

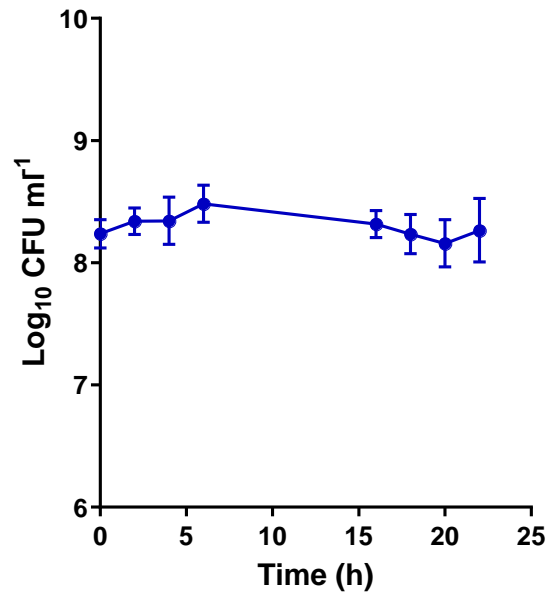
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Human serum triggers antibiotic tolerance in *Staphylococcus aureus*

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Supplementary information

Supplementary Figures 1-15



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34 **Supplementary Fig. 1. *S. aureus* CFU counts do not change during incubation in human serum.** Log₁₀
35 CFU ml⁻¹ during incubation of TSB-grown *S. aureus* in human serum for 22 h. Graph represents the
36 geometric mean ± geometric standard deviation of three independent experiments. Data were
37 analysed by one-way ANOVA and no statistically significant differences were observed (log₁₀ CFU ml⁻¹
38 at time points vs at 0 h). Source data are provided as a Source Data file.

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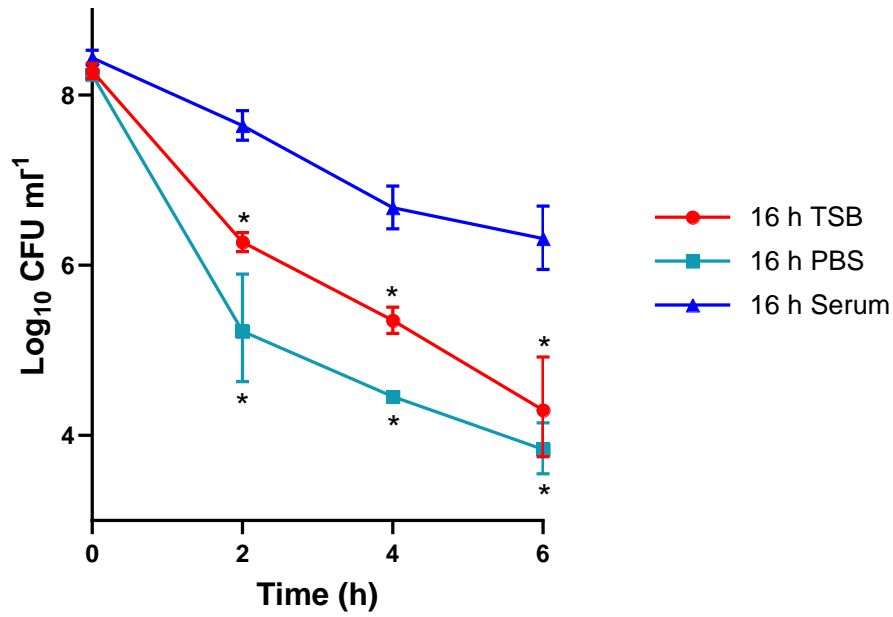
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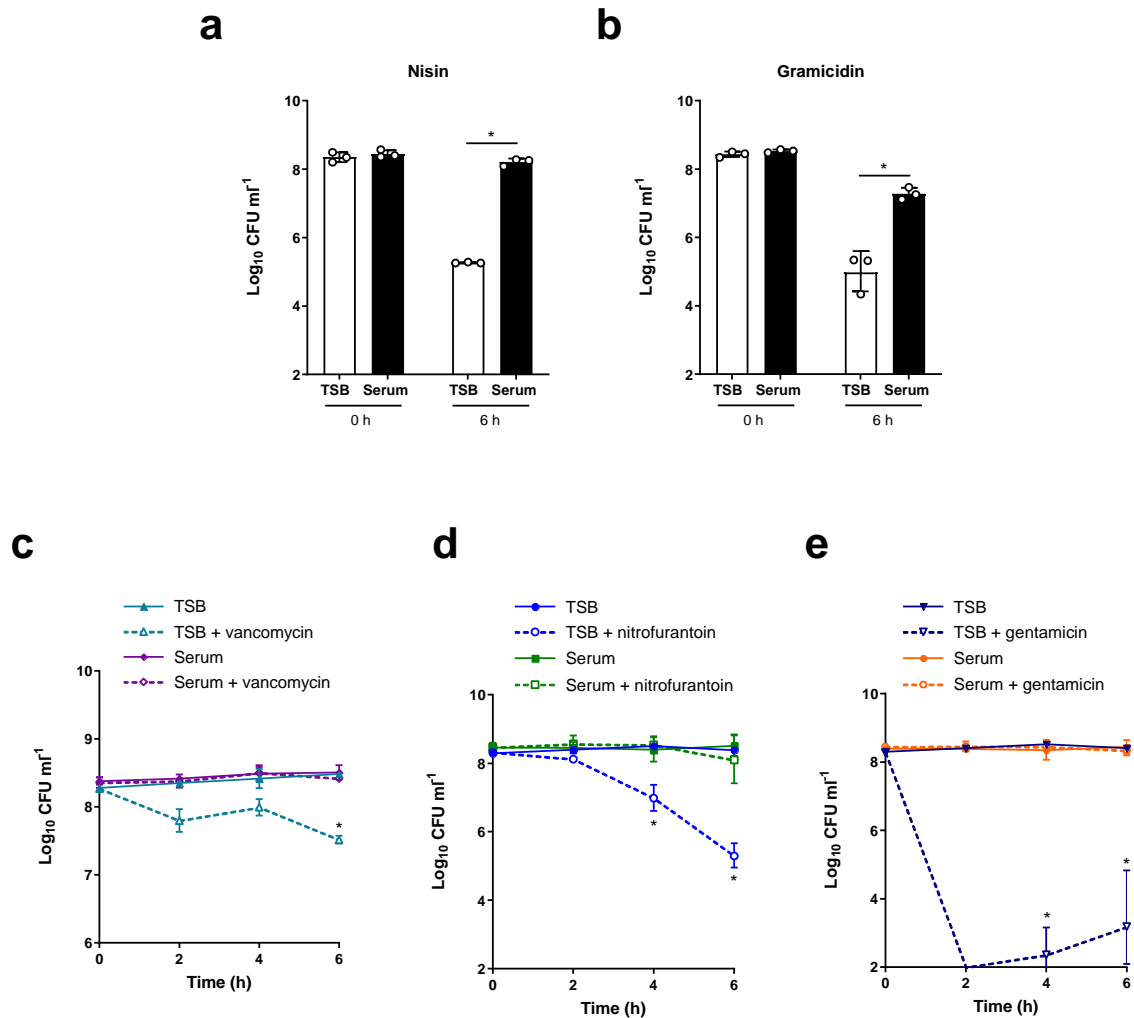
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55 **Supplementary Fig. 2. Incubation of *S. aureus* in serum but not TSB or PBS for 16 h results in**
 56 **tolerance towards daptomycin.** TSB-grown *S. aureus* cultures were incubated for 16 h in TSB, PBS or
 57 serum, adjusted to 2×10^8 CFU ml⁻¹ where necessary and resuspended in fresh serum containing 80
 58 $\mu\text{g ml}^{-1}$ daptomycin. Log₁₀ CFU ml⁻¹ was measured over 6 h. Graph represents the geometric mean \pm
 59 geometric standard deviation of three independent repeats and data were analysed by two-way
 60 ANOVA with Tukey's *post-hoc* test (serum-adapted vs PBS/TSB-adapted at each time point). * P =
 61 0.0016 (TSB 2 h), 0.0065 (TSB 4 h), 0.022 (TSB 6 h), 0.0292 (PBS 2 h), 0.0075 (PBS 4 h), 0.0023 (PBS 6
 62 h). Source data are provided as a Source Data file.

