Staphylococcus aureus inactivates daptomycin by releasing membrane phospholipids

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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 >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 phospholipid, 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.

S. aureus encodes multiple virulence factors, many of which are controlled by Agr[5,6], a quorum-sensing system encoded by a 4 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*, hypothesised 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 Fig. 1, 2). By contrast, whilst 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 hours 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 *agrA* or *agrC* mutants complemented with the relevant genes on plasmids

(Supplementary Fig. 3). After the initial period of killing, CFU 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 MIC and MBC values (Fig. 1c, Supplementary Table 1, 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 that reported previously[19], daptomycin treatment reduced the size of the wild-type population 15-fold by 8 h, 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 daptomycin susceptibility of the whole population. Using various ratios of wild-type and *agrA* mutant *S. aureus,* we found that larger *agr*-mutant sub-populations resulted in greater survival of both the Agr-defective strain and the wild-type (Fig. 1e, 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; 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 *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, whilst 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 blebbing without lysis[20-24]. Therefore, we hypothesised 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, Supplementary Fig. 11) and we confirmed the presence of membrane phospholipid via thin-layer chromatography (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; 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 cardiolipin 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

112 daptomycin, whilst CL did not (Fig. 2e). Together, these data confirm that released membrane PG
113 was responsible for inactivating the antibiotic.

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

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

127 We then determined why *S. aureus* lacking a functional Agr system, but not wild-type,
128 inactivated daptomycin. The simplest explanation was that the wild-type did not release lipids in
129 response to daptomycin. However, exposure of wild-type *S. aureus* to daptomycin resulted in
130 release of phospholipid at similar levels and composition to the Δ*agrA* mutant, demonstrating that
131 the Agr quorum-sensing system does not influence lipid-release (Supplementary Fig. 12, 14, 15;
132 supplementary table 2). Using pull-down assays, we discovered that daptomycin bound to
133 phospholipid in culture supernatant from Δ*agrA* but not wild-type *S. aureus* (Supplementary Fig. 15).
134 Because Agr triggers the secretion of numerous products[5,6], we hypothesised that these prevented
135 the binding of daptomycin by phospholipid from wild-type bacteria. In support of this hypothesis,
136 we discovered that spent culture supernatant from wild-type bacteria prevented Δ*agrA* bacteria
137 from inactivating daptomycin (Supplementary Fig. 16). Further analysis determined that the agent

138 responsible was heat-sensitive and passed through a 3 kDa cut-off filter, indicative of a small peptide
139 (Supplementary Fig. 16). AgrA regulates the expression of 7 small peptide cytolysins known as
140 phenol soluble modulins (PSMs), categorised into alpha (PSMα1-4) or beta (PSMβ1,2) families[27,28].
141 Given the surfactant properties of PSMs we hypothesised that they prevented the sequestration of
142 daptomycin by phospholipids.

143 Mutants in both the USA300 and SH1000 backgrounds lacking PSMα cytolysins inactivated
144 daptomycin and survived exposure to the antibiotic in a similar manner to *agr*-mutants (Fig. 3a,b)
145 (Supplementary Fig. 17). By contrast, PSMβ-defective mutants were impaired in daptomycin
146 inactivation and were as susceptible to the antibiotic as the wild-type, despite releasing similar levels
147 of phospholipid to the Δ*psm*α mutant (Fig. 3a,b,c; Supplementary Fig. 11). Supplementation of the
148 culture medium with a mixture of synthetic PSMα, but not PSMβ, peptides restored killing of
149 Δ*psm*αβ mutants (Fig. 3d,e; Supplementary figure 18). Of all the PSMs, PSMα1 was most effective at
150 preventing daptomycin inactivation by the USA300 Δ*psm*αβ mutant and was also able to prevent the
151 inactivation of daptomycin by USA300 Δ*agrA* mutant and purified PG, most likely via its surfactant
152 properties (Fig. 3f; Supplementary fig. S18). To test whether PSMα1 bound to released phospholipid
153 we measured the haemolytic activity of the toxin in the presence of shed membrane phospholipid,
154 or purified PG. The presence of phospholipid completely inhibited the haemolytic activity of PSMα1,
155 indicating direct binding of the cytolysin to the phospholipid (Supplementary Fig. 19). Based on these
156 findings, we hypothesised that PSMα peptides enhanced daptomycin-mediated killing of *S. aureus*
157 by preventing antibiotic sequestration by the released phospholipid. Using BoDipy-tagged
158 daptomycin, we found that wild-type *S. aureus* steadily accumulated daptomycin over time (Fig. 3g).
159 By contrast, binding of daptomycin to *S. aureus* Δ*agrA* decreased over time. In the presence of
160 PSMα1 peptide, however, daptomycin bound strongly to the Δ*agrA* mutant, demonstrating that this
161 cytolysin promoted binding of the antibiotic to bacteria, as observed for the wild-type (Fig. 3g).

