Staphylococcal host adaptation compromises neutrophil killing by concealing bound opsonins with peptidoglycan

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Keywords: *Staphylococcus aureus* / neutrophil / antibody / peptidoglycan / opsonophagocytosis / immune evasion

Running title: Cell wall accumulation conceals opsonins

Summary

The bacterial pathogen *Staphylococcus aureus* adapts to the host environment by increasing the thickness of its cell wall. However, the impact of cell wall thickening on susceptibility to host defences is unclear. Here, we show that bacteria grown in culture medium were rapidly opsonised by antibody and complement upon incubation with human serum, resulting in efficient phagocytosis and killing by human neutrophils. However, as bacteria adapted to serum, the resulting increase in cell wall thickness led to the concealment of bound opsonins and a corresponding reduction in phagocytosis and killing by neutrophils. Partial digestion of accumulated peptidoglycan using the enzyme lysostaphin restored opsonin exposure, phagocytosis and killing, whilst the antibiotic fosfomycin blocked accumulation of peptidoglycan and maintained the susceptibility of *S. aureus* to opsonophagocytic killing by neutrophils. These findings reveal that the adaptation of *S. aureus* to the host environment significantly reduces the ability of the immune system to detect and kill this pathogen through concealment of bound opsonins via peptidoglycan accumulation.

Introduction

Staphylococcus aureus frequently infects wounds caused by surgery or insertion of intravenous access devices [1,2]. These infections can result in *S. aureus* seeding into the bloodstream, leading to bacteraemia and subsequent metastatic dissemination to sites including the heart, bones and joints [1,3,4,5]. Despite antibiotic therapy and a potent immune response, *S. aureus* infections have a high rate of relapse and frequently become chronic or recurrent [3,5].

Neutrophils are a key host defence against *S. aureus* infection and are recruited to the site of infection from the bloodstream [6,7,8,9,10,11]. The detection of *S. aureus* by neutrophils is largely dependent upon opsonisation of bacteria by bound antibody and complement, which is enabled in most people by the presence of antibodies against a range of different staphylococcal surface proteins [10,11,12,13]. The binding of neutrophils to opsonins on the surface of *S. aureus* occurs via dedicated receptors and triggers phagocytosis of the pathogen followed by the subsequent exposure of ingested bacteria to a raft of bactericidal products including reactive oxygen species, antimicrobial peptides and proteases [11,12].

To combat the threat posed by neutrophils, *S. aureus* has evolved numerous mechanisms of evading opsoninic complement and antibody [12,13,14,15]. For example, *S. aureus* produces two immunoglobulin binding proteins, Spa and Sbi, that reduce antibody-mediated opsonisation, whilst the production of proteins such as SCIN, Efb and CHIPS reduces complement deposition, activation

63 and detection by immune cells [12-24]. As such, the bacterial cell surface is a critically important
64 determinant in immune detection of *S. aureus* and efforts by the pathogen to evade surveillance and
65 killing by host defences.

66 The staphylococcal cell envelope is a dynamic structure that responds to host-induced stresses [25-
67 28]. Consequently, *S. aureus* has a thicker cell wall *in vivo* than when growing *in vitro* [29], a phenotype
68 that is replicated when staphylococci are exposed to human serum or present within endothelial or
69 osteoblast cells [30,31,32]. In the case of serum, cell wall thickening is triggered when *S. aureus* detects
70 the presence of the host defence antimicrobial peptide LL-37 via the GraRS two component system
71 [30].

72 Host-induced changes to the cell wall are important for the ability of the pathogen to cause and sustain
73 infection. Cell wall thickening has been shown to reduce susceptibility to antibiotics, whilst mutant
74 strains lacking various cell wall synthetic enzymes are less virulent in infection models [29,30,33].
75 However, it is unknown whether host-induced changes to the bacterial cell wall affect the detection
76 and killing of *S. aureus* by the host immune system. To address this, we examined the impact of host-
77 induced changes to the staphylococcal cell envelope on subsequent interactions of *S. aureus* with
78 neutrophils. This revealed that cell wall thickening constitutes a previously unrecognised mechanism
79 of immune evasion that functions by concealing bound opsonins and thereby reducing
80 opsonophagocytic killing.

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82 ## Results

83 ### Host adaptation reduces killing of *S. aureus* by neutrophils

84 To understand the impact of host adaptation on staphylococcal susceptibility to host defences, we
85 either grew bacteria to exponential phase in tryptic soy broth (TSB) to represent standard laboratory
86 conditions or allowed bacteria to adapt to host conditions using a previously described *ex vivo* human
87 serum model that triggers cell wall thickening [30] (Fig. 1A). We then measured the survival of bacteria
88 prepared under each condition during incubation with purified *ex vivo* human neutrophils from male
89 and female healthy donors in the presence of serum to provide antibody- and complement-mediated
90 opsonisation [34,35]. We examined four distinct wild type *S. aureus* strains to represent both
91 methicillin resistant (USA300, Col) and methicillin susceptible (SH1000, Newman) organisms
92 [36,37,38,39].

93 For all four of the *S. aureus* strains tested, exponential phase bacteria were efficiently killed over time,
94 with <5% of bacteria remaining viable after 2 hours incubation with neutrophils (Fig. 1B,C,D,E).

