Host-induced cell wall remodelling impairs opsonophagocytosis of *Staphylococcus aureus* by neutrophils

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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 as bacteria adapted to serum, the resulting increase in cell wall thickness led to a reduction in the exposure of bound antibody and complement and a corresponding reduction in phagocytosis and killing by neutrophils. The exposure of opsonins bound to protein antigens or LTA were most significantly reduced, whilst opsonisation by IgG against wall teichoic acid or peptidoglycan was largely unaffected. Partial digestion of accumulated cell wall in host adapted cells using the enzyme lysostaphin restored opsonin exposure and promoted phagocytosis and killing. Concordantly, the antibiotic fosfomycin inhibited cell wall remodelling and maintained full susceptibility of *S. aureus* to opsonophagocytic killing by neutrophils. These findings reveal that the adaptation of *S. aureus* to the host environment reduces the ability of the immune system to detect and kill this pathogen through reduced exposure of protein- and LTA-bound opsonins via cell wall remodelling.

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 structures, including wall and lipoteichoic acids (WTA, LTA), peptidoglycan, capsular polysaccharide and proteins [10,11,12,13,14,15,16,17,18]. Whilst the precise abundance of antibody against each of the major surface structures varies from person to person, antibody against each macromolecule has been demonstrated to be sufficient to trigger opsonophagocytosis [14,15,16,17,18,19,20].

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

62 raft of bactericidal products including reactive oxygen species, antimicrobial peptides and proteases
63 [11,12].

64 To combat the threat posed by neutrophils, *S. aureus* has evolved numerous mechanisms of evading
65 opsonic complement and antibody [12,13,21,22]. For example, *S. aureus* produces two
66 immunoglobulin binding proteins, Spa and Sbi, that reduce antibody-mediated opsonisation, whilst
67 the production of proteins such as SCIN, Efb and CHIPS reduces complement deposition and activation
68 and detection by immune cells [12,13,21,22,23,24,25,26,27,28,29,30,31]. As such, the bacterial cell
69 surface is a critically important determinant in immune detection of *S. aureus* and efforts by the
70 pathogen to evade surveillance and killing by host defences.

71 The staphylococcal cell envelope is a dynamic structure that responds to host-induced stresses
72 [32,33,34,35]. Consequently, *S. aureus* has a thicker cell wall *in vivo* than when growing *in vitro* [36], a
73 phenotype that is replicated when staphylococci are exposed to human serum or present within
74 endothelial or osteoblast cells [37,38,39,40]. In the case of serum, cell wall thickening is triggered
75 when *S. aureus* detects the presence of the host defence antimicrobial peptide LL-37 via the GraRS
76 two component system [37]. This results in significantly greater quantities of both peptidoglycan and
77 WTA in the cell wall, relative to bacteria grown in laboratory culture medium [37].

78 Host-induced changes to the cell wall are important for the ability of the pathogen to cause and sustain
79 infection. Cell wall thickening has been shown to reduce susceptibility to antibiotics, whilst mutant
80 strains lacking various cell wall synthetic enzymes are less virulent in infection models
81 [32,34,35,37,40]. However, it is unknown whether host-induced changes to the bacterial cell wall
82 affect the detection and killing of *S. aureus* by the host immune system. To address this, we examined
83 the impact of host-induced changes to the staphylococcal cell envelope on subsequent interactions of
84 *S. aureus* with neutrophils. This revealed that cell wall thickening constitutes a previously
85 unrecognised mechanism of immune evasion that functions by significantly reducing the exposure of
86 opsonins bound to proteins and LTA and thereby reducing opsonophagocytic killing.

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88 ## Results

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

90 To understand the impact of host adaptation on staphylococcal susceptibility to host defences, we
91 either grew bacteria to exponential phase in tryptic soy broth (TSB) to represent standard laboratory
92 conditions or allowed bacteria to adapt to host conditions using a previously described *ex vivo* human
93 serum model that triggers cell wall thickening [37] (Fig. 1A). This model uses pooled human serum,

94 which avoids variability in anti-staphylococcal antibody levels between donors [14]. Since the serum
95 is not heat inactivated, it contains functional immunoglobulins and complement components.

96 In addition to triggering cell wall thickening via the GraRS system, serum also suppresses both the
97 growth of *S. aureus* and activation of the Agr quorum-sensing system that regulates expression of
98 many virulence factors [37,41,42,43,44,45,46,47,48].

99 We then measured the survival of bacteria prepared under each condition during incubation with
100 purified *ex vivo* human neutrophils from male and female healthy donors in the presence of serum to
101 provide antibody- and complement-mediated opsonisation [50,51]. We examined four distinct wild
102 type *S. aureus* strains to represent both methicillin resistant (USA300, Col) and methicillin susceptible
103 (SH1000, Newman) organisms [52,53,54,55].

104 For all four of the *S. aureus* strains tested, exponential phase bacteria were efficiently killed over time,
105 with <5% of bacteria remaining viable after 2 hours incubation with neutrophils (Fig. 1B,C,D,E).
106 However, host-adapted bacteria survived at levels up to 5 times greater than that seen for exponential
107 bacteria for all strains (Fig. 1B,C,D,E). In addition to demonstrating that host adaptation reduces
108 staphylococcal susceptibility to host immune defences, the high level of consistency observed across
109 all four strains indicated that this is a conserved phenotype.