162 Daptomycin shares functional similarities with cationic antimicrobial peptides (CAMPs), and
163 we therefore hypothesised that lipid shedding may also confer protection against these membrane-

164 acting antimicrobials. We found that lipid shedding by the Δ*agrA* mutant was triggered by both nisin
165 and melittin, which target the staphylococcal membrane, and that PG inactivated the antimicrobial
166 peptides (Supplementary Fig. 20). These findings suggest that CAMPs may have provided a selection
167 pressure for the evolution of the phospholipid-shedding response to membrane damage.
168 It has been reported previously that β-lactam antibiotics at sub-inhibitory concentrations
169 promote daptomycin activity against wild-type *S. aureus* by increasing the binding of the lipopetide
170 to the bacterial membrane[29,30]. We decided, therefore, to determine if this combination therapy
171 could prevent treatment failure of infection caused by the presence of *agr*-mutant *S. aureus*. A sub-
172 inhibitory concentration of the β-lactam antibiotic oxacillin enhanced killing of the *S. aureus* Δ*agrA*
173 mutant by daptomycin, by reducing the rate of lipid shedding and therefore prolonging the activity
174 of the lipopeptide antibiotic (Fig. 4a,b,c,). By contrast, oxacillin did not show synergy with
175 daptomycin against wild-type *S. aureus*, despite reducing lipid release, presumably because the lipid-
176 shedding mechanism is already compromised by PSM production (Fig. 4d,e,f). Similarly to the Δ*agrA*
177 mutant alone, daptomycin was significantly more effective against mixed populations of wild-type *S.*
178 *aureus* and Δ*agrA* mutant in the presence of oxacillin both *in vitro*, and in an *in vivo* model of
179 invasive infection, suggesting that daptomycin/β-lactam combination therapy would reduce
180 treatment failure due to *agr*-mutant *S. aureus* (Fig. 4g,h,i,j).
181 In conclusion, this work has identified membrane phospholipid shedding as a rapid
182 and transient defence utilised by *S. aureus* against an antibiotic of last resort, (Supplementary Fig. 21
183 and extended discussion in the Supplementary data file). However, this defence mechanism is
184 compromised by Agr-regulated toxins, providing a selective advantage to bacteria with *agr*-
185 inactivating mutations during daptomycin exposure. Furthermore, there are several host factors
186 which inhibit Agr activity[14], indicating that wild-type bacteria may successfully employ this defensive
187 system during certain types of infection. To combat the sequestration of daptomycin by
188 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.

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Author Contributions

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

Competing Financial Interests statement.

The authors declare no competing financial interests.

Figure Legends

Figure 1. Agr-defective *S. aureus* survives daptomycin exposure by inactivating the antibiotic. a, survival of USA300 wild-type (WT) or **b,** SH1000 WT or *agr* mutants over 8 h in the presence of 20 µg ml^{-1} daptomycin (n=3 in duplicate, for WT vs *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 vs *agrA* or *agrC* $P < 0.0001$ at indicated time points (*). **d,** CFU counts of USA300 WT or Δ*agrA* mutant after 8 h in the peritoneal cavity of mice, treated with 20 µg ml^{-1} daptomycin (+) or PBS only (-) (* $P < 0.05$, NS $P > 0.05$, each circle represents a single mouse, for WT groups n=9, 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,** CFU counts from the peritoneal cavity of mice infected with wild-type *S. aureus* or a 1:1 mixture of wild-type 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 wild-type or Δ*agrA* mutant exposed to the antibiotic using a zone of inhibition assay (**g**), or bactericidal assay (**h**)(n=3 in duplicate, WT vs *agrA* supernatant $P < 0.0001$ at indicated time points (*). Data in panels **a, b, c, g** and **h** were analysed using a two-way ANOVA with Dunnett's post-hoc test. Data in panels **d** and **f** were analysed by Mann-Whitney test. Where shown, error bars represent the Standard Deviation of the mean.

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 ^{o}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 vs 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 vs 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 vs 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 vs indicated lipid concentrations (*) $P < 0.0001$). **e,** daptomycin MIC/MBC of wild-type (WT) or *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 vs indicated lipids (*) $P < 0.0001$. **f,** lipid shedding from USA300 Δ*agrA* mutant exposed to 20 μg ml^{-1} daptomycin at 37 ^{o}C (CTL), 4 ^{o}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 vs TSB containing inhibitors or at 4 ^{o}C $P < 0.0001$ (*)). Data in panels **a, c, d, e** and **f** were analysed using a one-way ANOVA with Tukey's post-hoc test. Data in panel **b** were analysed using a 2-way ANOVA with Dunnett's post-hoc test. Where shown, error bars represent the standard deviation of the mean.

