Inactivation of the monofunctional peptidoglycan glycosyltransferase SgtB allows *Staphylococcus aureus* to survive in the absence of lipoteichoic acid

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Running title: Characterization of LTA negative *S. aureus* strains

Abstract

The cell wall of *Staphylococcus aureus* is composed of peptidoglycan and the anionic polymers lipoteichoic acid (LTA) and wall teichoic acid. LTA is required for growth and normal cell morphology in *S. aureus.* Strains lacking LTA are usually only viable when grown under osmotically stabilizing conditions or after the acquisition of compensatory mutations. LTA negative suppressor strains with inactivating mutations in *gdpP*, resulting in an increase in intracellular c-di-AMP levels, have been described previously. Here, we sought to identify factors other than c-di-AMP that allow *S. aureus* to survive without LTA. LTA-negative strains able to grow in un-supplemented medium were obtained and found to contain mutations in *sgtB, mazE, clpX* or *vraT*. The growth improvement through mutations in *mazE* and *sgtB* was confirmed by complementation analysis. We also show that an *S. aureus sgtB* transposon mutant, inactivated for the monofunctional peptidoglycan glycosyltransferase SgtB, displays a two-fold increase in the MIC towards a number of cell wall-targeting antibiotics, suggesting that alteration in the peptidoglycan structure could help bacteria compensate for the lack of LTA. Muropeptide analysis of peptidoglycan isolated from a WT and *sgtB* mutant strains did not reveal any drastic alternations in the peptidoglycan structure. In contrast, the peptidoglycan isolated from an LTA-negative *ltaS* mutant strain showed a significant reduction in the fraction of highly crosslinked peptidoglycan, which was partially rescued in the *sgtB*/*ltaS* double mutant suppressor strain. Taken together, these data point towards an important function of LTA in cell wall integrity through its requirement for proper peptidoglycan assembly.

Importance

The bacterial cell wall acts as primary defence against environmental insults such as changes in osmolarity. It is also a vulnerable structure as defects in its synthesis can lead to growth arrest or cell death. The important human pathogen *Staphylococcus aureus* has a typical Gram-

50 positive cell wall, which consists of peptidoglycan and the anionic polymers lipoteichoic acid
51 (LTA) and wall teichoic acid. Several clinically relevant antibiotics inhibit the synthesis of
52 peptidoglycan; hence it and teichoic acids are considered attractive targets for the development
53 of new antimicrobials. We show that LTA is required for efficient peptidoglycan crosslinking
54 in *S. aureus* and inactivation of a peptidoglycan glycosyltransferase can partially rescue this
55 defect, altogether revealing an intimate link between peptidoglycan and LTA synthesis.
56

57 **Introduction**

58 *Staphylococcus aureus* is a Gram-positive bacterium found as commensal on the skin and nasal
59 passages of healthy individuals. However, this bacterium is also an important human pathogen
60 causing hospital- as well as community-acquired infections, such as serious skin infections,
61 osteomyelitis and endocarditis (1-3). Of major concern is the increasing resistance of this
62 organism to a large number of clinically relevant antibiotics (4). A number of virulence factors
63 contribute to the successful host colonization, immune evasion and acquisition of nutrients
64 within the host (5). Many of these factors are either secreted proteins or other extracellular
65 proteins closely associated with the bacterial cell envelope.

66 The cell envelope is essential for bacterial survival and pathogenesis but also a target
67 of a number of important antimicrobials. It functions as barrier and protects bacteria from
68 environmental insults but at the same time needs to allow the passage of solutes and nutrients
69 as well as sense changes in the external environment (6). *S. aureus* has a typical Gram-positive
70 cell envelop, which consists of a cytoplasmic membrane that is surrounded by a thick
71 peptidoglycan layer (7). The peptidoglycan layer is a dynamic macromolecular structure that
72 undergoes constant cycles of polymerisation and hydrolysis to allow bacteria to grow and
73 divide (6). It is composed of glycan chains made of alternating N-acetylglucosamine and N-
74 acetylmuramic acid residues connected by peptide bridges (8). This mesh-like sacculus is able

75 to protect the cell from environmental threats and at the same time withstand the high internal
76 osmotic pressure (9). The final steps of peptidoglycan synthesis are catalyzed by enzymes
77 named penicillin binding proteins (PBPs) and a coordinated action of these enzymes is crucial
78 for the survival of the cell. PBPs with glycosyltransferase activity polymerize the glycan chains
79 while the ones with transpeptidase activity catalyze the formation of the peptide cross-bridges.
80 *S. aureus* encodes four PBPs, of which PBP1, which has transpeptidase activity, and the
81 bifunctional PBP2, which has transpeptidase and glycosltransferase activity, are the minimal
82 requirement for cell survival. In methicillin-resistant *S. aureus* (MRSA) strains, the alternative
83 PBP2A, which has transpeptidase activity, is needed for β-lactam resistance in addition to the
84 glycosyltransferase activity of PBP2. Moreover, additional non-essential proteins have been
85 identified that are involved in peptidoglycan synthesis such as the monofunctional
86 glycosyltransferases SgtA and SgtB (also named Mgt) (10). Previous studies have shown that
87 although SgtA and SgtB have glycosyltransferase activity *in vitro*, only SgtB can support the
88 growth of *S. aureus* in the absence of the main glycosyltransferase PBP2. However, this is not
89 possible in the presence of β-lactam antibiotics when an interaction between PBP2 and PBP2A
90 is needed (11, 12).
91 In Gram-positive bacteria, the peptidoglycan layer is interspersed by a plethora of proteins and
92 cell wall polymers named teichoic acids. Teichoic acids are further categorised into
93 lipoteichoic acids (LTA), anchored to the outer leaflet of the cytoplasmic membrane via a lipid
94 moiety, and wall teichoic acids (WTA) covalently attached to the peptidoglycan (13). Teichoic
95 acids form an important part of the cell wall and contribute to the physical and chemical
96 properties of the cell wall, and to the binding of divalent cations (14, 15). LTA is an anionic
97 polymer made up in *S. aureus* of glycerolphosphate repeating units that are further decorated
98 with D-Alanine residues (16). Most proteins required for its synthesis have now been identified
99 and extensively studied over the years. One of the key enzymes required for LTA synthesis is

100 the lipoteichoic acid synthase enzyme LtaS (17, 18). This enzyme polymerizes the LTA
101 backbone chain on the outside of the cell using the glycerolphosphate head group of the
102 membrane lipid phosphatidylglycerol as substrate (18, 19). LTA is indispensable for the growth
103 of *S. aureus* under standard laboratory growth conditions, highlighting its important
104 physiological role (18, 20). Previous studies indicated a function for LTA in helping to direct
105 the cell-division machinery (21), in controlling autolysin activity (20), for biofilm formation
106 (22), in mediating interactions with host cell receptors (23), in controlling susceptibility and/or
107 resistance to antimicrobial peptides and in maintaining cation homeostasis (15). *S. aureus ltaS*
108 mutants, which lack the complete LTA polymer, can in some strain backgrounds be constructed
109 at low growth temperature (20). It has also been shown that LTA-deficient *S. aureus* strains
110 are viable when grown under osmotically stabilizing conditions in medium containing 7.5%
111 NaCl or 40% sucrose (20, 24). However, the bacteria display severe morphological defects
112 including an enlarged cell size, clustering and cell division defects, even under those conditions
113 permissive for growth. Bacteria can also readily acquire compensatory mutations allowing
114 them to grow in un-supplemented medium and improving their cell morphology defects (24).
115 The majority of compensatory mutations previously observed were in *gdpP* leading to
116 inactivation of the c-di-AMP phosphodiesterase GdpP (24). The resulting increase in cellular
117 c-di-AMP levels allowed the bacteria to survive the cell wall stress caused by the absence of
118 LTA, which is now believed to be due to changes in the osmotic balance in the cell (24).
119 However, compensatory mutations that rescue the growth of an LTA negative *S. aureus* strain
120 were not only found in *gdpP* but also in other genes. As part of the current study, we sought to
121 identify these genes and further characterize the encoded proteins to gain additional insight
122 into why LTA is essential for the growth of *S. aureus* and potentially uncover novel cellular
123 functions for proteins involved in cell wall assembly or maintenance. Using a suppressor screen
124 approach, we found that inactivation of the monofunctional peptidoglycan glycosyltransferase