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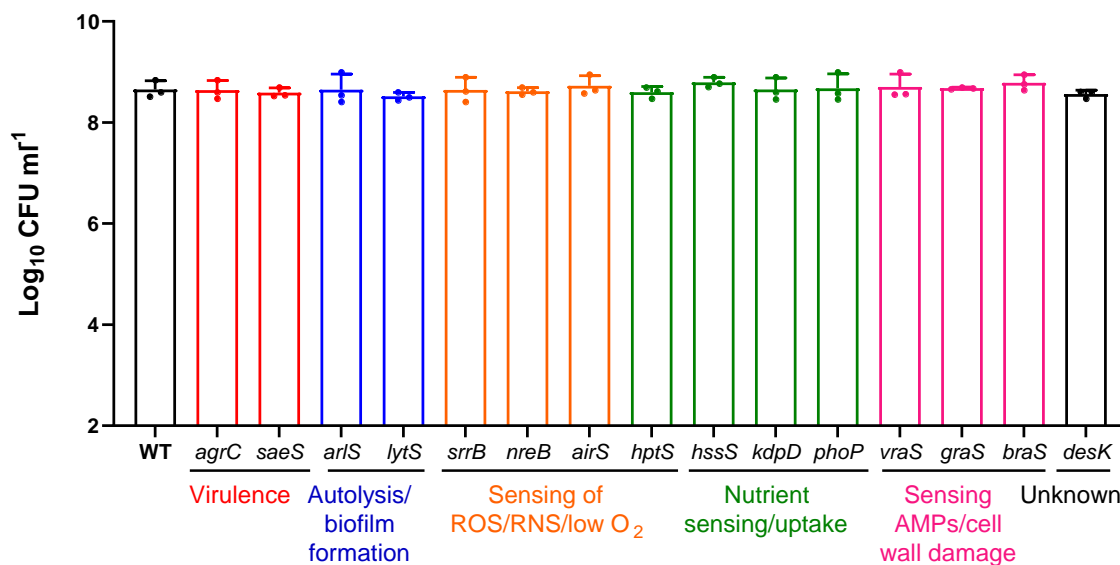


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 65 **Supplementary Fig. 3. Incubation of *S. aureus* in serum results in tolerance towards antimicrobials**
 66 **from various classes.** Log₁₀ CFU ml⁻¹ of TSB-grown and serum-adapted cultures of *S. aureus* USA300
 67 WT after/throughout a 6 h incubation in serum with 128 μg ml⁻¹ nisin (a), 160 μg ml⁻¹ gramicidin (b),
 68 160 μg ml⁻¹ vancomycin (c), 160 μg ml⁻¹ nitrofurantoin (d) or 40 μg ml⁻¹ gentamicin (e). Graphs
 69 represent the geometric mean ± geometric standard deviation of three (a, b, d, e) or two (c)
 70 independent replicates. Graphs in a – b were analysed by two-way ANOVA with Sidak's *post-hoc* test
 71 (* for a, P < 0.0001. For b, P < 0.0001. Graphs in c – e were analysed by two-way ANOVA with Tukey's
 72 *post-hoc* test; TSB-grown compared to serum-adapted at each time-point. (* for c, P = 0.0103. For d,
 73 P = 0.0197 (4 h), 0.0257 (6 h). For e, P = 0.0122 (4 h), 0.0491 (6h)). In e, some data points fell below
 74 the limit of detection of 100 CFU ml⁻¹. Source data are provided as a Source Data file.