Figure 3. Alpha phenol soluble modulins prevent daptomycin inactivation by *S. aureus*. **a,** survival of USA300 wild-type or *psm* mutants during exposure to 20 μg ml^{-1} daptomycin and **b,** the activity of the antibiotic in associated culture supernatants (in both cases n=3 in duplicate, for WT vs 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,** the quantity of lipid shed from USA300 wild-type 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 wild-type *S. aureus* in the absence of peptides is included for comparison (n=3 in duplicate, for Δ*psm*αβ without peptides vs 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,** the activity of daptomycin in culture supernatants from the experiment described in **d** (n=3 in duplicate, for Δ*psm*αβ without peptides vs 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 vs PG with PSMα1 $P < 0.01$). **g,** binding of BoDipy-labelled daptomycin to wild-type, Δ*agrA* mutant or Δ*agrA* mutant in the presence of PSMα1 (n=3 in triplicate, for WT vs *agrA* $P < 0.001$ at the indicated time points (*). Data in panels **a, b, c, d, e** and **g** were analysed were analysed using a 2-way ANOVA with Dunnett's post-hoc test. Data in panel **f** was analysed using a student's *t*-test. Where shown, error bars represent the standard deviation of the mean.

Figure 4. Oxacillin prevents lipid shedding and daptomycin inactivation. a,b,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**) or **b,** lipid release or **c,** daptomycin activity measured over 8 h (n=3 in duplicate, for TSB containing daptomycin only vs daptomycin + oxacillin $P < 0.001$ at the indicated time points (*). **d,e,f,** USA300 wild-type exposed to 0.25 μg ml^{-1} of oxacillin, daptomycin (20 μg ml^{-1}) or both antibiotics in combination and survival (**d**) or **e,** lipid release or **f,** daptomycin activity measured after 8 h (n=3 in duplicate, for TSB containing daptomycin only vs daptomycin + oxacillin $P < 0.05$ at the indicated time points (*). **g,h,i,** USA300 wild-type and Δ*agrA* at a 1:1 ratio were exposed to oxacillin, daptomycin or both antibiotics in combination and survival (**g**) or **h,** lipid release or **i,** daptomycin activity measured over 8 h (n=3 in duplicate, for TSB containing daptomycin only vs daptomycin + oxacillin $P < 0.0001$ at the indicated

368 time points (*). **j,** CFU counts from the peritoneal cavities of mice after 8 h treatment with oxacillin,
369 daptomycin, neither or both antibiotics in combination (* $P < 0.05$, NS $P > 0.05$, each circle
370 represents a single mouse, for each group n=5). Data in panels **a-j** were analysed by 2-way ANOVA
371 with Dunnett's post-hoc test. Where shown, error bars represent the standard deviation of the
372 mean.

Methods

Bacterial culture

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 RPM). Where required, culture medium was supplemented with kanamycin (50 μg ml^{-1}), chloramphenicol (10 μg ml^{-1}) or tetracycline (10 μg ml^{-1}). Staphylococcal CFU were enumerated by serial dilution and plating onto tryptic soy agar (TSA). *E. coli* was grown in Luria Broth at 37 °C, with shaking at 180 RPM 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 down-stream of the coding regions to be deleted. 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 electro-competent *S. aureus* USA300 LAC[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 2 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 minimum inhibitory and bactericidal concentrations (MIC/MBC)

MICs were determined using the broth microdilution protocol[35,36]. Stationary-phase *S. aureus* grown in TSB was adjusted to 5×10^5 CFU 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 >1000-fold reduction in CFU 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 CFU ml^{-1} and incubated statically at 37 °C in air for 18 h. Bacterial CFU 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 CFU 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 ^oC with shaking (180 RPM) and bacterial viability determined by CFU counts. Stationary-phase bacteria were used as this is when Agr is active *in vitro*. Most experiments used mono-cultures of strains but some utilised mixtures of wild-type and *agr*-mutant bacteria, with the inoculum maintained at a total of 10^8 CFU 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 beta-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 x *g*, 5 min) and filtration through a 0.2 μM filter.

