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***Enterococcus faecalis* and pathogenic streptococci inactivate daptomycin by releasing phospholipids**

Elizabeth V. K. Ledger¹, Vera Pader¹ and Andrew M. Edwards^{1*}

¹ MRC Centre for Molecular Bacteriology and Infection, Imperial College London, Armstrong Rd, London, SW7 2AZ, UK.

* For correspondence:

Tel: 0044 (0)207 594 2072

Fax: 0044 (0)207 594 3096

a.edwards@imperial.ac.uk

30 **Summary**

31 Daptomycin is a lipopeptide antibiotic with activity against Gram-positive bacteria. We have
32 shown previously that *Staphylococcus aureus* can survive daptomycin exposure by releasing
33 membrane phospholipids that inactivate the antibiotic. To determine whether other
34 pathogens possess this defence mechanism, phospholipid release and daptomycin activity
35 were measured after incubation of *Staphylococcus epidermidis*, Group A or B streptococci,
36 *Streptococcus gordonii* or *Enterococcus faecalis* with the antibiotic. All bacteria released
37 phospholipid in response to daptomycin, which resulted in at least partial inactivation of the
38 antibiotic. However, *E. faecalis* showed the highest levels of lipid release and daptomycin
39 inactivation. As shown previously for *S. aureus*, phospholipid release by *E. faecalis* was
40 inhibited by the lipid biosynthesis inhibitor platensimycin. In conclusion, several pathogenic
41 Gram-positive bacteria, including *E. faecalis*, inactivate daptomycin by releasing
42 phospholipids, which may contribute to the failure of daptomycin to resolve infections caused
43 by these pathogens.

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60 **Manuscript text**

61 Daptomycin is a lipopeptide antibiotic used as a last resort in the treatment of infections
62 caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant
63 enterococci (VRE) [1-3]. The use of daptomycin is becoming more common, with prescriptions
64 increasing 72 % between 2012 and 2015 in the UK [4]. Daptomycin is the only lipopeptide
65 antibiotic used clinically and functions in a similar manner to antimicrobial peptides [5]. The
66 antibiotic inserts into the membrane of Gram-positive bacteria by targeting
67 phosphatidylglycerol, where it forms oligomeric complexes [6-8]. The precise mechanism by
68 which the antibiotic kills bacteria is unclear, but involves depolarisation of the bacterial
69 membrane and inhibition of cell wall biosynthesis without causing lysis [8-13]. Although
70 daptomycin resistance is rare, treatment failure occurs in up to 30 % of staphylococcal
71 infections and 23 % of enterococcal infections [14,15]. Failure rates are highest in invasive
72 infections such as bacteraemia or osteomyelitis, with rates of 24 % and 33 % respectively,
73 resulting in poor patient prognoses [14]. Understanding the reasons for this treatment failure
74 is crucial to improving the effectiveness of daptomycin treatment.

75 We recently discovered that *S. aureus* has a transient defence mechanism against
76 daptomycin, which contributed to treatment failure in a murine model of invasive infection
77 [16]. In response to the antibiotic, phospholipids were released from the cell membrane
78 which sequestered daptomycin and abrogated its bactericidal activity [16]. Phospholipid
79 release occurred via an active process, which was blocked by the lipid biosynthesis inhibitor
80 platensimycin [16,17]. In addition to daptomycin, phospholipid shedding also provided
81 protection against the antimicrobial peptides nisin and melittin, suggesting a general defence
82 against membrane-targeting antimicrobials [16].

83 It is currently unknown whether other Gram-positive bacteria release phospholipids
84 in response to daptomycin, although membrane vesicles have been observed on the surface
85 of *E. faecalis* cells exposed to daptomycin [18]. In addition, there is growing evidence that
86 other Gram-positive pathogens, including Group A streptococci (GAS) and group B
87 streptococci (GBS), release phospholipids from their surfaces in the form of extracellular
88 vesicles [19,20]. Production of these membrane vesicles is increased in the presence of
89 antimicrobials and, at least for GAS, are rich in phosphatidylglycerol, which was shown to be
90 essential for daptomycin inactivation by *S. aureus* [16,19,21]. Therefore, we hypothesised

91 that phospholipid release is a common strategy amongst Gram-positive pathogens to resist
92 membrane-acting antimicrobials.