125 SgtB allows *S. aureus* to grow in the absence of LTA. We further show that peptidoglycan
126 crosslinking is drastically reduced in the absence of LTA, which can be partially restored upon
127 inactivation of SgtB. This in turn might strengthen the peptidoglycan layer and, in this way,
128 contribute to the observed growth rescue.
129
130 **Results**
131 **Identification of *S. aureus* suppressor strains able to grow in the absence of LTA in a c-**
132 **di-AMP-independent way**. LTA-deficient *S. aureus* strains are viable when grown under
133 osmotically stabilizing conditions in medium containing 7.5% NaCl or 40% sucrose or in un-
134 supplemented medium after the acquisition of compensatory mutations (20, 24). The majority
135 of compensatory mutations previously observed were in *gdpP* leading to inactivation of the c-
136 di-AMP phosphodiesterase GdpP (24). In the same study, suppressor strains with mutations
137 outside the *gdpP* gene were noted (24). In order to characterize suppressor strains with
138 mutations in genes other than *gdpP* and to gain further insight into the cellular function of LTA,
139 a larger suppressor screen was performed and the *ltaS* mutant strains LAC*Δ*ltaS::erm*
140 constructed in medium supplemented either with sucrose or NaCl were plated on un-
141 supplemented TSA plates. A number of independently obtained suppressor colonies were
142 subsequently passed 4 times in fresh TSB to further improve their growth. Next, the
143 chromosomal DNA was isolated from 80 suppressor strains and those lacking mutations in
144 *gdpP* (coding for the c-di-AMP hydrolase) and *dacA* (coding for the c-di-AMP cyclase
145 enzyme) were identified by determining the sequences of these two genes. Of 80 colonies
146 screened, 17 had no mutation in *gdpP* nor *dacA,* and 7 of these were selected for further
147 analysis. The absence of LTA in the suppressor strains was confirmed by western blot analysis
148 (Fig. 1A) and relative cellular c-di-AMP levels were determined using a previously described
149 competitive ELISA assay (25, 26) (Fig. 1B). In contrast to the *gdpP* mutant or LTA negative

150 suppressor strain with a mutation in *gdpP*, which showed the expected increase in c-di-AMP
151 levels, the 7 suppressor strains chosen for further analysis did not show an increase in the
152 cellular c-di-AMP concentration (Fig. 1B). Of note, as the relative c-di-AMP levels were
153 assessed using a competitive ELISA assay, a decrease in the A_{490} reading corresponds to an
154 increase in c-di-AMP level (Fig. 1B). Next, genomic variations in the seven suppressor strains
155 compared to the original *ltaS* mutant strains were identified by whole genome sequencing.
156 Mutations were found in *sgtB* (SAUSA300_1855), coding for the monofunctional
157 glycosyltransferase SgtB and mutations in this gene arose independently in three suppressor
158 strains (Table 1). Another strain had a mutation in SAUSA300_1254, coding for a hypothetical
159 membrane protein as well as SAUSA300_RS11150, encoding MazE, the antitoxin component
160 of a type II toxin-antitoxin module (Table 1). In the original study by Corrigan *et al.* (24),
161 mutations found in SAUSA300_1254 were proposed to be accessory and required to further
162 improve the growth of the *gdpP* mutant suppressor strains. Consistent with a previous report
163 (2), a large deletion in *clpX* (SAUSA300_1621), encoding a protein forming part of an ATP-
164 dependent protease, was observed in one strain (Table 1). Finally, a mutation in *vraT*
165 (SAUSA300_1867), coding for the membrane protein VraT and forming part of the VraRST
166 three-component system, was identified. For one suppressor strain no single nucleotide
167 polymorphism was identified with a high confidence score, probably due to limited sequence
168 coverage. The mutations identified through the whole genome sequencing approach were
169 subsequently confirmed by fluorescent automated resequencing of the respective genomic
170 region. Some of the mutations observed in *sgtB*, as well as the mutations in *vraT*, *clpX* and
171 *mazE* result in frameshift mutations and introduction of premature stop codons, suggesting that
172 the absence of the eoncoded proteins compensates for the lack of LTA.
173

174 **Phenotypic characterization of the LTA negative *S. aureus* suppressor strains**. In a
175 previous study, it was shown that *S. aureus* cells can grow without LTA in the absence of ClpX
176 (2), hence we did not further characterize the *clpX* mutant suppressor strain obtained as part of
177 this study. Instead our further analysis focused on suppressor strains with mutations in novel
178 genes, which are strains with mutations in *mazE*, *sgtB* or *vraT*. MazE, the antitoxin component
179 of a type II TA module, is part of the *sigB* regulon and has been shown to be essential for full
180 activity of the alternative sigma factor SigB (27). SigB and members of its regulon enable
181 bacteria to respond rapidly to environmental and antibiotic stresses and also play a role in cell
182 envelope homeostasis (28, 29). Various studies have investigated the role of the VraTSR three-
183 component regulatory system and this system has been reported to be involved in the induction
184 of the cell wall stressosome, mainly in the presence of cell wall targeting antibiotics (30-32).
185 Interestingly, the monofunctional glycosyltransferase SgtB, also identified as part of our
186 screen, is one of the proteins belonging to the cell wall stressosome whose expression is
187 regulated by VraTSR independent of the presence of cell wall targeting antibiotics (30). To
188 further characterize the suppressor strains, we first confirmed the growth improvement of
189 strains S2-*mazE*, S4-*sgtB* and S7-*vraT* when propagated in TSB. All three suppressor strains
190 grew significantly better compared to the original *ltaS* mutant strain and their growth rate was
191 only slightly reduced compared to the WT LAC* stain (Fig. 2A). Next, the cell morphology of
192 the WT, original *ltaS* mutant and the three suppressor strains was assessed by microscopy
193 following staining of the peptidoglycan with fluorescently labelled vancomycin. The cell
194 morphology of the suppressor strains was considerably improved and in particular for
195 suppressor strain S2-*mazE* the division site was correctly placed in most cells (Fig. 2B). Next,
196 the susceptibility of the suppressor strains to a number of cell wall targeting antibiotics was
197 determined. Although the growth and cell morphology of the suppressor strains were improved,
198 these strains remained hyper-sensitive to the β-lactam antibiotic oxacillin (Table 2). The

199 susceptibility to the cell wall or membrane targeting antibiotics lysostaphin, nisin, vancomycin
200 or daptomycin was slightly reduced in the suppressor strains with the exception of suppressor
201 strain S2-*mazE*, which showed a slight increase in resistance to lysostaphin and daptomycin
202 (Table 2). Finally, the growth of the three different suppressor strains was assessed on plates
203 containing Congo red. Congo red is an anionic azo dye traditionally used for the detection of
204 biofilms in *Staphylococcus*. However, at higher concentrations it inhibits the growth of *S.*
205 *aureus* and has been used in the past to indicate differences in the cell wall integrity of different
206 *S. aureus* strains (33). Recently, the target of Congo red has been established as the LTA
207 synthase enzyme LtaS (34). Hence our suppressor strains, which are deleted for *ltaS* and are
208 able to grow in the absence of LTA, should be resistant to this dye. To test the susceptibility of
209 the suppressor strains, serial dilutions of overnight cultures were spotted on TSA plates
210 containing 0.1% Congo red (Fig. 2C). The suppressor strains carrying mutations in *mazE* and
211 *sgtB* were significantly more resistant to Congo red than the WT strain, indicating that
212 inactivation of either one of these genes is indeed sufficient to bypass the LTA essentiality.
213 However, the suppressor strain with the mutation in *vraT* grew poorly on the Congo red plates,
214 suggesting that this strain might not be a bona-fide suppressor strain and hence this strain was
215 not further investigated.
216
217 **Introduction of SgtB or MazE in the respective suppressor strain results in the expected**
218 **growth arrest**. The result of the whole genome sequencing analysis suggested that inactivation
219 of MazE or SgtB is sufficient to allow *S. aureus* to grow in the absence of LTA. Introduction
220 of a wild-type copy of *mazE* or *sgtB* into the respective suppressor strain should revert this
221 phenotype and be lethal for the suppressor strains when grown in un-supplemented medium
222 but should not have an effect when the bacteria are propagated in medium supplemented with
223 40% sucrose. In order to test this, plasmids piTET-*mazE* or piTET-*sgtB,* allowing for

224 anhydrotetracycline (Atet) inducible gene expression, were introduced into the respective
225 suppressor strains. As control, these plasmids were also introduced in the WT LAC* strain.
226 Serial dilutions of these different strains were spotted onto TSA plates containing 100 ng/ml
227 or 200 ng/ml Atet for the expression of *mazE* or *sgtB* expression, respectively. As expected for
228 a successful complementation, the expression of *mazE* prevented the growth of the suppressor
229 strain S2-*mazE* (Fig. 3A) and the expression of *sgtB* prevented the growth of the suppressor
230 strain S4-*sgtB* (Fig. 3B) on TSA but had no effect when the bacteria were spotted on medium
231 supplemented with 40% sucrose (Fig. 4). The result of this complementation analysis supports
232 the notion that inactivation of SgtB or MazE is sufficient to allow *S. aureus* to grow in the
233 absence of LTA.
234
235 **Growth characterization and antibiotic resistance of an *S. aureus* LAC* *sgtB* mutant**.
236 Inactivation of MazE likely has pleotropic effects due to its involvement in the activity of the
237 alternative sigma factor SigB and the reason as to why its inactivation allows *S. aureus* to grow
238 in the absence of LTA could be indirect. Hence, we next focused in trying to gain a better
239 understand of the cellular function of the monofunctional peptidoglycan glycosyltransferase
240 SgtB and how its inactivation allows *S. aureus* to survive in the absence of LTA. To this end,
241 the *sgtB* mutant strain LAC**sgtB::tn* was constructed by moving the genomic region with a
242 transposon insertion in *sgtB* from the Nebraska transposon mutant library strain NE596 (35)
243 into the LAC* background. SgtB is one of two monofunctional peptidoglycan
244 glycosyltransferases encoded in the *S. aureus* genome and the protein can polymerize
245 peptidoglycan glycan chains *in vitro* (36). Although it is dispensable for the growth of *S.*
246 *aureus*, SgtB becomes necessary for bacterial survival in the absence of the main
247 glycosyltransferase PBP2 (10, 11, 37). Consistent with these previous observations, no
248 difference was observed in the cell growth and morphology of the *sgtB* mutant *S. aureus* strain