In other assays, bacteria were pelleted by centrifugation (17,000 x *g*, 5 min), the supernatant removed and the bacteria resuspended in an equal volume of filter sterilised 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 CFU ml^{-1}) in TSB across the surface. The spread bacterial inoculum was allowed to air dry and the wells 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 4 perpendicular points. In some cases, fresh daptomycin was added to culture supernatants, incubated for 0-8 h at 37 °C and antibiotic activity determined as described above. In other experiments, culture supernatants were pre-treated 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 prior to 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, United Kingdom) were infected via the intraperitoneal route with 2.5×10^7 CFU USA300 LAC or a 1:1 mixture of wild-type and Δ*agrA* mutant USA300 LAC (total of 2.5×10^7 CFU). For the indicated experiment, wild-type bacteria were resuspended in spent culture supernatant from either wild-type of *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 (i.e. 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 sacrificed by cervical dislocation and death confirmed by severing the femoral artery. The peritoneal cavity was washed with PBS and CFU counts 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 prior to undertaking the experiment using power analysis based on *in vitro* data[45]. Tubes containing the inocula and antibiotic solutions were blinded prior to 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 2 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 CFU ml^{-1} and incubated in a Polarstar Omega multiwell plate reader at 37 ^oC with shaking (500 RPM)[31,34]. The plate reader was programmed to take OD_{600} readings every 30 min for 17 h. Simultaneously, GFP fluorescence from the *agr*-P3 promoter was measured using an excitation filter of 485 nm and an emission filter of 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 2-fold dilution in fresh TSB before the addition of sheep blood to 2%. Supernatant/blood mixtures were incubated statically at 37 ^oC for 1 h before intact blood cells were removed by centrifugation. The degree of red cell lysis was determined by measuring A_{450}[31]. Blood incubated in TSB only served as a negative control, whilst 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 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 pull-down 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 ^oC to remove unbound desthiobiotin using a Float-A-Lyser G2 device (Spectrum labs) with a molecular weight cut-off of 0.1 kDa. The antibiotic activity of the labelled daptomycin was confirmed using a MIC assay and LC-MS analysis of labelled daptomycin was employed to confirm labelling of the antibiotic (data not shown). For the pull-down assay spent culture supernatant (1 ml) was incubated with 20 µg ml^{-1} desthiobiotin-labeled daptomycin for 1 h at 37 ^oC with end-over-end mixing at 10 RPM. 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 ^oC. Beads were then allowed to settle for 10 min before centrifugation at 500 x *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 was done before the beads were resuspended in elution buffer (100 µl, 4 mM biotin) followed by incubation at 80 ^oC for 20 min. TSB was then added to the beads and elution buffer, mixed by pipetting and beads 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 ^oC 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 LC-MS analysis of labelled daptomycin was employed to confirm labelling of the antibiotic (data not shown). Bacterial cells were incubated in TSB containing the labelled antibiotic for 0-8 h, as described for killing assays before they were washed 3 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 1 sample volume of chloroform:methanol (2:1, v/v). The samples were mixed by vortexing and then centrifuged (17,000 x *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, v/v). 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}) 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 v/v) as described above. Samples were then subjected to 2D thin layer chromatography, firstly 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 visualised 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 ^{o}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 1:1:8 ratio by volume) at 37 ^{o}C for 2 h, and quantification 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 culture supernatant from USA300 WT or Δ*agrA* mutant using FM-4-64 and reference to a standard plot generated using purified phosphatidylglcyerol.

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 measured as described in the Methods section for the determination of antibiotic bactericidal activity. After 8 h bacteria were pelleted by centrifugation (17,000 x *g*, 5 min), washed twice in TSB and resuspended in 3 ml fresh TSB, before incubation at 37 ^{o}C with shaking (180 RPM) for 16 h to stationary phase. The activity of daptomycin in the culture supernatants was determined by the zone of inhibition assay (daptomycin activity determination in the Methods section). 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 measured. We also determined the daptomycin MIC of recovered bacterial populations as described in the Methods section. At the end of the second period of daptomycin exposure, the daptomycin activity was determined and bacteria transferred into antibiotic-free TSB. This assay was repeated for 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, UK 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 comprised of the reverse sequence of PSMα1 (PSM-rev; KGTFQEILSKIVKIIGAIIGM) was synthesised. Unless otherwise indicated, peptides were used in assays at 10 μM, which was based on concentrations described for stationary phase cultures of USA300[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 3 or more independent experiments and error bars represent the SD of the mean. For multiple comparisons, data were analysed using a one-way 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 <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, 2-tailed Student's *t*-test. Where there are significant differences in values, these are described in the legend and indicated on the figure (*). Non-significant differences are only highlighted for animal studies but 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. CFU 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 used for comparisons.