93 Given the increasing use of daptomycin to treat enterococcal infections, the primary
94 aim of this work was to determine whether enterococci release membrane phospholipids that
95 inactivate the antibiotic. We also examined pathogenic streptococci, and *S. epidermidis*, as
96 the rising tide of antibiotic resistance may necessitate the use of daptomycin to tackle these
97 bacteria in the future.

98 We initially determined the daptomycin minimum inhibitory concentration (MIC) for
99 a representative panel of Gram-positive pathogens: *S. aureus* SH1000 [22], *S. epidermidis*
100 ATCC 12228 [23], GAS strain A40 [24]; GBS strains 515 [25] and COH1 [26]; *S. gordonii* strain
101 Challis [27]; and *E. faecalis* strains JH2-2 [28] and OG1X [29]. Bacteria were grown in Muller
102 Hinton Broth containing calcium (0.5 mM) and MIC determined by the broth microdilution
103 approach [30]. The most susceptible species were the pathogenic GAS strain A40 (0.125 µg
104 ml⁻¹), and GBS strains 515 (0.5 µg ml⁻¹) and COH1 (0.5 µg ml⁻¹), whilst *S. aureus* (1 µg ml⁻¹), *S.*
105 *epidermidis* (1 µg ml⁻¹), *S. gordonii* Challis (2-4 µg ml⁻¹) *E. faecalis* strains OG1X (2 µg ml⁻¹) and
106 JH2-2 (4 µg ml⁻¹) were the least susceptible.

107 To determine whether *E. faecalis* or streptococci respond to daptomycin by releasing
108 membrane phospholipids, we exposed streptococci and enterococci (10⁸ CFU ml⁻¹) to various
109 supra-MIC concentrations of the antibiotic (5-40 µg ml⁻¹) in Brain-Heart Infusion (0.5 mM
110 CaCl₂) broth at 37 °C under static conditions with 5% CO₂ and measured bacterial survival,
111 antibiotic activity and phospholipid release (Fig. 1c-h). Staphylococci were also exposed to
112 daptomycin (5-40 µg ml⁻¹), but in tryptic soy broth (TSB) containing 0.5 mM CaCl₂ at 37 °C with
113 shaking (180 RPM) (Fig. 1a,b).

114 For all strains, there was a dose-dependent decrease in survival after 8 h exposure to
115 daptomycin, as assessed by CFU counts (Fig. 1a-h). However, as expected from the MIC data,
116 survival of the two enterococcal strains, the staphylococci and *S. gordonii* was greater than
117 survival of GAS or GBS strains at every concentration of daptomycin examined (Fig. 1a-h).

118 Using the phospholipid-reactive fluorescent dye FM-4-64 (Life Technologies), we
119 found that daptomycin triggered phospholipid release from all of the bacteria examined,
120 albeit to differing levels. The quantity of phospholipid released was much greater for
121 staphylococci than the other species examined (Fig. 1i-p). However, for both staphylococci
122 and streptococci, the quantity of phospholipid released was lowest when the daptomycin

123 concentration was highest, suggesting that the antibiotic may have killed the bacteria before
124 they could release the lipid (Fig. 1i-p). By contrast, the enterococci released high levels of
125 phospholipid in the presence of the highest concentrations of daptomycin (Fig. 1o,p). This
126 may indicate different daptomycin concentration thresholds for triggering of phospholipid
127 release.