249 LAC**sgtB::tn* as compared to the WT LAC* strain (Fig. 5A and 5B). Our results indicate that
250 inactivation of SgtB allows *S. aureus* to survive in the absence of LTA, therefore the *sgtB*
251 mutant strain should no longer be sensitive to the Congo red dye. To test this, serial dilutions
252 of the WT, the *sgtB* mutant and a complementation strain were spotted on TSA plates
253 containing 0.1% Congo red, which inhibits the LtaS enzyme. Indeed, the *sgtB* mutant strain
254 was considerably more resistant to Congo red than the WT strain and this phenotype could be
255 complemented by introducing a functional copy of *sgtB* into the mutant strain (Fig. 5C).
256 Moreover, the *sgtB* mutant strain showed increased resistance to the cell wall or membrane
257 targeting antibiotics oxacillin, lysostaphin, nisin and daptomycin as compared to the WT strain
258 and this phenotype could be complemented by introducing a functional copy of *sgtB* (Table 2).
259 Taken together, while no growth or drastic morphological differences were observed between
260 the WT and the *sgtB* mutant strain, deletion of *sgtB* leads to increased Congo red dye and
261 antibiotic resistance in our strain background.
262
263 **Inactivation of SgtB leads to an increase in peptidoglycan cross-linking in an LTA-**
264 **negative *S. aureus* strain**. Since SgtB is involved in peptidoglycan synthesis and the *sgtB*
265 mutant displayed increased antibiotic resistance, we hypothesised that its deletion could
266 somehow "strengthen" the cell wall through alterations in the peptidoglycan structure. In order
267 to investigate this, the muropeptide profiles of peptidoglycan isolated from the WT LAC*, the
268 *sgtB* mutant strain LAC**sgtB::tn*, and the *ltaS/sgtB* mutant suppressor strain S4-*sgtB* was
269 determined following the growth of these strains in TSB. In addition, the muropeptide profile
270 was also determined for peptidoglycan isolated from these three strains as well as the original
271 *ltaS* mutant strain following growth in TSB supplemented with 40% sucrose (Fig. 6). The WT
272 and *sgtB* mutant strains showed very similar and typical *S. aureus* muropeptide profiles (Fig.
273 6A). Quantification of the monomeric, dimeric, trimeric and higher oligomeric peptidoglycan

274 fragments showed that the peptidoglycan is highly crosslinked in both strains with
275 approximately 70% of the UV absorbing material found in the higher oligomeric fraction (Fig.
276 6B and 6C). No drastic difference was found in the muropeptide profile between the WT and
277 *sgtB* mutant strain and growth of these strains in TSB supplemented with 40% did also not
278 drastically alter the muropeptide profile as compared to the profile from the TSB grown strains
279 (Fig. 6). In contrast to the WT and *sgtB* mutant strain, a smaller amount of higher-oligomeric
280 peptidoglycan material was observed for the *ltaS* suppressor strain S4-*sgtB* both in TSB and
281 TSB sucrose medium (Fig 6.). But perhaps most notably, a comparison of the muropeptide
282 profile of peptidoglycan isolated from the original *ltaS* mutant and the *ltaS/sgtB* suppressor
283 strain S4-*sgtB* after growth in TSB sucrose medium showed that, while the amount of
284 crosslinked peptidoglycan was reduced in both strains compared to the WT, the peptidoglycan
285 in the suppressor strain was more crosslinked as compared to the original *ltaS* mutant strain
286 (Fig 6B and 6C). Taken together, these data highlight that deletion of *ltaS* leads to a drastic
287 reduction in the amount of crosslinked peptidoglycan in *S. aureus*. Inactivation of SgtB in a
288 WT strain does not drastically affect the amount of crosslinked peptidoglycan, as one might
289 have expected based on the observed increase in resistance to cell wall active antibiotics.
290 However, in an LTA negative background strain, inactivation of SgtB leads to an increase in
291 peptidoglycan crosslinking, which might explain why an LTA negative strain can growth in
292 the absence of this monofunctional peptidoglycan glycosyltransferase.
293

294 **Discussion**

295 The anionic LTA polymer is a core component of the cell wall, essential for survival and a
296 number of studies have shown its importance in various cell processes (see review (38)).
297 Phenotypes caused by the depletion of LTA in *S. aureus* are misplacement and incomplete
298 formation of cell division septa, enlargement of the cells, together ultimately leading to cell

299 lysis (18, 24). However, how LTA mediates these roles is still unknown. In previous work, it
300 has been shown that LtaS and other core LTA synthesis proteins physically interact with early
301 and late stage cell division proteins as well as with a number of peptidoglycan synthesis
302 proteins (39). This indicates that LTA synthesis enzymes might at least transiently be part of
303 multi-enzyme complexes which might help to coordinate LTA synthesis with peptidoglycan
304 synthesis and cell division (39). We have previously shown that *S. aureus* mutants producing
305 increased intracellular c-di-AMP levels can survive without LTA (24). It is now believed that
306 at high c-di-AMP levels and through the c-di-AMP-dependent regulation of potassium and
307 osmolyte transporters, the internal turgor pressure in the cell might be reduced so that the
308 compromised LTA-depleted cell wall can sustain the the internal pressure (24, 40-42). Indeed,
309 as part of the current study we show that the absence of LTA leads to a drastic reduction in the
310 amount of cross-linked peptidoglycan in *S. aureus* (Fig. 6). This is consistent with the idea that
311 in the absence of LTA the cell wall is likely less able to sustain the high internal turgor pressure,
312 which might also contribute to the increased β-lactam sensitivity of LTA-negative strains
313 observed in this and previous studies (Table 2) (2, 24). The reason for the reduced amount of
314 peptidoglycan crosslinking is currently not clear but could potentially be due to mislocalization
315 of PBPs in the absence of LTA or PBPs having reduced enzyme activity since the proper ion
316 homeostasis cannot be maintained within the cell wall in the absence of LTA.

317 The aim of this study was to further elucidate the role of LTA in cell wall assembly and
318 potentially uncover additional proteins involved in the maintenance of cell wall integrity. A
319 suppressor screen followed by whole genome sequencing revealed mutations in genes coding
320 for ClpX, SgtB, MazE and VraT (Table 1) that can bypass the essentiality of LTA. Further
321 experimentation indicated that the strain with the mutation in *vraT* might not be a *bona fide*
322 suppressor strain. On the other hand, complementation analysis and growth assays on agar
323 plates containing the azo dye Congo red that prevents the growth of *S. aureus* by inhibiting the

324 LtaS enzyme (34) confirmed that inactivating mutations in *sgtB* and *mazE* could bypass the
325 essentiality of LTA. In a previous study it was found that an *S. aureus clpX* mutant readily
326 acquires inactivating mutations in *ltaS* resulting in the generation of LTA negative strains (2).
327 Conversely and consistent with this previous work, in this study we found in one of our LTA-
328 negative suppressor strains a large deletion in *clpX* (Table 1). ClpX is a protein folding
329 chaperon, which recognizes and targets proteins for degradation to the ClpP protease
330 component. In the absence of ClpX, *S. aureus* cells become smaller, show increased production
331 of autolysins and bacteria have a severe growth defect at temperatures of 30˚C or lower (2).
332 The introduction of loss of function mutations in the gene coding for the LTA synthase LtaS
333 in a *clpX* mutant alleviates some of these effects. This is perhaps due to LTA depletion having
334 an opposite effect that is leading to an increase in cell size and decreased in autolysis as reported
335 in some previous publications (2, 20).

336 Mutations in the monofunctional glycosyltransferase SgtB were the most prevalent
337 mutations that arose in our suppressor screen. Previous studies have shown that SgtB is not
338 essential for the survival of the cell and as reported in this and a previous study, an *sgtB* mutant
339 strain does not show any obvious growth or morphological differences as compared to a WT
340 strain under standard growth conditions (Fig. 5) (11). Interestingly, we found that the *sgtB*
341 mutant strain LAC**sgtB::tn* was 2-fold more resistant to a number of cell-targeting antibiotics
342 and this phenotype could be complemented by introduction of a wild-type copy of *sgtB* (Table
343 2). These results are in accordance with previous reports, where strains with mutations in genes
344 that compensate for the lack of LTA, such as *gdpP* and *clpX* mutant strains, also show increased
345 oxacillin resistance (2, 24).