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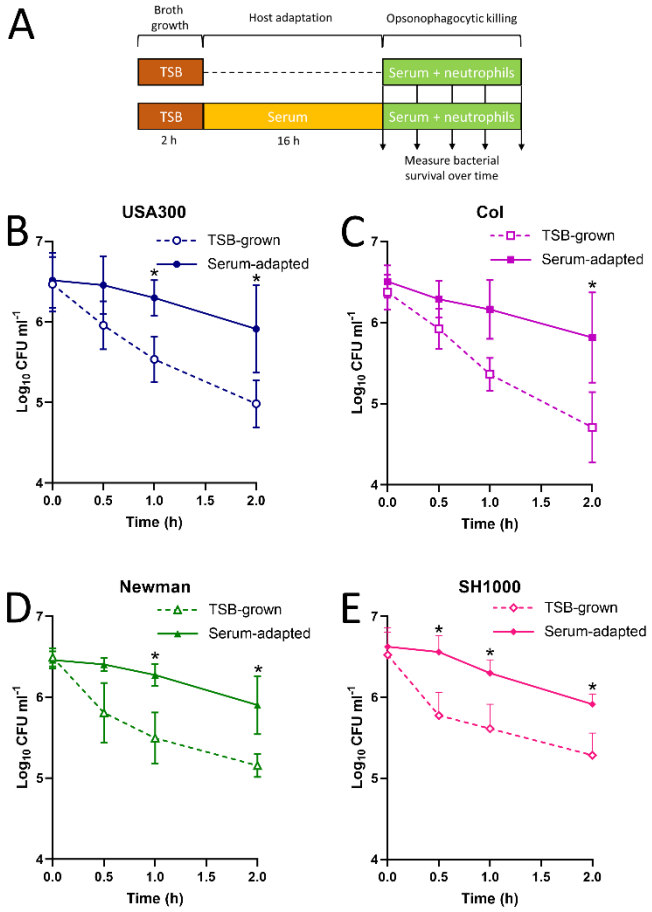

Figure 1. Incubation in human serum promotes tolerance to killing by neutrophils. Bacteria were grown in broth or allowed to adapt to human serum before incubation with human neutrophils in the presence of serum and staphylococcal survival measured over time (**A**). Survival of TSB-grown and serum-adapted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

Host adaptation reduces opsonin exposure and opsonophagocytosis

Having found that host adaptation reduced staphylococcal susceptibility to host defences, we next determined the mechanism(s) responsible. Given the consistency in survival data across all four strains examined, we focussed on the USA300 lineage since it is both well characterised and clinically important [52].

We started by assessing whether the increase in survival of host-adapted bacteria was due to impaired phagocytosis, using two distinct assays. Bacteria were grown in broth or host-adapted by incubation in serum, before washing in PBS and then incubation with neutrophils in the presence of fresh serum to enable opsonisation (Fig. 2A). The first phagocytosis assay was a flow cytometry-based approach

130 that determined how many fluorescently-labelled bacteria were associated (or not) with neutrophils
131 [56] (Supplementary figure S1). This revealed that the majority of both broth-grown and host-adapted
132 *S. aureus* were phagocytosed after 30 min incubation with neutrophils. However, whilst <3% of broth-
133 grown bacteria remained unphagocytosed, >20% of host-adapted bacteria were free from neutrophils
134 (Fig. 2B). This finding was replicated in a second phagocytosis assay that measured the viability of free
135 and neutrophil-associated bacteria [57], with >10% of host-adapted *S. aureus* unphagocytosed
136 compared with <1% of broth-grown *S. aureus* cells (Fig. 2C; Supplementary figure S2). Combined, these
137 two assays demonstrated that host-adapted *S. aureus* were significantly better at evading
138 phagocytosis than broth-grown bacteria.

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

145 To understand why more host-adapted *S. aureus* were able to evade phagocytosis compared with
146 broth-grown cells, we examined the degree of opsonisation of bacteria by antibody and complement
147 using western blotting. In keeping with previous work [58,59], for this experiment we used a mutant
148 strain of USA300 lacking Spa and Sbi to avoid interference caused by these immunoglobulin binding
149 proteins (Supplementary Fig. S3). Broth-grown or host-adapted bacteria were washed in PBS and then
150 incubated, or not, in fresh serum to enable opsonin binding as used in the opsonophagocytosis assays
151 described above (Fig. 1A) before detection of bound antibody and complement component C3 (Fig.
152 2D).

153 Despite their reduced phagocytosis by neutrophils, there was more antibody and complement bound
154 to host-adapted cells than to broth-grown, suggesting that a lack of bound opsonins did not explain
155 the immune evasion phenotype of host-adapted bacteria (Supplementary Fig. S4).

156 To understand why host-adapted cells had high levels of bound antibody and complement but low
157 levels of phagocytosis, bacteria were prepared as described above for opsonophagocytosis assays and
158 then the levels of surface-exposed antibody and the complement component C3 quantified using flow
159 cytometry (Fig. 2D; Supplementary Fig. S5). Broth-grown bacteria that had been exposed to PBS
160 instead of serum acted as a negative control and confirmed that antibodies used in the assay did not
161 bind non-specifically to *S. aureus* cells (Fig. 2D,E,F). We then showed that, as expected, broth-grown

162 bacteria that were incubated in human serum for 30 min were very strongly bound by both IgG and
163 the complement component C3 (Fig. 2E,F).

164 Next, we examined host-adapted bacteria and found that they had a significantly reduced level of
165 exposed opsonins, compared with TSB-grown cells, regardless of whether they had been incubated in
166 fresh serum for 30 min or not (Fig. 2E,F). Therefore, despite prolonged incubation in serum and high
167 levels of bound antibody and complement (Supplementary Fig. S4), host-adapted cells had
168 significantly reduced exposure of opsonins on their cell surface relative to broth-grown bacteria that
169 had been opsonised.