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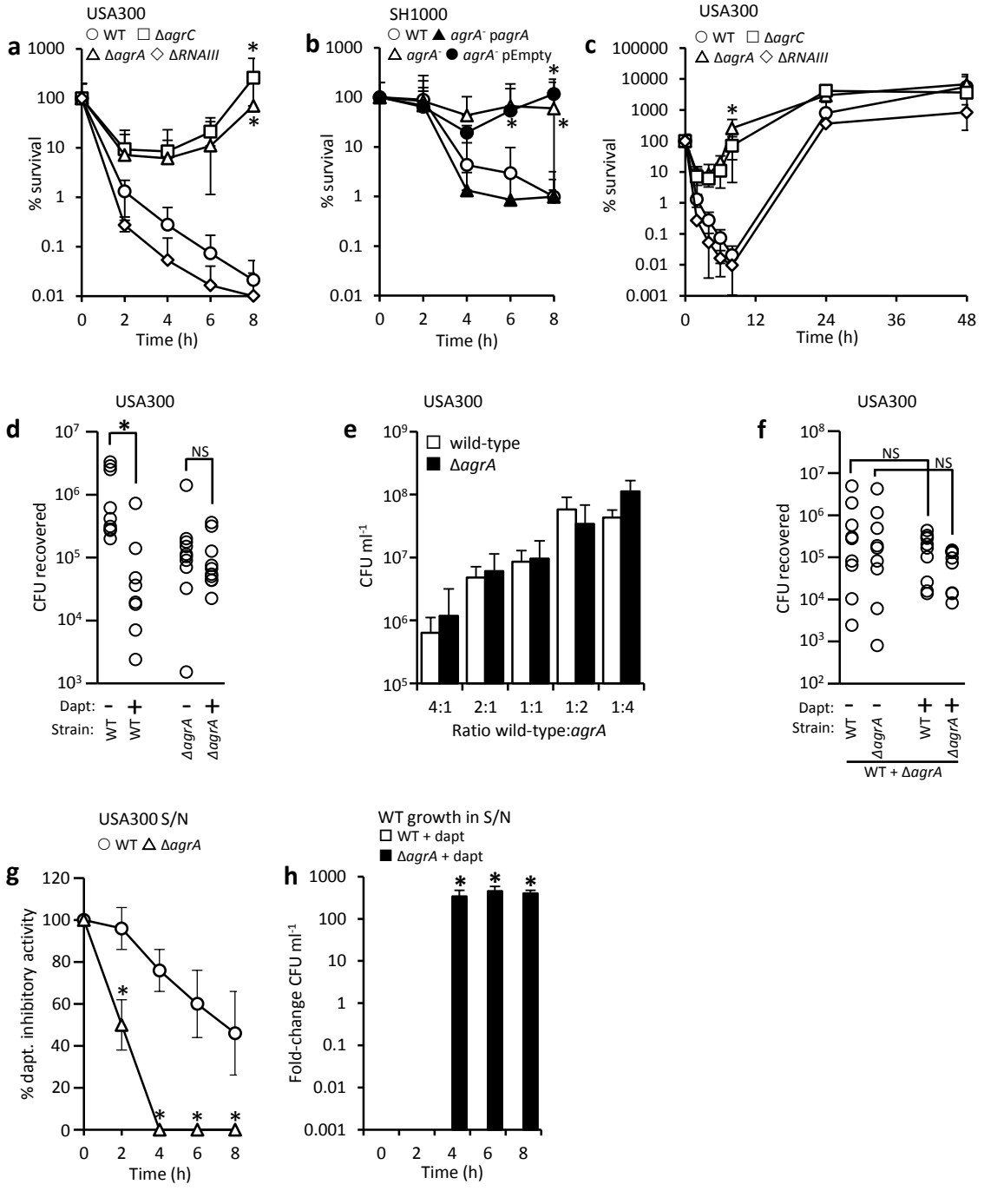
a
USA300
○ WT □ ΔagrC
△ ΔagrA ◇ ΔRNAIII
% survival
Time (h)
b
SH1000
○ WT ▲ agrA⁻ pagrA
△ agrA⁻ ● agrA⁻ pEmpty
% survival
Time (h)
c
USA300
○ WT □ ΔagrC
△ ΔagrA ◇ ΔRNAIII
% survival
Time (h)
d
USA300
CFU recovered
NS
Dapt:
Strain:
WT
ΔagrA
e
USA300
□ wild-type
■ ΔagrA
CFU ml⁻¹
4:1 2:1 1:1 1:2 1:4
Ratio wild-type:agrA
f
USA300
NS
CFU recovered
Dapt:
Strain:
WT + ΔagrA
g
USA300 S/N
○ WT △ ΔagrA
% dapt. inhibitory activity
Time (h)
h
WT growth in S/N
□ WT + dapt
■ ΔagrA + dapt
Fold-change CFU ml⁻¹
Time (h)

Figure 1.