346 The increased resistance to cell wall-targeting antibiotics of the *sgtB* mutant prompted
347 us to investigate the peptidoglycan structure of an *sgtB* mutant in more detail, as we
348 hypothesized that in its absence changes such as an increased crosslinking might be observed

349 that could potentially explain the increased resistance. However, we could not detect any
350 drastic changes in the muropeptide profile of an *sgtB* mutant strain as compared to a WT strain
351 (Fig. 6). On the other hand, the original *ltaS* mutant strain showed a drastic reduction of around
352 30% in the higher oligomeric crosslinked peptidoglycan (Fig. 6). But perhaps most
353 importantly, the peptidoglycan isolated from the *sgtB/ltaS* double mutant S4-*sgtB* suppressor
354 strain showed an increase in peptidoglycan crosslinking as compared to the original *ltaS* mutant
355 strain (Fig. 6). We speculate that this increase in peptidoglycan cross-linking could potentially
356 strengthen the cell wall to better sustain the high internal turgor pressure and be at least in part
357 responsible for the observed growth improvement. Bacterial two-hybrid studies have indicated
358 that the *S. aureus* SgtB protein interacts with SgtA, PBP1, PBP2 and PBP2A (11). Therefore,
359 SgtB and the main glycosyltransferase PBP2 might compete for substrate during peptidoglycan
360 biosynthesis and inactivation of the former might increase the substrate availability and activity
361 of the latter. This might in turn aid in the delivery of substrate to the transpeptidase domain of
362 the bifunctional PBP2 enzyme resulting in increased crosslinking. Alternatively, SgtB could
363 also affect the function of PBP4, which has been shown to be responsible for hyper-
364 crosslinking of the staphylococcal peptidoglycan (43). Clearly, more experiments are needed
365 to clarify the complex nature of peptidoglycan biosynthesis in *S. aureus* and changes observed
366 in the absence of LTA.

367 Mutations in MazE could potentially also be tied in with changes in the cell wall
368 structure of *S. aureus*. The MazEF type II toxin-anitoxin system is part of the *sigB* regulon and
369 has been shown to be required for full activity of the alternative sigma factor SigB (27). It has
370 also been reported that overexpression of σ^B causes cell wall thickening in *S. aureus* and
371 increased resistance to cell wall-targeting antibiotics (28). Therefore, inactivation of MazE, as
372 observed in one of our suppressor strains, could potentially also affect cell wall homeostasis
373 via its effect on SigB.

374 In summary, our results suggest that in the absence of LTA, peptidoglycan in *S. aureus*
375 becomes less cross-linked, which might weaken the cell wall and become less able to sustain
376 the high internal turgor pressure, ultimately leading to cell lysis. The suppressor mutations
377 obtained in *sgtB* (and perhaps also in some of the other genes observed in our screen) help the
378 cell survive this detrimental effect by altering and strengthening the cell wall, likely allowing
379 the cell wall to again better withstand the high internal turgor pressure.
380
381 **Materials and Methods**
382 **Bacterial strains and culture conditions**. All bacterial strains used in this study are listed in
383 Table 3. *Escherichia coli* strains were cultured in Luria Bertani (LB) medium and
384 *Staphylococcus aureus* strains in tryptic soya broth (TSB) at 37°C with aeration, unless
385 otherwise stated. When appropriate the growth medium was supplemented with antibiotics and
386 inducers as follow: for *E. coli* cultures: ampicillin (Amp) 100 μg/ml, chloramphenicol (Cam)
387 10 μg/ml, and kanamycin (Kan) 30 μg/ml; for *S. aureus* cultures: chloramphenicol (Cam) 10
388 μg/ml for plasmid selection and 7.5 μg/ml for chromosomally integrated plasmid selection,
389 erythromycin (Erm) 10 μg/ml and kanamycin (Kan) 90 μg/ml. The inducer anhydrotetracycline
390 (Atet) was used at a concentration of 100 or 200 ng/ml in agar plates and 50 ng/ml in broth.
391
392 **Plasmid and strain construction**. Strains and primers used in this study are listed in Tables 3
393 and 4, respectively. Strain LAC**sgtB::tn* was generated by phage transduction using phage
394 Φ85 and transducing the *sgtB* region containing a transposon insertion in *sgtB* from the
395 Nebraska transposon mutant library strain NE596 (35) into strain LAC*. Plasmids piTET-*sgtB*
396 and piTET-*mazE* for anhydrotetracycline (Atet) inducible expression of *sgtB* and *mazE* in *S.*
397 *aureus* were generated by amplifying *sgtB* or *mazE* from LAC* chromosomal DNA using
398 primers pair ANG2268/ANG2269 or ANG2743/ANG2744, respectively. The PCR products

399 and plasmid piTET were digested with AvrII and SacII, ligated and then transformed into *E.*
400 *coli* XL1-Blue yielding strain XL1-Blue piTET-*sgtB* (ANG3926) and XL1-Blue piTET-*mazE*
401 (ANG4727). The plasmids were shuttled through *E. coli* strain IM08B (ANG3927 and
402 ANG4728) and subsequently electroporated into LAC* yielding strains LAC* piTET-*sgtB*
403 (ANG4056) and LAC* piTET-*mazE* (ANG4729) or into the respective suppressor strains
404 yielding strains S4-*sgtB* piTET-*sgtB* (ANG4058) and S2-*mazE* piTET-*mazE* (ANG4730). The
405 plasmid pCL55-*sgtB* for complementation and expression of *sgtB* from its native promoter was
406 generated by amplifying the *sgtB* gene including its native promoter region from LAC*
407 chromosomal DNA using primers ANG2270 and ANG2271. The PCR product and plasmid
408 pCL55 were digested with EcoRI and BamHI, ligated and then transformed into *E. coli* XL1-
409 Blue yielding strain XL1-Blue pCL55-*sgtB* (ANG4708), shuttled through IM08B (ANG3923)
410 and subsequently electroporated into the *sgtB* mutant strain yielding strain LAC**sgtB::tn*
411 pCL55-*sgtB* (ANG4711). WT LAC* or mutant *S. aureus* strains with the empty vectors piTET
412 or pCL55 (see Table 3) were used as control strains in several experiments. The sequences of
413 all plasmid inserts were verified by automated fluorescence sequencing at GATC
414 Biotechnology.
415
416 **Bacterial growth curves**. *S. aureus* LAC* and the indicated *ltaS* suppressor strains were
417 grown overnight in TSB medium containing the relevant antibiotic. The original *ltaS* mutant
418 strains were grown in TSB containing either 7.5% NaCl or 40% sucrose. Overnight cultures
419 were washed three times in TSB and diluted to a starting OD_{600} of 0.05. Cultures were
420 incubated at 37°C with aeration and OD_{600} values determined at 2 h intervals. WT LAC* and
421 the *sgtB* mutant strain LAC**sgtB::tn* were grown overnight in TSB or TSB supplemented with
422 10 µg/ml Erm. The next day, bacteria were washed three times in TSB and diluted to a starting
423 OD_{600} of 0.05 and 200 µl placed into a 96-well microtiter plate. Growth was monitored for 10h

424 using a SPECTROSTARnano plate reader (BMG Labtech). All growth curves were performed
425 in triplicates with average values and standard deviations plotted.
426
427 **Fluorescence microscopy analysis**. Cells from 1 ml overnight culture were collected by
428 centrifugation, washed 3 times in PBS pH 7.4 and 150 µl of these cell suspensions applied to
429 polylysine (0.1%, w/v) treated coverslips. Bacteria-coated coverslips were incubated with 100
430 µl of a 1 µg/ml BODIPY-vancomycin (Molecular Probes) solution in H_2O for 20 min, washed
431 and mounted on glass slides containing 20 µl Vectashield (Vector Laboratories). Slides were
432 viewed under a Zeiss Axio Imager A2, using a GFP filter set and images were captured with
433 an AxioCam MRc Rev.3 camera and analyzed using the Zen Pro 2012 SP2 software. The
434 experiment was performed in triplicate.
435
436 **Minimum Inhibitory Concentrations (MIC) determination**. Overnight cultures of WT, the
437 *sgtB* mutant and complementation stain as well as the indicated suppressor strains were grown
438 over night in TSB medium. The next day, cultures were washed in PBS pH7.4 buffer adjusted
439 to an OD_{600} of 0.05 in Müller Hinton broth and 100 µl of these suspensions were incubated in
440 96-well plates with 2-fold dilutions of various antimicrobials at the following starting
441 concentrations: oxacillin 1.024 mg/ml or 1 µg/ml as appropriate, daptomycin 32 µg/ml,
442 lysostaphin 16 µg/ml, vancomycin 32 µg/ml, nisin 50 µg/ml and penicillin G 51.2 µg/ml or 0.2
443 µg/ml as appropriate. Oxacillin and daptomycin containing wells were supplemented with 2%
444 (w/v) NaCl and 0.23 mM $CaCl_2$, respectively. Plates were incubated at 37°C for 15 hours with
445 shaking. MICs were determined as the concentration of antibiotic at which growth was
446 inhibited by >75% compared to growth without the antibiotic.
447