170 Taken together, these experiments revealed that host-adapted bacteria are better able to survive
171 exposure to neutrophils than broth-grown *S. aureus* because they are less likely to be phagocytosed,
172 in keeping with the lower surface exposure of bound IgG and complement.

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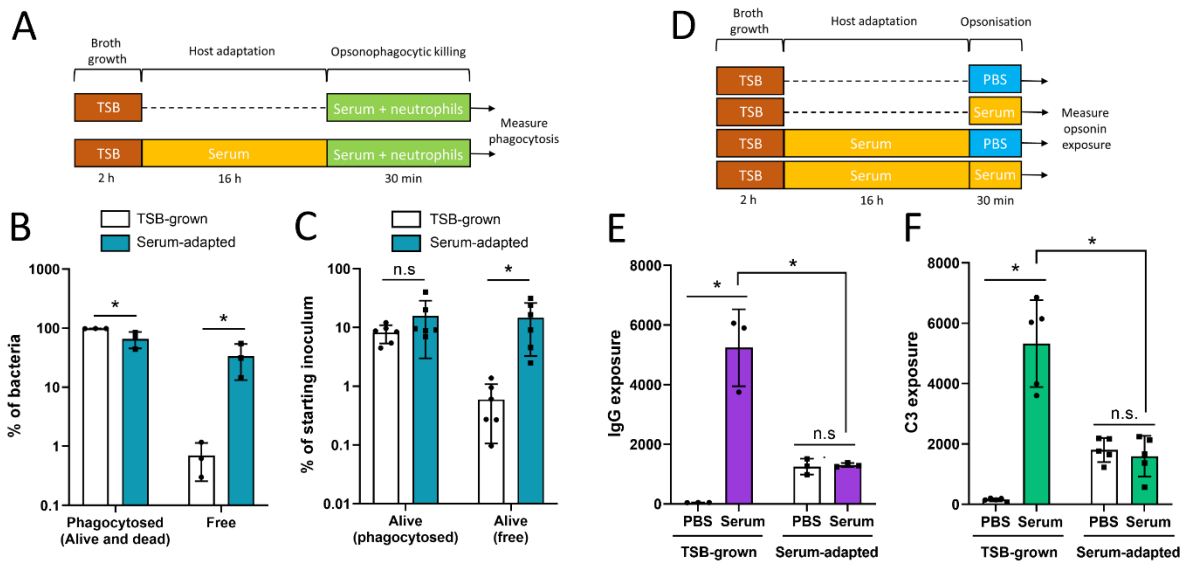

Figure 2. Incubation in human serum reduces opsonophagocytosis and surface-exposed IgG and complement. TSB-grown and serum-adapted cultures of *S. aureus* USA300 were incubated with purified human neutrophils for 30 min (**A**) before the percentage of neutrophil-associated (phagocytosed) and non-neutrophil 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 IgG bound to LTA and protein

Since the cell envelope of *S. aureus* accumulates peptidoglycan and WTA during incubation in serum [37], we tested whether this concealed some of the bound antibody and complement. To do this, host-adapted bacteria were incubated for 20 min with a range of sub-lethal concentrations of the enzyme lysostaphin, which cleaves peptidoglycan, to partially remove the cell wall. The lysostaphin was then removed by washing and bacterial viability confirmed by CFU counts. This limited cell wall digestion resulted in a significant, dose-dependent increase in exposure of bound IgG and complement, demonstrating that some of the bound opsonins were concealed by the accumulation of cell wall polymers during incubation in serum (Fig. 3A,B).

We then tested whether the concealment of bound IgG by accumulated cell wall explained the reduced phagocytosis of host adapted bacteria relative to TSB-grown *S. aureus*. In keeping with increased IgG and complement exposure, limited lysostaphin treatment increased the phagocytosis of host-adapted *S. aureus* by neutrophils (Fig. 3C,D).

208 Since human serum contains IgG that recognises multiple *S. aureus* surface structures, we next sought
209 to understand whether the reduced opsonisation observed for serum-adapted bacteria was specific
210 to a particular antibody target. Bacteria were grown in TSB and then incubated briefly in serum (30
211 min) or for 16 h to enable adaptation. Surface exposed IgG was then eluted from bacteria and assessed
212 for its binding to each of the major surface structures by ELISA.

213 Host-adapted bacteria had similar levels of anti-WTA IgG on their surface compared to exponential
214 phase bacteria and only slightly lower levels of anti-peptidoglycan IgG (2.5-fold difference). However,
215 surface exposure of IgG targeting other surface structures was greatly reduced in host-adapted
216 bacteria compared to exponential phase cells, with anti-LTA IgG 9-fold lower, anti-membrane-
217 associated proteins 18-fold lower and cell wall-associated proteins 48-fold lower (Fig. 3E). We did not
218 examine capsular polysaccharide in these assays as USA300 is deficient in this polymer [52]. As such,
219 the lower surface IgG exposure in host-adapted cells compared to those in exponential phase is
220 primarily due to a loss of exposure of antibody bound to LTA and surface proteins.

221 Partial digestion of peptidoglycan using lysostaphin restored surface exposure of IgG bound to LTA
222 and proteins to similar levels observed for TSB-grown bacteria (Fig. 3E). Therefore, accumulation of
223 cell wall in host-adapted bacteria preferentially conceals IgG bound to LTA and surface proteins, whilst
224 exposure of anti-WTA and anti-peptidoglycan remains strongly exposed.