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85 **Supplementary Fig. 4. TCS-deficient mutants survive as well as WT in human serum.** Log₁₀ CFU ml⁻¹
86 of *S. aureus* JE2 WT and mutants defective for the sensor kinase components of TCS after 16 h
87 incubation in human serum. Data represent the geometric mean ± geometric standard deviation of
88 three independent experiments. Data were analysed by one-way ANOVA and no statistically
89 significant differences were observed demonstrating there were no differences in survival in serum
90 between the WT and any of the mutants. Source data are provided as a Source Data file.

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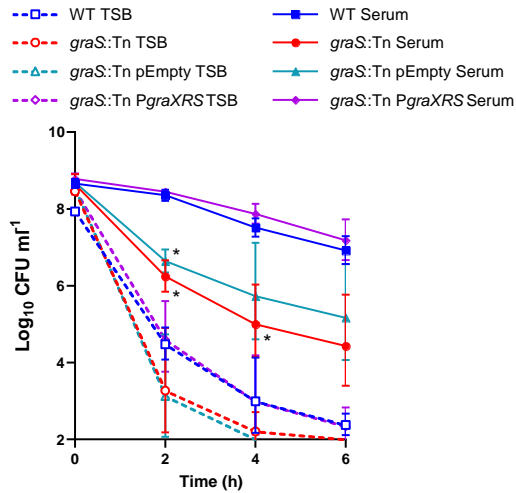
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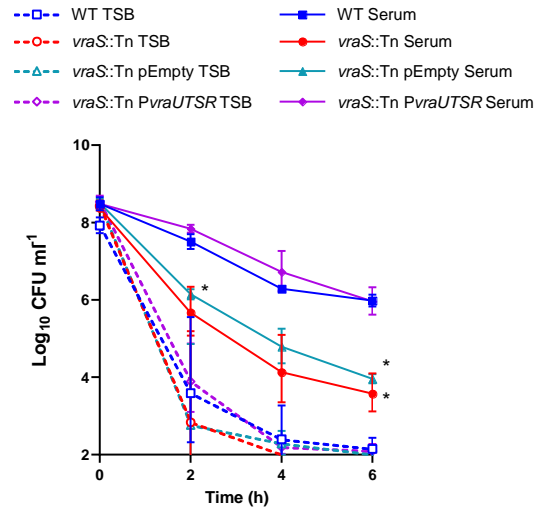
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108 **Supplementary Fig. 5. Complementation of the *vraS::Tn* and *graS::Tn* mutants restores tolerance to**

109 **WT levels.** Log₁₀ CFU ml⁻¹ of TSB-grown and serum-adapted cultures of *S. aureus* JE2 WT, *graS::Tn* and

110 *graS::Tn* complemented with pCN34 or P*graXRS* (a) and WT, *vraS::Tn* and *vraS::Tn* complemented with

111 empty pCN34 or P*vraUTSR* (b) over 6 h incubation in serum with 80 μg ml⁻¹ daptomycin. Graphs

112 represent the geometric mean ± geometric standard deviation of three independent experiments. *

113 for a, P = 0.0008 (2 h *graS::Tn* pEmpty Serum), 0.0019 (2 h *graS::Tn* Serum), 0.0279 (4 h *graS::Tn*

114 Serum). For b, P = 0.002 (2 h *vraS::Tn* pEmpty Serum), 0.0002 (6 h *vraS::Tn* pEmpty Serum), 0.0198 (6

115 h *vraS::Tn* Serum) determined by two-way ANOVA with Dunnett's *post-hoc* test, serum-adapted WT

116 compared to serum-adapted mutants at each time-point. Some data points fell below the limit of

117 detection of 100 CFU ml⁻¹. Source data are provided as a Source Data file.