448 **LTA detection by western blot**. LTA extraction and detection by western blot was performed
449 as previously described (44). Briefly, samples were prepared from 1 ml overnight cultures
450 normalized based on OD_{600} readings, that is cells from 1 ml culture with an OD_{600} of 6 were
451 suspended in 90 µl 2 × SDS protein sample buffer. 10 µl of these samples were separated on
452 15% SDS-polyacrylamide gels and the material subsequently transferred to PVDF membranes.
453 LTA was detected using the monoclonal polyglycerolphosphate-specific LTA antibody
454 (Hycult Biotechnology) at a 1:4,000 dilution and the HRP-conjugated anti-mouse IgG antibody
455 (Cell Signaling Technologies, USA) at a 1:10,000 dilution. The blots were developed by
456 enhanced chemiluminescence using ClarityTM Western ECL Blotting Substrate (Bio-Rad) and
457 imaged using the ChemiDoc Touch Imaging System (Bio-Rad). Western blots were performed
458 in triplicates and a representative result is shown.
459
460 **Whole genome sequencing**. WT LAC*, LAC*Δ$ltaS_N$, LAC*Δ$ltaS_S$, and the indicated
461 suppressor strains were cultured overnight at 37°C, cells harvested and genomic DNA
462 extracted. Genome sequencing was performed by MicrobesNG (University of Birmingham)
463 using an Illumina platform and a 250 bp paired end read kit. Sequence analysis was performed
464 using the CLC Genomics work bench software package. First the LAC* reads were aligned
465 against the published USA300 FPR3757 genome sequence (RefSeq accession number
466 NC_007793.1), assembled into a reference contig and the USA300 FPR3757 annotation
467 transferred onto the LAC* sequence. Next, the Illumina reads for the original *ltaS* mutant
468 strains LAC*Δ$ltaS_S$ and LAC*Δ$ltaS_N$ as well as the seven different suppressor strains were
469 mapped onto the assembled LAC* sequence and high frequency (> 65%) and good quality base
470 changes identified using the CLC Genomics Workbench software package. Genomic
471 alternations found in the suppressor strains but not present in the original *ltaS* mutant strain are
472 summarized in Table 1. The Illumina short read for the WT LAC* has been previously

473 published (25) and has been deposited in the European Nucleotide Archive under the study
474 accession number PRJEB14759. The Illumina reads for the *ltaS* mutants and *ltaS* suppressor
475 strains were deposited in the European Nucleotide Archive under the study accession number
476 PRJEB14723.
477
478 **Peptidoglycan isolation and analysis.** Overnight cultures of *S. aureus* strains LAC*, S4-*sgtB*
479 and LAC**sgtB::tn* were prepared in TSB or TSB supplemented with 40% sucrose and of the
480 original *ltaS* mutant strain LAC*Δ$ltaS_N$ (ANG2134) in TSB supplemented with 40% sucrose.
481 The next day, cells were back-diluted in 2 litres of the same growth medium to an OD_{600} of
482 0.05. The cultures were grown at 37°C until mid an OD_{600} of approximately 1.5, cooled on ice
483 and cells were collected by centrifugation. Peptidoglycan was purified and digested with
484 mutanolysin as previously described (24, 45). HPLC analysis of the digested peptidoglycan
485 material was performed as described previously described (45) and the muropeptide profiles
486 were determined for each strain and growth condition in triplicates. For the quantification of
487 monomeric, dimeric, trimeric and higher oligomeric peptidoglycan material, the peaks were
488 integrated. The total peak area for each muropetide profile was determined and set to 100%
489 and the % monomeric, dimeric, trimeric, and higher oligomeric peaks calculated and the
490 average values and standard deviation from the three profiles determined and plotted.
491
492 **c-di-AMP quantification by competitive ELISA.** Five ml TSB were inoculated with a single
493 colony of *S. aureus* LAC* (WT), strain LAC* *gdpP::kan,* US3 (LAC*Δ$ltaS_S$*::erm* suppressor
494 with mutation in *gdpP*) as well as the different LTA-negative suppressor and the tubes were
495 incubated for 8 hours at 37°C. Next, the cultures were back-diluted to an OD_{600} of 0.05 in 10
496 ml TSB and grown for 15 hours at 37°C. Bacterial cells from these cultures were collected by
497 centrifugation, cell lysates prepared and the cellular c-di-AMP levels determined using a

498 previously described competitive ELISA method (25, 26). However, in place of determining
499 c-di-AMP levels based on a standard curve the A_{490} values were directly compared. To this
500 end, the A_{490} reading obtained for samples derived for the WT strain was set to 100% and the
501 % values calculated for the samples derived from the other strains. Thee independent
502 experiments were performed with three technical replicates and the average values and
503 standard deviation of the % A_{490} values determined for each strain as compared to the WT
504 strain. Of note, since this is a competitive ELISA, and a decrease in A_{490} readings represents
505 an increase in cellular c-di-AMP levels.
506

507 **Acknowledgments**

508 This research was supported by the European Research Council grant 260371 and the
509 Wellcome Trust grant 100289 to AG as well as the UK Medical Research Council (MRC)
510 within the AMR Cross-council initiative Collaborative Grant MR/N002679/1 to WV. The
511 Illumina sequencing was performed by MicrobesNG, which was supported by the BBSRC
512 grant BB/L024209/1.

513 **Table 1: Genomic variations detected in the suppressor strains as compared the original**
514 ***ltaS* mutant strains**

Strain[1]	Ref. pos.[2]	Type[3]	Ref.[4]	Allele[5]	Freq.[6]	Av. Qual.[7]	Annotations	Amino acid change[8]
ANG3711 S1-*sgtB*	2013626 2013387- 2013622	SVN DEL	T	A	100	38.5	SAUSA300_1855, monofunctional glycosyltransferase SgtB SAUSA300_1855, monofunctional glycosyltransferase SgtB	Asn11Tyr
ANG3712 S2-*mazE*	1376094 1376096 1376098 2183630- 2183639	INS SNV REP DEL	- C C TATC GGA AAA	G A AG ----------	100 100 100	38.5 38.7 38.4	SAUSA300_1254, hypothetical membrane protein SAUSA300_RS11150, Antitoxin component of a type II TA module mazE	Ile291FS Ser292Tyr Pro293fs
ANG3713 S3-*clpX*	1770928 1770932 1771105 1770937- 1771100	SNV SNV SNV DEL	T T A	A C C	100 100 100	38.8 38.9 38.2	SAUSA300_1621, ATP-dependent protease ATP-binding subunit ClpX	Glu148Val Thr147Ala Val89Gly
ANG3714 S4-*sgtlB*	2013139	SNV	A	C	100	38	SAUSA300_1855, monofunctional glycosyltransferase SgtB	Leu173*
ANG3716 S6	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
ANG3717 S7-*vraT*	2023171	SNV	A	C	100	38.8	SAUSA300_1867, membrane protein Yvfq/VraT	Leu71*
ANG3718 S8-*sgtB*	2013542	INS	-	T	92.6	38.6	SAUSA300_1855, monofunctional glycosyltransferase SgtB	Ser39fs

515 [1]Suppressors S1, S2, S7 and S8 were derived from strain LAC*Δ$ltaS_S$ (ANG2135) and
516 suppressor strains S3, S4 and S6 were derived from strain LAC*Δ$ltaS_N$ (ANG2134).
517 [2]Reference position indicates base number in the LAC* reference genome, which was
518 assembled as previously described (25). [3]Type of mutation with DEL indicating deletion, SNV
519 indicating single nucleotide variation, INS indicating insertion and REP indicating
520 replacement. [4]Ref. indicates base in the LAC* reference genome. [5]Allele indicates base in the
521 sequenced strain. [6]Freq. indicates frequency of the specific variant found in the sample reads.
522 [7]Av. Qual. denotes the average base quality score at the indicated position. [8]Amino acid change
523 in the encoded protein in LAC* versus the sequenced strain with fs indicating a frame shift and
524 * a premature stop codon.
525

526 **Table 2: MIC values for different antibiotics given in µg/ml**

	LAC*	**S2-*mazE***	**S4-*sgtB***	**S7-*vraT***	***sgtB::tn***	***sgtB::tn* pCL55**	***sgtB::tn* pCL55-*sgtB***
oxacillin	31.25	0.25	0.125	0.25	62.5	62.5	31.25
lysostaphin	0.25	0.5	0.0625	0.125	0.5	0.5	0.25
nisin	4	1	2	2	8	8	2
vancomycin	2	1	0.5	2	2	2	2
daptomycin	2	4	1	2	4	4	2

527

528 The MIC was defined as antibiotic concentration that leads to > 75% growth inhibition as

529 compared to growth without antibiotic.