95 However, host-adapted bacteria survived at levels 5-10 times greater than that seen for exponential
96 bacteria for all strains (Fig. 1B,C,D,E). In addition to demonstrating that host adaptation reduces
97 staphylococcal susceptibility to host immune defences, the high level of consistency observed across
98 all four strains indicated that this is a conserved phenotype.

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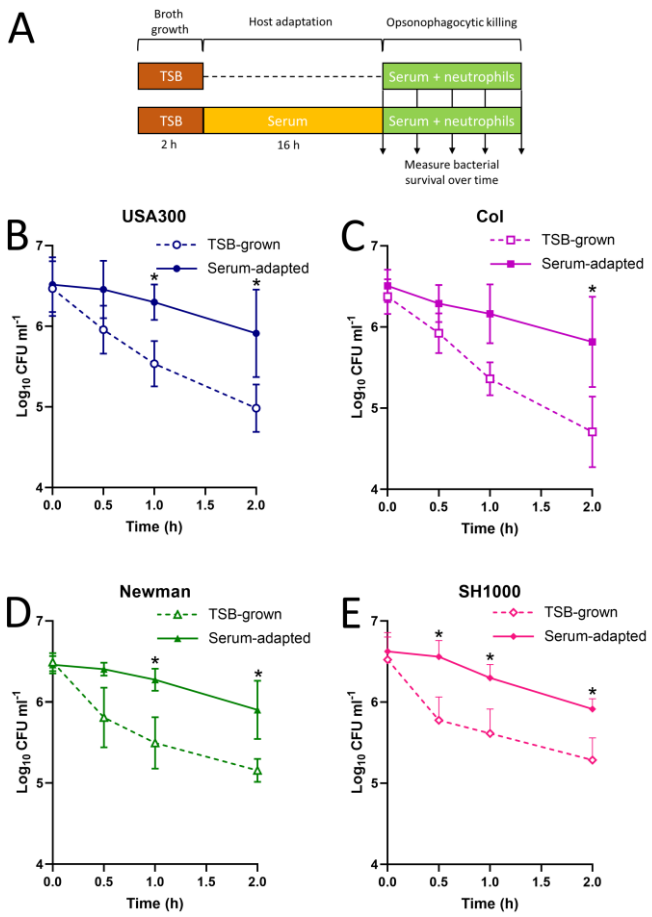

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102 **Figure 1. Incubation in human serum promotes tolerance to killing by neutrophils.** Bacteria were
103 grown in broth or allowed to adapt to human serum before incubation with human neutrophils in the
104 presence of serum and staphylococcal survival measured over time (**A**). Survival of TSB-grown and
105 serum-adapted cultures of *S. aureus* USA300 (**B**), Col (**C**), Newman (**D**) and SH1000 (**E**) during a 2 h
106 incubation with purified human neutrophils. Data represent the geometric mean ± geometric standard
107 deviation of at least three independent biological replicates. Data were analysed by a two-way ANOVA
108 with Sidak's *post-hoc* test (*, $P < 0.05$; Serum-adapted vs TSB-grown at indicated time-points).
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112 **Host adaptation reduces opsonin exposure and opsonophagocytosis**

113 Having found that host adaptation reduced staphylococcal susceptibility to host defences, we next
114 determined the mechanism(s) responsible. Given the consistency in survival data across all four strains
115 examined, we focussed on the USA300 lineage since it is both well characterised and clinically
116 important.

117 We started by assessing whether the increase in survival of host-adapted bacteria was due to impaired
118 phagocytosis, using two distinct assays. Bacteria were grown in broth or host-adapted by incubation
119 in serum, before washing in PBS and then incubation with neutrophils in the presence of fresh serum
120 to enable opsonisation (Fig. 2A). The first phagocytosis assay was a well-established flow cytometry-
121 based approach that determined how many fluorescently-labelled bacteria were associated (or not)
122 with neutrophils [35,40]. This revealed that the majority of both broth-grown and host-adapted *S.*
123 *aureus* were phagocytosed after 30 min incubation with neutrophils. However, whilst <3% of broth-
124 grown bacteria remained unphagocytosed, >20% of host-adapted bacteria were free from neutrophils
125 (Fig. 2B). This finding was replicated in a second phagocytosis assay that measured the viability of free
126 and neutrophil-associated bacteria [41], with >10% of host-adapted *S. aureus* unphagocytosed
127 compared with <1% of broth-grown *S. aureus* cells (Fig. 2C). Combined, these two assays
128 demonstrated that host-adapted *S. aureus* were significantly better at evading phagocytosis than
129 broth-grown bacteria.

130 An additional finding was that there were equal numbers of viable host-adapted and broth-grown *S.*
131 *aureus* cells associated with neutrophils. This indicated that host-adapted *S. aureus* were as
132 susceptible to the microbicides produced by the neutrophils as broth-grown cells (Fig. 2C). Therefore,
133 we concluded that the enhanced survival of host-adapted bacteria compared with broth-grown
134 bacteria (Fig. 1) was due to enhanced evasion of phagocytosis, rather than resistance to the
135 antibacterial products of neutrophils.