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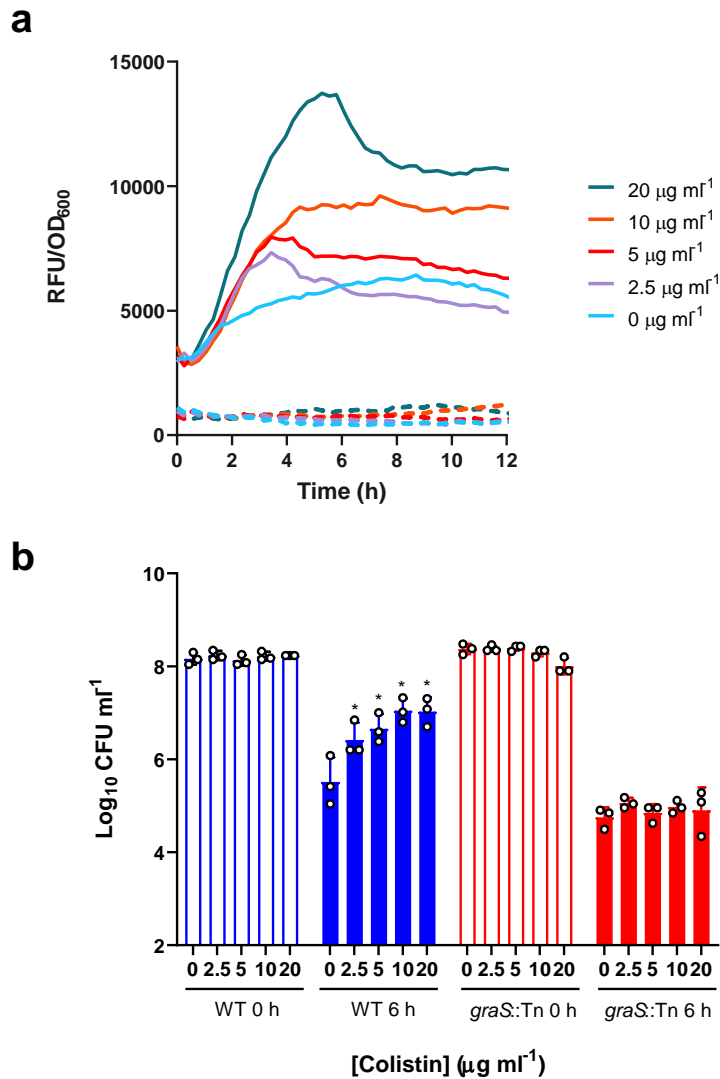
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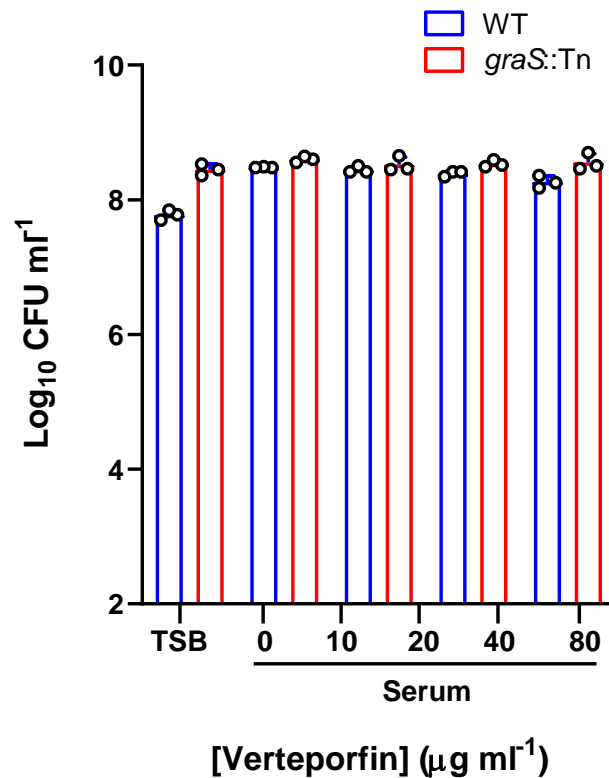


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127 **Supplementary Fig. 6. Colistin activates GraRS signalling and triggers daptomycin tolerance.** TSB-
 128 grown cultures of *S. aureus* JE2 WT (solid lines) and the *graS::Tn* mutant (dashed lines) containing
 129 *PdltA-gfp* were exposed to various concentrations of colistin (2.5 – 20 µg ml⁻¹) in RPMI 1640 (a). GFP
 130 fluorescence (RFU) and OD₆₀₀ were measured every 15 min for 12 h. Fluorescence values were divided
 131 by OD₆₀₀ measurements to normalise for changes in cell density. *S. aureus* JE2 WT and *graS::Tn* mutant
 132 were each incubated for 16 h in RPMI 1640 only or RPMI 1640 supplemented with indicated
 133 concentrations of colistin before determination of CFU counts prior to (0 h) and after (6 h) exposure
 134 to 80 µg ml⁻¹ daptomycin (b). Graph in a represents the mean of three independent experiments and
 135 error bars have been omitted for clarity. Graph in b represents the geometric mean ± geometric
 136 standard deviation of three independent experiments. *, P = 0.0001 (2.5 µg ml⁻¹), < 0.0001 (5 µg ml⁻¹),
 137 < 0.0001 (10 µg ml⁻¹), < 0.0001 (20 µg ml⁻¹) determined by two-way ANOVA with Dunnett's *post-hoc*
 138 test, RPMI 1640 and colistin compared to RPMI 1640 only. RFU, relative fluorescent units; OD₆₀₀,
 139 optical density at 600 nm. Source data are provided as a Source Data file.

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144 **Supplementary Fig. 7. Exposure of *S. aureus* to verteporfin does not affect bacterial survival in**
 145 **serum.** Log₁₀ CFU ml⁻¹ of *S. aureus* JE2 WT grown in TSB or after 16 h incubation in human serum with
 146 indicated concentrations of verteporfin. Data represent the geometric mean ± geometric standard
 147 deviation of three independent experiments. Data were analysed by two-way ANOVA and no
 148 statistically significant differences were observed between bacteria incubated in serum ± verteporfin
 149 confirming that verteporfin does not compromise *S. aureus* survival in serum in the absence of
 150 daptomycin. Source data are provided as a Source Data file.