128 To determine whether phospholipid release resulted in the inactivation of
129 daptomycin, the activity of the antibiotic in the culture supernatants was measured using a
130 previously described zone of inhibition assay [16] (Fig. 1q-x). Daptomycin was inactivated to
131 varying degrees by the bacteria, depending on the concentration of the antibiotic used.
132 However, both staphylococcal strains, both enterococcal strains, *S. gordonii* and the GAS
133 strain completely inactivated daptomycin at 5 $\mu\text{g ml}^{-1}$, but GBS strains only partially
134 inactivated the antibiotic at this concentration. At 10 $\mu\text{g ml}^{-1}$ daptomycin, only the
135 staphylococci, *S. gordonii* and the enterococci showed significant inactivation of the antibiotic
136 and at a concentration of 20 $\mu\text{g ml}^{-1}$ daptomycin, only staphylococci and enterococci
137 inactivated the antibiotic to any significant degree, with a loss of 30-60% of antibiotic activity.
138 However, despite triggering phospholipid release, at 40 $\mu\text{g ml}^{-1}$ daptomycin there was
139 relatively little (<20%) inactivation of the antibiotic by any of the bacteria tested. Therefore,
140 phospholipid release is finite and can be overcome with a sufficiently high dose of
141 daptomycin.

142 These data extend our previous finding that *S. aureus* releases phospholipid in
143 response to daptomycin and that this results in inactivation of the antibiotic by revealing a
144 very similar phenotype for *S. epidermidis*. These data also support the previous observation
145 that *E. faecalis* releases phospholipid in response to daptomycin [18], and show that this
146 phospholipid release correlates with daptomycin inactivation and bacterial survival.
147 Streptococci, particularly *S. gordonii*, also released phospholipid and inactivated daptomycin,
148 albeit less efficiently than *E. faecalis*. Therefore, daptomycin-induced phospholipid release
149 appears to be a conserved mechanism across Gram-positive pathogens.

150 Next, we wanted to explore whether the mechanism of phospholipid release and
151 daptomycin inactivation by enterococci and streptococci was similar to that of *S. aureus*.
152 Therefore, we undertook further experiments with *E. faecalis*, which was most efficient of the
153 enterococci and streptococci at releasing phospholipid and inactivating daptomycin, and *S.*
154 *aureus*, in which daptomycin-triggered phospholipid release has been well characterised [16].

155 In *S. aureus*, daptomycin-triggered phospholipid release is an active process that
156 requires energy, as well as protein and lipid biosynthesis [16]. To determine whether
157 phospholipid release by *E. faecalis* exposed to daptomycin was occurring via an active
158 process, or simply a consequence of damage caused by the antibiotic, bacteria were exposed
159 to the antibiotic in the presence or absence of a sub-inhibitory concentration of the
160 phospholipid biosynthesis inhibitor platensimycin [17]. As described previously, exposure of
161 *S. aureus* to daptomycin ($10 \mu\text{g ml}^{-1}$) resulted in increased phospholipid in the supernatant
162 but this was significantly reduced in the presence of platensimycin at half the MIC ($0.25 \mu\text{g}$
163 ml^{-1}) (Fig. 2a). Similarly, phospholipid was released upon exposure of *E. faecalis* to daptomycin
164 ($10 \mu\text{g ml}^{-1}$), but this was blocked when platensimycin was present at half the MIC ($0.5 \mu\text{g ml}^{-1}$) (Fig. 2b). The presence of platensimycin prevented *S. aureus* from inactivating daptomycin
166 (Fig. 2c) and significantly reduced the ability of *E. faecalis* to inactivate daptomycin (Fig. 2d).
167 This confirmed that daptomycin-induced phospholipid release by *E. faecalis* is an active
168 process that requires *de novo* lipid biosynthesis and is not simply a consequence of membrane
169 damage caused by the antibiotic. The ability of platensimycin to block phospholipid release
170 and prevent daptomycin inactivation by *E. faecalis* also provided strong evidence that, as for
171 *S. aureus*, daptomycin activity is blocked by the phospholipid in the supernatant. However, it
172 was necessary to rule out an alternative hypothesis; that the loss daptomycin inactivation was
173 simply due to binding of the antibiotic to the bacterial surface.