225 Finally, we showed that increasing opsonin exposure via partial lysostaphin digestion of the cell wall,
226 with the enzyme washed away before incubation with immune cells, rendered host adapted
227 staphylococci as susceptible to neutrophil-mediated killing as broth-grown bacteria (Fig. 3F).

228 Taken together, the experiments described here demonstrate that host-adapted *S. aureus* cells are
229 bound by high levels of antibody and complement but the accumulation of cell wall conceals some of
230 these bound opsonins, reducing phagocytosis and killing by neutrophils.

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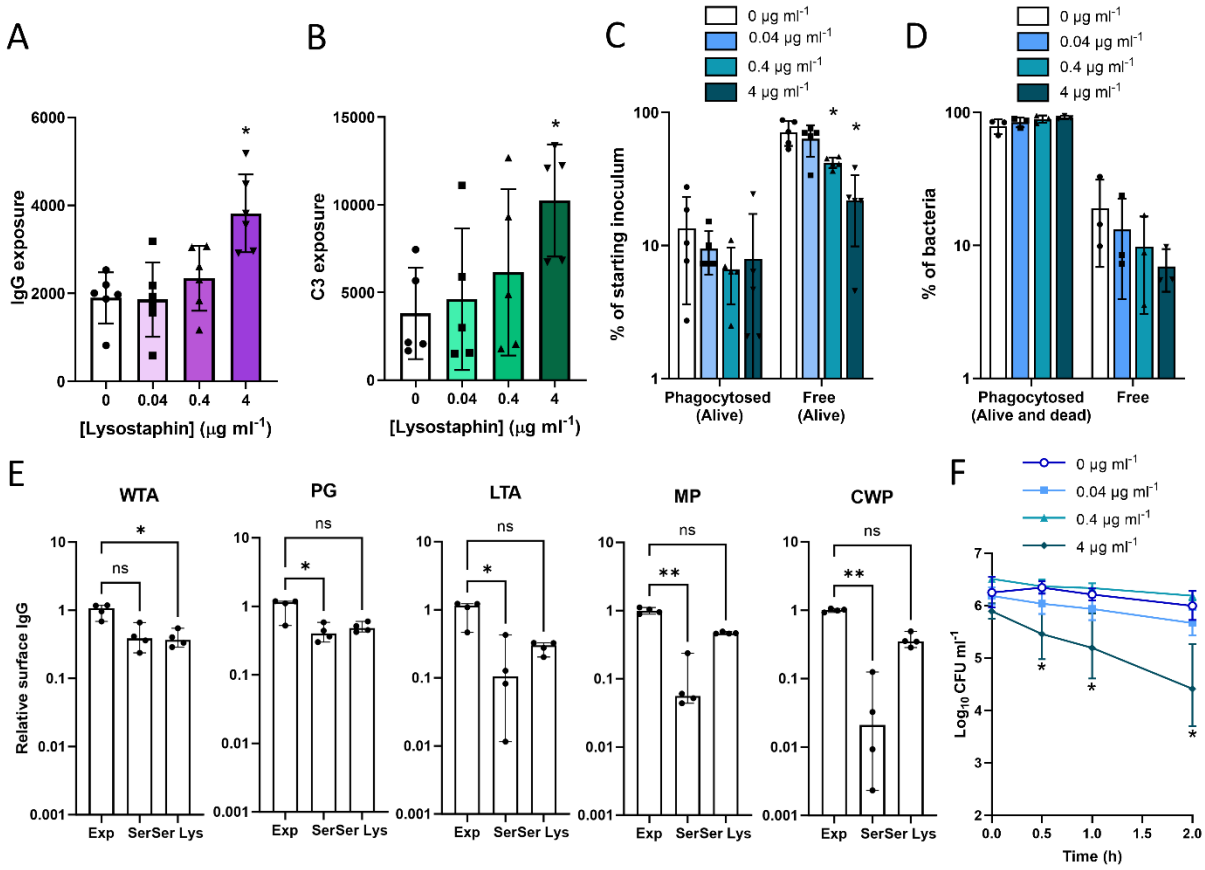

Figure 3. Cell wall accumulation impairs opsonophagocytosis by concealing bound opsonins. Host-adapted cultures of *S. aureus* USA300 were treated with indicated concentrations of lysostaphin for 20 min to partially digest the cell wall before levels of surface-exposed (**A**) IgG and (**B**) complement 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**) Binding ligands of IgG eluted from the surface of *S. aureus* grown to exponential phase and then incubated in serum for 30 min (Exp), allowed to adapt to serum (Ser) or serum-adapted cells that were subsequently subjected to lysostaphin treatment as determined by ELISA. Antibodies were assessed for binding to wall teichoic acid (WTA), peptidoglycan (PG), lipoteichoic acid (LTA), membrane-associated proteins (MP) or cell wall-associated proteins (CWP). (**F**) Survival of lysostaphin-treated cells during a 3 h incubation with purified human neutrophils. Lysostaphin was washed away before incubation with neutrophils.

Data in **A** – **D** represent the mean ± standard deviation of the indicated number of independent biological replicates. 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**, **D** and **F** were analysed by two-way ANOVA with Dunnett's *post-hoc* test (*, $P \leq 0.05$; Lysostaphin-treated vs non-lysostaphin treated). Data in **E** represent the median ± 95% CI of four independent biological replicates and were analysed by Kruskal Wallis test and Dunn's *post-hoc* test to establish statistically significant differences between groups (**, $P < 0.01$; *, $P < 0.05$; ns, $P \geq 0.05$ for the indicated comparisons). Data in **F** represent the geometric mean ± geometric standard deviation of 4 independent experiments.