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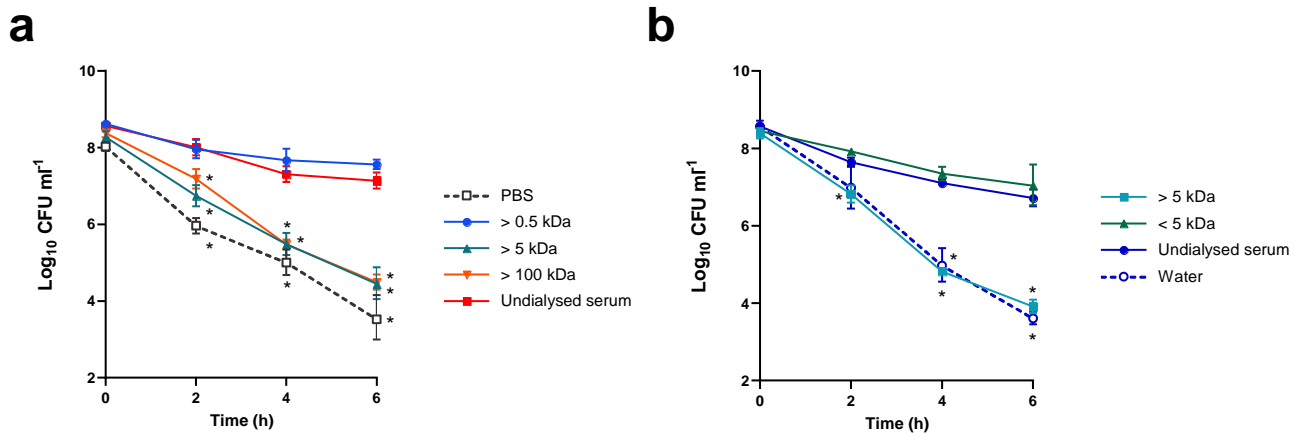
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164 **Supplementary Fig. 8. A 0.5 – 5 kDa fraction of serum is necessary and sufficient to trigger**
165 **daptomycin tolerance.** Serum was fractionated by dialysis against PBS using membranes with various
166 molecular weight cut-offs. Next, CFU ml⁻¹ were determined for *S. aureus* JE2 WT which had been
167 incubated for 16 h in serum fractions, or in PBS only or unfractionated serum, then exposed to 80 µg
168 ml⁻¹ daptomycin in fresh human serum over 6 h (a). Log₁₀ CFU ml⁻¹ of *S. aureus* JE2 WT which had been
169 incubated for 16 h in < 5 kDa or > 5 kDa serum fractions, or water only or undialysed serum, then
170 exposed to 80 µg ml⁻¹ daptomycin in fresh human serum over 6 h (b). Graphs represent the geometric
171 mean ± geometric standard deviation of three independent experiments and were analysed by two-
172 way ANOVA with Dunnett's *post-hoc* test. (* for a, P = 0.0373 (>100 kDa 2 h), 0.0016 (>100 kDa 4 h),
173 0.0003 (>100 kDa 6 h), 0.0106 (>5 kDa 2 h), 0.0037 (>5 kDa 4 h), 0.0053 (>5 kDa 6 h), 0.0008 (PBS 2 h),
174 0.0033 (PBS 4 h), 0.0121 (PBS 6 h). For b, P = 0.0233 (>5 kDa 2 h), 0.0009 (>5 kDa 4 h), 0.0002 (>5 kDa
175 6 h), 0.0206 (Water 4 h), 0.0002 (Water 6 h) comparing undialysed serum vs serum
176 fractions/PBS/water). Source data are provided as a Source Data file.