174 To measure binding of daptomycin to bacteria, daptomycin was labelled with the
175 Bodipy fluorophore (Life Technologies) as described previously [11,16]. As reported
176 previously, a killing assay with *E. faecalis* indicated that the labelled antibiotic had slightly
177 altered bactericidal activity relative to unlabelled daptomycin [11] (Fig 3a). However, as
178 described above for unlabelled antibiotic (Fig. 1q,x), the activity of the antibiotic decreased
179 after incubation with *E. faecalis* or *S. aureus* (Fig. 3b), confirming that the Bodipy label does
180 not significantly affect the interaction of the antibiotic with the bacteria studied.

181 After 8 h incubation with Bodipy-daptomycin, bacterial cells were pelleted and the
182 fluorescence of both the cells and the supernatants was measured separately using a Tecan
183 microplate reader with excitation at 502 nm and emission at 510 nm. Antibiotic attachment
184 to the *E. faecalis* cellular fraction was similar for both Bodipy-daptomycin concentrations
185 examined, suggesting saturated binding to cells (Fig. 3c). However, most of the antibiotic
186 remained in the supernatant (Fig. 3d). By comparison, Bodipy-daptomycin bound *S. aureus*

187 more strongly than *E. faecalis*, with higher levels of fluorescence associated with bacterial
188 cells and a corresponding drop in the fluorescence of the supernatant (Fig. 3c,d). This
189 difference in antibiotic binding may explain why the daptomycin MIC of the *E. faecalis* strains
190 used here (2-4 $\mu\text{g ml}^{-1}$) is higher than that of the *S. aureus* strain examined (1 $\mu\text{g ml}^{-1}$) and why
191 daptomycin triggers greater phospholipid release from staphylococci than enterococci.

192 Together, these data confirmed that the loss of daptomycin activity in *E. faecalis*
193 cultures was not due to binding of the antibiotic to the bacterial surface or the plastic vessels
194 used in the assays. However, as a final confirmation that phospholipid released from *E.*
195 *faecalis* inactivated daptomycin, we exposed the bacterium to daptomycin (5 $\mu\text{g ml}^{-1}$) to
196 trigger phospholipid release, collected the cell-free culture supernatant and added a second
197 dose of the antibiotic (5 $\mu\text{g ml}^{-1}$). The culture supernatant containing the released
198 phospholipids significantly reduced the activity of the second dose of daptomycin (by ~25%,
199 Fig. 3e). Therefore, as described for *S. aureus*, the release of phospholipids by *E. faecalis* in
200 response to daptomycin inactivates the antibiotic. The data described above also indicate that
201 several species of streptococci release phospholipids in response to daptomycin, which
202 inactivate the antibiotic, albeit to a lesser extent than *E. faecalis* or *S. aureus*.

203 Streptococci and enterococci cause a range of serious diseases, including septicaemia
204 and endocarditis, which can be treated by daptomycin, especially when the pathogen is multi-
205 drug resistant or the patient has a β -lactam allergy [1,31]. The presence of this defence
206 mechanism in a variety of clinically-relevant Gram-positive bacteria indicates that it is
207 conserved and could be a viable target to improve the effectiveness of daptomycin therapy
208 against these pathogens.

209 In this work, we focussed on daptomycin because it is a last resort antibiotic and is
210 associated with high rates of treatment failure. However, whilst daptomycin use is increasing,
211 it is very unlikely to have provided the selection pressure for the evolution of the phospholipid
212 release defence mechanism described here and previously [16]. Since cationic antimicrobial
213 peptides (cAMPs) act via a similar mechanism to daptomycin in targeting the Gram-positive
214 cell membrane [5] we hypothesise that these host defence molecules have likely driven the
215 evolution of phospholipid release as a defence mechanism.