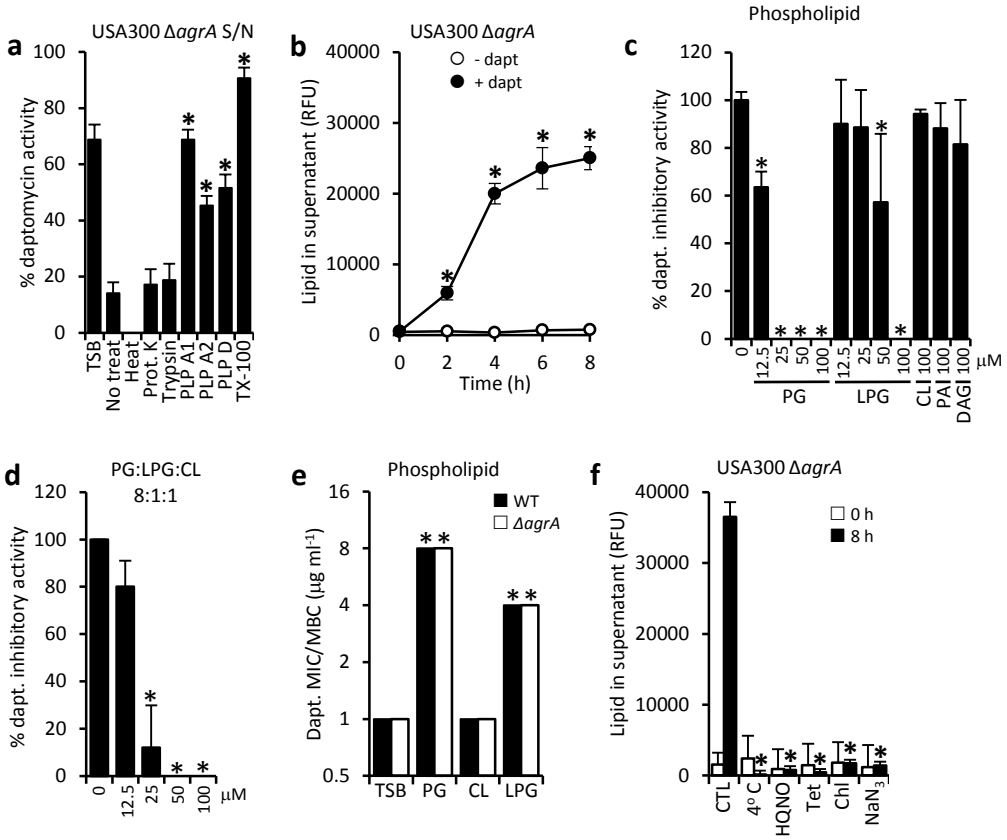
a
USA300 ΔagrA S/N
% daptomycin activity
TSB
No treat
Heat
Prot. K
Trypsin
PLP A1
PLP A2
PLP D
TX-100
b
USA300 ΔagrA
- dapt
+ dapt
Lipid in supernatant (RFU)
Time (h)
c
Phospholipid
% dapt. inhibitory activity
μM
PG
LPG
CL 100
PA 100
DAGI 100
d
PG:LPG:CL
8:1:1
% dapt. inhibitory activity
μM
e
Phospholipid
WT
ΔagrA
Dapt. MIC/MBC (μg ml-1)
TSB
PG
CL
LPG
f
USA300 ΔagrA
0 h
8 h
Lipid in supernatant (RFU)
CTL
4° C
HQNO
Tet
Chl
NaN3

Figure 2.