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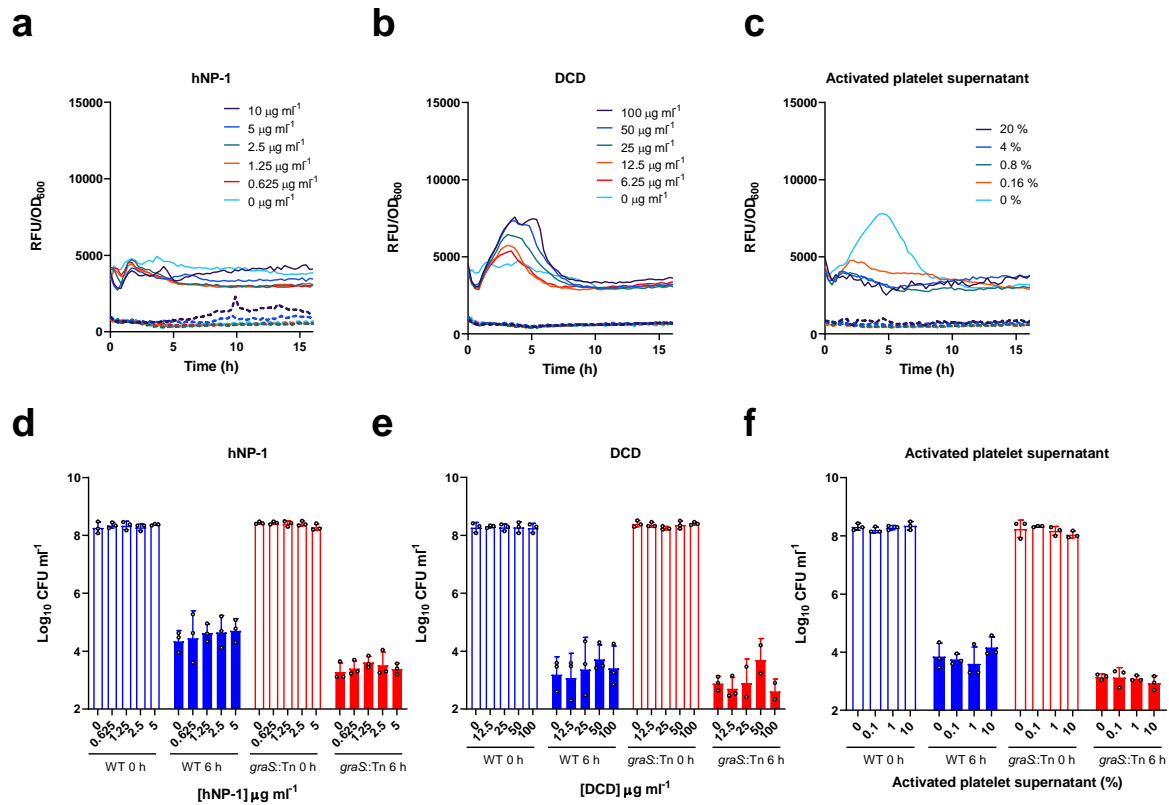
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189 **Supplementary Fig. 9. Human neutrophil peptide-1, dermcidin and platelet AMPs do not activate**
 190 **GraRS signalling or induce daptomycin tolerance.** TSB-grown cultures of *S. aureus* JE2 WT (solid lines)
 191 and the *graS::Tn* mutant (dashed lines) containing *PdltA-gfp* were exposed to a range of
 192 concentrations of hNP-1 (a), DCD (b) or activated platelet supernatant (c) in RPMI 1640 and GFP
 193 fluorescence (RFU) and OD₆₀₀ were measured every 15 min for 16 h. Fluorescence values were divided
 194 by OD₆₀₀ measurements to normalise for changes in cell density. *S. aureus* JE2 WT and *graS::Tn* mutant
 195 were pre-incubated in RPMI 1640 containing hNP-1 (d), DCD (e) or activated platelet supernatant (f)
 196 for 16 h before CFU ml⁻¹ were determine before (0 h) and after (6 h) exposure to 80 μg ml⁻¹
 197 daptomycin. Graphs in a – c represent the mean of at least two independent experiments with error
 198 bars omitted for clarity. Graphs in d – f represent the geometric mean ± geometric standard deviation
 199 of three independent experiments. Data in d – f were analysed by two-way ANOVA. No statistically
 200 significant differences were observed (RPMI 1640 + AMPs compared to RPMI 1640 alone). Source data
 201 are provided as a Source Data file.

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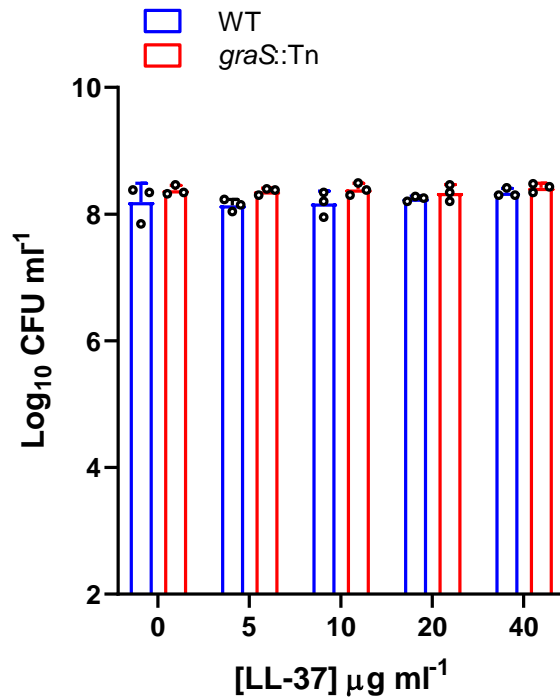
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209 **Supplementary Fig. 10. Exposure of *S. aureus* to LL-37 does not affect bacterial survival.** Log₁₀ CFU
 210 ml⁻¹ of *S. aureus* JE2 WT after 16 h incubation in RPMI 1640 with indicated concentrations of LL-37.
 211 Data represent the geometric mean ± geometric standard deviation of three independent
 212 experiments. Data were analysed by two-way ANOVA and no statistically significant differences were
 213 observed (RPMI 1640 + LL-37 compared to RPMI 1640 alone). Source data are provided as a Source
 214 Data file.

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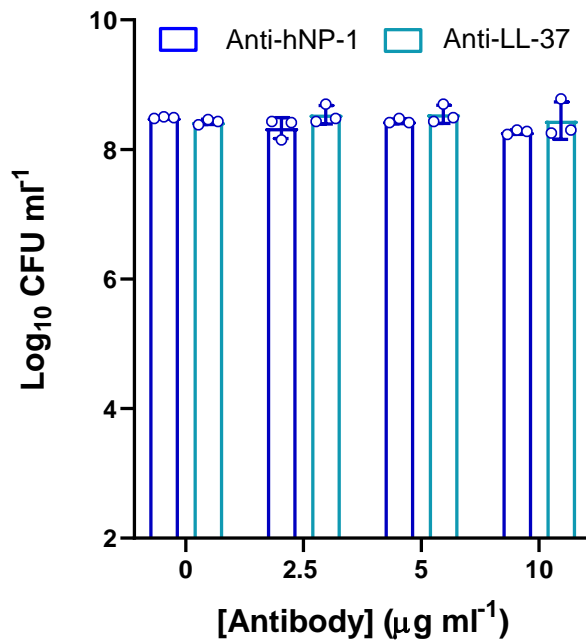
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229 **Supplementary Fig. 11. Exposure of *S. aureus* to anti-hNP-1 or anti-LL-37 antibodies did not affect**
230 **bacterial survival.** Log₁₀ CFU ml⁻¹ of *S. aureus* JE2 WT after incubation for 16 h in human serum
231 supplemented with indicated concentrations of antibodies against hNP-1 or LL-37. Data represent the
232 geometric mean ± geometric standard deviation of three independent experiments. Data were
233 analysed by two-way ANOVA and no statistically significant differences were observed. Source data
234 are provided as a Source Data file.