136 To understand why host-adapted *S. aureus* were phagocytosed much less efficiently than broth-grown
137 cells, we examined the degree of opsonisation of bacteria by antibody and complement using western
138 blotting. In keeping with previous work [42,43], for this experiment we used a mutant strain of USA300
139 lacking Spa and Sbi to avoid interference caused by these immunoglobulin binding proteins. Broth-
140 grown or host-adapted bacteria were washed in PBS and then incubated, or not, in fresh serum to
141 enable opsonin binding as used in the opsonophagocytosis assays described above (Fig. 1A) before
142 detection of bound antibody and complement component C3 (Fig. 2D).

143 Despite their reduced phagocytosis by neutrophils, there was more antibody and complement bound
144 to host-adapted cells than to broth-grown, suggesting that a lack of bound opsonins did not explain
145 the immune evasion phenotype of host-adapted bacteria (Supplementary Fig. S1).

146 To understand why host-adapted cells had high levels of bound antibody and complement but low
147 levels of phagocytosis, bacteria were prepared as described above for opsonophagocytosis assays and
148 then the levels of surface-exposed antibody and the complement component C3 quantified using flow
149 cytometry (Fig. 2D). Broth-grown bacteria that had been exposed to PBS instead of serum acted as a
150 negative control and confirmed that antibodies used in the assay did not bind non-specifically to *S.*
151 *aureus* cells (Fig. 2D,E,F). We then showed that, as expected, broth-grown bacteria that were
152 incubated in human serum for 30 min were very strongly bound by both IgG and the complement
153 component C3 (Fig. 2E,F).

154 Next, we examined host adapted bacteria and found that they had a very low level of exposed
155 opsonins, regardless of whether they had been incubated in fresh serum for 30 min or not (Fig. 2E,F).
156 Therefore, despite prolonged incubation in serum and high levels of bound antibody and complement
157 (supplementary Fig. S1), host-adapted cells had significantly reduced exposure of opsonins on their
158 cell surface relative to broth-grown bacteria that had been opsonised.

159 A further conclusion from these findings was that host adapted bacteria did not have any immuno-
160 reactive epitopes on their cell surface, since the second incubation in fresh serum did not enhance
161 opsonin exposure above that seen for the PBS control (Fig. 2E,F).

162 Taken together, these experiments revealed that host-adapted bacteria are better able to survive
163 exposure to neutrophils than broth-grown *S. aureus* because they are less likely to be phagocytosed,
164 apparently due to weak surface exposure of bound IgG and complement.

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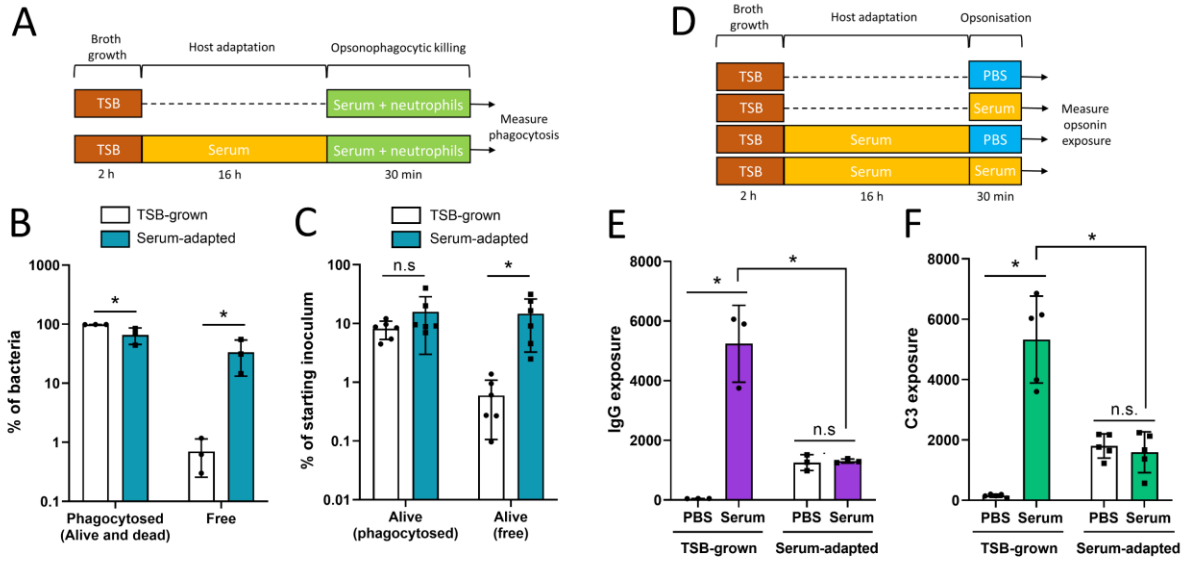

Figure 2. Incubation in human serum reduces opsonophagocytosis and surface-exposed IgG and complement. TSB-grown and serum-adapted cultures of *S. aureus* USA300 were incubated with purified human neutrophils for 30 min (**A**) before the percentage of neutrophil-associated (phagocytosed) and non-neutrophil associated (free) bacteria were determined by (**B**) flow cytometry and (**C**) CFU counts. TSB-grown and serum-adapted *S. aureus* cells were incubated for 30 min with PBS or 10 % serum (**D**) before the levels of surface-exposed bound (**E**) IgG and (**F**) C3 were determined by flow cytometry. Data in **B** and **C** show values for each independent experiment, with bars representing the mean ± standard deviation. In **E** and **F**, each biological repeat is represented by the median fluorescence value of 10,000 bacterial cells. Data in **B, C, E, F** were analysed by two-way ANOVA with Sidak's *post-hoc* test (*, $P < 0.05$; n.s, $P \geq 0.05$ for the indicated comparisons).