216 The discovery of phospholipid release in several Gram-positive pathogens expands our
217 growing appreciation of broad-spectrum extracellular defence mechanisms that protect
218 bacteria against antibiotics or host defences. For example, previous work has shown that the

219 production of outer-membrane vesicles by *E. coli* can protect against membrane-acting
220 antimicrobials such as polymixin E and colistin [32], whilst another report revealed that
221 lipochalins released by *Burkholderia* can sequester several different antibiotics [33]. These
222 findings underline the complex nature of innate antibiotic resistance, but also provide
223 opportunities for mechanistic insight and improved therapeutic approaches. For example, in
224 this report and previously, we have shown that inhibition of phospholipid biosynthesis using
225 platensimycin prevents the inactivation of daptomycin by both *S. aureus* and *E. faecalis* [16].
226 Although platensimycin has not entered clinical trials due to poor pharmacokinetic properties
227 [17,34], other inhibitors of lipid biosynthesis are in clinical development [35]. Therefore, the
228 use of daptomycin in combination with lipid biosynthesis inhibitors may provide an effective
229 way of enhancing treatment outcomes compared to the lipopeptide antibiotic alone.

230 In summary, we have demonstrated that *Enterococcus faecalis* releases phospholipids
231 in response to daptomycin via an active mechanism requiring *de novo* lipid biosynthesis and
232 that these phospholipids inactivate daptomycin. Pathogenic streptococci also appear to be
233 capable of inactivating daptomycin by releasing phospholipids, indicating that this mechanism
234 is conserved amongst Gram-positive pathogens.

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247 **References**

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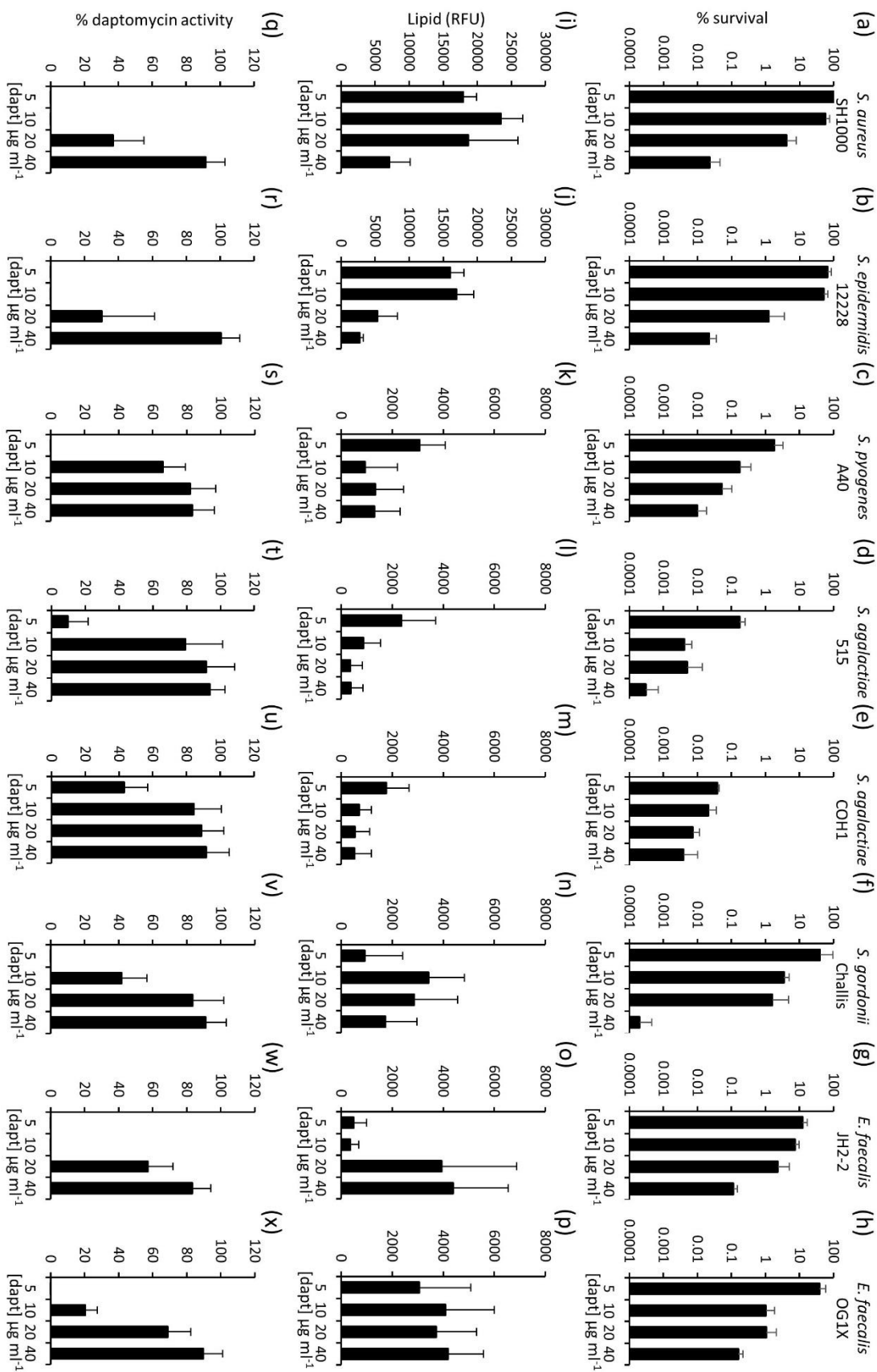
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386 **Figures and figure legends**