530

531 **Table 3: Bacterial strains used in this study**

Strain name	Description	Reference
	***Escherichia coli* strains**	
XL1-Blue	Cloning strain; TetR - ANG127	Stratagene
IM08B	*E. coli – S. aureus* sutttle strain - ANG3723	(46)
ANG3732	IM08B pCL55; AmpR	(42)
ANG3926	XL1-Blue piTET-*sgtB*; AmpR	This study
ANG3927	IM08B piTET-*sgtB*; AmpR	This study
ANG3928	IM08B piTET; AmpR	(47)
ANG4708	XL1-Blue pCL55-*sgtB*; AmpR	This study
ANG4707	IM08B pCL55-*sgtB*; AmpR	This study
ANG4727	XL1-Blue piTET-*mazE*; AmpR	This study
ANG4728	IM08B piTET-*mazE*; AmpR	This study
	***Staphylococcus aureus* strains**	
LAC*	LAC*; Erm sensitive CA-MRSA LAC strain; ANG1575	(48)
NE596	JE2*sgtB::tn*; NARSA library transposon mutant; ErmR – ANG3930	(35)
ANG1961	LAC**gdpP::kan,* KanR	(24)
ANG2134	LAC*Δ*ltaS*$_N$*::erm* pCN38; Isolated on 7.5% NaCl; CamR ErmR	(24)
ANG2135	LAC*Δ*ltaS*$_S$*::erm* pCN38; Isolated on 40% Sucrose; CamR ErmR	(24)
ANG2137	LAC*Δ*ltaS*$_N$*::erm* suppressor S6 original colony; ErmR	(24)
ANG2140	LAC*Δ*ltaS*$_S$*::erm* suppressor S7 original colony; ErmR	(24)
ANG2143	LAC*Δ*ltaS*$_S$*::erm* suppressor S8 original colony; ErmR	(24)
ANG2434	LAC*Δ*ltaS*$_S$*::erm* suppressor with *gdpP* mutation (pass 4) (short: US3); ErmR	(24)
ANG3694	LAC*Δ*ltaSs::erm* suppressor S1 original colony; ErmR	This study
ANG3698	LAC*Δ*ltaSs::erm* suppressor S2 original colony; ErmR	This study
ANG3703	LAC*Δ*ltaSs::erm* suppressor S3, original colony; ErmR	This study
ANG3707	LAC*Δ*ltaSs::erm* suppressor S4, original colony; ErmR	This study
ANG3711	LAC*Δ*ltaS*$_S$*::erm* suppressor S1 pass 4 (short: S1-*sgtB*); ErmR	This study
ANG3712	LAC*Δ*ltaS*$_S$*::erm* suppressor S2 pass 4 (short: S2-*mazE*); ErmR	This study
ANG3713	LAC*Δ*ltaS*$_N$*::erm* suppressor S3 pass 4 (short: S3-*clpX*); ErmR	This study
ANG3714	LAC*Δ*ltaS*$_N$*::erm* suppressor S4 pass 4 (short: S4-*sgtB*); ErmR	This study
ANG3716	LAC*Δ*ltaS*$_N$*::erm* suppressor S6 pass 4 (short: S6); ErmR	This study
ANG3717	LAC*Δ*ltaS*$_S$*::erm* suppressor S7 pass 4 (short: S7-*vraT*); ErmR	This study
ANG3718	LAC*Δ*ltaS*$_S$*::erm* suppressor S8 pass 4 (short: S8-*sgtB*); ErmR	This study
ANG4054	LAC* piTET, CamR	(47)
ANG4056	LAC* piTET-*sgtB*; CamR	This study

ANG4057	S4-*sgtB* piTET; ErmR CamR	This study
ANG4058	S4-*sgtB* piTET-*sgtB*; ErmR CamR	This study
ANG4059	LAC**sgtB::tn*; ErmR	This study
ANG4710	LAC**sgtB::tn* pCL55; ErmR CamR	This study
ANG4711	LAC**sgtB::tn* pCL55-*sgtB*; ErmR CamR	This study
ANG4725	S2-*mazE* piTET; ErmR CamR	This study
ANG4729	LAC* piTET-*mazE*; CamR	This study
ANG4730	S2-*mazE* piTET-*mazE*; ErmR CamR	This study

532

533 **Table 4: Primers used in this study**

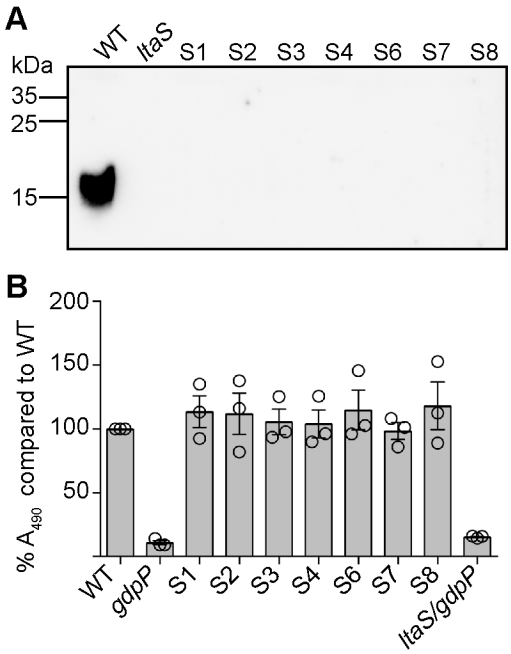
Primer ID	Name	Sequence
ANG2229	sausa300_1867 Fw seq	GAAGCATTTCAAATTCAATGTGCAAG
ANG2230	sausa300_1867 Rv seq	GAAGCATTTCAAATTCAATGTGCAAG
ANG2231	sgtB Fw seq	GTCGTCTCTATTGGCATTTAATAGGG
ANG2232	sgtB Rv seq	AATAATGAAGTACTAATCAAGTGGC
ANG2233	sausa300_1254 Fw seq	CATTTTGAAGAAAGGATTAATCAA
ANG2234	sausa300_1254 Rv seq	AATAATGAAGTACTAATCAAGTGGC
ANG2235	clpX Fw seq	GAAGGAACTAAAGAAGATTAATCTTC
ANG2236	clpX Rv seq	CTGGATATTGTTCTTCTTTTACTGCAC
ANG2268	5-AvrII-sgtB-pCL55iTet	CATGCCTAGGTTAAAAGAAGGAGCAAACGCATG
ANG2269	3-SacII-sgtB-pCL55iTet	CATGCCGCGGTTAACGATTTAATTGTGACATAGCC
ANG2409	5- EcoRI-promSgtB-pCL55	CATGGAATTCAAGTATTGTGGTTATCGATTG
ANG2410	3- BamH1-promSgtB-pCL55	CATGGGATCCTTAACGATTTAATTGTGACATAGCC
ANG2740	5-mazE SNP seq	AGATAATCATAGAGAAAGTCCACAGTCG
ANG2741	3-mazE SNP seq	TAAGTACGTCAGTTTTTCTTTCAATCGT
ANG2743	5-AvrII-mazE-pCL55iTet	CATGCCTAGGGGAATCAATTGGAGGTTCTCATATG
ANG2744	3-SacII-mazE-pCl55iTet	CATGCCGCGGTCATTCATTCGTTGAATTAGAAGAT AAATATG

534 Restriction site sequences are underlined.

535
536

537 **Figures and Legends**

538



539
540
541

542 **Figure 1: Detection of LTA by western blot and c-di-AMP using a competitive ELISA**

543 **method.** (A) Detection of LTA by western-blot. Cell extracts were prepared from LAC* (WT),

544 an *ltaS* mutant (strain ANG2135) and the 7 *ltaS* suppressor strains (S1-S4 and S6-S8) and

545 separated on a 15% PAA gel. LTA was subsequently detected by western blot using a

546 monoclonal polyglycerolphosphate specific antibody. A representative result from 3

547 independent experiments is shown. (B) c-di-AMP detection by competitive ELISA.

548 Cytoplasmic extracts were prepared from LAC* (WT), the high c-di-AMP level control strains

549 LAC* *gdpP::kan* (*gdpP*) and US3 (*ltaS/gdpP*) as well as from the 7 *ltaS* suppressor strains

550 (S1-S4 and S6-S8). c-di-AMP amounts were determined and compared to the WT strain by

551 ELISA. Of note, as this is a competitive ELISA assay, lower A_{490} readings are obtained for

552 samples with higher c-di-AMP levles. The A_{490} reading obtained for the sample derived from

553 the WT strain was set to 100% and % A_{490} reading calculated for the test strains. The average

554 % values and standard deviations from three independent experiments (with three technical

555 replicates) are plotted.