Cell wall accumulation impairs opsonophagocytosis by concealing bound opsonins

Since *S. aureus* accumulates peptidoglycan during incubation in serum, we tested whether this concealed bound antibody and complement. To do this, host-adapted bacteria were incubated with a range of concentrations of the enzyme lysostaphin, which cleaves peptidoglycan. This resulted in a dose-dependent increase in exposure of bound IgG and complement (Fig. 3A,B), and a corresponding increase in phagocytosis of host-adapted *S. aureus* by neutrophils (Fig. 3C,D). Furthermore, lysostaphin treatment of host-adapted *S. aureus* rendered them as susceptible to neutrophil-mediated killing as broth-grown bacteria (Fig. 3E).

Combined these findings indicated that peptidoglycan accumulation was responsible for the concealment of bound opsonins (Fig. 3F). However, it did not determine whether peptidoglycan-bound proteins or WTA were also required. We ruled out a role for peptidoglycan-bound proteins by

198 showing that host-adapted mutants defective for SrtA or SrtB, which cannot anchor proteins to
199 peptidoglycan, survived incubation with neutrophils as well as host-adapted wild type bacteria
200 (Supplementary Fig. S2A,B). We also showed that serum-induced accumulation of WTA did not
201 contribute to immune evasion using tunicamycin to block the synthesis of this polymer [30]
202 (Supplementary Fig. S2C).

203 Taken together, the experiments described here demonstrate that host-adapted *S. aureus* cells are
204 bound by high levels of antibody and complement but the accumulation of cell wall peptidoglycan
205 conceals these bound opsonins, enabling bacterial evasion of phagocytosis and killing by neutrophils.

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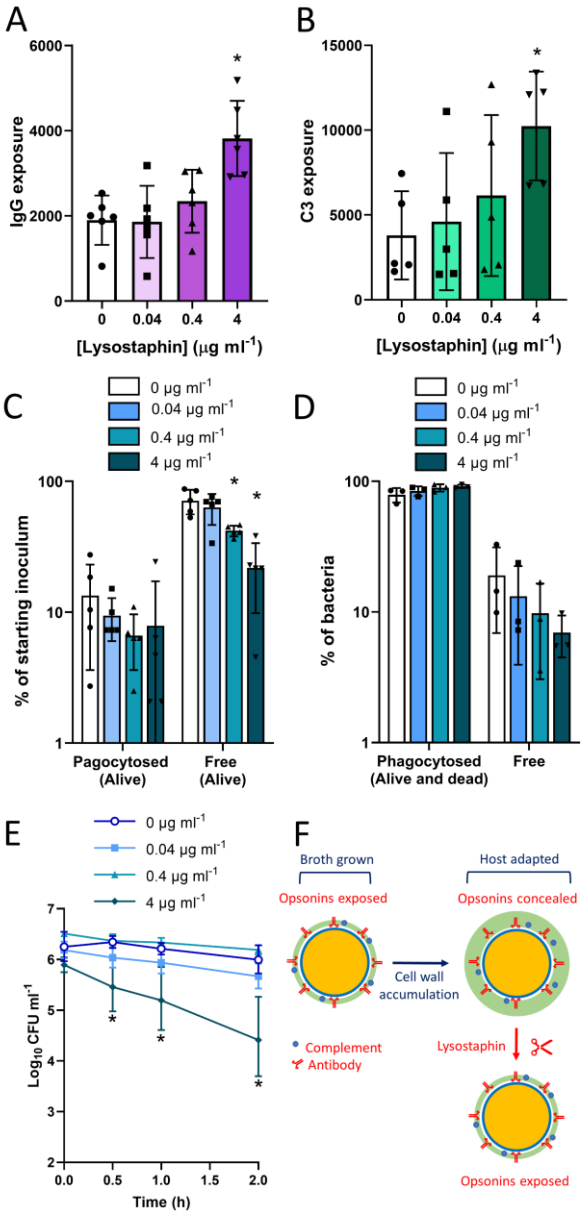

Figure 3. Cell wall accumulation impairs opsonophagocytosis by concealing bound opsonins. Host-adapted cultures of *S. aureus* USA300 were treated with indicated concentrations of lysostaphin for 20 min to partially digest the cell wall before levels of surface-exposed (**A**) IgG and (**B**) complement component C3 were determined by flow cytometry. Additionally, these cells were incubated with purified human neutrophils for 30 min before the percentage of neutrophil-associated (phagocytosed) and non-neutrophil associated (free) bacteria were determined by (**C**) CFU counts and (**D**) flow cytometry. (**E**) Survival of lysostaphin-treated cells during a 3 h incubation with purified human neutrophils. Summary diagram of the effect of lysostaphin on host-adapted bacteria, with lysostaphin-mediated degradation of the cell wall exposing bound opsonins that had been obscured by accumulated peptidoglycan (**F**).

Data in **A** – **D** represent the mean ± standard deviation of the indicated number of independent biological replicates. Data in **E** represent the geometric mean ± geometric standard deviation of 4 independent experiments. In **A** and **B**, each biological repeat is represented by the median fluorescence value of 10,000 bacterial events. Data in **A** and **B** were analysed by one-way ANOVA with Dunnett's *post-hoc* test. Data in **C** – **E** were analysed by two-way ANOVA with Dunnett's *post-hoc* test (*, $P \leq 0.05$; Lysostaphin-treated vs non-lysostaphin treated).