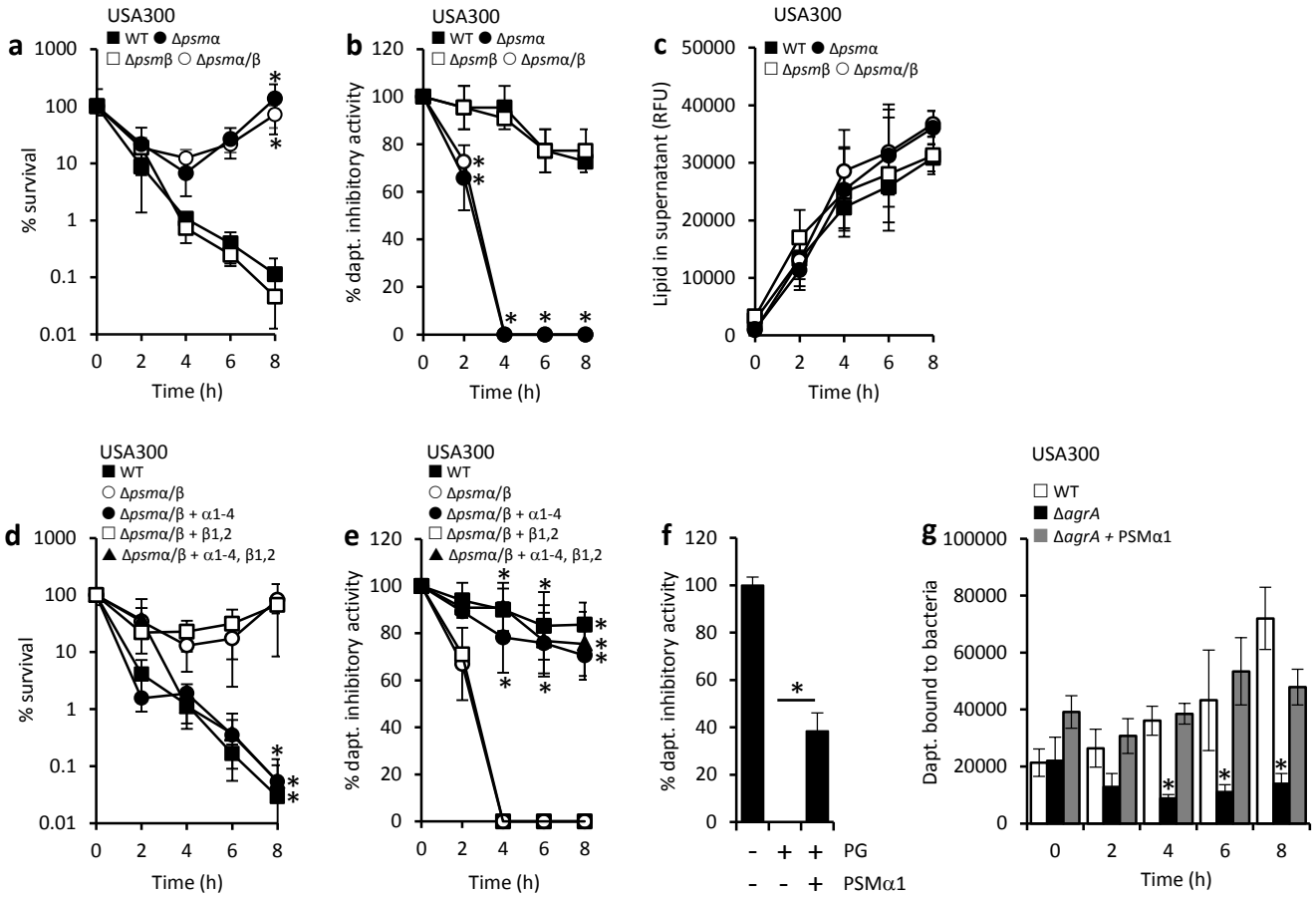
a
USA300
WT Δpsmα
Δpsmβ Δpsmα/β
% survival
Time (h)
b
USA300
WT Δpsmα
Δpsmβ Δpsmα/β
% dapt. inhibitory activity
Time (h)
c
USA300
WT Δpsmα
Δpsmβ Δpsmα/β
Lipid in supernatant (RFU)
Time (h)
d
USA300
WT
Δpsmα/β
Δpsmα/β + α1-4
Δpsmα/β + β1,2
Δpsmα/β + α1-4, β1,2
% survival
Time (h)
e
USA300
WT
Δpsmα/β
Δpsmα/β + α1-4
Δpsmα/β + β1,2
Δpsmα/β + α1-4, β1,2
% dapt. inhibitory activity
Time (h)
f
% dapt. inhibitory activity
PG
PSMα1
g
USA300
WT
ΔagrA
ΔagrA + PSMα1
Dapt. bound to bacteria
Time (h)

Figure 3.