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389 **Fig. 1.** Streptococci and enterococci release phospholipid and inactivate daptomycin.
390 (a-h), percentage survival of bacteria after 8 h incubation in broth containing the indicated
391 concentrations of daptomycin. (i-p) the concentration of phospholipid in culture supernatants
392 of bacteria exposed to daptomycin as determined by reactivity with a fluorescent dye (RFU:
393 relative fluorescence units). Note the different Y-axis scale for staphylococci vs other bacteria.
394 (q-x) relative percentage of daptomycin activity remaining in culture supernatants of bacteria
395 exposed to daptomycin for 8 h. The activity of daptomycin incubated in culture medium only
396 for 8 h was taken to be 100 %. For all data, the mean of 4 independent experiments are shown,
397 and error bars represent the standard deviation of the mean.

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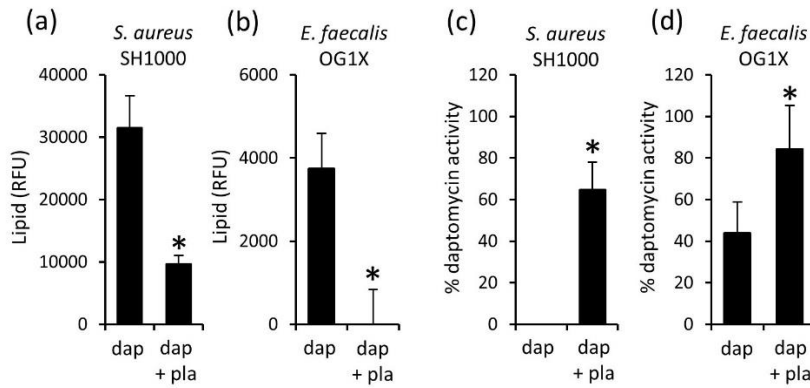
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426 **Fig. 2.** *De novo* lipid biosynthesis is required for enterococcal inactivation of daptomycin.

427 Phospholipid concentration (RFU) in culture supernatants from *S. aureus* (a) or *E. faecalis*

428 OG1X (b) incubated for 8 h in media containing daptomycin ($10 \mu\text{g ml}^{-1}$) only (dap) or both

429 daptomycin and 0.5 X MIC platensimicin (dap + pla). (c,d) relative % daptomycin activity in

430 supernatants from cultures described in (a) and (b), respectively. Data in (a) and (b) were

431 analysed using a one-way ANOVA with Tukey's post-hoc test. Data in (c) and (d) were analysed

432 by a Student's *t*-test. * $P < 0.05$.

