr*) locus in geographically diverse *Staphylococcus aureus* isolates with reduced susceptibility to vancomycin. *Antimicrob. Agents Chemother.* **46,** 1492–1502 (2002).
8. Fowler, V. G. Jr *et al.* Persistent bacteremia due to methicillin-resistant *Staphylococcus aureus* infection is associated with *agr* dysfunction and low-level *in vitro* resistance to thrombin-induced platelet microbicidal protein. *J. Infect. Dis.* **190,** 1140–1149 (2004).

9. Mwangi, M. M. *et al.* Tracking the *in vivo* evolution of multidrug resistance in *Staphylococcus aureus* by whole-genome sequencing. *Proc. Natl Acad. Sci. USA* **104,** 9451–9456 (2007).
10. Traber, K. E. *et al. agr* function in clinical *Staphylococcus aureus* isolates. *Microbiology* **154,** 2265–2274 (2008).
11. Laabei, M. *et al.* Evolutionary trade-offs underlie the multi-faceted virulence of *Staphylococcus aureus*. *PLoS Biol.* **13,** e1002229 (2015).
12. Schweizer, M. L. *et al.* Increased mortality with accessory gene regulator (*agr*) dysfunction in *Staphylococcus aureus* among bacteremic patients. *Antimicrob. Agents Chemother.* **55,** 1082–1087 (2011).
13. Paulander, W. *et al.* Antibiotic-mediated selection of quorum-sensing-negative *Staphylococcus aureus*. *mBio.* **3,** e00459 (2013).
14. Painter, K. L., Krishna, A., Wigneshweraraj, S. & Edwards, A. M. What role does the quorum-sensing accessory gene regulator system play during *Staphylococcus aureus* bacteremia? *Trends Microbiol.* **22,** 676–685 (2014).
15. Credito, K., Lin, G. & Appelbaum, P. C. Activity of daptomycin alone and in combination with rifampin and gentamicin against *Staphylococcus aureus* assessed by time–kill methodology. *Antimicrob. Agents Chemother.* **51,** 1504–1507 (2007).
16. Marco, F. *et al.* Daptomycin is effective in treatment of experimental endocarditis due to methicillin-resistant and glycopeptide-intermediate *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **52,** 2538–2543 (2008).
17. Jiang, J. H. & Peleg, A. Y. Daptomycin-nonsusceptible *Staphylococcus aureus*: the role of combination therapy with daptomycin and gentamicin. *Genes* **6,** 1256–1267 (2015).
18. Rauch, S. *et al.* Abscess formation and alpha-hemolysin induced toxicity in a mouse model of *Staphylococcus aureus* peritoneal infection. *Infect. Immun.* **80,** 3721–3732 (2012).
19. Mortin, L. I. *et al.* Rapid bactericidal activity of daptomycin against methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* peritonitis in mice as measured with bioluminescent bacteria. *Antimicrob. Agents Chemother.* **51,** 1787–1794 (2007).
20. Wale, L. J., Shelton, A. P. & Greenwood, D. Scanning electron microscopy of *Staphylococcus aureus* and *Enterococcus faecalis* exposed to daptomycin. *J. Med. Microbiol.* **30,** 45–49 (1989).
21. Cotroneo, N., Harris, R., Perlmutter, N., Beveridge, T. & Silverman, J. A. Daptomycin exerts bactericidal activity without lysis of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **52,** 2223–2225 (2008).
22. Muraih, J. K., Pearson, A., Silverman, J. & Palmer, M. Oligomerization of daptomycin on membranes. *Biochim. Biophys. Acta.* **1808,** 1154–1160 (2011).
23. Chen, Y. F., Sun, T. L., Sun, Y. & Huang, H. W. Interaction of daptomycin with lipid bilayers: a lipid extracting effect. *Biochemistry* **53,** 5384–5392 (2014).
24. Mishra, N. N. & Bayer, A. S. Correlation of cell membrane lipid profiles with daptomycin resistance in methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **57,** 1082–1085 (2013).
25. Silverman, J. A., Mortin, L. I., VanPraagh, A. D. G., Li, T. & Alder, J. Inhibition of daptomycin by pulmonary surfactant: *in vitro* modeling and clinical impact. *J. Infect. Dis.* **191,** 2149–2152 (2005).
26. Manning, A. J. & Kuehn, M. J. Contribution of bacterial outer membrane vesicles to innate bacterial defense. *BMC. Microbiol.* **11,** 258 (2011).
27. Wang, R. *et al.* Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat. Med.* **13,** 1510–1514 (2007).
28. Queck, S. Y. *et al.* RNAIII-independent target gene control by the *agr* quorum-sensing system: insight into the evolution of virulence regulation in *Staphylococcus aureus*. *Mol. Cell* **32,** 150–158 (2008).
29. Rand, K. H. & Houck, H. J. Synergy of daptomycin with oxacillin and other β-lactams against methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **48,** 2871–2875 (2004).
30. Berti, A. D. *et al.* Penicillin binding protein 1 is important in the compensatory response of *Staphylococcus aureus* to daptomycin-induced membrane damage and is a potential target for β-lactam–daptomycin synergy. *Antimicrob. Agents Chemother.* **60,** 451–458 (2015).
31. Pader, V., James, E. H., Painter, K. L., Wigneshweraraj, S. & Edwards, A. M. The Agr quorum-sensing system regulates fibronectin binding but not hemolysis in the absence of a functional electron transport chain. *Infect. Immun.* **82,** 4337–4347 (2014).
32. Monk, I. R., Shah, I. M., Xu, M., Tan, M. W. & Foster, T. J. Transforming the untransformable: application of direct transformation to manipulate genetically *Staphylococcus aureus* and *Staphylococcus epidermidis*. *mBio.* **3,** e00277 (2012).
33. Nicod, S. S. *et al.* Systematic mutational analysis of the LytTR DNA binding domain of *Staphylococcus aureus* virulence gene transcription factor AgrA. *Nucleic Acids Res.* **42,** 12523–12536 (2014).
34. James, E. H., Edwards, A. M. & Wigneshweraraj, S. Transcriptional downregulation of *agr* expression in *Staphylococcus aureus* during growth in human serum can be overcome by constitutively active mutant forms of the sensor kinase AgrC. *FEMS Microbiol. Lett.* **349,** 153–162 (2013).
35. Jorgensen, J. H. *et al.* in *Manual of Clinical Microbiology* Ch. 118, 1526–1543 (ASM Press, 1999).
36. Wiegand, I., Hilpert, K. & Hancock, R. E. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protoc.* **3,** 163–175 (2008).
37. Diederen, B. M., van Duijn, I., Willemse, P. & Kluytmans, J. A. *In vitro* activity of daptomycin against methicillin-resistant *Staphylococcus aureus*, including heterogeneously glycopeptide-resistant strains. *Antimicrob. Agents Chemother.* **50,** 3189–3191 (2006).
38. Sader, H. S., Fritsche, T. R. & Jones, R. N. Daptomycin bactericidal activity and correlation between disk and broth microdilution method results in testing of *Staphylococcus aureus* strains with decreased susceptibility to vancomycin. *Antimicrob. Agents Chemother.* **50,** 2330–2336 (2006).
39. Lefort, A. *et al.* Bactericidal activity of gentamicin against *Enterococcus faecalis in vitro* and *in vivo*. *Antimicrob. Agents Chemother.* **44,** 2077–2080 (2000).
40. Louie, A. *et al.* Pharmacodynamics of daptomycin in a murine thigh model of *Staphylococcus aureus* infection. *Antimicrob. Agents Chemother.* **45,** 845–851 (2001).
41. Safdar, N., Andes, D. & Craig, W. A. *In vivo* pharmacodynamic activity of daptomycin. *Antimicrob. Agents Chemother.* **48,** 63–68 (2004).
42. Rybak, M. J. The pharmacokinetic and pharmacodynamic properties of vancomycin. *Clin. Infect. Dis.* **42,** S35–S39 (2006).
43. Joukhadar, C. *et al.* Lack of bactericidal antagonism or synergism *in vitro* between oxacillin and vancomycin against methicillin-susceptible strains of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **54,** 773–777 (2010).
44. Johnson, P. J. & Levin, B. R. Pharmacodynamics, population dynamics, and the evolution of persistence in *Staphylococcus aureus*. *PLoS Genet.* **9,** e1003123 (2013).
45. Faul, F., Erdfelder, E., Lang, A.-G. & Buchner, A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav. Res. Methods* **39,** 175–191 (2007).
46. Zhou, X. *et al.* Bacterial division. Mechanical crack propagation drives millisecond daughter cell separation in *Staphylococcus aureus*. *Science* **348,** 574–578 (2015).
47. Bligh, E. S. & Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37,** 911–917 (1959).
48. Nahaie, M. R., Goodfellow, M., Minnikin, D. E. & Hájek, V. Polar lipid and isoprenoid quinone composition in the classification of *Staphylococcus*. *J. Gen. Microbiol.* **130,** 2427–2437 (1984).
49. Mukhopadhyay, K. *et al. In vitro* susceptibility of *Staphylococcus aureus* to thrombin-induced platelet microbicidal protein-1 (tPMP-1) is influenced by cell membrane phospholipid composition and asymmetry. *Microbiology* **153,** 1187–1197 (2007).
50. Rouser, G., Siakotos, A. N. & Fleischer, S. Quantitative analysis of phospholipids by thin-layer chromatography and phosphorus analysis of spots. *Lipids* **1,** 85–86 (1966).