265 **Antibiotic-mediated inhibition of peptidoglycan accumulation maintains opsonin exposure and**
266 **efficient opsonophagocytosis**

267 To further test whether host adaptation reduced phagocytosis via cell wall-mediated concealment of
268 bound opsonins, and to explore potential therapeutic approaches to enhance neutrophil-mediated
269 killing, we first used the antibiotic fosfomycin to block the accumulation of peptidolgycan during host-
270 adaptation, as we have done previously [37]. This antibiotic targets MurA, which catalyses the
271 production of the peptidoglycan precursor UDP *N*-acetylmuramic acid in the cytoplasm [60]. This
272 inhibits peptidoglycan synthesis and prevents serum-induced cell wall thickening from occurring and
273 has been used clinically in anti-staphylococcal combination therapies [37,61,62].

274 As observed previously, the adaptation of bacteria to serum resulted in a significant reduction in
275 opsonisation, as determined by exposure of IgG and complement, relative to broth-grown bacteria
276 (Fig. 4A,B). However, the presence of fosfomycin in serum significantly reduced opsonin concealment,
277 maintaining IgG and complement exposure at similar levels to that seen for broth-grown bacteria (Fig.
278 4A,B).

279 Further analysis of IgG exposure confirmed that fosfomycin treatment preserved the exposure of IgG
280 bound to all major surface structures relative to bacteria that had not been treated with the antibiotic
281 (Supplementary Fig. S6). Similar findings occurred with another inhibitor of peptidoglycan synthesis,
282 oxacillin, which acts on penicillin binding proteins [63], whereas antibiotics that targeted fatty acid
283 biosynthesis (AFN-1252) or DNA gyrase (ciprofloxacin) did not increase IgG exposure relative to host
284 adapted cells that had not been exposed to antibiotics [64,65] (Supplementary Fig. S6). Therefore, in
285 support of our previous findings, we concluded that the accumulation of peptidoglycan during host
286 adaptation significantly reduces the exposure of IgG bound to LTA and surface proteins.

287 The increased exposure of IgG and complement on the surface of bacteria incubated in serum
288 containing fosfomycin, restored phagocytosis to levels seen with broth-grown bacteria, as determined
289 by both phagocytosis assays (Fig. 4C,D). Furthermore, bacteria that were adapted in serum with
290 fosfomycin were killed by neutrophils as efficiently as broth-grown bacteria, whereas host-adapted
291 bacteria not exposed to fosfomycin survived at significantly higher levels (Fig. 4E). In keeping with our
292 analysis that AFN-1252 did not prevent IgG concealment during host adaptation, this antibiotic did not
293 promote phagocytosis of host-adapted *S. aureus* (Supplementary Fig. S7).

294 *S. aureus* anchors proteins to peptidoglycan via sortase enzymes (SrtA and SrtB) and so we assessed
295 whether the impact of fosfomycin on host adaption was due to interference with this process.
296 However, host-adapted mutants defective for SrtA or SrtB, which cannot anchor proteins to

297 peptidoglycan, survived incubation with neutrophils as well as host-adapted wild type bacteria
298 (Supplementary Fig. S8A,B).

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

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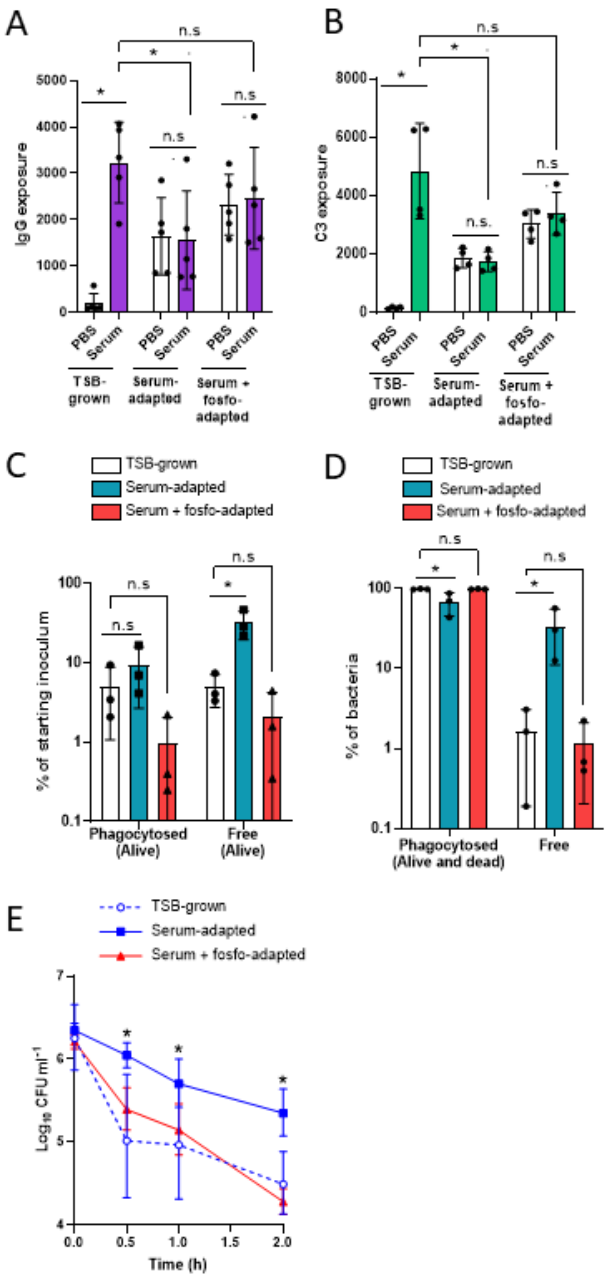