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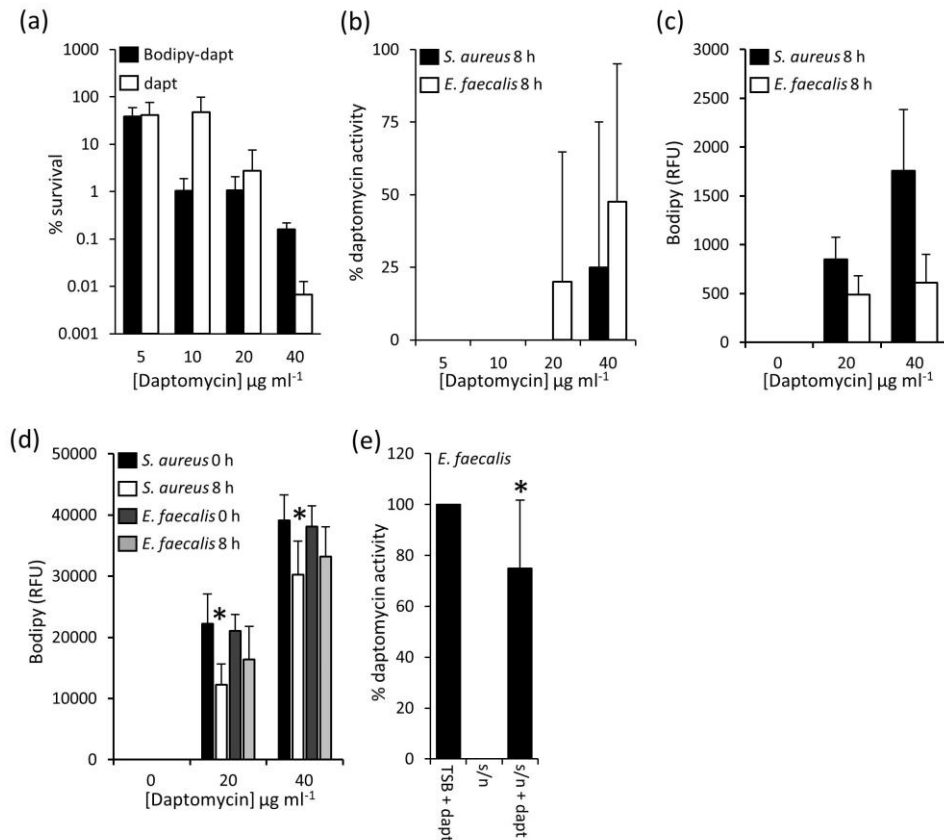
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445 **Fig. 3.** Loss of daptomycin activity in supernatant is not due to antibiotic binding to bacteria.

446 (a) percentage survival of *E. faecalis* OG1X incubated with various concentrations of

447 daptomycin (dapt) or Bodipy-daptomycin (Bodipy-dapt) for 8 h. (b) relative percentage

448 daptomycin activity in culture supernatants described in (a). (c) binding of Bodipy-daptomycin

449 to *S. aureus* or *E. faecalis* OG1X after 8 h incubation in media containing the indicated

450 concentration of the labelled antibiotic. (d) quantification of Bodipy-daptomycin (RFU)

451 remaining in culture supernatants from *S. aureus* or *E. faecalis* OG1X, after 8 h incubation with

452 Bodipy-daptomycin as described in (c) * indicates significantly different from 0 h time point.

453 (e) relative percentage activity of daptomycin (5 µg ml⁻¹) activity in TSB only (TSB + dapt), in

454 the supernatant from *E. faecalis* incubated with daptomycin for 8 h (s/n) and after the addition

455 of 5 µg ml⁻¹ daptomycin to the supernatant from *E. faecalis* incubated with daptomycin for 8

456 h (s/n + dapt).

457 Data in (d) and (e) were analysed by a two-way ANOVA with Tukey's post-hoc test. Graphs

458 show the mean average and, where shown, error bars represent the standard deviation of the

459 mean. For each panel *P<0.05.