242 **Antibiotic-mediated inhibition of cell wall accumulation maintains opsonin exposure and efficient**
243 **opsonophagocytosis**

244 To further test whether host adaptation reduced phagocytosis via peptidoglycan-mediated
245 concealment of bound opsonins, and to explore potential therapeutic approaches to enhance
246 neutrophil-mediated killing, we carried out host adaptation in the presence of a sub-lethal
247 concentration of the antibiotic fosfomycin. This antibiotic inhibits peptidoglycan synthesis and
248 prevents cell wall thickening from occurring and has been used clinically in anti-staphylococcal
249 combination therapies [30,44,45].

250 As above, the adaptation of bacteria to serum resulted in a significant reduction in opsonisation, as
251 determined by exposure of IgG and complement, relative to broth-grown bacteria (Fig. 4A,B).
252 However, the presence of fosfomycin in serum significantly reduced opsonin concealment,
253 maintaining IgG and complement exposure at similar levels to that seen for broth-grown bacteria (Fig.
254 4A,B). In keeping with this, bacteria adapted to serum in the presence of fosfomycin were
255 phagocytosed as efficiently as broth-grown bacteria, as determined by both phagocytosis assays (Fig.
256 4C,D). Furthermore, bacteria that were adapted in serum with fosfomycin were killed by neutrophils
257 as efficiently as broth-grown bacteria, whereas host-adapted bacteria not exposed to fosfomycin
258 survived at significantly higher levels (Fig. 4E).

259 Taken together, these findings provided additional evidence that cell wall accumulation conceals
260 bound opsonins, which in turn compromises phagocytosis and killing by neutrophils. They also indicate
261 that the antibiotic fosfomycin, in addition to its own antibacterial activity, may aid the clearance of
262 infection by preventing concealment of opsonins.

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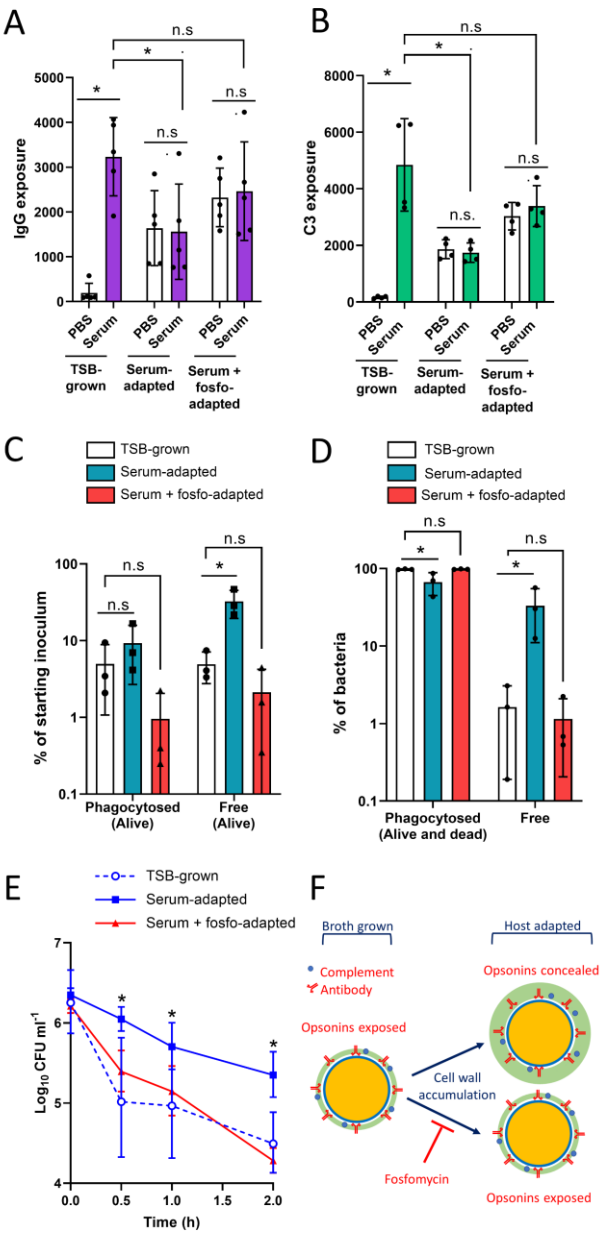

Figure 4. Antibiotic-mediated inhibition of cell wall accumulation maintains opsonin exposure and efficient opsonophagocytosis. TSB-grown, serum-adapted and serum + fosfomycin-adapted cultures of *S. aureus* USA300 were incubated for 30 min with PBS or 10 % serum before the levels of surface-exposed (**A**) IgG and (**B**) complement component C3 were determined by flow cytometry. Additionally, these cells were incubated with purified human neutrophils for 30 min before the percentage of neutrophil-associated (phagocytosed) and non-neutrophil associated (free) bacteria were determined by (**C**) CFU counts and (**D**) flow cytometry. (**E**) Survival of TSB-grown, serum-adapted and serum + fosfomycin-adapted cultures during a 2 h incubation with purified human neutrophils. Summary diagram of the effect of fosfomycin on opsonin exposure during host-adaptation, with antibiotic-mediated inhibition of peptidoglycan accumulation preserving the exposure of bound antibody and complement (**F**). Data in **A** – **D** represent the geometric mean ± geometric standard deviation of the