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322 **Figure 4. Antibiotic-mediated inhibition of cell wall accumulation maintains opsonin exposure and**
323 **efficient opsonophagocytosis.** TSB-grown, serum-adapted and serum + fosfomycin-adapted cultures
324 of *S. aureus* USA300 were incubated for 30 min with PBS or 10 % serum before the levels of surface-
325 exposed (**A**) IgG and (**B**) complement component C3 were determined by flow cytometry. Additionally,
326 these cells were incubated with purified human neutrophils for 30 min before the percentage of
327 neutrophil-associated (phagocytosed) and non-neutrophil associated (free) bacteria were determined
328 by (**C**) CFU counts and (**D**) flow cytometry. (**E**) Survival of TSB-grown, serum-adapted and serum +
329 fosfomycin-adapted cultures during a 2 h incubation with purified human neutrophils. Summary
330 diagram of the effect of fosfomycin on opsonin exposure during host-adaptation, with antibiotic-

331 mediated inhibition of peptidoglycan accumulation preserving the exposure of bound antibody and
332 complement (**F**). Data in **A** – **D** represent the geometric mean ± geometric standard deviation of the
333 indicated number of independent biological replicates. Data in **E** are presented as the geometric mean
334 ± geometric standard deviation of three independent experiments. In **A** and **B**, each biological repeat
335 is represented by the median fluorescence value of 10,000 bacterial events. Data in **A** and **B** were
336 analysed by two-way ANOVA with Tukey's *post-hoc* test. Data in **C** – **E** were analysed by two-way
337 ANOVA with Dunnett's *post-hoc* test (*, $P < 0.05$; n.s, $P \geq 0.05$; comparisons are indicated in **A** – **D** and
338 serum/serum + fosfomycin-adapted vs TSB-grown at each time-point in **E**).

339

340 **Discussion**

341 The binding of antibody and complement to the bacterial cell surface enables the detection and
342 destruction of pathogens by phagocytic immune cells [10,11,13]. The data presented here
343 demonstrate that *S. aureus* can conceal a subset of bound opsonins via cell wall accumulation,
344 significantly reducing opsonophagocytosis and killing by neutrophils, a previously unrecognised
345 mechanism of immune evasion (Supplementary Fig. S9).

346 Cell wall remodelling occurs in response to host stresses and provides protection against antibiotics
347 and host defence peptides. It involves the accumulation of peptidoglycan and WTA [36,37,66] and it
348 is therefore unsurprising that exposure of antibody targeting these two polymers was least affected
349 by host adaptation. By contrast, the exposure of antibodies bound to surface proteins and LTA was
350 significantly reduced by host adaptation, in keeping with their localisation within the cell wall itself
351 [67].

352 Previous work indicated that WTA can block antibodies from binding to antigens within the cell wall
353 [68]. Although WTA accumulates in the wall during host adaptation, it is currently unknown whether
354 this contributes to concealment of IgG bound to LTA or proteins. Unfortunately, since cell wall
355 accumulation is dependent upon D-alanine labelled WTA [37], we could not use a WTA-deficient
356 mutant to explore the role of this polymer in reducing opsonin exposure. However, our work did show
357 that inhibition of peptidoglycan accumulation preserved antibody exposure and opsonophagocytic
358 killing by neutrophils, demonstrating a key role for cell wall accumulation.

359 During host adaptation, peptidoglycan accumulation is due to a combination of peptidoglycan
360 synthesis and inhibition of autolytic activity [37]. Recent work has revealed that mutants lacking the
361 Atl autolysin have defective surface exposure of staphylococcal surface proteins, inhibiting their
362 recognition by reactive antibodies [69]. Exposure of surface proteins was restored using enzymatic

363 digestion of peptidoglycan, providing additional evidence that peptidoglycan accumulation can
364 obscure surface antigens and prevent their detection by antibodies. However, the impact of
365 concealment of surface proteins on opsonophagocytosis has not been investigated previously.

366 Several experimental vaccines have been developed based on surface proteins in an attempt to
367 generate high serum titres of opsonising antibody. Unfortunately, despite very promising data from
368 animal infection experiments, none of these vaccines have shown efficacy in humans [70]. Several
369 plausible reasons for this discrepancy have been proposed, including the host-specificity of
370 staphylococcal immune evasion factors and previous staphylococcal infection directing the host
371 towards non-protective immunity [14,70,71].

372 Another difference between model infection of animals and natural infection in humans is the
373 physiological state of the bacteria. For many animal infections, bacteria are grown in TSB immediately
374 prior to administration into the animal and will therefore have high levels of multiple protein antigens
375 exposed on their surface, which facilitates rapid opsonophagocytosis [72,73,74,74,75]. By contrast,
376 natural invasive infection typically begins with colonisation of superficial sites such as an inserted IV
377 catheter and so bacteria may be host-adapted when they enter the bloodstream and are thus less well
378 recognised by antibodies targeting surface proteins [76,77,78,79,80]. As such, the addition of WTA as
379 a vaccine antigen may provide a reasonable level of protection against bacteria that have accumulated
380 cell wall and thus have reduced exposure of surface proteins.

381 Previous work has indicated that the thickened cell wall associated with vancomycin resistance
382 reduces staphylococcal susceptibility to intracellular killing by neutrophils [81]. However, our data did
383 not show a difference in staphylococcal survival within neutrophils, with similar numbers of
384 intracellular viable broth-grown and host-adapted bacteria. Instead, the survival advantage of host
385 adaptation appeared to be due to enhanced evasion of phagocytosis.