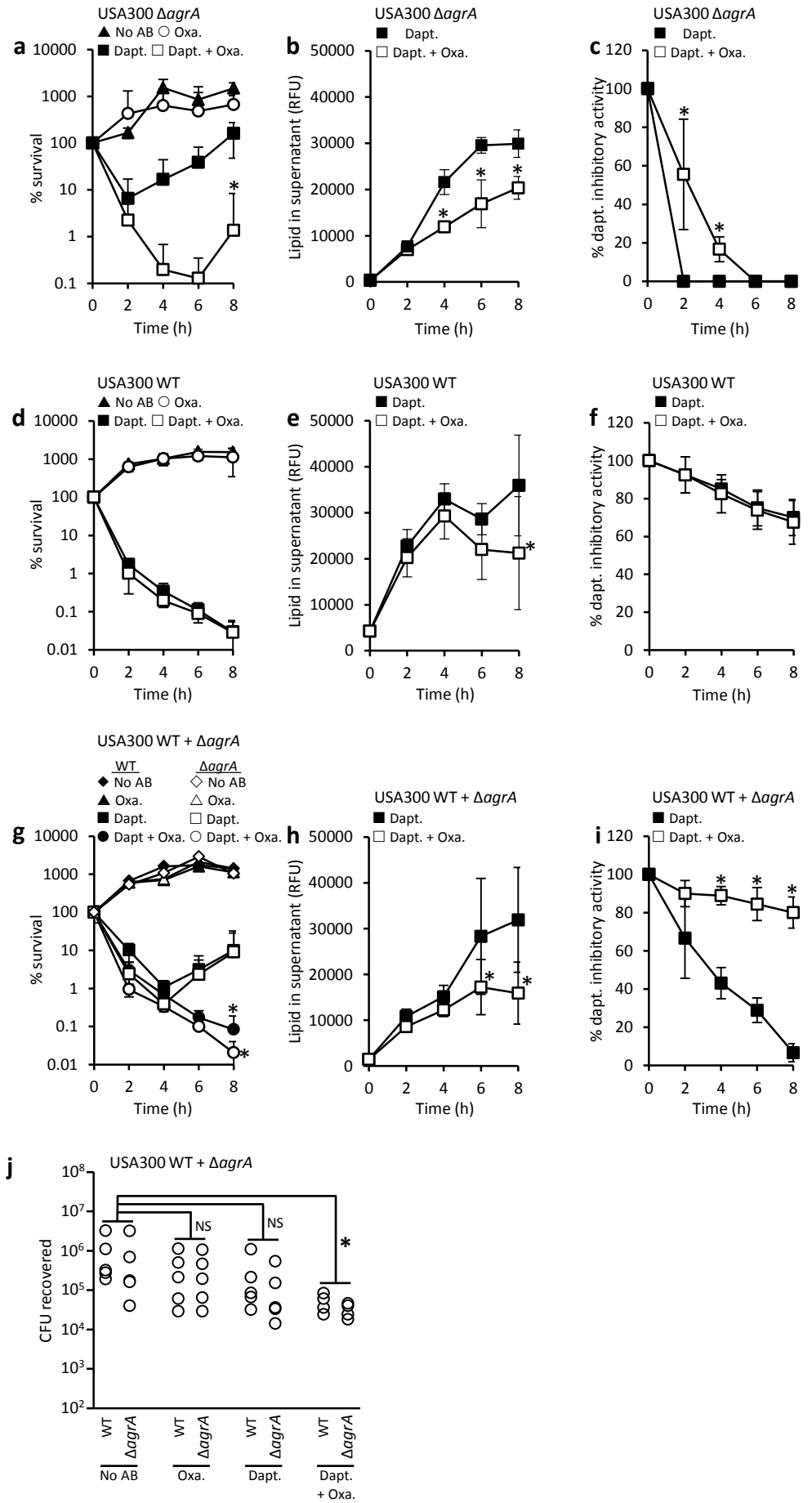
a
USA300 ΔagrA
▲ No AB ○ Oxa.
■ Dapt. □ Dapt. + Oxa.
% survival
Time (h)
b
USA300 ΔagrA
■ Dapt.
□ Dapt. + Oxa.
Lipid in supernatant (RFU)
Time (h)
c
USA300 ΔagrA
■ Dapt.
□ Dapt. + Oxa.
% dapt. inhibitory activity
Time (h)
d
USA300 WT
▲ No AB ○ Oxa.
■ Dapt. □ Dapt. + Oxa.
% survival
Time (h)
e
USA300 WT
■ Dapt.
□ Dapt. + Oxa.
Lipid in supernatant (RFU)
Time (h)
f
USA300 WT
■ Dapt.
□ Dapt. + Oxa.
% dapt. inhibitory activity
Time (h)
g
USA300 WT + ΔagrA
WT
◆ No AB
▲ Oxa.
■ Dapt.
● Dapt + Oxa.
ΔagrA
◇ No AB
△ Oxa.
□ Dapt.
○ Dapt. + Oxa.
% survival
Time (h)
h
USA300 WT + ΔagrA
■ Dapt.
□ Dapt. + Oxa.
Lipid in supernatant (RFU)
Time (h)
i
USA300 WT + ΔagrA
■ Dapt.
□ Dapt. + Oxa.
% dapt. inhibitory activity
Time (h)
j
USA300 WT + ΔagrA
CFU recovered
NS
NS
WT
ΔagrA
No AB
Oxa.
Dapt.
Dapt. + Oxa.

Figure 4.