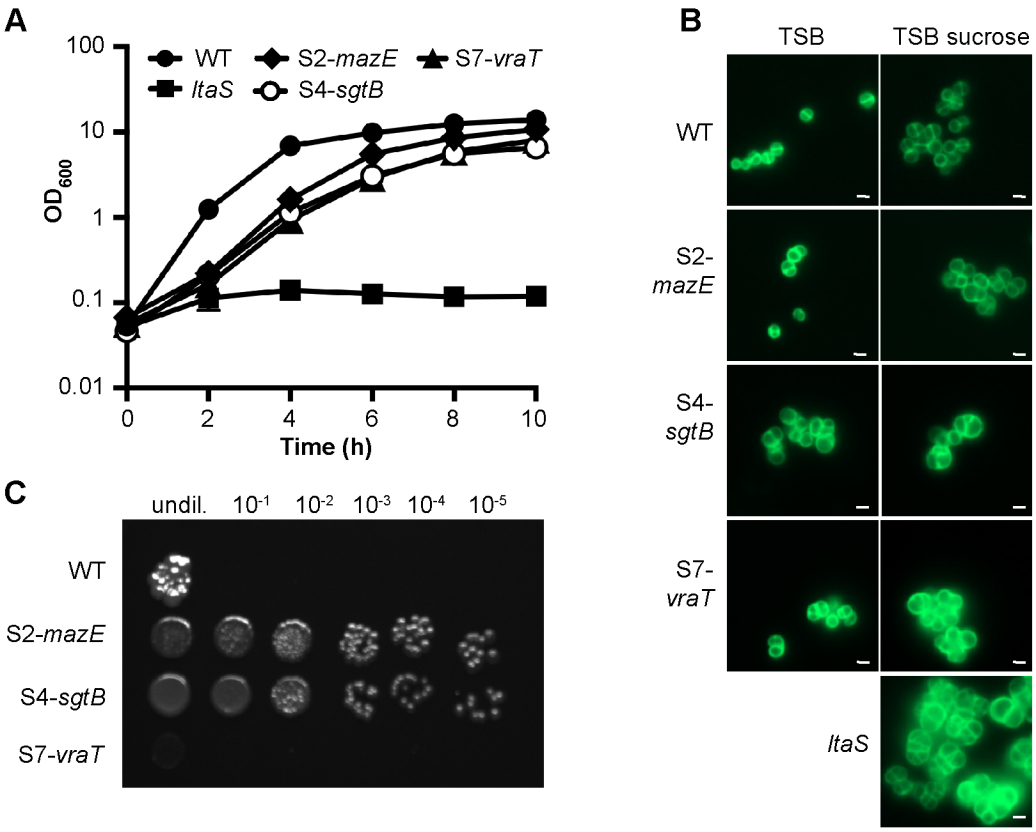
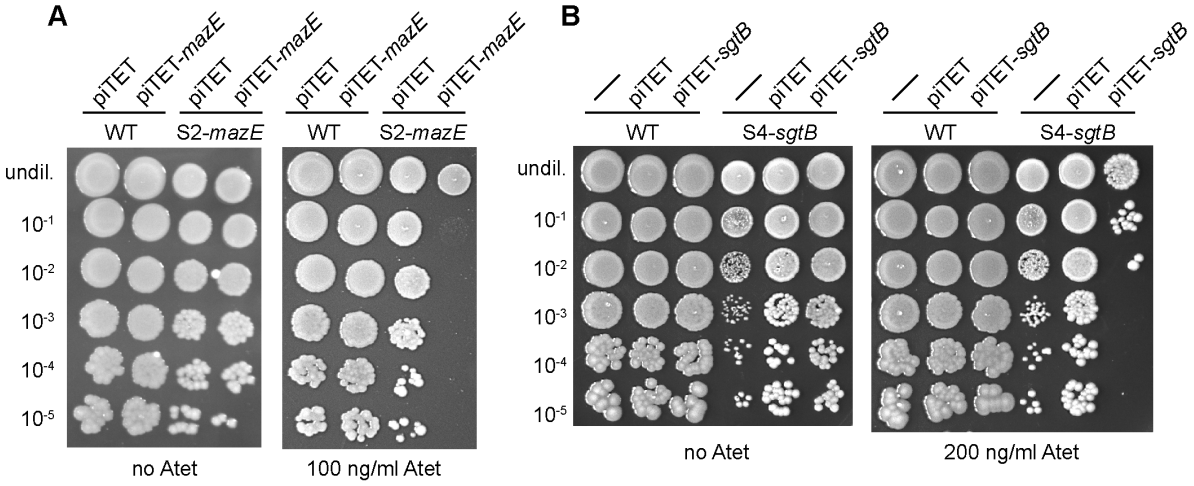


Figure 2: Growth and cell morphology of WT and mutant *S. aureus* strains. (A) Bacterial growth curves. LAC* (WT) and the suppressor strains S2-*mazE,* S4-*sgtB* and S7-*vraT* were grown overnight in TSB and the original *ltaS* mutant strain (ANG2135) in TSB with 40% sucrose. Next day, bacterial cells were washed and diluted in TSB to an OD_{600} of 0.05 and the bacterial growth was subsequently monitored over a period of 10 hours. The average OD_{600} readings from three experiments were plotted. (B) Microscopic analysis. Bacterial cells from overnight cultures of the same strains as used in panel A were washed, back-diluted and grown to mid-log phase in TSB or TSB with 40% sucrose as indicated. The bacterial cells were subsequently stained with BODIPY-vancomycin and viewed by fluorescent microscopy. (C) Analysis of bacterial growth on Congo red-containing TSA plates. Overnight cultures of WT and the indicated suppressor strains were serially diluted and spotted on TSA plates supplemented with 0.1% Congo red and the plates incubated for 48h at 37˚C. For B and C, a representative result of three independent experiments is shown.

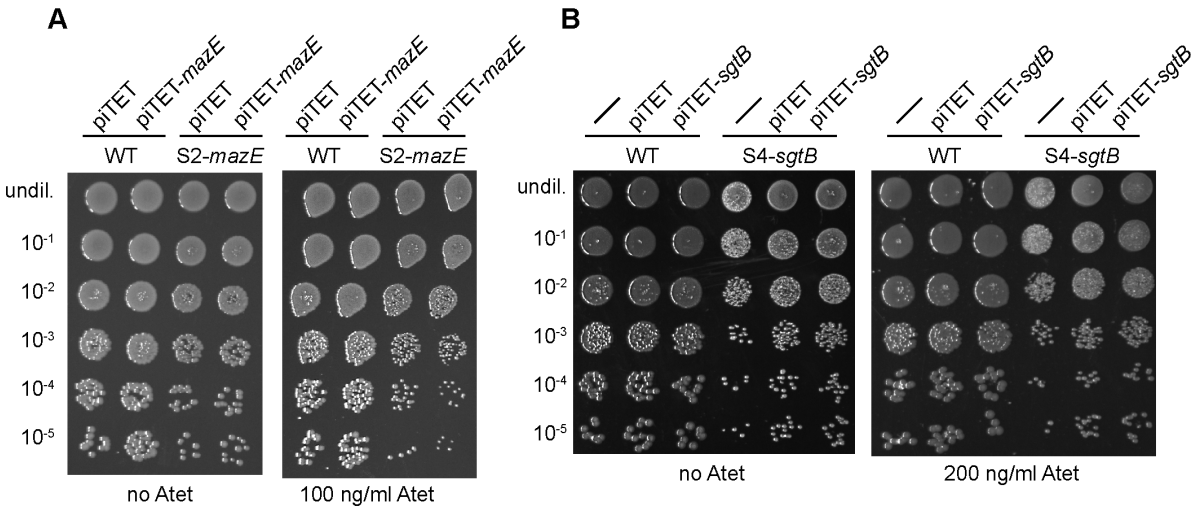
571



572
573
574 **Figure 3: Growth complementation analysis.** TSA plates with spot dilutions. (A) *S. aureus*
575 strains LAC* piTET, LAC* piTET-*mazE*, S2-*mazE* piTET or S2-*mazE* piTET-*mazE* were
576 grown overnight in TSB supplemented with chloramphenicol, washed twice in PBS and serial
577 dilutions spotted on TSA plates supplemented with 7.5 ng/ml chloramphenicol without (left
578 panel) or with 100 ng/ml Atet (right panel). (B) *S. aureus* strains LAC* (WT), LAC* piTET,
579 LAC* piTET-sgtB, S4-*sgtB*, S4-*sgtB* piTET or S4-*sgtB* piTET-*sgtB* were grown and samples
580 prepared as described in A, however without chloramphenicol selection, and dilutions were
581 spotted on TSA plates (left panel) or TSA plates supplemented with 200 ng/ml Atet (right
582 panel). Representative plate images from three independent experiments are shown.