386 We do not yet know if these findings are applicable to other Gram-positive pathogens. However, since
387 previous work has shown that serum triggers cell wall thickening in *Enterococcus faecalis* and viridans
388 group streptococci, it is possible that our findings with *S. aureus* represent a broadly conserved
389 mechanism of immune evasion [82].

390 Cell wall thickening in *S. aureus* is triggered by bacterial sensing of the host defence antimicrobial
391 peptide LL-37 via the GraRS system [37]. Since LL-37 is present in most tissues and amongst the earliest
392 host responses to infection or trauma [81,82,83], we hypothesise that *S. aureus* has evolved to sense
393 this AMP as an early indicator that it is subject to immune attack and provides an opportunity to
394 employ defensive measures against the impending arrival of neutrophils. In support of this hypothesis,

395 GraRS, the two-component system that detects LL-37, is activated in the early stages of staphylococcal
396 skin colonisation, whilst *S. aureus* mutants lacking GraRS are significantly less virulent than wild type
397 strains in invasive infection models [84,85,86].

398 In addition to providing protection against opsonophagocytosis, LL-37 exposure triggers reduced
399 susceptibility to the antibiotics daptomycin and vancomycin [37,87], suggesting *S. aureus* employs
400 strategies that are broadly protective against the twin threats of host immunity and antibiotic therapy.
401 This is similar to our previous work showing that induction of the *S. aureus* general stress response
402 regulated by the alternative sigma factor SigB can promote survival of bacteria exposed to host
403 defences and various classes of antibiotics [88]. Further support for the link between *S. aureus*-
404 immune interactions and antibiotic tolerance comes from studies showing that oxidative stress
405 conferred by phagocytic cells reduce staphylococcal susceptibility to antibiotics [89,90].

406 Whilst the immune response may compromise the efficacy of antibiotic therapy under certain
407 circumstances, our study also highlights how antibiotics and the immune response can work
408 synergistically by showing that fosfomycin blocked LL-37 induced cell wall thickening and thereby
409 maintained exposure of bound opsonins, leading to efficient opsonophagocytic killing. Additionally,
410 previous work has suggested that fosfomycin also promotes killing of *S. aureus* via enhanced
411 production of the neutrophil oxidative burst [91]. However, whilst we exposed *S. aureus* to fosfomycin
412 in serum, this was removed by washing prior to incubation with neutrophils and thus does not explain
413 the enhanced killing effect observed in our assays. This strongly suggests that there are at least two
414 mechanisms by which fosfomycin and neutrophils synergise against *S. aureus* and a greater
415 understanding of this may contribute to more effective therapeutic approaches that reduce the high
416 incidence of relapsing or chronic staphylococcal infections [92].

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

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426 ## Materials and methods

427 ### Bacterial strains and growth conditions

428 Bacterial strains used in this study are shown in Table 1. Strains were grown at 37 °C on tryptic soy
429 agar (TSA) or in tryptic soy broth (TSB) with shaking (180 r.p.m.) supplemented with erythromycin (10
430 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	93
JE2 *srtB*::Tn	JE2 strain defective for sortase B, which anchors NPQTN motif-containing IsdC protein to peptidoglycan	93
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

431

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

433

434 ### Construction of strains

435 The JE2 *sbi*::Tn/*spa*::*kan* double mutant was constructed via transduction of the kanamycin resistance
436 marker from Newman *spa*::*kan* [94] into the *sbi*::Tn mutant present in the NARSA transposon mutant
437 library [93] using ϕ11.

438

439 ### IgG Fc binding assay

440 The Fc portion of human Immunoglobulin G (1 mg, Abcam) was labelled with biotin (Thermo Scientific
441 EZ-Link Sulfo-NHS-Biotin) before removal of unbound biotin by dialysis. Labelled Fc portion was then
442 incubated with PBS washed bacterial cells for 30 min (10 µg protein and 10^9 CFU *S. aureus* in 1 ml PBS).
443 Unbound immunoglobulin fragment was removed by three rounds of washing with PBS before cells
444 were incubated with streptavidin-alkaline phosphatase for 30 min. Cells were then washed with three
445 rounds of PBS before incubation in 200 µl p-Nitrophenol phosphate substrate solution for ELISA
446 (Merck) for 10 min. Cells were then pelleted by centrifugation and the supernatant recovered and A_{405}
447 determined.

448 **Generation of broth-grown and host-adapted bacterial cultures**

449 To generate broth-grown bacteria, cultures were grown for 16 h in TSB to stationary phase. These
450 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
451 reached.

452 To generate host-adapted bacteria, broth-grown cultures were centrifuged (3,200 x *g* for 10 min),
453 resuspended in an equal volume of human serum from human male AB plasma (Sigma) and incubated
454 for 16 h at 37 °C. As *S. aureus* is unable to replicate in human serum, these cultures were also at 10^8
455 CFU ml^{-1} [37,47,48,49]. Where appropriate, serum was supplemented with a sub-lethal concentration
456 of fosfomycin (64 µg ml^{-1}), oxacillin (128 µg ml^{-1}), ciprofloxacin (160 µg ml^{-1}) or AFN-1252 (0.15 µg ml^{-}
457 ^{1}). These concentrations were chosen based on previous work that showed they were the maximum
458 concentration that did not affect staphylococcal viability in serum [37].
459
460 Where appropriate, the cell walls of serum-adapted cultures were degraded by lysostaphin. To do this,
461 1 ml aliquots of serum-adapted bacteria were washed in PBS and resuspended in 1 ml PBS
462 supplemented with indicated concentrations of lysostaphin (between 0.04 and 4 µg ml^{-1}). Bacteria
463 were incubated statically for 20 min at 37 °C before being washed by 3 rounds of centrifugation in
464 PBS.