284 indicated number of independent biological replicates. Data in **E** are presented as the geometric mean
285 ± geometric standard deviation of three independent experiments. In **A** and **B**, each biological repeat
286 is represented by the median fluorescence value of 10,000 bacterial events. Data in **A** and **B** were
287 analysed by two-way ANOVA with Tukey's *post-hoc* test. Data in **C** – **E** were analysed by two-way
288 ANOVA with Dunnett's *post-hoc* test (*, $P < 0.05$; n.s, $P \geq 0.05$; comparisons are indicated in **A** – **D** and
289 serum/serum + fosfomycin-adapted vs TSB-grown at each time-point in **E**).

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291

292 **Discussion**

293 The binding of antibody and complement to the bacterial cell surface enables the detection and
294 destruction of pathogens by phagocytic immune cells [10,11,13]. The data presented here
295 demonstrate that *S. aureus* can conceal bound opsonins via cell wall accumulation, significantly
296 reducing phagocytosis and killing by neutrophils. Crucially, once bound opsonins were concealed, it
297 was not possible to restore full opsonisation with fresh serum, demonstrating that host-adapted cells
298 with thickened cell walls have reduced exposure of immune-reactive epitopes.

299 Cell wall thickening is due to a combination of peptidoglycan synthesis and inhibition of autolytic
300 activity [30]. Recent work has revealed that mutants lacking the Atl autolysin have defective surface
301 exposure of staphylococcal surface proteins, inhibiting their recognition by reactive antibodies [46].
302 Exposure was restored using enzymatic digestion of peptidoglycan, supporting our finding that
303 peptidoglycan accumulation can obscure surface antigens and prevent their detection by antibodies.

304 Previous work has indicated that the thickened cell wall associated with vancomycin resistance
305 reduces staphylococcal susceptibility to intracellular killing by neutrophils [47]. However, our data did
306 not show a difference in staphylococcal survival within neutrophils, with similar numbers of
307 intracellular viable broth-grown and host-adapted bacteria. Instead, the survival advantage of host
308 adaptation appeared to be entirely due to enhanced evasion of phagocytosis.

309 We do not yet know if these findings are applicable to other Gram-positive pathogens. However, since
310 previous work has shown that serum triggers cell wall thickening in *Enterococcus faecalis* and viridans
311 group streptococci, it is possible that our findings with *S. aureus* represent a broadly conserved
312 mechanism of immune evasion [48].

313 Cell wall thickening in *S. aureus* is triggered by bacterial sensing of the host defence antimicrobial
314 peptide LL-37 via the GraRS system [30]. Since LL-37 is present in most tissues and amongst the earliest

315 host responses to infection or trauma [49,50,51], we hypothesise that *S. aureus* has evolved to sense
316 this AMP as an early indicator that it is subject to immune attack and provides an opportunity to
317 employ defensive measures against the impending arrival of neutrophils (Supplementary figure S3).
318 In support of this hypothesis, GraRS, the two-component system that detects LL-37, is activated in the
319 early stages of staphylococcal skin colonisation, whilst *S. aureus* mutants lacking GraRS are
320 significantly less virulent than wild type strains in invasive infection models [52,53,54].

321 In addition to providing protection against opsonophagocytosis, LL-37 exposure triggers reduced
322 susceptibility to the antibiotics daptomycin and vancomycin [30,55], suggesting *S. aureus* employs
323 strategies that are broadly protective against the twin threats of host immunity and antibiotic therapy.
324 This is similar to our previous work showing that induction of the *S. aureus* general stress response
325 regulated by the alternative sigma factor SigB can promote survival of bacteria exposed to host
326 defences and various classes of antibiotics [56]. Further support for the link between *S. aureus*-
327 immune interactions and antibiotic tolerance comes from studies showing that oxidative stress
328 conferred by phagocytic cells reduce staphylococcal susceptibility to antibiotics [57,58].

329 Whilst the immune response may compromise the efficacy of antibiotic therapy under certain
330 circumstances, our study also highlights how antibiotics and the immune response can work
331 synergistically by showing that fosfomycin blocked LL-37 induced cell wall thickening and thereby
332 maintained exposure of bound opsonins, leading to efficient opsonophagocytic killing. Additionally,
333 previous work has suggested that fosfomycin also promotes killing of *S. aureus* via enhanced
334 production of the neutrophil oxidative burst [59]. However, whilst we exposed *S. aureus* to fosfomycin
335 in serum, this was removed by washing prior to incubation with neutrophils and thus does not explain
336 the enhanced killing effect observed in our assays. This strongly suggests that there are at least two
337 mechanisms by which fosfomycin and neutrophils synergise against *S. aureus* and a greater
338 understanding of this may contribute to more effective therapeutic approaches that reduce the high
339 incidence of relapsing or chronic staphylococcal infections [60].

340 In summary, we show that *S. aureus* cells are heavily opsonised upon initial exposure to serum.
341 However, *S. aureus* responds to serum by accumulating peptidoglycan, which conceals bound
342 opsonins, reducing phagocytosis and killing by neutrophils.