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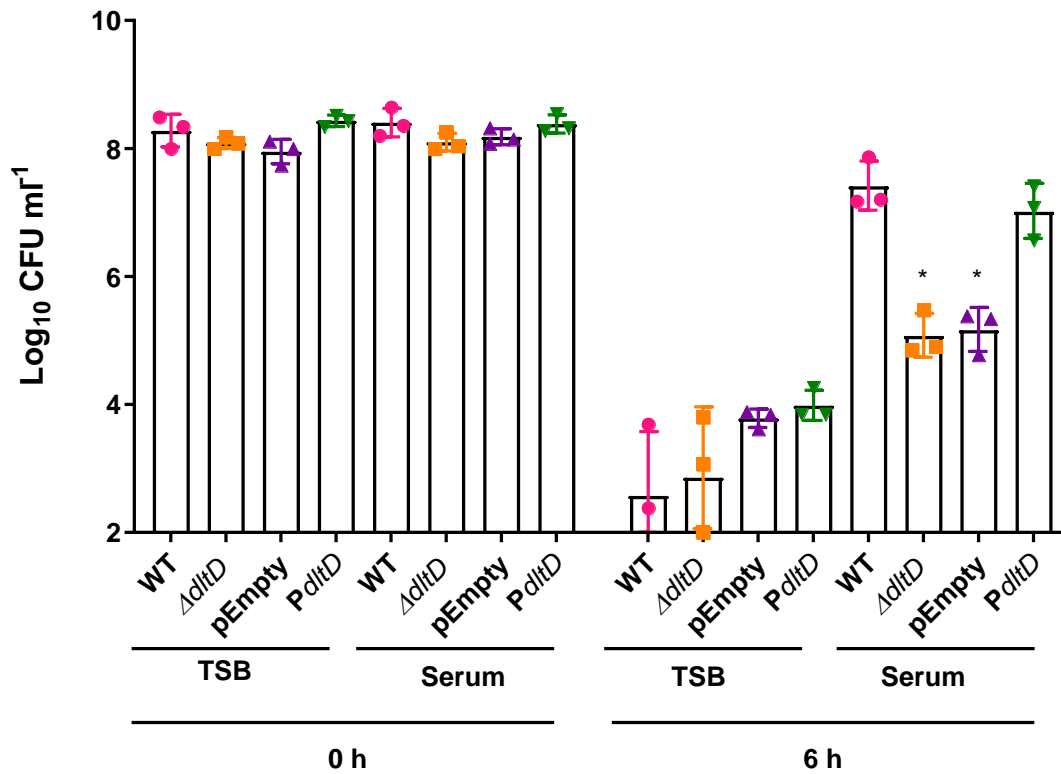
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246 **Supplementary Fig. 12. Complementation of $\Delta dltD$ mutant with WT $dltD$ restores tolerance to WT**
 247 **levels.** Log₁₀ CFU ml⁻¹ of TSB-grown and serum-adapted cultures of WT, the $\Delta dltD$ mutant or $\Delta dltD$
 248 mutant complemented with either pCN34 or $PdltD$ after a 16 h incubation in serum (0 h) and after
 249 exposure to daptomycin in serum (6 h). Graph represents the geometric mean \pm geometric standard
 250 deviation of three independent experiments (one data point fell below the limit of detection of 100
 251 CFU ml⁻¹). Data were analysed by two-way ANOVA with Dunnett's *post-hoc* test (* $P < 0.0001$ ($\Delta dltD$
 252 serum 6 h), < 0.0001 (pEmpty serum 6 h) comparing serum-adapted WT with serum-adapted
 253 mutants). Source data are provided as a Source Data file.

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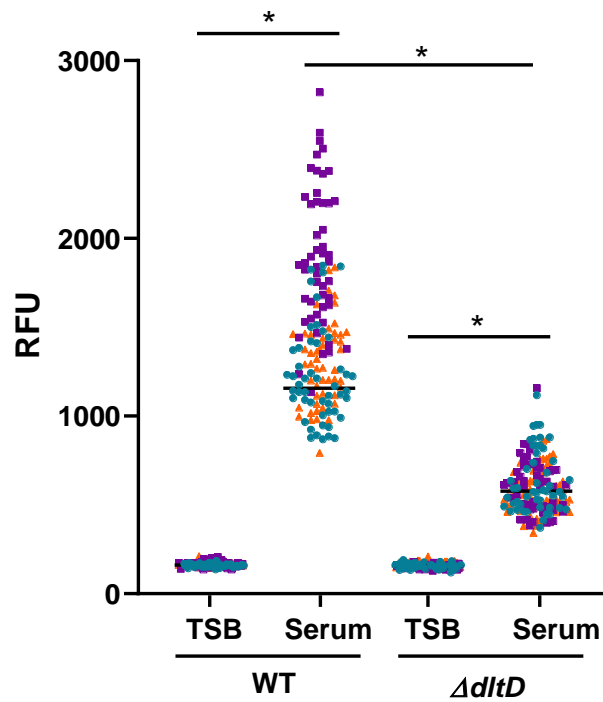
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266 **Supplementary Fig. 13. The $\Delta dltD$ mutant incorporates less HADA than WT during serum adaptation.**

267 The fluorescence of individual TSB-grown and serum-adapted WT and $\Delta dltD$ cells was quantified.
 268 Graph represents the fluorescence of 50 cells per biological replicate (150 cells in total) with the mean
 269 of the three replicates indicated. Each biological replicate is depicted in a different colour. Data were
 270 analysed by Kruskal Wallis test (* P = < 0.0001 (WT TSB vs Serum), < 0.0001 (WT Serum vs $\Delta dltD$
 271 Serum), < 0.0001 ($\Delta dltD$ TSB vs $\Delta dltD$ Serum)). Source data are provided as a Source Data file.