Acknowledgements

The authors thank the following for providing bacterial strains, phage or reagents: J.M. Van Dijl (University Medical Center Groningen), M. Otto (NIH, Bethesda), R. Massey (University of Bath), M. Horsburgh (University of Liverpool), T. Foster (Trinity College Dublin) and the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) Program under NIAID/NIH contract no. HHSN272200700055C. L. Haigh (Imperial College) is thanked for analysing modified daptomycin samples. A. Nobbs (University of Bristol) is acknowledged for helpful discussions and comments on the manuscript. A.M.E. acknowledges funding from the Department of Medicine, Imperial College. S.W. acknowledges funding from the BBSRC and Wellcome Trust. K.L.P. is supported by a PhD studentship from the Faculty of Medicine, Imperial College London. S.H. is supported by a scholarship from the Inlaks Shivdasani Foundation. T.B.C. is a Sir Henry Dale Fellow jointly funded by the Wellcome Trust and Royal Society (grant no. 107660/Z/15/Z).

Author contributions

V.P., S.W., T.B.C. and A.M.E. designed the experiments. V.P., S.H., T.B.C. and A.M.E. performed experiments. K.L.P. generated and characterized mutants. V.P., S.H., T.B.C. and A.M.E. analysed data. All authors contributed to the writing of the manuscript.

Additional information

Supplementary information is available for this paper.

Reprints and permissions information is available at www.nature.com/reprints.

Correspondence and requests for materials should be addressed to A.M.E.

How to cite this article: Pader, V. *et al. Staphylococcus aureus* inactivates daptomycin by releasing membrane phospholipids. *Nat. Microbiol.* **2,** 16194 (2016).

Competing interests

The authors declare no competing financial interests.