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347 ## Materials and methods

348 ### Bacterial strains and growth conditions

349 Bacterial strains used in this study are shown in Table 1. Strains were grown at 37 °C on tryptic soy
350 agar (TSA) or in tryptic soy broth (TSB) with shaking (180 r.p.m.) supplemented with erythromycin (10
351 µg ml^{-1}) or kanamycin (90 µg ml^{-1}) when required.

Strain	Relevant characteristics	Reference/source
USA300 JE2	LAC strain of the USA300 lineage of community acquired MRSA cured of plasmids	36
JE2 *sbi*::Tn *spa*::*kan*	JE2 strain defective for immunoglobulin-binding proteins Spa and Sbi	This study
JE2 *srtA*::Tn	JE2 strain defective for sortase A, which anchors LPxTG motif-containing proteins to peptidoglycan	61
JE2 *srtB*::Tn	JE2 strain defective for sortase B, which anchors NPQTN motif-containing IsdC protein to peptidoglycan	61
SH1000	*rsbU*+ derivative of laboratory strain 8325-4	37
Col	Early hospital-associated MRSA isolate, commonly used in laboratory experiments	38
Newman	Clinical isolate, methicillin susceptible, used widely in animal and laboratory experiments	39

352

353 **Table 1.** Strains used in this study.

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355 ### Construction of strains

356 The JE2 *sbi*::Tn/*spa*::*kan* double mutant was constructed via transduction of the kanamycin resistance
357 marker from Newman *spa*::*kan* [62] into the *sbi*::Tn mutant present in the NARSA transposon mutant
358 library [61] using ϕ11.

359 ### Generation of broth-grown and host-adapted bacterial cultures

360 To generate broth-grown bacteria, cultures were grown for 16 h in TSB to stationary phase. These
361 were then diluted to 10^7 CFU ml^{-1} in fresh TSB and incubated for 2 h at 37 °C until 10^8 CFU ml^{-1} was
362 reached.

363 To generate host-adapted bacteria, broth-grown cultures were centrifuged (3,200 x *g* for 10 min),
364 resuspended in an equal volume of human serum from human male AB plasma (Sigma) and incubated
365 for 16 h at 37 °C. As *S. aureus* is unable to replicate in human serum, these cultures were also at 10^8
366 CFU ml^{-1}. Where appropriate, serum was supplemented with a sub-lethal concentration of fosfomycin
367 (8 µg ml^{-1}) or tunicamycin (128 µg ml^{-1}).

368 Where appropriate, the cell walls of serum-adapted cultures were degraded by lysostaphin. To do this,
369 1 ml aliquots of serum-adapted bacteria were washed in PBS and resuspended in 1 ml PBS
370 supplemented with indicated concentrations of lysostaphin (between 0.04 and 4 µg ml^{-1}). Bacteria
371 were incubated statically for 20 min at 37 °C before being washed by 3 rounds of centrifugation in
372 PBS.

373 **Purification of neutrophils**

374 Ethical approval for drawing and using human blood was obtained from the Regional Ethics Committee
375 and the Imperial NHS Trust Tissue Bank (REC Wales approval no. 12/WA/0196 and ICHTB HTA license
376 no. 12275). Neutrophils were extracted from 45 ml human blood, collected in heparin tubes to prevent
377 coagulation. Blood (15 ml) was carefully layered over 30 °C PolymorphPrep (20 ml) and centrifuged
378 for 1 h at 500 x *g* to separate the different cell types. Neutrophils were collected, washed with Hanks
379 balanced salt solution (HBSS) and adjusted to 5 x 10^6 cells ml^{-1} in HBSS.

380 **Determination of bacterial killing by neutrophils and phagocytosis by CFU counts**

381 Neutrophils were adjusted to 5 x 10^6 cells ml^{-1} in HBSS supplemented with 10 % human serum, 0.1
382 mM $CaCl_2$ and 0.1 mM $MgCl_2$. In the case of lysostaphin-treated bacteria, 10 % serum was omitted
383 from the HBSS.

384 TSB-grown/host-adapted bacteria were generated as described above, washed three times in PBS and
385 then added to neutrophils at 5 x 10^6 CFU ml^{-1}. Tubes were incubated with end-over-end mixing at 37°
386 C for 3 h and at each time-point (0, 0.5, 1 and 2 h) aliquots were removed, serially diluted 10-fold in
387 PBS and plated to enumerate CFU ml^{-1}.

388 In addition, the number of phagocytosed/unphagocytosed bacteria was also enumerated at the 0.5 h
389 time-point. A 500 µl aliquot of the neutrophil/bacteria mixture was taken and centrifuged at 500 x *g*
390 for 1 min to pellet the neutrophils, along with any neutrophil-associated bacteria. The supernatant
391 (containing unphagocytosed bacteria) was serially diluted 10-fold in PBS and plated for CFU counts
392 and the pellet was resuspended in 500 µl PBS, serially diluted 10-fold in PBS and plated for CFU counts.
393 The CFU ml^{-1} values in the pellet and the supernatant were divided by the CFU ml^{-1} of the starting
394 inoculum to generate the percentage of CFU ml^{-1} phagocytosed and unphagocytosed, respectively.

395 **Measurement of phagocytosis by flow cytometry**

396 To measure phagocytosis by flow cytometry neutrophils and bacteria were prepared as described
397 above except that immediately before bacteria were added to the neutrophils the bacteria were
398 incubated with 10 µg ml^{-1} fluorescein isothiocyanate for 30 min at room temperature and then washed
399 three times in PBS.