465 **Purification of neutrophils**

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

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

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

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

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

487 To validate the experimental conditions used in this assay, two control experiments were run. Firstly,
488 bacteria were prepared as described above were subjected to centrifugation and the CFU counts pre-
489 and post-centrifugation quantified to determine whether bacteria were pulled out of suspension
490 under these conditions. Secondly, to understand whether bacteria associated with neutrophils were
491 intracellular, bacteria were incubated with neutrophils as described above, before subsequent
492 incubation with or without lysostaphin (40 µg ml^{-1}) to kill extracellular bacteria. Neutrophils were then
493 washed and CFU counts determined before washing to remove the lytic enzyme. In some experiments
494 neutrophils were lysed with Triton X-100 (0.1%) to determine whether this affected recovery of CFU.

495

496 **Measurement of phagocytosis by flow cytometry**

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

501 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 %
502 human serum, 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$. In the case of lysostaphin-treated bacteria, 10 %

503 serum was omitted from the HBSS. After a 30 min incubation at 37˚C with end-over-end mixing in the
504 dark, cultures were fixed by the addition of an equal volume of 4 % paraformaldehyde (PFA). Samples
505 were then analysed by flow cytometry using an Amnis CellStream. Bacteria were detected using the
506 488 nm laser and at least 10,000 bacterial events were recorded. Events with FITC ≥ 2 x 10^3 were
507 counted as bacteria. Events with an FCS of ≥ 3000 were counted as phagocytosed and < 3000 were
508 counted as unphagocytosed/free.

509

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

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

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

523 Cultures of TSB-grown and serum-adapted bacteria (1 ml at 10^8 CFU ml^{-1}) were prepared as described
524 above, washed by three rounds of centrifugation in PBS (13,000 x *g* for 1 min) and resuspended in 100
525 µl PBS. A *spa/sbi* double mutant was used to prevent non-specific antibody binding. Lysostaphin (10
526 µg ml^{-1}) was added and bacteria incubated statically for 1 h at 37˚C. Sample buffer (187.5 mM Tris-
527 HCl (pH 6.8), 6 % SDS, 30 % glycerol, 0.03 % bromophenol blue and 15 % beta-mercaptoethanol; 50
528 µl) was added and samples were incubated at 95˚C for 10 min before 15 µl was loaded onto 10 %
529 polyacrylamide gels. Gels were run in Tris-Glycine running buffer (25 mM Tris, 192 mM glycine, 0.1 %
530 SDS, pH 8.4) at 100 V for 10 min followed by 200 V for 50 min before being transferred onto PVDF
531 membranes (10 V for 60 min). Membranes were blocked for 1 h at room temperature in 5 % milk and
532 1 % BSA in TBST. IgG was detected using 1:10000 dilution of donkey anti-human IgG conjugated to
533 HRP (Abcam) and C3 was detected by 1:5000 dilution of rabbit anti-C3 (Abcam) followed by 1:10000
534 dilution of goat anti-rabbit IgG conjugated to HRP (Abcam). Blots were developed using SuperSignal

535 West Pico PLUS chemiluminescent substrate (Thermo scientific) and imaged using the Bio-rad
536 ChemiDoc MP imaging system.
537
538 **Characterisation of IgG bound to cells**
539 Bacteria were grown to exponential phase and incubated in serum for 30 min or allowed to adapt for
540 16 h, followed or not by partial cell wall digestion using lysostaphin as described above. Cells (10^8)
541 were washed three times in PBS before bound antibody was eluted using 200 µl antibody elution
542 buffer (Pierce) for 5 min. Cells were then removed by centrifugation and the eluted antibody solution
543 neutralised with 100 µl protein A binding buffer (Pierce).

544 To determine the binding ligands of bound antibodies, 10 µg purified cell surface components LTA
545 (Sigma), WTA [37], peptidoglycan [37], membrane proteins [91] or cell wall proteins [91] were
546 immobilised onto the wells of a Nunc Maxisorp ELISA plate by incubation at 4 ^{o}C for 16 h. Remaining
547 binding sites were blocked with PBS containing 3 % bovine serum albumin before addition of the
548 eluted antibody samples (200 µl). Wells containing eluted antibodies were incubated at ambient
549 temperature for 1 h, washed three times with PBS and then 200 µl PBS containing anti-human
550 antibodies conjugated to alkaline phosphatase (Abcam, 1:2000 dilution) was added for 1 h. Wells were
551 again washed three times with PBS and bound alkaline phosphatase quantified using a p-Nitrophenol
552 phosphate substrate solution for ELISA (Merck) and A_{405} readings.

553

554 **Statistical analyses**

555 CFU counts were $\log_{10}$ transformed and displayed as the geometric mean ± geometric standard
556 deviations [95]. Other data are displayed as the mean ± standard deviation or median ± 95 % CI. For
557 all experiments, three or more independent replicates were performed as indicated by individual data
558 points. Data were analysed by one-way ANOVA, two-way ANOVA, or Kruskal Wallis, with appropriate
559 post-hoc multiple comparison test as detailed in figure legends using GraphPad Prism (V8.0).

560

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Contributions and study design

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

Conflict of interest

The authors declare no conflict of interest.

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