583

584



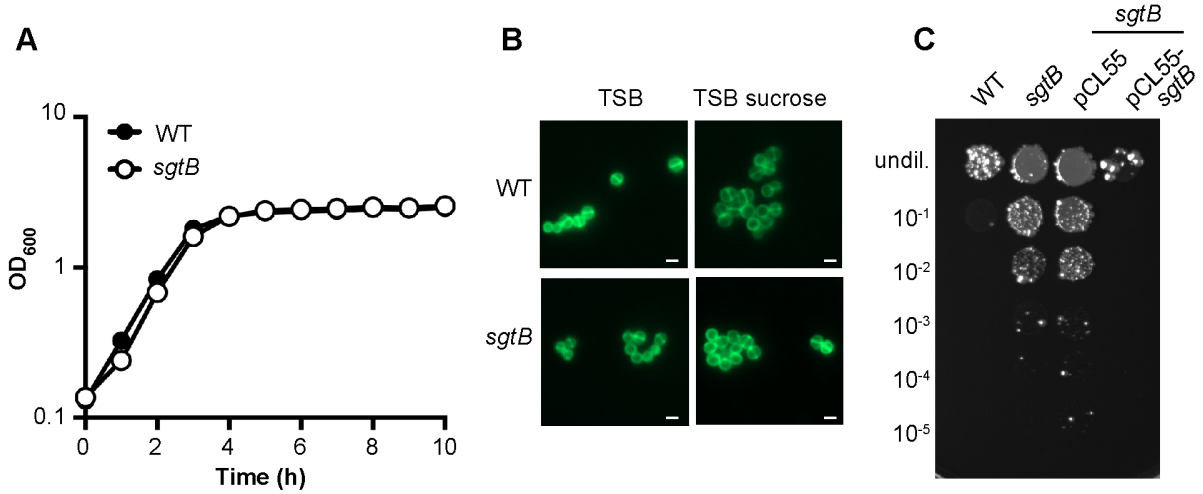
585

586

587 **Figure 4: Growth complementation analysis.** TSA sucrose plates with spot dilutions. (A) *S.*
588 *aureus* strains LAC* piTET, LAC* piTET-*mazE,* S2-*mazE* piTET or S2-*mazE* piTET-*mazE*
589 were grown overnight in TSB supplemented with chloramphenicol, washed twice in PBS and
590 serial dilutions spotted on TSA 40% sucrose plates supplemented with 7.5 ng/ml
591 chloramphenicol without (left panel) or with 100 ng/ml Atet (right panel). (B) *S. aureus* strains
592 LAC* (WT), LAC* piTET, LAC* piTET-*sgtB,* S4-*sgtB,* S4-*sgtB* piTET or S4-*sgtB* piTET-
593 *sgtB* were grown and samples prepared as described in A, however without chloramphenicol
594 selection, and dilutions were spotted on TSA 40% sucrose plates (left panel) or TSA 40%
595 sucrose plates supplemented with 200 ng/ml Atet (right panel). Representative plate images
596 from three independent experiments are shown.

597

598



599
600

601 **Figure 5: Growth and cell morphology of *S. aureus* strain LAC*sgtB::tn.** (A) Bacterial
602 growth curves. Overnight cultures of LAC* (WT) and strain LAC*sgtB::tn (sgtB) were diluted
603 in TSB to an OD_{600} of 0.05 and the growth monitored over a period of 10 hours using a plate
604 reader. The average OD_{600} values and standard deviation from three independent experiments
605 are plotted. (B) Microscopy analysis. *S. aureus* strains LAC* (WT) and LAC*sgtB::tn (sgtB)
606 were grown overnight in TSB or TSB supplemented with 40% sucrose, stained with BODIPY-
607 vancomycin and viewed under a fluorescence microscope. (C) Susceptibility to Congo red.
608 Overnight cultures of strains LAC* (WT) and the *sgtB* mutant strains LAC*sgtB::tn,
609 LAC*sgtB::tn pCL55 as well as the complementation strain LAC*sgtB::tn pCL55-sgtB were
610 serially diluted and aliquots spotted on TSA plates supplemented with 0.1% Congo red. For
611 panels B and C, representative result from three independent experiments are shown.

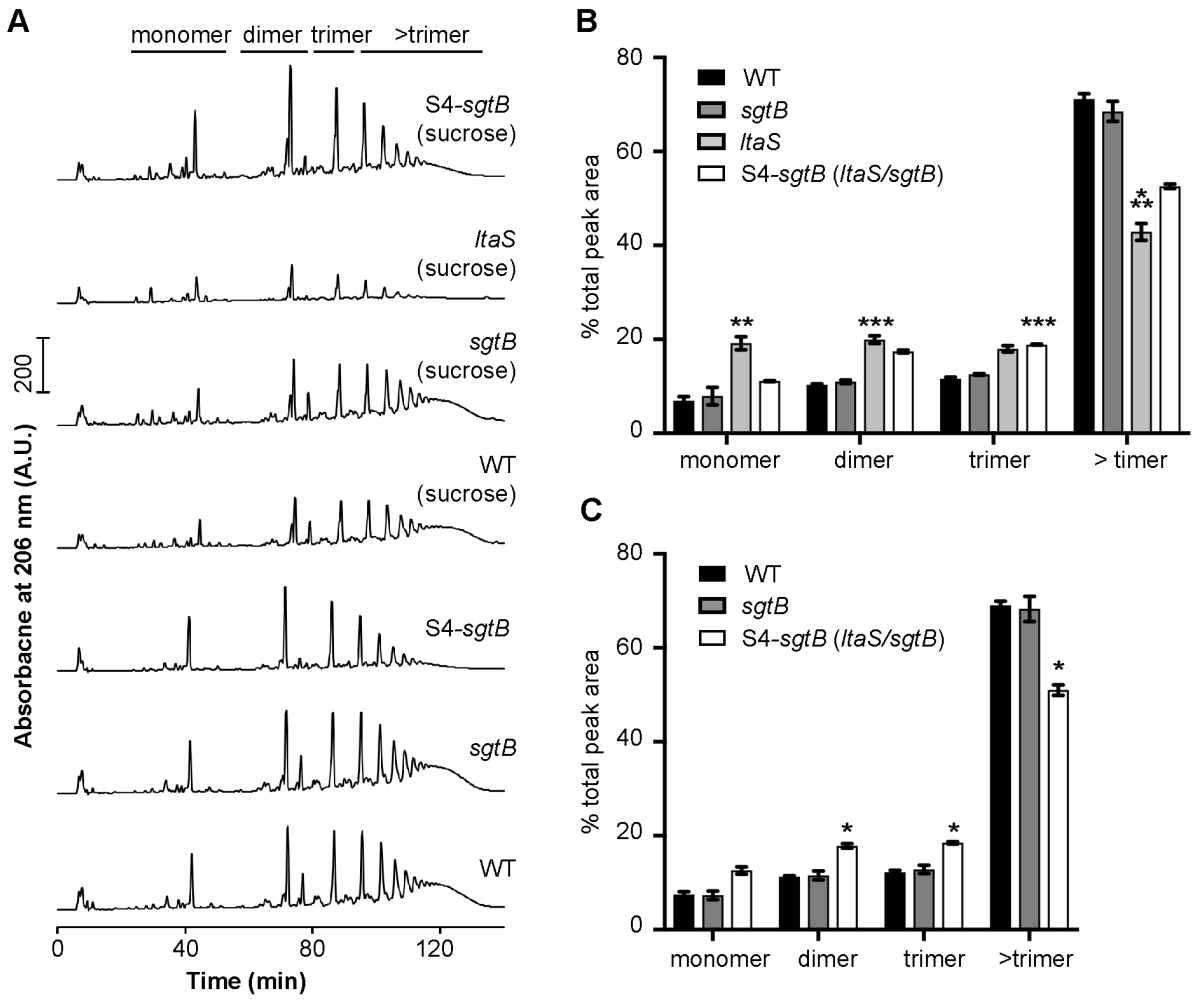


Figure 6: Peptidoglycan analysis of WT and mutant *S. aureus* strains. (A) HPLC profile of mutanolysin digested peptidoglycan. Peptidoglycan was isolated and digested with mutanolysin as described in the materials and methods section from *S. aureus* strains LAC* (WT), LAC**sgtB::tn* (*sgtB*) and the LTA negative suppressor strain S4-*sgtB* following growth in TSB medium or from *S. aureus* strains LAC* (WT), LAC**sgtB::tn* (*sgtB*), the LTA negative suppressor strain S4-*sgtB* as well as the original *ltaS* mutant strain (ANG2135) following growth in TSB 40% sucrose medium. Monomeric, dimeric, trimeric and > trimeric peptidoglycan fragments are indicated above the graphs and a representative profile from three independent samples is shown. (B-C) Quantification of the different peptidoglycan peaks. The peaks corresponding to monomeric, dimeric, trimeric and > trimeric peptidoglycan fragments were integrated and quantified for the strains used in panel A. The combined peak area was set

625 to 100% for each strain and the average values and standard deviations from the three
626 independent peptidoglycan isolations were plotted in panel B, for the strains grown in TSB
627 sucrose medium and in panel C for the strains grown in TSB medium. Statistical analysis to
628 identify differences in monomer, dimer, trimer or > trimer fractions between WT and mutant
629 strains was performed in Prism (GraphPad) using a Kruskal-Wallis test followed by a Dunn's
630 multiple comparison test. Adjusted p-values <0.1 are indicated by a single asterisk (*), adjusted
631 p-values < 0.05 by a double asterisk (**) and adjusted p-values < 0.01 by a triple asterisk (***).
632
633

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