400 As above, 5 x 10^6 CFU ml^{-1} bacteria were added to 5 x 10^6 cells ml^{-1} in HBSS supplemented with 10 %
401 human serum, 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$. In the case of lysostaphin-treated bacteria, 10 %
402 serum was omitted from the HBSS. After a 30 min incubation at 37˚C with end-over-end mixing in the
403 dark, cultures were fixed by the addition of an equal volume of 4 % paraformaldehyde (PFA). Samples
404 were then analysed by flow cytometry using an Amnis CellStream. Bacteria were detected using the
405 488 nm laser and at least 10,000 bacterial events were recorded. Events with FITC ≥ 2 x 10^3 were
406 counted as bacteria. Events with an FCS of ≥ 3000 were counted as phagocytosed and ≤ 3000 were
407 counted as unphagocytosed/free.

408 **Measurement of IgG and complement surface exposure by flow cytometry**

409 TSB-grown and serum-adapted cultures were prepared as described above. A *spa/sbi* double mutant
410 was used to prevent non-specific antibody binding. Aliquots (500 µl) were incubated for 30 min at
411 room temperature in either PBS or 10 % human serum. Samples were washed by three rounds of
412 centrifugation in PBS (13,000 x *g* for 1 min) and blocked for 1 h in 4 % BSA in PBS. Samples were washed
413 once in PBS before IgG was detected with a 1:1000 dilution of goat anti-human IgG antibody labelled
414 with the BV421 fluorophore (Jackson ImmunoResearch) or C3 was detected with a 1:1000 dilution of
415 goat anti-human C3 $F(ab')_2$ labelled with FITC (Protos Immunoresearch). Antibody incubations were
416 carried out statically for 1 h at room temperature in the dark. Samples were washed with PBS by three
417 rounds of centrifugation (13,000 x *g* for 1 min) and fixed in 4 % PFA. Samples were analysed by flow
418 cytometry using an Amnis CellStream. IgG was detected using the 405 nm laser and C3 using the 488
419 nm laser. At least 10,000 bacterial events were recorded and the median value recorded.

420 **Measurement of IgG and complement by western blotting**

421 Cultures of TSB-grown and serum-adapted bacteria (1 ml at 10^8 CFU ml^{-1}) were prepared as described
422 above, washed by three rounds of centrifugation in PBS (13,000 x *g* for 1 min) and resuspended in 100
423 µl PBS. A *spa/sbi* double mutant was used to prevent non-specific antibody binding. Lysostaphin (10
424 µg ml^{-1}) was added and bacteria incubated statically for 1 h at 37˚C. Sample buffer (187.5 mM Tris-
425 HCl (pH 6.8), 6 % SDS, 30 % glycerol, 0.03 % bromophenol blue and 15 % beta-mercaptoethanol; 50
426 µl) was added and samples were incubated at 95˚C for 10 min before 15 µl was loaded onto 10 %

427 polyacrylamide gels. Gels were run in Tris-Glycine running buffer (25 mM Tris, 192 mM glycine, 0.1 %
428 SDS, pH 8.4) at 100 V for 10 min followed by 200 V for 50 min before being transferred onto PVDF
429 membranes (10 V for 60 min). Membranes were blocked for 1 h at room temperature in 5 % milk and
430 1 % BSA in TBST. IgG was detected using 1:10000 dilution of donkey anti-human IgG conjugated to
431 HRP (Abcam) and C3 was detected by 1:5000 dilution of rabbit anti-C3 (Abcam) followed by 1:10000
432 dilution of goat anti-rabbit IgG conjugated to HRP (Abcam). Blots were developed using SuperSignal
433 West Pico PLUS chemiluminescent substrate (Thermo scientific) and imaged using the Bio-rad
434 ChemiDoc MP imaging system.

435 **Statistical analyses**

436 CFU counts were log10 transformed and displayed as the geometric mean ± geometric standard
437 deviations [63]. Other data are displayed as the mean ± standard deviation. For all experiments, three
438 or more independent replicates were performed as indicated by individual data points. Data were
439 analysed by one-way ANOVA or two-way ANOVA with appropriate post-hoc multiple comparison test
440 as detailed in figure legends using GraphPad Prism (V8.0).

441

442 ## Acknowledgements

443 We thank the blood donors, without whom this study would not have been possible. Joan Geoghegan
444 (University of Birmingham) and Angelika Grundling (Imperial College London) are thanked for
445 providing strains. EVKL was supported by a Wellcome Trust PhD Studentship (203812/Z/16/Z). We
446 acknowledge the technical support and the use of equipment from the SAFB Flow Cytometry Facility
447 (Imperial College London). AME acknowledges funding from the Rosetrees Trust and from the Imperial
448 NIHR Biomedical Research Centre, Imperial College London. All authors acknowledge the provision of
449 strains by the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) Program: under
450 NIAID/ NIH Contract No. HHSN272200700055C.

451

452 ## Contributions and study design

453 EVKL and AME designed the experiments, conducted the experiments, analyzed the data, and wrote
454 the manuscript. The funders had no role in the study design, interpretation of the findings or the
455 writing of the manuscript.

456

Conflict of interest

The authors declare no conflict of interest.

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