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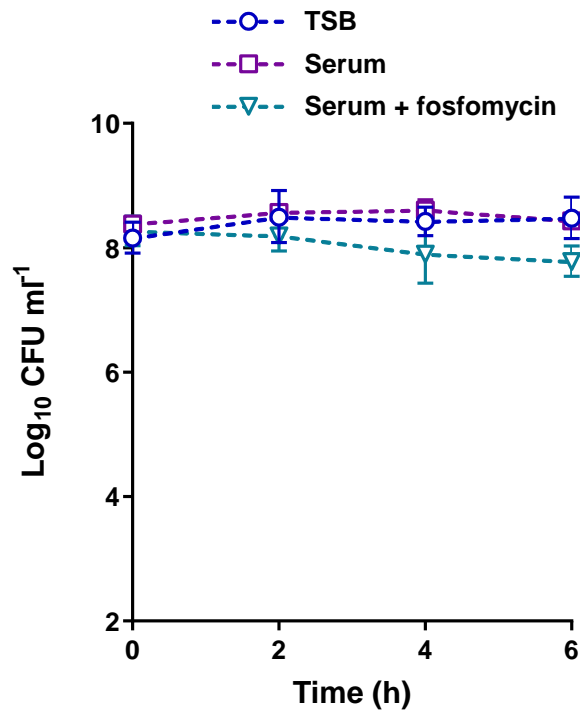
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288 **Supplementary Fig. 14. Inhibition of peptidoglycan synthesis during serum adaptation did not affect**
289 **bacterial viability.** Log₁₀ CFU ml⁻¹ over 6 h of TSB-grown *S. aureus* or cultures which had been
290 incubated in serum supplemented, or not, with 64 μg ml⁻¹ fosfomycin for 16 h. Graph represents the
291 geometric mean ± geometric standard deviation of three independent experiments. No statistically
292 significant differences were observed (two-way ANOVA, log₁₀ CFU ml⁻¹ at each time-point vs 0 h).
293 Source data are provided as a Source Data file.

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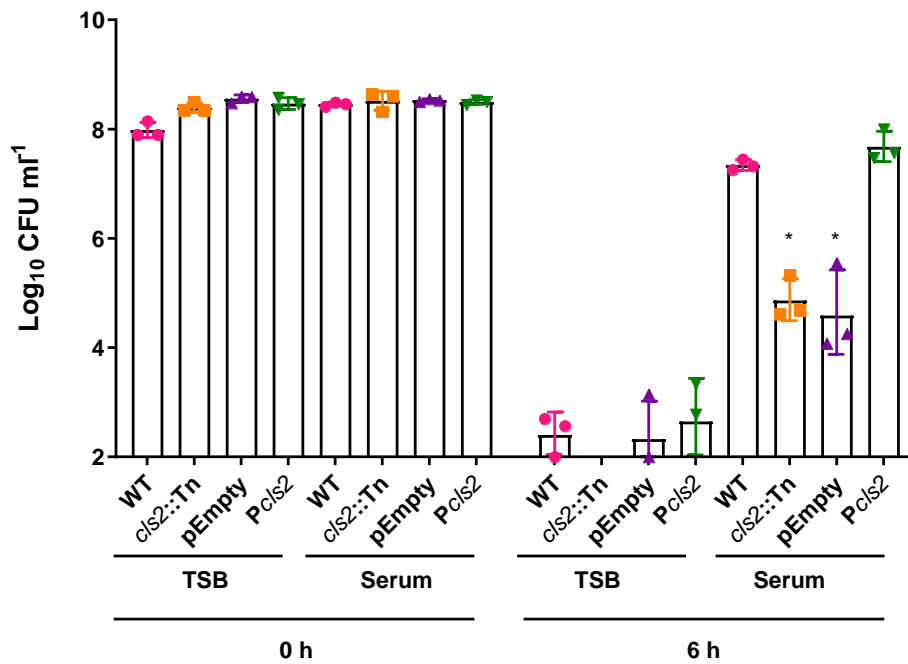
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309 **Supplementary Fig. 15. Complementation of the *cls2*::Tn mutant restores daptomycin tolerance.**

310 Log₁₀ CFU ml⁻¹ of TSB-grown and serum-adapted cultures of *S. aureus* JE2 WT, *cls2*::Tn or *cls2*::Tn
311 complemented with empty pCN34 or *Pcls2* before (0 h) or after (6 h) exposure to 80 μg ml⁻¹
312 daptomycin in serum. Graph represents the geometric mean ± geometric standard deviation of three
313 independent experiments (some data points fell below the limit of detection of 100 CFU ml⁻¹). Data
314 were analysed by two-way ANOVA with Dunnett's *post-hoc* test (* P < 0.0001 (*cls2*::Tn serum 6 h), <
315 0.0001 (pEmpty serum 6 h) comparing serum-adapted WT with serum-adapted mutants). Source data
316 are provided as a Source Data file.